# Molecular Mechanism of Action of Antioxidant Biomolecules from Higher Plants

A thesis submitted to the
**Department of Biotechnology** of **University of Mysore**
in fulfillment of the requirement for the degree of

## Doctor of Philosophy
## in
## BIOTECHNOLOGY

**By**

**Jyothi Maria Veigas** **M.Pharma**

Under the supervision of
**Dr. Bhagyalakshmi Neelwarne**

**Plant Cell Biotechnology Department**
**Central Food Technological Research Institute**
**(A Constituent Laboratory of Council of Scientific and Industrial Research, New Delhi)**
**MYSORE - 570 020, INDIA**

**May 2009**

**Jyothi Maria Veigas**
Senior Research Fellow

Department of Plant Cell Biotechnology

CFTRI, Mysore-570 020

E-mail: veigasmaria@gmail.com

# DECLARATION

I, **Jyothi Maria Veigas**, declare that this thesis entitled **"Molecular Mechanism of Action of Antioxidant Biomolecules from Higher Plants"** is the result of research work carried out by me under the supervision of **Dr. Bhagyalakshmi Neelwarne** at Plant Cell Biotechnology Department of Central Food Technological Research Institute, Mysore- 570 020, India, during the period of April 2005 to March 2009. I am submitting this thesis for the award of **Doctor of Philosophy (Ph.D.)** degree in **BIOTECHNOLOGY** of the University of Mysore.

I further declare that this thesis has not been submitted by me for the award of any other degree / diploma of this or any other University.

Place : Mysore
Date : 07/05/2009

**Jyothi Maria Veigas**
(Candidate)

**Dr. Bhagyalakshmi Neelwarne**

Scientist

Department of Plant Cell Biotechnology

E-mail: pcbt@cftri.res.in

blakshmi_1999@yahoo.com



# CERTIFICATE

This is to certify that the thesis entitled **"Molecular Mechanism of Action of Antioxidant Biomolecules from Higher Plants"** submitted by **Ms. Jyothi Maria Veigas** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology** is the result of research work carried out by her under my guidance in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** during the period of April 2005 to March 2009.

Place: Mysore
Date: 07/05/2009

**Bhagyalakshmi Neelwarne**
(Research Supervisor)

*To*

*My mother who was always there*

*and*

*My father who could not be.....*

**Abstract**

Oxidative stress is characterized by an altered antioxidant defense and is associated with human pathologies. High intake of fruits and vegetables has reduced risk of degenerative diseases due to the presence of antioxidant phytochemicals that react with reactive species which otherwise might lead to damage in the body. The present study investigated the potentials of anthocyanins and carotenoids derived from *Syzygium cumini* fruits and *Delonix regia* flowers respectively, as antioxidants, evaluated through chemical methods and animal cell models by monitoring antioxidant enzymes and the respective mRNAs.

The fruits of *S. cumini* contain anthocyanins (230mg/100g DW) which are identified to be glucoglucosides of malvidin, petunidin and delphinidin. The anthocyanin extract of *S. cumini* (SCA) shows strong antioxidant activities *in vitro* at very low concentrations up to 5ppm. The flowers of *D.regia* are a unique combination of hydrophilic (anthocyanins) and lipophilic (carotenoids) compounds. Fresh petals of *D. regia* contain 825mg and the oven dried petals contained 580mg/100g DW of total anthocyanins and 660mg/kg DW of β-carotene. The major carotene in the carotenoid fraction (CF) was β-carotene (50% of total) whereas the xanthophyll fraction (XF) was identified to have lutein, zeaxanthin, β-cryptoxanthin and astaxanthin. The carotenoids and anthocyanins from the two sources showed varying degrees of antioxidant activities depending on the assay system used.

The purified fraction of *S. cumini* contains solely mixture of anthocyanins and gives intense hue in solutions of low pH. It is highly colored at pH 2.0 with the color intensity reducing with an increase in pH, which however is stable up to pH 5.0 with no further color loss during the 45 days of study. The incorporation of the pigment in the pharmaceutical syrup gives it a pinkish hue which remains so with a minimal loss at the end of 8 weeks suggesting its potential to be a stable natural color apart from its antioxidant activity.

The antioxidant activity of the extracts of *S. cumini* and *D. regia* were studied using a biological model such as the isolated rat hepatocytes to further establish their usefulness as effective oxidative stress neutralizers. Epigallocatechin gallate (EGCG) and quercetin were used as standard antioxidants for comparison. Independent exposure of isolated rat hepatocytes to carbon tetrachloride ($CCl_4$) and *tert*-butyl hydroperoxide (TBH) brought about significant cellular injury marked by cell death, increased lipid peroxidation and decreased glutathione (GSH) content. Cells show differential response to oxidative stress. While antioxidant enzyme activities were radically reduced by $CCl_4$ it was increased in

TBH-exposed cells with a consequent increase in mRNA expression. In $CCl_4$ model, SCA acts chiefly via the glutathione redox system by elevating the cellular glutathione and activity of glutathione peroxidation by 2-fold while having no significant effect on catalase and superoxide dismutase. In TBH model SCA reversed the TBH-induced increase in enzyme activities and their mRNA expression with little effect on glutathione peroxidase activity at higher concentrations. Carotenoids of *D. regia* were protective at low concentrations of up to 100ppm beyond which they had no significant effect. The $CCl_4$-induced reduction in superoxide dismutase and catalase activities were further reduced by CF while XF had no effect. EGCG and CF further reduced the $CCl_4$-induced reduction in CAT and SOD activity while effect of XF on these two enzymes is not significant. The carotenoids extracts and EGCG confer protection mainly by increasing cellular GSH content and reducing lipid peroxidation. Carotenoid fractions, EGCG and quercetin increased TBH-induced increase in enzyme activity but reduced their mRNA expression suggesting a post transcriptional regulation of the enzymes. Lipid peroxidation was significantly abrogated by carotenoids fractions as well as EGCG and quercetin. These observations indicate that while SCA has a direct effect on AOEs, carotenoids and standards appear to act via mechanisms independent of these enzymes.

The antioxidant activity of the pigment extracts were further elucidated using a continuously growing stable cell line, Hep3B. In addition to the carotenoids extracts, anthocyanin rich fraction of *D. regia* (DRA) was also assessed in this study. TBH caused a significant increase in superoxide dismutase activity while reducing catalse and glutathione peroxidase activity in Hep3B cells and caused cytotoxicity via apoptosis associated with reduced Bcl-2/Bax ratio and increased caspase-3 activation. All the extracts and standards protected from TBH-induced cell death, the order of protection being SCA=DRA>EGCG>quercetin>XF>CF as measured by MTT assay and LDH leakage. The extracts alleviated the TBH-induced reduction in GSH content and increase in lipid peroxidation. *In vitro* DNA damage was prevented by the anthocyanin extracts as well as EGCG and quercetin while no significant protection was offered by carotenoids extracts, which may be attributed to their low solubility in aqueous medium. The anthocyanin extract of *S. cumini* significantly reversed the stress induced alterations in antioxidant enzyme chiefly via modulation of the transcription of the same. Among the two carotenoids fractions of *D. regia,* XF was more effective compared to the CF in preventing the cell death in Hep3B cells exposed to TBH. The anthocyanin extract also brought about significant protection in terms of cell viability, lipid peroxidation and GSH

content. The carotenoids extracts and the standards (EGCG) reversed the TBH-induced increase in antioxidant enzyme activity and their expression. The activity of GPx appears to be post transcriptionally regulated in Hep3B cells pretreated with quercetin, EGGC (10μM) and SCA since an increase in activity was not associated with an increased expression of its mRNA. A transcriptional regulation of CAT and SOD was obvious in cells pretreated with extracts as well as standards except DRA (100ppm) and quercetin (10μM). Since SCA was found to act via modulation of AOEs, its effect on the stability of transcribed mRNA of these enzymes was studied by inhibiting the transcription followed by measuring the steady state mRNA level in a time dependent manner. TBH caused a radical reduction in steady state mRNA level of CAT and GPx. GPx was most susceptible to degradation by TBH and was almost completely degraded one hour after the addition of the transcription inhibitor, actinomycin D. SCA provided protection to CAT mRNA by almost completely inhibiting the action of TBH on the same. Pretreatment of SCA delayed the decay of GPx mRNA by about 3 h, as compared to 1 h in TBH treated cells. The results of the present study suggest that SCA provides protection to AOEs by increasing their stability and hence making them available for translation.

In addition to being cytoprotective under oxidative stress conditions, the carotenoids and anthocyanins were also inhibitory to cell growth on long term exposure indicating antiproliferative or growth inhibitory activity in carcinoma cells. Antiproliferative activity appeared to be mediated via induction of apoptosis as evidenced by microscopic examination using propidium iodide stain.

The overall results suggest that the extracts of *S. cumini* and *D. regia* offer protection to cells by improving cellular defense systems such as the antioxidant enzymes either directly or by enhancing their turn over by extending their mRNA half life; and also by directly quenching free radicals and probably reversing the damage by prooxidants and other toxins through regulation of transcription factors and signaling cascades involved in cell survival.

## Acknowledgement

This thesis is the result of years of research in Plant Cell Biotechnology Department. During this time I have been associated with a great number of people whose contribution in assorted ways to the research and the making of the thesis deserves special mention.

I am ever so grateful to ***Dr. Bhagyalakshmi Neelwarne***, supervisor of this thesis work, for introducing me to the colorful world of anthocyanins and carotenoids, for taking me under her wings and guiding me through this amazing journey to Ph.D. I thank her for all the opportunities she made possible for me during this time, the absolute positiveness in her and her belief in me.

I wish to express my heartfelt gratitude to ***Dr. V. Prakash***, Director, CFTRI, Mysore, for granting me the opportunity to utilize excellent facilities available at CFTRI.

I am grateful to ***Dr. G.A. Ravishankar***, Head, Department of Plant Cell Biotechnology, CFTRI, for his constant encouragement and support during the pursuit of my research work.

I would like to extend my gratitude to the members of my doctoral committee ***Dr. M.C. Varadaraj, Dr. Lokesh, Dr. P. Srinivasan, Dr. K.K. Bhat*** and ***Dr. Appu Rao*** for their constructive criticism and valuable suggestions which helped me make this thesis look better.

My sincere thanks to ***Dr. M.S. Narayan***, for helping me with the LC-MS analysis and interpretation of the data. I also thank him for being friendly and warm. I truly cherish the discussions and the tea sessions with him.

I wish to thank ***Dr. H.P. Ramesh***, for making work at Cell Culture Facility simpler and helping me with all the required facilities.

My heartfelt thanks to the staff of PCBT ***Dr. T. Rajasekaran, Dr. R. Sarada, Dr. P. Giridhar, Mrs. Karuna, Mr. Shivanna, Mr. Srinivas Yella, Palaksha, Shashi*** and ***Channe Gowda*** who have always been so helpful.

Thanks to ***Dr. Arun Chandrashekar*** for being a great human being and a friend. His high spirits and intelligence have been an inspiration to me.

I thank ***Dr. M.C Misra, Dr. NG Karanth, Dr. S.G. Prafulla, Dr. Avinash Sattur, Dr. M.S. Thakur*** and **Mr**. ***Somanna*** for their support and kindness during my tenure at FTBE department.

Special thanks to ***Dr. Muthukumar***, Department of Biochemistry and Nutrition, for providing me with rat livers, without which the third chapter would not be possible.

Warmest appreciation to my lab associates ***Sridhar, Thimmaraju, Lokesh, Gururaj, Dayanand, Manjunath, Sandesh, Parimalan, Saktivelu, Kathiresan, Ashwani Sharma, Ramakrishna, Vinod, Vanitha, Roohie, Namitha, Mahendra, Kumuda, Padmaja, Avinash, Santhosh, Simmi, Sridevi, Kalpashri, Kavitha, Harsha, Imthiyaz, Gurudatt*** and ***Akshataha*** for their friendship, help and support over the years.

Thanks to ***Shibin*** for being my software consultant, unpaid. Thanks for patiently attending to my troublesome laptop and I know I owe you a lot.

Thanks to ***Anila*** for being a good friend and helping me with sequencing and in a lot of other ways.

Thanks to ***Satish, Puneet*** and ***Richa***, for the help and support, particularly during my initial days at the Cell Culture Facility.

***Shabana***, for her friendship through the years. Thanks for the lunch time rendezvous' and of course the shopping times together.

Thanks to ***Praveen, Girish, Raghuraj, Chidanand, Raghavendra, Dina, Mable, Renuka, Ayyappan, Lohith, Vijay*** and ***Sivakumar*** for making my FTBE days memorable ones and for continuing to be good friends. I truly enjoyed being in that place.

Thanks to ***Dr. Uma Manjappara*** and ***Dr. Deo*** for being great friends and making breakfast times fun.

I am grateful to ***Mrs. Jaya***, S&P department, for her care and affection.

Thanks are due to ***Devraj***, ***Manu*** and ***Manjunath*** for introducing me to the Mysore Film Society, for screening great movies and for their friendship.

***Sagar***, for being there always. For laughing silly with me; lending me a shoulder during my difficult times; for giving me the comfort zone to talk about everything and about nothing. It was comforting just to know you are there. Thanks a lot for being my best friend.

Many thanks to ***Abiraj*** for being a wonderful friend, sending me the research papers every time I asked for one, without complaining. Thanks a lot Abi. Without you, this thesis wouldn't have been what it is now.

***Satish*** for being my guardian, literally, and lending me a helping hand despite his busy schedule and for the good talks and the occasional coffee times.

I owe my heartfelt thanks to ***Parvathi***, for giving me space in her beautiful and a very comfortable home and for continuing to be a very good friend.

I acknowledge the timely help and cooperation of staff of supporting departments: Stores and Purchase, CIFS, FOSTIS, HRD, Computer Center and Administration.

The delicious breakfast at the Canteen cannot go unmentioned, for it made my life much simpler, sparing me from that extra time in the kitchen.

I sincerely acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, INDIA for the Senior Research Fellowship.

My deepest sense of gratitude goes to my family. Thank you mother for being there always, for being my friend, particularly at moments I most needed you, without you knowing it. My sister Sheela for being my best friend through the years and helping me in a lot of ways. My sisters, brothers, brothers-in-law, sisters-in-law, "Belthangady cousins", "NJ cousins", aunts and uncle for believing in me, supporting me and advising me through out; and my most adorable nieces and nephews, for making life more beautiful.

Last but not the least, thanks be to God for all that I am today and for the tests of life which have made me emerge much stronger and more confident.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF ILLUSTRATIONS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAPH | 2'azo bis (2-methylpropionamidine) dihydrochloride |
| AOE | Antioxidant enzyme |
| AP-1 | Activator protein-1 |
| ARE | Antioxidant response element |
| ATBC | Alpha tocopherol beta carotene |
| ATP | Adenosine triphosphate |
| Bax | Bcl-2 associated X protein |
| Bcl-2 | Antiapoptotic protein of B-cell lymphoma-2 family |
| BHA | Butylated hydroxyl anisole |
| BHT | Butylated hydroxyl toluene |
| CAT | Catalase |
| $CCl_3^{\bullet}$ | trichloromethyl radical |
| $CCl_4$ | Carbon tetrachloride |
| cDNA | Complimentary Deoxyribonucleic Acid |
| CF | Carotene fraction of *Delonix regia* flowers |
| $CO_2$ | Carbon dioxide |
| Conc. | Concentration |
| Cu-Zn SOD | Copper Zinc superoxide dismutase |
| CVD | Cardiovascular disease |
| CYP450 MFOS | Cytochrome P450 mixed function oxidases |
| DAPI | 4′6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DPPH | α, α-diphenyl-β-picryl hydrazyl |
| DRA | Anthocyanin rich extract of *D. regia* |
| DTNB | 5,5-dithiobis-2-nitrobenzoic acid |
| DTT | Dithiothreitol |
| DW | Dry weight |
| EDTA | Ethylene diamine tetra acetic acid |
| EGCG | Epigallocatechin gallate |
| ESI | Electrospray ionization |
| EtOAc | Ethyl acetate |
| FBS | Fetal bovine serum |
| FRAP | Ferric reducing ability of plasma |
| g | Gram |
| GAE | Gallic acid equivalent |
| $GCS_h$ | Heavy subunit of γ-glutamylcysteine synthetase |
| GPx | Glutathione peroxidase |
| GSH | Reduced glutathione |
| GSSG | Glutathione disulfide or oxidized glutathione |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| HCl | Hydrochloric acid |
| Hep3B | Human derived hepatocellular carcinoma |

| | |
|---|---|
| HepG2 | Human derived hepatocellular carcinoma |
| HPLC | High performance liquid chromatography |
| kg | Killo gram |
| LDH | Lactate dehydrogenase |
| LDL | Low density lipoprotein |
| LOOH | Lipid peroxide |
| M | Molar |
| m/z | Mass to charge ratio |
| MDA | Malondialdehyde |
| MeOH | Methanol |
| mg | Milli gram |
| mL | Milli liter |
| mM | Milli molar |
| Mn SOD | Manganese superoxide dismutase |
| mRNA | Messenger ribonucleic acid |
| MS | Mass spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NF-κB | nuclear factor kappa B |
| nM | Nano Molar |
| NO | Nitric oxide |
| Nrf2 | nuclear factor erythroid 2 p45 (NF-E2)-related factor |
| $O_2$ | Oxygen |
| $O_2^{-\bullet}$ | Superoxide anion |
| OD | Optical density |
| $OH^{\bullet}$ | Hydroxyl anion |
| $ONOO^-$ | peroxynitrite anion |
| $OOCCl_3$ | trichloromethyl-peroxyl radical |
| PARP | Poly(ADP)-ribose polymerase |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PI | Propidium iodide |
| ppm | Parts per million |
| RNA | Ribonucleic acid |
| RNAse | Ribonuclease |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SCA | Anthocyanin rich fraction of *Syzygium cumini* |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SOD | Superoxide dismutase |
| SPE | Pulp extract of *S. cumini* fruits. |
| $T_{acy}$ | Total anthocyanins |
| TAE | Tris-acetic acid-EDTA |
| TBA | Thiobarbituric acid |

| | |
|---|---|
| TBH | *Tertiary*- butyl hydroperoxide. |
| TCA | Trichloro acetic acid |
| TE | Tris EDTA |
| TFA | Trifluoro acetic acid |
| U/mg | Units per milli gram |
| UV | Ultra violet |
| v/v | Volume per volume |
| XF | Xanthophylls fraction of *D. regia* flowers |
| XRE | Xenobiotic response element |
| µg | Micro gram |
| µL | Micro liter |
| µM | Micro molar |
| °C | Degree centigrade |

# *General Introduction and Review of Literature*

## G.1 Introduction

Diets rich in fruits and vegetables have been associated with reduced risk of diseases such as cancer, coronary heart disease, neurodegeneration, macular degeneration, diabetes etc (Hertog *et al.,* 1995; Youdim *et al.,* 2001; Ramassamy, 2006). While these diseases have been linked to reduced antioxidant defense and increased oxidative stress, the antioxidants present in fruits and vegetables have chiefly been considered responsible for inhibiting the free radical mediated reactions thus protecting the body against the damaging effects of the free radicals and other reactive species.

Every living aerobic organism is a subject of "oxygen paradox". The super molecule- oxygen, on which the very existence of aerobic life forms depends, is also a dangerously harmful compound. The dangers imposed by oxygen, upon all life forms, arise from its electron structure which predisposes it to reduction by a univalent pathway (Fridovich, 1999). The univalent reduction of oxygen generates reactive intermediates the excessive accumulation of which leads to oxidative stress and has been implicated in a number of degenerative processes such as mutagenesis, cancer, atherosclerosis, congestive heart diseases, chronic inflammatory diseases, macular degeneration, cataract, disorders of central nervous system such as Alzheimer's dementia, Parkinson's disease including the ageing process (Davies, 1995).

### G. 2 Free radicals/ reactive species

Free radicals are unstable compounds containing one or more unpaired electrons in their outer valence shell. They absorb electrons from a surrounding molecule thus causing damage to that particular molecule and in the process become chemically stable ((Halliwell and Gutteridge, 1995). The radical nature of molecular oxygen gives way to some important oxidation/reduction chemistry. Oxygen atom has an unpaired electron in its outer valence shell and molecular oxygen has two unpaired electrons thus making them free radicals. The reductive environment of the cellular milieu provides ample opportunities for oxygen to undergo unscheduled univalent reduction thus generating reactive intermediates such as superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide ($H_2O_2$) and the highly reactive hydroxyl radical ($OH^{\bullet}$) (Davies, 1995). Other oxidants relevant to human pathology are hypochlorous acid (HOCl), peroxynitrite (OONO), reactive aldehydes, lipid peroxides, lipid radicals and nitrogen oxides. Most of the reactive species such as $O_2^{-\bullet}$, $OH^{\bullet}$ and NO are radicals with an unpaired electron in their outer valence shell while the others like $H_2O_2$ and $OONO^{\bullet}$ are not radicals but are potentially reactive (Kojda and Harrison, 1999). The surplus reactive species in the living cells leads to oxidative stress.

**Fig. 1.** summarises the univalent reduction of molecular oxygen and the key events involved in generation of reactive oxygen species and sites of action of antioxidant enzymes.

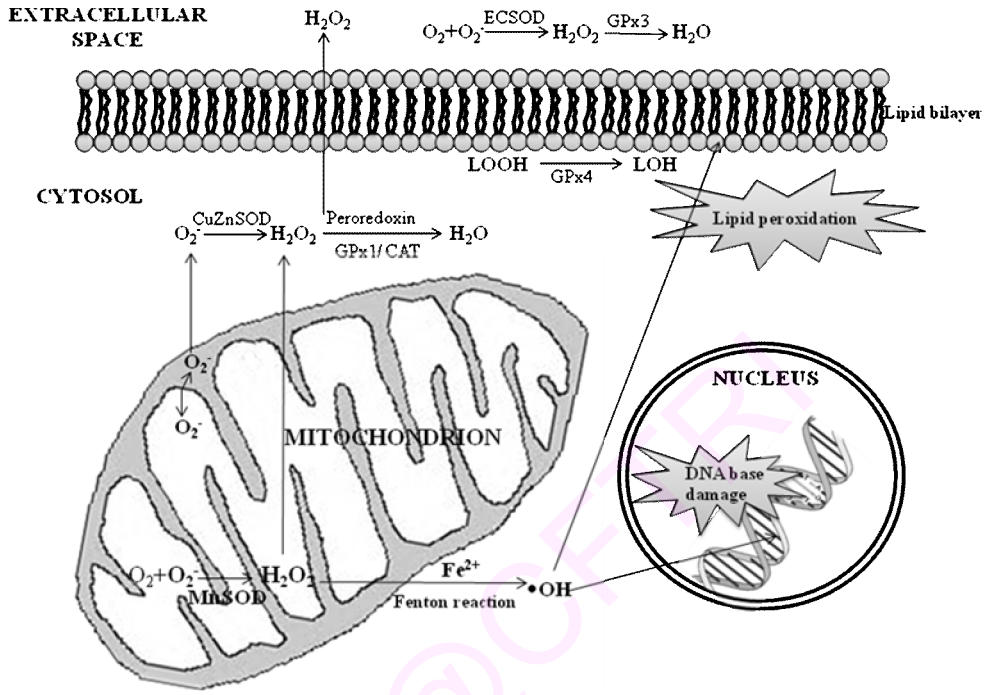



Fig. 1. Events occurring in the univalent reduction of molecular oxygen and action of antioxidant enzymes within a cell. $H_2O_2$ = hydrogen peroxide; $O_2$ = oxygen; $O_2^{\cdot}$ = superoxide anion; ECSOD = extracellular supeoxide dismutase; $H_2O$ = water; LOOH = lipid peroxide; LOH = lipid hydroxide; GPx = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase; •OH = hydroxyl radical; $Fe^{2+}$ = ferrous ion.

### G. 3 Oxidative stress

The body has an inherent ability to overcome the ravaging effects of the free radicals generated within the cell by generating an assortment of hydrophilic and lipophilic antioxidant molecules and enzymes and also through other antioxidant molecules assimilated through diet. Under normal physiological conditions, a homeostasis exists between the reactive species and the antioxidant defense. When the levels of reactive species surpass the antioxidant capacity of the cells, as does in a pathological state, this homeostasis is altered and oxidative stress ensues. In recent years these reactive species have been implicated in a wide range of degenerative processes and diseases such as mutagenesis (Kamiya, 2003), cell transformation and cancer (Waris and Ahsan, 2006), atherosclerosis, arteriosclerosis (Kojda, 1999; Martinet *et al.,* 2004), cardiovascular disorders (Touyz, 2004), chronic inflammatory diseases such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis; acute

inflammatory problems such as wound healing (Symons and King, 2003; Gelderman *et al.,* 2007; Novo and Parola, 2008); photo-oxidative stresses to the eye such as cataract, central nervous system disorders such as Alzeimer's dementia, Parkinsons disease (Knight, 1997) and a wide variety of age-related disorders and the ageing process itself (Lee *et al.,* 2006). Because free radicals are implicated in all these processes, minimizing and neutralizing their activity with antioxidants may allow us to live longer and healthier lives, look and feel better, and reduce or eliminate the risk of certain physiological disorders and diseases. Some of these oxidation-linked diseases and syndromes can be aggravated, perhaps even initiated, by various proxidants, which can be either biologically/biochemically generated or derived from the environment.

The major endogenous source of oxygen radicals under physiological conditions is the mitochondrial electron transport chain chiefly generating superoxide anion (Chance *et al.,*. 1979). Other sources of oxygen radical generation are the phagocytic cells such as nutrophils, monocytes and macrophages during phagocytosis and auto-oxidation of biological molecules (Babior, 1992; Kappus and Dipplock, 1992; Rice-Evans and Bruckdorfer, 1992). The exogenous sources of free radicals are radiation, tobacco smoke, environmental pollutants, detergents, ozone etc.

**G. 4 Antioxidants**

Antioxidants are substances that reduce, neutralise and prevent the damage done to body by free radicals. Several of the available definitions are based both on exact chemical terminology or terminology based on phenomena. In more precise chemical terms, Britton (1995) defined that, to be an effective antioxidant, a molecule such as a carotenoid would have to remove these radicals from the system either by reacting with them to yield harmless products or by disrupting free radical chain reactions. Tsuchihashi *et al.,* (1995) proposed that the antioxidant potency is determined by several factors such as intrinsic chemical reactivity of the antioxidant toward the radical, site of generation and reactivity of the radicals, site of antioxidant, concentration and mobility of the antioxidant at the microenvironment, stability and fate of antioxidant-derived radical, and interaction with other antioxidants. Halliwell (1990) defined biological antioxidants as "molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules".

There are an infinite number of antioxidants which act by different mechanisms to neutralize the effect of free radicals. These antioxidants may be classified as

- Antioxidant enzymes- Catalyse the breakdown of free radicals

- Chain-breaking antioxidants- donate or receive an electron to inhibit the free radicals from generating more free radicals
- Metal binding proteins- prevent metal ions from forming free radicals.

Recent times have seen a radical shift in interest towards naturally derived antioxidants as sources of biologically active compounds due to their health promoting and disease preventing properties. Synthetic antioxidants had occupied the major antioxidant market for the past few decades. The possible toxicity as well as general consumer rejection has led to the decrease in the use of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) (Wong *et al.,* 2006). Therefore, it is important as well as critical to search for radical scavengers from natural sources to enable the development of new drugs and the prevention of several diseases (Halliwell & Gutteridge, 1999; Valko *et al.,*, 2004). Antioxidants scavenge and minimize the formation of oxygen-derived species and inhibit oxidative damage induced by free radicals. They also recover the level of intracellular antioxidants (vitamins, methionine, glutathione and glutathione-related minerals) (Ip *et al.,* 1991; Schrauzer, 1992). Hence, these antioxidants may be particularly important in diminishing cumulative oxidative damage. Recently, several reports have suggested that plant foods may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxidative damage in vitro and in vivo (Ruch *et al.,* 1989; Tsuda *et al.,* 1994). Therefore, much attention has been focused on natural antioxidants.

**G. 5 Antioxidant molecules in the diet**

Essentially human body is equipped with a strong antioxidant defense both through externally supplemented diet and in the form of endogenous antioxidant enzymes and other biomolecules. The innate antioxidant defense of the body may not suffice for the defense against excess free radicals generated. Supplementing the diet with antioxidants is an important factor in shielding against oxidative insult. Therefore, antioxidants are potentially the most important part of human nutrition. Fruits and vegetables and plant extracts are incredibly essential as they clean up the free radicals as they circulate in blood and enter the tissues thus curtailing the damaging effects of free radicals. A number of antioxidants form a part of the diet viz., vitamin C, vitamin E, plant derived compounds such as polyphenols, carotenoids, flavonoids etc. Natural coloring compounds have been gaining significance in biology and medicine due to their potential health benefits. Since the present thesis is mainly focused on antioxidant mechanisms of natural colors, carotenoids and anthocyanins, the review of literature on natural antioxidants is limited to discussion on these two classes of compounds.

### G. 6 Natural colors

One of the attributes that judge the quality and visual acceptance of any consumable formulation mainly depends on its color characteristics. Color is an important element in deciding the appeal of the product it is incorporated into. With increasing reports on toxicities of synthetic colors, interest has shifted towards the use of natural colors. In India, Rule 26 of The Prevention of Food Adulteration Act, 1954 (PFA) and The Prevention of Food Adulteration Rules, 1955 & 1999 permit only those colors whether isolated from natural sources or produced synthetically in food items (**Table 1**).

In India, the use of anthocyanin as colorant is not approved as yet. In the USA, 4 of the 26 approved colorants are anthocyanin based viz., grape skin extract, grape color extract, fruit juice and vegetable juice. In the European Union all anthocyanin-derived colorants are recognized as natural colorants under classification E163 (Wrolstad, 2003).

Apart from their coloring properties, most natural colors are biologically active molecules having therapeutic and diagnostic value. For example, curcumin has been reported to be a digestive aid and curative against stomach disorders. It is also been proved as a promising molecule against cancer (Piper *et al.,* 1998).

Table 1. Natural colors permitted to be used in food in India and USA (Kapoor, 2006)

| **Compound** | **Country** | **Color** |
|---|---|---|
| Beta-carotene | India/USA | Yellow/Orange |
| Carotene | USA | Orange |
| β-apo-8' carotenal | India | Yellow/Orange |
| Methylester of β-apo-8, carotenoic acid | India | Yellow/Orange |
| Ethylester of β -apo-8' carotenoic acid | India | Yellow/Orange |
| Canthaxanthin | India | Yellow/Orange |
| Chlorophyll | India/USA | Green |
| Chlorophyllin | USA | Green |
| Riboflavin (Lactoflavin) | India | Yellow |
| Caramel | India | Brown |
| Annatto | India/USA | Yellow/Orange |
| Saffron | India | Orange |
| Curcumin (turmeric) | India/USA | Yellow |
| Capsanthin | USA | Red/Orange |
| Beetroot | USA | Pink/blue/red |
| Carmine | USA | Red |
| Lycopene | USA | Reddish orange |
| Carminic Acid | USA | Orange/Red |
| Lutein | USA | Yellow |
| Anthocyanin | USA | Red/Purple |
| Vegetable carbon | USA | Black |

In the recent years a tremendous interest in carotenoids and anthocyanins as food colors has made researchers look into newer sources for these pigments. Therefore there is much scope to introduce alternative sources of natural colors in food and pharmaceuticals; but according to legislation, there exist a need for their extensive safety evaluation study through systematic pharmacological and clinical trials.

### G.6.1 Carotenoids

Carotenoids comprise a diverse class of natural pigments that are of interest for pharmaceuticals, coloring food and animal feed and nutrient supplements (Schmidt-Dannert *et al.,* 2000) due to their high antioxidant activity (Sies *et al.,* 1992). They form one of the largest classes of natural pigments and are responsible for red-yellow-orange colors of most fruits and vegetables.

Carotenoids are defined by their chemical structure. They are composed of 40-carbon polyene chain and depending on the presence of terminal cyclic end-group and oxygen containing functional group are classified respectively as:

- carotene hydrocarbons and
- xanthophylls (oxygenated derivatives of carotene hydrocarbons),

Naturally occurring carotenes mostly occur in all-trans form. Molecular structures of some of the physiologically important carotenoids are depicted in **Fig.2.**

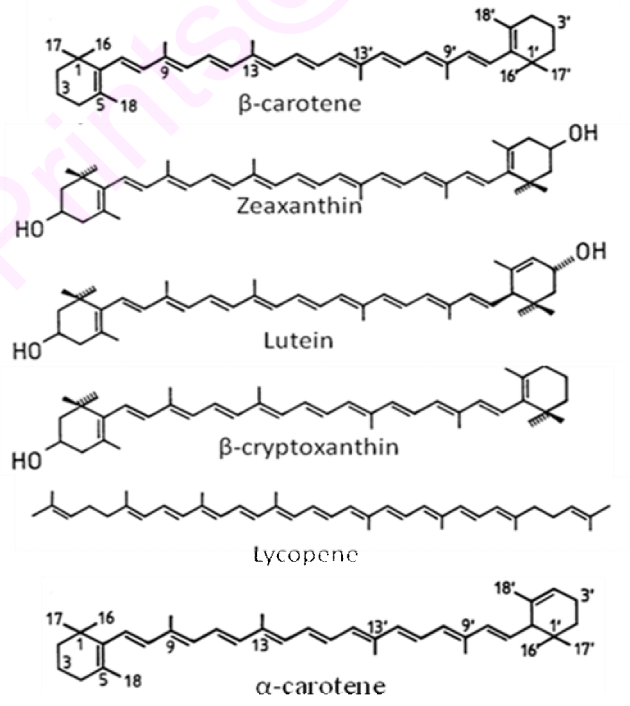



Fig. 2. Chemical structures of some physiologically important carotenoids

The structure of a carotenoid ultimately determines the potential biological function that the pigment may have. The characteristic pattern of alternating single and double bonds in the polyene backbone of carotenoids allows them to absorb excess energy from other molecules, while the nature of the specific end groups on carotenoids may influence their polarity. The former may account for the antioxidant properties of biological carotenoids, while the latter may explain the differences in the ways that individual carotenoids interact with biological membranes (Britton, 1995). The extensive conjugated double bond system serves as the light-absorbing chromophore thus imparting red, yellow or orange color to the compound (Rodriguez-Amaya, 2001). The extensive conjugated double bonds system is attributed to the singlet oxygen quenching capacity of carotenoids, the activity increasing with the increase in number of double bonds (Foote *et al.,* 1970).

**G.6.1.1 Role in biology and medicine**

While xanthophylls are predominant in green leafy vegetables, almost all carotenes are found in yellow vegetables. Carotenoids such as β-carotene, α-carotene and cryptoxanthin act as provitamin A compounds, being converted into vitamin A *in vivo*. β-carotene and lutein are the most common carotene and xanthophyll known. The role of carotenoids in the prevention of chronic diseases and their biological actions are depicted in **Fig. 3**.

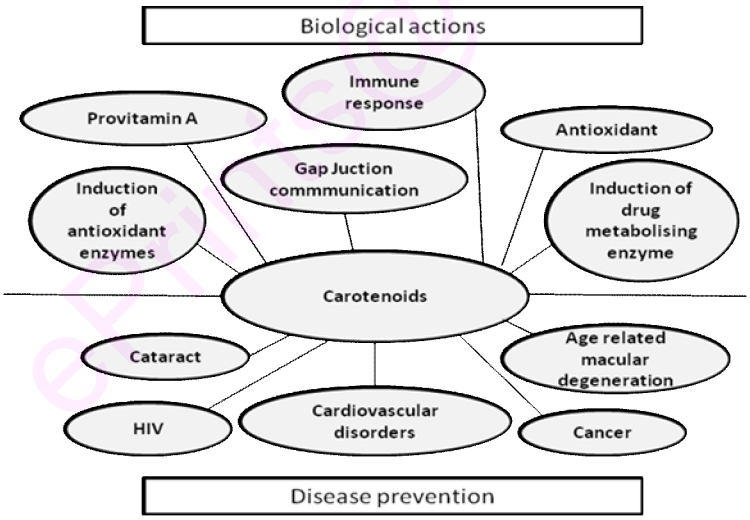



Fig. 3. Role of carotenoids in the prevention of chronic diseases.

Carotenoids are potent antioxidants quenching singlet oxygen and acting as chain breaking antioxidants (Burton, 1989), carotene hydrocarbons being more efficient singlet oxygen quenchers compared to the xanthophylls (Cantrell *et al.,* 2003). Carotenoids have been reported to have cancer chemopreventive activities in different cell and animal models. Lutein and zeaxanthin have been found to be independently associated with the reduced risk of age-

related macular degeneration (SanGiovanni *et al.,* 2007). Carotenoids have received much importance since the report of Peto *et al.,* (1981) who suggested the role of β-carotene from fruits and vegetables as a protective agent against cancer. Apart from their provitamin-A activity and antioxidative properties, carotenoids are responsible for a wide range of intercellular activities such as communication, immune response, neoplastic transformation, growth control and regulation of cellular levels of the enzymes that detoxify carcinogens (Pryor *et al.,* 2000). Epidemiological studies indicate an inverse relationship between consumption of fruits and vegetables rich in carotenoids and risk of cancer and cardiovascular diseases which is attributed to their antioxidant activity through singlet oxygen quenching and deactivation of free radicals (Palozza and Krinsky, 1992; Burton, 1989). Low plasma carotenoids concentration is often used as an indicator for those at risk of chronic diseases (Foy *et al.,* 1999). Such indices are based on the direct association between the intake of carotenoid-rich fruit and vegetables, plasma and tissue concentration of carotenoids and the development of chronic disease states, particularly cardiovascular diseases and cancer of various organs (Southon *et al.,* 2001). Plasma xanthophylls are inversely related to the indices of oxidative DNA damage and lipd peroxidation (Haegele *et al.,* 2000). Natural antioxidants are more favorably accepted and recognized by higher animals than synthetic ones (Shi, 2001). The same is true with carotenoids, the natural forms of which primarily exist in the all-*trans* form (Rodriguez-Amaya, 2001) along with oxygenated carotenoids that have better efficacy over synthetic *all trans* forms (Heber, 2004). However, there have been contradictory reports about the effect of carotenoids, specifically, β-carotene. β-carotene, though protective against oxidative stress, is said to have increase the risk of lung cancer in smokers and workers occupationally exposed to asbestos (ATBC, 1994; Omenn, 1998). Therefore, it is essential to carry out a detailed study to assess the effectiveness and safety of carotenoids, to define the population that can benefit from carotenoids, specify the dose and length of treatment and also to establish whether mixtures of carotenoids rather than single carotenoids are more effective.

### G.6.2 Anthocyanins

Anthocyanins form the largest water soluble pigments in plants. These are chemically grouped under flavonoids family forming a group of phenolic compounds responsible for the pink-red-purple-blue colors of fruits and vegetables, some roots and tubers. Next to chlorophyll, they are the most important group of plant pigments visible to the human eye. Chemically anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of flavilium (2-phenylbenzopyrilium) salts.

The basic structure of the flavilium salt and the structures of most commonly occurring anthocyanins are depicted in **Fig. 4**.

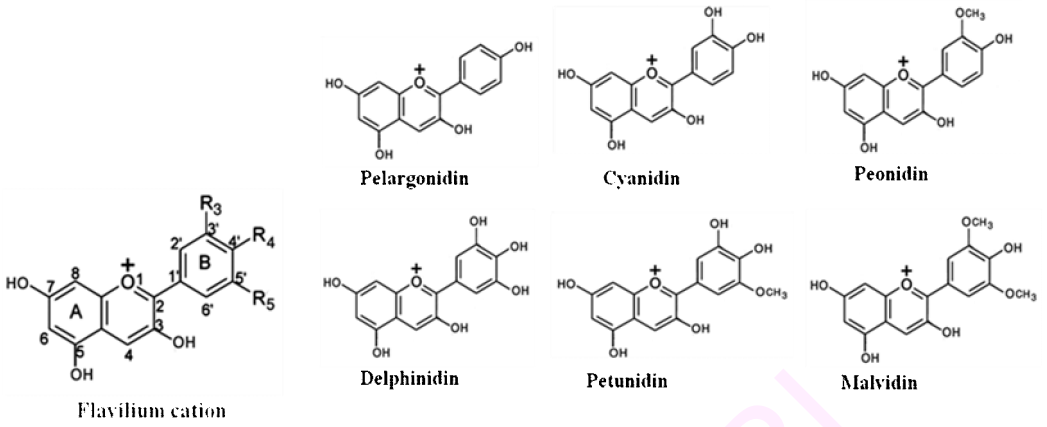



Fig. 4. Structures of the flavilium cation and six most commonly occurring anthocyanidins

Glycosidic substitutions occur at 3 or 5$^{th}$ position which may be acylated with aliphatic acids or cinnamic acid. This leads to a considerable structural variation giving rise to about 600 anthocyains which have been reported in nature (Andersen, 2002).

The chemical structure of the anthocyanin plays an important role in deciding the color and also the biological activity. The color of anthocyanins as well as their activity is regulated by the position, number and type of substitution on the flavilium cation. Stintzing *et al.,* (2002) reported that glycosidic substitution at 5$^{th}$ position of the cyanidin glycoside and acylation with cinnamic acids shifts the color to more purplish hue. Commercial anthocyanin colorants are mostly derived from fruits and vegetables the largest natural commercial source being the red grape skin. Other sources include elderberry, blackberry, raspberry, red cabbage, black carrot, purple corn, red raddish and pruple sweet potato (Kirca *et al.,* 2006). Anthocyanins from different sources vary from each other with respect to their color and stability. This is attributed to the difference in glycosidic substitution and the presence and type of acylating molecule. Due to the presence of positive charge, the flavylium cation is more susceptible for nucleophilic attack especially at the 4$^{th}$ position. Substitution at 4$^{th}$ position significantly reduces degradation by nucleophiles (Garcia-Viguera, 1999). The stability of anthocyanins is an important factor in deciding its potential as a natural colorant. Increasing the stability of a particular anthocyanin by chemical modification appears to be the best way to achieve this.

**G.6.2.1 Stability of anthocyanins**

They are relatively stable in the presence of light and heat and withstand pasteurization (65-85°C) and short heating at 120°C. However, stability of anthocyanins has been a major

concern pertaining to its incorporation into foods and beverages. The prime limitation of use of anthocyanins in food is their degradation due to reaction in the food product, processing time and temperature, exposure to light, oxygen, change in pH, presence of enzymes, structure of anthocyanins etc (Francis, 1989). This instability limits its use as a colorant in food products as compared to the synthetic dyes.

**G.6.2.1.1 Factors affecting anthocyanin stability**

**G.6.2.1.1.1 Temperature, Light and Oxygen**

Anthocyanins degradation is accelerated during the processing and storage of the product in the presence of high temperature. The color of the anthocyanins is reduced with the increase in temperature, exposure to light and this reduction is accelerated in the presence of oxygen. Thermal degradation follows first order kinetics and chalcone formation is presumed to be the first step in the process, eventually leading to browning especially in the presence of oxygen (Markakis, 1982; Ahmed *et al.,* 2007). A number of studies have been reported on the instability of anthocyanins in the presence of these factors (Fossen *et al.,*. 1998; Cevallos-Casal and Cisneros-Zevallos, 2004). However, low temperatures have been shown to increase the anthocyanins content in fresh strawberries and raspberries during storage (Kalt *et al.,* 1999).

**G.6.2.1.1.2 Effect of pH**

Anthocyanins are sensitive to pH and their hue is determined by the same in solution. The structural transformation of anthocyanins with the change in pH is represented in **Fig. 5**.

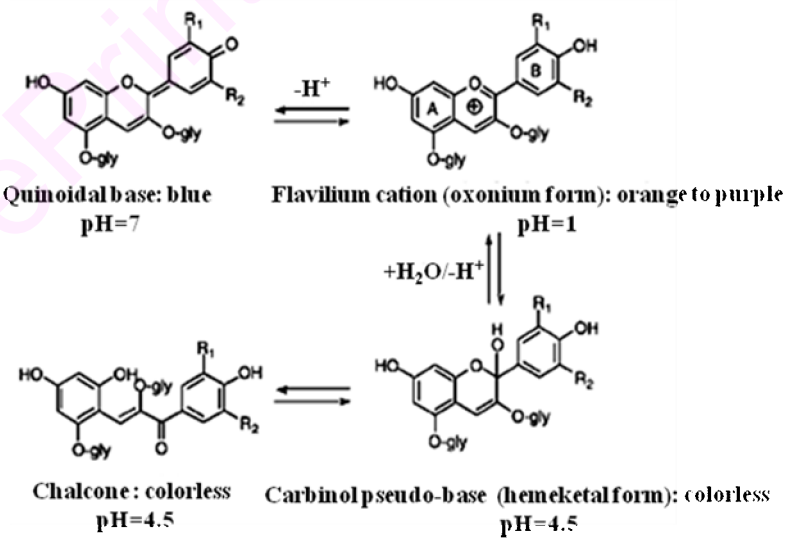



Fig. 5. Structural transformation of anthocyanins with change in pH

Anthocyanins are most stable at acidic pH (1-3) exhibiting red color with a reversible change to blue/violet at higher pH values (Cabrita *et al.,* 2000). At pH 1-3 the anthocyanin exists as a flavylium cation. With an increase in the pH, the kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to the acidic hydroxyl groups of the aglycone. The first reaction gives a colorless carbinol pseudobase, which can undergo ring opening to a chalcone pseudobase. The second reaction gives rise to quinoidal bases. At still higher pH values between 6 and 7, further deprotonation of the quinoidal bases occur with the formation of purplish, resonance-stabilised quinonoid anions (Fossen *et al.,* 1998).

**G.6.2.1.1.3 Miscellaneous factors affecting anthocyanin stability**

Inter-molecular copigmentation is one of the factors deciding the color intensity and stability of anthocyanins. Co-pigmentation with rose petal phenolics enhanced the color stability of strawberry anthocyanins (Mollov *et al.,* 2007). Chemical modification of anthocyanin rich extracts of black rice using various carboxylic acids has shown to increase the color and stability of the anthocyanins (Yawadio *et al.,* 2007). Black carrot anthocyanins are more stable due to the presence of acylating groups as compared to their orange counterparts (Cevallos-Casals and Cisneros-Zevallos, 2004). There have been contradictory reports on the stability of anthocyanins in the presence of ascorbic acid, the latter being known to increase degradation of anthocyanins in several model systems (Garcia-Vigeura and Bridle, 1999; de Rosso and Marcadante, 2007). However, Sarma *et al.,* (1997) observed that anthocyanins protect ascorbic acid against metal induced oxidation by forming ascorbic acid-metal-anthocyanin complexes thus conferring mutual protection against metal induced oxidation (Grommeck and Markakis, 1964). Presence of diglucosidic substitution confers higher stability to anthocyanins than monoglucosidic substitution (Markakis 1982; Mazza and Miniati, 1993). Acylation of the sugar residue on the aglycone significantly enhances the stability of anthocyanins (Giusti and Wrolstad, 1996). High sucrose content is found to enhance the stability of anthocyanins in solutions (Pi-Jen *et al.,* 2004) perhaps by increasing the water activity.

**G.6.2.2 Biological significance**

A substantial amount of literature points to the fact that anthocyanins posess positive health benefits owing to their antioxidant properties. They have been implicated in prevention and alleviation of a number of pathophysiological conditions. The “French paradox” –an inverse correlation between wine consumption in French population and cardiovascular diseases- is largely attributed to the presence of anthocyanins in the red wine. Anthocyanins are a part of normal diet which comes with the consumption of fruits and vegetables. Daily dietary intake

of anthocyanins is estimated to be 12.5mg/day per person in the United States (Wu *et al.,* 2006). However, depending upon the nutrition habits, it may go up to 200mg/day (Kuhnau *et al.,* 1976).

Anthocyanins are known to possess excellent antioxidant properties (Kong *et al.,* 2003). Anthocyanins from different sources have been reported to inhibit lipid peroxidation, platelet aggregation (Ghiselli *et al.,* 1998), possess anti-tumor, antimutagenic (Yoshimoto *et al.,* 2001), hepatoprotective (Obi *et al.,* 1998) and cardioprotective (Leifert and Abeywardena, 2008) properties. Anthocyanin extracts from chokeberry and bilberry at low doses have been found to inhibit ROS and at higher concentrations induced endothelium-dependent vasorelaxation in porcine arteries suggesting a beneficial effect in vascular disorders (Bell and Gochenaur, 2006). Administration of black currant and bilberry anthocyanins improves visual acquity and enhances night vision in animals and humans (Nakaishi *et al.,* 2000; Muth *et al.,* 2000) while black currant anthocyanins have also been shown to stimulate rhodopsin regeneration (Matsumoto *et al.,* 2003) in the retina. Though some researchers report the degradation of anthocyanins in vivo, anthocyanins have been found to be absorbed unmodified from the diet (Cao *et al.,* 2001; Nielsen *et al.,* 2003). Anthocyanins are capable of forming co-pigments with DNA thus offering mutual protection against hydroxyl radicals generated by Fenton reaction (Sarma and Sharma 1999). This can be a positive attribute in increasing cell survival under oxidative stress and may have physiological implications in preserving cellular functions and thus preventing its damage.

Several mechanisms of actions have been proposed for anthocyanins including their free radical scavenging activity, metal ion chelation, inhibition of lipid peroxidation, DNA copigmentation etc. In vitro and in vivo trials have demonstrated the potential of anthocyanins to prevent cancer cell proliferation and inhibit tumor formation (Hou, 2003; Kang *et al.,* 2003; Koide *et al.,* 1997; Meiers *et al.,* 2001). Hou *et al.,* (2004) revealed that anthocyanins inhibit tumorigenesis by blocking activation of a mitogen-activated protein kinase pathway. This report provided the first indication of a molecular basis for why anthocyanins display anticarcinogenic properties. In other research, fruit extracts with significant anthocyanin concentrations proved to be effective against various stages of carcinogenesis (Smith *et al.,* 2000; Kang *et al.,* 2003; Bomser *et al.,* 1996; Kandil *et al.,* 2000), but the individual role of anthocyanins versus other components was not determined.

**G.7 Endogenous antioxidants**

Despite the continued onslaught of human body by the reactive oxygen and nitrogen species, the body continues to strike a balance between the oxidative stress and the antioxidant

defenses. All aerobic forms are equipped with an armory of endogenous and exogenous antioxidants which fight against the oxidative insult.

Endogenous antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase comprise a principal system built in all aerobic organisms to defend oxidative stress. It is reported that increased activity of these antioxidant enzymes play a key role in increasing the longevity in humans (Mecocci *et al.,* 2000). The antioxidant enzymes efficiently counteract the reactive species generated and aid in maintaining the intracellular redox homeostasis. Apart from the antioxidant enzymes, there are other endogenous molecules such as glutathione and selenium which are important in the normal functioning of antioxidant enzymes (Nakane *et al.,* 1998). Metal binding proteins such as lactoferrin, transferrin (iron-binding proteins) and ceruloplasmin (Cu-binding protein) bind to transition metal ions that otherwise participate in the formation of reactive species (Halliwell and Gutteridge, 1999) thus playing an important role in the amelioration of pathological states (Levy and Viljoen, 1995; Floris *et al.,* 2000; Halliwell *et al.,* 1988) especially in conditions associated with inflammation.

### G.7.1. Antioxidant enzymes

The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are primary antioxidant enzymes that protect cells from damage caused by ROS.

#### G.7.1.1 Superoxide dismutase (SOD)

Superoxide dismutase is the primary defense against damage caused by $O_2^{\bullet-}$ and its reactive progeny. Superoxide radicals generated by the respiratory chain through the reduction of molecular oxygen are highly damaging to the cells. Superoxide radical is also formed by the univalent reduction of oxygen during various enzymatic reactions (Fee *et al.,* 1975) and leukocyte phagocytosis. However, within the cell, superoxide radicals produced are physiologically inactivated by the mitochondrial superoxide dismutase to molecular oxygen and $H_2O_2$ which is further inactivated by the $H_2O_2$–inactivating enzymes (Fridovich, 1999; Mates, 2000).

Three forms of SODs have been identified and characterized in humans: cytosolic Cu-Zn SOD, mitochondrial Mn SOD and extracellular EC SOD.

Cu-Zn SOD is the major intracellular SOD and is a dimeric protein (32kDa) with two identical subunits. Each subunit contains a Zn atom and a Cu atom. Zinc atom helps stabilize the protein while Cu is responsible for the activity of the enzyme that is, conversion of two

superoxide molecules into $H_2O_2$ and $H_2O$ (Rotilio *et al.,* 1972) displaying a diffusion limited pseudo first order reaction kinetics ($k= 3x10^9\ M^{-1}\ s^{-1}$). It is present in the cytoplasm and nucleus where it mainly functions as a superoxide scavenger (Crapo *et al.,* 1992). The loss or dysfunction of either Cu-Zn SOD or Mn SOD has been associated with ROS-mediated pathologies. For example, mutated Cu-Zn SOD proteins have been linked to instances of amyotrophic lateral sclerosis (Cleaveland and Rothstein, 2001) while loss of Mn SOD has been associated with neonatal death. Postnatal exposure to ambient oxygen results in increased susceptibility to mitochondrial oxidative injury of neurons of the central nervous system, cardiac myocytes and other metabolically active sites in Mn SOD deficient mice (Lebovitz *et al.,* 1996). Lack of Cu-Zn SOD in knock out mice renders them susceptible to development of liver tumors (Elchuri *et al.,* 2005). A recent study identified Cu-Zn SOD as the chief regulator of growth factor signaling, especially in endothelial and tumor cells (Jaurez *et al.,* 2008) their growth. This makes it a potential therapeutic target for the inhibition of angiogenesis and tumor growth. Liochev and Fridovich (1991; 1994) proposed that an excess SOD should decrease the steady-state level of $O_2^{\bullet-}$ with no subsequent increase in the endogenous $H_2O_2$ formation. This was supported by Teixeira *et al.,* (1998), where cell variants over-expressing Cu-Zn SOD were shown to exhibit low steady state levels of $H_2O_2$, even under conditions of oxidative stress. They demonstrated that the reduction in steady state levels of $H_2O_2$ is in fact, a direct effect of reduction of $O_2^{\bullet-}$ due to the higher cytosolic activity of the enzyme aconitase. Several natural products cause the induction of antioxidant enzymes in higher animals. Modest induction of SOD has been found to be associated with reduction in the age-related increase of lipid peroxidation products in human plasma (Nelson *et al.,* 2006). Transgenic over-expression of SOD and CAT in *Drosophila melanogaster* significantly increased its life span (Repine 1991). Over-expression of SOD in mammals has been found to be linked with increase in longevity (Ku *et al.,* 1993). Nelson *et al.,* (2006) propose a fundamentally different approach of safely and modestly inducing the antioxidant enzymes *in vivo* by administration of antioxidant supplements in moderate doses. This may prove to be a powerful tool in studying oxidative stress and diseases associated therein.

**G.7.1.2 Glutathione peroxidase (GPx)**

Glutathione peroxidases (GPxes) are a group of selenocysteine containing proteins playing a central role in the reduction of $H_2O_2$ and a wide range of organic hydroperoxides to water and their corresponding alcohols respectively (Mills, 1957; Flohe and Gunzler 1985) and acts as peroxynitrite reductase (Sies *et al.,* 1997). The catalytic reactions effected by GPx are shown below:

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} 2\,H_2O + GSSG$$

$$ROOH + 2\,GSH \xrightarrow{GPx} ROH + H_2O + GSSG$$

The glutathione is regenerated from the GSSG by the enzyme glutathione reductase. The catalytic site in GPx is identified to be the selenocysteine moiety (Forstrom *et al.,* 1978). Depending on their cellular location and substrate specificities, four isozymes of GPxes are recognized in higher organisms viz., cellular (GPx1), gastrointestinal (GPx2), extracellular or plasma (GPx3) and phospholipid (GPx4) glutathione peroxide. Among the four, GPx1 is the most abundant. Mills (1957), for the first time showed the presence and action of GPx in erythrocytes, where it was found to inhibit the oxidative breakdown of hemoglobin in the presence of $H_2O_2$. Ever since then this family of enzymes has drawn attention of many researchers pertaining to its importance in health and diseases. Gpx activity is primarily dependent on selenium (Se). Selenium deficiency is directly associated with decreased enzyme activity and GPx protein (Takahashi *et al.,* 1986) as well as its cytoplasmic mRNA abundance (Moriarty *et al.,* 1998). The proof for its protective effects against oxidative stress is innumerable. The knock-out mice which lack GPx1 are sensitive to oxidative stress by xenobiotics such as diquat, paraquat, $H_2O_2$ etc and eventually die of excess oxidative damage (Fu *et al.,* 1999; Cheng *et al.,* 1998; de Haan *et al.,* 1998). The activity of GPx1 is regulated by a signaling pathway that is activated in response to oxidative stress. The c-Abl and Arg SH3 domains of the respective tyrosine kinases bind to the proline-rich sites on GPx1, thus stimulating its activity and this association is controlled by the cell's oxidant level (Cao *et al.,* 2003). GPx is particularly important in those cells, whose mitochondria lack the enzyme catalase, where it does most of the detoxification of generated $H_2O_2$ (Bai and Cederbaum, 2001). The protective role of GPx1 has been unequivocally established in various models (particularly in vivo models) such as ischemia/reperfusion injury (Yoshida *et al.,* 1997; Crack *et al.,* 2001), virus-induced myocarditis (Beck *et al.,* 1998), endotoxemia (Jaeschke *et al.,* 1999), diabetes-associated atherosclerosis (Lewis *et al.,* 2007) and pro-oxidant induced neurotoxicity (Klivenyi *et al.,* 2000). Sies *et al.,* (1997) demonstrated its GSH dependent reduction of peroxynitrite to nitrite which attenuates the peroxynitrite mediated protein nitration in human fibroblast lysates. This might have physiological relevance in inhibiting the peroxynitrite induced apoptosis in cells. They later demonstrated that in vivo, GPx acts differently by actually accentuating peroxynitrite-induced apoptosis which is perhaps due to the ineffective enzymatic reduction of peroxynitrite in culture where it encounters more reactive peroxynitrite intermediates such as $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (Fu *et al.,* 2001). Therefore, it appears that the activity of the enzyme mainly depends on the type of oxidant and hence the

judicial supplementation of antioxidant must be considered, keeping in mind the implications of such compounds under different stress conditions.

**G.7.1.3 Catalase**

The whole process of free radical generation and their decomposition is a concerted action involving a battery of detoxifying proteins named antioxidant enzymes. The superoxide that is formed during the respiratory chain is converted to $H_2O_2$ by superoxide dismutase. The $H_2O_2$, if not removed interacts with transition metal ions such as $Fe^{2+}$ (Fenton reaction) producing highly reactive hydroxyl radical. This is effectively avoided by the action of catalase which decomposes $H_2O_2$ to molecular oxygen and water in tandem with GPx. Catalase can decompose $H_2O_2$ catalysed by two different modes of enzymatic activity: the catalytic mode of activity (reaction 1) and the peroxidative mode of activity (reaction 2) (Keilin and Hartree 1945; Oshino *et al.,* 1973).

Catalase is a homotetrameric heme containing protein (Chance *et al.,* 1979). The role of CAT in defending cells and tissues against oxidative stress has been studied extensively. Overexpression of CAT renders cells more resistant to toxicity of $H_2O_2$ and oxidant-induced injury from exposure to hyperoxia (Erzurum *et al.,* 1993; Yoo *et al.,* 1994). Most cells are lacking in their mitochondrial CAT (Bai and Cederbaum 2001). In such cells, mitochondrial superoxide anion and hydrogen peroxide are dismutated by SOD and GPx and the significant amount of $H_2O_2$ that diffuses into the cytosol, despite the action of SOD and Gpx in mitochondria, is detoxified by the cytosolic CAT. Since mitochondria are the main sources of reactive oxygen species, a consequence of oxidative phosphorylation, they are highly susceptible to oxidative damage. Mitochondrial overexpression of CAT has been found to provide better protection than cytosolic overexpression against $H_2O_2$ induced lung injury (Arita *et al.,* 2005). It is also shown to increase the median life span in experimental animals associated with delayed onset of cardiac lesions and cataract development and reduction in oxidative damage, $H_2O_2$ production, $H_2O_2$-induced aconitase inactivation and mitochondrial DNA lesions (Schriner et al., 2005). Catalase knock out mice show normal and healthy signs of development but show varying degrees of sensitivity to oxidative damage depending on the tissue and oxidant type (Ho et al., 2004). Aging is characterized by progressive decline in physiological functioning of multiple organ systems and a steady increase in chronic degenerative diseases. Overexpression of mitochondrial CAT in aging mice is associated with

reduction in a number of age-related pathologies such as malignant non-hematopoietic tumor burden, reduced cardiac lesions and reduced systemic inflammation. However, it had no effect on hematopoietic neoplasia or glomerulopathy (Treuting et al., 2008). The results support the oxidative theory of ageing and its delay by manipulation of the antioxidant environment of the organism (Harman, 2006). Therefore, antioxidant intervention may be a viable target for the prevention or delay of some, if not all, age-related diseases.

**G. 7.2 Glutathione**

Glutathione is a small molecule that plays key regulatory roles in metabolic and cell-cycle-related functions (Poot *et al.,* 1995; Adamson *et al.,* 1996) and is responsible for detoxification of ROS by acting as peroxide scavengers and regulating the redox state of cells. This cysteine containing tripeptide (γ-glutamyl cysteinyl glycine), which is found in milli molar concentrations in animal cells, provides the principal intracellular defense against oxidative stress (Shan *et al.,* 1990) and participates in detoxification of xenobiotics (Thomas, 1993) aiding in the maintenance of intracellular redox status. Depletion of GSH is one of the chief causes of cell death. For example, overdose of acetaminophen (paracetamol) results in hepatic and renal failure and ultimately in death due to cellular GSH depletion (Thomas, 1993) indication that it is an essential component of the human immune response. Low intracellular glutathione levels in antigen-presenting cells correlate with defective processing of antigen with disulfide bonds, indicating that this thiol may be a critical factor in regulating productive antigen processing (Short *et al.,* 1996). Externally supplemented GSH has negligible systemic bioavailability. Therefore synthesis of GSH in conditions of its deficiency is enhanced by means of its precursors such as cystine. Natural products have been suggested to enhance in vivo GSH synthesis in a number of cell models by increasing the level of γ-glutamyl-cyseinyl-synthetase, a rate limiting enzyme in GSH synthesis (Rimbach *et al.,* 2001). Therefore, supplementing with antioxidant biomolecules that stimulate the synthesis of GSH in vivo may be a rational way of overcoming GSH deficiency.

**G.8 Measurement of antioxidant capacity**

The study of the free radicals and antioxidants in biology is creating a medical revolution that promises a new age of health and disease management. From prevention of the oxidative reactions in foods, pharmaceuticals and cosmetics to the role of ROS in chronic degenerative diseases and aging challenges continue to emerge from difficulties associated with methods used in evaluating antioxidants in vitro and in vivo. The accurate assessment of antioxidant action of biomolecules is far from real due to the accompanying set backs such as the specificity and selectivity of antioxidant towards free radicals and vice versa. However, measurement of antioxidant capacity of a particular compound is of utmost importance in

considering its potential in antioxidant therapy. A number of methods have been designed and developed over the past several decades to assay antioxidants.

### G.8.1 In vitro antioxidant assays

Several in vitro methods have been developed to measure the antioxidant property of foods and biological samples (Wayner *et al.,* 1985; Cao *et al.,* 1993; Schlesier *et al.,* 2002; Bohm and Schlesier, 2004; Prior *et al.,* 2005). However, there is no single standardized method which gives the best picture of the antioxidant property of a substance. Most of the methods described measure the inhibition of free radical generation or neutralisation of the generated free radicals by the antioxidant. The methods are chiefly based on technologies using different free radical generators, target molecules and endpoints. Therefore, a particular compound which shows high antioxidant activity in one assay does not necessarily show the same effect in the other. This may be attributed to the complex nature of the phytochemicals, redox potential of the compound and the assay medium, the type of free radical generated, polarity of the antioxidant and the nature of interaction between antioxidant and the reactive species. Therefore, a test compound is subjected to a battery of tests involving different conditions of medium and free radicals in order to testify its efficacy against different types of oxidative stress conditions.

The most common methods are mentioned here and the working principles of the methods used in the present study are discussed in the respective chapters.

The quenching of the stable free radical α, α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) has been the most common and the first test used to establish a compound's antioxidant property. Blois (1958) for the first time demonstrated the use of $DPPH^{\bullet}$ as a stable radical to assess free radical quenching property. However, it has the disadvantage that it cannot be correlated to conditions of physiological pH. It was observed that the antioxidants showed different $IC_{50}$ values when tested in methanol and buffered methanol (Sharma and Bhat, 2009) indicating its non-reliability under non-physiological pH. Recently a high throughput relative DPPH radical scavenging assay was developed which can be used for both hydrophilic and lipophilic compounds without the use of solubilising agents (Cheng *et al.,* 2006) thus making it a useful technique for diverse class of compounds.

Another assay that is most commonly used is based on the iron reducing capacity of the antioxidant, called the ferric reducing/antioxidant power (FRAP) assay, originally designed to test the ferric reducing capacity of plasma (Benzie *et al.,* 1996). This assay measures the ability of antioxidants to reduce $Fe^{3+}$ to $Fe^{2+}$ (Prior *et al.,* 2005). FRAP is the only assay that directly measures the antioxidants or reductants while most of the other assays measure the

inhibition of reactive species in the reaction mixture (Halvorsen *et al.,* 2002) which might be a function of type of reactive species.

Hydroxyl radical is a highly reactive free radical and is implicated in free radical mediated pathologies damaging almost every known biomolecule. It binds to nucleotides in DNA and causes strand breaks by abstraction of a hydrogen atom from deoxyribose molecule (Balasubramanian *et al.,* 1998) leading to carcinogenesis, mutagenesis and cytotoxicity. The ability of the hydroxyl radical to cause deoxyribose degradation is studied by generating these radicals through Fenton reaction between $H_2O_2$ and the ferrous salt in the presence of ascorbic acid (Walling *et al.,* 1975; Halliwell *et al.,* 1987). However, this method cannot be applied to those compounds which can directly react with $H_2O_2$ or are strong iron chelators such as tannic acid and phytic acid (Cheng *et al.,* 2003).

Lipid peroxidation induced by oxidants and oxidative stress generates an assortment of lipid oxidation products that may initiate a chain reaction propagating the production of more free radicals that damage biomolecules. The most abundant byproduct of lipid peroxidation is malondialdehyde (MDA) resulting from the peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acid (Esterbauer *et al.,* 1991). Level of MDA is a fairly sensitive marker of oxidative stress and the antioxidant status. At low pH and elevated temperature, MDA readily participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), 1:2 MDA:TBA adduct. These facts, along with the availability of facile and sensitive methods to quantify MDA (as the free aldehyde or its TBA derivative), have led to the routine use of MDA determination and, particularly, the "TBA test" to detect and quantify lipid peroxidation in a wide array of sample types (Janero, 1990). Therefore prevention of lipid peroxidation is an essential process in all aerobic organisms since lipid peroxidation products cause damage to DNA and other biological macromolecules.

Other more common methods are oxygen radical absorbing capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) etc. The antioxidant capacity is chiefly contributed by the population of phenolic compounds and there is a clear correlation between the phenolic content and antioxidant capacity as shown in other studies (Miliauskas *et al.,* 2004) and in our own study.

The in vitro chemical antioxidant assays, though provide significant information regarding the antioxidant property of phytochemicals, fail to demonstrate in vivo action. Most of the antioxidant methods used do not reflect the intracellular physiological conditions and therefore, do not consider the bioavailability, uptake and metabolism of the antioxidants. Since the biological systems are much more complex rather than just the mixtures of oxidant and antioxidant (as in the case of a chemical in vitro test), the antioxidant compound may

operate *via* multiple mechanisms involving a number of physiological factors such as enzymes and other biomolecules; therefore, the relevance of chemical in vitro methods to human body is questionable. Hence, the use of models that best mimic the cellular environment is advised in order to get a better picture of the protective effect of a particular compound in question.

### G.8.2 Use of cell culture models in antioxidant research

In agreement with the above argument, the mechanisms of action of antioxidants extend beyond the scavenging property in disease prevention and health promotion. Since animal models and human studies are more expensive, time consuming and involve ethical issues regarding their use, there is a need for cell culture models to support antioxidant research for the initial screening of foods, dietary supplements (Liu and Finley, 2005) and other pharmaceutically important molecules. Several short-term in vitro models have been used in cytoprotective and cytotoxic studies. However, despite the advantages of cellular models being rapid and inexpensive, majority of them have limitation of not bearing the enzyme machinery required for activation and/or detoxification of oxidants. Different approaches to overcome these problems and their advantages and limitations are summarised in **Table 2.** (Mersch-Sundermann *et al.,* 2004). In general, cells and cell lines capable of endogenously expressing xenobiotic metabolizing enzymes seem to be better than other cells to study the cytoprotective, antigenotoxic or cogenotoxic effect of xenobiotics.

The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, not surprisingly, cells of liver origin are widely used in biomedical research involving xenobiotic metabolism including genotoxicity and antioxidant studies. Table 2 outlines the advantages and disadvantages of different approaches to cell culture studies.

#### G.8.2.1 Primary rat hepatocytes

The liver is the major organ since it performs an astonishingly large number of tasks of regulating several functions of the body in higher animals and humans. The main functions are metabolism of carbohydrates, lipids, proteins, drugs and xenobiotics that are ingested by the body. Use of primary cultured hepatic cells is an established method to study mechanisms of hepatotoxicity and carcinogenesis, drug metabolism as well as in a number of biochemical investigations under defined conditions in vitro (Moldeus *et al.,* 1978; Williams *et al.,* 1982). The isolated rat hepatocytes have been shown to retain most of the characteristic features of intact tissue including the permeability characteristics (Moldeus *et al.,* 1978). However, the cytochrome P450 system, which is the chief drug metabolizing apparatus in the liver, rapidly declines in isolated rat hepatocytes in cultures (Wortelboer *et al.,* 1991; Richert *et al.,* 2002)

which make it unsuitable for long term studies on xenobiotics induced cytotoxicity. It was also reported that the antioxidant defense of the isolated rat hepatocytes declined with time (Richert *et al.,* 2002). However, the culture conditions have been developed with the supplementation of hormones and other nutrients which can retain the activity of cytochrome P450 for up to 72 hrs (Grant *et al.,* 1985). Therefore, in the present study freshly isolated rat hepatocytes were used for the study of stress induced cytotoxicity and its amelioration by antioxidants such as anthocyanins and carotenoids. McQueen and Williams (1987) suggested that the ability of the hepatocytes to fight the xenobiotics stress varies from species to species thus suggesting the use of multi-species testing which permits a more complete assessment of genotoxicity.

Table 2. Advantages and disadvantages of different approaches used in cytotoxicity/protective studies.

| **Method** | **Advantages** | **Limitations** |
|---|---|---|
| Primary cells | • Retains native metabolic competence with all xenobiotics metabolizing enzymes.<br>• Can be differentiated under controlled culture conditions<br>• Unmodified from the parent tissue except for enzymatic or physical dissociation<br>• Represent the variability by polymorphism | • Undergo only limited number of cell divisions<br>• Limited availability of tissue resections<br>• Difficult to achieve a single cell type in culture |
| Genetically engineered cells | • Highly useful for mechanistic studies<br>• Availability of isogeni control cells without the introduced cDNA and therefore enzyme specific activation can be analysed | • Not appropriated to test unknown compounds<br>• The use of cell lines containing single transgenic enzymes gives a false picture of metabolism of a compound under in vivo conditions |
| Cell lines with endogenous enzyme activity | • Could be propagated for several generations<br>• Easy to maintain | • Limited array of promutagen activating enzymes<br>• Enzyme expression shows strong inter-species differences |

### G.8.2.2 Use of transformed cells

Due to the instabilities of the antioxidant status and cytochrome P450 oxidase system in primary cultured hepatocytes, it is advisable to use a system that is more stable, mimics most of the human *in vivo* conditions and gives reproducible results. The present study utilizes hepatoma cells to assess the cytoprotective and cytotoxic effects of anthocyanins and carotenoids. Use of human liver in bioavailabity, biotransformation and toxicity studies is limited due to issues related to ethical concerns. There are very few studies in literature that made use of human livers (Dai and Cederbaum, 1995; Nicod *et al.,* 1997; Holownia *et al.,* 1997). Under such situations cell lines offer an attractive possibility of working with cells of human origin. Cell lines are gaining considerable impetus especially in the field of pharmaco-toxicology for pre-validaiton and validation studies (Batt *et al.,* 1995) of bioactive molecules. However, these cell lines cannot be used for biotransformation and metabolic studies because their pattern of gene expression of drug-metabolising enzymes is distinct from in vivo conditions (Wilkening *et al.,* 2003). Though a number of human derived cell lines have been developed, most genotoxic and other studies mainly make use of HepG2 and Hep3B cells because they most closely retain the activities and substrate specificities of enzymes typical for humans and secrete most of the plasma proteins (Knasmuller *et al.,* 2004). HepG2, originally isolated by Aden *et al.,* (1979) from a primary hepatoblastoma of an 11-year-old Argentine boy, is the most versatile one. This cell line has been identified to be an ideal cell model to study, genotoxicity, antigenotoxicity, mutagenecity and cytotoxicity of chemical compounds (Knasmuller *et al.,* 1998; Mersch-Sundermann *et al.,* 2003) because of the increased sensitivity of enzymes in HepG2 towards genotoxicants (Knasmuller *et al.,* 2004). Hep3B is a hepatocarcinoma cell line derived from 8 year old juvenile Black male and contains an integrated surface antigen for Hepatitis B virus (Knowles *et al.,* 1980). These cell lines retain many of the specialized functions normally lost by primary hepatocytes in culture such as secretion of the major plasma proteins (Knowles and Aden, 1983). A study on the relative sensitivities of HepG2 and Hep3B towards dietary and life-style related DNA carcinogens revealed that HepG2 was more sensitive compared to the Hep3B cells (Majer *et al.,* 2004). A careful observation of the data obtained in their study showed that though Hep3B cells differ substantially from HepG2 cells in terms of their sensitivity towards genotoxicants tested, they are not completely insensitive, at least towards most of the agents used. It was found insensitive only to $As_2O_3$. Hep3B was more sensitive towards drug metabolizing enzymes such as CYP1A1 and NAT1 compared to HepG2; while it was less sensitive to most of the enzymes, other cytochrome P450 enzymes such as CYP1A2, 2B1 and 2E1 and NAT2 were not detected in both (Majer *et al.,* 2004). Apparently, the works of Majer

*et al.,* (2004) mainly focus on the response of cells to genotoxicity. A compound can render toxicity to cells by mechanisms independent of DNA damage involving various other mechanisms. In such cases their theory cannot be completely relied upon. Moreover, the number of micronuclei in control (untreated cells) was always higher in HepG2 cells than that in Hep3B cells (Majer *et al.,* 2004) suggesting a more close resemblance of Hep3B to normal human cells where the number of micronuclei in healthy cells are presumably lesser. This assumption is supported by Manov *et al.,* (2002) who recommend Hep3B cell line over HepG2, owing to their preservation of ultrastructural characters during different stages of the experiment. Therefore, it is suggested that though Hep3B does not completely resemble HepG2 cells in cellular functions, it can still be used as a working model for testing cytotoxic and cytoprotective phytochemicals with significant response. Apparently these two cell lines can be used for cytoprotective as well as anticancer studies since they more closely resemble the normal human hepatocytes in most ways while retaining the property to multiply under the culture conditions.

A thorough survey of recent literature revealed that a Hep3B is used as a cell model in number of studies involving phytochemicals for their cytoprotective/cytotoxic activity and also in metabolic and gene expression studies. **Table 3** summarises few of the studies carried out using this cell line.

Table 3. Examples for use of Hep3B as cell culture model

| **Putative protective compound** | **End point mediator** | **End point(s)** | **Reference** |
|---|---|---|---|
| N-acetyl cysteine | Acetaminophen | Apoptosis | Manov *et al.,* 2002 |
| Anthocyanidins | -- | Apoptosis | Yeh and Yen, 2005 |
| Resveratrol/quercetin | Interleukins (inflammatory cytokines) | C-reactive protein expression | Kaur *et al.,* 2007 |
| Lycopene | -- | Cell growth inhibition | Park *et al.,* 2005 |
| γ-tocotrienol | -- | Apoptosis | Sakai *et al.,* 2006 |
| Trichostatin A | Nickel | Cytotoxicity/DNA damage | Kang *et al.,* 2005 |
| None | Cadmium | Apoptosis | Lemarie *et al.,* 2004 |
| Andrographolide | -- | Cell growth inhibition and/or apoptosis | Ji *et al.,* 2007 |
| Carboxyfullerin | TGF-β | Inhibition of apoptosis | Huang *et al.,* 1998a |
| Ascorbic acid | Iron | Ferritin translation | Toth *et al.,* 1995. |

Taking these facts into consideration, the present thesis employed the use of Hep3B cells to study the cytoprotective action of anthocyanins and carotenoids against *tert*-butylhydroperoxide-induced oxidative stress. The initial study was designed with HepG2 cells considering its advantages over Hep3B; however, the failure to obtain a monolayer of cells under our laboratory conditions made the choice of Hep3B over HepG2 inevitable.

### G.9 Induction of oxidative stress and choice of oxidative model

The body is constantly exposed to a plethora of chemicals which create a oxidative stress environment within the cell. Though the body has its own mechanism to cope with these harmful reactive species, an excess generation of the same can result in oxdative damage and ensuing pathologies. Many chemicals are used in vitro to simulate an in vivo toxicity conditions. For example doxorubicin in cardiac toxicity (Choi *et al.,* 2007), carbon tetrachloride and other haloalkanes (Yu *et al.,* 2002), chloroform and azathioprine in hepatotoxicity (Lee and Farrell, 2001), hydrogen peroxide (Alia *et al.,* 2005) and tert-butyl hydroperoxide in toxicities of various cells including hepatic cells (Garcia-Cohen *et al.,* 2000; Alia *et al.,* 2006b).

Liver is body's major organ where most of the drug metabolism and biotransformation occurs. Ironically, the metabolic products of detoxification reactions that protect other tissues from effects of the primary toxicant can be destructive to the liver when in excess or chronically present. Due to this reason, it is highly susceptible to chronic exposure to drugs, environmental toxicants and other xenobiotics. Therefore, the major consequence of ingestion of xenobiotics through food, water and air is an indirect effect on virtually all other organs. Therefore the present study utilized two well-known stress inducing agents such as carbon tetrachloride in primary rat hepatocytes and *tert*-butyl hydroperoxide in primary rat hepatocytes and hepatocarcinoma cells.

#### G.9.1 Carbon tetrachloride

Carbon tetrachloride, a by-product of sewage and drinking water chlorination, is one of the major hepatotoxins which is metabolized in the liver by the CYP2E1 isozyme of cytochrome P450 by reductive dehalogenations (Johansson and Ingelman-Sundberg, 1985; Noguchi *et al.,* 1982) resulting in the reactive trichloromethyl radical ($CCl_3^{\bullet}$) and subsequently resulting in an even more reactive form, the trichloromethyl-peroxyl ($^{\bullet}OOCCl_3$) radical (Ruch *et al.,*., 1986). The reactions occurring during the metabolism of $CCl_4$ are shown below:

$$CCl_4 + e^- \longrightarrow {}^{\bullet}CCl_3 + Cl^- \qquad (1)$$

$${}^{\bullet}CCl_3 + O_2 \longrightarrow OOCCl_3 \qquad (2)$$

The lipid radical reacts with molecular oxygen the products of which fuel a series of reactions ultimately resulting in the initiation of membrane lipid peroxidation and a consequent liver injury (Slater, 1984). Therefore, carbon tetrachloride forms an important tool in elucidating the mechanisms of action of hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death and carcinogenicity (Weber *et al.,* 2003). Similarly it is employed as an important model to study the effect of a number of hepatoprotective agents in conditions of toxicity (Sen *et al.,* 2007; Parola *et al.,* 1992; Fariss *et al.,* 1993).

Administration of carbon tetrachloride ($CCl_4$) to rodents and cultured cells is a widely used model to study mechanisms of hepatic injury. It causes hepatocyte injury that is characterized by centrilobular necrosis that is followed by hepatic fibrosis in intact livers. Johnston and Kroening (1998) suggest an early death of cultured rat hepatocytes which may be due to a direct effect of $CCl_4$ on their intracellular membranes independent of its metabolism. Compounds derived from natural sources have been proved to be effective against $CCl_4$ toxicity in a number of cell and animal models. Anthocyanins of *Hibiscus rosasinensis* significantly protected the rat liver from $CCl_4$–induced injury as evidenced by the liver marker enzymes (Obi *et al.,* 1998). A protein isolate from the *Phyllanthus nirurii*, an indigenous, plant with hepatoprotective properties is shown to reverse $CCl_4$-induced alterations in liver toxicity marker enzymes as well as antioxidant enzymes catalase and superoxide dismutase (Bhattacharjee and Sil, 2007). Carotenoids, the lipid soluble pigments abundant in plants show excellent antioxidant activities in vitro which may be manifested in its protective role against oxidative stress induced organ damage and other pathologies. Kang *et al.,* (2001) reported the protective effect of astaxanhin against $CCl_4$-mediated liver toxicity in rats through reduction in lipid peroxidation and reversing the alteration in antioxidant status. It is apparent from all these studies that these natural compounds mainly act through the antioxidant enzyme system which perhaps involves the control by the cytochrome P450 mixed function oxidases. For these reasons, $CCl_4$ is often used in experiments associated with toxicity of the liver and its amelioration by hepatoprotective agents.

### G.9.2 *Tert*-butyl hydroperoxide (TBH)

Measurement of antioxidant activity by phytochemicals is an important means of establishing its therapeutic potential. It is also crucial that a particular antioxidant in question be effective against a diverse class of free radicals and free radical inducing agents. After ascertaining these in vitro, using chemical tests, it is imperative to use isolated cell models under physiological conditions to check their efficacy in the presence of a number of cellular factors. Therefore two different free radical generators were used to induce oxidative stress in

isolated rat hepatocytes to prove that the antioxidants tested are effective against diverse classes of free radicals.

Most short-term cell culture studies use $H_2O_2$ and TBH to induce pharmacological oxidative stress status in cells (Goya *et al.,* 2007; Baldwin and Barrett, 1998; Cuello *et al.,* 2007).

*Tert*-butyl hydroperoxide is an organic hydroperoxide which decomposes to other alkoxyl and peroxyl radicals in a reaction aided by metal ions that can generate ROS including $H_2O_2$. It is a precursor in the formation of malondialdehyde, a lipid peroxidation product and therefore, decomposition of TBH accelerates lipid peroxidation chain reactions, induces cell toxicity by damage to DNA, depletes cellular GSH and protein thiols resulting in the alteration of intracellular calcium homeostasis, cell damage and apoptosis (Lötscher *et al.,* 1979; Sandstrom, 1991; Ochi, 1988; Ochi, 1990).

In the present study TBH was chosen over $H_2O_2$ to induce stress due to the findings of several researchers that $H_2O_2$ *per se* does not evoke a significant stress response in cells (Alia *et al.,* 2005) as compared to organic hydroperoxides. Alia *et al.,* (2005) propose that this differential response might be due to the fact that the cell has a very effective mechanism to detoxify hydrogen peroxide through the action of catalase, since this is a product naturally occuring in the cell. In contrast, the organic hydroperoxide such as TBH does not have a specific detoxifying element within the cell, and requires endogenous thiol molecule glutathione for detoxification (Alia *et al.,* 2005). Therefore, the present thesis utilizes TBH as another free radical generator in order to study the cell's response to oxidative stress chiefly in terms of antioxidant enzymes and their modulation in the presence of antioxidants.

**G.10 Measurement of cytotoxicity in cell culture**

Determination of cell viability is an important parameter in studying the biological safety of a compound and also to study the protective effect of the same under toxic states of cells. Several cytotoxicity screening methods are commonly used which include MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Cell Titre Blue (CTB) assay, Neutral red uptake assay, methylene blue uptake assay, lactate dehydrogenase (LDH) leakage assay etc.

MTT is a yellow water soluble tetrazolium dye that is cleaved by the mitochondrial dehydrogenases to purple water insoluble formazan crystals which can be solubilised with dimethyl sulfoxide (Mossman, 1983; Edmondson *et al.,* 1988). The amount of formazan formed is a direct measure of viable cells. This method was used in the present study due to its reproducibility and ease with working.

CTB assay is based on the ability of cells to reduce the non-flrourescent resazurin in to fluorescent resorufin, by the mitochondrial and cytosolic enzymes allowing the detection of cell growth and viability by spectrofluorimetry (O'Brein *et al.,* 2000). Increased fluorescence gives an account of viable cells. The enzymes that are thought to participate in CTB or MTT reduction include alcohol and aledhyde oxidoreductases, NADPH quinine reductase, flavin reductase, NADH dehydrogenase and cytochromes (O'Brein *et al.,* 2000).

Neutral red uptake assay is a simple colorimetric assay which makes use of the ability of viable cells to incorporate and bind the suprovital dye in the lysosomes (Zhang *et al.,* 1990) which is then extracted in a suitable solvent and absorbance read using a spectrophotometer.

Another method makes use of the inability of the dead cells to take up certain dyes such as trypan blue, eosin or propidium (Strober, 199). The cells treated with the dye are observed under the microscope and the number of cells accumulating the dye is visually scored. This is a tedious and time consuming method compared to the other tests. All the above methods require the cell membranes to be intact and the cells metabolically active, to be able to take up or exclude the dye.

Cell toxicity in cell culture can also be evaluated by the leakage of intracellular enzyme lactate dehydrogenase (LDH) into the culture medium. LDH is a cytoplasmic enzyme that is released into the medium upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity. Recent studies suggest that LDH is a more reliable and accrate marker of cytotoxicity since damaged cells lose their integrity resulting in the leakage of cell content (Grivell *et al.,* 1996). The assay is dependent on the oxidation of NADH which is marked by the disappearance of absorbance at 340nm, due to the conversion of pyruvate to lactate by NADH (Howell et al, 1979). The assay also makes use of the oxidation of lactate to pyruvate which then reacts with the tetrazolium salt INT to form the water soluble dye which is measured by colorimetry (Decker and LohmannMatthes, 1988). However, this method was not used in the present study.

**G. 11 Measurement of apoptosis**

Apoptosis is a highly regulated process involving a battery of signaling events including the caspases, apoptotic family of proteins and other transcription factors. Oxidative stress, leading to the formation of free radicals is often implicated in ensuing cell death, either apoptotic or necrotic. Necrosis is caused by catastrophic toxic and traumatic events with passive cell swelling, injury to cytoplasmic organelles, rapid collapse of internal homeostasis leading to membrane lysis, release of cellular contents and ultimatelyresilting in inflammation (Kerr and Harmon, 1991; Swartz *et al.,* 1993). On the contrary, apoptosis is a programmed cell death

which is characterized by cell shrinkage, membrane blebbing, nuclear fragmentation and chromatin condensation (Kerr *et al.,* 1972; Bursch *et al.,* 1992). Events of cell death within a cell can be monitored by microscopic fluorescence imaging using specific fluorochromes such as propidium iodide (PI), acridine orange, ethidium bromide, 4′6-diamidino-2-phenylindole (DAPI), bisbenzimide, annexin V etc., either alone or in combination. While the first three dyes specifically bind to nucleic acids (Zamai *et al.,* 2001; Collins *et al.,* 1997; Willingham, 1999) annexin V is specific to phosphotidyl serine, the molecules that become exposed to the outer plasma membrane leaflet due to the loss of its asymmetry, a hall mark of early apoptosis (van Engeland *et al.,* 1998). The fluorescent dye PI is one of the most commonly used ones and can be used to detect apoptosis in adherent cells (Zamai *et al.,* 2001).

Estimation of caspase activation is another method of detection of apoptosis. Caspases are a family of cysteinyl proteases considered to be executive factors for apoptsis. There are two types of caspases, the initiator caspases (caspase 8 and 9) and the effector caspases (caspase 3, 6). It has been believed that activation of caspase is induced through two pathways. In the first pathway, binding of cytochrome c released from mitochondria to Apf-1 participates in the activation of caspase-9. In the second pathway, binding of death ligand to death receptors such as Fas and TNF receptor participates in the activation of caspase-8. Caspase-8 and caspase-9 activate caspase-3 (Strasser *et al.,* 2000; Zou *et al.,* 1999) and the activated caspase-3 celaves poly (ADP-ribose) polymerase (PARP) (Boulares *et al.,* 1999) which is used as an indicator of apoptosis. The final outcome of these proteolytic cascades is the specific cleavage of a wide variety of substrates that are implicated in apoptosis.

On the other hand, the Bcl-2 family of proteins exerts antiapoptotic or proapoptotic activity through altering the mitochondrial membrane permeability (Tsujimoto and Shimizu, 2000). The proto oncogene Bcl-2, first recogni zed at the chromosomal breakpoint of (14:18)-bearing human B-cell lymphomas, is one such gene. The product Bcl-2 protein of this gene has been localized to mitochondria, endoplasmic reticulum, and the membranes of the nuclear envelope (Tyurina *et al.,*. 1997). Bcl-2 is known to regulate mitochondrial membrane potential and be tightly associated with the mitochondrial pore transition, pore regulation, the release of cytochrome c and other apoptosis inducing factors (Shimizu *et al.,* 1999). Bax is a pro-apoptotic protein of the Bcl-2 protein family that resides in the outer mitochondrial membrane. It is controversial whether Bax promotes cell death directly through its putative function as a channel protein or incdirectly by inhibiting cellular regulators of the the cell death proteases-caspases. Recent reports have indicated that after exposure to certain apoptotic stimuli Bax is translocated from the cytoplasm to the mitochondrial membrane and

that this process is essential for cell death to occur and found the same results in post-mortem dopaminergic neurons of Parkinson disease (Hartmann *et al.,*., 2001). Antiapoptotic factors such as Bcl-2 inhibit cell death by stabilizing the mitochondrial membrane, while pro-apoptotic ones such as Bax and Bid induce cell death by increasing the mitochondrial membrane permeability leading to the release of cytochrome C (Shimizu *et al.,* 1999; Saito *et al.,* 2000).

An estimation of activity and content of Bax and Bcl-2 gives an indication of the apoptotic potential of the compounds. It is generally measured by estimating the ratio of Bax to Bcl-2. A higher ratio suggests proapoptotic nature while the reverse signifies antiapoptitic property. The oxidative stress inducing agent may cause cell death either by apoptosis or necrosis. Tert-butyl hydroperoxide used in this study is known to cause cell death by apoptosis in cells through mitochondrial pathway related to upregulation of Bax and Bid, downregulation of Bcl-2, caspace activation, enhanced mitochondrial membrane potential, DNA fragmentation and increased release of cytochrome C (Prasad *et al.,* 2007; Piret *et al.,* 2004). Various investigations have shown that a number of phytochemicals with antioxidant properties also possess cytotoxic effect (Gopalakrishnan and Kong, 2008; Leung *et al.,* 2007), especially in carcinoma cells, which is effected via apoptotc pathways involving Bcl-2 family of proteins (Cheng *et al.,* 2007). In the present thesis, the ability of anthocyanins and carotenoids to upregulate the Bcl-2/Bax mRNA ratio was elucidated in order to establish their antiapoptotic property in the presence of oxidative stress.

While apoptotic cell death mediated by antioxidants is desirable in cancer chemotherapy, necrosis is considered to lead to pathologic states. Similarly antioxidant can interfere at different points of signaling and abrogate the oxidative stress induced cell death. Therefore, cumulative information on the morphological changes, activation of caspases and expression of apoptotic proteins gives a broad picture of the state of the cells under oxidative stress status and its amelioration by antioxidants.

**G. 12 Measurement of antioxidant enzymes activity and their gene expression**

As discussed in the earlier sections, ROS are continuously generated in the body as a result of metabolic reactions as well due to exposure to certain chemicals. While an optimum amount of ROS is required to maintain a normal redox balance, an excess can be harmful to the body. The human cell is generally well equipped to cope with the normally encountered levels of reactive species with its antioxidant enzymes and glutathione redox system. There is growing evidence that presence of small, continuous stimulus such as ROS actually induces the expression of these enzymes as a defense mechanism (Vina *et al.,* 2006). Exposure of human endothelial cells to hyperoxia increases the mRNA levels of CAT, GPx and SOD (Jornot and

Junod, 1992). Therefore, the measurement of changes in endogenous antioxidant enzyme activity and gene expression is considered a fairly sensitive biomarker of the cellular oxidative stress.

A large number of genes have evolved in eukaryotes with the rationale of providing protection to cells from the toxic effects of xenobiotics and other toxic chemicals. In oxidative stress conditions, certain antioxidant enzymes and their genes are regulated and the type and extent of such response depends upon the type of toxicity. Therefore it is imperative to understand the roles of these genes in determining the cellular responses to oxidative stress. A number of biochemical models have been developed over a period of several decades including the development of enzyme knock-out (or null) models (Ho 2002; de Haan *et al.,* 2003), which play a pivotal role in establishing the role of oxidants and antioxidants and other drug molecules in maintenance of health and alleviation of disease states.

The changes in antioxidant enzyme activity may arise from transcriptional and/or translational regulation of gene expression. A study on the effect of $H_2O_2$ and paraquat on the antioxidant status of rat astroglial cells revealed a transcriptional regulation of MnSOD and a post transcriptional regulation of CAT gene expression (Rohrdanz *et al.,* 2001). The transcription of antioxidant enzymes is controlled by certain transcriptional regulators which are activated in response to oxidative stress. Mouse GPx and CAT genes possess putative binding motifs for the transcriptional regulators NF-κB and AP-1, which are involved in the up regulation of GPx and CAT in response to oxidative stress (Zhou *et al.,* 2001). Two characteristic regulatory elements called, the antioxidant responsive element (ARE) and xenobiotics responsive element (XRE), present on the promoter region of the CuZnSOD gene of human liver cells induce up regulation of this gene under oxidative stress (Park and Rho, 2002). Therefore it is suggested that the antioxidant enzymes can be transcriptionally induced by the above transcription factors in combination with or independently of these two regulatory elements to form a defense against oxidative stress (Alia *et al.,* 2005) while a posttranscriptional regulation cannot be ruled out. Shull *et al.,* (1991) observed that the antioxidant enzymes are selectively induced when exposed to different ROS generating systems. For example, exposure of tracheobronchial epithelial cells *in vitro* to $H_2O_2$ induced a significant elevation in the steady state mRNA expression of CAT while that of MnSOD and GPx remained unaltered. However, an exposure to xanthine/xanthine oxidase system induced the expression of MnSOD with no effect on the other antioxidant enzymes (Shull *et al.,* 1991). Therefore, an understanding of the different regulatory elements involved in gene expression in oxidative stress conditions and their reversal by antioxidants is important in developing antioxidant therapy.

Evidence is mounting on the usefulness of dietary antioxidants in alleviating the ROS-induced pathologies. Consumption of high amounts of fruits and vegetables is reported to increase erythrocyte GPx activity which is correlated to the non-nutritive antioxidants such as carotenoids, anthocyanins and flavonoids (Dragsted *et al.,* 2004). A recent report suggests that anthocyanins induce phase II antioxidant enzymes through activation of antioxidant response element (ARE) upstream of antioxidant genes (Shih *et al.,* 2007). Dietary supplementation with β-carotene significantly enhanced the activity of GPx and moderately enhanced activity of SOD in rats with ischemic reperfusion injury (Codoñer-Franch *et al.,* 2008). Difference in longevity between males and females is correlated to the lower reactive oxygen species and higher expression of antioxidant enzymes in females which in turn is attributed to estrogen which up regulates antioxidant expression through activation of estrogen responsive elements (Boras *et al.,* 2003, 2005; Pinto *et al.,* 1968). Over expression of antioxidant enzymes is associated with an increase in life span. Up regulation of SOD and CAT in transgenic *Drosophila melanogaster* increased the lifespan by about 50% (Orr *et al.,* 1994; Sun and Tower, 1999) which is again higher in females (Spencer *et al.,* 2003). The finding by Rabinovitch's team (Schriner *et al.,* 2005) revealed an increase in murine life span by targeted overexpression of mitochondrial catalase which comes with additional benefits of delayed cardiac pathology and cataract, reduced oxidative damage and mitochondrial deletions. Therefore, targeting such specific sites to over express antioxidant genes by dietary antioxidant intervention may indeed help extend a healthy life span in humans.

**G. 13 Stability of mRNA**

The consequences of RNA damage and its repair have been gaining importance in the recent past. Considering that there is generally more RNA in a cell than DNA, it is likely that there will be significant damage to cellular RNA when cells are exposed to cytotoxic substances (Bellacosa, 2003). Though RNA damage may be a positive factor in anticancer chemotherapy, its repair by the cell signifies a much important role in genotoxic stress. Factors that cause damage to DNA damage RNA as well due to their structural similarity. In fact, RNA is more amenable to certain types of free radical attack than is DNA owing (i) its widespread cytoxolic distribution (ii) its single standed structure (iii) the absence of protective histones (iv) absence of advanced repair mechanisms and (v) the presence of free oxygen group. This has been demonstrated both in *vivo* and *in vitro* (Hofer, 2005; 2006) and mRNA damage may result in abnormal protein translation whereas tRNA and rRNA damage could result in dysfunction of protein synthesis (Martinet *et al.,* 2004).

Modulation of gene expression at the level of mRNA stability has emerged as an important regulatory paradigm. Concentration of an mRNA is a function of its rate of synthesis and rate

of degradation. Therefore, the half-life of mRNA in the cell is an important determinant of gene expression and its translation into the protein product (Ross, 1995).

Transcriptional regulation of antioxidant enzymes is well known. CAT gene expression in mouse skeletal muscles is transcriptionally regulated (Luo *et al.,* 2003), which is associated with NK-κB and AP-1 factors (Zhou *et al.,* 2001). However, post transcriptional regulation is becoming evident especially through stabilization of the mRNA transcripts. For example, rise in CAT mRNA levels after exposure to low levels of $H_2O_2$ is found to be post transcriptionally regulated through increased stabilisation of the same (Sen *et al.,* 2005) in V79 fibroblasts. Similarly, enhanced expression of CAT gene in hyperoxia-exposed rat lung is connected to the increased CAT mRNA stability (Clerch *et al.,* 1991). Therefore, a change in the abundance of a particular RNA may reflect a change in the transcriptional activity of the gene, a change in the rate of turnover of the specific RNA or both. The rate of degradation of a specific RNA molecule can be measured by blocking the synthesis of all RNA molecules. Stability of mRNA can be measured by blocking its synthesis with a transcription inhibitor, isolating cytoplasmic RNA at different time intervals and monitoring the rate of loss of a particular transcript with a gene specific probe. The most commonly used transcription inhibitors are actinomycin D, α-amanitin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and cordycepin(Ross 2004). However each inhibitor has its own disadvantages in that α-amanitin does not enter all cells while actinomycin D also inhibits translation and affects ATP pools (Peltz *et al.,* 1991). Also, the results obtained with different methods differ considerably. Stability of mRNA molecules along with transcription, mRNA processing, translational efficiency and post translational modification is an important determinant of protein levels in the cells.

Increased stability of mRNA transcripts generally means a steady state translation into the protein product. This property is desirable in antioxidant therapy especially in cases where an oxidative stress results in reduction in antioxidant defense. Drugs that increase the stability of antioxidant enzyme transcripts are especially advantageous in order to increase the resistance towards oxidative insult. While most studies on effect of dietary antioxidants on the expression of antioxidant enzymes is limited to their up-regulation and down regulation, study on their effect on stability of the respective mRNA transcripts is lacking. Therefore, an attempt was made in the present work to study the effect of anthocyanin pigments on the stability of mRNAs of CAT, SOD and GPx as altered by oxidative stress.

**G. 14 Measurement of DNA damage**

Active oxygen species such as $O_2^{-\bullet}$, $H_2O_2$, $OH^{\bullet}$ can damage almost all cell components including the DNA leading to single strand breaks, double strand breaks or chromosomal

aberrations (Halliwell and Gutteridge, 1995) that is manifested in mutagenesis, carcinogenesis, and aging. The chief sources of reactive species that account for much of the DNA damage in biological system are the Fenton system, ionizing radiation and nuclease activation (Aruoma and Halliwell, 1998) through the generation of $OH^{\bullet}$. A number of factors have been identified to be damaging to DNA, all mediated through free radical generation. The chief factors include ionizing radiation, transition metal ions, cigarette smoke and other chemical carcinogens (Medvedev and Leschenko, 2008; Kobayashi *et al.,* 1988; Jackson *et al.,* 1987). Mechanism of damage involve abstractions and addition reactions by free radicals leading to carbon-centered sugar radicals and $OH^{\bullet}$ or $H^{\bullet}$ adduct radicals of heterocyclic bases (Dizdaroglu *et al.,* 2002). The Fenton reaction is the main source of $OH^{\bullet}$ radicals which react with DNA and the sugar, deoxyribose, in multiple ways resulting in the production of a plethora of modified bases (Aruoma *et al.,* 1989; Dizdaroglu *et al.*, 1991). $Fe^{3+}$-citric acid, a physiological iron ion complex has been evidenced to produce small amounts of $OH^{\bullet}$ in the presence of $H_2O_2$ vitro, the rate of which is greatly increased in the presence of ascorbic acid (Hu and Shih, 1997). In vitro, $OH^{\bullet}$ can be generated by a Fe (III)-EDTA-Ascorbate-$H_2O_2$ system, which causes significant base modifications in DNA (Aruoma *et al.,* 1989). However, this method cannot be used for ascorbic acid and other phytochemicals that act as prooxidants which would otherwise promote oxidative DNA damage in this system (Aruoma, 2003). Elevated levels of DNA damage have been witnessed in various disease states and it has been hypothesized that such an elevation may be a causative factor in the etiology of such diseases. An active DNA repair system is critical for preventing the occurrence of mutations leading to carcinogenesis and other pathologies. Natural products play a pivotal role in conferring protection to DNA thus preventing their base modification or single/double strand breaks. Flavonoids such as naringenin from grape fruit, EGCG from green tea (Sartippour *et al.,* 2006), quercetin, an almost ubiquitous flavonoid and carotenoids have received much interest in this area due to their proven DNA protecting activity and consequent cancer chemo preventive property.

**G. 15 Dietary antioxidants and cancer chemoprevention**

Carcinogenesis is a multistage process consisting of apparently three major steps: initiation, promotion and progression and in human it takes many years for a normal cell to turn in to complete malignancy (Moolgavkar 1978; Sporn 1991). Reports emerge with the proof that this process can be delayed or completely prevented by dietary intervention. It is interesting to note that the phytochemicals which show cytoprotective activity in normal cells can also be potent inhibitors of cancer or tumor development. What really sets apart their differential effects in abnormal cancer cells *vs* normal cells is their ability to induce apoptotic pathways to

delay cancer in abnormal cancer cells and at the same time manipulate levels of metabolizing enzymes and induce detoxifying enzymes rendering them non-toxic to normal cells (Gopalakrishnan and Kong, 2008). The dietary polyphenols and carotenoids and other phytoconstituents have demonstrated reduction in risk of cancers of breast, prostate, colon, stomach, skin, liver etc. (Craig *et al.,* 1999; Hercberg *et al.,* 1998; Kelloff *et al.,* 1999; Knekt *et al.,* 1997). EGCG and quercetin, the most important flavonoids in medicine possess both cytoprotective and cytotoxic properties and have been found to confer protection in various cancer cell and animal models (Sartippour *et al.,* 2007). **Table 4** summarises the selected reports on the anticancer properties of phytochemicals used in this study.

Inhibition of free radical generation or scavenging the free radicals generated by other biochemical reactions is an important factor in the prevention of cellular damage. While the body is designed to detoxify and allay itself of harmful effects of exotoxins, it may not be sufficiently prepared to handle the onslaught of pollutants and toxic substances predominant in the environment. In addition, refining of foods generally leads to deficiency in essential nutrients involved in detoxification process. Therefore, supplementing the diet with such compounds which enhance antioxidant defense is essential in order to prepare the body against oxidative insult. Several natural products have been proven to be effective against cellular damage either by acting as antioxidants or as positive modulators of other cellular machineries (Semiz and Sen 2007; Sakr 2007). Anthocyanins and carotenoids have especially occupied a place in the antioxidant market due to their innumerable health benefits as already discussed earlier. Therefore, addition of newer and more economical sources of these compounds to the existing list is desirable. In this direction we chose two plant species which are less exploited and are abundant sources of these two classes of compounds. They are:

- *Syzygium cumini* fruits for anthocyanins
- *Delonix regia* flowers for carotenoids.

Table 4. Summary of anticancer properties of flavonoids and carotenoids

| **Class** | **Compound** | **Anticancer activity** |
|---|---|---|
| Flavonoids | Anthocyanins | Delphinidin, cyanidin, and petunidin have been shown to inhibit chemical carcinogen-induced AP-1 transcriptional activity and cell transformation in JB6 cells (Hou *et al.,* 2004). Anthocyanins inhibit tumerigenesis by blocking the activation of mitogen-activated protein kinase (MAPK) pathway and c-jun $NH_2$ terminal kinase (JNK) which is ROS dependent (Hou *et al.,* 2004; Feng *et al.,* 2007). Anthocyanins from black raspberries prevent oesophageal tumors in rats probably by downregulating carcinogen-induced nuclear factor-κB (NF-κB) and activator protein-1 expression (Wang *et al.,* 2009). While being non toxic to normal cells, anthocyanins induce peroxide accumulation and apoptosis in HL-60 cells (Feng *et al.,* 2007). |
| | EGCG | In tumor cells EGCG induces production of $H_2O_2$ creating an oxidative stress environment (Long *et al.,* 1999; Yang *et al.,* 2000) which triggers an apoptotic pathway that is distinct from chemical or Fas-mediated pathways and acts through activation of mitogen-activated protein kinases, c-Jun N-terminal kinase and p38, and the caspase cascade (Kong *et al.,* 1998; Yang *et al.,* 2000; Balasubramanian *et al.,* 2002; Saeki *et al.,* 2002; Chen *et al.,* 2003). |
| | Quercetin | At low doses (0.1-5μM) shows cytoprotection while at higher doses (50-100 μM) it is clearly cytotoxic (Alia *et al.,* 2006a). Inhibits cell proliferation by downregulating the cell cycle genes such as CDC6, CDK4 and cyclin D, inducing cell cycle arrest and by upregulating tumor suppressor genes (van Erk *et al.,* 2005). It stimulated proliferation of colon carcinoma cells at lower concentrations while at higher concentrations inhibits the same (Dihal *et al.,* 2006; Uwe *et al.,* 2004) |

| | | |
|---|---|---|
| Carotenoids | β-carotene | β-carotene induces ROS production and activation of NF-κB, by a redox mechanism which is accompanied by inhibition in cell growth and induction of apoptosis through over expression of c-myc, an apoptosis inducing protein, in leukemia and colon cancer cell lines (Palozza *et al.,* 2003). It has also been found to exhibit anticancer activity in breast cancer cells by downregulation of cycloxygenase-2 (COX2) mediated by the upregulation of peroxisome-proliferator activated receptor-γ (PPAR-γ) and modulating the expression of its downstream components ultimately leading to apoptosis through ROS production (Cui *et al.,* 2007). However, β-carotene consumption in high doses is presumed to increase risk of ling cancer in smokers and asbestos workers (ATBC 1994; Omenn *et al.,* 1996). |
| | Lycopene | A significant inverse correlation has been found between consumption of tomatoes, a rich source of lycopene, and cancers such as that of prostate, breast, cervical, ovarian, liver and other organs (Giovannocci *et al.,* 1995; La Vecchia 1997; Giovannocci 1999). In prostate cancer, it acts by substantially reducing the levels of prostate specific antigen and also oxidative DNA damage (Giovannocci *et al.,* 1995). |
| Xanthophylls | Lutein | Selectively induces apoptosis in transformed mammary cells by increasing the BCl2/Bax ratio (Sumantran *et al.,* 2000), expression of p53 and reducing angiogenesis in the tumors (Chew *et al.,* 2003). |
| | Zeaxanthin | There are not many studies proving its role in anticancer studies; however, it has been reprted that zeaxanthin and luetien protect the retina of the eye from the damaging effects of light reducing the risk of age-related macular degeneration and cataract (Trumbo and Ellwood, 2006). |
| | β-cryptoxanthin | High levels of β-cryptoxanthin are associated with a reduced risk of lung cancer according to a Chinese cohort study (Yuan *et al.,* 2003). |

## G. 16 *Syzygium cumini* Skeels (Fam: Myrtaceae)

*Syzygium cumini* is a tropical fruit bearing tree native to Inida, Burma, Ceylon and Andaman Islands (Morton, 1987). The tree yields berries (**Fig. 6**) which are also known as black java plum, Indian black plum, jambolan or jamun. The edible berries are round or oblong (upto 2 inches long) and turn from green to light-magenta and then dark purple or nearly black when ripe. The pulp is purple or white, very juicy, and normally encloses a single, oblong, green or brown seed, up to The fruit is usually astringent and the flavor varies from acid to fairly sweet. The skin is thin, smooth, glossy, and adherent.

**Fig. 6.** Photograph depicting part of *S. cumini* plant with fruits, flowers and leaves. Inset shows the fully ripe berries.

**Food Uses**

Jambolans of good size and quality, having a sweet or subacid flavor and a minimum of astringency, are eaten raw and may be made into tarts, sauces and jam. The white-fleshed jambolan has adequate pectin and makes a very stiff jelly unless cooking is brief. The more common purple-fleshed fruits yield richly colored jelly but are deficient in pectin and require the addition of a commercial jelling agent or must be combined with pectin rich fruits. The fruits are also used to make syrup.

**History in medicine**

The fruits of *S. cumini* possess various medicinal properties and are used in Ayurveda as a stomachic, astringent, antiscorbutic, diuretic antidiabetic, in chronic diarrhea and enlargement of spleen (Morton, 1987; Nadkarni, 1954; Achrekar *et al.*, 1991). The fruit concentrate of *S. cumini* has a very long history of use for various medicinal purposes and currently has a large market for the treatment

of chronic diarrhea and other enteric disorders, including its use as an antimicrobial (Migliato, 2005). The leaves are found to reduce radiation induced DNA damage in cultured human peripheral blood lymphocytes (Jagetia & Baliga, 2002). Different parts of the tree such as the leaves, seeds and bark are shown to posess antidiabetic properties (Sridhar *et al.,* 2005) while some reports contradict the same (Teixeira *et al.,* 1997). The fruit pulp has been reported to be antihyperglycemic (Achrekar *et al.,*, 1991). A recent study by Li *et al.,* (2009a) suggests an anticancer effect of jamun fruit extract in breast cancer cells. In Ayurveda, the juice of the ripe fruit, or a decoction of the fruit, or jambolan vinegar, is administered in cases of enlargement of the spleen, chronic diarrhea and urine retention. Water-diluted juice is used as a gargle for sore throat and as a lotion for ringworm of the scalp. Extracts of bark and seeds, in liquid or powdered form, are given orally to patients with diabetes mellitus or glycosuria. During the past decade a number of research papers have published on the antihyperglycemic action of the seeds of this fruit. The hypoglycemic activity of jambolan extracts is contradictory. While most studies report the hypoglycemic effect of the seeds (Sridhar *et al.,* 2005; Sharma *et al.,* 2006) there are other reports refuting them (Teixeira *et al.,* 1997). Though different parts of this species are used in herbal formulations, a very few reports are available on the systematic characterization of chemical components of the fruit.

**G. 16.1 Chemistry of fruits**

While the fruit has gained immense use in Ayurveda a detailed study on the chemistry of the same is lacking. The fruit peel owes its deep purple color to the presence of anthocyanins. There have been different reports regarding the anthocyanin composition of the fruits. A recent study reported a total phenolic and total anthocyanin content of 3.9g/kg and 1.3g/kg of fruit respectively (Benherlal and Arumughan, 2007). According to one of the earliest reports the deep purple color of the fruit is due to anthocyanins namely delphinidin-3-gentiobioside and malvidin-3-laminaribioside along with petunidin-3-gentiobioside (Venkateswarulu, 1952). Sharma and Seshadri (1955) reported the presence of cyanidin diglycoside and glycosides of petunidin and malvidin. Another recent report on *S. cumini* Lamarck revealed the presence of malvidin-3-glucoside and petunidin-3-glucoside in the Brazilian variety (Lago *et al.,* 2004). The fruit is also rich in other nutritive elements such as proteins, carbohydrates, vitamins, calcium, iron, phosphorous etc. along with antioxidants such as ascorbic acid and other phenolics (Morton, 1987).

The present thesis partly deals with the study of characterisaiton of anthocyanins from fruit peel of *S. cumini* and the antioxidant potential of the anthocyanin rich extract of the same, elucidated

through chemical methods and cultured cell models. This study was carried out due to the limited literature available on this plant source with reference to these two subjects.

### G. 17 *Delonix regia* Rafin. (Fam: Leguminosae)

*Delonix regia* Rafin. Bojer *ex* Hook (Syn. *Poinciana regia*) commonly known as 'Gul Mohr' or the falmbouyant flame tree (**Fig. 7**), is a striking ornamental tree producing abundant clusters of orange-red flowers during early summer.

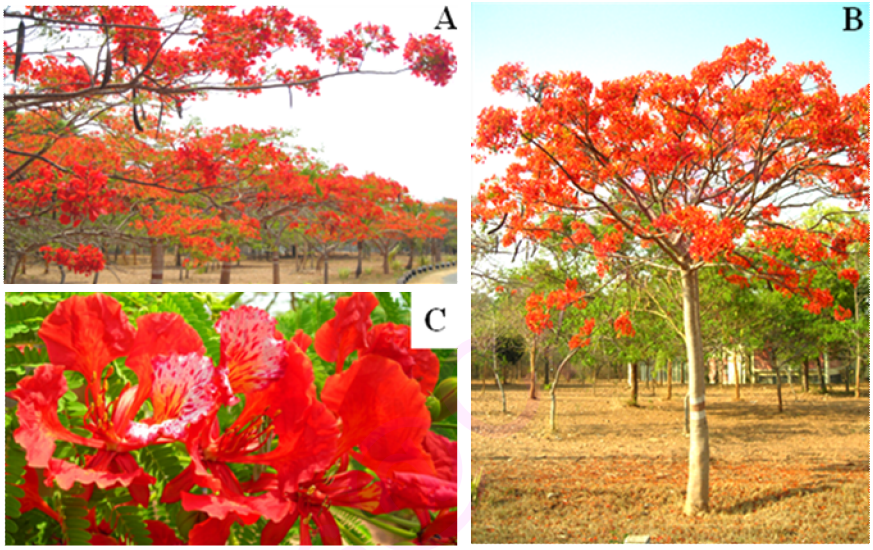



**Fig. 7.** Avenue of *D. regia* trees in full bloom (A); Single tree (B); a close shot of the flowers showing the abundance of flowers.

Flowers are eaten fresh due to their sweet-sour-astringent taste and are reported to be used as an anthelmintic (Anonymous, 1952; 1956). They are also used in culinary preparations by rural people of Asia, far-East and Australia. Though the chemical composition (Gupta *et al.,* 2005) and medicinal properties (Sethuraman *et al.,* 1986) have been well established for the other species of *Delonix* (*D. elata*), only a very few preliminary reports are available on the chemistry and biological potential of *D. regia* (Seetharam *et al.,* 2002). The methanolic extract of *D. regia* Gamble was reported to have antioxidant properties (Aqil *et al.,* 2006). The most recent report on anthocyanin composition of the flowers describe the presence of cyanidin-3-O-glucoside and 3-O-rutinosides of cyanidin and pelargonidin (Adje *et al.,* 2008). Four flavanoids isolated from the flowers of *D. regia* have been reported to show strong antioxidant activity against various free radicals (Su and Fan, 1997). Saleh *et al.,* (1976) reported the presence of two anthocyanins – cyanidin-3-glucoside and cyanidin-3-gentiobioside – in the flowers whereas the carotenoids of different floral parts of *D. regia* were first reported by Jungalwala and Cama (1962). They studied

the carotenoids of different floral parts in detail using the chromatographic and spectrophotometric techniques and reported the presence of an array of carotenoids. That was the first, most comprehensive and the only study on the carotenoids of *D. regia* floral parts. β-carotene was identified as the major carotenoid among the 29 carotenoids identified in the flower petals. Others included γ-carotene, rubiaxanthin, phytoene, lycopene isomers, lutein, zeaxanthin, phytofluene with several epoxy carotenoids (Jungalwala and Cama, 1962).

Apart from the above literature, no significant amount of literature is availale on the biological significance of the flowers of *D. regia*. Since the fowers are an affluent source of carotenoids with high concentrations of β-carotene, it can be considered a source of natural color and a provitamin A supplement. A unique feature of these flowers is the presence of carotenoids as well as anthocyanins in high amounts. Therefore, this flower was chosen in order to explore its potential as a natural color and to establish its antioxidant potential in cell free and cell based assays. With the available literature and background information, it is obvious now that formation of reactive oxygen species is balanced out by antioxidant defenses and augmenting this defense by antioxidant therapy could therefore provide a potential means to treat conditions in which the formation of reactive oxygen species exceeds the capability of natural protective mechanisms.

Therefore the present study was focused on elucidating the antioxidant mechanisms exerted by the phytochemicals from the above two sources, for which the following objectives were considered:

## G. 18 Objectives

1. Identifying effective antioxidant components in fruits of *S. cumini* and flowers of *D. regia*, using *in vitro* assays and cell models.
2. Developing experimental model to elucidate the damage/changes caused to antioxidant enzymes- catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) - in cell preparations of rat liver.
3. Using the experimental model developed, reassessment of the selected antioxidative compounds for their mechanism of action in modulating the changes caused to mRNA of the antioxidant enzymes.
4. Development of a pharmaceutical formulation by incorporation of a natural pigment and study its stability in the formulation.

The work done is presented in the following chapters

- Charcterisation and in vitro antioxidant activity
- Stability of anthocyanins pigment of *S. cumini*
- Bioefficacy studies in primary animal cell culture
- Studies on expression of antioxidant enzymes and antiproliferative activity

# Chapter 1

# *Characterisation of biomolecules and in vitro antioxidant activity*

**Summary**

Natural colors from fruits of *Syzygium cumini* (anthocyanins) and flower petals of *Delonix regia* (carotenoids and anthocyanins) were identified, characterized and sequentially isolated extracts were evaluated for their biological effects. The antioxidant capacity of the extracts were tested using *in vitro* models like DPPH$^{\bullet}$ scavenging, reducing power assay, scavenging of hydroxyl radical and nitric oxide and lipid peroxidation in different tissue homogenates and human plasma. Total content of anthocyanins being high (230mg/100g DW) the three anthocyanins of *S. cumini* fruit peel were identified as glucoglucosides of delphinidin (1), petunidin (2) and malvidin (3) by HPLC-ESI-MS. The anthocyanin extract showed 78.2% DPPH$^{\bullet}$ scavenging at 2.5ppm, while BHA exhibited only 41.6% activity at the same concentration, thus proving to be an efficient free radical scavenger than the widely used BHA. One ppm of the extract was equivalent to 3.5 μM ascorbic acid as estimated by reducing power assay and inhibition of rat brain lipid peroxidation was 94.4% at 5.0ppm concentration. It was almost equally active in all the biological models except human erythrocyte ghost cells where it showed only 48% inhibition at 5.0ppm. Fresh petals of *D. regia* were found to contain 825mg and the oven dried petals contained 580mg/100g DW of anthocyanins and 660mg/kg DW of β-carotene. The major carotene in the carotene fraction was β-carotene which amounts to 76% of total carotenoids and 50% of carotene fraction. Xanthophyll fraction was tentatively identified to have lutein, zeaxanthin, β-cryptoxanthin and astaxanthin. The flower petals were successively extracted with different solvents and assayed for antioxidant activity. All the extracts, except for the hexane extract, showed over 90% quenching of DPPH$^{\bullet}$ at 250 ppm. Hydroxyl radicals were effectively scavenged (>90%) at 25-100 ppm by all the extracts with the exception of crude pigment extract and the xanthophyll fraction. Most of the extracts of *D. regia* were effective in countering the actions of free radicals and lipid peroxidation in the experimental models. The various antioxidant activities were compared with standard antioxidants such as BHA, BHT, gallic acid and ascorbic acid depending on the experimental model. The results of the present study have established that floral petals of *D. regia* are rich in pigments and potential antioxidants holding a great promise for food and pharmaceutical applications.

**Publications**

**Veigas JM**, Narayan MS, Laxman PM, Neelwarne B. 2007. Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *Syzygium cumini* Skeels. Food Chem. 105; 619-627.

**Jyothi M. Veigas**, Narayan MS, Chidambaramurthy KN, Neelwarne B. 2007. Antioxidative efficacies of floral petal extracts of *Delonix Regia* Rafin. Int. J. Biomed. Pharma. Sci. 1(1); 73-82.

## 1.1 Introduction

The role of antioxidants ingested through diet in protecting the body against oxidative stress has been a topic of continuing interest in the field of biology and medicine. Innumerable reports point at the antioxidant potentials of natural compounds. It has been estimated that 80% of the world's population rely primarily on natural food supplements or drugs (Zhu *et al.,* 2004). Fruits and vegetables have been the chief source of antioxidant biomolecules. Diverse classes of compounds possess different degrees of antioxidant activity depending on the surrounding oxidative milieu. Flavonoids are a class of compounds of varied biological properties such as antioxidative, antimicrobial and possibly anticarcinogenic and cardioprotective effects. Flavonoids comprise flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Carotenoids are another important and diverse class of natural pigments that are of interest for pharmaceuticals, coloring food and animal feed and nutrient supplements (Schmidt-Dannert *et al.,* 2000) due to their high antioxidant activity (Sies *et al.,* 1992; Serrano *et al.,* 2005). Identifying newer, more economical sources of natural pigments with strong antioxidative bioefficacies are currently needed for application in food and pharmaceutical industry. Therefore, the present study was aimed at analyzing the two less exploited sources viz., fruits of *Syzygium cumini* and flowers of *Delonix regia* rich in anthocyanins and carotenoids respectively, for their potential health benefits.

***Syzygium cumini*** is a tropical tree bearing large amounts of deep purple berries (**Fig. G. 6).** The fruits, also called as Indian black plum or Java plum are edible having various medicinal properties and are used in Ayurveda as a stomachic, astringent, antiscorbutic, diuretic, antidiabetic, in chronic diarrhea and enlargement of spleen (Achrekar *et al.,* 1991; Morton, 1987; Nadkarni, 1954). The fruit concentrate of *S. cumini* has a long history of use for various medicinal purposes and currently has a large market for the treatment of chronic diarrhea and other enteric disorders, including its use as an antimicrobial (Migliato, 2005). The seeds have been the subject of intense research as they show anti-diabetic property. Though different parts of this species are used in herbal formulations, reports on systematic characterization of anthocyanins and bio-efficacy of the fruit is lacking. Therefore, the present study focuses on the characterization of anthocyanins using a more powerful and accurate tool such as the HPLC, coupled to ESI–MS which involves minimal sample preparation.

***Delonix regia*** is a tropical flowering tree (**Fig. 7**) bearing abundant flowers during summer. The flamboyant nature of the flowers is mainly attributed to the presence of mainly carotenoids followed by anthocyanins. In fact, this co-existence of carotenoids and anthocyanins is quite rate among plants. Though the chemical composition (Gupta *et al.,* 2005) and medicinal properties

(Sethuraman *et al.,* 1986) have been well established for the other species of Delonix (*D. elata*), only a very few preliminary reports are available on the chemistry and biological potential of *D. regia* (Seetharam *et al.,* 2002). The carotenoids were studied in detail for the first time by Jungalwala and Cama (1962) in different floral parts while the anthocyanins were reported to be glycosides of cyanidin by Saleh *et al.,* (1976). The present study aims at the partial characterization of some of the major carotenoids present in the flower petals.

Furthermore, in addition to their colorful characteristics, anthocyanins and carotenoids are known to possess excellent antioxidant properties (Kong *et al.,* 2003; Pryor *et al.,* 2000) due to their capacity to quench free radicals. The reactive species generated vary in their chemical nature. A number of methods have been developed to measure the antioxidant activity of natural compounds and for comparing antioxidant activity of various classes of compounds. These include, oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), total oxidant scavenging capacity, trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging activity, lipid peroxidation assay, hydroxyl radical scavenging assay and NO scavenging assay (Chen *et al.,* 1999; Sanchez-Moreno *et al.,* 1999). A single antioxidant assay is not representative of the ability of an antioxidant in question. This is because effectiveness of a particular method depends on the type of free radical involved and the chemical nature of antioxidant compound. Therefore it is necessary to carry out a series of tests engaging different free radicals or reactive species to determine the efficiency of antioxidant against different oxidative stress conditions.

There is considerable anecdotal and epidemiological evidence that dietary anthocyanins and polyphenols confer preventive and therapeutic roles in a number of human diseases. For similar reasons the consumption of red wine, rich in anthocyanins, is believed to lower cardiovascular diseases and related mortality (de Lorgeril *et al.,* 2006). A growing body of literature points to the importance of natural antioxidants from many plants that may be used to reduce oxidative damage in the human body (Tsuda *et al.,* 2004; Dominguez *et al.,* 2005). In addition to their individual effects, the antioxidants interact in synergy imparting "sparing effects" in which one antioxidant protects another against oxidative destruction (Langseth, 1995). For these reasons, there is an upsurge of research in finding newer sources of effective natural antioxidants. The present study focuses on the antioxidant capacity of pigments extracted from fruits of *S. cumin* and dried flowers of *D. regia* through chemical and biological models.

## 1.2 Materials and Methods

### 1.2.1 Collection of plant material

#### *1.2.1.1 Syzygium cumini* fruits

Fully ripe berries were obtained from the local market; the peel was manually separated and immediately transferred to solvent for pigment extraction.

#### *1.2.1.2 Delonix regia* flowers

The flowers of *D. regia* in full bloom were collected from the trees grown in campus during May-June and the flowers were separated, dried at 45°C ± 1°C until they reached constant weight and either used immediately or stored in an airtight container for 2-3 months at -20ºC, during which time all the extractions were completed.

### 1.2.2 Preparation of extracts

#### 1.2.2.1 Preparation of anthocyanin extracts

Anthocyanins were extracted with 0.1% HCl in ethanol (Francis, 1986) by way of soaking the plant material in a 10-fold volume of the solvent for 3 h on an orbital shaker set at 100 rpm (25°C ± 1). After filtration, the residue was repeatedly extracted until the filtrate obtained was nearly colorless. The extracts were combined and concentrated in a Buchi Rotavapor (Flawil, Switzerland) under vacuum at 30°C (±1°C), and partitioned against ethyl acetate before application onto an Amberlite XAD-7 column (Andersen *et al.,* 1995). The column eluate was concentrated to dryness, lyophilized, stored in aliquots and is used for quantification, characterization and bioactivity studies.

#### 1.2.2.2 Preparation of the successive extracts of *D. regia* flowers

The oven-dried petals were powdered using a kitchen blender and 20 g (dry weight) were subjected to successive extraction in a Soxhlet extractor using solvents in increasing order of their polarity starting from hexane followed by ethyl acetate (EtOAc), acetone, methanol (MeOH) and water ($H_2O$). The individual extracts were concentrated at 40°C ± 1°C under reduced pressure (as per manufacturer's instruction for the respective solvent) using Buchi rotavapour (Flawil, Switzerland), dried in a vacuum oven and stored at -20ºC until further use.

#### 1.2.2.3 Preparation of carotenoids rich fractions of *D. regia* flowers

A flow chart for the preparation of pigment extracts is outlined in **Fig. 1.1.**

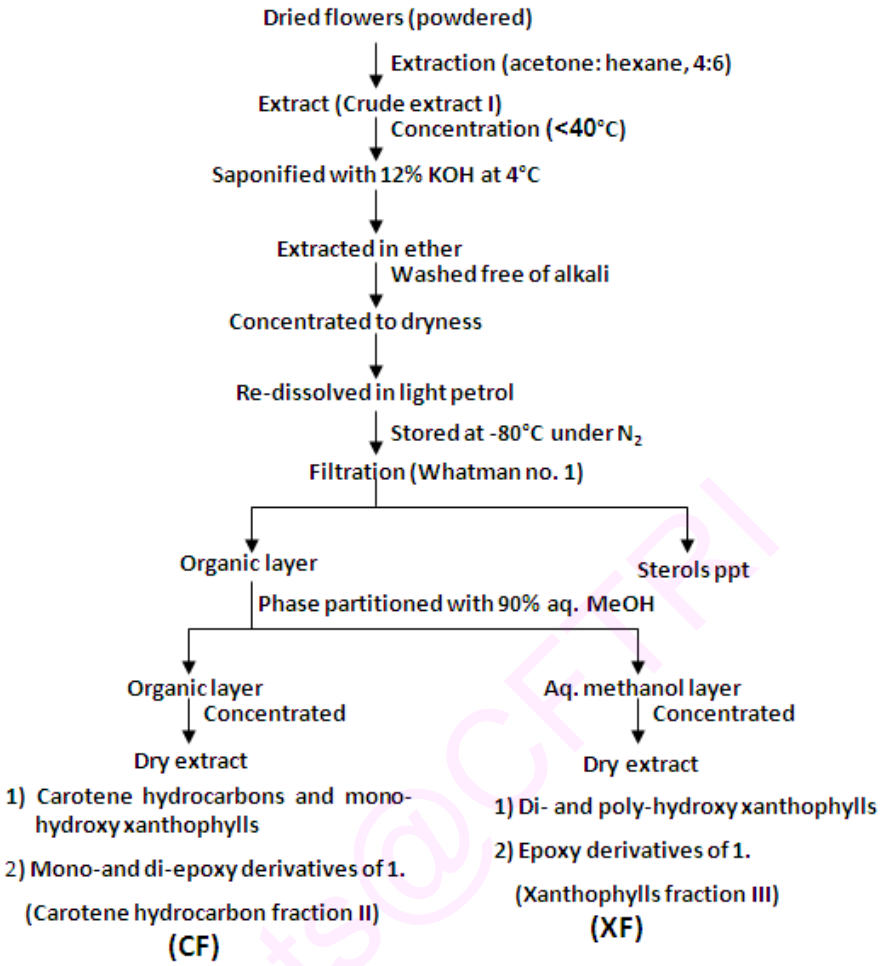



**Fig. 1.1.** Flow chart outlining the steps involved in preparation of carotene hydrocarbon fraction (CF) and xanthophylls fraction (XF).

### 1.2.3 Estimation of phytoconstituents

#### 1.2.3.1 Quantification of total anthocyanin content

Total anthocyanins ($T_{acy}$) content was determined by a pH differential method (Lee *et al.,* 2005). One gram of the plant material was extracted in methanol acidified with 1% HCl at room temperature on an orbital shaker set at 100rpm for 6 hrs. The mixture was centrifuged at 7000 rpm (9300×g) for 10 min and the supernatant decanted into a 50.0 mL volumetric flask. The pellet was re-extracted thrice, the supernatants combined and the volume was made up with methanol. One mL each of the solution was diluted with pH 1.0 buffer (1.86 g KCl dissolved in distilled water and adjusted to pH 1.0 ± 0.05 with HCl and diluted to 1.0 L with distilled water in a volumetric flask) and pH 4.5 buffer (0.4 M sodium acetate adjusted to pH 4.5 ± 0.05 with HCl) respectively and the OD of the two solutions was measured at 520 and 700 nm against a distilled water blank. A similar measurement was made using suitably diluted lyophilized anthocyanin

extracts. The concentration of $T_{acy}$ was expressed as cyanidin-3-glucoside equivalents using the following formula:

$$T_{acy}\ (\%\ w/w) = (A \times MW \times DF \times V \times 100) / \varepsilon \times l \times W$$

where: $A = (A_{520nm} - A_{700nm})$ pH 1.0 $- (A_{520nm} - A_{700nm})$ pH 4.5; MW = 449.2 g $mol^{-1}$ for cyanidin-3-glucoside; DF = dilution factor; V = total volume of the extract; $\varepsilon$ = 26900 molar extinction coefficient, in $L \times mol^{-1} \times cm^{-1}$, for cyanidin-3-glucoside; l = path length in cm; W= sample weight (mg).

#### 1.2.3.2 Estimation of total phenolics

Total phenolics were determined as per the method described by Velioglu *et al.,* (1998) with a slight modification. Briefly, 1 g of the sample was extracted with 80% methanol containing 1% HCl at room temperature on an orbital shaker set at 100 rpm for 6 hrs. The mixture was centrifuged at 7000 rpm (9000g) for 10 min and the supernatant decanted into a 50.0 mL volumetric flask. The pellet was re-extracted thrice, the supernatants were pooled and the volume was made up with methanol. An aliquot of the extract (25 µL) was mixed with 225 µL methanol and 1.0 mL Folin-Ciocalteu reagent (0.2 M) and allowed to stand at room temperature (25ºC ± 0.5ºC) for 5 min. One mL of 6% w/v sodium carbonate solution was added to the mixture followed by incubation for 90 min at room temperature and the absorbance was measured at 725 nm. A calibration curve of gallic acid was prepared (ranging from 0.001-0.01 mg/mL). Results were determined from regression equation of the calibration curve and expressed as mg gallic acid equivalents per gram (mg GAE $g^{-1}$) of the sample. The method was also applied to the successive extracts of *D. regia*, to select the solvent that was most suitable for the extraction of polyphenols.

#### 1.2.3.3 Determination of total carotenoids in flowers of *D. regia*

An accurately weighed powder of dried *D. regia* flower petals was extracted thrice with acetone. The extracts were pooled and made up to 100.0mL with acetone in a volumetric flask. Optical density (OD) of the suitably diluted solution was measured at 450nm using UV-visible spectrophotometer (Shimadzu 160A, Japan). Total carotenoids content was measured using the following formula (Rodriguez-Amaya, 2001):

Carotenoid content (mg/sample) = (OD at 450nm x V x DF x 10)/2500.

#### 1.2.3.4 Determination of β-carotene in flowers of *D. regia*

Carotenoids content was determined by the method described in AOAC International (1993). Briefly, 2.5 g of the dried sample was blended for 5 min with acetone:hexane (4:6) containing 0.1 g of magnesium carbonate and centrifuged at 8000 rpm for 10 min. The residue was washed with two 25 mL portions of acetone followed by one 25 mL portion of *n*-hexane. The extracts were combined and washed with water to remove acetone. The upper layer was placed in a 100.0 mL volumetric flask containing 9 mL acetone and the volume was adjusted with hexane. The optical density of the suitably diluted solution was measured at 436 nm using UV-visible spectrophotometer (Shimadzu 160A, Japan).

Concentration of β-carotene was calculated from the formula:

$$C = (OD \text{ at } 436nm \times 454) / (196 \times L \times W)$$

Where, C represents concentration of β-carotene (mg $lb^{-1}$), L represents the path length (cm) and W represents the weight of the sample in g $mL^{-1}$ of final dilution. (C x 2.2 gives the concentration in mg $kg^{-1}$).

#### 1.2.3.5 HPLC quantification of β-carotene in *D. regia* flowers

The extract obtained in section 1.3.4 was analysed by HPLC using an isocratic mobile phase consisting of acetonitrile:methanol:dichloromethane (7:2:1v/v/v). The flow rate was set at 1.0 mL $min^{-1}$. Chromatogram was acquired at 450nm.

Synthetic *all-trans*-β-carotene was used as the standard and a calibration curve was prepared ranging from 0.125µg to 0.5µg β-carotene under the conditions mentioned above.

Concentration of β-carotene in the extract was calculated using the regression equation of the calibration curve ($y=3.746x + 0.805$; $R^2=0.99$) and expressed as g/kg β-carotene on dry weight basis.

### 1.2.4 Identification of biomolecules and characterisation

#### 1.2.4.1 Paper chromatography of anthocyanins

Partially purified anthocyanins were separated on Whatman No. 3 chromatographic paper. Known volumes of the extract were applied directly on paper and descending chromatography was carried out in butanol: acetic acid: water (4:1:5v/v/v).

#### 1.2.4.2 HPLC–MS Analysis of anthocyanins

The partially purified samples as well as anthocyanins separated on paper, were diluted suitably with methanol, filtered through a 2µm membrane filter (Millipore, USA) and analysed by HPLC,

using a Waters Alliance 2695 HPLC equipped with an auto sampler and coupled with a Waters 2696 photodiode array detector and a Q-TOF Ultima™ mass spectrometer, utilizing the electro spray ionization (ESI–MS) interface (Waters Corporation, Manchester, UK). The chromatographic separation was performed on a Wakosil II $C_{18}$ reverse phase stainless steel column, 250 x 4.6 mm i.d., 5 µm (SGE, Australia) with a guard column of the same material. The mobile phase (0.6 ml $min^{-1}$) consisted of (A) water/acetonitrile (95:05, v/v) and (B) water/acetonitrile (50:50, v/v) adjusted to pH 2.5 (with TFA). The gradient was: 0 min, 15%B; 0–20 min, 15–30%B; 20–25 min, 30–35%B; 25–35 min, 35–40%B; 35–42 min, 40%B; 42–43 min, 40–100%B; 43– 48 min, 100B; and 48–49 min, 100–15%B, followed by 5 min for equilibrium at 15%B. Chromatograms were acquired at 520 nm. Samples (20 µl) were analysed in duplicate. Positive ion spectra of the column eluate were recorded in the range of m/z 20–2000 at a scan rate of 2 s/cycle under the following conditions: collision energy 10.0; capillary voltage 35 V; cone voltage 100 kV; source temperature 80°C; desolvation temperature 150°C; cone gas flow 0.4 l/min; desolvation gas flow (8.3 l/min). Argon was used as the collision gas. Data acquisition and processing was performed using MassLynx™ 4.0 SP4 software (Micromass).

#### 1.2.4.3 HPLC-MS analysis of phenolic compounds

In order to find out if the anthocyanin extract of *S. cumini* used for the study contains any other phenolics which might be responsible for the biological activity of the same, HPLC analysis of the extract as well as the ethyl acetate fraction (used for removing phenolics and flavonoids prior to elution on XAD column) of the extract was carried out. HPLC-MS analysis was carried out in negative ion mode. Chromatographic separation was performed on a Phenomenex Gemini 5 µm$C_{18}$110 A reverse phase column, 250 x 4.6 mm i.d. Mobile phase consisted of (A) 3% aqueous acetic acid and (B) methanol with a gradient of 100% A for 1 min, 56%B for 24 min and 100% A for the next 2 min at a flow rate of 1.0 ml/min. Chromatograms were acquired at 280 nm.

#### 1.2.4.4 Thin layer chromatography of carotenoids fractions of *D. regia* flowers

The pigment fractions (CF and XF) obtained after systematic fractionation were dissolved in hexane. The extract was applied in a line on pre-coated Silica gel G plates (Merck). The plate was developed in a TLC chamber containing the solvent system hexane: acetone (7:3v/v) and flushed with $N_2$ gas. A few crystals of BHA were added to the solvent system to prevent auto-oxidation of carotenoids.

The individual bands were scraped off, dissolved in petroleum ether and applied on Silica gel G plates as spots and developed under conditions mentioned above.

#### 1.2.4.5 HPLC-MS analysis of carotenoids of *D. regia*

The carotenoids in the two fractions were resolved on a Phenomenex Gemini 5 µm $C_{18}$110 A reverse phase column, 250 x 4.6 mm i.d. Carotene fraction was separated using the mobile phase acetone (A) and 9:1 v/v methanol: $H_2O$ (B) with a gradient of 20% A for 0.01 min increasing to 80% A in 25 min to 35 min and returning to 20% A in the next 5 min. The column was equilibrated at 20% A for 5 min.

Xanthophyll fraction was separated using an isocratic mobile phase consisting of acetonitrile:methanol:dichloromethane (71:22:7v/v/v). The flow rate in both the analyses was set at 0.6ml/min. Chromatograms were acquired at 450nm.

For LC-MS, positive ion mode ESI was used. Samples were analysed in duplicate.

### 1.2.5 In vitro antioxidant activities

#### 1.2.5.1 Free radical (DPPH$^{\bullet}$) scavenging activity

The antioxidant activities of extracts were measured on the basis of their ability to scavenge the stable DPPH$^{\bullet}$. Different concentrations of the extracts in methanol (2.0 mL) were treated with 0.5 mL of 0.5 mM methanolic solution of DPPH$^{\bullet}$. Absorbance at 517 nm was determined after 20 min and the percentage scavenging activity was calculated against a reagent blank (Blois, 1958).

#### 1.2.5.2 Total reduction capacity

The reduction of ferric to ferrous ion by the extracts is an indication of the potential antioxidant property. The reducing power of the extracts was determined by the method of Gulcin (2006). Different concentrations of the extracts in methanol (1.0 mL) were diluted with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and mixed with 2.5 mL 1% potassium ferricyanide. After incubation at 50°C for 20 minutes, 2.5 mL of 10% TCA were added to the mixture. 2.5 mL of the reaction mixture was diluted with an equal amount of distilled water and absorbance was measured at 700 nm after treatment with 0.5 mL of 0.1% $FeCl_3$. Increased absorbance of the reaction mixture indicates an increase in reduction capability.

#### 1.2.5.3 Assay of hydroxyl radical scavenging activity

This test is based on the oxidation of 2-deoxyribose by OH$^{\bullet}$ (formed by the Fenton reaction) and its degradation to malondialdehyde (MDA) (Chung *et al.,* 1997). Different concentrations of the extracts were made up to 1.2 mL with 0.1 M phosphate buffer (pH 7.4) in test tubes and treated with 0.2 mL each of Fe-EDTA solution (10 mM each of $FeSO_4.7H_2O$ and EDTA), 2-deoxyribose (10 mM), $H_2O_2$ (10 mM) and ascorbic acid (10 mM). The reaction mixture was incubated at 37°C for 1 hr and the reaction was stopped by adding 1 mL of ice cold 2% TCA. One mL of 1% TBA

solution was added to the reaction mixture and placed in a boiling water bath for 15 min, cooled on ice, centrifuged and absorbance of the supernatant was measured at 535 nm.

#### 1.2.5.4 Nitric oxide scavenging activity

Aqueous sodium nitroprusside (SNP) at physiological pH spontaneously generates nitric oxide, a very unstable species that reacts with oxygen to produce nitrite ions. The extent of nitrite ions formed is measured using Griess reagent. In the present study, 0.5 mL of 10 mM SNP in phosphate buffered saline (PBS, pH 7.4) was incubated with different concentrations of extract in PBS (2.0 mL) for 180 min. After the incubation period, 0.5 mL of the reaction mixture was removed and diluted with 0.5 mL of Griess reagent (1:1 mixture of 1% sulphanilamide in 2% phosphoric acid and 0.1% of *n*-naphthyl ethylenediamine dihydrochloride) (Marcocci *et al.,* 1994). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with *n*-naphthyl ethylenediamide (Ilavarasan *et al.,* 2005) was checked by measuring OD at 546 nm after 10 min incubation.

#### 1.2.5.5 Anti-lipid peroxidation assays

Lipid peroxidation was assessed (Halliwell & Gutteridge, 1989) in rat brain, liver, liver mitochondria, testes and human erythrocyte ghost cells.

#### 1.2.5.6 Preparation of tissue homogenate and liver mitochondria

For the preparation of substrate, brain, liver and testes were obtained from normal Wistar strain rats and washed in ice-cold saline. The liver was perfused with ice-cold saline before homogenization. A 10% w/v homogenate of the tissues was prepared separately in ice-cold 1.15% KCl using a Teflon Potter–Elvehjem glass homogeniser. The homogenate was used for determination of lipid peroxidation. Rat liver mitochondria were prepared according to the method of Hogeboom (1955). Mitochondrial fraction was suspended in 1.15% KCl, so as to contain approximately 1 mg of protein per ml of suspension.

#### 1.2.5.7 Preparation of human erythrocyte ghost

Small amounts of human erythrocytes were prepared from 10 ml of freshly drawn blood by sedimentation, at unit gravity, through 4 volumes of 0.75% (w/v) dextran T-500 at room temperature in Tris-buffered saline (TBS), pH 7.5 (10 mM Tris-HCl and 150 mM NaCl). The cells were then washed three times in 10 volumes of TBS supplemented with 10 mM glucose, before being resuspended to a 20% (v/v) suspension, with respect to the packed-cell volume (Kuhlman, 2000 ).

Protein estimation in all these preparations was done by Lowry's method (1951).

### 1.2.5.8 Measurement of lipid peroxidation by thiobarbituric acid (TBA) assay

An aliquot containing 500 µg protein equivalent was used for lipid peroxidation assay, as described by Halliwell and Gutteridge (1989). Briefly, aliquots of the tissue homogenates were treated with different concentrations of the extract in methanol (0.5 ml) followed by 1.0 ml each of 10 µM $FeSO_4$ and 0.1 M ascorbic acid and incubated at 37°C for 60 min. One ml each of 28% trichloroacetic acid (TCA) and 1% TBA were added to the reaction mixture and heated for 15 min at 95°C. After cooling on ice and centrifuging the samples, the absorbance of the supernatant was read at 532 nm. The reaction mixture without the extract served as a control.

### 1.2.5.9 AAPH induced plasma oxidation

AAPH induced plasma oxidation and its inhibition by the extracts was monitored by the method described by Ljubuncic *et al.,* (2005) with minor modification. Human plasma from healthy donors (obtained from a local hospital/health center) was diluted (1:2) with PBS, pH 7.4. Aliquots (1.0 mL) of diluted plasma were treated with different concentrations (1-25 ppm) of the extracts in PBS and incubated with 0.25 mL of freshly prepared 200 mM AAPH at 37ºC for 2 h. At the end of the incubation period, plasma oxidation was stopped by the addition of 0.5 mL of ice-cold 20% TCA and 0.5 mL 1% TBA was added to each tube followed by heating at 100ºC for 20 min. After cooling on ice, the samples were centrifuged at 10,000 rpm (13000 × *g*) for 5 min and absorbance of the supernatant was read at 532 nm. Blanks of each sample were prepared and assessed in the same way to exclude any contribution due to background. Results were expressed in terms of spectrophotometric readings of the samples at 532 nm against a reagent blank.

## 1.3 Results and Discussion

### 1.3.1 Quantification of phytoconstituents

#### 1.3.1.1 Total anthocyanins and total phenolics

The results of spectrophotometric analyses of the *S. cumini* extract revealed a total phenolic content of 596 mg GAE/100 g and an anthocyanin content of 230 mg/100 g fruit on a dry weight basis (**Table 1.1**). The total anthocyanins in *S. cumini* are equivalent to that of blue berries and black currants that contain 230 and 229 mg/100 g, on a dry weight basis, respectively and are 1.6-times higher than that of black berries (141 mg/100 g) (Moyer *et al.,*, 2002). The blue and black berries have recently been regarded as premier nutraceutical commodities of great commercial value. Since *S. cumini* trees are perennial, with enormous yields of berries, the information found through the present study appears to partly complement its application in various nutraceutical products.

Table 1.1. The total anthocyanins ($T_{acy}$) and total phenolics (TP) in the whole plant material and in the anthocyanin extracts of *S. cumini* fruits and *D. regia* flowers.

| Extract | $T_{acy}$ | TP (GAE) |
|---|---|---|
| *S. cumini* fruits | 230mg/100g DW | 596mg/100g DW |
| *S. cumini* peel extract | 100% | -- |
| *D. regia flowers* (freshly harvested) | 825mg/100g DW | |
| *D. regia* flowers (Fresh, oven dried) | 580 mg/100g DW | 1.9g/100g GAE DW |
| *D. regia extract* | 50.33% | -- |

Total phenolics as high as 1.9 g GAE/100g and anthocyanin content of 580 mg/100g (dw) were found where the anthocyanins contributed to ⅓rd of the total phenolics of flowers (**Table 1.1**). The yield and respective total phenolics of the extracts of *D. regia* is summarized in **Table 1.2**. The yield in methanol was highest (31.8 g % DW) having a total phenolic content of 24.6 mg GAE/g which is probably because of the exhaustive nature of Soxhlet extraction, resulting in maximum removal of the phenolic compounds from the flowers. It was also noted that when the phenolic contents of all the successive extracts were summed up, the value rose to 33.5 mg GAE/g (DW), which is equivalent to ¼ of that reported in tea (131.9 mg/g) (Turkmen *et al.,* 2006). In this context, it is worth recalling that tea polyphenols have recently gained enormous commercial importance in various anti-oxidant formulations. It was also interesting to note that the fresh flowers yield high anthocyanin content (825mg/100g DW) whereas the flowers that gradually fall off the tree yield 580mg/100g DW. The latter is equivalent to the total anthocyanin

content of freshly plucked flowers which were subsequently dried before analysis. Therefore, anthocyanin can be extracted from those flowers and petals that are dropped from the trees without harvesting the inflorescence and thus avoiding the damage to the trees. Another interesting point to note was that anthocyanin content was not altered subsequent to carotenoids extraction thus pointing to the fact that the two classes of compounds can be extracted successively, without altering the composition of each other.

Table 1.2. The extract yield in different solvents and the total phenolic content in each of the extracts of *D. regia*.

| **Extract** | **Yield (g% dw)** | **TP (mg GAE $g^{-1}$ extract)** |
|---|---|---|
| Hexane | 3.3 | 0.5±0.01 |
| EtOAc | 3.1 | 1.3±0.01 |
| Acetone | 3.9 | 2.3±0.02 |
| MeOH | 31.8 | 24.6±0.03 |
| Water | 5.2 | 4.8±0.03 |
| Crude pigment | 6.3 | nd |
| CF | 4.6 | nd |
| XF | 1.2 | nd |

Total phenolics are determined in triplicates for each extract. Data are expressed as mean ±SD. TP = total phenolics; nd = not detected: CF=carotene hydrocarbon fraction; XF=xanthophylls fraction.



### 1.3.1.2 Carotenoid content in flowers of *D. regia*

Petals were found to contain 940mg//kg of total carotenoids and 660 mg/kg of β-carotene (70% of total carotenoids) by spectrophotometric method. This was further confirmed by HPLC comparing with the synthetic *all-trans*- β-carotene. β-carotene was found to be the major peak in the extract. The identity of β-carotene was confirmed by spiking with standard β-carotene. The HPLC chromatograms of the standard β-carotene and that of the extract are represented in **Fig. 1.2**. Results of HPLC analysis showed β-carotene content of 667 mg/kg of the dried flower petals which is strikingly similar to that obtained by spectrophotometry. Spiking of the sample with standard β-carotene showed a single peak suggesting that the major peak identified in the extract is indeed β-carotene. HPLC analysis of total carotenoids reveal that β-carotene amounts to 76% of total carotenoids while lutein is 4% suggesting the reliability of the mehods used for carotenoids estimations. The need for β-carotene for alleviating the vision disorder and improve

immunity in children of developing countries is well recognized. Since there is a high demand for carotenoids in food, pharmaceutical and cosmetic industries, the petals of *D. regia* appear to be a potential source, provided the safety issues are sorted out.

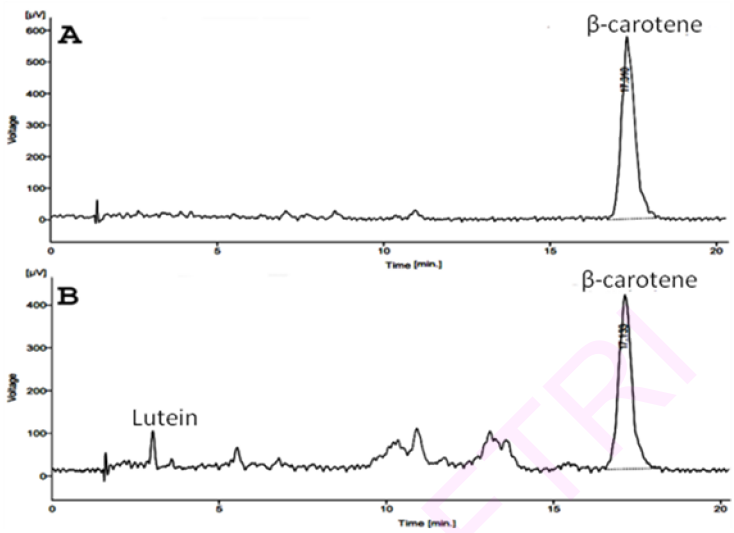



Fig. 1.2. HPLC chromatograms of (A) synthetic *all-trans*- β-carotene and (B) extract of *D. regia* flower petals. β-carotene was extracted according to the method described in AOAC International (1993) and analysed by HPLC as explained in section 1.3.5.

### 1.3.2 Characterisation of biomolecules

#### 1.3.2.1 Paper chromatography of anthocyanins

Paper chromatography of the lyophilized anthocyanin extract of *S. cumini* revealed the presence of three distinct mauve colored bands (**Fig. 1.3a**), well separated from each other while *D. regia* extract showed a single band (**Fig 1.3b**).

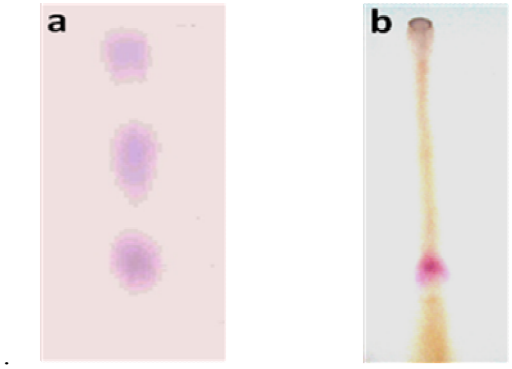



Fig. 1.3. Descending paper chromatograms of anthocyanin rich extracts of (a) *S. cumini* and (b) *D. regia*.

### 1.3.2.2 HPLC-MS analysis of anthocyanins

HPLC, coupled to MS, has been a powerful tool for the characterization of anthocyanins from various sources. A clear separation was achieved by direct injection of the partially purified extract in HPLC (**Fig. 1.4**).

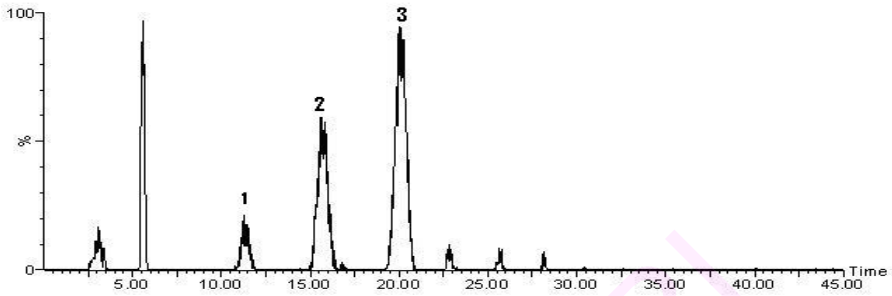



Figure 1.4. HPLC/MS separation of anthocyanins of *S. cumini*. 1) delphinidin-diglucoside; 2) petunidin-diglucoside; 3) malvidin-diglucoside. Detection was done at 520nm

Three major compounds were identified as glucoglucosides of delphinidin (1), petunidin (2) and malvidin (3), based on previous work (Venkateswarlu, 1952) and also supported by their respective MS. The major anthocyanins, corresponding to peak 1, 2 and 3, represented about 23%, 35% and 38%, respectively of the total peak area revealed at 520 nm. ESI–MS of each peak resulted in clear and characteristic fragmentation patterns (**Table 1.3**).

A typical ESI positive MS shows two ions: the protonated molecular ion $[M + H]^+$ and a fragment ion $[M + H - X]^+$ arising from loss of the sugar moiety. However, since the anthocyanins have a natural residual positive charge, one observes a true molecular ion $[M]^+$ and a fragment ion $[M-X]^+$ which is of the underivatised aglycone. The value of X, based on the difference between the molecular ion and fragment, gives a clue to the nature of the sugar molecule (Abdel-Aal *et al.,* 2006).

The compound 1 produced ions at m/z 627, m/z 465 and m/z 303. Similarly, compound 2 showed ions at m/z 641, m/z 479 and m/z 317 and compound 3 produced ions at m/z 627, m/z 493 and m/z 331 (**Table 1.3 and Fig. 1.5A-C**). This suggests that the aglycones are delphinidin (m/z 303), petunidin (m/z 317) and malvidin (m/z 331) for compounds 1, 2 and 3, respectively and the differences of m/z 162 and m/z 321 from the aglycone in the two fragments (in all three compounds) suggest the presence of 2 hexoses. The mass spectrum of these compounds revealed fragments resulting from the sequential loss of two glucose [$M^+$-162 Da] molecules. However, since both the sugar units are glucose (confirmed by paper chromatography of the acid hydrolysate in comparison with the authentic standards), it is not possible to determine the

sequence of their elimination (Oliveira *et al.,* 2001). It should be noted that in the absence of authentic anthocyanin standards whose structures are identified by proton or carbon-13 nuclear magnetic resonance spectrometry, the utilization of HPLC-MS cannot determine the points of attachment of the disaccharide units to the aglycone.

Table 1.3. Assignment of $M^+$ ions for the anthocyanins of S. cumini fruit peel and their fragments.

| **Compound** | **Retention time (min)** | **ESI(+)-MS/MS *m/z*** | | |
|---|---|---|---|---|
| | | **$M^+$** | **$[M^+$-Glu$]^+$** | **$[M^+$-Glu-Glu$]^+$** |
| Delphinidin-diglucoside | 11.2 | 627 (100) | 465 (40) | 303 (100) |
| Petunidin-diglucoside | 15.6 | 641 (90) | 479 (50) | 317 (100) |
| Malvidin-diglucoside | 19.8 | 655 (90) | 493 (50) | 331 (100) |

Values in parenthesis represent the relative abundance of the ions.

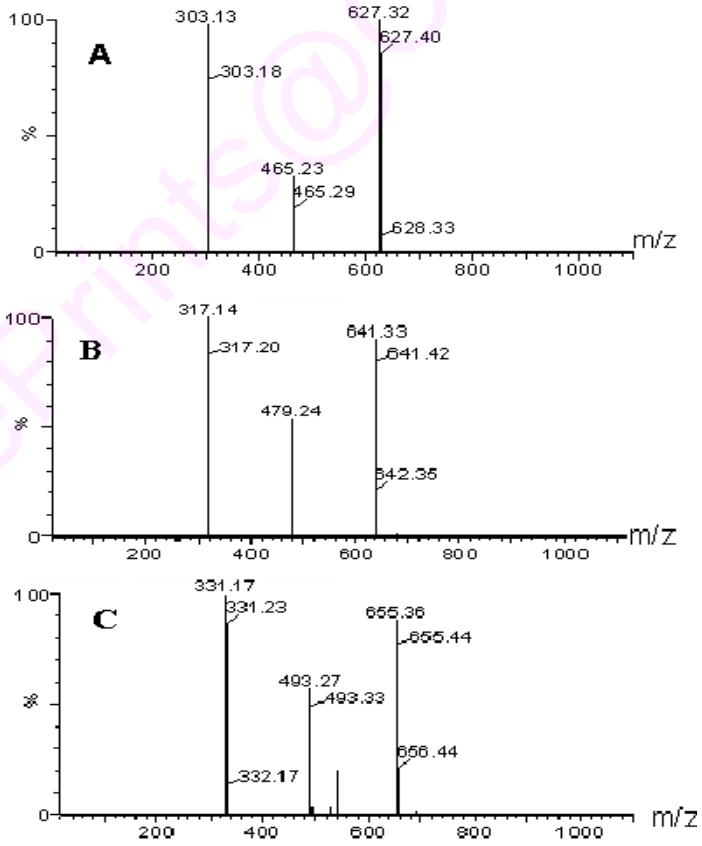



Fig. 1.5. ESI-MS spectra of A) delphinidin-diglucoside; B) petunidin-diglucoside; C) malvidin-diglucoside from *S. cumini* fruit peel extract.

Upon paper chromatography of the anthocyanin extract, only three anthocyanin pigments of intense mauve colour were observed. When the methanolic solutions of individual spots were analysed by ESI–MS, only the same three anthocyanins were observed as were identified in the partially purified total extract. Though cyanidin was detected through single ion monitoring, it could not be assigned to a specific peak in the chomatogram and a corresponding anthocyanin was not identified. However, a research paper published later than this work revealed the presence of two additional anthocyanins, 3, 5-diglucosides of peonidin and cyanidin (de Brito *et al.,* 2007) along with the ones identified in the present study. The anthocyanins identified were not acylated as confirmed by alkaline hydrolysis followed by HPLC-MS. This finding was further supported by the work of de Brito *et al.,* (2007).

**1.3.2.3 Presence of phenolics in the anthocyanin extract**

Anthocyanin extract obtained through column chromatography was a highly concentrated one and contained solely anthocyanins (**Table 1.1**). The anthocyanin extract was partitioned with ethyl acetate before its application on XAD-7 column. This was done in order to ensure a highly concentrated anthocyanin extract devoid of interfering phenolics and other compounds. The ethyl acetate fraction obtained was further concentrated and subjected to HPLC since most phenolics and flavonoids like quercetin are soluble in this solvent. In order to rule out the possibility of other phenolics being present in the anthocyanin extract, it was also subjected to HPLC. The HPLC pattern of the phenolic components of the anthocyanin extract and the ethyl acetate fraction are shown in Fig. 1.6a and b.

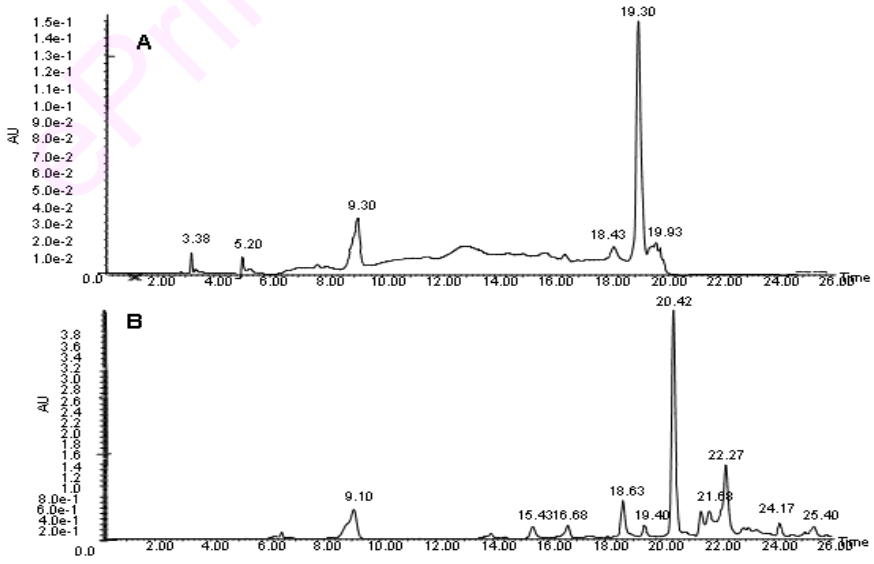



Fig. 1.6. LC/MS chromatograms of (a) anthocyanin extract (b) ethyl acetate fraction of anthocyanin extract. Chromatograms were recorded at 280nm.

The ethyl acetate fraction of the anthocyanin extract exhibited more peaks with higher intensity than did the anthocyanin extract. This suggests that most of the other phenolics were extracted into ethyl acetate fraction during the partial purification of anthocyanin extract before its application onto the Amberlite XAD-7 column. A comparison of the X-axes of the chromatograms of anthocyanin extract and the ethyl acetate fraction, analysed under identical conditions, shows that the height of the highest peak ($1.5e^{-1}$ on x-axis) corresponding to the phenolics in the anthocyanin extract was less than the most minimum value exhibited by the ethyl acetate fraction ($2e^{-1}$ on x-axis). This shows that most of the other phenolics and phenolic acids have been extracted into ethyl acetate during the partitioning prior to passage through XAD indicating that the anthocyanin extract of *S. cumini* contains negligible amounts of other phenolics and that the activity of the extract might be essentially due to the anthocyanins alone.

### 1.3.2.4 Thin layer chromatography of carotenoids fractions of *D. regia* flowers

Thin layer chromatography of XF and CF on silica gel G plates showed several distinct bands. Carotene fraction showed 3 visibly intense bands (**Fig 1.7a & 1.7b**) while the XF showed 6 major and 2 minor bands (**Fig 1.7c & 1.76d**).

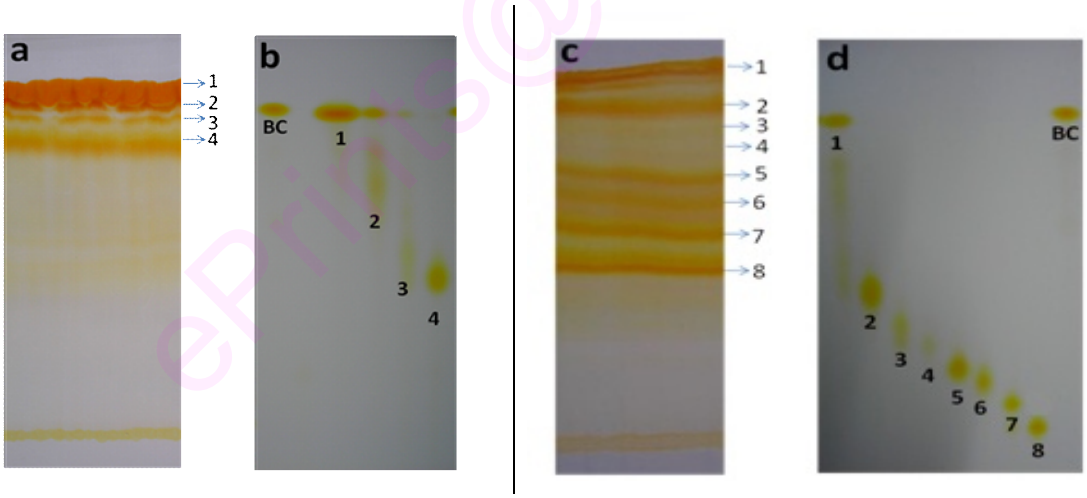



Fig. 1.7. Thin layer chromatography of CF (a & b) and XF (c & d) on silica gel G plates. The individual bands from respective plates were scraped off, dissolved in petroleum ether and spotted on TLC plates (b & d).

### 1.3.2.5 HPLC-MS analysis of carotenoids of *D. regia*

The carotenoids of different floral parts of *D. regia* were extensively studied by Jungalwala and Cama (1962). They identified the presence of β-carotene as the major carotenoids in the floral petals comprising of 34% of total carotenoids. Nine well characterized epoxycarotenoids were

identified in the petals along with a few unidentified epoxycarotenoids and other carotenoids. Anthers and pollen mainly contained xanthophylls, the chief xanthophylls being zeaxanthin (90% of total carotenoids in anthers). There has not been a more extensive study on the carotenoids of flower parts of *D. regia* elsewhere. The aim of characterizing carotenoids in the present study was mainly restricted to the two fractions (CF and XF) used for the activity studies. This was in order to establish a connection between the antioxidant activity and the specific carotenoids present in the particular fraction. However, no quantitative estimation was carried out for individual carotenoids identified. In the absence of standard carotenoids, only the major carotenoids are tentatively identified using fragmentation pattern of the compounds and comparing the same with available literature data. However, β-carotene was confirmed by comparing with the standard. The characteristic visible absorption spectra of most peaks were compared with the available literature (Azevedo-Meleiro and Rodriguez-Amaya, 2004) to substantiate the identity.

HPLC coupled to mass spectrometry has been an elegant solution for fast and unambiguous identification of carotenoids and is employed in analysis of various plant extracts and biological samples (Kurz *et al.,* 2008). Owing to their non-polar nature, carotenoids are resistant to ionization thus making their detection by mass spectrometry difficult. Several LC-MS techniques have been used for carotenoids analysis including continuous flow-fast atom bombardment (CF-FAB), electro-spray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The present study utilized the ESI mode of detection to identify the carotenoids present in the two fractions. ESI has an advantage of being both an ionization technique and a solvent removal interface. During electrospray, a fine mist of charged droplets is formed from the HPLC eluate at atmospheric pressure by spraying solution through a capillary electrode at high potential. As the charged droplets are electrostatically attracted towards the opening of the mass spectrometer, they encounter a cross-flow of heated nitrogen curtain gas that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. When the droplets shrink in size until the electrostatic repulsion between ions in each droplet exceeds the combined energy of salvation and surface tension, ions are ejected from the droplets into the gas phase or else the droplet disintegrates and releases analyte ions (van Breeman 1997). The HPLC-MS chromatogram of CF is shown in **Fig. 1.8**. This fraction consists of carotene hydrocarbons and mono hydroxyl xanthophylls along with their mono- and di-epoxy derivatives (**Fig. 1.1**). Three prominent peaks were observed in the HPLC chromatogram of CF. The major peak (3) was identified to be *all-trans*-β-carotene based on its molecular ion (m/z 536.94). This makes up 50% of the total area in the chromatogram. Due to the unusual ionisation process, electrospray of carotenoids produces abundant molecular cations ($M^{\bullet+}$) with little fragmentation and no

molecular anions (van Breeman 1997). The identity of peak 3 was confirmed by using the synthetic *all-trans*-β-carotene and also by spiking the extract with standard. It also showed the characteristic visible absorption spectrum at 450nm with λmax at 428, 455 and 480nm. Therefore, it appears that *all-trans*-β-carotene is the major carotenoid present in the CF of *D. regia*.

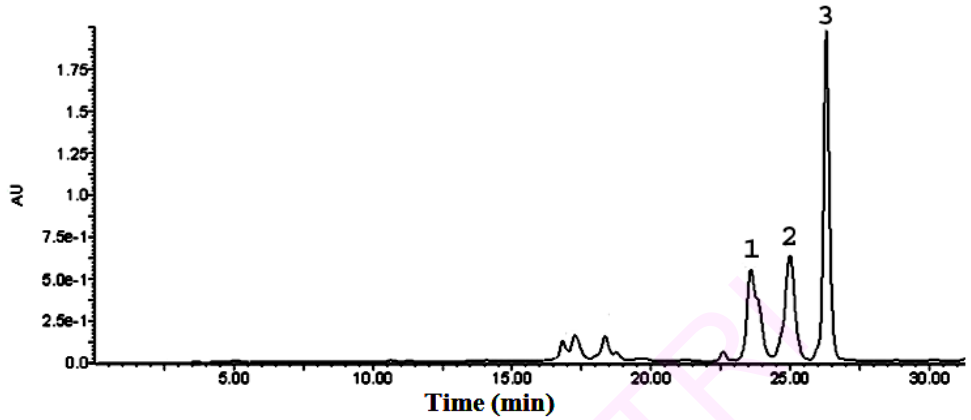



Figure 1.8. HPLC chromatogram of CF. The peaks are tentatively identified as (1) α-carotene (2) γ-carotene (3) β-carotene.

Recent years have seen an upsurge in interest towards *trans*-β-carotene. It is found to be preferentially absorbed over 9-*cis*-β-carotene in humans (Ben-Amotz and Levy, 1996) and ferrets (Erdman *et al.,* 1998). Beneficial effects of β-carotene have been extensively studied by various researchers and are discussed elsewhere in the thesis. Considering the high concentrations of β-carotene in the extract it can be an excellent source of provitamin A and hence can be used in nutraceutical and antioxidant formulations. Interestingly all the three peaks showed the same molecular ion at m/z 356 suggesting the presence of isomers. The peak 1 was tentatively assigned the structure of α-carotene chiefly based on its visible absorption spectrum with λmax at 425, 448 and 476nm which was in agreement with that of Azevedo-Meleiro and Rodriguez-Amaya (2004). Similarly a tentative identification of peak 2 was made as γ-carotene which showed a clear visible spectrum with λmax at 438, 462 and 494nm. Peak 1 and 2 constitute 20% and 22% respectively of the total area of the chromatogram (**Fig. 1.8**). However, the structures of peak 1 & 2 are not absolute. In the absence of authentic standards whose structure has been confirmed, it is not possible to assign an identity to a particular peak since ESI affords very little fragmentation and the isomers of β-carotene possess the same molecular mass as that of β-carotene.

The xanthophylls rich fraction is mainly composed of di- and poly-hydroxyl xanthophylls and their epoxy derivatives (**Fig. 1.1**). The HPLC-MS chromatogram of XF is shown in **Fig. 1.9**.

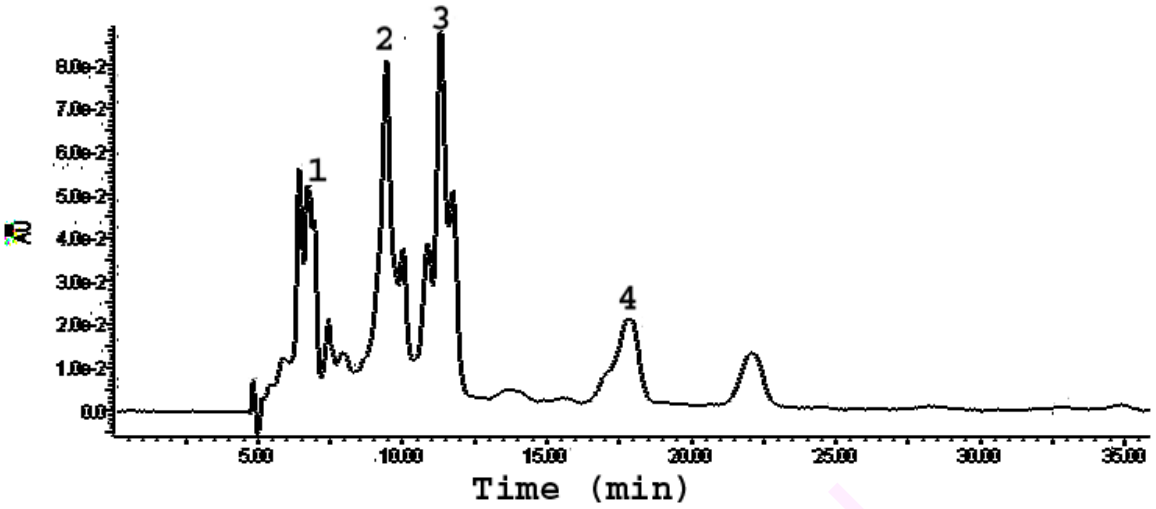



Figure 1.9. HPLC chromatogram of XF. The peaks are tentatively identified as (1) astaxanthin (2) lutein (3) zeaxanthin (4) β-cryptoxanthin

Several well resolved peaks were obtained when XF was analysed by HPLC. However, only a few of them are tentatively identified due to the ambiguity in the identification of the others. Four major peaks were tentatively identified based on their molecular mass and the retention time by comparing with the available data in the literature. Compound 1 was resolved at RT 6.7 min and showed a molecular mass of the potassium adduct of astaxanthin at 635 (M + K) and its molecular ion at m/z 596.17. Compound 2 had a retention time of 9.45 min showing a molecular ion at m/z 569.21 suggesting the presence of lutein followed by zeaxanthin (3) at 11.33 min which showed a molecular ion at m/z 569.20. Compound 4 eluted at 17.87min and was identified to be β-cryptoxanthin which showed a molecular ion at m/z 553.19. Based on the data available on the elution pattern of xanthophylls and comparing their corresponding molecular mass, the four major peaks were tentatively identified as astaxanthin, lutein, zeaxanthin and β-cryptoxanthin.

The first report on the carotenoids analysis of different flower parts of *D. regia* was made by Jungalwala and Cama (1962) who extensively studied the presence of an array of carotenoids and xanthophylls in the flower petals as well as other floral parts. They reported β-carotene as the major carotene hydrocarbon followed by γ-carotene which was nearly half that of β-carotene. However, they did not identify the presence of α-carotene. The major xanthophylls identified by Jungalwala and Cama (1962) were lutein and zeaxanthin and relatively small amounts of β-cryptoxanthin. This is in agreement with the present study where lutein and zeaanthin were found to be the major compounds. Astaxanthin was also tentatively identified in the present study, however was not reported earlier. The study however suggests that *D. regia* is a rich source of

mixture of pharmacologically important carotene hydrocarbons and xanthophylls and thus making it a potential source of carotenoids.

### 1.4 *In vitro* antioxidant activities

A detailed study on the antioxidant activities of different standard antioxidants was carried out before screening the extracts. Four well-known antioxidants were chosen viz., ascorbic acid (AA), gallic acid (GA), butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT). The standard antioxidants were tested using three antioxidant assay systems viz., DPPH radical scavenging assay, AAPH induced plasma oxidation assay and hydroxyl radical scavenging assay and the results are summarized in **Table 1.4.**

Table 1.4. Antioxidant properties of standard antioxidants as tested by different model systems.

| Assay system | Conc. (ppm) | % antioxidant activity | | | |
|---|---|---|---|---|---|
| | | AA | GA | BHA | BHT |
| **DPPH• model** | 1 | 17.4±0.9 | 41.5±0.1 | 22.5±0.6 | 13.2±2.9 |
| | 5 | 68.2±3.2 | 93.8±0.2 | 68.4±1.1 | 45.7±2.1 |
| | 10 | 96.1±0.4 | 94.1±0.1 | 88.5±0.7 | 71.4±0.7 |
| | 25 | 96.5±0.2 | 94.3±0.1 | 91.6±0.4 | 88.7±0.5 |
| | 50 | 96.5±0.1 | 94.8±0.2 | 91.3±0.2 | 91.3±0.5 |
| | 100 | 96.5±0.1 | 95.2±.1 | 91.3±0.6 | 91.7±0.1 |
| **AAPH - induced plasma oxidation** | 5 | 39.9±1.0 | 51.7±1.0 | 34.6±1.5 | 66.8±3.5 |
| | 10 | 50±0.5 | 63.6±1.0 | 59.8±0.5 | 71.7±0.5 |
| | 25 | 61.2±3.5 | 74.1±2.0 | 76.9±2.4 | 76.6±0.5 |
| | 50 | 63.9±0.5 | 71.4±0.2 | 83.1±1.6 | 79.7±1.0 |
| | 100 | 67.8±1.0 | 71.7±1.5 | 86.5±0.0 | 83.2±1.0 |
| **OH• scavenging assay** | 5 | * | 69.9±4.1 | 77.6±0.7 | 69.1±0.3 |
| | 10 | * | 81.6±0.5 | 84.1±0.2 | 85.9±0.1 |
| | 25 | * | 92.1±0.3 | 94.8±0.7 | 95.2±0.7 |
| | 50 | * | 97.2±1.0 | 98.7±0.3 | 99.4±0.1 |
| | 100 | * | 101.5±1.0 | 103.2±0 | 106.4±0.2 |

The antioxidant activities of the extracts of *S. cumini* and *D. regia* against various free radicals were analysed using different in vitro cell-free and cell based antioxidant model systems.

### 1.4.1 Free radical ($DPPH^{\bullet}$) scavenging activity

$DPPH^{\bullet}$ scavenging assay has been found useful for rapid analysis of free radical quenching efficacies of various plant extracts. $DPPH^{\bullet}$, a stable free radical with an unpaired electron, shows a strong absorption band at 517nm, its solution appearing deep violet color. As the electron becomes paired off, which happens in the presence of an antioxidant (electron/hydrogen donor),

the absorption vanishes (Blois, 1958). Thus, faster the reaction, more potent is the free radical scavenging ability.

In the present study, the anthocyanin extract of *S. cumini* extract quenched the DPPH• in a dose dependent manner (**Fig 1.10**).

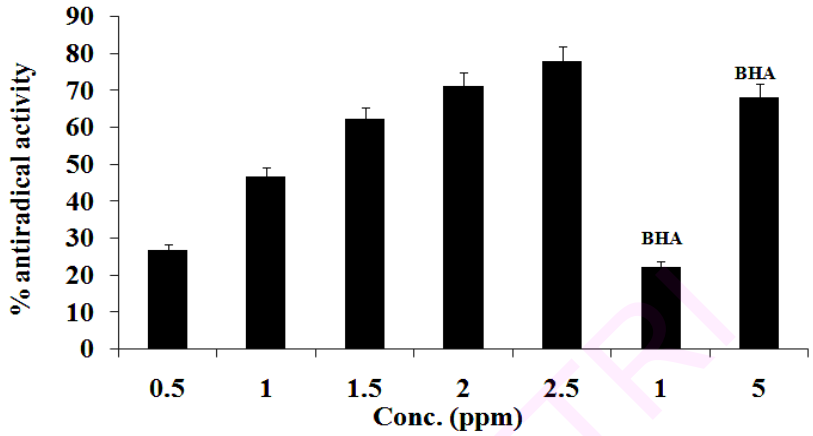



Fig. 1.10. DPPH• scavenging activity of anthocyanin-rich extract of *S. cumini* fruit peel. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$.

The *in vitro* activity was compared with the standard synthetic antioxidant butylated hydroxyl anisole (BHA). At 1.0 ppm the activity of *S. cumini* extract was 46.75% while BHA exhibited only 22.5% activity (50% lesser than that of the extract) at the same concentration (**Fig 1.10**). At 2 ppm, the activity of the anthocyanin extract was equivalent to that of 5 ppm BHA in scavenging DPPH• radical. This shows that *S. cumini* extract is a potent free radical scavenger compared to the widely used BHA. A recent study has reported a 50% DPPH• scavenging with 168µg/mL of the hot aqueous infusion prepared from fruit peels of *S. cumini* dried for 7 days (Banerjee *et al.,* 2005). However, our study establishes that the fresh fruit peel extract offers the same effect at as low as 1.39µg/mL suggesting that the drying and heating process applied in the preparation of infusion might lead to the loss of thermo-labile bioactives like anthocyanins and other polyphenols. Similarly, the antioxidant activity cannot be solely attributed to the anthocyanins since during the extraction of the skin other phenolics and flavonoids also come into solution. However, the extract used in the present study is a highly concentrated anthocyanin-rich one, without the interference of other class of compounds as evidenced by HPLC analysis (Veigas *et al.,* 2007). Therefore, the activity at extremely low concentrations can be endorsed to the anthocyanins.

The DPPH$^{\bullet}$ scavenging assay was carried out first to check the antioxidant property of the different extracts of *D. regia*. These flowers were found to be rich source of polyphenols, methanol extract yielding the highest polyphenol content (**Table 1.2**). Therefore, a comparison was made of the total phenolic content and DPPH scavenging property of methanol extracts of few well known plant extracts and are presented in **Table 1.5**, in order to provide information about *D. regia* in scavenging free radicals and the effect of polyphenols thereof.

Table 1.5. A comparison of the total phenolics (TP) and DPPH radical scavenging activity among different plant materials of known radical scavenging properties.

| **Plant species** | **Part used** | **TP(mg GAE/g)** | **Conc. (ppm)** | **DPPH scavenging** | **Reference** |
|---|---|---|---|---|---|
| *Delonix regia* | Flowers | 169.67 | 100 | 71.93% | (Aqil *et al.*,.,2006) |
| | Petals | 33.5 | 100 | 84.17% | (Present study) |
| *Camellia sinesnsis* | Leaves | 130.6 | 4.14 | 50% | (Turkmen *et al.,* 2006) |
| *Punica granatum* | Fruit peel | 44.0 | 100 | 95% | (Singh *et al.*,.2002) |
| *Vitis vinifera* | Pomace | 35.7 | 100 | 90% | (Murthy *et al.*,.,2002) |
| *Ginkgo biloba* | Extract (EGb761) | -- | 45.1 | 50% | (Silva *et al.*,.,2005) |
| *Panax notoginseng* | Root | -- | 3000 | 50% | (Zhao *et al.*,.,2006) |

Though the methanol extract (100ppm) of the flowers of *D. regia* showed about 72% DPPH scavenging activity in an earlier study (Aqil *et al.,* 2006), analysis in the present study showed that the acetone, methanol and water extracts exhibit more than 80% DPPH scavenging. However, the carotenoids pigment extracts were active only at concentrations above 250 ppm (**Fig.1.11**). This may be attributed to the non-polar nature of the carotenoids which perhaps fail to exert an antioxidant effect in a polar environment.

Comparison of the activity with the standard antioxidants such as ascorbic acid, gallic acid, BHA and BHT (**Table 1.4**) revealed that the water extract of *D. regia* at 100 ppm was equivalent to 50 ppm of almost all the standards. The $IC_{50}$ of the hexane, EtOAc, acetone, MeOH, $H_2O$, crude extract, carotene hydrocarbon fraction and xanthophyll fraction were respectively 213.6, 53.0, 72.9, 55.9, 29.5, 103.4, 228.7 and 366.3, all in ppm, when compared with BHA ($IC_{50}$ 3.6 ppm). All the samples inhibited radical formation in a concentration dependent manner with the water extract being the most active followed by EtOAc, MeOH and acetone extract (**Fig. 1.11**).

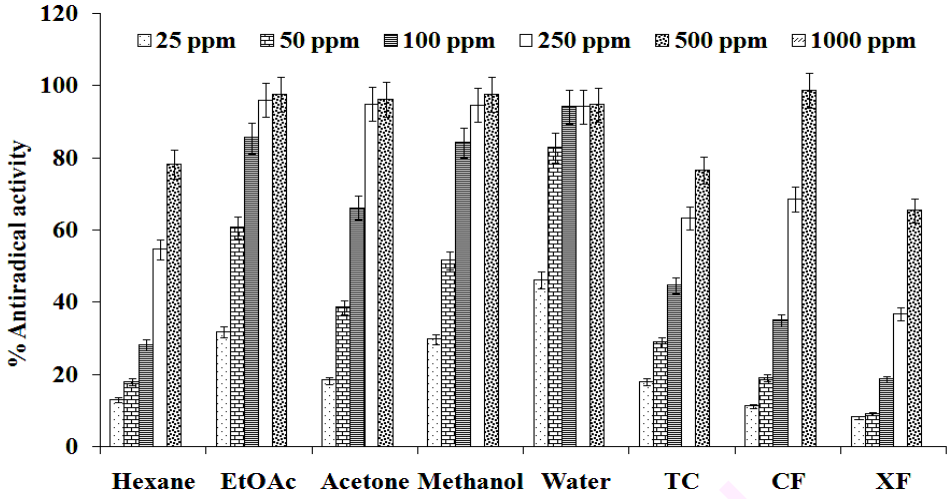



Fig. 1.11. DPPH• scavenging activity of extracts of *D. regia* flowers. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at p≤0.05. TC = total carotenoids; CF = carotene fraction; XF= xanthophyll fraction

The high antioxidant activity of water extract may be attributed to the presence of water-soluble antioxidants like phenolic acids. The relationship between the antioxidant activity of methanol extract and total phenolics of *D. regia* is represented in **Fig. 1.12**.

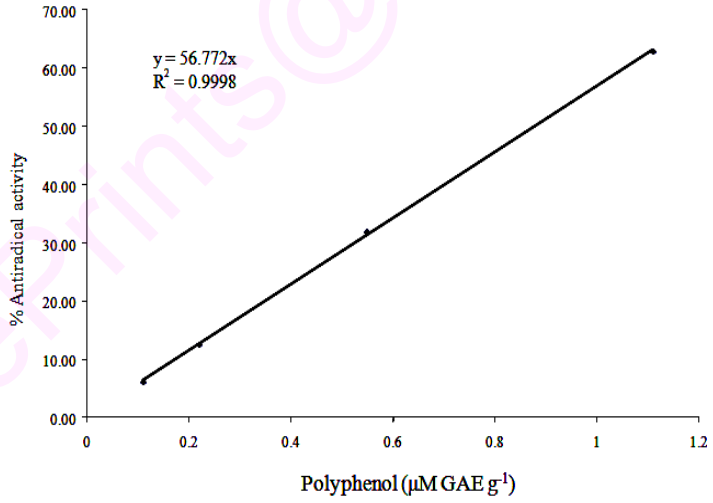



Fig. 1.12. Relationship between the polyphenol content and antioxidant activity of *D. regia*. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at p≤0.05. The polyphenol content is expressed as µM GAE/g of flowers.

There is a strong correlation between the antioxidant activities as measured by DPPH• radical scavenging and total polyphenol content ($R^2$ = 0.9998) suggesting that polyphenols are likely to contribute to the antiradical activity. This was especially so in methanol and water extracts, as

both are highly polar. In another similar study on black tea there was a strong correlation between antioxidant activity and polyphenol content ($R^2$ = 0.989) (Miliauskas *et al.,* 2004).

### 1.4.2 Reducing power assay

In this assay, the yellow color of the test solution changes to various shades of blue and green depending upon the reducing capacity of the test compound. The reduction capacity of the extracts is directly proportional to the green/blue color produced due to the reduction of $Fe^{3+}$/ferricyanide complex to the ferrous ($Fe^{2+}$) form. The $Fe^{2+}$ was monitored by measuring the formation of Perl's Prussian blue at 700nm (Gulcin *et al.*, 2005).

The reducing activity of *S. cumini* anthocyanins was compared with and expressed as ascorbic acid equivalents. Some investigators report a parallel association of the antioxidant activity with the development of reducing power (Duh, 1998). Reducing power of $T_{acy}$ of *Perilla pankinensis* has been found to increase with an increase in the anthocyanin concentration (Gulcin *et al.,* 2005). The $Fe^{3+}$- $Fe^{2+}$ transformation in the presence of the anthocyanin-rich extract of *S. cumini* increased with the increase in concentration of the extracts (**Fig 1.13**).

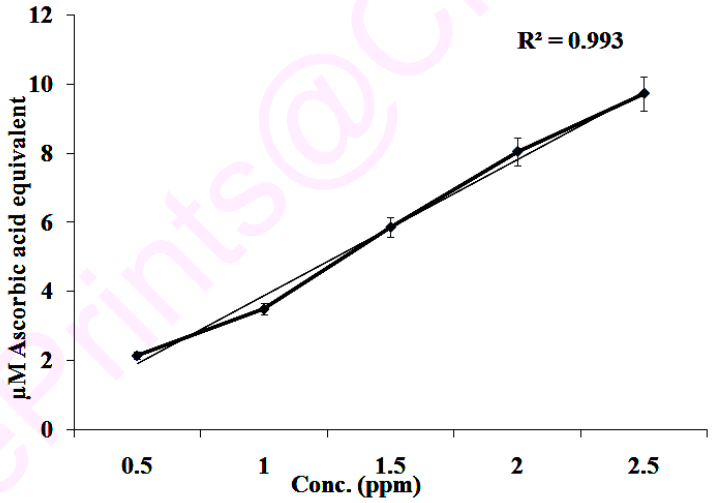



Fig. 1.13. Reducing power of anthocyanin rich extract of *S. cumini* fruit peel. Three independent experiments were performed in triplicates at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$.

It was observed that 1 ppm of the extract was equivalent to 3.5µM of ascorbic acid in bringing about $Fe^{3+}$- $Fe^{2+}$ transformation. The reducing potential of anthocyanins can also be ascribed to their metal chelating action. They are capable of forming anthocyanin-metal complexes with copigments such as ascorbic acid thus protecting ascorbic acid against metal-induced oxidation (Sarma *et al.,* 1997). Similarly, Chen and Ahn (1998) maintained that natural phenolics such as quercetin, rutin, catechin and caffeic acid acted as iron chelators. Therefore, it can be suggested

that anthocyanin extract of *S. cumini* acts mainly via iron chelation in imparting effective antioxidative potential.

A linear relationship between the DPPH radical scavenging capacity as well as the ferric reducing capacity and the anthocyanin concentration of the extract of *S. cumini* was observed with a regression of $R^2$=0.954 and $R^2$=0.993 respectively for the two assay methods. This shows that the activity is a factor of concentration of the anthocyanins. However, the activity may cease to increase after a concentration threshold is reached as happens in most cases. The present study has demonstrated that DPPH scavenging activity by standard antioxidant such as ascorbic acid, gallic acid, BHA and BHT reach a maximum at a concentration of 10, 5, 25 and 50 ppm respectively. An increase in concentration beyond this point does not increase the antioxidant capacity in vitro.

**Figure 1.14** represents the reducing power of the successive extracts and carotenoid extracts of *D. regia*. The $Fe^{3+}$-$Fe^{2+}$ transformation in the presence of the extracts increased in a concentration dependent manner. The reducing power of the standard/extracts is in the following order: EtOAc ≈ XF> ascorbic acid > MeOH > hexane > acetone > $H_2O$ > CF> crude extract. The reducing power of the extracts may be due to their hydrogen donating ability (Shimada *et al.,* 1992).

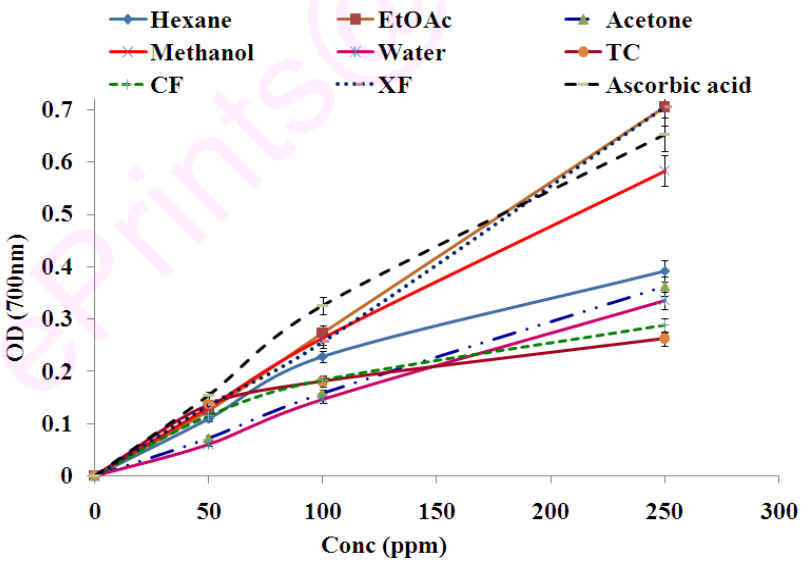



Fig. 1.14. Reducing power of extracts of *D. regia* flowers.Three independent experiments were performed in triplicate at each concentration and data presented as the average where the deviation was within 5%. TC = total carotenoids; CF = carotene fraction; XF= xanthophyll fraction.

### 1.4.3 In vitro lipid peroxidation

The introduction of phenolic compounds into the lipid-rich biomembranes effectively inhibits the lipid oxidation caused by the chain-propagating lipid peroxyl radicals and ferrous generating enzymatic systems present on them (Pazos *et al.,* 2006). Anthocyanins from various sources, being polyphenolic in nature, are known to cause similar effects against oxidative reactions (Cooke *et al.,* 2005) arising from endogenous and exogenous sources. Consumption of anthocyanins is known to increase serum antioxidant potential in experimental animals as well as in human subjects (Cao *et al.,* 1998; Mazza *et al.,* 2002). Furthermore, Ramirez-Tortosa *et al.,* (2001) reported that an anthocyanin-rich extract decreased hepatic lipid peroxidation in vitamin E-depleted rats. The use of homogenized liver/brain tissue works as a rapid and convenient bench top bio-assay method for preliminary analysis of the activity of the compounds of interest which can be further confirmed using *in vitro* cell culture and *in vivo* models. In the present study, the *S. cumini* extract was tested for its ability to inhibit the iron-induced lipid peroxidation in rat brain, liver, liver mitochondria, testes and human erythrocyte ghost cells. **Table 1.6** summarises the effect of *S. cumini* anthocyanins on iron induced lipid peroxidation in different tissue systems. The data indicated that the pigment extract was most effective (94%) against brain lipid peroxidation at 5.0 ppm compared with rat liver (83%), mitochondria (86%) and testes (72%) with erythrocyte ghost cells (48%) being least responsive to anthocyanin treatment. This could possibly be due to the involvement of a different mechanism in erythrocyte membranes, which may act as barriers to large molecules.

Table 1.6. Anti lipid peroxidation activity of anthocyanins rich extract of *S. cumini* fruit peel

| Conc.(ppm) | % Protection against lipid peroxidation | | | | |
|---|---|---|---|---|---|
| | **Brain** | **Liver** | **Testes** | **LM** | **EG** |
| 0.5 | 68.32±0.22 | 26.05±1.55 | 25.78±1.65 | 25.46±1.57 | 5.86±0.02 |
| 1 | 71.21±2.31 | 57.36±1.08 | 43.24±0.33 | 44.06±0.85 | 32.44±0.65 |
| 1.5 | 73.56±1.25 | 62.64±1.23 | 50.06±0.98 | 67.11±1.23 | 35.15±1.68 |
| 2.0 | 74.72±1.54 | 67.22±0.98 | 56.68±1.65 | 70.54±1.66 | 38.81±2.03 |
| 2.5 | 86.65±1.80 | 70.03±1.55 | 63.44±2.02 | 72.77±2.56 | 40.75±1.65 |
| 5.0 | 94.45±0.11 | 83.33±1.33 | 72.25±2.65 | 86.33±2.05 | 48.55±1.23 |

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD. LM=liver mitochondria; EG=Erythrocyte ghost cells.

From the data it can also be observed that the $EC_{50}$ of the extract was less than 0.5 ppm for brain homogenate while it was more than 5.0ppm for erythrocytes. This was intriguing given the fact that brain tissue is highly lipid rich while anthocyanins are not lipophilic. This may be explained by the direct interaction of the lipid radicals with the anthocyanins thus eliminating the peroxidative effect, which perhaps does not happen within a living system where the antioxidant molecules have to cross several lipid rich biological membranes. The homogenates of liver and liver mitochondria were almost equally protected against peroxidation by the anthocyanins extract indicating a possible similarity in the mechanism involved (**Table 1.6**). BHA showed approximately 80% activity against all the systems (data not shown), which is very close to but lower than that of the anthocyanins. The extent of protective effect exerted by the anthocyanins in different tissues is in the following order: rat brain>liver mitochondria> liver> testes> human erythrocyte ghost cells. Banerjee *et al.,* (2005) reported the requirement of a 400 times higher concentration (222μg/ml) of the hot water extract of dried peel of black plum to cause similar effect, with a further reduction of activity when the dried fruit peel was stored for 6 months at 40°C (Banerjee *et al.,* 2005). The lower efficacy in the latter study may be attributed to the loss of anthocyanins during storage at high temperature as well as during the heat processing involved in the preparation of the extract. This is affirmed by the fact that anthocyanins content in the dried fruit peel of *S. cumini* was drastically reduced during the 6-monts storage period (Banerjee *et al.,* 2005). The present study utilizes a partially purified, highly concentrated, freeze dried extract of *S. cumini* fruit peel which chiefly contains anthocyanins. This is one reason why the *S. cumini* extract shows high activity at substantially low concentrations when tested in vitro compared to the other reports (Banerjee *et al.,* 2005). Through the present study it has been clearly established that the fresh peel extract of *S. cumini* is a powerful antioxidant due to its high anthocyanin content. Being a natural color, it can be considered one of the healthy alternatives to synthetic dyes for applications in food, pharmaceutical and nutraceutical formulations.

In the past few decades, several epidemiological studies have revealed an inverse correlation between consumption of carotenoid rich foods and risk of several chronic diseases including cardiovascular and photosensitivity diseases, cataracts, age-related macular degeneration and some cancers (Palozza, 1998; Johnson, 2001). There are no evidences of toxic symptoms of β-carotene in normal subjects even under long-term administration at pharmacological doses. Conversely, β-carotene is known to act as a tumor promoter in subjects with pre-existing cancer lesions induced by chronic tobacco smoking (Diplock, 1997). According to Langseth (1995), carotenoids and vitamin C are the major contributors to the apparent cancer-protective effects of the foods. In the present study, the MDA formation, an index of lipid peroxidation, significantly

reduced when successive extracts were incubated with the tissue homogenates prepared from brain and kidney, in a concentration dependent manner with all the extracts except for hexane extract. *n*-hexane extract showed an inverse relation to the activity against brain lipid peroxidation (**Table 1.7**). The activity markedly reduced from 83.1% (at 1 ppm) to 42.9% (at 25 ppm) in brain homogenates. This trend was also noticed with kidney tissue where absolutely no activity was observed beyond 1 ppm (5.1%), which was marked by the very intense color owing to high concentration of MDA diadduct formation. This behavior can probably be correlated to the pro-oxidant activity of the extract which in turn may be due to its lipid rich nature especially when the response is non-linear with the concentration.

Table 1.7. Inhibition of rat brain lipid peroxidation by extracts of *D. regia*.

| Conc | % inhibition of brain lipid peroxidation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| (ppm) | BHA | Hexane | EtOAc | Acetone | MeOH | Water | TC | CF | XF |
| 1 | 85.2±0.2 | 83.1±1.7 | 58.3±2.3 | 59.6±1.7 | 57.6±1.1 | 43.3±2.8 | 20.4±1.6 | 33.7±4.2 | 25.4±0.7 |
| 5 | 87.3±0.1 | 76.8±1.9 | 69.8±2.2 | 68.9±0.7 | 69.8±1.9 | 51.4±2.1 | 33.7±0.8 | 66.0±0.9 | 40.1±3.9 |
| 10 | 87.7±0.1 | 67.3±1.1 | 73.6±0.6 | 71.7±1.0 | 73.7±2.4 | 64.8±3.5 | 45.4±2.5 | 75.5±0.7 | 45.6±0.1 |
| 25 | 88.8±0.3 | 42.9±2.7 | 79.4±3.2 | 80.8±0.8 | 81.9±2.8 | 77.8±2.1 | 53.0±2.3 | 77.2±2.2 | 70.8±2.9 |

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD. TC = total carotenoids; CF= carotene hydrocarbon fraction; XF = xanthophylls fraction.

However, it was observed that below 1 ppm the hexane extract exerted a significant effect against kidney lipid peroxidation. Maximum protection (81.9%) towards brain lipid peroxidation was offered by the methanol extract (81.9%) at 25 ppm, which was similar in effect to that of BHA at 1 ppm. This was followed by acetone (80.8%), ethyl acetate (79.4%) and water (77.8%). The carotene hydrocarbons fraction showed 83.3% protection against kidney lipid peroxidation followed by xanthophyll fraction (78.3%) and crude pigment extract (60.2%). Similarly, 77.3%, 70.8% and 53.0% respectively were registered for the three pigment extracts in the case of brain lipid peroxidation (**Tables 1.7 & 1.8**). While the successive extracts showed higher activity against brain lipid peroxidation, the pigment extracts exerted a greater protection against kidney lipid peroxidation. This is possibly due to the involvement of different mechanisms in the two tissue systems. Similarly, the water extract did not offer a promising protection against kidney lipid peroxidation unlike in other models. However, the exact reason for this behavior of the extracts in different systems needs further studies.

Table 1.8. Inhibition of rat kidney lipid peroxidation by extracts of *D. regia*.

| Conc. | % inhibition of kidney lipid peroxidation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| (ppm) | BHA | Hexane | EtOAc | Acetone | MeOH | Water | TC | CF | XF |
| 1 | 85.8±0.1 | 5.1±0.4 | 26.5±1.1 | 19.3±0.1 | 24.9±1.1 | 11.4±0.1 | 26.4±2.9 | 46.3±1.1 | 39.7±0.7 |
| 5 | 86.2±0.4 | -- | 40.8±0.1 | 24.2±0.2 | 33.4±1.3 | 13.1±0.4 | 38.8±8.5 | 67.6±2.2 | 59.7±0.8 |
| 10 | 86.5±0.2 | -- | 57.3±1.1 | 32.8±2.4 | 53.4±2.8 | 18.3±0.7 | 53.2±1.7 | 80.9±0.1 | 71.1±3.5 |
| 25 | 86.6±0.0 | -- | 71.9±0.0 | 58.1±0.1 | 74.1±0.6 | 27.4±0.1 | 60.2±2.1 | 83.2±0.0 | 78.3±0.8 |

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD. TC = total carotenoids; CF= carotene hydrocarbon fraction; XF = xanthophylls fraction.

In addition to the above assay models, three other antioxidant assay models were employed to elucidate the in vitro antioxidant mechanisms of *D. regia* flower extracts.

### 1.4.4 AAPH-induced lipid peroxidation

AAPH, a water-soluble azo-compound, thermally decomposes generating peroxyl radicals in the presence of oxygen at a constant rate thereby oxidizing plasma lipids (Abajo *et al.,* 2004). The extent of plasma oxidation was measured by thiobarbituric acid reactive substance assay. Increasing concentrations of EtOAc, acetone, MeOH and $H_2O$ extracts caused a significant concentration-dependent inhibition of AAPH-induced plasma oxidation reaching above 90% in case of EtOAc, acetone and $H_2O$ at the highest concentration tested (**Table 1.9**). Minimum inhibition of plasma oxidation was observed with hexane extract. The activity increased from 32.0% (25 ppm) to 42.1% (100 ppm) and reduced to 32.0% at 250 ppm. Similar behavior was noticed in crude pigment extract where the activity slightly increased from 25 ppm (26.2%) to 50 ppm (34.9%) and plunged to 2.9% at 250 ppm. This behavior of crude pigment extract was also observed in the $OH^{\bullet}$ scavenging assay. Unlike in the kidney and brain models, the methanol extract was less effective compared to the others. That is to say that there is no correlation between the total phenols and the activity in this system, indicating the possible involvement of compounds other than phenolics in bringing about inhibition of plasma oxidation.

The carotene hydrocarbon fraction showed nearly 66% activity at 250 ppm and the xanthophyll fractions did not significantly inhibit AAPH-induced plasma oxidation as compared to BHA. Summarized, the activity of hexane, EtOAc, acetone, crude pigment extract and xanthophyll fraction was nearly ⅓$^{rd}$ of that of BHA at 25ppm while that of MeOH, $H_2O$ and carotene hydrocarbon fraction was ⅔ $^{rds}$ of that of BHA.

Table 1.9. Inhibition of AAPH induced plasma oxidation by extracts of *D. regia*.

| Conc. | % Inhibition of AAPH-induced plasma oxidation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| (ppm) | BHA | Hexane | EtOAc | Acetone | MeOH | Water | TC | CF | XF |
| 25 | 76.9±2.4 | 32.0±0.8 | 28.1±1.6 | 34.3±0.8 | 50.5±1.6 | 50.1±0.8 | 26.2±2.46 | 44.7±4.1 | 26.2±2.5 |
| 50 | 83.1±1.6 | 37.6±0.8 | 74.7±4.0 | 55.6±0.8 | 70.2±0.8 | 73.0±1.6 | 34.8±1.64 | 55.8±0.0 | 29.6±0.8 |
| 100 | 86.5±0.0 | 42.1±0.8 | 92.6±0.8 | 81.5±0.8 | 73.0±1.6 | 93.3±0.0 | 27.3±2.46 | 62.8±1.6 | 34.3±0.8 |
| 250 | 87.6±0.0 | 32.0±0.8 | 95.5±1.5 | 93.2±1.6 | 74.7±0.8 | 96.6±1.6 | 2.9±0.82 | 65.7±0.8 | 38.4±1.6 |

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD. TC = total carotenoids; CF= carotene hydrocarbon fraction; XF = xanthophylls fraction.

### 1.4.5 Hydroxyl radical scavenging

Living tissues are constantly subjected to oxidative insults by various means resulting in the formation of oxygen radicals. In biochemical systems, $O_2^{\bullet -}$ and $H_2O_2$ react together to form the hydroxyl radical, $OH^{\bullet}$. This is the most reactive of all the free radicals and can attack and destroy almost all known biomolecules (Halliwell and Gutteridge, 1989; Chung *et al.,* 1997). The 2-deoxyribose oxidation method was used for the determination of hydroxyl radical scavenging ability of the extracts of *D. regia*. The addition of low concentrations of Fe (II) salts to deoxyribose causes rapid degradation of the sugar into a malondialdehyde like compound, which forms a chromogen with TBA. 2-deoxyribose is oxidized by $OH^{\bullet}$ that is formed by the Fenton reaction and degraded to malondialdehyde (Halliwell and Gutteridge, 1981). The extent of malondialdehyde formed and its inhibition by the extracts is measured at 520 nm. The $OH^{\bullet}$ scavenging efficacy of *D. regia* extracts were studied at different concentrations (5-100 ppm). The successive extracts were nearly as effective as BHA at 5 ppm against $OH^{\bullet}$-mediated damage while carotene hydrocarbon and xanthophyll fractions were needed at a higher concentration (above 25 ppm) to bring about the same effect (**Fig. 1.15**).

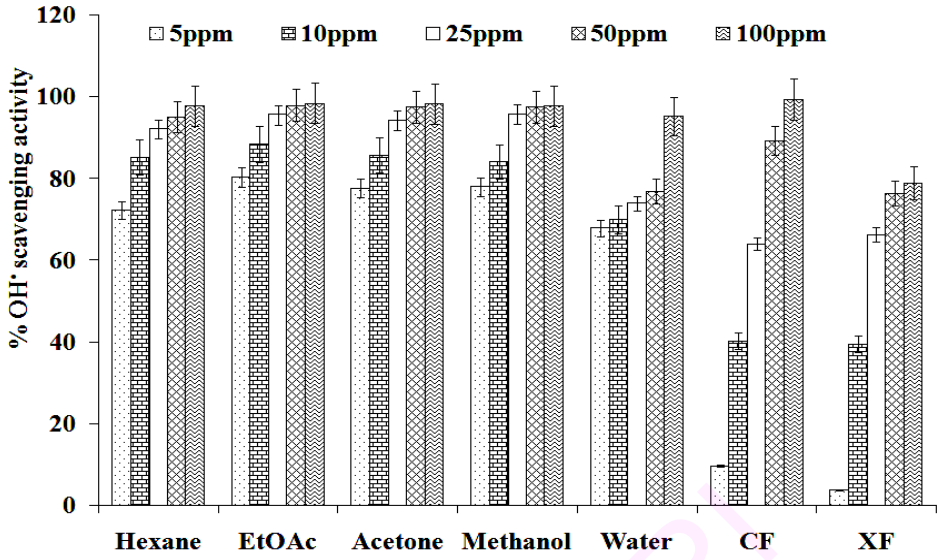



Fig. 1.15. Hydroxyl radical scavenging activity of extracts of *D. regia* flowers. Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD at $p \leq 0.05$. CF= carotene hydrocarbon fraction; XF = xanthophylls fraction

The crude pigment extract showed negative results at the concentrations tested. However, at 1.0 ppm it showed 94.5% activity (data not shown). The effective quenching of hydroxyl radicals by *D. regia* extracts may be attributed to the prevention of propagation of lipid peroxidation thereby reducing the rate of chain reaction, almost as efficiently as the vastly used antioxidants such as gallic acid, BHA and BHT (**Table 1.4**).

### 1.4.6 Nitric oxide scavenging

Nitric oxide (NO) or some related reactive nitrogen species accounts for the activity of endothelium derived relaxing factor (EDRF) responsible for vascular smooth muscle relaxation, acts as a neurotransmitter, prevents platelet aggregation and is a defense molecule of immune system against tumor cell, parasites and bacteria (Gulcin, 2006). Despite these positive effects, it is also a potentially toxic gas with free radical properties. NO has been reported to cause mutagenesis and deamination of DNA bases to form carcinogenic n-nitroso compounds. NO reacts rapidly and spontaneously with a superoxide anion ($O_2^{\bullet -}$) to form a peroxynitrite anion ($ONOO^-$), which is more toxic than $O_2^{\bullet -}$ or NO to biological systems and causes modification of proteins or nucleic acids causing deleterious health effects (Yen *et al.,* 2006). In the present study NO scavenging effect of the different extracts of *D. regia* (1-50 ppm) was investigated. Of the eight extracts tested, hexane extract and crude pigment extract showed similar results (**Table 1.10**) exhibiting 93.9% and 93.1% NO scavenging respectively at 50 ppm. At this concentration, the inhibitory activity of BHA, EtOAc, acetone, MeOH, $H_2O$, carotene

hydrocarbon fractions and the xanthophyll fraction were 56.7, 67.9, 71.2, 77.4, 73.6, 75.9 and 73.1%, respectively. The activity profile of the extracts almost matched at all the concentrations (**Table 1.10**).

Table 1.10. Nitric oxide scavenging activity of extracts of *D. regia*.

| Conc (ppm) | % NO scavenging activity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **BHA** | **Hexane** | **EtOAc** | **Acetone** | **MeOH** | **Water** | **TC** | **CF** | **XF** |
| 1 | 39.1±0.2 | 16.8±1.07 | 52.9±1.6 | 18.0±2.1 | 42.8±1.3 | 60.9±2.1 | 22.6±0.6 | 29.8±0.9 | 31.7±1.6 |
| 5 | 49.9±2.6 | 54.1±0.5 | 70.0±0.0 | 63.5±1.1 | 70.5±1.3 | 71.7±2.4 | 48.1±1.9 | 58.3±0.6 | 52.1±0.3 |
| 10 | 52.2±0.0 | 76.5±0.5 | 70.0±0.0 | 72.1±0.8 | 75.9±1.3 | 73.8±0.0 | 70.8±3.2 | 78.7±0.6 | 74.1±1.9 |
| 25 | 54.1±0.0 | 80.4±1.8 | 70.5±0.8 | 74.7±0.2 | 77.2±0.0 | 74.3±0.2 | 83.7±2.6 | 81.0±0.6 | 83.3±1.3 |
| 50 | 56.7±1.6 | 93.9±0.5 | 67.9±2.4 | 71.2±1.3 | 77.4±0.2 | 73.6±0.3 | 93.1±0.65 | 75.9±1.3 | 73.1±0.6 |

Three independent experiments were performed in triplicate at each concentration. Data are expressed as mean ± SD. TC = total carotenoids; CF= carotene hydrocarbon fraction; XF = xanthophylls fraction.

Thus the present study shows that all the extracts effectively suppress NO production under the prevailing conditions. The activity of the extracts against NO production was significantly better than that of BHA. The effect of the extracts on the accumulation of nitrite can also be a consequence of the reaction of the extract with other oxides of nitrogen, i.e. $NO_2$, $N_2O_3$, $N_2O_4$ and $OONO^-$, which are the possible intermediates in the oxidation of nitric oxide to nitrite (Marcocci *et al.,* 1994). Non-polar extracts such as that from EtOAc, acetone and carotene hydrocarbon fraction as well as the xanthophylls fraction showed a retarded free radical scavenging activity at the highest levels (50 ppm) tested. This behavior is probably because of the dual nature of diazotization and coupling reactions that fluctuate based on the availability of phenolic groups. NO scavenging properties of the extracts suggest that they can be potential therapeutic agents for the control of oxidative and non-oxidative damage caused by nitric oxide.

## 1.5 Conclusions

In conclusion, the present work provides a comprehensive picture of the chemical nature and antioxidant activity of anthocyanins present in the berries of *S. cumini* and carotenoids in *D. regia* flowers. Flowers of *D. regia* were found to contain high amounts of anthocyanins and carotenoids. The high antioxidant activities at low concentrations render them potential sources of antioxidants as well as natural colorants. Though the flowers of *D. regia* are successively extracted under high temperature conditions, persistence of high anti-oxidative activity indicates the presence of heat stable antioxidant phyto-constituents apart from the heat labile carotenoids

and anthocyanins. Unlike tea plants (one of the most sought-after polyphenol reserves) that require specific agro-climatic conditions to grow, *D. regia* is naturally abundant in tropics and consists of a variety of radical scavenging phyto-constituents. The unique combination of high amounts of both anthocyanins and carotenoids makes it an even more interesting source of antioxidants and natural colors. *S. cumini* extract is a rich source of anthocyanins whose content is equivalent to that of blue berries and black currants and higher than that of black berries, the widely acclaimed anthocyanin rich edible fruits that are extensively marketed in the nutraceutical arena. The *S. cumini* anthocyanins, like all the other anthocyanins, have the advantage of high solubility in aqueous mixtures, imparting an attractive color that makes their incorporation into numerous aqueous food and non-food formulations, including pharmaceuticals, easy.

# Chapter 2

## *Stability studies on anthocyanins of S. cumini*

## Summary

The fruit peel of S. *cumini* is a rich source of anthocyanins and the freeze dried, purified extract was found to contain purely anthocyanins. The present chapter was designed to study the stability of the anthocyanin pigment in aqueous solutions under different pH conditions as well as under conditions of dark and light. The anthocyanin pigment was also incorporated into a pharmaceutical formulation containing salbutamol as the active ingredient and the stability of the color studied at different temperatures for 8 weeks. The study revealed that the pigment was stable up to pH 5 despite a reduction in its hue at pH above 2 when stored at 10ºC. Though there was a reduction in color in anthocyanin solution at pH 3-5, it was found to be stable during the tested period of 45 days, where no further color loss was observed. Storage of the anthocyanin pigment in aqueous solution of pH 3 at temperatures ranging from 0ºC to 45ºC for 4 weeks in dark resulted in loss of up to 20% (at 10ºC). The extract was quite stable at 0ºC with 11% loss in 4 weeks, while the pigment loss in the antitussive formulation was only 13% at 30ºC at the end of 8 weeks. The high antioxidant activity and relatively high stability of the pigments make S. cumini a potential source of natural colorant as well as antioxidants.



**Publications**

**Veigas JM**, Narayan MS, Laxman PM, Neelwarne B. 2007. Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *Syzygium cumini* Skeels. Food Chem. 105; 619-627.

## 2.1 Introduction

One of the attributes that judge the quality and visual acceptance of food mainly depends on its color characteristics. Color is an important element in deciding the appeal of the product it is incorporated into. With an increase in understanding of the toxic consequences of synthetic dyes and colors, consumer attention has greatly shifted towards the use of natural colors in foods and beverages. The demand is on the rise for naturally derived red colors as an alternative to the most commonly used synthetic FD&C Red #40 (Giusti and Wrolstad, 2003).

Anthocyanins are the best-known natural red colorants used in foods (Bridle and Timberlake, 1997) and are approved as food colorants in some countries. In the USA, the grape skin extract, grape color extract, fruit juice and vegetable juice are exempt from certification (Wrolstad, 2004) and are used to color foods. Other approved natural colors include betanin, cochineal and carotenoids (Francis, 1986). High sensitivity of anthocyanins towards heat, light and pH has been the primary concern with regard to its application as a colorant in foods and beverages (Cevallos-Casal and Cisneros-Zevallos, 2004). The color stability of anthocyanin pigments are dependent on several factors including structure and concentration of pigments, pH, temperature, light intensity and quality, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation products and sulfur dioxide (Mazza and Miniati, 1993; Francis, 1986).

Their stability is generally compromised at elevated temperatures and pH. The anthocyanin color is most stable at low pH values of 1-3 where the anthocyanin exists in the form of a flavylium cation exhibiting a bright red color (Cabrita et al. 2000). An increase in pH almost always results in the loss of color and stability (Cevallos-Casal and Cisneros-Zevallos, 2004; Fossen *et al.,* 1998) except in one study where an increase in stability of petanin, an acylated anthocyanin, was witnessed at alkaline pH (Fossen *et al.,* 1998). This strengthens the existing reports that acylated anthocyanins are more stable compared to the non-acylated or simple glycosilated anthocyanins and hence can be used to color foods at higher pH. Presence of organic acid such as ascorbic acid and its degradation products in anthocyanin solutions speeds up the anthocyanin degradation (Poei-Langston and Wrolstad, 1981; Kirca *et al.,* 2006) while citric acid greatly increases anthocyanin stability (Lee *et al.,* 1996). Addition of sugars plays an important role in the stability of anthocyanins. A study by Wrolstad *et al.,* (1990) revealed that addition of sucrose (up to 40%) offered significant protection to anthocyanin pigment content and also against browning and polymeric color formation in frozen strawberries. However, some researchers report a degradative effect of sugar, especially sucrose at low concentrations, on anthocyanins (de Rosso and Mercadante, 2007).

This study was designed to understand the color changes occurring with the change in pH and the stability of the same during cold storage. Similarly, the stability of the color was studied at low pH at different temperatures under conditions of light and dark. Attempt was made to incorporate the anthocyanin extract into a pharmaceutical formulation and assess the color over a period of time.

## 2.2 Materials and methods

### 2.2.1 Stability of anthocyanin color in solutions

#### 2.2.1.1 Stability of color at different pH

Effect of pH on the stability of the anthocyanin was studied at 7 different pHs (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0). Citrate-phosphate buffers at these pHs were prepared and anthocyanin extract of *S. cumini* was added to a final concentration of 10mg/ml. Each colored solution was stored in two screw capped tubes in volumes of 10.0ml and stored at 10°C. The color intensities were measured as absorbance values of solutions at their respective absorption maxima recorded on a UV-160A spectrophotometer (Shimadzu Corp. Japan).

#### 2.2.1.2 Stability of color in aqueous solution

A 1% solution of the lyophilized anthocyanins extract was prepared in water whose pH was adjusted to 3.0. The solution was distributed in screw capped tubes in volumes of ten ml and stored under dark condition at 0, 10, 20, 30 and 45°C. Color stability was monitored by measuring the absorbance of the solution at an absorption maximum of 520nm. Each tube was used for one spectral measurement only, so as to minimize the contact with air.

### 2.2.2 Incorporation of anthocyanins extract in syrup and determination of color stability

Salbutamol syrup (antitussive) was prepared according to Kohli and Shah (1990). Anthocaynin extract in its lyophilized form was incorporated into the syrup at a final concentration of 0.1%. The stability of the color in the syrup was studied for 8 weeks under light (2000 Lux) and dark conditions at different temperatures (20, 30 and 45°C).

## 2.3 Results and Discussion

### 2.3.1 Stability of anthocyanin color in solutions

Stability of a colored compound is very important in retaining the appearance of the product in which it is incorporated and hence the customer acceptance, as well as the biological efficacy. Naturally occurring colorants, such as anthocyanins from black carrot, grape, red cabbage, are now frequently used in foodstuffs and pharmaceuticals due to their additional health benefits (Markakis, 1982). However, anthocyanins are relatively unstable, especially when non-acylated (Kirca *et al.,* 2007) and are prone to degradation in the presence of light, high temperature and varying pH (Fossen *et al.,* 1998). The fruit peel of *S. cumini* was found to be a rich source of anthocyanins and the lyophilized extract contained solely anthocyanins as evidenced by spectrophotometric and HPLC studies described in the earlier chapter. This chapter deals with the study of the stability of anthocyanin rich extract of *S. cumini* in aqueous solutions under different pH conditions. A pharmaceutical formulation at low pH was prepared with the extract as the coloring agent and the stability of the same was also studied under different light and temperature conditions.

#### 2.3.1.1 Effect of pH on color in aqueous solutions

There has been an upsurge of interest in the development of natural colors for application in food and pharmaceutical preparation due to the negative effects of synthetic dyes (Francis, 1989). Anthocyanins are one of the most promising natural colorants and are responsible for the reds, blues and violets of flowers, fruits and vegetables. The instability of anthocyanins caused by light, temperature and pH limits its use. The color of anthocyanins and its intensity is largely regulated by a change in pH of the matrix in which it is present. In the present study, anthocyanin extract of *S. cumini* was dissolved in aqueous solutions of different pH ranging from 2-8. One hour after the preparation of the solutions, the absorption maxima for each solution was determined. The subsequent measurements were made at their respective absorption maxima for 30 days. The change in color at different pH is depicted in **Fig. 2.1**.

The color of the solution changed from red to pink to purple to blue from pH 2 to pH 8. A steady decrease in color intensity was observed with an increase in pH up to 5 with a pinkish coloration. At pH 2, the solution exhibited an intense red color indicating the predominant existence of flavylium cationic form of the anthocyanin. As the pH of the solution was increased to 5, the color intensity reduced due to the hydration of the flavylium cation to yield a colorless carbinol

pseudo-base (Mazza and Miniati 1993). Complete discoloration was not observed at this pH indicating that most of the anthocyanin still exists in its flavylium cation form.

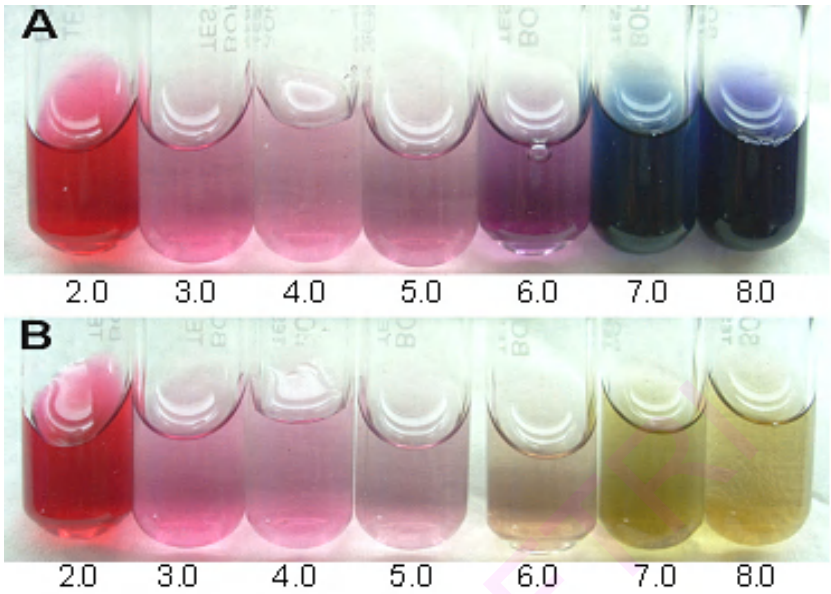



Fig. 2.1. Change in color of *S. cumini* anthocyanins in aqueous solutions at different pHs after 1h (A) and after 30 days (B) at 10°C, protected from light.

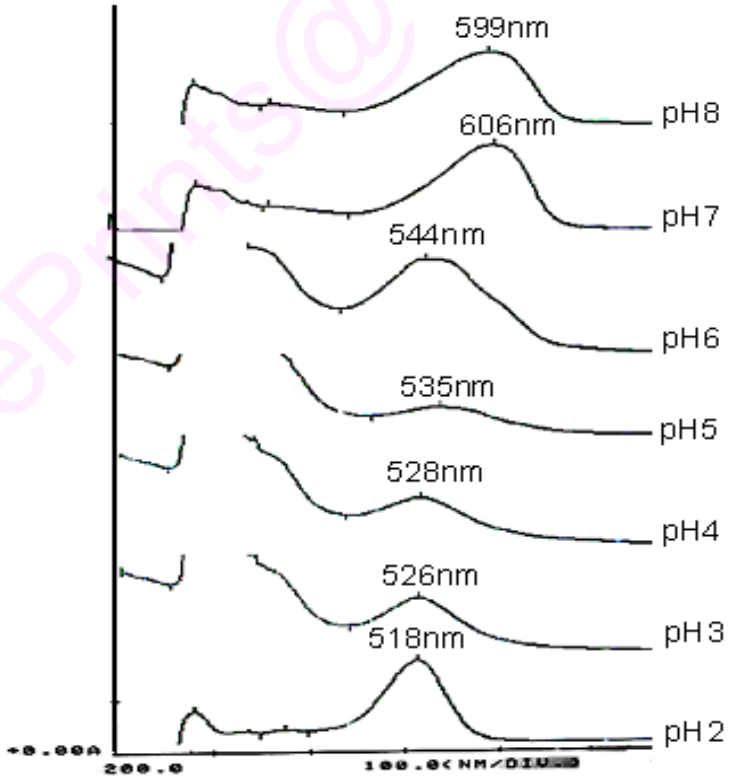



Fig. 2.2. Visible spectra of the *S. cumini* anthocyanin solution at different pH, measured 1 h after preparation of solutions.

The visible absorption maxima for the anthocyanin extract suffered a bathochromic shift on increase of pH from 2 to 8 (**Fig. 2.2**). The absorption maximum at pH 2 was 518nm. An increase in pH significantly induced a bathochromic shift in the $\lambda_{max}$ with a hyperchromic effect in color intensity. At pH 5 the $\lambda_{max}$ shifted to 535nm with a further increase to 607nm at pH 7. At pH 6, the solution exhibited a purple color and the color changed to blue at pH 7. A slight hypsochromic shift in $\lambda_{max}$ (599nm) was observed when the pH was raised from pH 7 to pH 8 with the color changing from greenish blue to intense blue shade. The proton transfer reactions related to the acidic hydroxyl groups of the aglycones gives rise to quinoidal bases, further deprotonation of which occurs above pH 6 with the formation of resonance stabilized quinonoid anions, which are purplish in color (Brouillard, 1988). The color of the anthocyanin solutions was retained to its original hue even after 30 days of storage at 10°C in dark at pH up to 5.0.

**Figure 2.3** depicts loss of color in solution at different pH over a period of 45 days. The absorption of the solutions (pH 2 to 5) on day 30 remained very close to the original absorption measured 1h after preparation of the solutions.

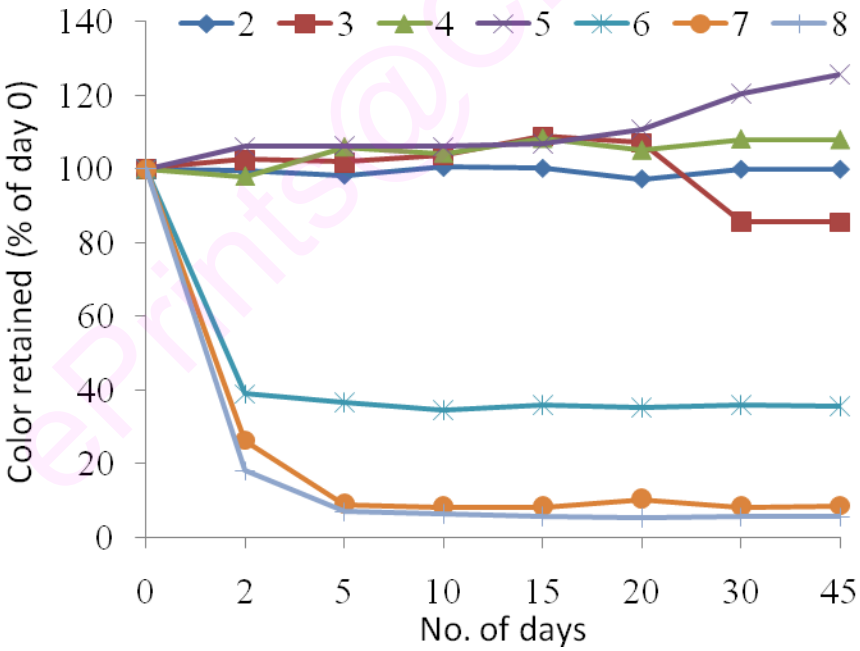



Fig. 2.3. Effect of pH on percentage color retention of *S. cumini* anthocyanin extract at different pH over a period of 45 days.

However, the color of the solution at pH 6 and above completely disappeared on day 2 (**Fig. 2.1**) perhaps due to the formation of unstable quinonoidal bases, responsible for the instability of color at high pH. Red cabbage anthocyanins have been shown to be stable at a wide pH range being pink at pH 3, violet at pH 5 and blue at pH 7. At pH 3, it was very stable up to a temperature of

60°C which was attributed to the presence of acyl groups which hinder the hydrolysis of the flavyliym cation to form the colorless carbinol base (Bridle and Timberlake, 1997).

The instability of anthocyanins of *S. cumini* extract at higher pH may be ascribed to the lack of acyl groups and complex sugars as evidenced by HPLC-MS studies. However, being stable at acidic pH, the anthocyanin pigments of this source may be effectively used to color foods and other formulations with pH values up to 5 where a rosy tinge is desired. The extract cannot be used in alkaline foods due to its high instability under these conditions.

**2.3.1.2 Stability in aqueous solution**

Anthocyanin stability is affected by temperature. The previous experiment revealed that the anthocyanin pigment is stable at pH 1-5 when stored at 10°C. Therefore this study was carried out to check its stability in a low pH solution at different temperatures. pH 3.0 was taken as the representative value and aqueous solution of the anthocyanin was stored in the dark at 0, 10, 20, 30 and 45°C for 4 weeks. The thermal degradation was studied during this period by measuring its absorbance at 520nm. Anthocyanins tend to lose color during storage at higher temperatures (Cisneros-Zevallos and Cevallos-Casal, 2004). Formation of chalcone is presumed to be the first step in thermal degradation of anthocyanins eventually leading to formation of brown products (Markakis *et al.,* 1957). **Figure 2.4** shows the loss of color in *S. cumini* anthocyanin solution stored at different temperatures over time. The color loss in the extract increased with an increase in temperature with time. At the end of 4 weeks color loss in the aqueous extract was 11.8% and 19.6% at 0°C and 10°C, respectively. This shows that the extract is more stable at low temperatures and best stored in frozen conditions.

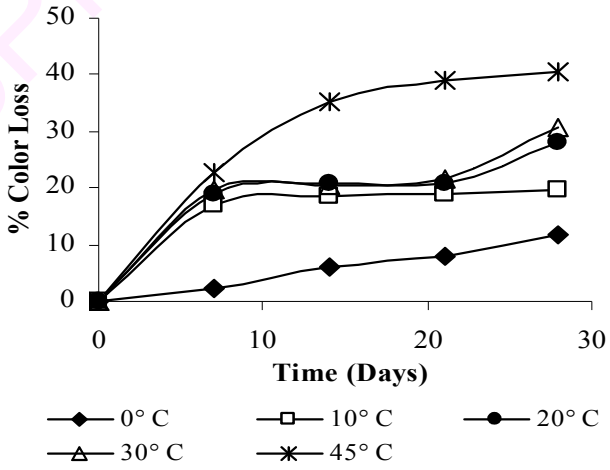



Fig. 2.4. Stability of anthocyanins rich extract of *S. cumini* fruit peel under different temperatures. Aqueous solution (1%) of the extract (pH 3.0) was stored in screw-capped vials. Samples from two of the vials were taken afresh every week and absorbance measured at 520nm.

Interestingly at 10°C the anthocyanin pigment loss was sudden and drastic amounting to 17% on day 7. Further storage for 4 weeks did not have significant effect on color loss (**Fig. 2.4**). The loss of color at 4$^{th}$ week was found to be 20%, a 3% rise from day 7which is negligible. Color loss in anthocyanin solution stored at 30°C was not significantly different from that stored at 10°C or 20°C at 21 days (3 weeks); however, further storage at 30°C gradually lead to 30% loss of color. A substantial loss in color intensity was noticed only when the solution was stored at 45°C showing a 35% loss in the second week and reaching 40% at the end of the study (4 weeks). The results suggest that the anthocyanin pigment of *S. cumini* is relatively stable at temperatures up to 30°C when stored in dark, though storage at lower temperatures is more beneficial.

### 2.3.2 Stability of anthocyanin color in syrup

The stability of the color in the syrup containing anthocyanin extract was studied at 20, 30 and 45°C in the dark and in the presence of a continuous illumination of 2000 Lux for 8 weeks. Figure 9 explains the effect of temperature and light on the stability of anthocyanin color when incorporated into syrup. Like in the case of extract, the color loss in the syrup was also temperature dependent. However, the color retention was relatively higher than that in the extract. Because, the color loss was only 13% at the end of 8 weeks at 20°C and 30°C (**Fig 2.5a-c**) when stored in dark whereas a 19.6% loss was witnessed with the aqueous extract when stored at 10°C. However, the exposure to light had negative effect on the retention of color in the syrup (**Fig 2.5a& b**) which was further accentuated at high temperature (**Fig 2.5c**). However, there was no observable difference in the color of the extract/ formulation to the naked eye.

The relatively high stability of the color in syrup may be attributed to its possible interaction with the syrup components. One such is the presence of sucrose at high concentrations. Presence of sucrose at concentrations above 40% has been found to increase the ability of anthocyanin to bind to water molecules thus favouring the stability of the same in sucrose solutions (Tsai *et al.,* 2004). The effect of other ingredients present in the syrup is not very clear. However in the present formulation the effect did not appear to be significant. Since, the syrups are generally stored in amber-colored containers; the pigment from *S. cumini* appears to be useful for similar formulations that are stored on the shelf at room temperature. However, the extract is more stable when stored at 0°C. Till date, no reports exist on the incorporation of anthocyanins as a coloring agent in pharmaceutical formulations except for one where red cabbage anthocyanin was tested to be a pH indicator in chlorbutol solution (Chigurupati *et al.,* 2002), a pharmaceutical preservative.

Since anthocyanins are hydrophilic compounds, they can be easily incorporated into syrups, which is one of the most accepted forms of pharmaceutical formulations. The high antioxidant

property of the pigments may confer additional prophylactic advantage, along with the therapeutic property of the active ingredient in the syrup. However, their interaction with other ingredients in the formulations needs further studies. Recent studies have suggested that copigmentation of anthocyanin with other compounds significantly augments the stability of the color. The copigment as such is colorless, but greatly enhances the color of the anthocyanin when added in a solotuin (Mazza and Brouillard, 1987, 1990).

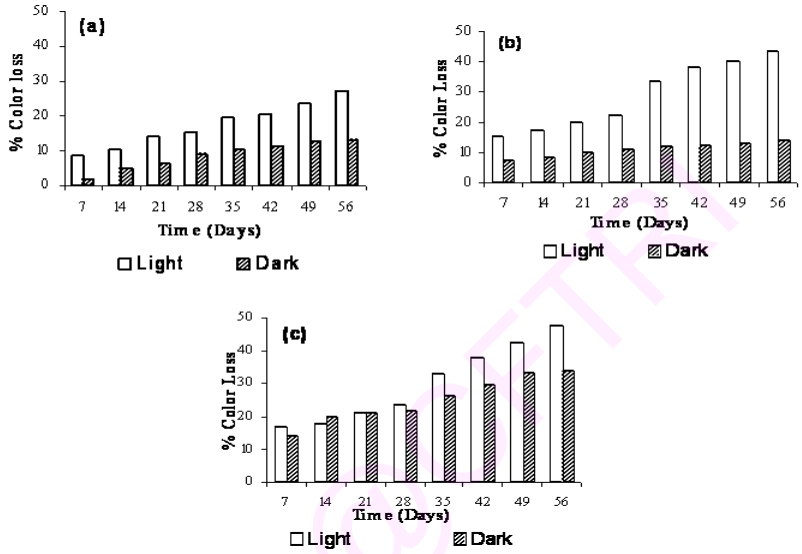



Figure 2.5. Loss of color in the salbutamol formulation when stored at (a) 20ºC (b) 30ºC (c) 45ºC under light (2000 Lux) and dark conditions. The formulation was stored in screw-capped vials. Samples from two of the vials were taken afresh every week and absorbance measured at 520nm.

Anthocyanins may form copigmentation with compounds like flavonoids, organic acids, amino acids, alkaloids, nucleotides, polysaccharides, metal ions or anthocyanins themselves (Mazza and Brouillard, 1990). Copigmentation with naturally derived flavones has shown hyperchromic effect and a bathochromic shift in the absorbance of the anthocyanins (Bakowska *et al.,* 2003) indicating that copigmentation not only stabilizes the anthocyanin color but also enhances its hue. Since the extract prepared in our study is a highly concentrated anthocyanin extract, without significant amounts of other interfering molecules, it is possible to increase the stability of its color by copigmentation. This may open avenues which will lead to its incorporation into various food and beverages and possibly in pharmaceutical formulations.

## 2.4 Conclusions

It is apparent from the results obtained in this study that anthocyanins of *S. cumini* are an excellent source of natural colors with relatively high stability. This attribute can be made use of especially in formulations with low pH values since the stability was found to be high at pH range

of below 5. The fruit pulp blend has been reported to be used in ice cream preparation at 10% concentration by Anjum *et al.,* (2000) and was found to retain physico-chemical and sensory properties. Similarly, the anthocyanin extract of *S. cumini* can be a great source of value added products due to its coloring properties and health benefits.

# Chapter 3

# *Bioefficacy studies in primary animal cell culture*

## Summary

Antioxidant activities of extracts of *S. cumini* and *D. regia* were further established using isolated rat hepatocytes *in vitro* under controlled culture conditions. In the present study anthocyanin extract (SCA) as well as the fruit pulp extract (SPE) of *S. cumini* were used along with the two carotenoids fractions (CF and XF) of *D. regia*. SCA suppressed $CCl_4$-induced LDH leakage by 54% at 50 ppm, thereby improving the cell viability by 39%. The SCA significantly reversed the $CCl_4$ induced changes in cellular GSH level, lipid peroxidation and activity of the antioxidant enzyme (AOE) glutathione peroxidase. Exposure of hepatocytes to SCA after $CCl_4$ treatment was found to elevate GSH and GPx activities by 2-folds, whereas the activities of catalase and superoxide dismutase were not significantly affected. SPE, though was protective against $CCl_4$-induced cell death, greatly depleted cellular GSH and increased GPx activity nearly 3-fold. Carotenoid fractions of *D. regia* were protective against $CCl_4$-induced cell death at low doses (up to 100ppm) beyond which they showed no effect. EGCG and CF further reduced the $CCl_4$-induced reduction in CAT and SOD activity while effect of XF on these two enzymes is not significant. However GSH content was significantly elevated in cells treated with the extracts and EGCG excepting SPE. Glutathione content was elevated in the presence of carotenoids extracts and was comparable to 0.1μM EGCG. XF showed greater protection against lipid peroxidation compared to CF. It appears that the extracts offer protection to rat hepatocytes against $CCl_4$-induced toxicity by mechanisms not solely dependent on AOEs.

Toxicity of TBH was characterized by 45% cell death with an increase in LDH leakage, reduction in GSH content, increased lipid peroxidation and increased activity and expression of AOEs. In addition to EGCG, quercetin was also used as a standard to compare the activity. SCA brought about a significant reduction in TBH-induced increase in lipid peroxidation and improved AOE activities and their mRNA expression with an increase in GSH content. Carotenoid fractions and standards further increased TBH-induced increase in enzyme activity but reduced their mRNA expression suggesting a posttranscriptional regulation of the enzymes. Lipid peroxidation was significantly abrogated by carotenoids fractions as well as EGCG and quercetin. These observations indicate that while SCA has a direct effect on AOEs, carotenoids and standards appear to act via mechanisms independent of these enzymes.



## Publication

**Veigas JM**, Shrivasthava R, Bhagyalakshmi N. 2008. Efficient amelioration of carbon tetrachloride induced toxicity in isolated rat hepatocytes by *Syzygium cumini* Skeels. extract. Toxicol. In Vitro 22(6); 1440-1446.

## 3.1 Introduction

Liver is the major organ in the body since it performs an astonishingly large number of tasks of regulating several functions in higher animals and humans. The major functions are metabolism of carbohydrates, lipids, proteins, drugs and xenobiotics that are consumed. Thus the liver is prone to injury due to the chronic exposure to drugs, environmental toxicants and other xenobiotics. Major consequence of this is an indirect effect on virtually all other organs. Therefore, dissecting the mechanisms of damage by ingested chemicals and its alleviation by antioxidant phytochemicals helps developing strategies for new drug formulations. Carbon tetrachloride ($CCl_4$) and *tert*-butyl hydroperoxide (TBH) are the most commonly used models of xenobiotics-induced liver toxicity (Yu *et al.,* 2002; Ruch *et al.,* 1986) and were employed in the present study to demonstrate the protective effect of antioxidants under chemically different oxidative stress conditions.

Due to the diverse chemical nature of prooxidants, all antioxidants may not protect cells against all kinds of free radicals and antioxidants may well be selective towards the type of free radicals they attack. The ability to act against different types of free radicals and toxins is a desirable property of any antioxidant. This is the basis for the choice of two chemically diverse oxidative agents in order to establish the activity of the said compounds against two different classes of free radical generators viz., $CCl_4$ and TBH. Carbon tetrachloride (Johansson and Ingelmann Sunderberg, 1985; Wolf *et al.,* 1980) and TBH (Thomson *et al.,* 1995) are metabolized in the liver by the CYP450 mixed function oxidase system (MFOS). Since longer incubation of isolated rat hepatocytes diminishes the CYP450 MFOS (Adzet *et al.,* 1987; Binda *et al.,* 2003), freshly isolated cells were used for the study.

Carbon tetrachloride, a by-product of sewage and drinking water chlorination, is a major hepatotoxin metabolized in the liver by the cytochrome P450 resulting in the reactive trichloromethyl radical ($CCl_3^{\bullet}$) and subsequently an even more reactive form, the trichloromethyl-peroxyl ($^{\bullet}OOCCl_3$) radical (Ruch *et al.,* 1986). In addition, $CCl_4$ generates free radicals which fuel a series of reactions ultimately resulting in the initiation of membrane lipid peroxidation and a consequent liver injury (Slater, 1984). Cytochrome P450 enzymes (CYP450) (Phase I mono-oxygenase enzymes) are widely known for their role in metabolism of drugs and xenobiotics (Moon *et al.,* 2006). Modulation of this enzyme system can influence the metabolism of xenobiotics producing effects that are pharmacologically and toxicologically significant. A number of plant constituents have been shown to modulate CYP450 enzyme system (Rodeiro *et al.,* 2008; Trudeau *et al.,* 2007). Dietary flavonoids and carotenoids especially play an important

role in alleviating xenobiotic-induced cell injury (Moon *et al.,* 2006; Vijayaraghavan *et al.,* 2008). The reactive metabolites formed via the involvement of CYP450 enzyme system trigger an antioxidant response in rat hepatocytes (Reinke *et al.,* 1988; Sipes *et al.,* 1977).

*Tert*-butyl hydroperoxide, a membrane permeant oxidant, has been extensively used to induce oxidative stress in different systems. It is known to be a precursor in the formation of malondialdehyde, the lipid peroxidation product. It is metabolized by two distinctive pathways. One pathway involves the cytochrome P450 leading to the toxic peroxyl and alkoxyl radicals (Rush *et al.,* 1985) and the other is a detoxification reaction involving glutathione peroxidase giving rise to *tert*-butyl alcohol and oxidized glutathione resulting in severe depletion of reduced glutathione and ultimately to death (Thor *et al.,* 1984; Sies *et al.,* 1972). Antioxidants such as quercetin and rutin have been demonstrated to protect the hepatocytes against TBH-induced lethality especially through the modulation of AOEs (Alia *et al.,* 2005; 2006b). Therefore, the present study was aimed at elucidating the regulation of AOEs in alleviating the TBH-induced cytotoxicity.

Inhibition of free radical generation or scavenging the free radicals generated by other biochemical reactions is an important factor in the prevention of cellular damage. While the body is designed to detoxify and allay itself of harmful effects of exotoxins, it may not be sufficiently prepared to handle the onslaught of pollutants and toxic substances predominant in the environment. In addition, refining of foods generally leads to deficiency in essential nutrients involved in detoxification process. Therefore, supplementing the diet with such compounds which enhance antioxidant defense is essential in order to prepare the body against oxidative insult. Several natural products have been proven to be effective against cellular damage either by acting as antioxidants or as positive modulators of other cellular machineries (Semiz and Sen, 2007; Sakr, 2007).

The literature on amelioration of xenobiotic-induced oxidative toxicity by nutritional antioxidants is not scarce. Carbon tetrachloride induced hepatic injury has been found to be effectively ameliorated by plant derived products (Rajesh and Latha, 2004; Martin-Aragon *et al.,* 2001). Anthocyanins (Lila 2004; Kong *et al.,* 2003) and carotenoids (Palozza and Krinsky, 1992; Paiva and Russell, 1999) from other sources have proven to be excellent antioxidants against various oxidative insults. Since carotenoids and anthocyanins are credited with high efficiency against oxidative stress in cell and animal models (Bestwick and Milne, 1999; Ben-Dor *et al.,* 2005), the current chapter was aimed at understanding the molecular mechanisms involved in the action of these two classes of compounds from *D. regia* flowers and *S. cumini* fruits respectively against free radical generators.

The study was systematically carried out in the following order:

- Ameliorative effects of the extracts on isolated rat hepatocytes against $CCl_4$-induced oxidative cytotoxicity
- Ameliorative effects of the extracts on isolated rat hepatocytes against TBH-induced oxidative cytotoxicity.

The study was mainly focused on dissecting the mechanisms involved in the modulation of AOEs and other oxidative markers in the presence of stress. In the second part, attempt has been made to study the transcriptional changes of AOEs in the presence of oxidative stress induced by TBH and its modulation by the antioxidants.

Among an array of extracts screened in the previous chapter, four were chosen for the present study: two each from *D. regia* and *S. cumini* and are designated in the following manner:

| **Extract** | **Sample ID** |
|---|---|
| Anthocyanin rich peel extract of *S. cumini* | SCA |
| Pulp extract of *S. cumini* | SPE |
| Carotene hydrocarbon fraction of *D. regia* | CF |
| Xanthophylls fraction of *D. regia* | XF |

The well known flavonoid antioxidants EGCG and quercetin were used as reference compounds for comparison of activity since their status as antioxidants is well documented (Shi *et al.,* 2000; Alia *et al.,* 2006a,b). Concentrations of flavonoids in general do not exceed 1-5µM/L plasma even when consumed in excess (Alia *et al.,* 2006a; Sugisawa and Umegaki, 2002). Preliminary studies indicated the protective effects of these two flavonoids at concentrations 0.1µM to 10µM against TBH-induced cell death. Though all the concentrations tested completely protected the cells form TBH-induced cell death, the lowest concentration (1µM) which prevented the cell death was used for further studies.

## 3.2 Materials and Methods

### 3.2.1 Preparation of rat primary hepatocytes

Rat primary hepatocytes were isolated from male Wistar rat (150-250 g) by two-step collagenase perfusion of the liver (Ying-Jie *et al.,* 1998). Cell viability, determined by trypan blue exclusion, was over 85%. The isolated rat hepatocytes were cultured in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution, 0.1 U/ml insulin and 0.01 μM dexamethasone for 3 hours at 37ºC in a humidified atmosphere of 95% $O_2$ and 5% $CO_2$.

### 3.2.2 Treatment of isolated rat hepatocytes for evaluation of toxicity of extracts

Cells (2 x $10^5$) were plated in 96 well plates for cell viability assessment and in 12 well plates for other assays. The cells were treated with different concentrations of extracts for 4 h at 37ºC in a humidified atmosphere of 95% relative humidity with 95% $O_2$ and 5% $CO_2$.

### 3.2.3 Treatment of isolated rat hepatocytes for $CCl_4$-toxicity studies

Cells (2 x $10^5$) were plated in 96 well plates for cell viability assessment and in 12 well plates for other assays. The cells were treated with 7 mM of $CCl_4$ for 1 h at the end of which the medium was replaced with fresh medium containing different concentrations (0-500ppm for cytotoxicity assessment; 0-300ppm for other biochemical assays) of the extracts. The cultures were subsequently incubated for further 3 hours at 37ºC in a humidified atmosphere of 95% relative humidity with 95% $O_2$ and 5% $CO_2$.

### 3.2.4 Treatment of isolated rat hepatocytes for TBH-toxicity studies

The cells were treated with different concentrations (0-500 ppm for cell viability; 0, 50 and 100 ppm for the other assays) of the extracts for 4 hours at the end of which the medium was replaced with fresh medium containing 2mM TBH. The cultures were subsequently incubated for further 2 hours at 37ºC in a humidified atmosphere of 95% relative humidity with 95% $O_2$ and 5% $CO_2$.

Treatments were performed with different concentrations of *S. cumini* anthocyanin extract (SCA), *S. cumini* pulp extract (SPE), carotene fraction (CF) and xanthophylls fraction (XF) of *D. regia* flowers and EGCG (standard antioxidant). The extracts and standard were dissolved in DMSO. The final concentration of the solvent did not exceed 0.5% in the treatments. DMSO at this concentration was found to be non-toxic to cells under the experimental conditions followed.

At the end of the treatment period, the cells were assessed for cell viability and other biochemical indices mentioned above such as membrane damage (LDH leakage), GSH content, level of lipid peroxidation and activities of AOEs such as catalase, superoxide dismutase and glutathione peroxidase.

### 3.2.5 Assessment of cell viability and integrity

Cell viability was assessed by MTT assay and lactate dehydrogenase (LDH) activity. LDH activity in the culture medium was measured as an index of plasma membrane integrity. The assay was carried out in 96 well plates. At the end of the treatment period, 50 µl of the medium from each treatment was removed for LDH leakage determination and treated with freshly prepared β-NADH solution (4.58 mg pyruvate, 5.32 mg NADH, and 7.49 $NaHCO_3$ in 10 ml 0.05 M potassium phosphate buffer, pH 7.4). The plates were incubated in dark for 20 minutes, after which the absorbance of the reaction mixture was measured at 340nm using an ELISA microtitre plate reader. Hepatocyte viability was expressed as the quantum fraction of LDH released into the culture medium relative to the total cellular activity observed after cell lysis with 0.1% v/v Triton-X 100 (Haidara *et al.,* 1999). To the remaining medium, MTT (15 µl of a 1mg/ml solution) was added and incubated for 4 hours at 37ºC. The medium was removed and the formazan crystals formed were dissolved in 200 µl of DMSO and the absorbance was measured at 570 nm using an ELISA microtitre plate reader (Neuman *et al.,* 1993). The data are expressed as percentage of control.

### 3.2.6 Preparation of cell lysate

After the treatment period, the cells were centrifuged at 5000x g for 5 min and the pellet was washed with ice-cold PBS, pH 7.4. The cell pellet was resuspended in PBS and sonicated with an ultrasonicator (Bandelin Sonopuls HD 2200, Berlin) for 3 cycles at 30sec/cycle. The cell extract was centrifuged at 12000x g for 20 min at 4°C and the supernatant was used for enzyme assays and GSH content determination. Total protein was estimated using Lowry's method (Lowry *et al.,* 1951).

### 3.2.7 Glutathione assay

Reduction in glutathione level was determined in the cell lysate by the method of Popat *et al.,* (2002) with slight modification. A 50 µl of the cell lysate was treated with 150 µL of DTNB (5,5-dithiobis-2-nitrobenzoic acid) reagent (12mM NADPH, 0.1mM DTNB, 50U/ml GSH reductase in 0.1 mM sodium phosphate buffer with 1 mM EDTA, pH 7.5) and absorbance was read at 415nm using an ELISA microtitre plate reader. The GSH content was expressed as nM/mg protein using a calibration curve prepared using known concentrations of reduced glutathione.

### 3.2.8 Lipid peroxidation

The cell lysate (0.5 ml) obtained after sonication was incubated with 1.0 ml of KCl (0.15 M) and 250 µl of 0.2 mM ferric chloride solution at 37˚C for 30 min. The reaction was stopped by adding 2.0 ml ice-cold mixture of 0.25 N HCl containing 15% trichloroacetic acid (TCA), 0.3%

thiobarbituric acid (TBA) and 0.05% butylated hydroxyl toluene (BHT) and was heated at 90˚C for 60 min. The samples were cooled and centrifuged at 7000×g for 5 min. The absorbance of the supernatant was measured spectrophotometrically at 535 nm and the results were expressed as percentage lipid peroxidation (Buege and Aust, 1978).

### 3.2.9 Antioxidant enzyme assays

Superoxide dismutase (SOD) activity in the cell lysate was measured by the inhibition of pyrogallol auto-oxidation (Kamendulis *et al.,* 1999). Briefly, 50 µl of the cell lysate was mixed with 100 µl of assay buffer (50 mM Tris HCl, 1 mM EDTA pH 8.2) and 50 µl of 0.2 mM pyrogallol. The absorbance was monitored at 420 nm for 3 min. One unit of SOD activity is defined as the amount of enzyme required to produce a 50% inhibition of pyrogallol auto-oxidation. Catalase activity was determined by spectrophotometry by measuring reduction in $H_2O_2$ absorbance at 240 nm (Aebi, 1984). The reaction mixture contained 500 µl of 30 mM $H_2O_2$, 400 µl of phosphate buffered saline (pH 7.4) and 100µl of cell lysate. One unit of catalase activity is defined as the amount of enzyme that decomposes1mM $H_2O_2$ per minute. Glutathione peroxidase activity of the cell lysate was determined spectrophotmetrically at 340 nm. Assay mixture was composed of 0.05 M phosphate buffer (pH 7.0), 1mM EDTA, 3U glutathione reductase, 1mM GSH, 0.2mM NADPH, 12 mM tertiary butyl hydroperoxide and cell lysate. Oxidation of NADPH was recorded at 340 nm at 15s intervals for 3 min. Oxidation of NADPH at 37°C was determined spectrophotometrically at 340 nm. One unit of activity was defined as the amount of GPx required to oxidize 1 mM of NADPH per min (Flohe and Gunzler, 1984).

### 3.2.10 Transcript analysis of genes of AOEs

Total RNA from hepatocytes was extracted using TRI REAGENT$^{TM}$ (Sigma-Aldrich, USA) according to the manufacturer's protocol which is shown in **Fig. 3.1**.

The resulting RNA pellet was dissolved in RNAse free water and total RNA was quantified by measuring the absorbance of a suitably diluted solution at 260nm. Total RNA was calculated using the following formula:

$$T_{RNA}\ (\mu g/\mu L) = OD_{260nm} \times \text{Dilution}$$

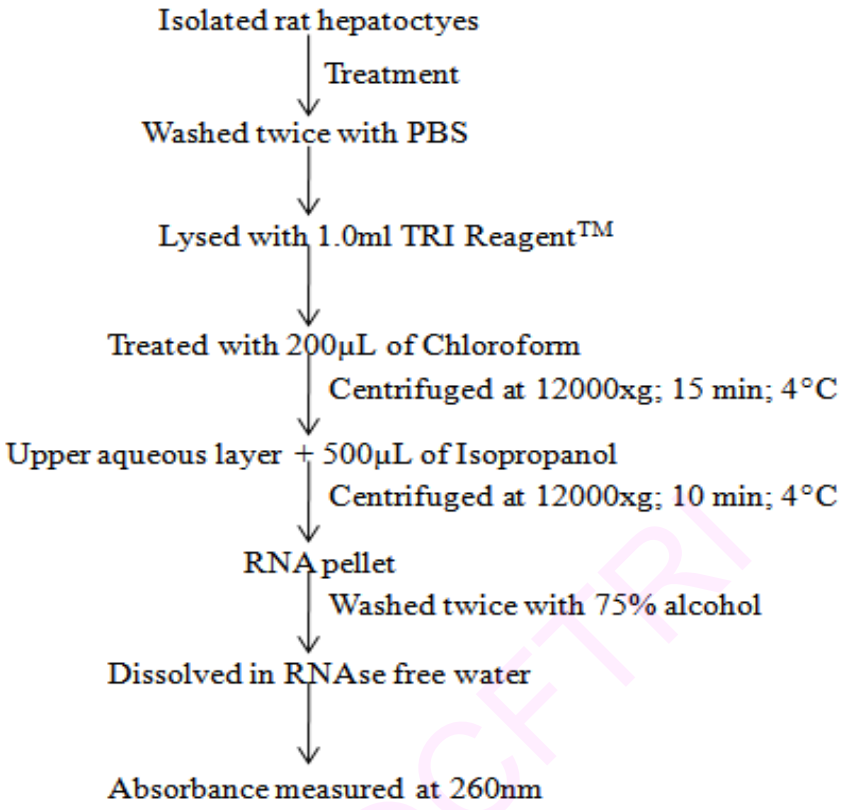



Fig. 3.1. Flow chart for isolation of total RNA from cells.

### 3.2.11 Reverse transcription of total RNA to obtain cDNA

First strand cDNA synthesis was carried out as shown in **Fig. 3.2**.

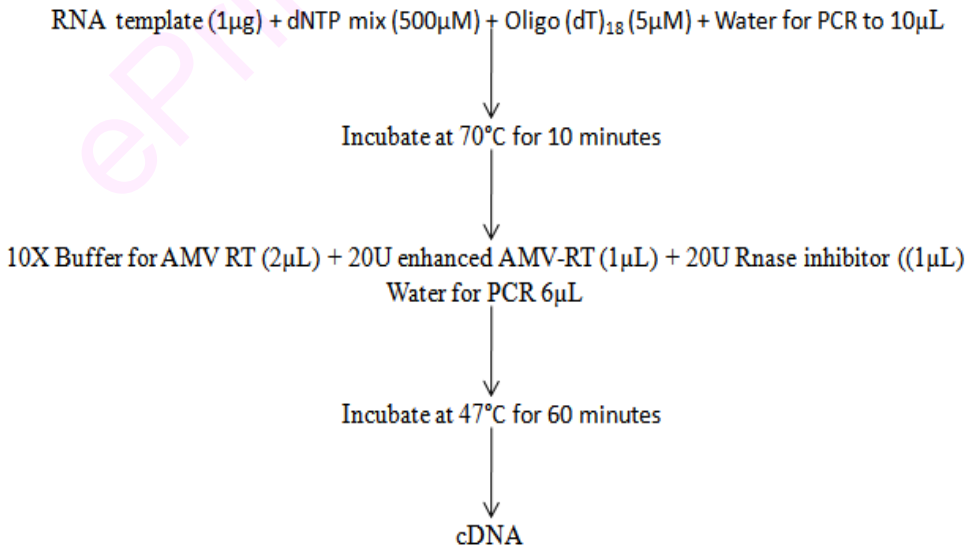



Fig. 3.2. Flow chart for reverse transcription of total RNA for cDNA synthesis.

### 3.2.12 PCR amplification of genes coding AOEs

The first strand cDNA obtained was used for PCR amplification of AOEs using gene-specific primers. To ascertain expression levels, Cu-Zn SOD, β-actin, GPx were amplified using the primer sequences reported by Bhor *et al.,* (2006). Primers were designed for CAT based on the available sequences from NCBI data base. The primer sequences and the product size of the gene amplified are shown in **Table 3.1**.

Table 3.1. Primer sequences and product sizes of the genes amplified from isolated rat hepatocytes

| Gene | Accession number | Primer (5′-3′) | | Product size (bp) |
|---|---|---|---|---|
| β-actin | V01217 | Forward | CCTGCTTGCTGATCCACA | 505 |
| | | Reverse | CTGACCGAGCGTGGCTAC | |
| CAT | M25669 | Forward | GATTGGCAGAGCCTGAAGTC | 219 |
| | | Reverse | AGGCTCTGAGGAACACTGGA | |
| CuZnSOD | X05634 | Forward | GCAGAAGGCAAGCGGTGAAC | 383 |
| | | Reverse | TCACACCACAAGCCAAGCGG | |
| GPx | M21210 | Forward | CTCTCCGCGGTGGCACAGT | 290 |
| | | Reverse | CCACCACCGGGTCGGACATAC | |

CAT: catalase; SOD: Superoxide dismutase; GPx: Glutathione peroxidase

PCR reaction mixtures contained cDNA equivalent to 0.1 µg total RNA, 0.5 µM each forward and reverse gene specific primers, 250 µM each dNTP, 1x Taq reaction buffer, 1U of Taq polymerase. PCR was performed for 35 cycles. The reaction conditions for CAT, SOD and β-actin were: initial activation at 94°C for 2 min; three-step cycling involving (a) denaturation at 94°C for 1min (b) annealing at 58°C for 30s, (c) extension at 72°C for 30s. Final extension was at 72°C for 10 min; and for GPx initial denaturation at 94°C for 30s and final extension for 7 min keeping the other conditions similar to the other genes. β-actin was used as the house keeping gene. The PCR product was loaded on to a 1.2% agarose gel and horizontal electrophoresis was performed in 10x TAE buffer for 2 h at 50V/cm. DNA in the gel was visualized under UV light after staining with 5µg/mL ethidium bromide solution in water and the images were documented using BioRad Chemidoc XRS gel documentation system (Hercules, CA, USA). Quantification of the bands was done using the E.A.S.Y Win32 imaging software provided by Herolab (GmbH Wiesloch). The amplified genes were cloned into pTZ57-R/T T-tail cloning vector (MBI Fermentas, GmbH) and sequenced (Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India). The sequences of genes amplified from isolated rat hepatocytes are presented in **Appendix A**.

### 3.2.13 Data analysis

All experiments were done in triplicates and the data presented are the averages of mean of three independent experiments with standard deviation. The data were analyzed by one-way analysis

of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and post-hoc mean separations were performed by Duncan's Multiple-Range Test at $p \leq 0.05$ (Harter, 1960).

## 3.3 Results and Discussion

Recent years have witnessed an outburst of interest in understanding the involvement of free radicals in the initiation and progression of pathologies, including carcinogenesis.

### 3.3.1 Effect of extracts on the viability of isolated rat hepatocytes

Before proceeding with the experiments, it was imperative to test the toxicity of the extracts on isolated rat hepatocytes in order to choose the non-toxic dose. Therefore, the cells were exposed to different doses (10-500ppm) of extracts for 4 h followed by MTT assay. The results of MTT assay showed that the extracts of *S. cumini* as well as that of *D. regia* were non-toxic to rat hepatocytes at the tested concentrations (**Fig. 3.3**).

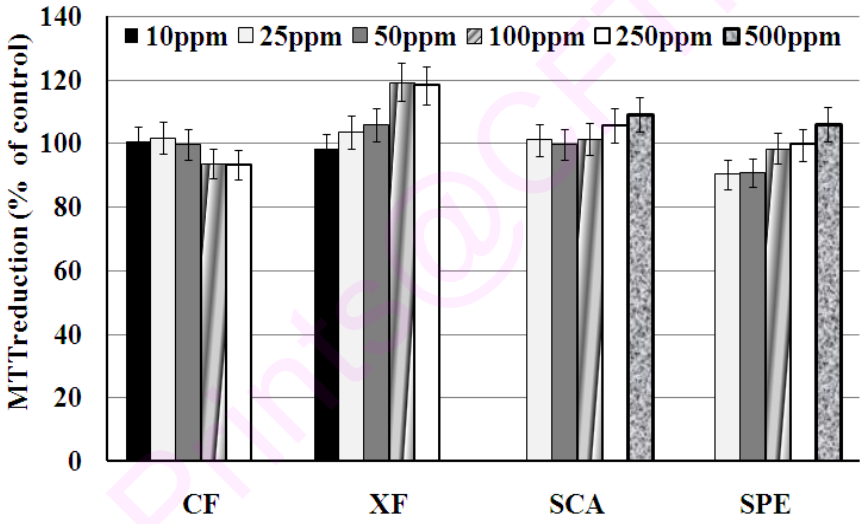



Fig. 3.3. Effect of the extracts on the viability of rat hepatocytes. Rat hepatocytes were isolated and incubated for 4 h with DMEM containing different concentrations of extracts; viability determined by MTT assay and expressed as percentage of control. Data are expressed as average ± SD of three independent experiments.

It was noticed that the extracts retained the viability of the isolated rat hepatocytes under the culture conditions and that the viability in rat hepatocytes treated with most of the extracts was more than that of the control, untreated cells (100%). This indicates that these extracts, especially XF and SCA (**Fig. 3.3**) provide protection against natural stress the cells might be experiencing during the mechanical isolation procedure and culture conditions, which might cause some of the cells to die. Based on the results, those concentrations at which the cell viability was retained to above 90% were selected for further studies.

## 3.3.2 AMELIORATION OF $CCL_4$-INDUCED TOXICITY

### 3.3.2.1 Cell Viability and Integrity

In order to examine the cytoprotective effects of anthocyanins and carotenoids against $CCl_4$-induced cytotoxicity, cytotoxic effect of $CCl_4$ was first evaluated in freshly isolated rat hepatocytes. Preliminary examination revealed that 7mM of $CCl_4$ inhibited the cell growth by 40-50% upon incubation for 1 h followed by incubation with fresh medium for 3 h. This concentration of $CCl_4$ was used to induce cytotoxicity in further experiments. Carbon tetrachloride is a potent hepatotoxic agent and according to the literature, it is the first compound ever to be tested as a liver toxicant (Cheeseman, 1995). It chiefly acts by forming reactive metabolites through the cytochrome P450 mixed function oxidase system (Ruch *et al.,* 1986). Although production and use of $CCl_4$ has reduced over the years due to increased awareness of its toxicity, certain population still remains at risk due to exposure water contamination, mainly chlorination, air pollution and occupational settings. Chronic exposure to $CCl_4$ has shown to induce liver damage, fatty degeneration and cirrhosis including fetal growth retardation in rats, guinea pigs and monkeys (Adams *et al.,* 1952; Prendergast *et al.,* 1967). Humans occupationally exposed to 45-100ppm of $CCl_4$ demonstrated symptoms including nausea, anorexia, vomiting, flatulence, epigastric discomfort or distention, depressive symptoms, headache or giddiness (Kazantzis *et al.,* 1960). Therefore, it is desirable that antioxidants which neutralize the harmful effects of such compounds be evaluated.

The viability of cells after treatment with and extracts was analysed by MTT assay. MTT assay is a test for metabolic competence of the cells and is based upon the assessment of mitochondrial performance. The conversion of yellow colored MTT to a purple formazan derivative by mitochondrial succinate dehydrogenase (Fry and Hammond, 1993) is a direct measure of cell viability.

**Figure 3.4** shows that EGCG and the extracts exert a differential effect against $CCl_4$-induced cytotoxicity. Carbon tetrachloride induced a 40% reduction in cell viability as compared to the untreated control cells. The viability was considerably alleviated to 85-99% in the presence of the extracts after $CCl_4$ treatment (**Fig. 3.4**). Similar effects were found with EGCG when used at levels 0.1, 1.0, 5.0 and 10.0 μM.

It was apparent from the results (**Fig 3.4**) that among the four extracts, SCA showed the highest cytoprotective activity. At 50ppm, it showed an activity equivalent to that of 0.1μM of EGCG. The carotenoid extracts, CF and XF showed a similar pattern where they were cytoprotective up to 100ppm, beyond which the viability of cells reduced dose dependently.

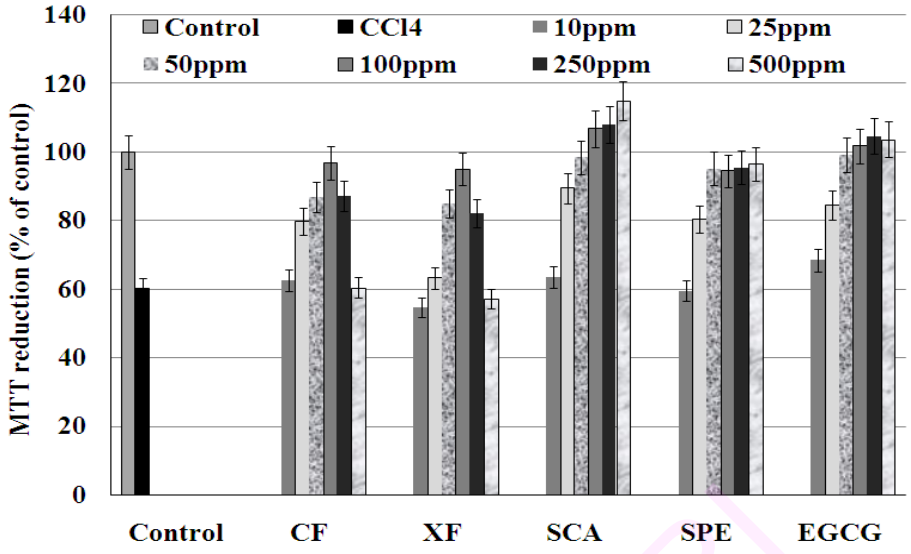



Fig. 3.4. Effect of extracts and EGCG on the $CCl_4$-induced cell death in isolated rat hepatocytes. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with different concentrations of extracts and EGCG* for 3 h. Cell viability was assessed by MTT assay. The concentrations of EGCG were in the order of 0.05, 0.075, 0.1, 1.0, 5.0 and 10.0 µM. Data correspond to the representative values from three independent experiments done in triplicate and are expressed as average ± SD.

The SCA at concentrations above 100ppm increased the cell viability beyond the control levels indicating that the anthocyanins render excellent protection against oxidative stress. Anthocyanins have a long history of use as antioxidants. Anthocyanins from different sources have consistently shown to be protective against in oxidative stress models. Anthocyanins from various sources were found to be protective against oxidant-mediated cell death in transformed and untransformed cells (Elisia and Kitts 2008; Chang *et al.,* 2006). The action may be attributed to their distribution in various cellular compartments (Youdim *et al.,* 2000) which perhaps extend an antioxidant effect within and around the cell.

Further, in order to confirm the results of MTT assay, intracellular LDH leakage was evaluated as a marker of cell membrane integrity and viability. While the control cells (untreated) showed less than 5% leakage of LDH, the $CCl_4$-exposed cells showed a substantially high value of 45% leakage of LDH (**Fig. 3.5).** Exposure of the $CCl_4$-treated cells to extracts and EGCG significantly brought down the levels of LDH leakage indicating the reversal of the damage induced by $CCl_4$ toxicity in primary rat hepatocytes (**Fig. 3.5**). This was in agreement with the results of MTT assay where treatment of the rat hepatocytes with extracts and EGCG post- $CCl_4$ exposure showed an increase in the viability. EGCG was most effective at the lowest concentration (0.1µM) and SCA at 250ppm was equivalent to the action of 1µM EGCG in terms of inhibition of LDH

leakage. Treatment of the rat hepatocytes with *S. cumini* extracts post- $CCl_4$ exposure showed an increase in the viability of the same. This was further substantiated by a consequent suppression of LDH leakage by SCA and SPE at concentrations ranging from 50-500 ppm suggesting protection against $CCl_4$ toxicity. The higher concentrations of EGCG and SPE increase LDH leakage although to an extent significantly lower than that of $CCl_4$ ($p<0.001$).

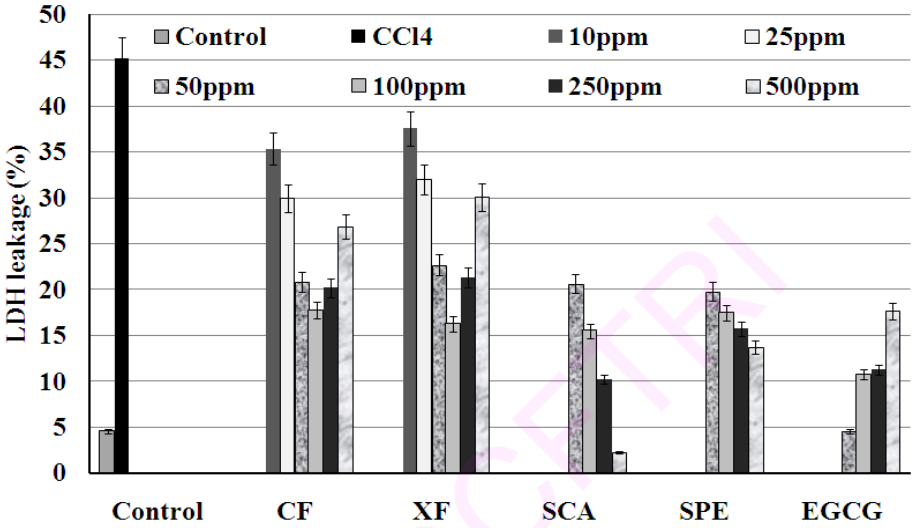



Fig. 3.5. Effect of extracts and EGCG on the $CCl_4$-induced leakage of LDH from isolated rat hepatocytes. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with different concentrations of extracts and EGCG* for 3 h. The concentrations of EGCG were in the order of 0.05, 0.075, 0.1 & 1.0μM. Data correspond to the representative values from three independent experiments done in triplicates and are expressed as average ± SD.

The cells treated with carotenoids extracts showed similar results as with MTT assay. That is, the LDH leakage reduced in a dose dependent manner up to 100ppm, beyond which there was a steady increase. Burton and Ingold (1984) postulated that carotenoids, particularly β-carotene, exert a prooxidant effect at conditions of high concentration and high oxygen tension. However, Krinsky and Johnson (2005) suggest that apparently the activity observed by Burton and Ingold (1984) is not prooxidant but that, the carotenoids show reduced activity at such conditions. It is also demonstrated that carotenoids show an antioxidant or pro-oxidant behavior depending upon the redox potential of the individual molecule and the inorganic chemistry of the cell (Schwartz 1996). Therefore, it may be presumed that carotenoids of *D. regia* show a reduced cellular protection at higher concentrations. A similar observation was made with the total carotenoids of *D. regia* against AAPH-induced plasma oxidation *in vitro*, where the inhibitory activity reduced with an increase in concentration of the carotenoids (**Table 1.9**). Similarly, a dose-dependent increase in LDH leakage by EGCG appears to be due to the cytotoxicity of the same at higher concentrations which is also observed by other reserachres. For instance, Chung *et al.,* (2007)

observed that EGCG had no cytotoxic effect up to a dose of 10μM in human neuroblastoma cells while a cytotoxic effect was observed above this dose which was evident by the increase in LDH leakage and a consequent decrease in MTT reduction. Similarly, the increased LDH leakage in EGCG treated cells at higher dose post-$CCl_4$ treatment was in agreement with the results of Chung *et al.,* (2007), where EGCG was shown to enhance the toxicity of rotenone, a pesticide, in human neuroblastoma cells. This further suggests that EGCG at still higher doses may actually exacerbate the xenobiotic-induced cell toxicity. Thus, the suppression of the LDH leakage as well as the mitigation of $CCl_4$-induced cell toxicity by the selected levels of extracts clearly establishes their role as cytoprotective agents against xenobiotics injury, the most potent being those from Jambolana fruit since these extracts did not impart any negative effects.

Based on the results of LDH and MTT assay, 50 and 100 ppm of the extracts and 0.1 and 1.0 μmol/l EGCG were chosen for further study since these concentrations provided sufficient protection (>85%) against $CCl_4$ induced cell death.

### 3.3.2.2 Glutathione content

Glutathione (GSH) is an important tripeptide that plays a vital role in the detoxification of exogenous and endogenous compounds, either by conjugating with an electrophilic drug or its toxic metabolites or by acting as a reducing agent in the metabolism of peroxides and free radicals (Popat *et al.,* 2002). It plays a central role in preventing lipid peroxidation since it stimulates GPx to scavenge organic and inorganic peroxides (Reed, 1990; Smith *et al.,* 1996). It is now established that oxidative injury generally leads to GSH depletion and a consequent, lipid peroxidation and ultimately cell death (Casey *et al.,* 1995; Mytilineou *et al.,* 2002). GSH depletion caused by overdose of toxins has been associated with renal and hepatic failure ultimately causing death (Herzenberg *et al.,* 1997; Thomas, 1993; Zimmerman and Maddrey, 1995).

Carbon tetrachloride induced death of cultured rat hepatocytes has been linked to a depletion of GSH content with an ensuing increase in lipid peroxidation or direct injury to proteins and nucleic acids (Bellomo and Orrenius, 1985; Miccadei *et al.,* 1988). **Figure 3.6** summarises the effect of extracts and EGCG on intracellular GSH content. A slight but significant ($p<0.05$) fall (13%) in the intracellular GSH levels was achieved by exposing the isolated rat hepatocytes with $CCl_4$. Treatment of $CCl_4$-treated cells with SCA substantially increased the GSH content which was higher at 50 ppm than 100 ppm. The increase was nearly 2-fold (6.46 nM/mg protein) at 50 ppm as compared to the GSH levels in cells treated with $CCl_4$ (3.67 nM/mg protein). The recovery response of the cells was highest with SCA followed by EGCG, CF and XF (**Fig. 3.6**).

However SPE caused a depletion of intracellular GSH levels. Since this fraction displays good antioxidant activity in various other tests and also since there was no reduction in the viability of cells treated with SPE, it can be assumed that the SPE offers protection through pathways other than that involving GSH. Since SPE treated cells show high GPx activity (**Fig. 3.10**), it is possible that cellular GSH is utilized as a substrate for GPx. Another practical and more meaningful explanation that can be offered here is the conjugation of the GSH by the phenolic acids (Galati *et al.,* 2006) present in the pulp extract (Anonymous, 1976), thus making the free GSH unavailable for the assay.

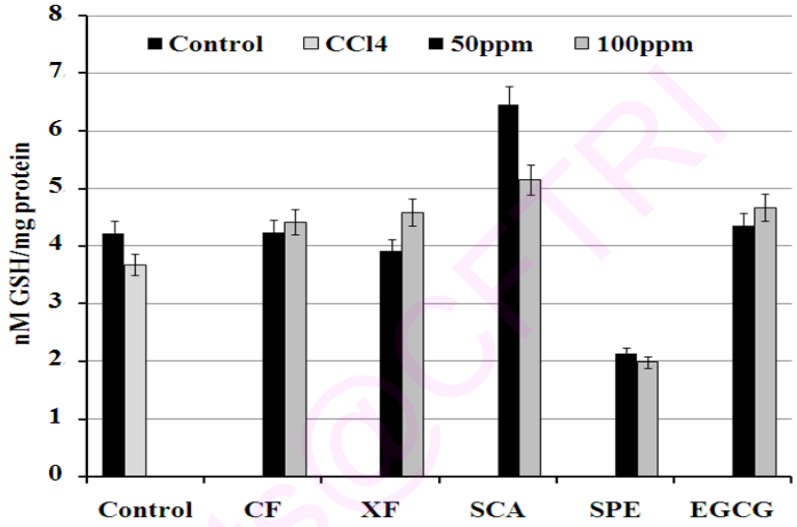



Fig. 3.6. Effect of extracts and EGCG on the GSH content of rat hepatocytes treated with $CCl_4$. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with 50ppm and 100ppm of extracts and 0.1 and 1.0 μM EGCG for 3 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

A similar observation was made with buthionine sulfoximine, which reduced GSH level in cultured rat hepatocyte without reducing the cell viability (Lee and Farrell, 2001). The SCA, CF, XF and EGCG apparently increased the GSH content as compared to the control levels (**Fig. 3.6**). The maintenance of GSH levels in the presence of oxidative stress may be due to the stimulation of the enzyme γ-glutamylcysteine synthetase and GSH synthetase by the antioxidant (Wu *et al.,* 2004). It has been postulated that certain phenolics are capable of stimulating GSH synthesis in vivo (Myhrstad *et al.,* 2002; Moskaug *et al.,* 2005). Rimbach *et al.,* (2001) reported the ability of *Gingko biloba* extract (EGb761) to induce GSH in mammalian cell lines. They observed a strong correlation between the increased expression of catalytic subunit of γ-glutamylcysteinyl synthetase, the rate-limiting enzyme in GSH synthesis. A similar mechanism may be expected here since EGb761 chiefly contains flavonoids along with gingkolides. Anthocyanins being

flavonoids in nature may cause analogous effects. Anthocyanins have been reported to increase and also prevent the depletion of GSH content under conditions of oxidative stress (Valcheva-Kuzmanova *et al.,* 2004; Weisel *et al.,* 2006). Recent reports also point to the ability of carotenoids to stimulate GSH synthesis *in vivo*. Carotenoids of saffron and β-carotene have shown to increase GSH synthesis by overexpressing γ-glutamyl cysteine synthase (the rate limiting enzyme in GSH synthesis) in different cell models and animal models (Ochiai *et al.,* 2007; Takeda *et al.,* 2008). EGCG is also known to reduce hepatic fibrosis via induction of *de novo* GSH synthesis in hepatic stellate cells in a time and dose dependent manner. The hepatic stellate cells were shown to exhibit a delayed response towards EGCG, with a very slight increase in GSH content during the first 5 h of incubation (Yumei *et al.,* 2006). A 10% increase in GSH content was observed at 1.0 μM on incubation for 3h with hepatic cells which perhaps increases with an increase in incubation time. This theory can also be extended to the cells' response to the extracts under the study and can be exploited in such cases of liver disorders, which however need further investigation. Alternatively, Chen *et al.,* (2004) have reported the protective effect of EGCG against $CCl_4$ induced hepatotoxicity in mice by down-regulating the nitric oxide synthase mRNA expression and inducible nitric oxide-derived prooxidants. This suggests that in $CCl_4$ toxicity, EGCG exerts protection via other pathways in addition to that involving GSH synthesis. The high GSH content may also be responsible for a direct scavenging of free radicals generated *in vivo* in addition to acting as a substrate to the enzyme GPx. The results of carotenoids of *D. regia* are indicative that they may also act by increasing the cellular GSH synthesis. However, this hypothesis needs a thorough investigation where an assessment at transcriptional levels of these enzymes under the influence of free radical generator and scavengers will throw light on the mode of action of the extracts.

#### 3.3.2.3 Lipid peroxidation

Lipid peroxidation is characteristically a free radical chain reaction initiated by the abstraction of a hydrogen atom from PUFA side chains. The initiation of lipid peroxidation is carried out in most cases by free radicals, which cause cellular injury by inactivating membrane enzymes and receptors, depolymerizing polysaccharides, cross-linking and fragmenting protein. As a result, the structure and fluidity of the membrane are damaged and normal cell function is lost. Malondialdehyde (MDA) is one of the main lipid peroxidation product, its elevated levels reflecting the degree of lipid peroxidation induced injury in hepatocytes (Ng *et al.,* 2005). The extent of MDA formation is a direct measure of oxidation and decomposition of polyunsaturated fatty acids and the percentage protection offered by the extracts against MDA formation has been

calculated in the present study. Carbon tetrachloride induced about 20% increase in cellular lipid peroxidation in isolated hepatocytes (46.25 ± 2.31%) as compared with the untreated cells (28.96 ± 1.44%). SCA (50 and 100ppm) and EGCG (1 µM) significantly reduced the $CCl_4$-induced lipid peroxidation in rat hepatocytes to near-normal levels (**Fig. 3.7**). However, SPE did not significantly alter the level of lipid peroxidation initiated by $CCl_4$. Earlier studies have established that the depletion of GSH by $CCl_4$ is followed by peroxidation of cellular lipids resulting in death of the hepatocytes (Miccadei *et al.,* 1988). This was further supported in our study where the $CCl_4$-exposed hepatocytes clearly exhibited a depletion of GSH content and a substantial increase in lipid peroxidation (**Fig. 3.6 & 3.7**). This suggests that the anthocyanins present in the extract may play an important role in preventing initiation and propagation of the lipid peroxidation process by scavenging the free radicals via the GSH (Martin-Aragon *et al.,* 2001).

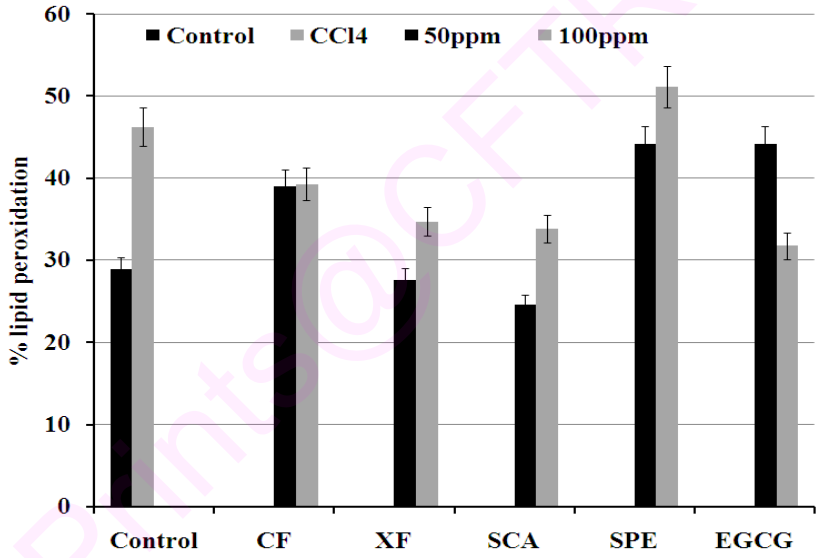



Fig. 3.7. Effect of extracts and EGCG on the inhibition of $CC_4$-induced lipid peroxidation in isolated rat hepatocytes. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with 50ppm and 100ppm of extracts and 0.1 and 1.0 µM EGCG for 3 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

A considerable reduction in lipid peroxidation was observed in cells treated with SCA, the extract being more effective at lower concentration. Probably this being an ideal situation, higher level of extract containing un-reacted anthocyanins having an absorption maximum of 535 may contribute for erroneous results. This has been indicated as one of the reasons for high lipid peroxidation value obtained at high concentrations of SCA and SPE, although this argument needs to be confirmed by detailed analytical studies. Contrarily, SPE showed a reduction in GSH content, not offering any protection against lipid peroxidation despite the high viability of cells (GSH content

is generally regarded as an index of cell death). However, a closer observation of earlier results of LDH leakage and viability shows that SPE does protect the cells against cell death. The ineffectiveness of the SPE against$CCl_4$-induced lipid peroxidation may be linked to the low cellular GSH content, while the reduction in cell death may be due to mechanisms independent of these two parameters. It is reported elsewhere that increase in lipid peroxidation is not always associated with an increase in cell death.

Carotenoids from various sources have demonstrated strong antioxidant activities in vitro. The antioxidant action of carotenoids is chiefly attributed to their singlet oxygen quenching and their ability to trap peroxyl radicals. The prevention of lipid peroxidation by carotenoids is credited to its singlet oxygen quenching ability (Stahl and Sies 1996). From **Fig. 3.7** it is obvious that CF and XF significantly reversed the effect of $CCl_4$. Carotenoids, the major exogenous antioxidants, are responsible for preventing lipid peroxidation induced by singlet oxygen or free radical initiators and an inverse relationship has been found between the β-carotene content and malondialdehyde formation in humans (Lepage *et al.,* 1996). It is suggested that the carotenoids, particularly β-carotene binds to the cell surface and confers protection against the singlet oxygen generated at its proximity (Bohm *et al.,* 1993). A similar action may be expected with other carotenoids species and this could possibly explain the inhibition of lipid peroxidation in hepatocytes treated with CF and XF by a related mechanism. The CF being rich in β-carotene (**Fig. 1.8**) may speak for the higher response as compared to the XF. However, xanthophylls are demonstrated to have antioxidant activities comparable to β-carotene (Di Mascio *et al.,* 1989). Remarkably consistent inverse relationships between plasma xanthophyll carotenoids and indices of oxidative damage have been reported earlier (Haegele *et al.,* 2000). Therefore, it is evident that both carotene hydrocarbons and their oxygenated counterparts are effective antioxidants thus playing a crucial role in thwarting the free radical mediated initiation and even progression of different pathologic events. It is suggested that complex mixtures of carotenoids alone or in combination with other antioxidants are more effective in their ability to inhibit lipid peroxidation and hence against oxidative stress-induced pathologies (Vitaglione *et al.,* 2004; Paiva and Russell, 1999). The two extracts are a mixture of several types of carotenoids and hence exert a positive effect on the overall physiological status of the cells. Under conditions of oxidative stress they perhaps act synergistically with each other thus ameliorating the degenerative effects induced by the xenobiotic or the prooxidant. From the results obtained in the present study it can be concluded that carotenoids of *D. regia* flowers exert protective effect against oxidative stress induced injury of hepatocytes and can be an economic source of carotenoids to be used both as natural colorant and antioxidant supplement.

### 3.3.2.4 Antioxidant enzyme assays

Cellular metabolism of molecular oxygen generates reactive species such as superoxide, $H_2O_2$ and hydroxyl radicals (Chance *et al.,* 1979). The AOEs play an important role in counteracting the reactive species generated within the body and aid in maintaining the intracellular redox homeostasis. It is also reported that increased activity of these AOEs play a key role in increasing the longevity in humans (Mecocci *et al.,* 2000). When the levels of reactive species surpass the antioxidant capacity of the cells, oxidative stress ensues (Halliwell, 1999). A number of factors contribute to the generation of free radicals in the body with a consequential advancement in pathology. They include exposure to environmental toxicants and pesticides, radiation, smoke, particulate matter and also cellular metabolic reactions. Carbon tetrachloride is one such agent and is known to cause a reduction in tissue's glutathione (GSH) content, suppress activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and increase lipid peroxidation with subsequent liver damage (Castro *et al.,* 1974; Ohta *et al.,* 1995).

Superoxide dismutase is an important enzyme and catalyses the first step in the antioxidant reaction. While overproduction of SOD has been reported to be beneficial in increasing the longevity of organisms, it is also found responsible for the deleterious effects perhaps as a result of increased $H_2O_2$ production (de Haan *et al.,* 1996; Amstad *et al.,* 1991; 1994). Compounds of natural origin have been reported to act against oxidizing agents chiefly via induction of detoxifying enzymes (Giudice and Montella 2006). Anthocyanins have demonstrated hepatoprotective effects against various tumor promoting agents such as $H_2O_2$, TBH, $CCl_4$, etc in cell and animal models (Obi *et al.,* 1998; Liu *et al.,* 2006; Shih *et al.,* 2007). The discussions on hepatoprotective effects generally are limited to the activities of hepatic marker enzymes, lipid peroxidation and cellular GSH status (Tsuda *et al.,* 2000; Valcheva-Kuzmanova 2004; Valentova *et al.,* 2007) while information on the action of these compounds on the AOEs is scarce (Igarashi *et al.,* 2000); however, this subject has been addressed in other tissues. Therefore, this study evaluated the effect of anthocyanin rich extract of *S. cumini* and carotenoids fractions of *D. regia* on the AOEs in isolated rat hepatocytes. **Fig. 3.8** depicts the effect of extracts on the activity of SOD post-$CCl_4$ treatment.

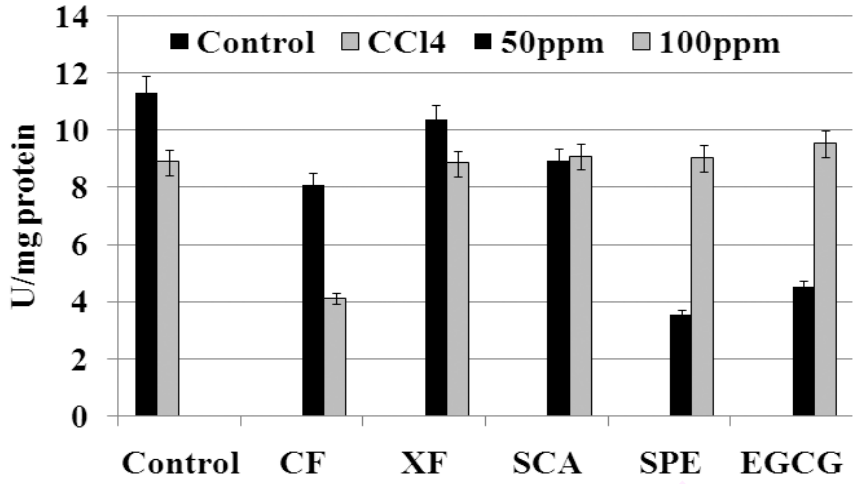



Fig. 3.8. Effect of extracts and EGCG on the activity of superoxide dismutase in isolated rat hepatocytes exposed to $CCl_4$. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with 50ppm and 100ppm of extracts and 0.1 and 1.0 μM EGCG for 3 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

Carbon tetrachloride significantly reduced the activity of SOD, a 22% reduction compared to control cells. None of the extracts showed significant protective effects except for XF at 50ppm which reversed the $CCl_4$-induced reduction in SOD to near normal levels. However, the extracts SCA and SPE as well as EGCG brought about a negligible 1-6% increase in the SOD activity brought down by $CCl_4$ but were inefficient in restoring the enzyme activity to the normal level. The CF completely failed to evoke any effect but further reduced the activity of the enzyme. **Figure 3.9** depicts the alteration of enzyme CAT in the presence of $CCl_4$ and the extracts.

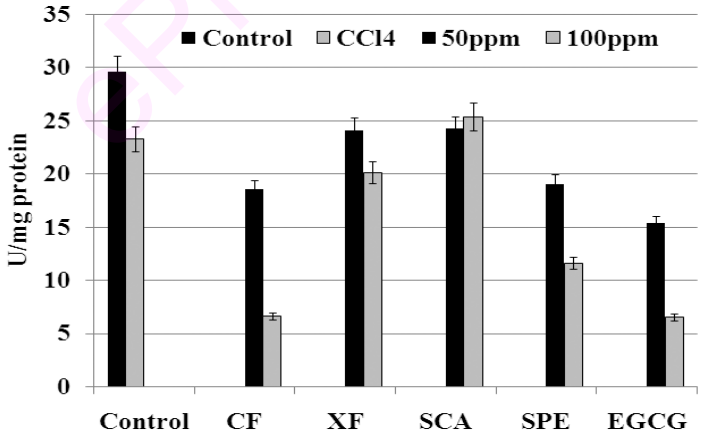



Fig. 3.9. Effect of extracts and EGCG on the activity of catalase in isolated rat hepatocytes exposed to $CCl_4$. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with 50ppm and 100ppm of extracts and 0.1 and 1.0 μM EGCG for 3 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

Catalase plays a major role as AOE in the mammalian system, by detoxifying hydrogen peroxide generated by SOD, various oxidases and other enzymatic and non-enzymatic auto oxidation of compounds (Eaton, 1989). Carbon tetrachloride brought about a 20% reduction in activity of CAT (23.30 ± 0.19 U/mg protein) as compared to the vehicle treated cells (29.60 ± 0.32 U/mg protein). It was observed that the CAT activity increased in a dose dependent manner when treated with the SCA while it was the reverse with CF and XF. This shows that the low concentrations of carotenoids are more protective towards xenobiotic injury to cells. Similar observations were also made with EGCG and SPE which showed higher activity at lower concentrations. However the CAT activity was well below that of $CCl_4$-treated cells at the tested concentrations further exacerbating the effect of $CCl_4$. Xanthophylls fraction and SCA were more effective in countering the action of $CCl_4$ on the activity of CAT, though the elevation in activity was only negligible. Carotenoids such as β-carotene mainly act by direct scavenging of free radicals generated. Since the CF, which has β-carotene as the chief carotenoid, shows very low CAT activity it presumably acts by a direct action on the metabolites of $CCl_4$ not involving CAT. EGCG, CF and SPE further reduced the CAT activity to 15.3, 18.51 and 18.9 U/mg protein respectively at 50 ppm (**Fig. 3.9**).

Another peroxide neutralising enzyme, GPx counteracts the free radicals using GSH as a substrate. As a result, GSH is oxidized to GSSG (oxidized glutathione), which in turn is reduced to GSH by glutathione reductase at the expense of NADPH, forming a redox cycle. **Figure 3.10** shows the effect of different extracts on AOEs activities in $CCl_4$ intoxicated rat hepatocytes. Carbon tetrachloride showed about 28% reduction in GPx activity (5.22 U/mg protein) as compared to the untreated cells (7.32 U/mg protein).

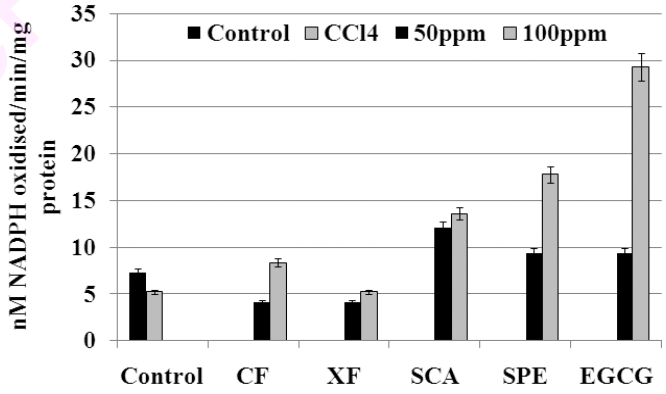



Fig. 3.10. Effect of extracts and EGCG on the activity of glutathione peroxidase in isolated rat hepatocytes exposed to $CCl_4$. The cells were treated with 7 mM of $CCl_4$ for 1 h followed by treatment with 50ppm and 100ppm of extracts and 0.1 and 1.0 μM EGCG for 3 h. Data

correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

Supplementing the cells with extracts significantly enhanced the GPx activity in a dose dependent manner. At 100 ppm, SCA and SPE showed an activity of 13.59 and 17.77 U/mg protein respectively. EGCG evoked a significant 4-fold increase in GPx activity in the isolated rat hepatocytes. It appears from the results that unlike the other two AOEs, GPx was highly regulated by CF than XF in isolated rat hepatocytes. A significant rise in the activity was noted at 100ppm of CF (8.36 Units) rising above the normal levels.

Carbon tetrachloride is commonly used to induce acute and chronic liver toxicity and to study mechanisms of the same in cell and animal models (Ruch *et al.,* 1986; Brattin *et al.,* 1985). In the present study the overall effects of $CCl_4$ on isolated rat hepatocytes show that it significantly reduces the cellular antioxidant defense by reducing the activities of CAT, SOD and GPx and decreasing the cellular GSH content and by increasing lipid peroxidation. This is in agreement with the earlier literature (Ruch *et al.,* 1986). Effect of plant derived flavonoids, anthocyanins, carotenoids and other non-nutritional elements on $CCl_4$-induced toxicity has been an object of intense research. Anthocyanins of different sources have been extensively studied for their protective action against $CCl_4$-induced toxicity in different tissues of rodents (Liu *et al.,* 2006; Obi *et al.,* 1998; Valcheva-Kuzmanova, 2004). However, most of these reports emphasise the importance of hepatic marker enzymes rather than the AOEs and there are very few reports concerning the effect of these anthocyanins on the AOEs in stress-induced cells. Similarly carotenoids have been shown to be cytoprotective in various oxidative stress models. Carotenoids of *Dunaliella salina* show strong protection against $CCl_4$-induced hepatotoxicity in experimental models by increasing the activities of CAT, SOD and GPx in addition to decreasing the activities of hepatic marker enzymes (Hsu *et al.,* 2008). Carotenoids such as □-carotene, β-carotene, lutein and lycopene showed protection against $CCl_4$-induced toxicity to hepatocytes in *in vitro* cultures by reducing the lipid peroxidation while they also increased the cell survival (Kim, 1995). Lycopene supplementation in congestive heart disease patients showed increased serum AOE activities as compared to those patients who were not supplemented (Bose and Agrawal, 2007). All these reports support the protective effects of carotenoids against oxidative injury. Therefore, the present study was aimed at elucidating the antioxidant mechanisms of anthocyanins of *S. cumini* fruits and carotenoids of *D. regia* flowers with EGCG being used as a reference compound. AOEs play an important role in maintaining the cellular redox homeostasis. Epidemiological and experimental studies indicate a direct relationship between the dietary antioxidants and enzymatic antioxidant defense and inverse relationship with coronary heart

disease, inflammation, oxidative stress and cancer (Hozawa *et al.,* 2007; Evans *et al.,* 1998). Women on a carotene depleted diet seemed to possess depressed erythrocyte SOD activity and increased lipid peroxidation (Dixon *et al.,* 1994) suggesting the need of dietary carotenoids not just as a provitamin A but also to enhance the resistance towards oxidation susceptibility. Superoxide dismutase is an important enzyme converting the superoxide ions to less reactive $H_2O_2$ and oxygen. Its activity was significantly inhibited by $CCl_4$ while most extracts did not offer significant protection to SOD except the xanthophylls rich fraction, XF, which resumed the activity to near normal level (**Fig. 3.8**). It appears that the results are in accordance with the well-known hypothesis that carotenoids particularly, β-carotene, are more effective at lower concentrations under conditions of preexisting cellular stress such as cigarette smoke (ATBC, 1994; Russell, 2004). This can be substantiated here because of the fact that the cells were pretreated with $CCl_4$ which induces a stress condition and the reactive intermediates of $CCl_4$ metabolism might initiate alteration in metabolism of carotenoids which are either less active or pro-oxidative. The enzyme CAT, which catalyses the peroxidation of $H_2O_2$ formed due to the reaction of SOD on superoxide anion, is an important defense mechanism of aerobes since $H_2O_2$ is constantly produced in the body as a result of metabolic reactions. The carotenoids and SCA evoked a CAT response similar to that of SOD. However, the response of CAT and SOD to SPE and EGCG was opposite where the SOD activity increased dose dependently, that of CAT reduced. That is to say that higher SOD activity was associated with a lower CAT activity. Glutathione peroxidase activity radically increased in cells treated with SCA, SPE and EGCG while it was marginal in CF and nil in XF (**Fig. 3.10**). A significant correlation can be made between the different biochemical parameters here. It is observed that a high SOD activity is associated either with an increased CAT activity or GPx activity but never both (**Fig. 3.8, 3.9 & 3.10**) except in the case of SCA. A high SOD is generally associated with an increased production of superoxide ion. However, the low SOD activity in $CCl_4$ treated cells may not be due to low superoxide ion concentration, but may be due to impairment in the activity of SOD due to the effect of $CCl_4$ on the protein. The high SOD activity in cells treated with extracts, especially XF and other phenolic extracts at 100ppm is a cellular adaptation to overcome or neutralize the superoxide anion in the cells, which in turn result in the production of $H_2O_2$. The $H_2O_2$ so produced is further detoxified both by CAT or GPx and perhaps by both in case of SCA. However, XF fails to elevate the GPx response to normal in rat hepatocytes pretreated with $CCl_4$. The EGCG mainly acts via stimulation of GSH synthesis and by increasing the GPx level to 4-fold (**Fig. 3.10**). In the present study, the protective effects of the XF and SCA may extend beyond inducing AOEs. The antioxidants form a mutual protection where an overproduction of

superoxide ion inactivates CAT and GPx while the $H_2O_2$ may inhibit SOD (Kono and Fridovich, 1982; Blum and Fridovich, 1985; Bray *et al.,* 1974). Therefore, under conditions of cellular oxidative stress the entire enzyme system may get inactivated at least in part due to the presence of unscavenged superoxide ion and $H_2O_2$. Under such circumstances, if SOD is induced, the concentrations of superoxide would decrease allowing partial recovery of CAT and GPx (Nelson *et al.,* 2006). Another factor to consider is the measurement of lipid peroxidation. 4-hydroxynonenal, a product of lipid peroxidation induces GPx synthesis. So, if high SOD activity imparts reduction of lipid peroxidation, it would result in low GPx synthesis as a consequence of decreased 4-hydroxynonenal production (Larini *et al.,* 2004). This perhaps explains the behavior of cells towards XF, where an increase in SOD activity with a consequent reduction in lipid peroxidation and GPx activity was observed. However, this hypothesis could not be applied to cellular response to SCA post $CCl_4$ treatment where an increased SOD and decreased lipid peroxidation did not yield high GPx activity.

From the literature it appears that carotenoids, particularly β-carotene, fail to exert a protective effect under pre-existing oxidative stress conditions especially in smokers. Evidences suggest a prooxidant action of carotenoids, especially when present at high concentrations, in smokers which is attributed to the altered metabolism of carotenoids to reactive intermediates (Wang *et al.,* 1999; Liu *et al.,* 2004). At the same time another study indicated that a high dose of β-carotene increased the lung pathology irrespective of smoking or non-smoking subjects, worsening in smoke exposed models, while a low dose β-carotene in smoke exposed ferrets provided protection against squamous metaplasia linking the paradoxical effects of β-carotene in incidence of lung cancer to its dose (Russell, 2004). A close scrutiny of the results obtained with the biochemical analyses in the present study suggests that at higher concentrations carotenoids, particularly the carotene fraction rich in β-carotene, indeed fail to provide protection against $CCl_4$-induced oxidative stress. This can also be attributed to the high oxygen partial pressure under the culture conditions (95% $O_2$ and 5% $CO_2$) which does not happen in vivo, thus in itself may be causing an oxidative environment (Halliwell, 2003). Therefore, a thorough in vivo investigation is necessary before arriving at a conclusion.

The pulp extract of *S. cumini* (SPE) did not show significant protection against AOEs and lipid peroxidation. Since MTT and LDH assays clearly show cytoprotection by SPE, there may be other mechanisms involved in cell protection rather than the involvement of GSH alone. The involvement of other enzymes such as glutathione reductase and respective transferase (not followed in this study) cannot be ignored since the glutathione redox system functions in tandem with each other to counterbalance the reactive species. The overall results show that the extracts,

especially SCA, mainly act via the glutathione redox system rather than through CAT and SOD enzyme system. A direct scavenging effect may not be ruled out. The failure of the extracts CF and SPE in completely restoring the SOD and CAT activities suggest that the two extracts reverse cell damage through biological pathways independent of these AOEs in $CCl_4$-induced toxicity. It is possible that they act via the glutathione redox system. The differential effects of extracts and EGCG indicate that they exert protective effect via different mechanisms.

## 3.3.3 AMELIORATION OF *tert*-BUTYL HYDROPEROXIDE-INDUCED TOXICITY

The previous section discussed the protective role of anthocyanins of *S. cumini* fruits and carotenoids of *D. regia* flowers against damaging effect of $CCl_4$ in isolated rat hepatocytes emphasizing on the AOEs. This section will elaborate on the mechanism of damage induced by yet another oxidant, *tert*-butyl hydroperoxide (TBH) and its amelioration by anthocyanins and carotenoids from selected sources. Due to the diverse chemical nature of prooxidants, all types of antioxidants may not protect cells against all kinds of free radicals and antioxidants may well be selective towards the type of free radicals they attack. The selectivity perhaps depends on the solubility of the oxidant and the pro-oxidant, their affinity to each other and also the cellular milieu in which they are present. Ability to act against different types of free radicals and toxins is a desirable property of any antioxidant. *Tert*-butyl hydroperoxide is one of the best described models for induction of oxidative stress status and investigation of mechanism of oxidative cell injury (Lazze *et al.,* 2003; Alia *et al.,* 2005). Therefore, the objective of the present study was to determine whether the anthocyanins of *S. cumini* and carotenoids of *D. regia* which possess strong antioxidant activity in vitro as described in the previous sections, can also protect the isolated rat hepatocytes against TBH induced oxidative stress.

*Tert*-butyl hydroperoxide is an organic hydroperoxide that is widely used to induce oxidative stress in various cell models (Lazzé *et al.,* 2003; Alia *et al.,* 2005). It is metabolised to free radical intermediates by cytochrome P-450 in hepatocytes, causes glutathione depletion and is one of the precursor molecules in the formation of malondialdehyde, a major lipid peroxidation product (Rush *et al.,* 1985; Garcia-Cohen, 2000). The toxicity of TBH is attributed to the butoxyl radicals generated due to Fenton reaction (Baker and He, 1991). *Tert*-butyl hydroperoxide (TBH) causes cytotoxicity through generation of reactive species, particularly superoxide ion, GSH depletion, lipid peroxidation, induction of DNA strand breaks and influx of calcium ions (Bellomo *et al.,* 1982; Ochi and Miyaura, 1989; Awe and Adeagbo, 2002; Gitika *et al.,* 2006).

### 3.3.3.1 Cell viability and integrity

Assessment of functional integrity of cells as a means to establish the absence of cytotoxicity involves a battery of well validated assays having considerable amount of historical control data. The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and lactose dehydrogenase (LDH) assays are well established and are widely used to assess the mitochondrial

competence and cell membrane integrity respectively (Ljubuncic *et al.,* 2005). In the previous section it was demonstrated that the extracts at the tested concentrations were non-toxic to isolated rat hepatocytes and based on other biochemical parameters, 50 and 100ppm of the extracts were chosen to elucidate their protective effect against TBH-induced oxidative toxicity.

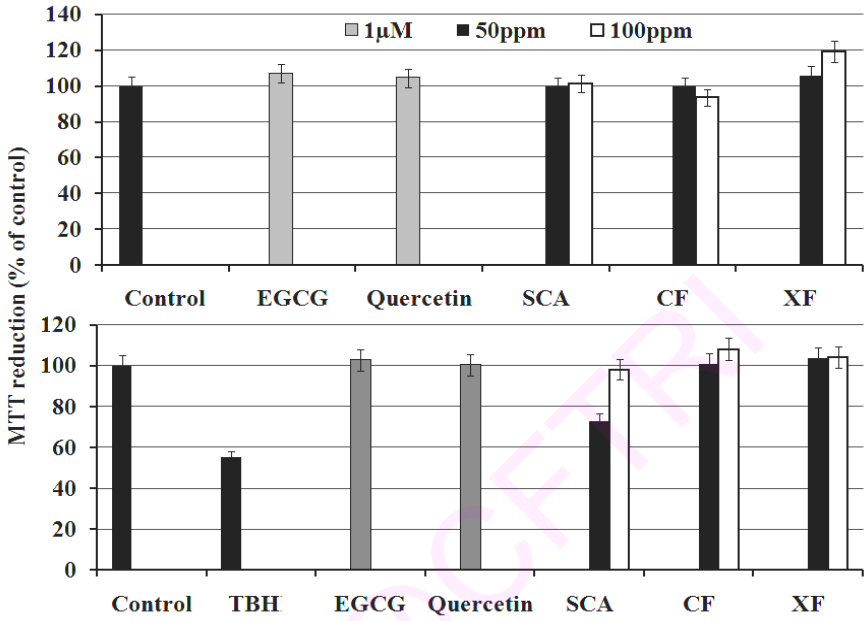



Fig. 3.11 Effect of extracts and standards on viability of rat hepatocytes in the presence (lower panel) and absence (upper panel) of TBH. The cells were treated with extracts for 4 h followed by treatment with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

A 2 h incubation of the cells with 2mM TBH in serum free culture medium resulted in 45% reduction in viability (**Fig. 3.11**) whereas pretreatment of the rat hepatocytes with different extracts for 4 h provided significant protection against TBH induced cytotoxicity.

The extracts provided protection in a dose dependent manner. The SCA at 50 and 100 ppm restored the cell viability to 73% and 98% respectively. The standard antioxidants EGCG and quercetin as well as the carotenoids extracts of *D. regia*, the CF and XF retained the viability above that of control cells. It may be noted from the **Fig. 3.11** that the EGCG, quercetin, SCA and XF when fed to cells in the absence of stress maintained the cell survival above the control levels. The elevation of cell viability by these compounds is possible due to their protection against the natural stress the cells are subjected to during the isolation and culture conditions of 95% oxygen. This is a positive attribute of the extracts suggesting that they may actually increase the survival time of cells or delay their death under normal conditions.

Lactate dehydrogenase (LDH) is an enzyme that exists in many tissues and organs, such as heart, muscle, kidney, liver etc. Intracellular LDH leakage, as a result of the plasma membrane breakdown and a concomitant alteration in its permeability, was evaluated as a marker of cell membrane integrity and viability. LDH leakage was used as an indicator of cytotoxicity induced by TBH in rat hepatocytes in culture. A 2 h exposure of isolated rat hepatocytes to 2mM TBH evoked a significant increase in LDH activity (60% compared to 34% in control) in cell culture medium indicating cell damage (**Fig 3.12**).

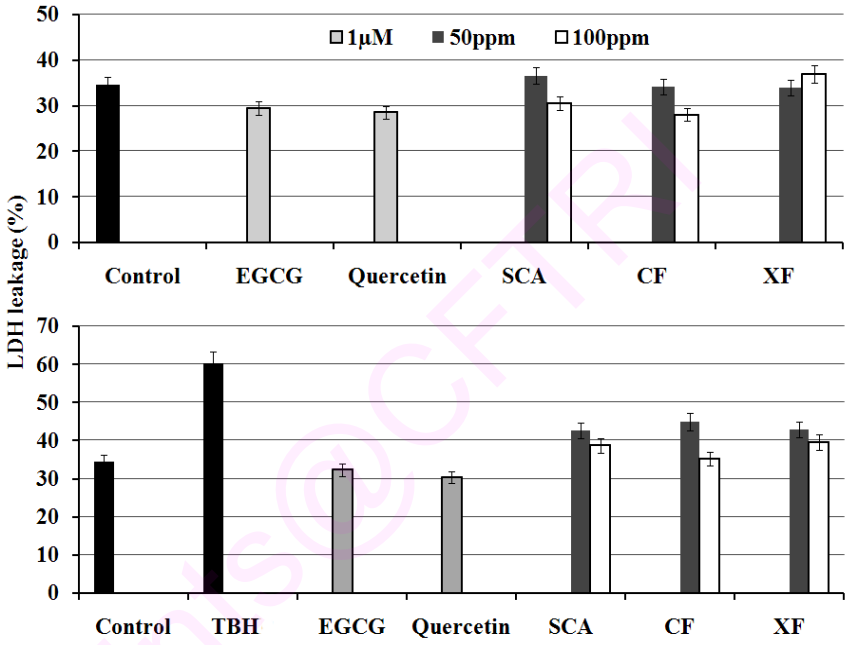



Fig. 3.12. Effect of extracts and standards on LDH leakage in rat hepatocytes in the presence (lower panel) and absence (upper panel) of TBH. The cells were treated with extracts for 4 h followed by treatment with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

The extracts as such did not cause significant LDH leakage and its concentration was maintained at the basal level in all treatments. However, pretreatment of the hepatocytes with extracts and standards caused a substantial reduction in LDH leakage which was dose dependent (**Fig. 3.12**). *Tert*-butyl hydroperoxide is an extensively used oxidative model to study the mechanism of hepatotoxicity and to screen hepatoprotective antioxidants (Hwang *et al.,* 2005; Lee *et al.,* 2005a; Lin *et al.,* 2000b) where LDH leakage, glutathione content and lipid peroxidation were the primary end points studied. Treatment of rat hepatocytes with different classes of antioxidants has proven to reduce TBH-induced cytotoxicity. Pretreatment of SCA caused a 30-35% reduction in TBH-induced LDH leakage at the tested concentrations of 50 and 100ppm. The results are in agreement with other reports on cytoprotective effects of anthocyanins. Pretreatment of primary

rat hepatocytes with anthocyanins of dried *Hibiscus sabdariffa* flowers significantly inhibited the LDH leakage induced by TBH (Wang *et al.,* 2000). EGCG and quercetin at 1µM concentration were non-cytotoxic and provide complete protection to cells against TBH induced cell death. EGCG (Hou *et al.,* 2007; Yao *et al.,* 2008) and quercetin (Muthukumaran *et al.,* 2008; Alia *et al.,* 2006b) have been shown to be cytoprotective in different cell and animal models against various chemical insults. The LDH level in EGCG and quercetin pretreated cells was below that of untreated cells thus further supporting its protective effect. The extracts at 100 ppm compared well with 1µM of EGCG and quercetin in terms of cell viability and LDH leakage in TBH-challenged cells.

### 3.3.3.2 Glutathione content

Glutathione, a low molecular thiol present in millimolar concentrations in animal cells is an important member of the body's primary line of defense and plays key regulatory roles in metabolic reactions and progression of cell-cycle (Shan *et al.,* 1990; Poot *et al.,* 1995; Huang *et al.,* 2001). Depletion of GSH has been implicated in hepatic and renal injury caused by overdose of drugs like paracetamol (Thomas 1993). *Tert*-butyl hydroperoxide is a substrate for glutathione peroxidase and results in rapid depletion of glutathione in hepatocytes (Bellomo *et al.,* 1982) chiefly via metabolism by GPx and therefore is a suitable model to study glutathione depletion and consequent cytotoxicity in cells.

The present study showed a significant reduction (35% compared to control) in GSH content in TBH treated rat hepatocytes. The cells treated with 50 ppm of anthocyanin extract in the absence of TBH showed GSH content comparable to the control, untreated cells. The SCA replenished the GSH content in a dose dependent manner. A 37% increase in GSH content was observed at 50ppm as compared to the TBH treated cells whereas at 100ppm it raised above that of untreated cells indicating its high antioxidant activity (**Fig. 3.13**).

When the isolated rat hepatocytes were exposed to the extracts and standards, no significant difference was observed in GSH content between the control and EGCG/quercetin treated cells. However the extracts showed an induction of GSH in rat hepatocytes to an extent of 25-33%, highest induction being observed at 50ppm of XF. Pretreatment with the extracts and standards showed varied effects on the thiol status of the cells. All the three extracts elevated the TBH-induced reduction in GSH content close to the control levels. At 100ppm, the SCA, CF and XF elevated the GSH above normal levels. It is not clear if there is induction of GSH in the presence of oxidative stress since the GSH concentration in cells was not above that when treated with the extract alone. It appears that the induction of GSH synthesis in cells in the presence of

anthocyanins and carotenoids is an adaptation of cells to cope up with the upcoming oxidative stress conditions.

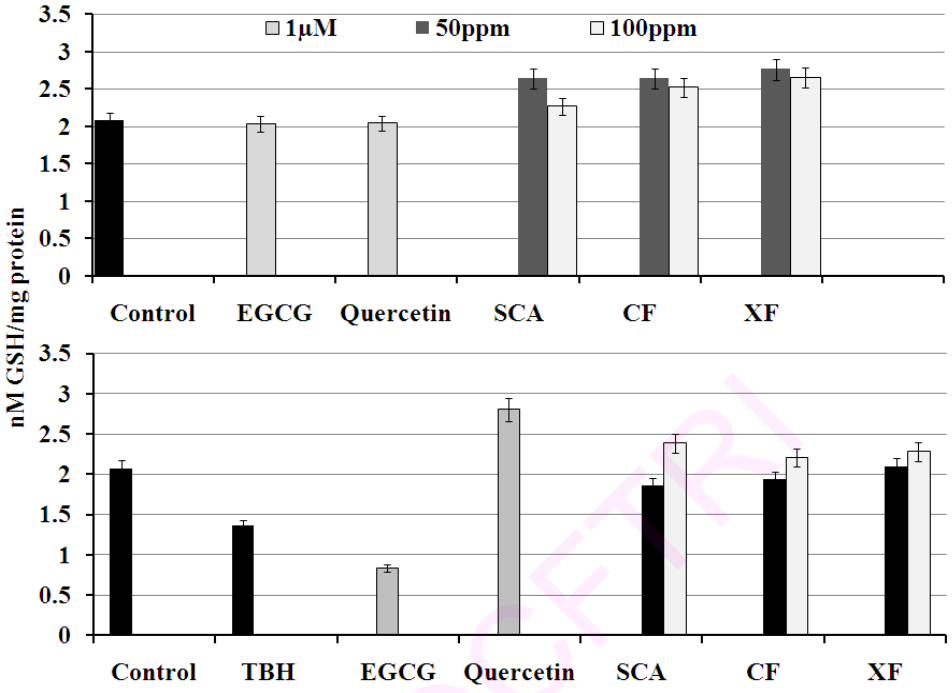



Fig. 3.13. Effect of extracts and standards on GSH content in rat hepatocytes in the presence (lower panel) and absence (upper panel) of TBH. The cells were treated with extracts for 4 h followed by treatment with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

Earlier in this chapter it was discussed in detail the reports suggesting the ability of carotenoids and anthocyanins from different sources to enhance GSH biosynthesis in vivo by stimulating over-expression of γ-glutamyl cysteine synthase. A comparison of cells response to extracts with that to EGCG and quercetin suggest that the EGCG may show cytoprotection via mechanisms independent of GSH. It significantly reduced the GSH pool to a level 60% below that of TBH treated cells. Similar observations were made where EGCG was found to have no effect on GSH induction (Steele *et al.,* 2000) and pre-treatment of PC12 cells with EGCG caused a significant reduction in GSH content post exposure to lead acetate while other tea catechins did not share this behavior (Chen *et al.,* 2004). This suggests that the effect of different polyphenols on the thiol status of the cell perhaps varies with their chemical structures and/or is related to the regulation of certain gene expression properties. On the contrary, Yumei *et al.,* (2006) reported EGCG exerts its antioxidant effect through induction of GSH in rat hepatic stellate cells thus exhibiting antifibrogenic property. However, the study utilised much higher concentrations of EGCG (10-80µM) than it was used in our study (1µM). Moreover, in our own study described earlier in this

chapter, EGCG had a protective effect on $CCl_4$-induced reduction in cellular GSH content (**Fig. 3.6**) by replenishing it to above control levels. Therefore, one may presume that effect of EGCG on GSH content perhaps depends on the type of cell/tissue, presence and absence of oxidative stress, the chemical nature of the oxidant and the concentration of EGCG itself. Contrary to the effect of EGCG on GSH content, quercetin caused a 2-fold increase in GSH content in cells exposed to TBH (**Fig. 3.13**). One of the mechanisms of protection against oxidative stress by quercetin is suggested to be through increase in GSH content (Ishige *et al.,* 2001). Most flavonoids have been reported to increase GSH content *in vivo* by transactivation of the heavy subunit of γ-glutamylcystein synthetase ($GCS_h$), a rate limiting enzyme in GSH synthesis. Myhrstad *et al.,* (2002) observed quercetin-induced GSH synthesis via induction of antioxidant response elements (ARE) and electrophile response elements (EpRE) located on the $GCS_h$ promoter regions. Therefore, a rise in GSH content in hepatocytes pretreated with quercetin and also SCA, CF and XF may also be due to the increased synthesis of GSH as a defense response to increased oxidative stress induced by TBH. Reduced cellular glutathione is associated with a variety of pathological processes. For example, apoptosis in neural cells preceded rapid increase in ceramide production as a consequence of depletion in GSH pool (Beaver and Waring, 1995; Yoshimura *et al.,* 1999). Therefore, phytochemical antioxidants which can stimulate *de novo* GSH synthesis can play a central role in protecting against various oxidative stress related pathologies.

#### 3.3.3.3 Lipid peroxidation

Lipid peroxidation is one of the major causes of cell injury which is mediated by the inactivation of membrane enzymes and receptors, depolarisation of polysaccharides and protein cross-linking ligand fragmentation (Luqman and Rizvi, 2006). Though TBH causes severe lipid peroxidation, it does not seem to be the main mechanism by which it causes cell injury or death (Rush *et al.,* 1985). However, most studies targeted at understanding the protective mechanisms of phytochemicals against TBH-induced toxicity use lipid peroxidation as one of the primary end points. In the present study, the cells exhibited 2-fold increase (34.15%) in lipid peroxidation when exposed to TBH as compared to that of untreated cells (15.11%). While the extracts and standards did not cause significant lipid peroxidation when applied alone to isolated rat hepatocytes, they substantially reduced the extent of lipid peroxidation brought about by TBH to near control level. Anthocyanins from different sources and also pure anthocyanins have been reported to inhibit lipid peroxidation induced by various oxidants. *Tert*-butyl hydroperoxide induced increase in lipid peroxidation has been found to be effectively abrogated by anthocyanins of hibiscus in rat livers and isolated rat hepatocytes (Wang *et al.,* 2000). Anthocyanidins isolated

from pomegranate fruits were shown to inhibit lipid peroxidation induced by $H_2O_2$ in rat brain homogenates (Noda *et al.,* 2002). Youdim *et al.,* (2000) reported the incorporation of elderberry anthocyanins in to the endothelial cells inhibiting the oxidative stress induced by various stressors. They demonstrated that incorporation of anthocynins is considerably higher in plasma membrane compared to the cytosol. The surface action of the anthocyanins with the oxidative free radicals perhaps protects the cells against membrane lipid peroxidation. Therefore the absorption of anthocyanins by the cells appears to be an important factor in conferring protection. **Figure 3.14** gives an account of the effect of extracts and standards on cellular lipid peroxidation in the absence and presence of TBH-induced oxidative stress. The SCA at 100 ppm reduced the lipid peroxidation to 13.26% as against a 34.15% in TBH-treated cells. The anthocyanin extract at 50 and 100ppm was equivalent to 1µM of EGCG and quercetin in their activity (**Fig. 3.14**).

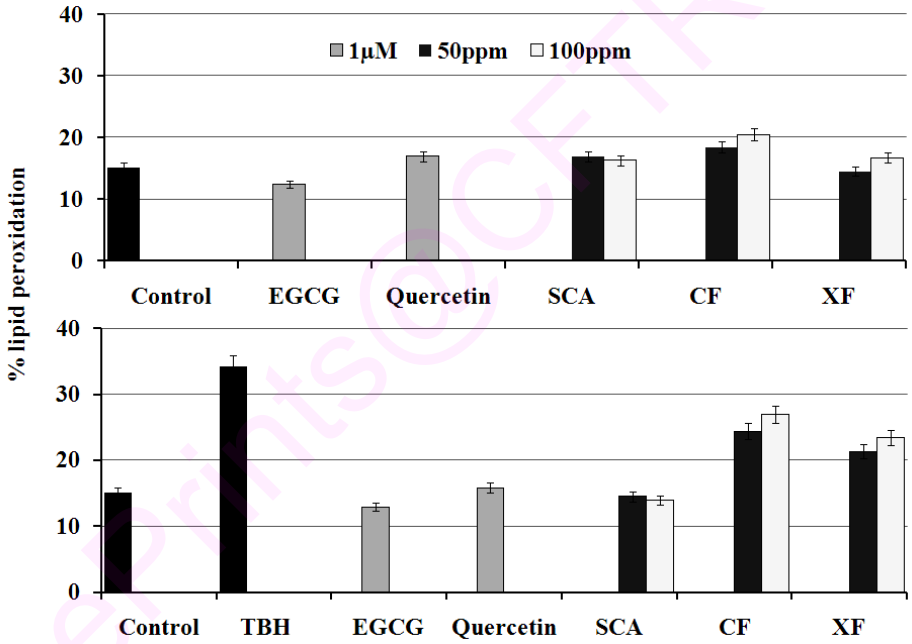



**Fig. 3.14.** Effect of extracts and standards on lipid peroxidation in rat hepatocytes in the presence (lower panel) and absence (upper panel) of TBH. The cells were treated with extracts for 4 h followed by treatment with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicate and are represented as average ± SD.

Pretreatment with carotenes and xanthophylls exert a substantial protection to hepatocytes against ensuing oxidative stress. It is evident from **Fig. 3.14** that both CF and XF protect the cells against TBH-induced lipid peroxidation. Apparently the xanthophyll fraction provides more protection as compared to the carotene fraction. This difference may be attributed to the differntial absorption of these compounds in biological systems. Carotenes and xanthophylls show differential abilities to scavenge free radicals in vitro, xanthophylls being more effective than carotenes (Miller *et al.,*

1996). *In vivo*, certain xanthophylls have been shown to be preferentially absorbed and distributed in cells compared to the carotenes and other xanthophylls. For example, astaxanthin, lutein and zeaxanthin were found to be absorbed more effeciently than α and β-carotene from the intestinal lumen suggesting a greater bioavailability of xanthophylls (Gartner *et al.,* 1996; O'Sullivan *et al.,* 2007). Lutein uptake was greater than that of zeaxanthin in human epthelial lens cells and protected against UV-B induced lipid peroxidation (Chitchumroonchokchai *et al.,* 2004). Therefore, the activity of carotenoids in biological systems appears to be a factor of the polarity of the compounds and hence its absorption and bioavailability. The XF contains compounds such as lutein and zeaxanthin, which perhaps is the reason for its higher efficiency in inhibiting lipid peroxidation.

#### 3.3.3.4 Antioxidant enzyme activity and expression

The cellular AOEs play an important role in the defense against oxidative insult. Alterations in the activity of these enzymes are considered an important biomarker of oxidative stress response (Sies, 1993). It is a general consensus that oxidative stress results in an increased activity of AOEs such as catalase, glutathione peroxidase and superoxide dismutase. Shull *et al.,* (1991) reported a selective AOE inductive response of tracheobronchial epithelial cells to different oxidative insults. The AOE response depends on the toxin either upregulating or downregulating the same. For instance, while toxins like $CCl_4$, alcohol, paraquat and microcystine, a microbial toxin lower the cells' AOE activities (Martin-Aragon *et al.,* 2001; Igarashi *et al.,* 2000; Pushpakiran *et al.,* 2004), others like TBH and rotenone increase the activity of the enzyme in response to the stress (Sanchez-Reus, 2007; Alia *et al.,* 2005). However, stress response of a particular cell or tissue type may vary with the ensuing pathology or the type of oxidative stress. For example, activity of the AOEs varied with tissue type in streptozotocin induced diabetic rats. While the activities of specific AOEs were up regulated in certain tissues, they were down regulated in others (Kakkar *et al.,* 1995) suggesting that the degree of stress response is perhaps tissue specific. The activity of AOEs were found to decrease in $CCl_4$-exposed isolated rat hepatocytes as described earlier in this chapter. Further studies were carried out to elucidate the cellular response to yet another oxidant, TBH in terms of AOE activity and their gene expression and the same is discussed in the following sections.

##### 3.3.3.4.1 Superoxide dismutase

Superoxide dismutase is a component of the primary line of defense against oxidative stress and comprises of Cu/ZnSOD, MnSOD and ECSOD. They catalyse the dismutation of superoxide anion into oxygen and the reactive $H_2O_2$. The peroxide is further inactivated by CAT and/or GPx

and therefore, SOD acts upstream of GPx and CAT. Biological importance of SODs has been implicated in health and disease conditions that are extensively reviewed by several researchers (Mates, 2000; Johnson, 2002). Cu-Zn SOD is the major intracellular SOD and is an important factor in determining the antioxidant status of a cell. Lack of or the presence of mutated Cu-Zn SOD has been implicated in pathologies such as familial amyotropic lateral sclerosis (Rosen *et al.,* 1993). TBH, in the present study, evoked a significant increase (1.5-fold) in SOD response when isolated rat hepatocytes were subjected to oxidative stress by exposing them to 2mM TBH for 2 h (**Table 3.2**). Studies have shown that anthocyanins are capable of inducing SOD and CAT enzymes in vivo (Chiang *et al.,* 2006). However the cells, when treated with the SCA alone, did not show a significant difference in SOD activity as compared to the untreated cells. In fact higher concentration of SCA reduced the activity of the enzyme by about 14% at 100 ppm.

**Table 3.2**. Effect of the extracts, EGCG and quercetin on the activity of the enzyme SOD in the presence and absence of TBH in isolated rat hepatocytes*.

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 894.65 ± 42.35 | | | |
| TBH (2mM) | | | 1295.12 ± 40.42 | |
| SCA | 819.61 ± 24.82 | 776.93 ± 39.58 | 932.18 ± 32.77 | 1031.45 ± 53.28 |
| CF | 3557.87 ± 53.91 | 2305.99 ± 42.48 | 3969.93 ± 48.02 | 1991.11 ± 40.12 |
| XF | 1032.52 ± 19.84 | 1124.49 ± 27.33 | 1271.99 ± 34.71 | 1476.68 ± 38.35 |
| **1µM** | | | | |
| EGCG | 1334.96 ± 33.28 | | 1200.58 ± 45.34 | |
| Quercetin | 1414.97 ± 52.45 | | 1433.82 ± 48.34 | |

* The cells were treated with extracts for 4 h followed by with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

Pretreatment of isolated rat hepatocytes with anthocyanin extract brought about a significant reversal (30% and 20% at 50 and 100 ppm respectively) in TBH-induced increase in SOD activity (**Table 3.2**). Interestingly tremendous increase in activity of SOD was observed when the isolated rat hepatocytes were treated with carotenoids of *D. regia* in the absence of TBH. A similar observation was made with EGCG and quercetin. Pretreatment with CF at 50ppm further increased the SOD activity at both the concentrations, the activity being more intense at 50 ppm. In the previous section we noticed a similar behavior where the CCl4-induced reduction in SOD activity was further reduced by CF. While quercetin had no effect on TBH induced SOD, EGCG brought about a 9% reduction in the enzyme activity which was not very significant (**Table 3.2**).

Products of natural origin have been shown to induce AOEs in various cell and animal models. It was noted that EGCG and quercetin significantly increased the activities of all the three AOEs, quercetin being a more powerful AOE inducer. Quercetin has shown to induce a powerful SOD response in hepatoma cells in vitro (Alia *et al.,* 2005). However, in the presence of TBH stress the activity was brought back to near normal despite a high activity exerted when present alone (Alia *et al.,* 2005, 2006b). The SOD response of cells in the presence of TBH stress was not different from that in the absence of it as evidenced in the present study. The activities were on par with that in the TBH exposed cells. This behavior was seen with all the AOEs where the standard flavonoids as well as carotenoids stimulated significant increase in the activity. The increased antioxidant response by the carotenoids and the flavonoids employed in the present study may be an adaptive mechanism by the cell to cope up with the ensuing stress. Several reports point to the ability of natural compounds to induce AOEs. It appears from our results that pretreatment of the flavonoids and carotenoids offers better protection against an ensuing oxidative stress than against a pre-existing oxidative stress.

The relative mRNA level of SOD in isolated rat hepatocytes and a representative gene expression profile of the anthocyanins and standards assayed by semiquantitative RT-PCR is represented in F**ig. 3.15**

.

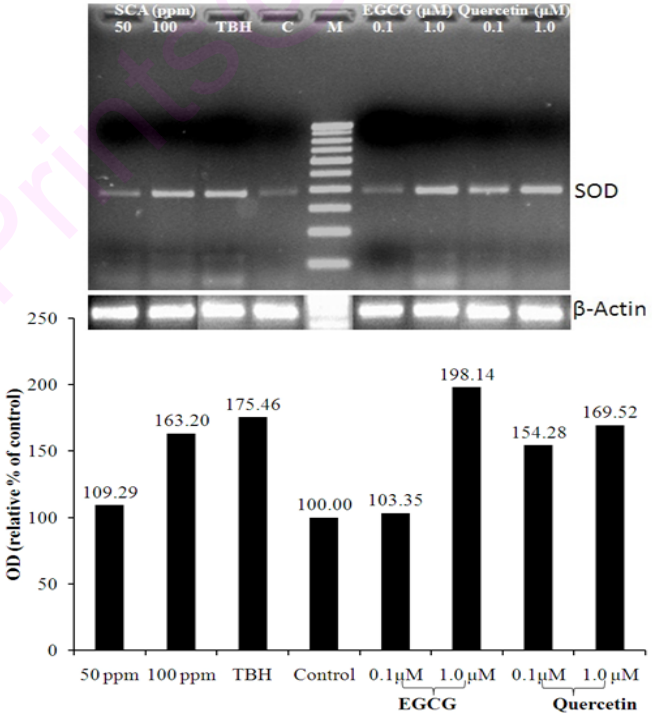



Fig. 3.15. Effect of SCA and standards on the expression of SOD mRNA in isolated rat hepatocytes exposed to TBH stress. Expression of enzyme mRNA was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of

sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

The activity of the SOD increased with a concomitant increase in the mRNA expression in TBH treated cells and cells pretreated with SCA indicating a transcriptional regulation of the enzyme. Oxidative stress is associated with increased or decreased gene expression depending on the type of oxidative stress. Rotenone, a neurotoxin, induced transcription of SOD, CAT and GPx in rat brains (Sanchez-Reus *et al.,* 2007). Anthocyanins of red potato flakes were found to upregulate rat hepatic SOD mRNA (Han *et al.,* 2006) enhancing hepatic antioxidant system. It was observed that TBH induced a dramatic increase in mRNA of SOD in rat hepatocytes which was parallel to the increase in the activity (**Fig. 3.15**). In cells treated with 50 ppm SCA, the mRNA expression was significantly reduced to a near normal level but at 100 ppm, the change was not significant. Similarly, the transcript levels of cells treated with quercetin also remained high without substantial attenuation in TBH induced increase in the same. This was in contrast to a recent study which revealed that quercetin liposomes administered to rotenone intoxicated rats reduced the rotenone induced increase in mRNA transcript levels (Sanchez-Reus, 2007). However their results of activity were in agreement with the present study where quercetin induced SOD activity which was not significantly different from that by TBH. Therefore it appears that in both the studies quercetin activity is post-transcriptionally regulated irrespective of the amount of mRNA that is transcribed.

The influence of carotenoids of *D. regia* flowers on the gene expression of SOD is represented in **Fig. 3.16**. The CF showed a dose dependent reduction in mRNA abundance in hepatocytes exposed to TBH. A substantial reduction in the transcript levels of SOD was also observed with XF with no obvious difference between the two doses. Discrepancies were observed between the activity and mRNA expression of SOD. There was a 3-fold increase (compared to TBH treated cells) in the activity when cells were pretreated with 50 ppm of CF while the mRNA expression remained below TBH control levels (**Table 3.2 and Fig. 3.16**).

The increased SOD transcript level with a consequent increase in the activity in TBH exposed cells might be a cell's way of responding to an external oxidative stimulus in an effort to neutralize the damaging effects of same. However, the increased activity of SOD although appears beneficial for protecting against oxidative stress, it may actually speed up the $H_2O_2$ formation and thus intensifying the oxidative stress (Scott *et al.,* 1987). Therefore, an effective defense requires balanced increments in AOEs involved in detoxification of reactive species generated by each of them. It is discussed in the later sections with respect to consequent

increases in activities of CAT and GPx which detoxify $H_2O_2$ to non-toxic molecules thus helping maintain cellular redox homeostasis.

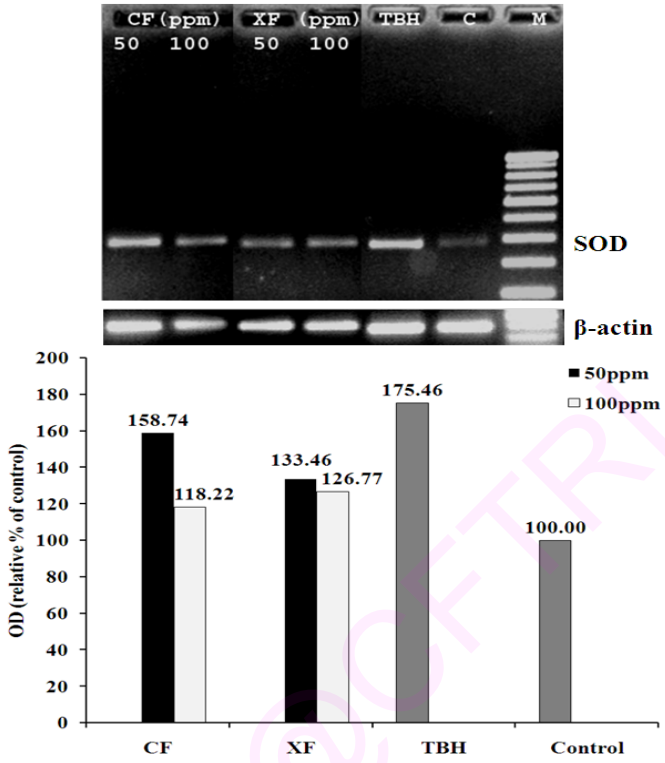



Figure 3.16. Effect of carotenoids fractions of *D. regia* on the expression of SOD mRNA in isolated rat hepatocytes exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

### 3.3.3.4.2 Catalase

Catalase plays a pivotal role in antioxidant defense. Studies on end-stage failing heart has revealed an up regulation of catalase as a compensatory means for increased oxidative stress (Dieterich *et al.,* 2000). Treatment with TBH significantly stimulated the activity of the AOEs in isolated rat hepatocytes. A 2-fold increase in CAT activity was induced by TBH (**Table 3.3**). The activity in control, untreated cells was relatively low compared to the cells treated with the anthocyanins either in presence or absence of TBH. This may be due to the reduction in catalase activity and its mRNA expression with time in cultured rat hepatocytes (Rohrdanz and Kahl 1998). The SCA at both the concentration elevated the CAT activity which was insignificant, maintaining the activity close to the basal level. However, pretreatment with SCA significantly inhibited the TBH-induced increase in CAT activity. There was no considerable difference in the

action of the extract at the two concentrations applied. The extract inhibited the action of TBH by about 50% (**Table 3.3**) at both the concentrations with no significant difference between the two.

**Table 3.3**. Effect of the extracts, EGCG and quercetin on the activity of the enzyme CAT in the presence and absence of TBH in isolated rat hepatocytes.

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 6.08 ± 0.23 | | | |
| TBH | | | 15.75 ± 1.48 | |
| SCA | 6.78 ± 0.37 | 6.91 ± 0.15 | 8.23 ± 0.35 | 8.94 ± 0.66 |
| CF | 16.89 ± 2.14 | 14.53 ± 2.45 | 16.51 ± 1.84 | 14.44 ± 2.01 |
| XF | 13.76 ± 0.99 | 18.11 ± 1.92 | 17.12 ± 1.88 | 26.84 ± 2.96 |
| **1µM** | | | | |
| EGCG | 18.13 ± 1.05 | | 13.43 ± 0.94 | |
| Quercetin | 16.84 ± 2.45 | | 14.18 ± 0.94 | |

The cells were treated with extracts for 4 h followed by with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

It was interesting to note that EGCG, quercetin and the carotenoids fractions radically increased the CAT activity a response equivalent to or higher than that to TBH (**Table 3.3**). However, pretreatment with EGCG, quercetin and CF (100ppm) brought down the elevated CAT activity only slightly while XF brought about a four-fold increase in activity compared to control. In vitro antioxidant studies have shown that EGCG is a potent antioxidant compared to other catechins (Hara 1994; Igarashi *et al.,* 1999). At low, physiological concentrations (<10µM), EGCG has been shown to be cytoprotective (Sugisawa and Umegaki 2002). EGCG induces the production of $H_2O_2$ in vivo. The increase in CAT activity in cells treated with EGCG is perhaps a cell's means of defending itself from the harmful effects of $H_2O_2$. However, at doses above 100 µM, EGCG induces chromosomal damage due to the production of $H_2O_2$ (Sugisawa and Umegaki 2002). From the present study it appears that the anthocyanins and EGCG offer protection against oxidative stress through unrelated mechanisms.

The mRNA transcript levels of CAT gene in cells treated with SCA and their relative abundance with respect to the control cells and a comparison with EGCG and quercetin is depicted in **Fig. 3.17**.

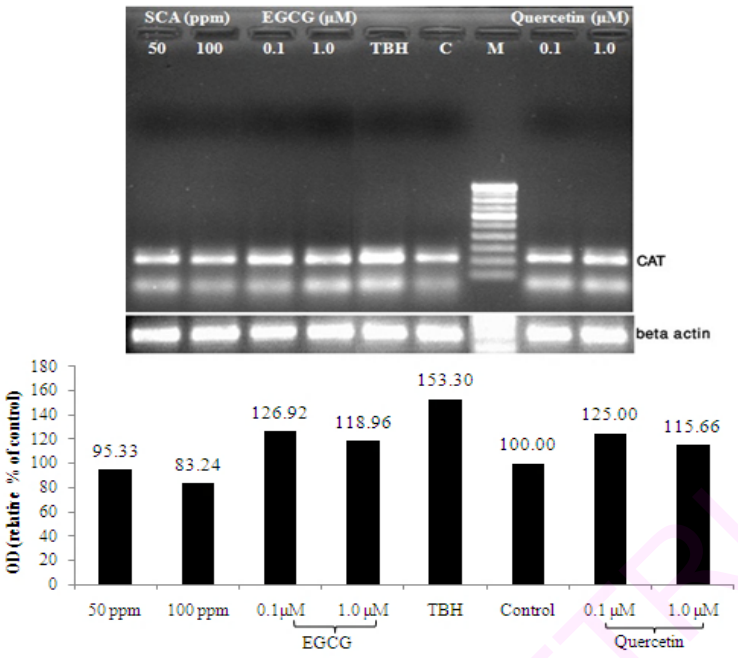



Fig. 3.17. Effect of SCA and standards the expression of CAT mRNA mRNA in isolated rat hepatocytes exposed to TBH stress. Expression of enzyme mRNA was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

TBH significantly induced (2-fold) the CAT activity (**Table 3.3**) as well as the expression of enzyme (**Fig 3.17**). A 53% increase in CAT mRNA transcript was achieved post TBH exposure of cells for 2 h compared to untreated cells. Oxidative stress is associated with an increased expression of AOE mRNA transcripts in rat hepatocytes (Rohrdanz *et al.,* 2000) and in other cell lines (Tate *et al.,* 1990; Shull *et al.,* 1991). Rohrdanz *et al.,* (2001) demonstrated an increase in mRNA transcript level when rat brain astrocytes were exposed to $H_2O_2$ and paraquat. However, Alia *et al.,* (2006b) demonstrated a no difference in mRNA expression of CAT in HepG2 cells treated with TBH from control cells despite an increase in the activity under oxidative stress. They also observed a further attenuation of CAT expression in cells treated with quercetin prior to TBH exposure. However, the results of present study differed significantly in that, quercetin reduced the TBH-induced CAT expression significantly (despite its stimulatory action on enzyme activity) but it did not reduce the expression level below that of control (**Table 3.3 and Fig. 3.17**). The effect of the treatment of isolated rat hepatocytes with carotenoids fractions of *D. regia* flowers on the mRNA transcript level of CAT gene prior to TBH exposure is summarized in **Fig. 3.18**.

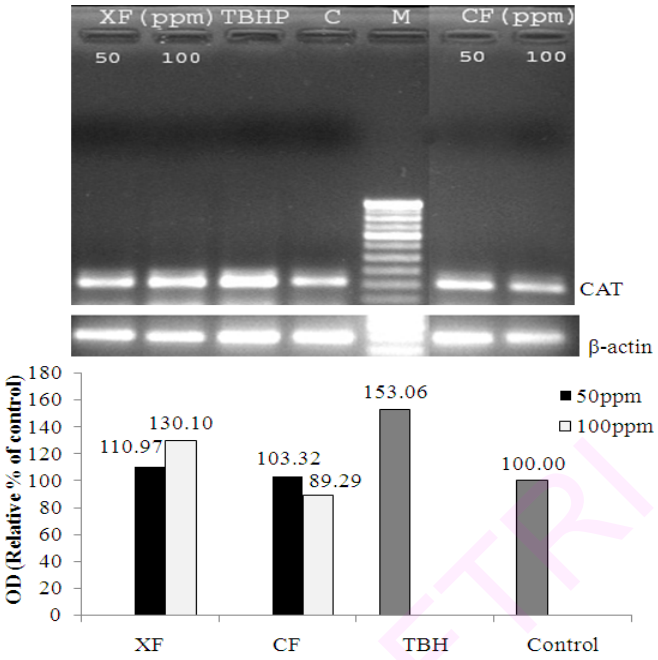



Fig 3.18. Effect of carotenoids of *D. regia* on the expression of CAT mRNA in isolated rat hepatocytes exposed to TBH stress. Expression of enzyme mRNA was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

The elevated expression of the CAT was considerably attenuated by CF followed by XF. CAT is known to be post-transcriptionally regulated after oxidative stress. While TBH had no effect on CAT activity in Chinese hamster V79 cells (Ochi, 1990), it significantly induced CAT activity in HepG2 cells without affecting the mRNA expression (Alia *et al.,* 2006b). In the present study, though CF and XF increased the activity of the enzyme drastically, a parallel increase in mRNA transcript was not observed. Apparently, the activity and expression of AOEs are not inter-related. Conceivably there are transcriptional and/or post transcriptional regulatory mechanisms operating at different levels depending upon the tissue type and/or the oxidant molecule. However, in the present study, a dose dependent antagonism was observed in cells pre-treated with anthocyanin extract of *S. cumini* both in terms of activity and transcription. The transcript levels of CAT were significantly reduced compared to that in TBH treated cells. EGCG, quercetin and the carotenoids extracts at the tested concentration reduced the mRNA expression of CAT compared to TBH though they did not have any effect on the TBH-induced activity of the same.

From the results obtained above it appears that the carotenoids fractions act via similar mechanisms adopted by EGCG and quercetin with respect to their action on CAT enzyme.

#### 3.3.3.4.3 Glutathione peroxidase (GPx)

Glutathione peroxidase is an important seleno-enzyme participating in detoxification of $H_2O_2$ downstream of SOD. A 4.3-fold increase in GPx activity was observed when cells were challenged with TBH as compared with the untreated cells. There have been contradictory reports on the effect of TBH on activity of GPx in cells. While Ochi *et al.,* (1990) report the inhibitory effect on GPx, there are others who claim an opposite effect (Alia *et al.,* 2005; 2006a; Garcia-Alonso *et al.,* 2006). It is possible that this difference is due to the difference in the type of cell line they used in their individual experiments. This enzyme was found to be inhibited by 50µM of TBH in vitro (Pigeolet *et al.,* 1990). However, this inhibition is perhaps due to the direct effect of the organic hydroperoxide on the purified enzyme in vitro. A direct contact of the protein with TBH may not occur *in vivo*, due to the presence of biological barriers and other cellular factors which might protect the protein against the inhibitory activities of the oxidant. Another theory suggests that the induction of molecular homolysis of hydroperoxides by unsaturated fatty acids of membrane lipids leads to lipid auto-oxidaiton producing lipid hydroperoxides resulting in more free radicals. Therefore, the free radicals derived from such processes or some consequent mon-radical products may serve as inducers of the glutathione enzyme system rather than the hydroperoxide substrates (Munkres and Colvin, 1976). Therefore, what exactly is the reason for the difference in GPx activity in different cell types under TBH stress is still unknown. In the present study, the anthocyanin extract of *S. cumini* itself had a stimulating effect on GPx activity. The activity increased 2.6 and 3.6-fold when cells were treated with 50 and 100ppm extract respectively. Though the extract, at 50ppm increased the activity, it significantly reduced the TBH-induced activity by about 70% (**Table 3.4**). However, 100 ppm of the anthocyanins was ineffective against GPx activity enhanced by TBH but instead it further increased the activity of the enzyme.

The phospholipid hydroperoxide GPx is considered to be involved in protection of lipid biomembranes against oxidative peroxidation (Masella *et al.,* 2005). The increased reduction in lipid peroxidation by the extracts and standards appears to be the result of increased action of GPx thus conferring enhanced protection against TBH induced lipid peroxidation. When the cells were exposed to anthocyanin extracts alone, the lipid peroxidation levels were much lower than the control levels with a consequent increase in GPx activity. This suggests that activity of GPx and extent of lipid peroxidation are inversely proportional.

Table 3.4. Effect of the extracts, EGCG and quercetin on the activity of the enzyme GPx in the presence and absence of TBH

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 54.92 ± 2.43 | | | |
| TBH (2mM) | | | 239.81 ± 21.56 | |
| SCA | 143.37 ± 11.45 | 201.31 ± 18.44 | 81.23 ± 6.49 | 246.42 ± 5.38 |
| CF | 533.23 ± 18.92 | 592.65 ± 21.71 | 626.51 ± 13.67 | 640.57 ± 11.48 |
| XF | 506.57 ± 15.24 | 784.37 ± 30.84 | 465.31 ± 21.22 | 424.53 ± 16.73 |
| **1µM** | | | | |
| EGCG | 175.98 ± 14.34 | | 267.95 ± 22.19 | |
| Quercetin | 246. 05 ± 19.38 | | 270.21 ± 28.34 | |

* The cells were treated with extracts for 4 h followed by treatment with with 2mM TBH for 2 h. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

EGCG and quercetin had a similar effect on GPx as they had on CAT activity. That is, when the cells were exposed to EGCG/quercetin in the absence of TBH a tremendous increase in GPx activity was observed which was not attenuated in the presence of TBH. A like behavior was observed when isolated rat hepatocytes were treated with CF and XF either alone or treated prior to TBH exposure. A 10-fold increase in GPx activity compared to control was observed with CF while it was 14-fold when cells were treated with XF at 10ppm. The activity was further increased post-TBH treatment in cells treated with these extracts. It was particularly high in CF treated cells. Natural products have known to induce AOEs in vivo. Green tea is reported to increase GPx activity in livers of alcohol intoxicated aged rats. It significantly increased the GPx activity in rat liver as compared to vehicle treated, control rats (Augustyniak, 2005). EGCG being the chief constituent of green tea may be partly responsible for increased GPx activity and this can be correlated to the protective action of EGCG on isolated rat hepatocytes in the present study. Flavonoids and other polyphenols have been credited with the ability to enhance antioxidant capacity of living cells (Ishige *et al.,* 2001; Rodrigo *et al.,* 2002). Despite the contradictory reports on the regulation of AOE activities, the polyphenols appear to confer protection against oxidative stress perhaps via mechanisms independent of or in combination with other pathways.

**Figure 3.19** shows the effect of pretreatment of SCA and standards on TBH-induced alterations in GPx mRNA transcripts. The transcript analysis of GPx gene in hepatocytes treated with TBH revealed an up-regulation of the same by about 44%.

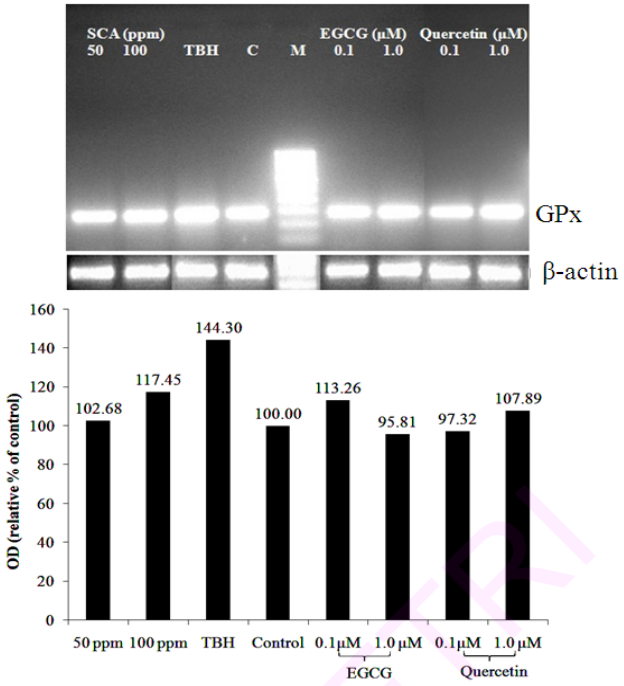



Fig. 3.19. Effect of SCA and standards on the expression of GPx mRNA in isolated rat hepatocytes exposed to TBH stress. Expression of enzyme mRNA was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

It was observed that pretreatment with SCA significantly reduced the mRNA abundance of GPx gene despite an increase in activity at 100 ppm. The expression of GPx is under complex regulation that includes transcriptional (Merante *et al.,* 2002), post-transcriptional (Baker *et al.,* 1993) and translational mechanisms (Driscoll and Copeland, 2003). Though there was an increase in the activity of the enzyme, no increase in the steady state mRNA level was observed with SCA nor with EGCG and quercetin suggesting the activity is pos-transcriptionally regulated.

The carotenoid extracts of *D. regia* flowers did not alter the transcription of GPx mRNA, though the activity was significantly increased (**Fig. 3.20**). There was a many fold increase in the GPx activity in rat hepatocytes pretreated with CF and XF. When the cells were exposed to CF and XF alone, they evoked a 10- and 14-fold increase in activity respectively at 100ppm. The activity, however, was not normalized in the presence of oxidative stress where TBH further enhanced the CF-induced increase in GPx activity, but that of XF was slightly attenuated but was still 2-fold higher than TBH-treated cells. The transcript abundance in cells treated either with CF or XF remained close to basal levels. EGCG and quercetin, though increased the activity several folds compared to control as well as TBH treated cells, had no apparent effect on mRNA expression of

the enzyme. Quercetin at 1µM had an effect similar to that of EGCG, increasing the GPx activity by about 5-fold (compared to control) in the presence and absence of TBH while the mRNA expression was not altered.

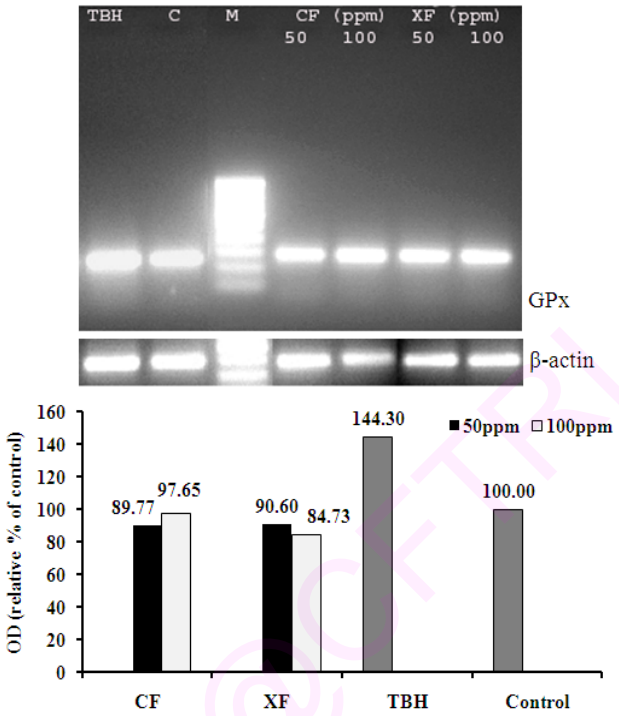



Fig. 3.20. Effect of *D. regia* carotenoids on the expression of GPx mRNA in isolated rat hepatocytes exposed to TBH stress. Expression of enzyme mRNA was determined by semiquantitative RT-PCR in isolated rat hepatocytes treated for 4 h with noted concentrations of sample and standards followed by 2 h exposure to 2mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as percentage of control.

This result is in contrast to the results of Alia *et al.,* (2006a) where quercetin alone did not induce a significant change in the activity of GPx but increased the mRNA expression significantly in hepatoma cells at similar concentration tested in our study. Similarly, a reduction of TBH-induced increase in GPx activity was reported by quercetin (Alia *et al.,* 2006b) which is again in contrast to the current study. Nevertheless, the results of the present study is in agreement with Rohrdanz *et al.,* (2003), who demonstrated a decrease in mRNA expression of GPx by quercetin but lacked data on activity.

Anthocyanins from the other sources have been shown to increase the mRNA expression of AOEs in different cell and animal models (Han *et al.,* 2006; Chiang *et al.,* 2006). Apparently, the anthocyanin extract of *S. cumini* fruits provide protection to the cells by increasing the activity of GPx when treated alone which is also proven by the present study. Interestingly in the presence of

oxidative stress, the activity of this enzyme was significantly reduced at 50 ppm. Though these AOEs are an important factor in counterbalancing the oxidative stress, increased SOD has been shown to accelerate $H_2O_2$ formation in living cells (Scott *et al.,* 1987). The present study demonstrates an induction of AOEs when the cells were treated with the standards such as EGCG and quercetin and induction of GPx by the anthocyanin extract. Despite an increased enzyme activity, the lipid peroxidation levels remained low with a concomitant increased GSH content, thus conferring protection to the cells. Several other studies report such phenomena by various other naturally derived phytochemicals (Nelson *et al.,* 2006; Wang and Ballington, 2007). A likely explanation that can be given here is that, these substances act primarily by direct induction of these enzymes. However, the effect of EGCG is debatable here since it showed high AOE activities and a low cellular GSH content. It is reported elsewhere that EGCG even at µM concentrations showed and oxidative stress enhancing effects as evidenced by the generation of $H_2O_2$ (Elbling *et al.,* 2005). They observed that the toxic effect of EGCG was significantly higher than that of the generated $H_2O_2$ amounts added exogenously suggesting that the toxic effects of EGCG cannot be fully ascribed to $H_2O_2$ generation but may be due to the formation of superoxide. A very high activity of SOD and its expression may be correlated with the toxicity. However, in light of several other reports which demonstrate the antioxidant effects of EGCG (Augustyniak *et al.,* 2005; Reddy *et al.,* 2008), particularly at low concentrations, such a claim has to be viewed with care and much deliberation.

TBH is known to act chiefly via production of free radicals and reactive oxygen species thus leading to an increased lipid peroxidation and other consequences. The increased activity of the AOEs may be considered secondary in this case and may be attributed to an adaptive response of the cell to cope with the TBH-induced increase in oxidative stress. The overall results suggest that keeping the cells′ antioxidant defense active without inflicting an oxidative stress status is beneficial in fighting against the possible onslaught from the reactive species for which judicious levels of natural extracts appear helpful.

### 3.3.3.5 Conclusions

Isolated rat hepatocytes show extensive damage when exposed to 2mM TBH as evidenced by increased cell death, LDH leakage and lipid peroxidation and reduction in cellular GSH content due to the overwhelming oxidative stress. The antioxidants tested in the present study particularly the anthocyanins of *S. cumini* effectively reverse the effect of TBH in terms of enhancing the viability and normalizing GSH, lipid peroxidation and antioxidant activity both in $CCl_4$- and TBH-induced toxicity models. The effects of SCA were comparable with that of EGCG and quercetin in terms cell viability and lipid peroxidation but not with respect to their response to

AOEs. EGCG and quercetin significantly enhanced the AOE activities whether in the presence of oxidative stress or not. However, this was not the case with SCA which effectively reversed the toxin-induced alterations in enzyme activities in both models.

The carotenoids, particularly the CF, behaved in a manner similar to EGCG and quercetin in most ways by enhancing the AOE activities. But unlike EGCG, CF and XF did not cause depletion of cellular GSH. Quercetin was different from EGCG in a way that though it enhanced the TBH-induced enzyme activity, it significantly enhanced the GSH pool which perhaps is the reason for the protection of cells against oxidative stress. And as discussed earlier, the increased AOE activity in case of quercetin and carotenoids may be an adaptive mechanism to combat the oxidative reactive species generated in case of an oxidative stress condition.

# Chapter 4

# *Effects of extracts on human hepatoma cell line: Studies on expression of antioxidant enzymes and cell proliferation*

## Summary

The antioxidant potentials of the extracts of *S. cumini* and *D. regia* were further elucidated using Hep3B cell line. In addition to the extracts and standards used in the previous chapter, anthocyanin rich extract of *D. regia* was also tested for its cytoprotective activity. All the extracts and standards protected from TBH-induced cell death, the order of protection being SCA=DRA>EGCG>quercetin>XF>CF as measured by MTT assay and LDH leakage. The extracts elevated the GSH content above the control level indicating an ability to stimulate cellular GSH synthesis. TBH induced a 2-fold increase in lipid peroxidation which was prevented by the extracts as well as EGCG and quercetin, SCA being the most powerful inhibitor of lipid peroxidation. Anthocyanins from both the sources and standards conferred protection against Fenton mediated oxidative damage to isolated DNA, whereas carotenoids provided only a negligible protection, perhaps due to their low solubility in aqueous medium. Morphological analysis of the cells revealed apoptotic characters in TBH exposed cells which was significantly prevented by the extracts. This was further confirmed by the reduced Bcl2-Bax ratio in TBH treated cells and the increase in the ratio in cells pretreated with the extracts and standards. TBH also induced a 2-fold induction of caspase-3 activation, a hallmark of apoptosis. All the extracts and standards inhibited the caspase-3 activation almost completely and the inhibition was nearly equivalent to that by the pancaspase inhibitor zVAD-fmk. Study on the activities and mRNA expression of antioxidant enzymes (AOE) showed a significant induction of SOD activity and reduction of CAT and GPx by TBH. SOD activity appeared to be transcriptionally regulated since an increased mRNA expression was also noticed. However, the reduction in CAT and GPx mRNA expression was not proportional to their activity in TBH treated cells. Contrary to their response in isolated rat hepatocytes, carotenoid fractions of *D. regia*, EGCG and quercetin reversed the TBH-induced changes in the AOE activities to a considerable extent. The activity of GPx appears to be posttranscriptionally regulated in Hep3B cells pretreated with quercetin, EGGC (10μM) and SCA since an increase in activity was not associated with an increased expression of its mRNA. A transcriptional regulation of CAT and SOD was obvious in cells pretreated with extracts as well as standards except DRA (100ppm) and quercetin (10μM). Since SCA was found to act via modulation of AOEs, its effect on the stability of transcribed mRNA of these enzymes was studied by inhibiting the transcription followed by measuring the steady state mRNA level in a time dependent manner. TBH caused a radical reduction in steady state mRNA level of CAT and GPx. GPx was most susceptible to degradation by TBH and was almost completely degraded one hour after the addition of the transcription inhibitor, actinomycin D. SCA provided protection to CAT mRNA by almost completely inhibiting the action of TBH on the same. Pretreatment of SCA delayed the decay of GPx mRNA by about 3 h, as compared to 1 h in TBH treated cells. The results of the present study suggest that SCA provides protection to AOEs by increasing their stability and hence making them available for translation.

The extracts were found to be toxic to cells when exposed for an extended period of time, indicating antiproliferative or growth inhibitory activity in carcinoma cells in addition to their protective effect against oxidative stress. Incubation of the Hep3B cells for 24 to 72 h suggested their antiproliferative activity mainly by induction of apoptosis as evidenced by microscopic examination using propidium iodide stain.

The overall results suggest an antioxidant activity mediated protection by extracts of *S. cumini* and *D. regia* against oxidative stress which is also mediated by regulation of apoptotic machinery and an antiproliferative activity in carcinoma cells, especially on long term exposure.

## 4.1 Introduction

The information regarding the role of natural products in deactivating the harmful effects of reactive species is not new. Nature has been an abundant source of unique and complex biologically active organic compounds among which, flavonoids and carotenoids have been reported to be key elements in preventing free radical mediated reactions (Popatovich and Kostyuk, 2003; Krinsky and Johnson, 2005). Some of the phenolic acids and related compounds have proven to induce a cascade of antioxidative processes involved in cellular functions both in cytoplasm and in the nucleus. Aerobic life forms are constantly exposed to a variety of oxidizing agents. While some amounts of oxidant are essential for life, overproduction of the same may be fatal leading to intractable pathological conditions such as cancer and CVD (Block *et al.,* 1992; Willet, 1994; 2002). Such oxidative insults can be prevented to a certain extent by bioactive molecules such as flavonoids and carotenoids present in fruits and vegetables.

Earlier part of this report describes the antioxidant roles of anthocyanins of *S. cumini* and carotenoids of *D. regia* using in vitro chemical assays as well as using primary cultured rat hepatocytes. In vitro chemical assays do not give a complete picture of the antioxidant potentials of a compound since it lacks the intricacies of cellular milieu. Primary cultures of isolated rat hepatocytes have been extensively used in antioxidant and toxicity studies (Lewerenz *et al.,* 2003; Rubiolo *et al.,* 2008). Though primary cultures mimic in vivo conditions to the closest extent such cells are hard to maintain and are short lived. Therefore, continuously growing hepatic cell lines are the best alternatives for such studies. Such cell lines are preferable in view of their unlimited life span in culture and the stability of the drug-metabolising enzymes in comparison with primary cultures of hepatocytes (Nicod *et al.,* 1997). Consequently, the hepatocellular carcinima cell line Hep3B was chosen because it most closely retains the activities and substrate specificities of enzymes typical for humans and secrete most of the plasma proteins (Knasmuller *et al.,* 2004).

The endogenous AOEs such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) form the first line of defense in mammalian system against oxidative insults. Several studies have proved that the levels of mRNAs coding for these enzymes as well as their activity are altered in conditions of diseases. It has been observed that exposure to agents that lead to oxidative stress also lead to an increase in the mRNA content of certain AOEs. Thus, studying the status/quantum of relevant mRNA can reveal damage/protection conferred by antioxidative food molecules at genetic level and hence study of mRNA can be used to develop specific model systems. Therefore, the effect of antioxidants studied in the earlier chapters

against TBH induced oxidative stress in Hep3B cells was further elucidated in terms of AOE activities, their expression and mRNA stability.

### 4.1.1 Measurement of mRNA stability

Oxidative stress increases the levels of ROS within the cell which triggers an oxidative stress response through the redox-sensitive regulatory molecules to inhibit the cellular injury (Camhi *et al.,* 1995). Oxidative stress generally results in an increased AOE activity to prepare the cell against oxidative injury; however, the increased activity is not always associated with an increased gene expression and vice versa (Shull *et al.,* 1991; Clerch *et al.,* 1998; Rohrdanz and Kahl, 1998; Franco *et al.,* 1999; Cyrne *et al.,* 2003). While mRNAs for various proteins are transcribed and continuously released into the cytoplasm, they are available for translation for a specific period. Therefore, their stability is a very important index of the turnover of these enzymes. Extension of half-life invariably is an indication of ensuring mRNA availability for translation. Thus, delay of mRNA decay via different mechanisms offered by exogenous antioxidant pigments would unravel the latter's efficacy in conferring protection for mRNA. Since there are no reports available on the effect of natural antioxidants on the mRNA stability of AOEs this study focused on the mRNA stability in the presence of TBH stress and its modulation by anthocyanins of *S. cumini* fruits. Measuring the extent of mRNA degradation rates has usually been accomplished by inhibiting the synthesis of all cell mRNAs with actinomycin D, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, cordycepin or α-amanitin and measuring the rate at which the mRNA of interest disappears (Ross, 1995). Although it is well known that actinomycin D and other global inhibitors of mRNA synthesis interfere with diverse cell processes and often produce artifacts in measuring mRNA degradation rates, the absence of alternative methods has led to their continued and widespread use.

### 4.1.2 Antioxidants as chemopreventive agents

It is important to note that the phytochemicals which show cytoprotective activity in the presence of oxidative stress in normal cells may also possess chemopreventive properties in proliferating/ tumor cells. The characteristic differential effect is chiefly due to their ability to induce apoptotic machineries to encumber cancer in abnormal cells and simultaneously modulating the detoxifying and antioxidant genes, thus making them non-toxic to normal cells. There have been reports of such dual effects of natural compounds in the literature (Garcia-Alonso *et al.,* 2006; Alia *et al.,* 2005; 2006a, 2006b). Taking this into consideration, the present study was focused on elucidating the antiproliferative potentials of the selected antioxidant fractions along with their cytoprotective effects against oxidative stress.

With the results obtained in the previous chapters, this study was designed to further establish the antioxidant and antiproliferative potential of the extracts of *S. cumini* and *D. regia* in a human derived hepatocarcinoma cell line. In the present study, in addition to the carotenoids extracts of *D. regia*, anthocyanin rich extract was also tested for its activity.

## 4.2 Materials and methods

### 4.2.1 Cell culture and cell treatments

Human hepatocarcinoma cell line (Hep3B) was obtained from National Centre for Cell Sciences, Pune, India. Cells were grown on monolayers in DMEM medium supplemented with 10% heat inactivated fetal bovine serum and 1x antibiotic antimycotic solution (Sigma-Aldrich) at 37ºC in a humidified atmosphere of 95% relative humidity with 95% $O_2$ and 5% $CO_2$. Cells were cultured in 96-well plates for viability assays and in 12-well plates for biochemical analyses. For DNA and RNA isolation, cells were grown in 25cm$^2$ culture flasks.

Cells at 80% confluence were treated with different concentrations of SCA, DRA, CF, XF, EGCG and quercetin in serum free DMEM and incubated at 37ºC in a humidified atmosphere of 5% $CO_2$ for 4 h. The medium was replaced with fresh serum free medium with or without 0.5mM TBH and further incubated for 3 h.

**The following parameters were carried out as explained in Chapter 3.**

**4.2.2** Viability measurement by MTT assay and LDH leakage measurement

**4.2.3** Measurement of cellular GSH

**4.2.4** Measurement of lipid peroxidation

**4.2.5** Activity of AOEs (CAT, SOD and GPx)

**4.2.6** Isolation of total RNA and reverse transcription

Other analyses were carried out as described below:

### 4.2.7 Isolation of DNA for DNA protection assay

2x10$^5$ cells were seeded in 25cm$^2$ culture flasks in DMEM supplemented with 10% FBS and 1x antibiotic antimycotic solution. Cells at 80% confluence were used for isolation of DNA. The cells were detached by trypsinisation and centrifuged at 6000xg at 4°C for 5 min. The pellet was washed twice with PBS and resuspended in 250µL of lysis buffer (0.5%, w/v, SDS, 10mm EDTA, 50mM Tris–HCl, pH 8) containing 0.2M sucrose. Lysis was carried out at 55°C for 30 min by addition of 25µL of 10% SDS. To the cooled mixture 200µL of 3M sodium acetate was added and incubated on ice for 60 min. Centrifuged at 15000xg for 10 min; the supernatant was transferred to a fresh tube containing 200µL TE buffer and 2µL of DNAse free RNAse solution and incubated at room temperature for 30min followed by an incubation with 20U of proteinase

K for 10 min. To the resulting mixture, 500 µL of chloroform: isoamyl alcohol (24:1) was added, vortexed and centrifuged at 12000xg. The upper layer was carefully separated and mixed with 500 µL ice-cold absolute alcohol and 80 µL 3M sodium acetate followed by incubation at 4°C overnight. Centrifuged at 15000xg for 15 min and the supernatant was discarded. The pellet was washed with 70% ethanol twice to remove traces of salts. The pellet was dried under air-current till alcohol odor ceases. The DNA was dissolved in TE buffer and used for DNA damage assay.

#### 4.2.7.1 Treatment of isolated DNA and agarose gel electrophoresis

In the present study isolated DNA was subjected to oxidative damage by Fenton reaction and its protection by the antioxidants was established *in vitro*. Briefly, the reaction mixture contained 10µL DNA in TE buffer (pH 8.0), 6µL of Fenton reagent (mixture of 1mM each of $FeSO_4.7H_2O$:Acsorbic acid:$H_2O_2$ in TE buffer) with or without 1µL of the antioxidant of different concentrations. The samples were dissolved in DMSO and control contained 1µL DMSO instead of the antioxidant. The reaction mixture was incubated in dark at 37°C for 2.5 hrs. The experiment was also carried out by incubating the isolated DNA with the same concentrations of antioxidant compounds in the absence of Fenton reagent in order to check their effect on DNA. The entire contents were loaded on 0.8% agarose gel and horizontal electrophoresis was performed in 10x TAE buffer for 2 h at 50V/cm. DNA in the gel was visualized under UV light after staining with 5µg/mL ethidium bromide solution in water and the images were documented using BioRad Chemidoc XRS gel documentation system (Hercules, CA, USA)

### 4.2.8 Morphological analysis of Hep3B cells

Hep3B cells were seeded at a density 2 x $10^4$ in 6-well plates and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. At 80% confluence, the cells were treated with different concentrations of aforementioned extracts and incubated for 4 h in serum free DMEM. The medium was then replaced by a fresh serum free medium containing 0.2mM TBH and further incubated for 3 h. The morphological changes of cells were observed under an inverted microscope (Olympus) and documented.

The fluorescence microscopic observations were made using the standard protocol. Hep3B cells were seeded at a density 2 x $10^4$ in 6-well plates containing sterile microscopic glass cover slips and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. At 80% confluence, the cells were treated with the different concentrations of aforementioned extracts and incubated for 4 h in serum free DMEM. The medium was then replaced by a fresh serum free medium containing 0.5mM TBH and further incubated for 3 h. At the end of the treatment the cover slips were

carefully removed, washed with PBS (pH 7.4), stained with propidium iodide (1mg/mL in PBS) and observed under a fluorescence microscope and documented.

### 4.2.9 Measurement of Caspase-3 activity

Caspase-3 activity was measured using CaspACE$^{TM}$ Assay System, Colorimetric (Promega Corp., USA) following manufacturer's protocol. Accordingly, Hep3B cells were treated with different concentrations of the extracts for 4 h followed by 0.5mM TBH for 3 h. For inhibited apoptosis sample, Caspase-3 inhibitor, Z-VAD-FMK (50ng/mL) was added to cells along with TBH. At the end of the treatment period cells were washed with ice-cold PBS (pH7.4) and harvested by trypsinisation and washed again with ice-cold PBS. The cells were lysed by freeze-thawing after suspension in lysis buffer. Cell lysates were clarified by centrifugation at 12000xg for 15min at 4°C and Caspase-3 activity in the supernatant was assayed by colorimetric assay. An aliquot of the cell lysate (equivalent to 50µg protein as assayed by Lowry's method) was mixed with 32µL Caspase assay buffer, 2µL DMSO, 10uL DTT (100mM) and volume was made up to 100µL with deionised water. The mixture was incubated with 2µL of 10mM DEVD-pNA (Caspase-3 substrate) for 4 h at 37°C and the color developed was measured at 405nm.

### 4.2.10 PCR amplification of Bcl-2 and Bax by semiquantitative RT-PCR

Total RNA was isolated and first strand cDNA for PCR amplification was synthesized by the method explained in Chapter III (**Fig.3.1 & 3.2**). Primers were designed based on the available sequences from NCBI data base. The primer sequences and the product size of the gene amplified are shown in **Table 4.1**.

Table 4.1. Primer sequences and product sizes of Bcl-2 and Bax genes amplified from Hep3B cells

| Gene | Accession number | Primer (5´-3´) | | Product size (bp) |
|---|---|---|---|---|
| β-actin | NM_001101 | Forward | TGGACATCCGCAAAGACCTG | 166 |
| | | Reverse | CCGATCCACACGGAGTACTT | |
| BAX | NM_138765 | Forward | CCCTTTTGCTTCAGGGTTTC | 471 |
| | | Reverse | CTCAGCCCATCTTCTTCCAG | |
| BCl2 | NM_000657 | Forward | ATGTGTGTGGAGAGCGTCAA | 151 |
| | | Reverse | CTCAGCCCAGACTCACATCA | |

PCR reaction mixtures contained cDNA equivalent to 0.1 µg total RNA, 0.5 µM each forward and reverse gene specific primers, 250 µM each dNTP, 1x Taq reaction buffer, 1U of Taq polymerase. PCR was performed for 35 cycles. The reaction conditions for amplification were: initial activation at 94°C for 2 min; three-step cycling involving (a) denaturation at 94°C for 30s

(b) annealing at 57.7°C for 1 min, (c) extension at 72°C for 30s. Final extension was at 72°C for 10 min; β-actin was used as the house keeping gene.

### 4.2.11 Analysis of stability of mRNA of antioxidant enzymes in Hep3B cells treated with *S. cumini* anthocyanins.

The Hep3B cells (2 x $10^5$) were seeded in sterile 25$cm^2$ flasks and incubated in a humidified atmosphere of 95% relative humidity with 95% $O_2$ and 5% $CO_2$. The cells at 80% confluence were treated with anthocyanin extracts (50 an 100 ppm) for 4 h. The medium was replaced by fresh medium containing 0.5mM TBH and incubated under standard culture conditions for further 3 h. To determine the mRNA decay, the medium was replaced with fresh medium containing actinomycin D (5µg/mL) at the end of the treatment period and RNA was isolated using TRI reagent (explained in Chapter 3) at different time points of 0, 1, 2, 3, 4 and 6 hrs. Ideally decay curves should have time points that extend beyond the half-life of mRNA in question. However, durations beyond 6 h were not possible in the present study since Hep3B cells showed signs of deterioration due to toxicity of actinomycin D, particularly in those cells exposed to TBHP in the absence of anthocyanins.

#### 4.2.11.1 PCR amplification of AOE genes by semiquantitative RT-PCR

The first strand cDNA obtained by reverse transcription of total RNA was used for PCR amplification of AOEs using gene specific primers. Primers were designed based on the available sequences from NCBI data base. The primer sequences and the product size of the gene amplified are shown in **Table 4.2**.

Table 4.2. Primer sequences and product sizes of CAT, SOD and GPx genes amplified from Hep3B cells

| Gene | Accession number | Primer (5´-3´) | | Product size (bp) |
|---|---|---|---|---|
| β-actin | NM_001101 | Forward | TGGACATCCGCAAAGACCTG | 166 |
| | | Reverse | CCGATCCACACGGAGTACTT | |
| CAT | NM_001752 | Forward | TGGAAAGAAGACTCCCATCG | 633 |
| | | Reverse | CCAACTGGGATGAGAGGGTA | |
| CuZnSOD | NM_000454 | Forward | GAAGGTGTGGGGAAGCATTA | 404 |
| | | Reverse | GGGCCTCAGACTACATCCAA | |
| GPx | NM_002084 | Forward | ATGCTGGCAAATACGTCCTC | 577 |
| | | Reverse | TGGAGACACGGATTTCTTCC | |
| | | Reverse | CTCAGCCCAGACTCACATCA | |

CAT: catalase; SOD: Superoxide dismutase; GPx: Glutathione peroxidase

PCR amplification of genes for AOEs was carried out under conditions explained in (**4.2.10**). The entire PCR product was loaded into wells of 1.2% agarose gel and electrophoressed in 1X TAE

buffer (pH 8.0) at 100V/cm for 1 h. The band in the gel was visualized under UV light after staining with 5µg/mL ethidium bromide solution in water and images were digitised and documented using BioRad Chemidoc XRS gel documentation system (Hercules, CA, USA) and quantification was done using the E.A.S.Y Win32 imaging software provided by Herolab (GmbH Wiesloch). The amplified genes were cloned into pTZ57-R/T T-tail cloning vector (MBI Fermentas, GmbH) and sequenced (Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India). The sequences of genes amplified from Hep3B cell line are presented in **Appendix B**.

### 4.2.12 Antiproliferative activity of extracts of *S. cumini* and *D. regia*

Antiproliferative activity of the extracts and standards was determined by incubating the Hep3B cells with different concentrations of extracts and standards for 24, 48 and 72 h in serum free medium followed by MTT assay. Further confirmation was done by means of fluorescence microscopy of Hep3B cells (explained in section 4.2.8) treated with extracts and standards for 24 h.

## 4.3 Results and discussions

The previous chapter discussed the effect of $CCl_4$ and TBH-induced oxidative stress on antioxidant system in isolated rat hepatocytes and their counterbalance with anthocyanin extract of *S. cumini* and carotenoids of *D. regia*. The present chapter discusses in detail the effect of TBH on a continuously growing human cell line, Hep3B. Hep3B is a hepatocellular carcinoma cell line derived from 8 year old juvenile Black male and contains an integrated surface antigen for Hepatitis B virus (Knowles *et al.,* 1980). These cell lines retain many of the specialized functions normally lost by primary hepatocytes in culture such as secretion of the major plasma proteins (Knowles and Aden, 1983) and is sensitive towards many genotoxicants (Majer *et al.,* 2004). Therefore, this study was designed to have a better understanding of the molecular mechanisms involved in TBH-induced cytotoxicity and its reversal or amelioration by the anthocyanins and carotenoids.

### 4.3.1 Cell viability and integrity

Cell viability was measured by the ability of viable cells to convert MTT, the yellow tetrazolium dye in to purple formazan by mitochondrial dehydrogenases. None of the extracts from *S. cumini* and *D. regia* were found to be cytotoxic at the tested concentrations on short treatment period of 4 h. The cells treated with the anthocyanin and carotenoids extracts showed slight growth enhancing properties where the cell viability was higher compared to that in control untreated cells (**Fig. 4.1**). The standard antioxidants EGCG and quercetin appear non-toxic up to concentrations of 10µM where they show stimulatory action on cells beyond which they are

cytotoxic. The concentration of TBH required to kill 50% of Hep3B cells when incubated for 3 h was found to be 0.5mM and the same was used for further assays. Treatment of Hep3B cells with TBH caused significant reduction (50%) in viable cells with a consequent increase in LDH leakage (**Fig 4.1 & 4.2**). Pretreatment of the Hep3B cells with antioxidants substantially reduced the TBH-induced cytotoxicity in a dose dependent manner. The SCA did not afford significant protection up to 50 ppm; however, at 100 and 250 ppm, the viability was restored to near control levels. Pretreatment with anthocyanin rich extract of *D. regia* (DRA), showed a steady dose dependent increase in viability in cells exposed to TBH with an 80% viability restored at 50 ppm. The concentration which rendered highest protection was 250 ppm for both SCA and DRA where viability was restored to 97%. Quercetin and EGCG showed similar activities at 10µM exhibiting approximately 90% viability in cells treated with TBH. However, both the standards showed reduced protection above 20 µM indicating that they may actually enhance the toxicity of oxidant.

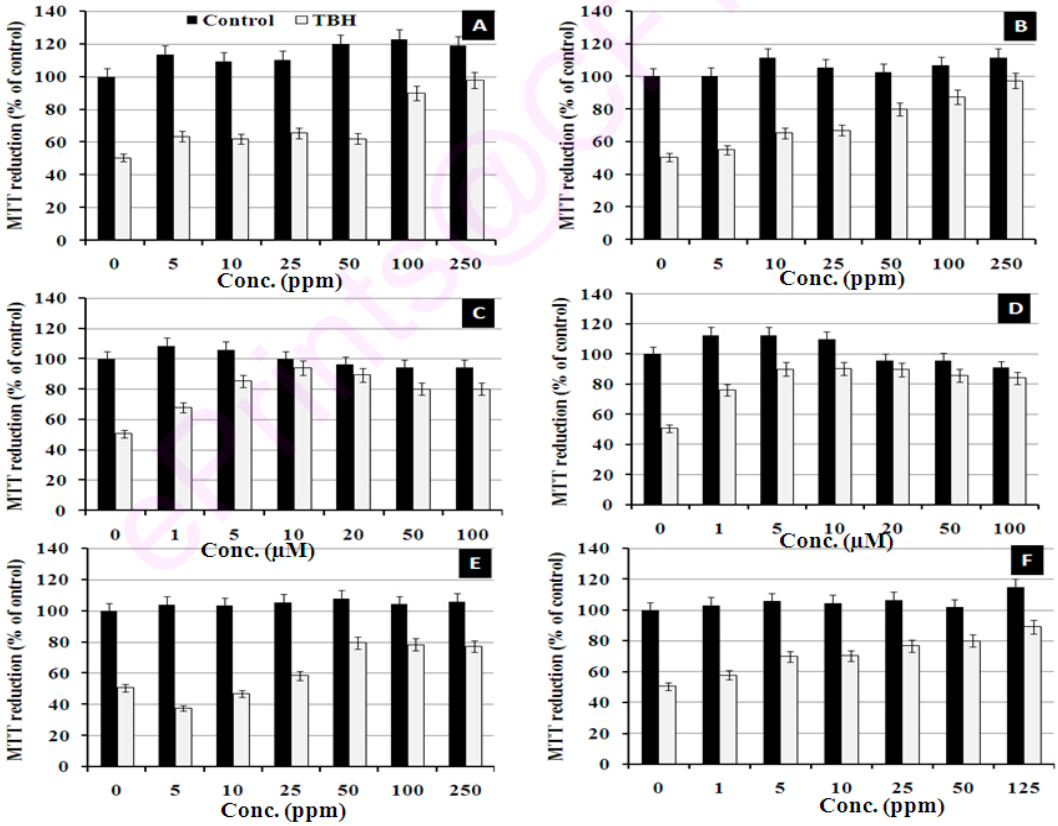



Fig. 4.1. Effect of the SCA (A), DRA (B), EGCG (C), quercetin (D), CF (E) and XF (F) on viability of Hep3B cells in the presence and absence of TBH stress. Hep3B cells were treated with the extracts for 4 h followed by with 0.5mM TBH for 3 h. Viability was measured by MTT assay. Data are expressed as average ± SD of three independent experiments.

The CF showed a dose dependent increase in cell viability at doses up to 50 ppm (maximum 80% viability) beyond which there was no significant difference in protection against stress-induced cell death. The XF also showed a dose dependent increase in viability reaching a maximum of 90% at 125 ppm. **Figure 4.2** shows the effect of antioxidant extracts on membrane integrity as assessed by LDH leakage in Hep3B cells in the presence and absence of TBH. The results of LDH assay are in agreement with that of MTT assay where the viability was inversely associated with LDH leakage. A dose dependent reduction of TBH-induced increase in LDH leakage was observed when cells were pretreated with SCA, DRA and XF. The release of LDH in EGCG treated cells reduced with dose up to 10µM and a steady increase was observed beyond this, may be due to the cytotoxic effects at higher concentrations. However, though quercetin showed cytotoxicity at concentrations of 20µM and above, there was no significant increase in LDH release. *Tert*-butyl hydroperoxide is known to cause cell death by apoptosis through induction of oxidative stress (Kim *et al.,* 1998; Langley *et al.,* 1993). The increased LDH leakage in a cell culture system is generally a sign of membrane disintegration as a result of necrosis *in vitro*, given the fact that it lacks the phagocytic machinery which would otherwise engulf the apoptotic cells preventing the cell contents to be leaked in to surrounding medium (Rao *et al.,* 2005).

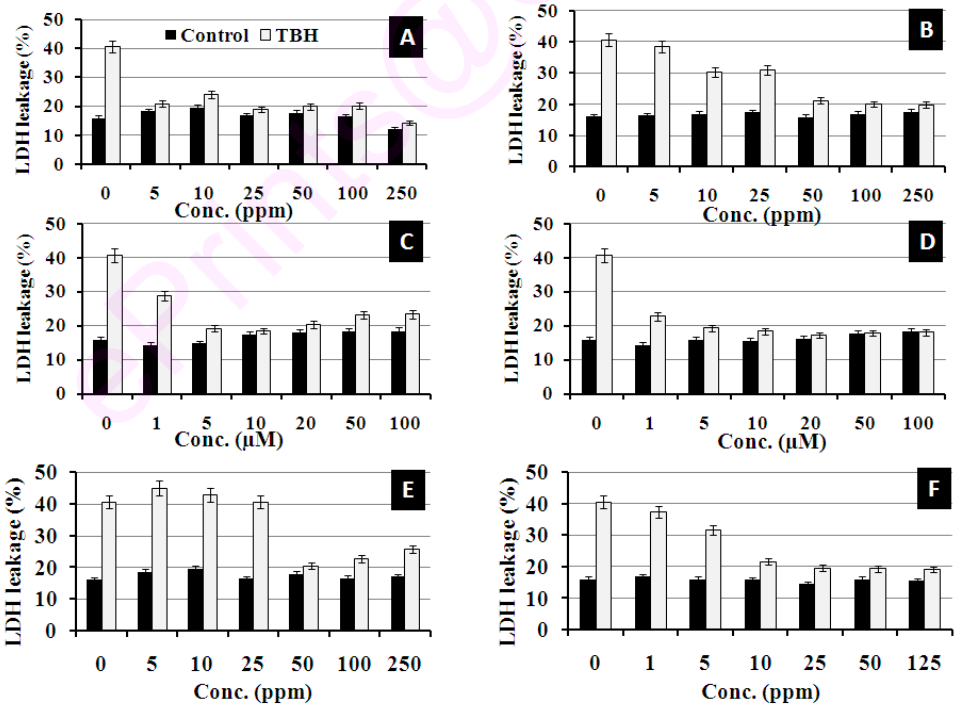



Fig. 4.2. Effect of the SCA (A), DRA (B), EGCG (C), quercetin (D), CF (E) and XF (F) on leakage of lactate dehydrogenase enzyme from Hep3B cells in the presence and absence of TBH stress. Hep3B cells were treated with the extracts for 4 h followed by with 0.5mM TBH for 3 h. Data are expressed as average ± SD of three independent experiments.

It was observed that in the absence of oxidative stress, the antioxidant extracts from *S. cumini* and *D. regia* were non-toxic to cells at the tested concentrations. All the extracts, to an extent, had a stimulatory effect on the growth where the cell viability was higher than that in control cells. The highest stimulatory activity was observed when Hep3B cells were treated with SCA alone which showed a 20% increase in viability of cells compared to control. The CF at higher concentrations failed to provide protection against TBH-induced cytotoxicity and further aggravated the toxicity by increasing cell death in Hep3B cells. This fraction of *D. regia* is predominantly composed of β-carotene. The toxic behavior of this extract is justified by other reports where β-carotene has been shown to act as prooxidant or show no antioxidant activity. Reports suggest the oxidative effects of β-carotene in the presence of an existing oxidative stress condition such as high oxygen tension (Palozza *et al.,* 1997). The loss of antioxidant activity by CF in the presence of oxidative stress may be due to the formation of β-carotene peroxyl radical (β-COO•) as hypothesized by Burton and Ingold (1984) or due to the autooxidation of β-carotene by which it loses its antioxidant property (Kennedy and Lieber, 1992). Excess intracellular oxidative stress causes β-carotene to undergo oxidative degradation leading to the formation of carotenoids cleavage products which are more pro-oxidative than antioxidative (Siems *et al.,* 2002; Alija *et al.,* 2006). A similar observation was made in the previous chapter where β-carotene rich CF failed to protect the isolated rat hepatocytes from $CCl_4$-induced toxicity further substantiating the ineffectiveness of the same. Therefore it is conceivable that in conditions of oxidative environment created by TBH or for that matter any other oxidant, the reactive species might be so overwhelming that CF may lose its scavenging property and either becomes inactive or prooxidant owing to its high β-carotene content.

The increased LDH leakage and reduced viability of Hep3B cells treated with EGCG and quercetin either alone or treated before TBH exposure may be attributed to the cytotoxic effects of these two compounds at higher concentrations. There are reports suggesting the dual effects of EGCG and quercetin and several other plant products including carotenoids (Schwartz, 1996). Quercetin, at cellular level, was shown to be cytoprotective at low concentrations while at high concentrations, cytotoxic (Negre-Salvayre and Salvayre, 1992). The cytotoxicity of quercetin at higher concentration is due to the formation of metabolites such as semiquinone radical ultimately resulting in the formation of toxic $H_2O_2$ (Jovanovic, 1994). There is evidence that quercetin can act as pro-oxidant upon oxidative activation to free radical intermediate semiquinone which can further react with $O_2$ to generate superoxide (Metodiewa *et al.,* 1999). Similarly, EGCG, the most abundant catechin from green tea shows an analogous dose dependent behavior as reported in many a findings. While it is non-toxic to rat hepatocytes at low

concentrations, at concentrations above 10µM it was found to be cytotoxic (Collins *et al.,* 2007). There have been contradictory reports on the activity of EGCG. It has been reported to be a potent antioxidant protecting cells against various oxidative injuries due to its iron chelation and scavenging properties (Higuchi *et al.,* 2003; Ramirez-Mares and de Mejia, 2003). On the contrary, Elbling *et al.,* (2005) suggest that EGCG does not act as antioxidant even at the lowest concentrations tested, but instead acts as prooxidant. However, the concentrations used in their study were much too high to be physiologically relevant. And it is reported from other studies that EGCG at concentrations above 10µM is cytotoxic (Collins *et al.,* 2007). This was evident in the present study where EGCG above 10µM was clearly cytotoxic irrespective of the presence or absence of oxidative stress (**Fig. 4.1 & 4.2**).

Keeping the above results in view, further studies were carried out with lowest concentrations of samples and standards which retain the cell viability above 80% in TBH exposed cells according to MTT assay.

### 4.3.2 GSH content

Cytototoxicity of TBH has been chiefly attributed to depletion of GSH and increase in phosphorylase *a* activity with an associated increase in lipid peroxidation and membrane damage (Ochi, 1988; Buc-Calderon *et al.,* 1991). Glutathione is crucial in protecting the cells against ravaging effects of reactive species which are reduced by GSH in the presence of GPx resulting in oxidation of GSH to GSSG which in turn is reduced back to GSH by glutathione reductase at the expense of NADPH, thus maintaining an adequate cellular GSH pool. In the present study, TBH reduced the GSH content by 32% as compared to the control cells (**Fig. 4.3**) and this reduction was directly associated with death of Hep3B cells (**Fig. 4.1**). Hep3B cells exposed to the tested concentrations of extracts of *S. cumini* and *D. regia* showed an increase in GSH content in the absence of oxidative stress (**Fig. 4.3**). A similar pattern was observed in isolated rat hepatocytes treated with the extracts at the same concentrations without TBH (**Fig. 3.13.**).

A significant rise in GSH content was observed when Hep3B cells were exposed to SCA and DRA, the rise being 32% and 40% respectively, as compared to control cells. The significance of GSH in maintaining cellular homeostasis has been discussed in the earlier chapter. It is once again evident from this result that the extracts and standards perhaps stimulate *de novo* GSH synthesis *in vivo* by acting on the enzyme γ-glutamyl cystiene synthetase, the rate limiting enzyme in GSH biosynthesis.

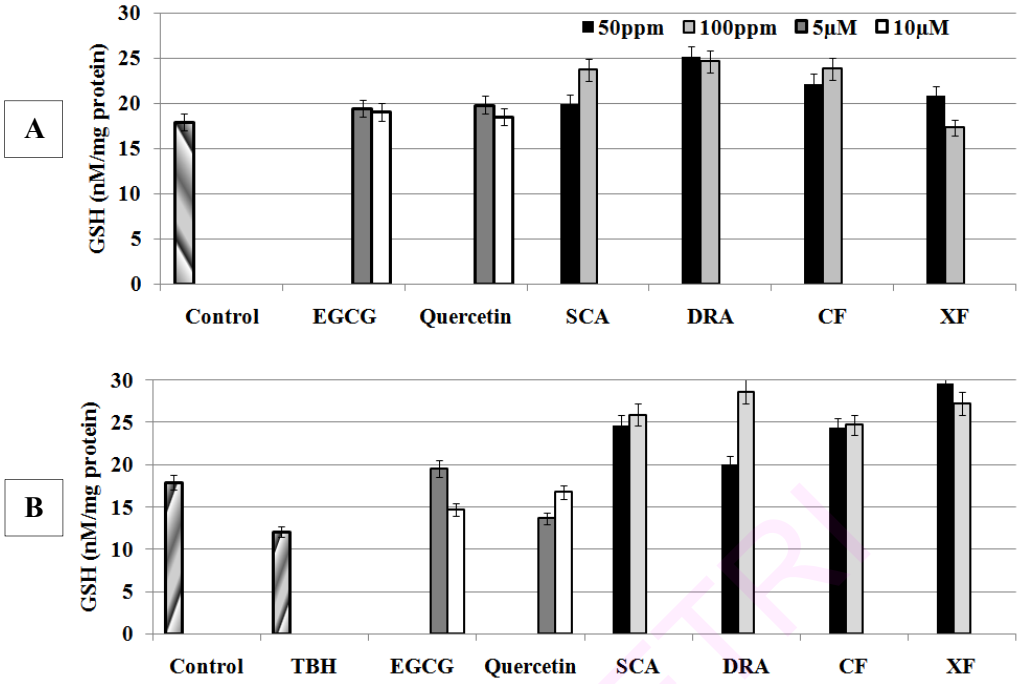



Fig. 4.3. Effect of the SCA, DRA, EGCG, quercetin, CF and XF on cellular GSH content in Hep3B cells in the absence (A) and presence (B) of TBH stress. Hep3B cells were treated with the extracts for 4 h followed by with 0.5mM TBH for 3 h. Data are expressed as average ± SD of three independent experiments.

Pretreatment of the Hep3B cells with the extracts of *S. cumini* and *D. regia* retained the GSH pool even after exposure to TBH. The effect of anthocyanins and carotenoids was more pronounced compared to the standard antioxidants EGCG and quercetin. Quercetin, though showed a dose dependent increase in GSH content in TBH-exposed cells, failed to replenish the GSH completely. It was observed that EGCG at 10µM failed to revive the GSH pool to control levels suggesting that higher concentrations of the same may be fatal to cells. Interestingly, XF, though caused a slight reduction in GSH content when applied at 100 ppm in the absence of oxidative stress, showed a remarkable response in the presence of TBH, enhancing the cellular GSH to 30nM/mg protein compared to the 18nM/mg protein in control cells (**Fig. 4.3**). As indicated earlier, carotenoid cleavage products have been found to induce depletion in mitochondrial GSH content with a consequent increase in malondialdehyde formation (Siems *et al.,* 2002). This is possibly the case with XF where higher concentrations of the same perhaps leads to increased depletion of GSH and hence cell death. However, the reason for increased GSH content by XF in the presence of oxidative stress is not known and is intriguing. There is ample evidence of some antioxidants acting as prooxidants at high concentrations. However, they may act as antioxidants at the same concentrations when present along with an externally applied prooxidant or free

radical generating agent. The elevation of GSH content in cells pretreated with extracts suggests the possibility of stimulation of *in vivo* GSH synthesis.

Glutathione deficiency is implicated in ageing and pathogenesis of many diseases such as Alzheimer's disease, Parkison's disease, liver disease, cystic fibrosis, sickle cell anemia, HIV, AIDS, cancer, stroke and diabetes (Wu *et al.,* 2004). Brain membrane damage associated with hydroxyl radical, peroxynitrite and reactive aldehydic products of lipid peroxidation was prevented by elevated GSH levels *in vivo* (Pocernich *et al.,* 2001; Subramaniam *et al.,* 1997). Similarly, a shift in the GSH/GSSG redox towards the oxidizing state activates various signaling pathways including protein kinase B, protein phosphatases 1 and 2A, calcineurin, NF-κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase-1, and mitogen-activated protein kinase, thereby reducing cell proliferation and inducing apoptosis (Sen, 2000). Flavonoids from Gingko have been associated with reversing of age related reduction in mitochondrial GSH (Sastre *et al.,* 1998). It is apparent from the results (**Fig. 4.3**) that the anthocyanins as well as carotenoids increase the GSH content irrespective of the presence of oxidative stress. The enhanced GSH content in Hep3B cells during the pretreatment with extracts and standards prepare the cell against the oxidative onslaught that is to ensue. It is evident from previous studies that several natural products induce *in vivo* GSH synthesis. For example, the extracts of *Gingko biloba* inhibited apoptosis, induced a significant induction of GSH and γ-glutamyl cystiene synthetase mRNA in mice exposed to ROS and various cell lines (Schindowski *et al.,* 2001; Rimbach *et al.,* 2001). Ingestion of compounds that enhance the cellular GSH pool may confer protection against oxidative stress induced neuronal disorders and other age related disorders. Therefore, knowledge of nutritional regulation of GSH synthesis and metabolism is critical for development of effective strategies to improve health and treat diseases.

### 4.3.3 Lipid peroxidation

The earliest studies on the effect of organic hydroperoxides such as TBH or cumene hydroperoxide on isolated rat hepatocytes suggested a rapid oxidation of GSH accompanied with peroxidation of membrane lipids and loss of cell viability (Rush *et al.*, 1985; Bellomo *et al.,* 1982; Hill and Burk, 1984). TBH forms alkoxyl and peroxyl radicals both of which react with unsaturated lipids to form lipid peroxides (Buc-Calderon *et al.,* 1991). Exposure of Hep3B cells to 0.5mM TBH induced a significant rise in lipid peroxidation (2-fold compared to control). The extracts and standards completely abolished the lipid peroxidation induced by TBH. The SCA was highly effective and reduced the lipid peroxidation level even below that of control level by three fold (**Fig. 4.4**).

There is accumulating evidence about the inhibitory effects of non-nutritive phytochemicals on the lipid peroxidation *in vivo*. Supplementation of β-carotene led to the reduction in malondialdehyde level in children with cystic fibrosis (Lepage *et al.,* 1996). β-carotene binding proteins have been identified on the cell surface which specifically bind to β-carotene and thus confer protection against singlet oxygen generated in its proximity (Bohm *et al.,* 1993).

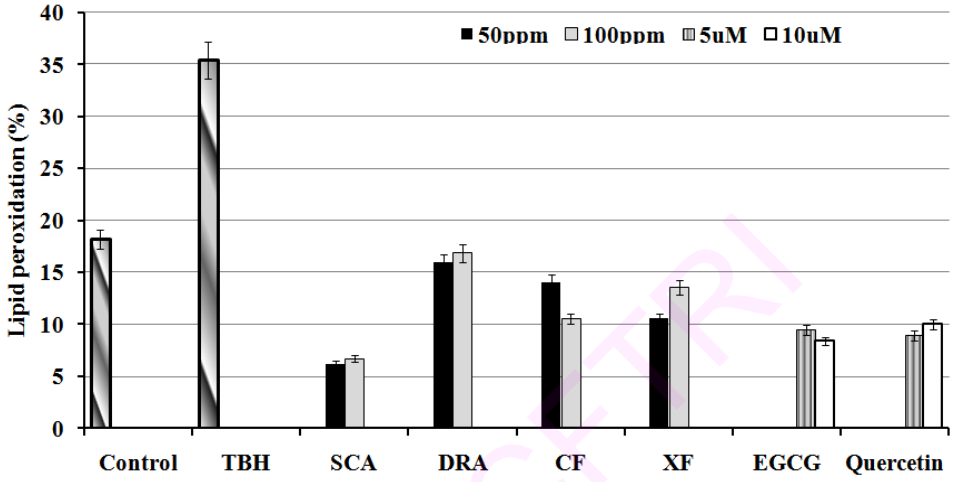



Fig. 4.4. Effect of the SCA (A), DRA (B), EGCG (C), quercetin (D), CF (E) and XF (F) TBH-induced cellular lipid peroxidation in Hep3B cells. Hep3B cells were treated with the extracts for 4 h followed by with 0.5mM TBH for 3 h. Data are expressed as average ± SD of three independent experiments.

Catechins and quercetin have the ability to localize near the surface of phospholipid bilayer thus making it effective against aqueous free radical (Terao *et al.,* 1994). The structure of the flavonoid also makes an impact on the free radical scavenging property of the same. The presence of phenolic hydroxyl groups on the aromatic ring of anthocyanins and other flavonoids is an important factor determining their antioxidant activity. The rapid donation of hydrogen atom to lipid peroxyl radical results in the formation of the polyphenol phenoxyl radical which is stabilized by further donation of another hydrogen or by reacting with another radical (van Acker *et al.,* 1996). The SCA contains three anthocyanins of delphinidin, petunidin and malvidin where delphinidin contains the highest number of hydroxyl groups, helping scavenge the peroxyl radicals generated.

### 4.3.4 DNA damage

Oxidative DNA damage is an inevitable phenomenon under physiological conditions which is initiated by the ROS generated through metabolic reactions. However, the body has developed several well established mechanisms to overcome the DNA damage under normal conditions. However, when the ROS is generated in excess due to exposure to chemical and physical insults,

DNA damage may overwhelm the repair capacity. The •OH is the primary free radical that causes extensive DNA damage. It is produced as a result of interaction between reactive species such as $H_2O_2$ and $O_2•^-$ with transitional metal ions (iron and copper, in particular) in metal-catalysed Haber-Weiss reaction which makes use of Fenton chemistry (Kehrer, 2000). Ferrous sulfate is the typical iron compound used in Fenton reagent (FR). In the present study, damage to DNA was evaluated in isolated DNA from Hep3B cells using Fenton reaction. Ascorbic acid causes DNA single strand breaks in the presence of $H_2O_2$ in ferritin rich human neuroblastoma cells thus causing cell death (Bruchelt *et al.,* 1991). This property was used to induce damage in isolated DNA from Hep3B cells and the protective effect of anthocyanins and carotenoids from *S. cumini* and *D. regia* respectively was studied in vitro.

The effect of the anthocyanin extracts of *S. cumini* and *D. regia* as well as carotenoids fractions of *D. regia* on DNA integrity was studied in the presence and absence of Fenton reagent and the results are summarized in **Fig. 4.5**. It is apparent that none of the extracts as well as EGCG and quercetin caused damage to isolated DNA when treated alone. The mixture of $H_2O_2$ and ferrous sulfate causes DNA damage (Alamaas *et al.,* 1997) and the Fe(II) induced DNA damage is enhanced by ascorbic acid (Hu and Shih,1997). The •OH causes DNA strand breaks and addition of •OH scavenger such as mannitol significantly reduces the increased DNA damage (Rieth *et al.,* 1992). If left unchecked, the •OH-mediated DNA damage may lead to activation of poly(ADP-ribose) polymerase a key enzyme in the initiation of apoptosis (Heller *et al.,* 1995).

While it is prevalent that the DNA lesions are removed by specific cellular mechanisms such as the base excision repair (Cooke *et al.,* 2006), whether natural products are capable of repairing the already damaged DNA is not very well known. However, a study by Chakraborty *et al.,* (2004) suggests a DNA repair efficiency of certain natural products in cultured cells. The incubation of isolated DNA with FR caused a significant DNA damage evidenced by streaks and increased electrophoretic mobility, though laddering was not observed (**Fig. 4.5**). $H_2O_2$ is known to cause single strand breaks in DNA, a toxicity mediated in the presence of iron. The SCA and DRA both significantly prevented the •OH induced DNA damage at all tested concentrations (1-60ppm). There is ample evidence for the DNA protective effects of anthocyanins both *in vivo* and *in vitro*. Consumption of anthocyanin-phenolic rich juice was found to be associated with a significant reduction in oxidative DNA damage in the peripheral blood mononuclear cells of healthy probands (Weisel *et al.,* 2006) and in patients on hemodialysis (Spormann *et al.,* 2008). Anthocyanins have been shown to exert antiproliferative effects in cell cultures and exhibit anti-

inflammatory and vasoprotective activities in animal models. Lazze *et al.,* (2003) demonstrated effective inhibition of DNA base oxidation by anthocyanins in rat smooth muscle cells in vitro.

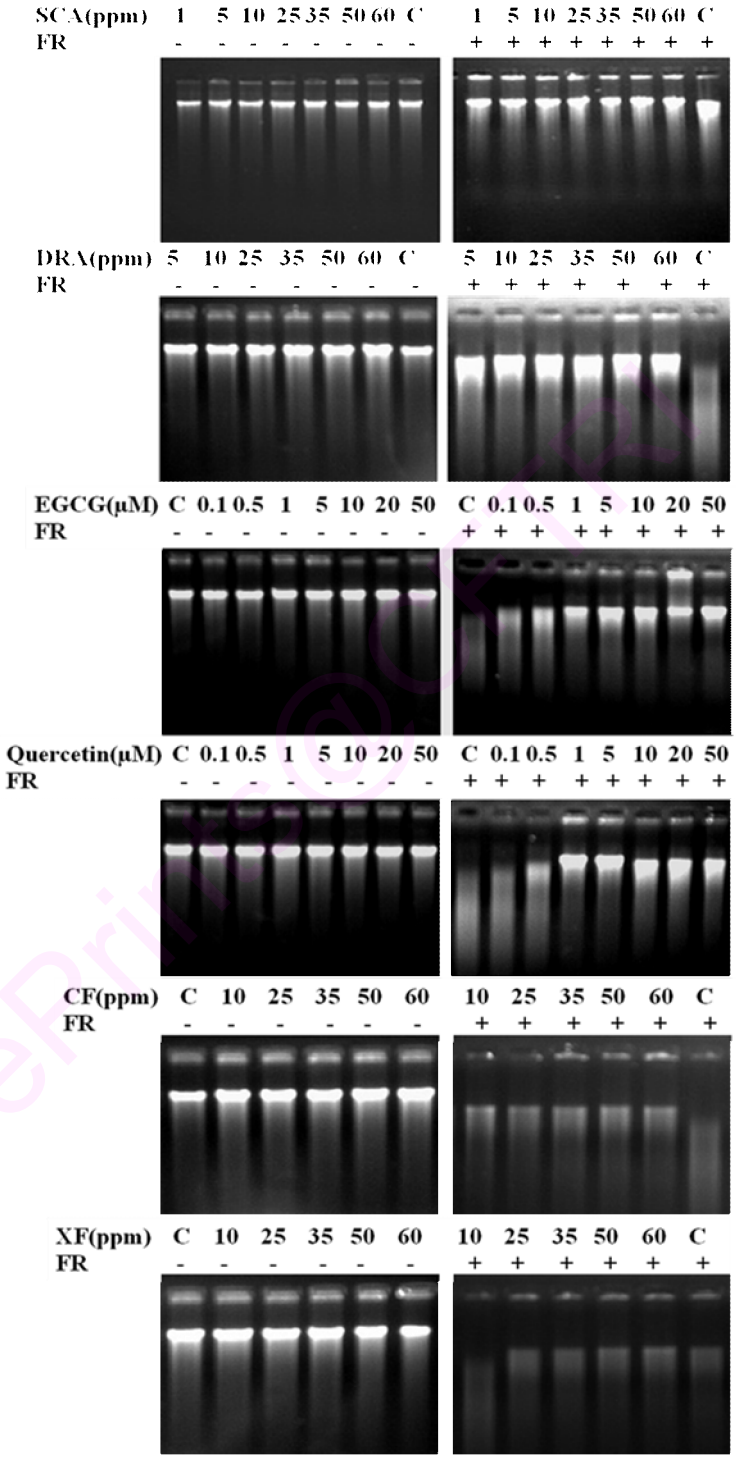



Fig. 4.5. Effect of extracts and standards on DNA damage in the presence and absence of iron/ascorbate/$H_2O_2$.

Although most of the experimental evidence attributes the antioxidant activity of anthocyanins and flavonoids to their radical scavenging (van Acker *et al.,* 1996), metal chelating (Sarma *et al.,* 1997; Somaatmadja *et al.,* 1964) and hydrogen donating (Rice-Evans *et al.,* 1997) ability, a more complex mechanism may not be ruled out. They are also able to form copigmentation with other compounds conferring mutual protection against oxidative degradation (Sarma *et al.,* 1997; Del Pozo-Insfran, 2007). Treatment of calf thymus DNA with cyanidin formed a cyanidin-DNA copigmentation complex conferring a mutual protection against oxidative damage by •OH generated via Fenton reaction (Sarma and Sharma, 1999). This suggests that anthocyanin-DNA copigmentations might have physiological functions as a possible defense against oxidative DNA damage. Anthocyanins are known to be absorbed intact (Youdim *et al.,* 2000) and distributed in various organs such as liver, eye and brain (Kalt *et al.,* 2008) though the anthocyanins differ in their metabolic fate in vivo (Wu *et al.,* 2006). Therefore, the anthocyanins may afford protection to cell by forming DNA complexes by a direct interaction with the phosphate back bone of DNA as hypothesized by Sarma and Sharma (1999) which stabilizes the adduct by strong hydrogen bonding. However, it is likely that the anthocyanins confer protection to DNA via complex formation rather than free radical scavenging action given the fact that plants containing anthocyanins and flavonoids show reduced UV-induced photo damage (Stapleton and Walbot, 1994) compared to those which do not. Since it is reported that anthocyanins are found in the cytosol in cultured endothelial cells (Youdin *et al.,* 2000), it is likely that they form complexes with the DNA thus protecting it against possible oxidative damage.

The standard antioxidants used in the present study, EGCG and quercetin, protected DNA against oxidative damage only at concentrations above 1µM. Both the standards did not show any significant DNA damage when applied alone (**Fig. 4.5**). EGCG and quercetin have been considered excellent chemopreventive agents by several researchers. Quercetin and delphinidin were shown to intercalate DNA and RNA duplexes with minor external binding to the major or minor groove and the backbone phosphate group, stability of adduct being higher for quercetin than delphinidin. However antioxidant potential was found to be higher for delphinidin (Kanakis *et al.,* 2007). EGCG and quercetin at lower concentrations up to 10µM prevent oxidative chromosomal damage induced by $H_2O_2$, TBH and superoxide; but cause substantial DNA damage at higher concentrations above 100µM (Sugisawa and Umegaki 2002; Johnson and Loo 2000). In the present study EGCG and quercetin at concentrations up to 50µM showed significant inhibition in DNA damage. The effects of anthocyanin fractions of S. *cumini* fruits and *D. regia* flowers were comparable to the tested concentrations of EGCG and quercetin in terms of DNA protective ability. Studies on DNA protective effects of flavonoids such as EGCG and quercetin

suggest that they chiefly act via mechanisms involving iron chelation. It is proposed that compounds with a catechol group on the aromatic rings display strong cytoprotective effects suggesting that the presence of two hydroxyl groups *o*-position is essential for chelating action (Sestili *et al.,* 1998; 2002). They noted that the replacement of the catechol group of quercetin with two hydroxyl groups in the *m*-position abolished the chelating effect of quercetin thus further confirming the structure activity relationship.

The anthocyanin extract of *S. cumini* (SCA) is a combination of glucosides of malvidin, petunidin and delphinidin (Veigas *et al.,* 2007) whereas the anthocyanins of *D. regia* (DRA) are mainly composed of glycosides of cyanidin and pelargonidin (Adje *et al.,* 2008). It is apparent from their structures that all of the anthocyanin moieties possess a catechol group in the aromatic ring which can be attributed to the protection against Fenton mediated DNA damage owing to their strong iron chelating abilities.

The carotenoids fractions of *D. regia* flowers showed no significant protection against oxidative DNA damage (**Fig. 4.5**). The extracts CF and XF, at the tested concentrations (10-60ppm) did not completely reverse the DNA damage induced by Fenton reagent. However, it was observed that the electrophoretic mobility of the DNA treated with Fenton reagent was much higher than that treated with CF and XF suggesting that some amount of protection was conferred. Both the extracts caused no damage to DNA when treated in the absence of oxidative stress suggesting that CF and XF are not genotoxic at the tested concentrations. However, the failure of the two extracts to visibly protect the DNA from oxidative damage is perhaps due to their inability to react with the free radicals in the aqueous medium *in vitro* owing to their hydrophobic nature. Interestingly, the CF, despite being more hydrophobic than XF, was more protective against DNA damage. Experimental evidence shows that carotenoids play a key role in preventing oxidative DNA damage *in vitro* and *in vivo*. A randomized, double-blind, placebo controlled intervention study involving healthy post-menopausal women showed a significant reduction in endogenous DNA damage in subjects supplemented with carotenoids mixture containing β-carotene, lutein and lycopene (Zhao *et al.,* 2006). An inverse relationship between plasma xanthophylls levels and oxidative damage indices has been drawn where lymphocyte DNA damage product 8-OHdG was significantly reduced at high levels of β-cryptoxanthin and/or lutein (Haegele *et al.,* 2000). Therefore, though the carotenoids do not provide significant protection under in vitro conditions, their protective effects cannot be completely ruled out given the fact that the two fractions (CF and XF) have been consistently showing cytoprotective effects as evidenced by other assays.

### 4.3.5 Morphological analysis of Hep3B cells

In a cellular system two distinct forms of cell death exist: apoptosis and necrosis. For several years cell death due to chemical injury was believed to be chiefly a necrotic event. However, with the recognition of another cell death pathway, called the apoptosis, a better understanding of the mechanisms involved cytotoxicity is made possible. It is believed that apoptosis, perhaps is the major pathway involved in chemically induced cell death and necrosis might occur only in conditions of gross cell injury (Alison and Sarraf, 1995; Gill and Dive, 2000; Raffray and Cohen, 1997). Apoptosis appears to be a more realistic way of cell death *in vivo* since it provides a mechanism for disposal of damaged cells by means of phagocytosis, without perturbing the tissue environment.

*Tert*-butyl hydroperoxide is an organic hydroperoxide which causes cell death by oxidative stress induced apoptosis (Prasad *et al.,* 2007) which however was evident only at concentrations causing concomitant necrosis (Gomez-Lechon, 2002). Apoptosis is characterized by cell shrinkage, membrane blebbing, nuclear fragmentation and chromatin condensation, and is mediated by a series of signal transduction pathways (Vaux and Strasser, 1996). In contrast, necrosis is a result of a direct or physical damage to cells typified by cell swelling, injury to cytoplasmic organelles and rapid collapse of internal homeostasis leading to membrane lysis and release of cellular contents to the surrounding medium (Majno and Joris, 1995). While apoptotic cells are eliminated by the process of phagocytosis, necrotic cells are not, thus ultimately leading to inflammatory responses.

**Figures 4.6-4.11** represent the effect of natural color extracts as well as EGCG and quercetin on morphological changes induced by 0.5mM TBH in Hep3B cells. The panels A and G represent the morphology of control cells as observed under inverted microscope and florescent microscope, after staining with PI, respectively in all the figures while B and H represent the respective Hep3B cells treated with TBH.

The destruction of monolayer was observed clearly in Hep3B cells treated with 0.5mM TBH (**Fig. 4.6B**) which was not seen in untreated cells. The PI is a nuclear stain and cellular dye exclusion is a characteristic of cellular integrity. The nuclei appear bright red (white arrow in panel H) in Hep3B cells treated with TBH suggesting apoptotic mode of cell death induced by TBH. The cell population was also reduced to a great extent in TBH treated cells (**Fig. 4.6B & H**) as compared to the untreated ones (**Fig. 4.6A & G**). The overall distribution of the dye is larger in TBH treated cells suggesting loss of cellular integrity (**Fig. 4.6H**) which is substantiated earlier by the increased LDH leakage from cells (**Fig. 4.2**).

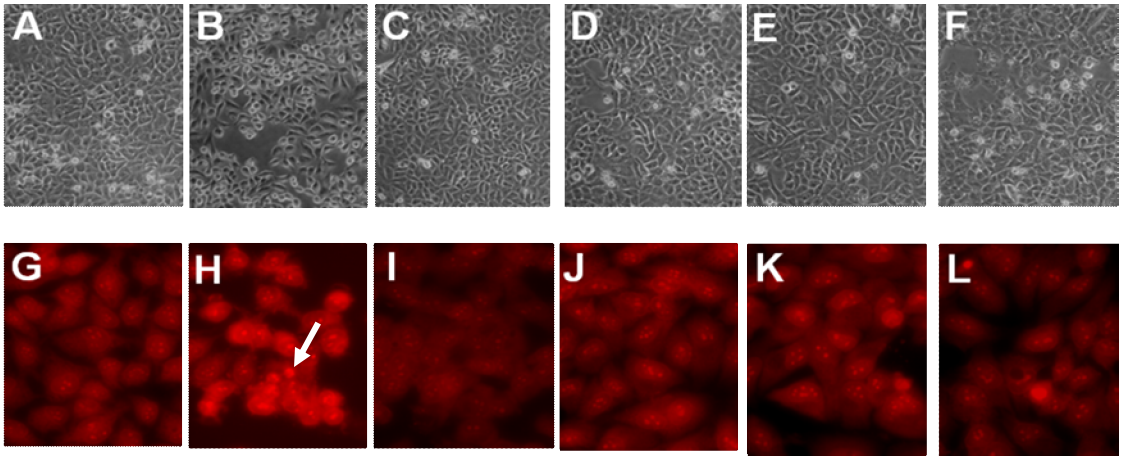



Fig. 4.6. Morphology of Hep3B cells treated with SCA and visualized under inverted microscope in control (A),TBH (B), 25ppm (C), 50ppm (D), 100ppm (E) and 250ppm (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 25ppm (I), 50ppm (J), 100ppm (K) and 250ppm (L).

Hep3B cells pretreated with SCA extracts significantly inhibited the toxic effects of TBH by normalizing the morphological characters which is evident in microscopy (**Fig. 4.6 C-F**). The uptake of PI stain was significantly reduced and number of apoptotic cells were diminished (**Fig. 4.6 I-L**). It was observed that chromatin condensation and nuclear fragmentation was inhibited by pretreating the cells with SCA. The morphology of SCA pretreated cells was similar to that of control cells.

The anthocyanin fraction of *D. regia* flowers showed typical morphological features of control cells. However at concentration of 250ppm the cellular morphology changed, showing toxic symptoms such as reduction in cell volume, cell shrinkage etc. with nuclei stained bright red and cells taking up more stain (**Fig. 4.7 F**). It appears that DRA is more effective at lower concentrations and at higher doses might aggravate the oxidative stress induced by xenobiotics.

The SCA is a mixture of 3-glucosides of delphinidin, petunidin and malvidin the latter being the major anthocyanin present (**Fig. 1.3**). The extract is assessed to have no major impurities and is found to contain solely anthocyanin (total cyanidin-equivalent anthocyanin content is found to be 100% of the freeze dried extract). The DRA is a mixture of anthocyanins (total cyanidin-equivalent anthocyanin content is found to be 50% of total freeze dried extract) along with other unidentified phenolic compounds. The anthocyanins of *D. regia* flowers were identified to be chiefly glycosides of cyanindin and pelargonidin (Adje *et al.,* 2008).

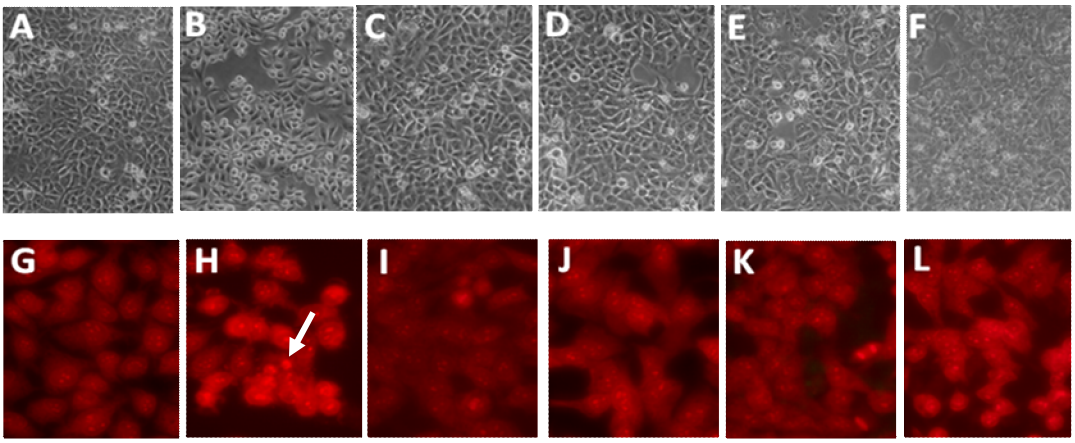



Fig. 4.7. Morphology of Hep3B cells treated with DRA and visualized under inverted microscope in control (A),TBH (B), 25ppm (C), 50ppm (D), 100ppm (E) and 250ppm (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 25ppm (I), 50ppm (J), 100ppm (K) and 250ppm (L).

Antioxidant activity of anthocyanins and most phenolics is well characterized *in vitro* and *in vivo*. The protective effect of anthocyanins against oxidative stress induced cytotoxicity is established in several cell lines and animal models. The purified anthocyanin extract from blackberry significantly inhibits the peroxyl radical induced apoptosis in Caco-2 cells (Elisia and Kitts, 2008). This is also supported in the present study where Hep3B cells treated with anthocyanins of *S. cumini* and *D. regia* offer protection to cells by diminishing the Fenton-mediated DNA damage more effectively than the carotenoids, EGCG and quercetin at as low as 5ppm (**Fig. 4.5**) as well as by upregulating the Bcl-2/Bax ratio which is discussed later in the following sections (**Fig. 4.13**).

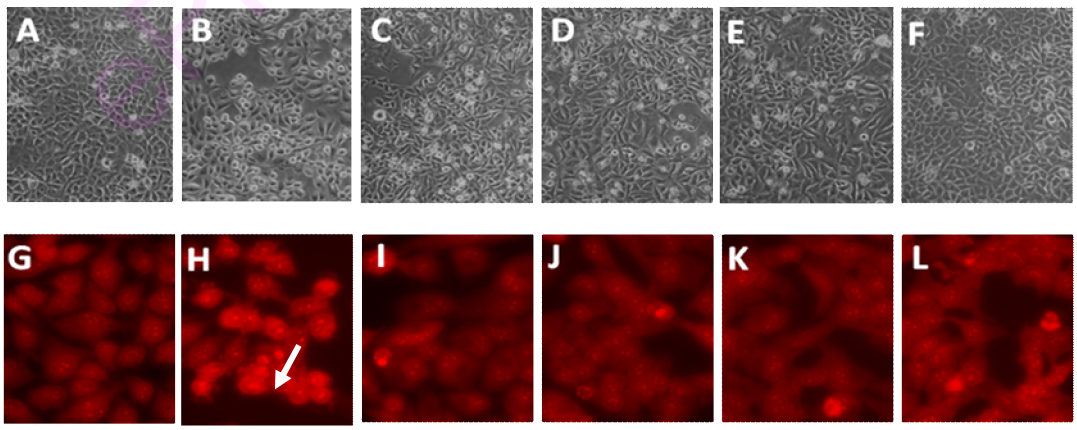



Fig. 4.8. Morphology of Hep3B cells treated with EGCG and visualized under inverted microscope in control (A),TBH (B), 1µM (C), 5µM (D), 10µM (E) and 20µM (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 1µM (I), 5µM (J), 10µM (K) and 20µM (L).

Hep3B cells pretreated with EGCG and quercetin showed a dose dependent revival in morphology (**Fig. 4.8 and 4.9**). The results are in accordance with the MTT assay (**Fig. 4.3**) where the maximum efficacy was observed up to 10µM beyond which both the compounds were toxic to cells either when present alone or in the presence of TBH stress (**Fig. 4.8 L & 4.9 L**).

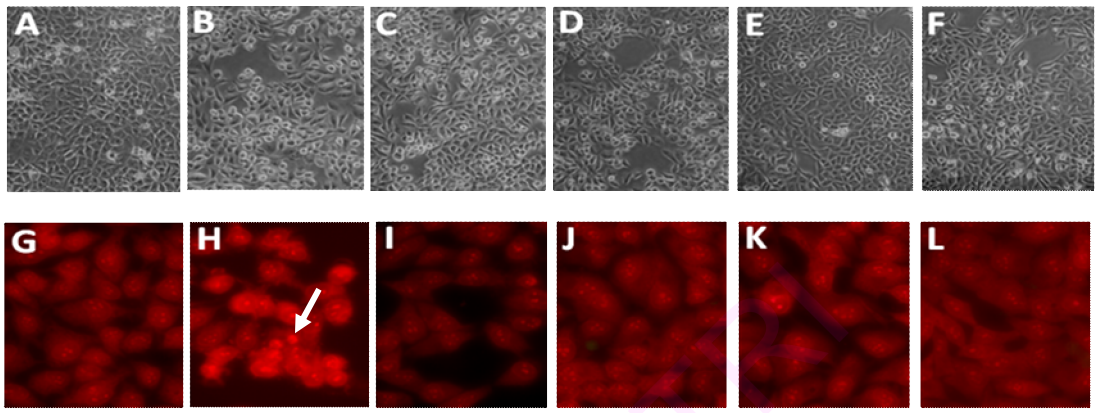



Fig. 4.9. Morphology of Hep3B cells treated with quercetin and visualized under inverted microscope in control (A),TBH (B), 1µM (C), 5µM (D), 10µM (E) and 20µM (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 1µM (I), 5µM (J), 10µM (K) and 20µM (L).

Both CF and XF maintained the normal morphology of the Hep3B cells in TBH exposed cells with cells exposed to XF showing a few bright nuclei (**Fig. 4.10 & 4.11**).

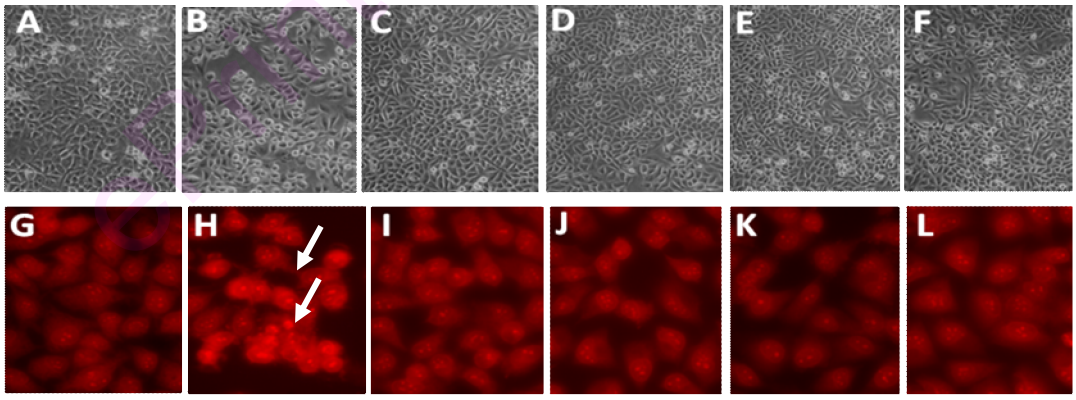



Fig. 4.10. Morphology of Hep3B cells treated with CF and visualized under inverted microscope in control (A),TBH (B), 25ppm (C), 50ppm (D), 100ppm (E) and 250ppm (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 25ppm (I), 50ppm (J), 100ppm (K) and 250ppm (L).

Though TBH caused significant cell death as assessed by MTT assay and LDH leakage, the population of apoptotic cells (cells showing condensed chromatin and fragmented nuclei) was not significant. However, the TBH treated Hep3B cells show a larger distribution of the propidium

iodide dye and show bright red color as compared to the control cells and cells treated with the extracts an standards prior to TBH exposure suggesting cytotoxicity.

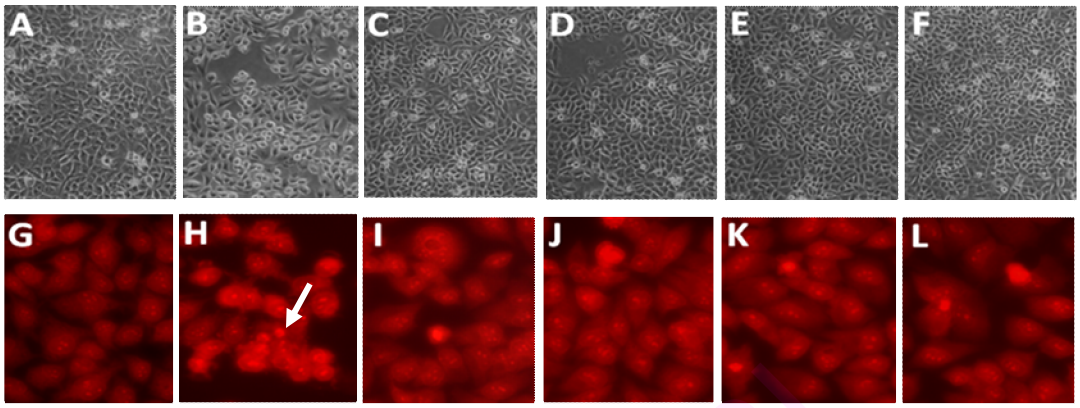



Fig. 4.11. Morphology of Hep3B cells treated with XF and visualized under inverted microscope in control (A),TBH (B), 25ppm (C), 50ppm (D), 125ppm (E) and 250ppm (E). Cells were stained with propidium iodide and observed under fluorescent microscope in control (G), TBH (H), 25ppm (I), 50ppm (J), 125ppm (K) and 250ppm (L).

The results of morphological analysis show that the TBH generated free radicals that cause cell death by apoptosis. The protection offered by the test compounds can be correlated to the ability of the cells to replenish the TBH-induced reduction in GSH (**Fig. 4.3**) and amelioration of lipid peroxidation. It is noted that all the compounds tested significantly reduced lipid peroxidation, SCA being the most effective (**Fig. 4.4**). Further experiments on the Caspase activity and gene expression of apoptotic and antiapoptotic proteins support cytotoxic effects of TBH and its reversal by the anthocyanins and carotenoids.

### 4.3.6. Measurement of Caspase-3 activity

Exposure of Hep3B cells to TBH caused a substantial loss of cell viability and the fluorescence microscopic analysis showed the occurrence of characteristic apoptosis process in such cells. The TBH-induced cytotoxicity was inhibited to a significant extent ($p \leq 0.5$) by the extracts and standards. The next question that arose was whether caspases were involved in the cell death response induced by TBH. Caspases are a family of cysteinyl proteases with central functions in apoptotic and inflammatory pathways (Lamkanfi *et al.,* 2002). They are called caspases due to their absolute requirement for cleavage after aspartic acid (Thornberry and Lazebnik, 1998). Caspases are of two types upstream initiator caspases (caspase-8 & -9 which cleave and activate other caspases) and downstream effector caspases (caspase-3 and -6 which cleave cellular substrates thereby disassembling cellular structures or inactivating enzymes) (Cryns and Yuan, 1998). Caspase-3 specifically cleaves at the C-terminal side of the aspartate residue of the amino

acid sequence DEVD (Asp-Glu-Val-Asp) and is a key apoptotic protein involved in the execution phase of multiple apoptotic pathways. Its deletion has been found to inhibit c-*myc* induced apoptosis and diabetes development without tumerigenesis (Radziszewska, 2009).

Caspase-3 activation is a key component of TBH-induced apoptosis. A three hour incubation of HepG2 cells with 0.5mM TBH has been found to show maximum caspase-3 activation which was effectively inhibited by zVAD-fmk (Piret *et al.,* 2004). TBH-induced apoptotic cell death is triggered by upregulation of intracellular signaling pathways involving caspase activation, increased mitochondrial membrane hyperpolarisation, cytochrome *C* release from mitochondria, increase in $Ca^{++}$ influx, cleavage of poly-(ADP-ribose) polymerase (PARP), DNA fragmentation, upregulation of Bax and downrgulation of Bcl-2 chiefly mediated through increased ROS (Piret *et al.,* 2004; Prasad *et al.,* 2007; Kim *et al.,* 1998).

In the present study a 2-fold induction of apoptosis was observed in Hep3B cells incubated with 0.5mM TBH for 3 h. zVAD-fmk is a predominant inhibitor of caspase-1 and caspase-3 activation and has been found to inhibit the cisplatin induced 25-fold increase in caspase-3 activation and apoptosis in rat proximal tubular cells (Yang *et al.,* 2004). TBH-induced caspase-3 activation was almost completely abolished by co-incubating the cells with 5ng/mL of the pancaspase inhibitor zVAD-fmk (**Fig. 4.12**) suggesting that TBH-induced apoptosis involves caspase-3 mediated mechanisms. All the extracts and EGCG and quercetin showed an activity similar to that of zVAD-fmk and there was no significant difference between different extracts in the amount of protection conferred against TBH-induced caspase-3 activation.

Activation of caspase-3 is an early regulatory event in apoptosis. It acts on a number of biological substrates such as PARP, DNA-dependent protein kinase, lamins, topoisomerases etc. (Tewari *et al.,* 1995; Okinaga *et al.,* 2007). Caspase-3 mediated PARP cleavage leads to DNA fragmentation which is a late event of apoptosis. However, the present study did not show the characteristic DNA laddering after TBH treatment (data not shown) which may be due to the short incubation time which perhaps is insufficient to initiate DNA fragmentation. This explanation seems credible in view of reports suggesting appearance of DNA fragmentation after several hours of caspase-3 activation. One such report demonstrates activation of caspase-3 at 8 h followed by DNA fragmentation at 16 h in the presence of cadmium in murine macrophages (Kim and Sharma, 2006). However, reduced Bax/Bcl-2 ratio and morphological analysis by inverted microscopy and fluorescence microscopy suggest clearly an apoptotic mode of cell death in TBH-exposed Hep3B cells.

Quercetin at 5µM and SCA at 25ppm completely inhibited the TBH-induced caspase-3 activation and the activity was comparable to each other and to control cells (**Fig.4.12 A & D**). A reduction in inhibition of caspase-3 activation was observed with an increase in dose of SCA. This suggests that the higher concentration of SCA may not be protective against caspase-3 activation or it may become toxic at higher concentrations. There was no difference of caspase-3 inactivation in Hep3B cells treated with different doses of DRA suggesting the inhibition is not dose-dependent (**Fig. 4.12 B**).

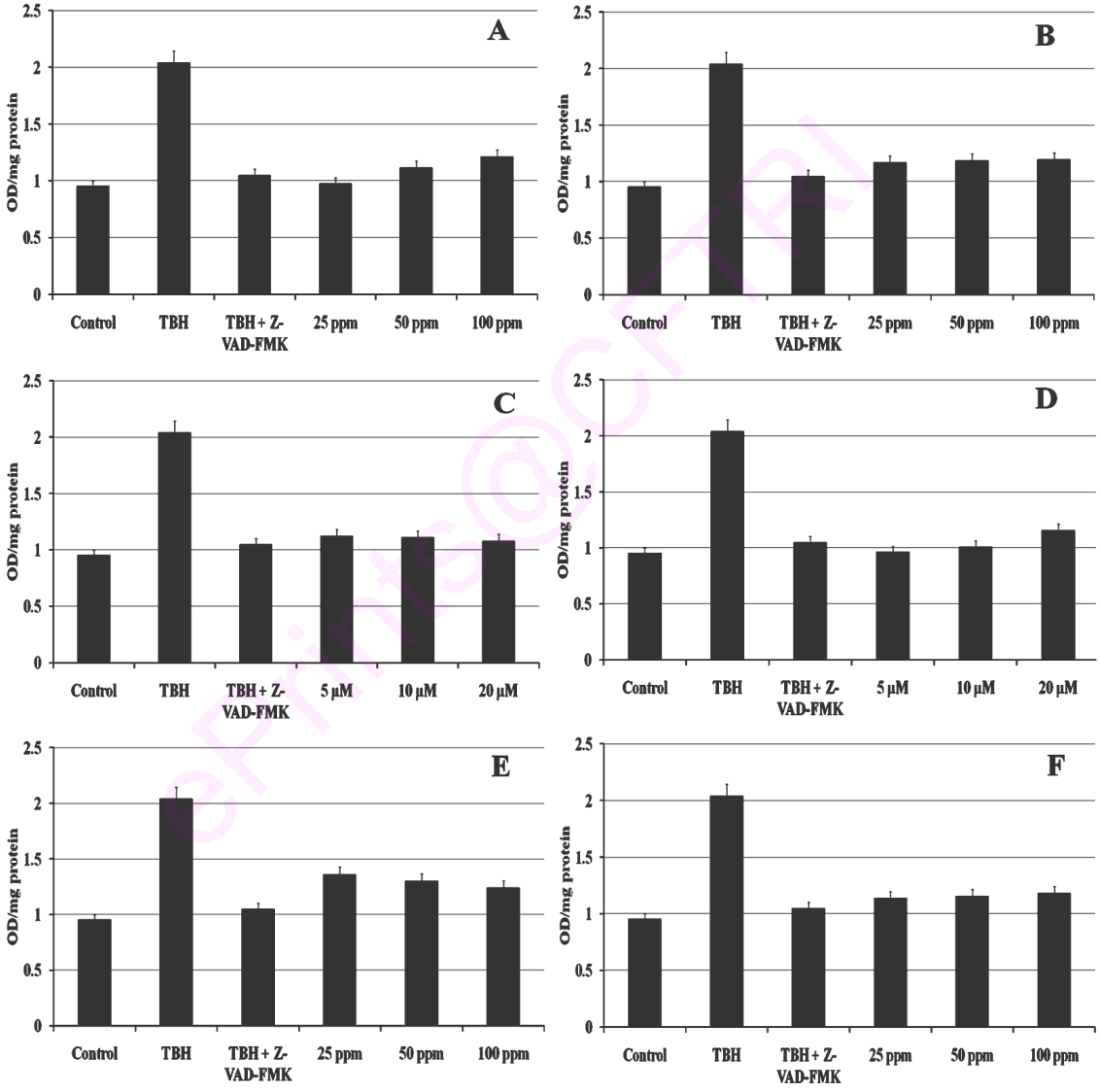



Fig. 4.12. Effect of extracts and standards on TBH-induced caspase activation in Hep3B cells. Hep3B cells were incubated with different concentrations of (A) SCA, (B) DRA, (C) EGCG, (D) quercetin, (E) CF and (F) XF for 3 h followed by exposure to 0.5mM TBH for 4 h. Caspase activity was assayed as described in section 4.2.9 and expressed as absorbance at 405nm/mg protein.

Several studies have indicated the benefits of natural compounds against chemical induced oxidative cytotoxicity. Cyanidin-3-O-glucopyranoside, an anthocyanin, has been shown to protect HCC cell line HepG2 and intestinal carcinoma cell line, Caco2 against aflatoxin B1 and ochratoxin A-induced cytotoxicity by inhibiting the caspase-3 activation and DNA damage (Guerra *et al.,* 2005). Anthocyanin rich extract of *Aronia melanocarpa* protects human umbilical cord epithelial cells against cholesterol induced apoptosis by reversing the upregulation of caspase-3 activation and downregulation of Bcl-2 expression (Zapolska-Downar *et al.,* 2008). In light of the available literature and the data obtained in this study it is obvious that anthocyanin rich extracts SCA and DRA protect Hep3B cell line against TBH-induced cytotoxicity by various mechanisms.

Among the carotenoids fractions of *D. regia*, XF showed a greater inhibition of caspase-3 activation where the activity at 25 and 50ppm (**Fig. 4.12 F**) was comparable to 5µM and 10µM of EGCG (**Fig.4.12 C**) once again substantiating the fact that the xanthophylls are more active than carotenoids. The chief constituents of XF are lutein, zeaxanthin and β-crypotxanthin. These three compounds have gained significant place in biology and medicine due to their diverse pharmacological actions. Lutein, a macular pigment, was found to alleviate stress-induced retinal ischemia-reperfusion injury by reducing the cell loss, number of apoptotic cells and poly (ADP-ribose) immunoreactivity, inhibition of caspase-3 activation, reducing the MDA levels and increasing cellular GSH (Dilsiz *et al.,* 2006; Li *et al.,* 2009). Xanthophylls, particularly lutein, zeaxanthin and β-cryptoxanthin have been studied extensively for their antioxidant and disease preventing properties (Ribaya-Mercado and Blumberg 2004). Inverse correlations between these xanthophylls and oxidative DNA damage, age related macular degeneration, incidence of cancer and cardiovascular disease have been reported (Dorgan *et al.,* 1998; Collins *et al.,* 1998). β-cryptoxanthin has been found to be preferentially accumulated in the human plasma compared to other carotenoids and acts as a ligand for the mammalian carotenoids binding protein (Rao *et al.,* 1997). A 100µM β-carotene protected rat hepatocytes against bile acid-induced cellular necrosis and apoptosis via signaling pathways involving reduction in caspase-3 activation, inhibition of mitochondrial cytochrome C release and prevention of mitochondrial pore transition. However, a lower concentration of β-carotene was associated with low inhibition against cell necrosis (Gumpricht *et al.,* 2004) suggesting that it is required at higher concentrations to confer adequate protection against oxidative stress. In the present study CF showed a dose dependent increase in inhibition of caspase-3 activation suggesting that β-carotene (the major compound present in CF) protects Hep3B cells against TBH-toxicity. The involvement of other two carotenes present in the extract may not be ruled out and hence the effect is mainly a concerted action of all the

carotenoids present. Results from intervention studies on β-carotene supplementation have not been very encouraging. There are reports claiming association of β-carotene with increased cancer risk (Cooper *et al.,* 1999), reduced cancer risk (Greenberg *et al.,* 1994) and no effect (Hennekens *et al.,* 1996) suggesting that it is not beneficial in the absence of the chemical spectrum provided by fruits and vegetables and that it serves mainly as a biomarker of fruit and vegetable consumption rather than being beneficial (Haegele *et al.,* 2000). The beneficial effects observed in the present study is probably due to the concerted effects of all the carotenoids since it is accepted that mixture of carotenoids may be more beneficial than single pure compounds because of their synergistic effects.

Among the tea catechins EGCG is known to be the strongest caspase inhibitor and was found to inhibit caspase-3 activation in D-galactosamine induced apoptosis in rat hepatocytes in addition to inhibiting the activation of caspase-7 and caspase-2 (Katunuma *et al.,* 2006). It was shown to protect the PC12 cells against $H_2O_2$ induced cytotoxicity through inhibition of caspase-3 activation and PARP cleavage along with upregulation of upstream signaling including P13K/Akt and GSK-3 pathways (Koh *et al.,* 2003). Quercetin is one of the most widely distributed flavonoids almost ubiquitous in its existence in the plant kingdom. It has been found to facilitate apoptosis in tumor cells and not in normal cells via suppression of an endogenous cytoprotective heat shock protein 70 (Hosokawa *et al.,* 1990) while others attribute the cytoprotective effect to signaling pathways involving inhibition of AP-1 (Yokoo and Kitamura, 1997). The antioxidant extracts of *S. cumini* and *D. regia* most closely resemble EGCG and quercetin in preventing TBH-induced apoptosis. It appears that these extracts exert their activity not just via the antioxidant action but also by regulating the signaling events involved in apoptotic cell death. This was further confirmed by their regulation of Bax/Bcl-2 ratio in the presence of oxidative stress.

### 4.3.7 Analysis of Bcl-2 and Bax gene expression

Apoptosis is a programmed mechanism of cell death involving a cascade of genes which are either up regulated or down regulated during the process (Steller, 1995; Williams, 1991). Various experiments indicate that apoptosis can be induced by oxidative stress induced by externally applied xenobiotics such as $H_2O_2$, organic hydroperoxides-TBH, cumene hydropeorixide etc (Cregan *et al.,* 1999; Mazlan *et al.,* 2006; Prasad *et al.,* 2007).

A number of genes belonging to the Bcl-2 family have been identified which play a pivotal role in apoptotic process. While few members such as Bcl-2, Bcl-xL, Mcl-1 and BAG-1 act as inhibitors of apoptosis, Bad, Bid, Bax, Bak, and Bik act as promoters of apoptosis. The present

study emphasized on the two genes of Bcl-2 family namely, the antiapoptotic Bcl-2 and the proapoptotic Bax. Both are localized in the outer mitochondrial membranes, nuclear envelope and edoplasmic reticulum where ROS are generated and are activated in response to external stimuli through signal transduction process involving several proteins.

The expression of Bcl-2 and Bax were studied in Hep3B cells treated with TBH and their modulation by pretreatment with the antioxidant extracts and standards. The organic hydroperoxide TBH is reported to cause apoptosis in immune cells by up regulation of proapoptotic proteins Bax and Bid and down regulation of the antiapoptotic Bcl-2 accompanied by release of cytochrome C (Prasad *et al.,*2007) suggesting a mitochondrial pathway. Low doses of oxidants like hydrogen peroxide are capable of inducing apoptosis. The expression profile and relative mRNA abundance of Bcl-2 and Bax with respect to β-actin along with the ratio of Bcl-2/Bax is depicted in **Fig. 4.13a**. Hep3B cells treated with TBH for 3 h showed radical increase in the Bax mRNA expression compared to the control. The increase was 45% compared to that in control, untreated cells. It was interesting to note that there was no considerable difference in the expression of either Bcl-2 or Bax between the control and TBH treated cells.

Over expression of anti-apoptotic proteins Bcl-2 and Bcl-xL is found to be of frequent occurrence in the development and progression of hepatocellular and other carcinomas (Robert and Gores, 2005). The high Bcl-2 expression perhaps is a mechanism developed by carcinoma cells in order to keep them proliferating. Although there was no significant change in the Bcl-2 mRNA expression in Hep3B cells treated with TBH, a significant increase in Bax was noticed, which perhaps is the major effector of apoptosis. Kim *et al.,* (1999) observed a no-change in Bcl-2 expression in human neuroblastoma cells exposed to TBH. However, they observed an increased expression of p53, a direct transcriptional activator of Bax genes (Miyashita *et al.,* 1994). The Bax mRNA expression in the present study may have been effected by an increased p53 expression as suggested by Kim *et al.,* (1999). The expression of Bcl-2 mRNA was significantly lower in Hep3B cells pretreated with the antioxidant extracts (except SCA at 50 ppm) as compared to either control or TBH treated cells. However, the mRNA expression of Bax was down regulated in Hep3B cells pretreated with extracts and standards compared to control and TBH treated cells (**Fig. 4.13**).

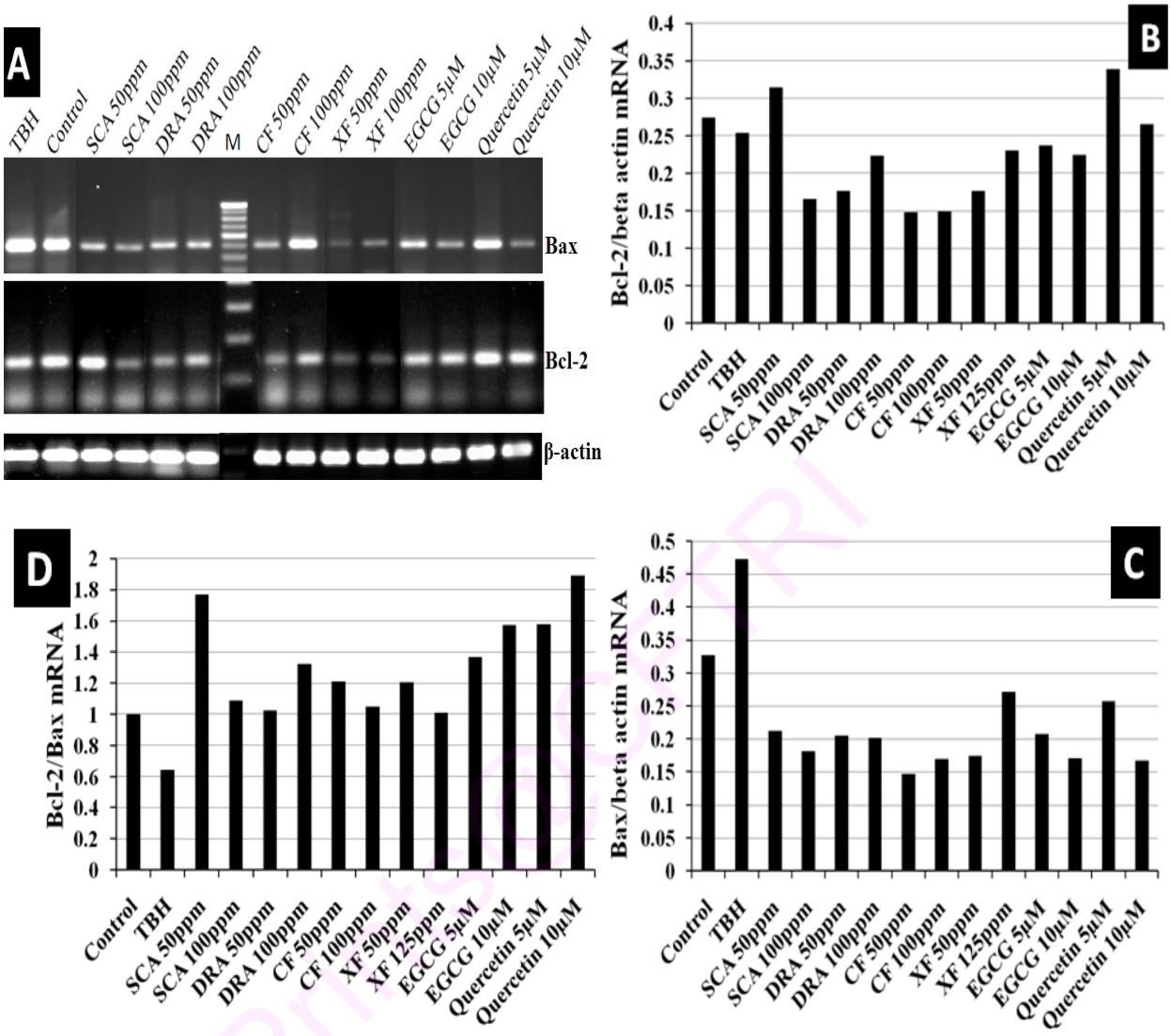



Fig.4.13. Effect of extracts and standards on the expression of Bax and Bcl-2 mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of extracts and standards followed by 3 h exposure to 05mM TBH. PCR amplified β-actin was used for loading normalization. Densitometric quantitations of the bands are expressed as ratio of the respective mRNA to β-actin mRNA. M indicates for 1000 bp molecular marker.

In a study concerning cytoprotection against oxidative stress induced cell death and toxicity, it is not unusual to expect a reduced expression of Bax and a concomitant increase in expression of Bcl-2; because in a perfect scenario where an antioxidant prevents the cell death caused by the oxidant, Bcl-2, an antiapoptotic gene is expected to increase in order to cope with the cytotoxic effect of the said oxidant. On the contrary, the present study showed a reduction in expression of Bcl-2 and Bax genes under all treatment conditions except in the case of SCA (50ppm) and quercetin (5µM), where an increase Bcl-2 expression was associated with a concomitant

reduction in expression of Bax (**Fig. 4.13 B&C**). In all the other treatments Bcl-2 expression remained below that of control and TBH treated levels while all the extracts and standards reduced the Bax expression to a large extent (**Fig. 4.13 B**).

Though Bcl-2 is considered antiapoptotic, its role has been met with controversies. Clinical studies have shown that there is no correlation between the level of Bcl-2 expression and the acute lymphoblastic leukemia (Coustan-Smith *et al.,* 1996). Over expression of Bcl-2 protein has shown to increase the half life of Bax protein (Miyashita *et al.,* 1995) and promote death of photoreceptor cells (Chen *et al.,* 1996) suggesting a proapoptotic gene-like function under certain circumstances. Shinoura *et al.,* (1999) suggest that while low level Bcl-2 expression was antiapoptotic, high level expression is proapoptotic, particularly to Fas-mediated apoptosis in glioma cells. Therefore, under such circumstances, the ratio of Bcl-2 to Bax mRNA expression may give a more accurate measure of pro- or antiapoptotic status of the cell. Resistance to radiotherapy of rectal adenocarcinoma tumors and prostate cancer was associated with an increased Bcl-2/Bax ratio (Scopa *et al.,* 2001) suggesting that Bcl-2/Bax ratio may serve as a potential molecular marker in prediction of tumor prognosis. Hep3B cells treated with TBH showed a 36% reduction the Bcl-2/Bax ratio shifting the balance towards proapoptotic status, which was effectively reverted back to normal levels by the extracts. The effect of EGCG and quercetin was dose dependent elevating the Bcl-2/Bax ratio to 57% at 10µM and 5µM respectively, compared to the control cells. The highest upregulation of the ratio was induced 10µM quercetin showing an elevation of 89% compared to untreated cells. It is intriguing to learn that mRNA expression of both Bcl-2 and Bax were downregulated in Hep3B cells treated with the extracts as compared to either TBH treatment or control (**Fig. 4.13 A-C**). While down regulation of Bax is a desirable property in conferring cytoprotection, reduction in Bcl-2 expression here is questionable. It is also noted that the Bcl-2 expression is high in TBH-treated cells, despite a high cytotoxicity as observed by MTT assay and microscopic analysis explained in earlier sections. According to Shinoura *et al.,* (1999), Bcl-2 at low expression levels acts as antiapoptotic and proapoptotic at high expression levels in Fas mediated apoptosis. However, since Hep3B cells are Fas negative, such a mechanism does not seem feasible in the present study. Therefore, it is possible that the Bcl-2 is either post transcriptionally or translationally regulated in cells treated with TBH implying that the cells may not able to express Bcl-2 protein to the control levels due to apoptosis, despite a high expression of the same. The high Bcl-2 in control cells was expected since overexpression of Bcl-2 is a characteristic many tumors and hepatocarcinoma cells possess (Oltersdorf *et al.,* 2005; Roberts and Gores, 2005; Yildiz *et al.,* 2008). The flavoinds EGCG ad quercetin have been known to inhibit H2O2 induced apoptosis in

human umbilical vein endothelia cells by upregulating Bcl2 and down regulating Bax expression (Choi *et al.,* 2003). The results of the study indicate that the anthocyanins, carotenoids, EGCG and quercetin chiefly act via the upregulation of Bcl-2/Bax mRNA ratio thus conferring protection to Hep3B cells against oxidative stress induced apoptosis.

### 4.3.8 Antioxidant enzyme activity and expression

Reactive oxygen species are implicated in various disorders including cardiovascular disorders, rheumatoid arthritis, cystic fibrosis, neurodegenerative diseases and cancer. A shift in the balance of antioxidant and the pro-oxidant towards an oxidative state has been proven to be a major root cause of these pathologies. These reactive species are kept in check by body's natural defense system by converting them to less toxic or non-toxic compounds. The body's first line of defense include the AOEs SOD, CAT, GPx along with other enzymes such as hemeoxygenase, glutathione reductase and glutathione transferase (Halliwell and Gutteridge, 1999) and the endogenous small molecules such as glutathione. AOEs play an important role in defense against free radical production as well as in repair of free radical mediated molecular damage (Matés, 2000). The AOEs are most effective when the reactive species generated are well within the limits at which they can neutralize them. However, in the presence of excess oxidative stress which happens under conditions of disease states, exposure to chemicals and xenobiotics, emotional and physical stress etc., the AOEs fail to exert a desired effect. Under such conditions, the AOE defense system can be reinforced by externally supplementing compounds with antioxidant properties.

Carotenoids, anthocyanins and other flavonoids such as EGCG and quercetin, the natural antioxidants found in abundance in fruits, vegetables and other plant sources, comprise the best known compounds capable of abrogating the free radical mediated biological reactions. The hepatocellular carcinoma cell line Hep3B is shown to express high levels of AOEs (Chern, 2002), thus making it an ideal model to study the stress and anti-stress parameters. The present study describes the effects of anthocyanins and carotenoids isolated from fruits of *S. cumini* and flowers of *D.regia* respectively on the AOEs SOD, CAT and GPx with reference to their activity and mRNA transcription in Hep3B cells and their comparison with the standard antioxidants EGCG and quercetin. The antioxidants were also tested in the absence of TBH to know if the extracts themselves have any effect on the enzyme activities.

#### 4.3.8.1 Superoxide dismutase

Superoxide dismutases are a family of metallo-proteins that convert the superoxide radical to molecular oxygen and hydrogen peroxide in an attempt to remove the cellular free radicals. While

a low level of $O_2^{\bullet-}$ is beneficial, particularly in converting the chain-propagating lipid peroxyl radical ($^{\bullet}$OOL) to relatively unreactive hydroperoxide in the presence of a proton (Liochev and Fridovich 1994), it can be toxic when present at higher levels. **Table 4.3** summarises the effect of TBH on the SOD status in Hep3B cells after a 3 h exposure and its modulation by pretreating the cells with antioxidants used in the study.

**Table 4.3**. Effect of SCA, EGCG and quercetin on the activity of the enzyme SOD (U/mg protein) in Hep3B cells in the presence and absence of TBH.

| Sample | **Control** | | **TBH** | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 2197.473 ± 107.12 | | | |
| TBH | | | 4447.82 ± 192.84 | |
| SCA | 2615.43 ± 83.94 | 2583.94 ± 97.38 | 3504.71 ± 122.48 | 3926 ± 163. 84 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 1905.17 ± 87.62 | 2076.93 ± 93.56 | 3345.39 ± 94.92 | 2933.81 ± 138.93 |
| Quercetin | 2183.933 ± 98.41 | 2345.316 ± 93.42 | 2632.34 ± 41.72 | 2823.542 ± 121.31 |

The activity of SOD was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

Exposure of Hep3B cells to 0.5mM TBH for 3 h caused a significant, 2-fold increase in SOD activity as compared to the control, untreated cells. None of the extracts or standards showed a significant difference in SOD activity compared to the control cells when applied in the absence of TBH. However, pretreatment of cells with these extracts showed a substantial reduction in TBH-induced increase in SOD activity. The tremendous increase in SOD activity in TBH treated cells appears to be a defense mechanism to cope with the ensuing stress. It is hypothesized that $O_2^{\bullet-}$ reduces ferric to ferrous iron which in turn reacts with TBH to yield tert-butyl alkoxyl radical. These radicals are highly reactive and can initiate lipid peroxidation ultimately causing cell death. The result was in agreement with that obtained by Alia *et al.,* (2006b) who observed a significant increase in the activity of SOD in HepG2 cells treated with TBH. However, they observed no significant difference in the expression of the enzyme CuZnSOD in TBH treated cells. There have been contradictory reports on the effect of TBH on expression of CuZnSOD. While Alia *et al.,* (2006b) observed no difference in its expression in HepG2 cells, another study showed a significant reduction in the expression of CuZnSOD in vascular beds of deoxycorticosterone acetate salt-induced hypertensive rats (Awe *et al.,* 2003). The activity and expression of SOD were found to be increased in testis of rats administered with TBH (Kaur *et al.,* 2006). This was

in agreement with the present study where an increase in SOD activity in Hep3B cells treated with TBH was associated with a slight increase in the mRNA expression of the same (**Fig. 4.14**).

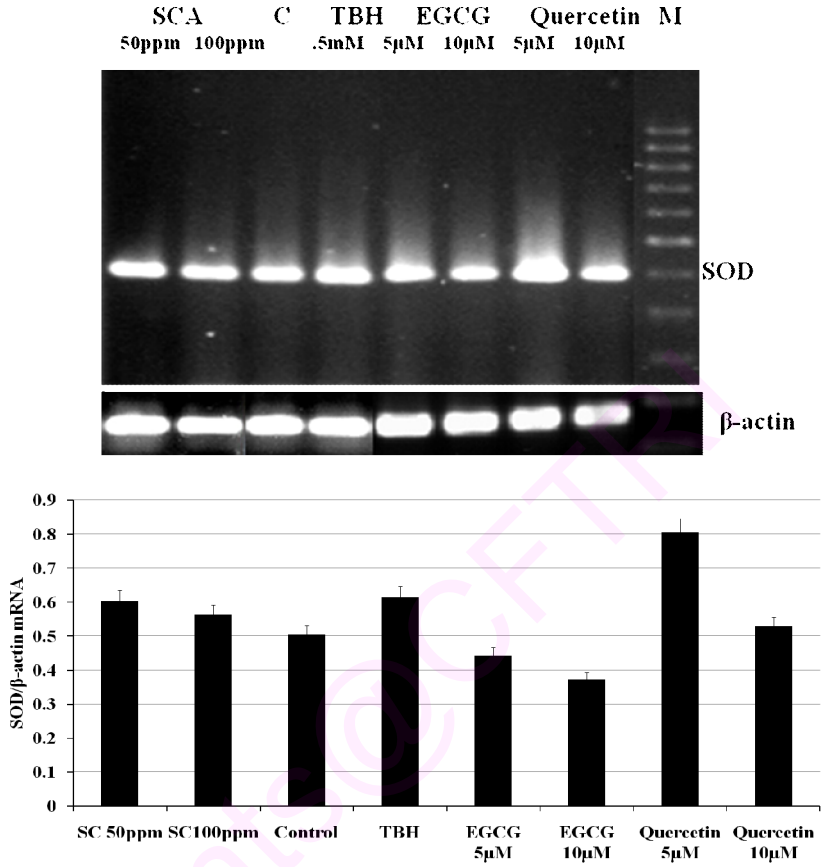



Fig. 4.14. Effect of SCA and standards on the expression of SOD mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitation of the bands are expressed as ratio of SOD mRNA to β-actin mRNA.

However, the increase was not parallel to the activity in the sense that, the activity was increased 2-fold while there was a slight but significant increase (20%) in the transcript level suggesting that a transcriptional regulation of the enzyme might be operating in the cells in response to oxidative stress. A similar observation was made by Teixeira *et al.,* (1998) who observed a 0.8-fold increase in the SOD expression with 2.3-fold increase in activity.

Though SOD is an efficient antioxidant, excess production of the same has been documented to induce toxic effects *in vivo* (Amstad *et al.,* 1991; Elroy-Stein *et al.,* 1986). The overexpression of CuZnSOD is also associated with pathologies such as Down's syndrome and Alzheimer's disease (Lohr, 1991; Kedziora and Bartosz, 1988). Similarly, stable or transient over expression of

CuZnSOD was found to worsen the TBH-induced apoptois in PC12 cells (Pias *et al.,* 2003). Formation of OH• from the decomposition of $H_2O_2$ was observed in the presence of high concentration of CuZnSOD exacerbating the oxidative stress induced damage while catalase abolished the toxicity (Gergel *et al.,* 1995).

In the present study, the 20% increase in SOD expression post TBH exposure appears to be lethal to the cells. The toxic effect of excess SOD is attributed to the over production of $H_2O_2$ resulting in increased oxidative damage by hydroxyl radicals (Ceballos-Picot *et al.,* 1992). However, there have been contradictions on whether SOD increases (de Haan *et al.,* 1996) or decreases (Teixeira *et al.,* 1998) $H_2O_2$ levels *in vivo*. Gardner *et al.,* (2002) developed a minimal mathematical model to explain this difference. Through this model they suggest that at normal SOD levels, the outcome depends on the ratio between the rate of processes that consume $O_2^{\bullet-}$ without forming $H_2O_2$ and those that consume $O_2^{\bullet-}$ with high ($\geq 1$) $H_2O_2$ yield. When this ratio is exceptionally low ($\leq 1$), the overexpression of SOD is expected to show a modest decrease in $H_2O_2$ production while a ratio higher than unity increases $H_2O_2$ production with an increase in SOD activity (Gardner *et al.,* 2002). The present study showed a 2-fold increase in SOD activity with a 1.2-fold increase in expression suggesting an involvement of high $H_2O_2$. This study leads to the presumption that there might be an increased formation of $H_2O_2$ in cells exposed to TBH considering its cytotoxic effects evidenced by morphological analysis and viability assays. A negative feed back mechanism may not be ruled out where increased $O_2^{\bullet-}$ production leads to the inactivation of the enzyme aconitase, thus reducing the flow of reducing equivalents into the respiratory chain and proton leakage by $O_2^{\bullet-}$ which decreases the reduction of respiratory chain components (Liu, 1997). Therefore, in the presence of a feed back inhibition, $O_2^{\bullet-}$ concentration is reduced with SOD overexpression. As a consequence, the inhibition is lifted and rate of $O_2^{\bullet-}$ generation is increased (Gardner *et al.,* 2002) thus increasing the activity of the enzyme. Additionally, Offer *et al.,* (2000) observed a bell shaped dose response curve with CuZnSOD suggesting a prooxidant effect of the enzyme at higher concentrations. They suggest that the outcome of high SOD is a low steady state $O_2^{\bullet-}$ rather than a significantly high $H_2O_2$ resulting in an oxidation of the target molecule by the CuZnSOD rather than protecting it. Therefore, the toxicity of TBH may partly be attributed to a high $H_2O_2$ with an additional prooxidant effect of the enzyme SOD.

Further, it is observed that pretreatment of Hep3B cells with SCA at 50ppm and 100ppm reduced the TBH-induced increase in SOD activity by 22% and 12% respectively. However, the extract had no effect on the activity when applied in the absence of TBH but showed activity that remained close to the basal level (**Table 4.2**). Pretreatment of Hep3B cells with SCA did not

significantly affect the expression of CuZnSOD mRNA. At 50ppm and 100ppm of SCA, the CuZnSOD expression was reduced by 2% and 9% respectively. Han *et al.,* (2006) report an increase in the expression of hepatic CuZnSOD in rats treated with red potato flake extract conferring higher antioxidant potential as measured by reduction in serum lipid peroxidation. However, in the absence of data on the expression of CuZnSOD in cells treated with SCA alone, it is not possible to derive a conclusion if the high expression is due to its own ability to enhance mRNA expression or due to its inability to negate the TBH-induced effects. However, presumably SCA acts via mechanisms independent of SOD enzyme.

The effect of *D. regia* extracts on TBH-induced alterations in SOD activity is summarized in **Table 4.4**. The treatments did not evoke a direct SOD response in terms of activity when the Hep3B cells were exposed to extracts in the absence of TBH. The DRA, extract being rich in anthocyanins along with other phenolics, significantly reduced the TBH-induced increase in SOD activity.

**Table 4.4**. Effect of the *D. regia* extracts, EGCG and quercetin on the activity of the enzyme SOD (U/mg protein) in Hep3B cells in the presence and absence of TBH

| Sample | **Control** | | **TBH** | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 2197.473 ± 107.12 | | | |
| TBH | | | 4447.82 ± 192.84 | |
| DRA | 1148.34 ± 42.59 | 1274.45 ± 36.43 | 1025.51 ± 32.51 | 934.34 ± 56.32 |
| CF | 2031.91 ± 94.31 | 1983.27 ± 64.55 | 3573.92 ± 132.01 | 3202.54 ± 145.63 |
| XF | 1948.83 ± 73.87 | 1703.44 ± 83.84 | 3160.32 ± 109.23 | 2793.33 ± 113.31 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 1905.17 ± 87.62 | 2076.93 ± 93.56 | 3345.39 ± 94.92 | 2933.81 ± 138.93 |
| Quercetin | 2183.933 ± 98.41 | 2345.316 ± 93.42 | 2632.34 ± 41.72 | 2823.542 ± 121.31 |

The activity of SOD was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

The SOD activity in DRA treated cells reduced 2-fold compared to control cells and 4-fold compared to TBH-treated cells. No significant difference in activity was observed irrespective of the presence or absence of oxidative stress post-DRA exposure (**Table 4.4**). The CuZnSOD mRNA expression profile of cells treated with DRA indicates a 27% reduction in expression at 50ppm, with no effect at 100ppm compared to TBH-treated cells. However, the activity reduced significantly with no significant difference between the two concentrations tested. No correlation could be drawn between the activity of the enzyme and the CuZnSOD mRNA expression.

Carotenoids of *D. regia* significantly attenuated the TBH-induced increase in CuZnSOD. The xanthophylls fraction brought about a 35% reduction in TBH-induced increase in the mRNA expression of the enzyme (**Fig 4.15**) while reducing the activity by about 20% compared to TBH treatment and no significant difference between the two tested doses (**Table 4.4)**. The CF reversed the elevated mRNA expression to control level at 50ppm which further reduced at 100ppm by 50% compared to TBH treatment. Consequently, the SOD activity reduced by 40% of TBH treated cells at both the doses. These results indicate effective amelioration of defense mechanisms of Hep3B cells by phenyl propanoids of *D. regia* to an extent much better than the well-established molecules such as EGCG and quercetin.

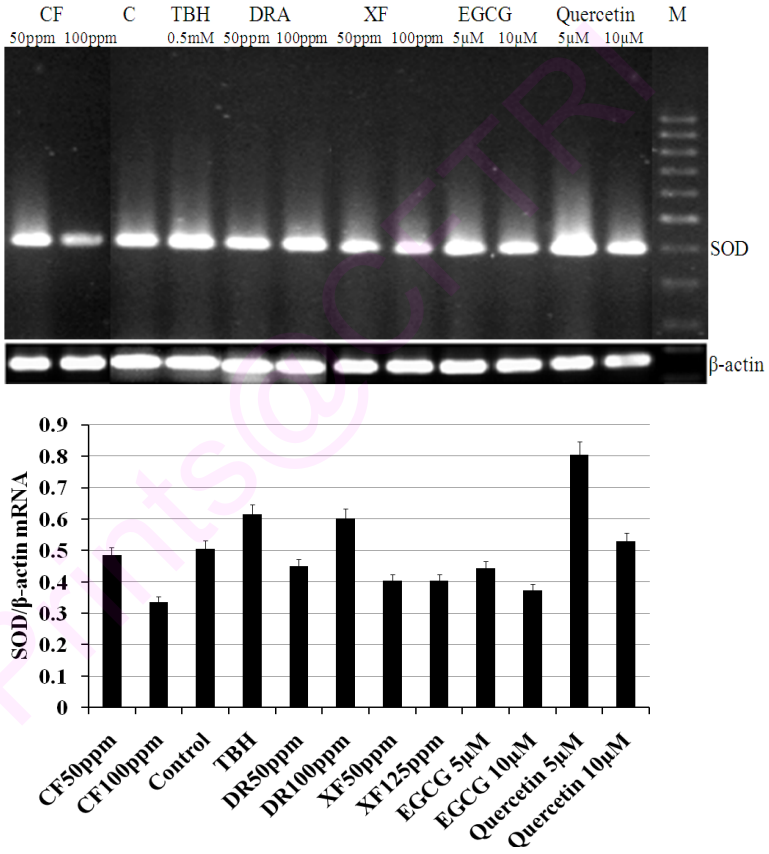



Fig. 4.15. Effect of *D. regia* extracts and standards on the expression of SOD mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitations of the bands are expressed as ratio of SOD mRNA to β-actin mRNA.

The standard antioxidants EGCG and quercetin alone did not significantly increase or decrease the activity of SOD. The TBH-induced activity was reduced by 25% and 35% respectively at 5µM and 10µM EGCG while quercetin showed a 40% reduction **(Table 4.4)**. Both the flavonoids

have been extensively studied for their antioxidant properties and there is plethora of relevant data available in the literature. A comparison of the effect of extracts on SOD activity in TBH stressed cells shows that activity of cells pretreated with SCA (50ppm) CF (at both the doses) and XF (50ppm) more closely resembled that with EGCG. XF at 100ppm showed an activity comparable with 10µM quercetin (40% reduction in TBH-induced SOD activity). The reduction in TBH-induced increase in SOD activity was associated with a consequent reduction of its transcript levels. All the extracts except DRA at 100ppm and quercetin at 5µM significantly reduced the increased mRNA transcript level of CuZnSOD. The theory of prooxidant effect of CuZnSOD (Offer *et al.,* 2000) may not hold good in the case of Hep3B cells pretreated with 100ppm DRA and 5µM quercetin. This is because the high CuZnSOD mRNA is not associated with a high enzyme activity suggesting that there does not exist a low steady state $O_2^{\bullet-}$ thus perhaps preventing the enzyme becoming prooxidant. The lack of relationship between the transcript level and activity also suggests that the enzyme is not transcriptionally regulated. It appears from the results that the extracts act at transcriptional level and perhaps also at post-transcriptional level, by reducing the enzyme available for activity. Most recent report on EGCG suggests its longevity effects in *Caenorhabitis elegans* under heat stress, which is mediated by an up regulation of SOD enzyme, which however was associated with an increase in heat shock proteins. (Zhang *et al.,* 2009). Therefore, in lieu of the contradictory data available on SOD expression and its effects under oxidative stress, it is important to consider related parameters, particularly the effects on other AOEs.

#### 4.3.8.2 Catalase

Catalase is a heme containing homotetrameric protein with a function of $H_2O_2$ detoxification. It is ubiquitously distributed in all aerobic organisms and is chiefly located in the cytosol, though evidence for its presence in mitochondria, the major source of ROS, is made available recently (Salvi *et al.,* 2007). Hydrogen peroxide produced through rapid conversion of $O_2^{\bullet-}$ by mitochondrial SOD produces the highly reactive OH• by interacting with transition metal ions of the respiratory complexes through Fenton reaction which plays an important role in pathologies, particularly those involving inflammatory conditions (Puppo and Halliwell, 1988). The $H_2O_2$, unless removed by the concerted action of CAT an GPx can create havoc by damaging the membrane integrity through peroxidation of phospholipid bilayer, induce permeablilty pore transition with the release of cytochrome C release and activation of pro-apoptotic cascade (Kowaltowski and Vercesi, 1999; Salvi *et al.,* 2003). Therefore, CAT is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of $H_2O_2$ (Ho *et al.,* 2004). The role of CAT in protecting the cells and tissues against oxidative stress has been studied

extensively. Mitochondrial CAT (MCAT) over expression is associated with effective defense against $O_2^{\bullet-}$ generators while cytosolic CAT over expression provides protection against $H_2O_2$ generators (Gurgul *et al.,* 2004). Over expression of MCAT in mice was reported to reduce the severity of age-related arteriosclerosis and increase genomic stability as indicated by a decrease in oxidative stress and mitochondrial deletions in heart and muscle tissues in association with an increase in median and maximum life span (Cutler, 2005). CAT has been reported as the predominant $H_2O_2$ removing enzyme in human erythrocytes. However, there have been conflicting reports on the decreased (Goth *et al.,* 2005; Abraham *et al.,* 2005), increased (Rukmini *et al.,* 2004) and no change (Cao *et al.,* 2004) in activity of CAT under different oxidative stress states. A high level of CAT activity is protective against oxidative damage. Natural antioxidants are credited with their ability to detoxify free radicals chiefly due to their reducing potentials. In the present study, it was observed that TBH significantly inhibited CAT activity in Hep3B cells by 30% compared to the control cells (**Table 4.5**).

**Table 4.5**. Effect of SCA, EGCG and quercetin on the activity of the enzyme CAT (U/mg prtein) in Hep3B cells in the presence and absence of TBH.

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | **50 ppm** | **100 ppm** | **50 ppm** | **100 ppm** |
| Control | 7.85 ± 0.23 | | | |
| TBH | | | 5.72 ± 0.18 | |
| SCA | 7.29 ± 0.09 | 8.19 ± 0.15 | 6.95 ± 0.06 | 7.38 ± 0.12 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 7.53 ± 0.11 | 8.34 ± 0.13 | 7.96 ± 0.08 | 7.29 ± 0.16 |
| Quercetin | 7.34 ± 0.31 | 8.32 ± 0.25 | 8.21 ± 0.08 | 7.89 ± 0.09 |

The activity of CAT was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

This observation was intriguing given the fact that TBH evoked a radical increase in SOD activity (200% compared to control). Theoretically, an increase in SOD activity results in an increased production of $H_2O_2$. In conditions of normal enzyme functioning, there should be an increased CAT activity in order to detoxify the $H_2O_2$ formed by SOD. However, this was not the case in the present study. A similar observation was made by Farombi *et al.,* (2004) where exposure of rats to TBH caused a significant reduction in erythrocyte CAT and GPx activity; however data on SOD was lacking. Though it is said that catalase is inhibited by $O_2^{\bullet-}$ (Kono and Fridovich, 1982), the theory does not seem to fit here due to the fact that the high activity of SOD in fact reduces the steady state level of $O_2^{\bullet-}$. However, if the inhibition is due to the low $O_2^{\bullet-}$ , reversal of

inhibition of CAT activity by the extracts would suggest that they do so by scavenging the $O_2^{\bullet-}$. Therefore, under the given circumstances, the inhibition of CAT activity appears to be due to damage to the proteins caused by the substrate hydroperoxide thus making them less active or inactive. The anthocyanin extract of *S. cumini* (SCA) when applied alone to Hep3B cells evoked a CAT response comparable to control cells, maintaining the activity close to the basal level. However, prior exposure of the cells to SCA restored the TBH-induced reduction in CAT activity by about 88% and 94% (at 50 and 100ppm respectively) of that of control cells (**Table 4.5**). The SCA compared well with EGCG and quercetin in terms of CAT activity.

**Fig. 4.16** represents the mRNA profile of CAT and its relative expression in Hep3B cells treated with SCA, EGCG and quercetin prior to exposure to TBH.

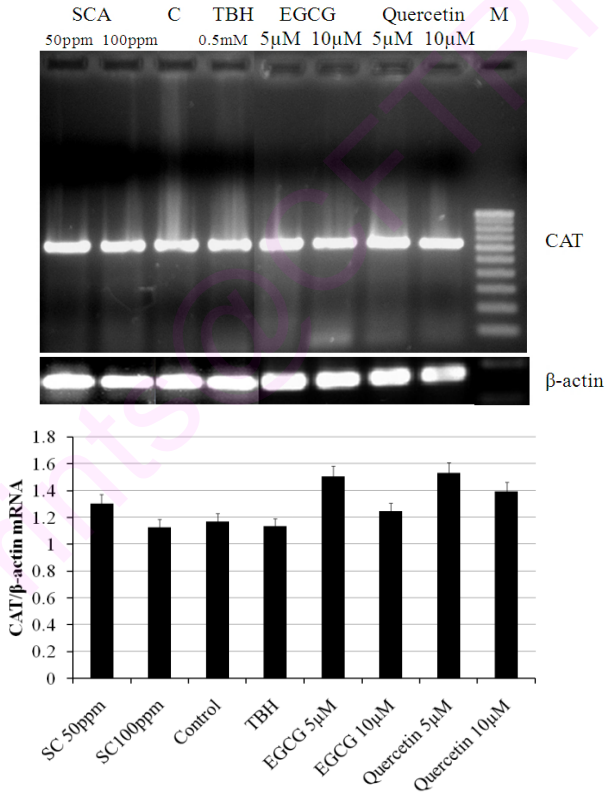



Fig. 4.16. Effect of SCA and standards on the expression of CAT mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitations of the bands are expressed as ratio of CAT mRNA to β-actin mRNA.

Though TBH induced a significant reduction in the activity of the enzyme, no change in the mRNA transcript level of the same was observed suggesting that the activity was not transcriptionally regulated. The reduction in activity appears to be an outcome of direct inhibition of the enzyme by the free radical substrates of the enzyme. The SCA had no effect on the

transcription of at 100ppm, but increased it by 15% at 50ppm. The highest stimulation of mRNA transcription was observed with 5µM of EGCG and quercetin which showed 30% increase compared to TBH treated cells (**Fig. 4.16**). This increase in mRNA transcript was associated with an increase in the activity of the enzyme.

The extracts of *D. regia* had differential effects on CAT activity. The anthocyanin rich fraction DRA, was slightly inhibitory when applied alone to Hep3B cells for 4 h. The inhibition was 25% and 15% at 50 and 100ppm respectively. The inhibition reduced with the increase in the dose. Pretreatment with DRA at 50ppm failed to ameliorate TBH-induced reduction in the enzyme activity, but at 100 ppm the activity was replenished to a significant extent (91% of that of control cells). The carotenoids extracts did not significantly alter the CAT activity in the absence of TBH stress, but elevated the TBH-induced reduction in CAT activity in the order of XF>CF. The effect of XF was comparable to that of EGCG. The highest activity was observed with quercetin followed by EGCG, XF, CF and DRA.

**Table 4.6**. Effect of the *D. regia* extracts, EGCG and quercetin on the activity of the enzyme CAT (U/mg protein) in Hep3B cells in the presence and absence of TBH

| Sample | **Control** | | **TBH** | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 7.85 ± 0.23 | | | |
| TBH | 5.72 ± 0.18 | | | |
| DRA | 6.04 ± 0.06 | 6.69 ± 0.11 | 5.58 ± 0.11 | 7.19 ± 0.21 |
| CF | 6.93 ± 0.01 | 7.29 ± | 6.53 ± 0.07 | 7.21 ±0.07 |
| XF | 7.84 ± 0.03 | 7.91 ± | 7.17 ± 0.15 | 7.36 ± 0.09 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 7.53 ± 0.11 | 8.34 ± 0.13 | 7.96 ± 0.08 | 7.29 ± 0.16 |
| Quercetin | 7.34 ± 0.31 | 8.32 ± 0.25 | 8.21 ± 0.08 | 7.89 ± 0.09 |

The activity of CAT was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

The CAT mRNA profile and their relative expression are depicted in **Fig. 4.17**. It can be derived that all the extracts elevated the mRNA transcript levels in a dose dependent manner and was parallel to the activities. This suggests that the extracts modulate the CAT activity by transcriptional regulation.

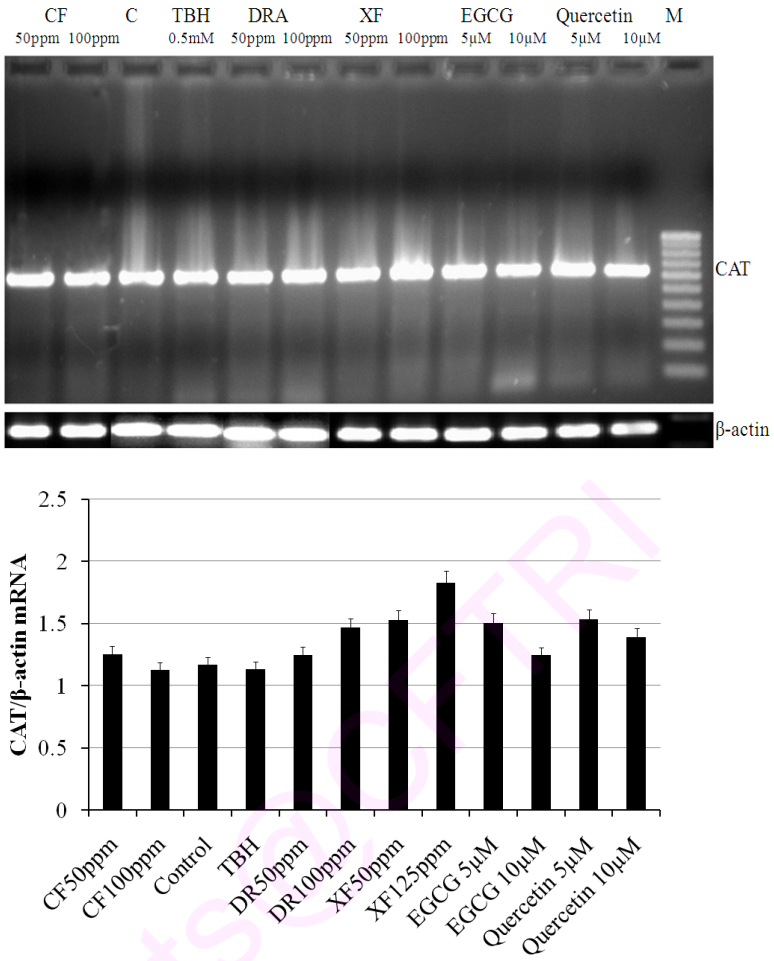



Fig. 4.17. Effect of *D. regia* extracts and standards on the expression of CAT mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitations of the bands are expressed as ratio of CAT mRNA to β-actin mRNA.

However, Hep3B cells treated with EGCG and quercetin showed a reduction in the mRNA with increase in dose. Overexpression of CAT was found to protect cells against $H_2O_2$ induced cell death (Mann *et al.,* 1997). Therefore, the overexpression of the enzyme in Hep3B cells pretreated with TBH may be considered a defense mechanism so as to counter the reactive species generated. It was noted that all the extracts caused a significant stimulation of mRNA transcripts. There are reports of phytochemicals of various sources stimulating the activity of AOEs such as CAT and SOD. Nelson *et al.,* (2006) have demonstrated the ability of a nutraceutical supplement to induce these two enzymes in human erythrocytes despite an absence of nuclei and lack of their ability to induce new synthesis of AOEs once they enter circulation. In the present study it was

observed that the extracts as well as the standards caused a significant induction of CAT either at one or both the doses tested (**Fig. 4.16 and 4.17**).

Considering the results of Nelson *et al.,*(2006), this observation is not surprising given the fact that liver cells possess nuclei and there is continuous synthesis of new protein and hence an induction of CAT. However, contrary to the results of Nelson *et al.,* (2006) the present study did not witness an induction of SOD after pretreatment of extracts to TBH exposed cells (with the exception of quercetin). This may be a defense mechanism adopted by the cell to over come SOD-inducing effect of TBH in order to minimize the cellular load of $O_2^{\bullet-}$ and/or $H_2O_2$.

Additionally several lines of evidence suggest that the expression of mRNAs are controlled by regulatory elements. CAT gene expression has been found to be transcriptionally regulated in mouse skeletal muscles through the involvement of NFκB and AP-1 (Luo and Rando, 2003). Other studies have described the importance of CCAAT binding factors to have influence on the transcriptional regulation of the catalase gene in mouse muscle and $H_2O_2$-adapted human leukemia HL60 cells (Nenoi *et al.,* 2001). It has been reported that upregulation of CAT is associated with activation of p38 mitogen activated protein kinase (Sen *et al.,* 2005). Therefore, the upregulation of CAT mRNA in Hep3B cells pretreated with the extracts and standards may be associated with a stimulation of these regulatory elements rather than a direct effect on transcription.

#### 4.3.8.3 Glutathione peroxidase

Glutathione peroxidase is a seleno-enzyme which converts the $H_2O_2$ produced by the action of SOD on $O_2^{\bullet-}$ to form water in a reaction that oxidizes GSH to the disulfide form, GSSG. Among the four types of GPxes that are identified, cellular Gpx is the most abundant in most cells. The results of the present study showed a marked decrease in the activity of the enzyme Gpx in Hep3B cells treated with 0.5mM TBH. The activity was reduced by 50% of that of control cells (**Tabel 4.7**).

GPx has been described as the most important enzyme for stabilizing oxidative reactions in cells and low GPx activity is particularly lethal under conditions of oxidative stress such as treatment with antineoplastic drugs (Remacle *et al.,* 1992). In cells with high susceptibility to lipid peroxidation, as is the case with TBH treatment, adequate GPx activity is required to neutralize the lipid hydroperxoides, breaking chain reaction of peroxidative damage and thus reduce the generation of free radicals.

**Table 4.7**. Effect of SCA, EGCG and quercetin on the activity of the enzyme GPx (U/mg protein) in Hep3B cells in the presence and absence of TBH.

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 1052 ± 48.56 | | | |
| TBH | | | 516.12 ± 22.41 | |
| SCA | 742.83 ± 28.33 | 704.78 ± 23.84 | 576.49 ± 18.93 | 569.99 ± 13.83 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 950.02± 33.11 | 972.27 ± 36.94 | 923.74 ± 38.38 | 850.38 ± 23.84 |
| Quercetin | 974.62 ± 30.15 | 991.33 ± 29.02 | 748.52 ± 19.84 | 829.08 ± 20.02 |

The activity of GPx was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

The reduced GPx activity in TBH-treated cells is associated with a 2-fold increase in lipid peroxidation (**Fig. 4.4**) suggesting the increased accumulation of lipid hydroperoxides in the absence of a mechanism to counteract the same. Similarly, an inverse relationship has been drawn between the GPx activity, GSH content and cellular lipid peroxidation. Inhibition of GSH synthesis in macrophages was associated with a significant reduction in cellular GSH content and GPx activity accompanied by a substantial elevation of LDL oxidation and peroxide levels (Rosenblat and Aviram 1998). In agreement with the results of Rosenblat and Aviram (1998), the present study witnessed a marked reduction in GPx activity (**Table 4.7**) and GSH (**Fig 4.3**) content with a sizeable amount of lipid peroxidation (**Fig. 4.3**) in Hep3B cells treated with 0.5mM TBH. Interestingly, treatment with SCA alone showed a 30% reduction in the activity of the GPx compared to the control cells and pretreatment of the same failed to evoke a significant GPx response in TBH-treated cells. Only a marginal 4% increase in GPx activity was observed in TBH-exposed cells pretreated with SCA (**Table 4.7**). A 22% reduction in mRNA expression was observed in TBH treated cells along with a 50% reduction in its activity compared to control (**Fig. 4.18**). The expression of GPx mRNA was found to be parallel with its activity, an indication of transcriptional regulation.

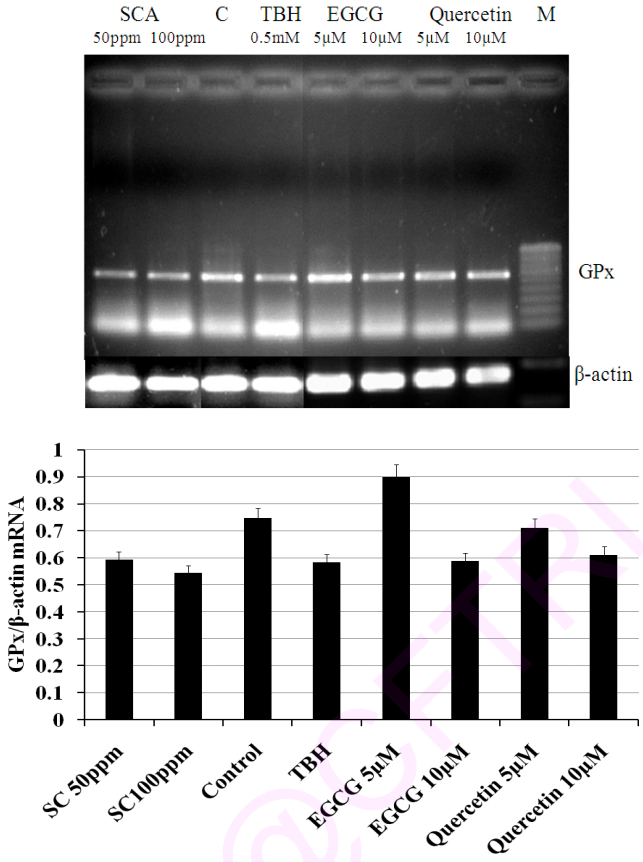



Fig. 4.18. Effect of SCA and standards on the expression of GPx mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitations of the bands are expressed as ratio of GPx mRNA to β-actin mRNA.

The extracts of *D. regia* and the standards showed no significant difference in GPx activity as compared to the control (**Table 4.8**). The activity was slightly reduced in Hep3B cells treated with these extracts and standards alone. The anthocyanin rich fraction, though failed to completely revive the GPx activity to normal levels, inhibited the TBH-induced reduction in GPx activity to a large extent in a dose dependent manner. The activity in DRA pretreated cells was found to be 62% and 77% at 50ppm and 100ppm respectively as compared to the 50% activity in TBH-treated cells. The carotenoids fractions moderately reversed the effect of TBH on GPx activity, CF being more efficient than XF in this case (XF was more effective in CAT activity). Among the two carotenoids fractions, CF at 100 ppm offered maximum protection by increasing the activity 66% as opposed to TBH-treatment.

**Table 4.8**. Effect of the *D. regia* extracts, EGCG and quercetin on the activity of the enzyme GPx (U/mg protein) in Hep3B cells in the presence and absence of TBH

| Sample | Control | | TBH | |
|---|---|---|---|---|
| | 50 ppm | 100 ppm | 50 ppm | 100 ppm |
| Control | 1052 ± 43.56 | | | |
| TBH | | | 516.12 ± 22.41 | |
| DRA | 786.77 ± 24.11 | 853.84 ± 31.82 | 661.32 ± 19.01 | 811.84 ± 27.84 |
| CF | 957.87 ± 41.71 | 983.34 ± 32.77 | 645.42 ± 23.88 | 693.29 ± 18.93 |
| XF | 896.95 ± 37.35 | 801.79 ± 24.38 | 603.55 ± 20.91 | 894.94 ± 27.09 |
| | **5µM** | **10µM** | **5µM** | **10µM** |
| EGCG | 950.02± 33.11 | 972.27 ± 36.94 | 923.74 ± 38.38 | 850.38 ± 23.84 |
| Quercetin | 974.62 ± 30.15 | 991.33 ± 29.02 | 748.52 ± 19.84 | 829.08 ± 20.02 |

The activity of GPx was determined in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. Data correspond to the representative values from three independent experiments done in triplicates and expressed as average ± SD.

**Figure 4.19** represents the mRNA profile and expression of the GPx enzyme with respect to β-actin. The reduction in GPx activity in TBH-treated cells is associated with a concomitant decrease in the expression of the same. The DRA, CF, XF and quercetin showed a dose dependent increment in the GPx activity (**Table 4.8**) and mRNA expression in TBH exposed cells with the exception of EGCG which showed a reduction in GPx mRNA with dose. Maximum transcription was achieved with 100ppm XF and 5µM EGCG. Both EGCG and quercetin caused a dose-dependent reduction in GPx mRNA expression (**Fig. 4.19**).

GPx is known to be regulated by signaling pathways involving c-Abl and Arg tyrosine kinases which directly bind to the proline-rich sites on GPx during oxidative stress thus stimulating its activity. It is reported that c-Abl and Arg tyrosine kinases are activated during oxidative stress and form complexes with GPx depending on the oxidative stress levels. Activation of c-Abl and Arg at non-lethal concentrations of $H_2O_2$ appears to play an antiapoptotic function in protecting cells against increased oxidative stress (Cao *et al.,* 2003). However, the constitutive association of GPx with c-Abl and Arg is disrupted at lethal concentrations of $H_2O_2$ causing a down regulation of GPx activity leading to cellular damage and apoptosis (Sun *et al.,* 2000). It was observed in the present study that the GPx activity was significantly reduced in the presence of TBH. The reduction in activity may be attributed to an increased SOD activity which results in an excess $H_2O_2$ load causing a disruption of GPx complexes with c-Abl and Arg tyrosine kinases and hence apoptotic cell death.

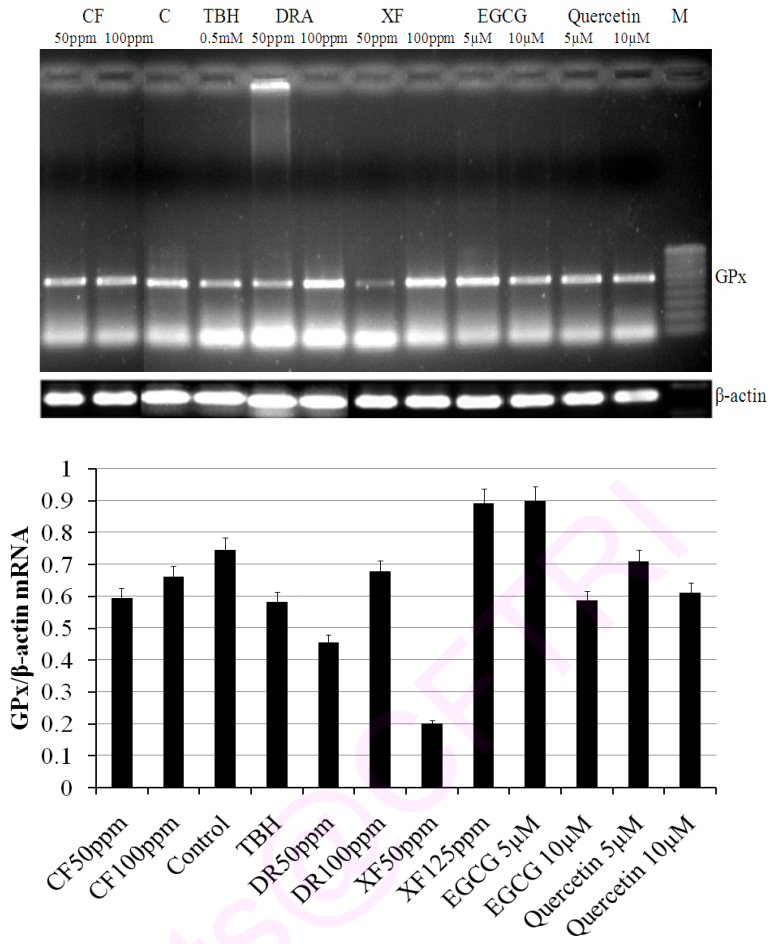



Fig. 4.19. Effect of *D. regia* extracts and standards on the expression of GPx mRNA in Hep3B cells exposed to TBH stress. mRNA expression of the enzyme was determined by semiquantitative RT-PCR in Hep3B cells treated for 4 h with noted concentrations of sample and standards followed by 3 h exposure to 0.5mM TBH. PCR amplified β-actin was used for loading normalisation. Densitometric quantitations of the bands are expressed as ratio of GPx mRNA to β-actin mRNA.

Similarly, the reversal of death in cells treated with the extract is associated with an increase in GPx activity in all the cases except with SCA, suggesting that such a mechanism may be involved in conferring protection to the cells against oxidative stress. The SOD activity in Hep3B cells treated with these extracts was also associated with a reduction in TBH-induced increase. There are reports of CAT being activated by c-Abl and Arg mediated phosphorylation (Bharti *et al.,* 1998). Therefore, the activity of antioxidant phytochemicals may depend on a number of signaling pathways in order to bring about a reduction in free radical load or to protect the cells against free radical induced oxidative stress.

The overall results of consequence of oxidative stress and antioxidants on the AOE suggest that each compound acts differently with respect to activity and expression of the enzyme. TBH has been shown to evoke significant cellular stress in other cell lines (Alia *et al.,* 2005, 2006b).

Dietary phytochemicals have been known to induce cellular defense enzymes in response to oxidative stress. This induction is believed to be mediated by the antioxidant response elements within the promoter regions of these genes. Two characteristic regulatory elements, the antioxidant responsive element (ARE) and xenobiotic responsive element (XRE) have been identified to be located in the promoter region of the CuZnSOD in human liver cells (Park and Rho 2002). In addition, putative binding motifs for NF-κB and AP-1, the transcriptional regulators activated in response to oxidative stress, are found on mouse GPx and CAT gene (Zhou *et al.,* 2001). Therefore, it appears that the antioxidants can be transcriptionally induced by the above factors in combination with or independently of the two regulatory elements as an oxidative stress response.

Effect of quercetin on oxidative stress-induced alterations in AOEs activity and expression is well documented. Rohrdanz *et al.,* (2003) showed that quercetin brings about a reduction in CuZnSOD, CAT and GPx mRNA expression at the concentrations tested in the present study (5 and 10μM). They also observed that pretreatment of H4IIE cells with quercetin protected against $H_2O_2$ induced oxidative stress without altering the enzyme expression concluding that quercetin acts via mechanisms independent of AOE. However, the present study clearly showed an amelioration of oxidative stress by quercetin via activity of AOE irrespective of whether the mRNA expression was altered or not. This suggests that the activity of AOE, in quercetin treated cells prior to TBH exposure, is regulated transcriptionally as well as post-transcriptionally. The study conducted by Rohrdanz *et al.,* (2003) lacked data on activity and the expression of mRNA is not a direct measure of the activity of its respective protein. Therefore, such data have to be measured with careful considerations while studying the antioxidant potential of the said compound. Similarly, Alia *et al.,* (2006b) reported no change in expression of AOE mRNA in the presence of TBH which was true only for CAT mRNA (**Fig. 4.16**) in the present study while the CuZnSOD was upregulated (**Fig. 4.14**) and GPx mRNA was downregulated (**Fig. 4.18**) in the presence of 0.5mM TBH incubated for 3 h. They report an upregulation of these enzymes by quercetin in TBH treated HepG2 cells (Alia *et al.,* 2006a) which is in agreement with the present study. Additionally, quercetin has been reported to be a potent inhibitor of lipoxygenase and cycloxygenase activities, Na-K-ATPAase, protein kinase C and various tyrosine kinases (Lipkin *et al.,* 1999). Quercetin, in addition to its well-known ability against cellular oxidative stress, it

has been reported to suppress both chemically induced and spontaneous formation of tumors in animals, through one or more of the above mentioned mechanisms (Lipkin *et al.,* 1999).

Frequent consumption of green tea, one of the most popular and widely consumed beverages, has been known to protect against development of various cancers according to numerous experimental and several population-based studies. Molecular mechanisms underlying chemopreventive effects exerted by green tea and its components have been extensively investigated which has resulted in the identification of EGCG as the functional ingredient. EGCG, the chief catechin of green tea is gaining significance as a cytoprotective as well as an anticancer agent. In the present study, it reversed the effect of TBH on AOEs by elevating the mRNA transcription of CAT and GPx. There is accumulating evidence to show that the redox sensitive transcription factor, nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2) is a key component that activates antioxidant response element (ARE)-mediated expression of many antioxidant and phase II detoxifying enzymes (Na and Surh, 2008). Treatment of human breast epithelial cells with EGCG was found to increase the ARE binding and transcriptional activity of Nrf2 and activate Akt and extracellular signal-regulated protein kinase1/2 (ERK1/2). Therefore, it is suggested that Nrf2 mediates EGCG-induced expression of AOEs possibly via Akt and ERK1/2 signaling, which may provide cells with acquired antioxidant defense capacity to survive the oxidative stress (Na and Surh, 2008; Sriram *et al.,* 2009).

When the two carotenoids fractions CF and XF were compared for their activities, XF was found to be more effective against TBH-induced alterations in AOE activities. Carotenoids have long been associated with reduced incidence of chronic degenerative diseases including cardiovascular disease and cancer owing to their antioxidant activity. Differential activities of carotenes and xanthophylls are well documented. From the chemical investigation it was observed that CF chiefly contained β-carotene along with its isomers. β-carotene is associated with an induction of CAT, SOD and GPx. This was noticed in the present study, though it was not as effective as the other extracts in elevating the reduced levels of GPx and CAT. Carotenoid treatment has been demonstrated to have been activating the translocation of cytosolic Nrf2 to nucleus resulting in the induction of phase II detoxifying enzyme through transactivation of ARE (Ben-Dor *et al.,* 2005). Differential effects of carotenes and xanthophylls may primarily be attributed to their bioavailability and their metabolism within cells. Xanthophylls are more bioavailable than β-carotene which could be the reason behind the better efficacy of xanthophylls in the present study. The main carotenoids present in XF were identified to be lutein, zeaxanthin and β-cryptoxanthin. All these carotenoids have been extensively studied and their beneficial effects have been proved by in vitro, in vivo and epidemiological studies as explained in the previous

chapter. Though CF provides protection to the Hep3B cells against TBH-induced toxicity, its action on AOE is not substantial compared to the other extracts and standards. This observation was in accordance with that of Bestwick and Milne (1999) who stated that β-carotene does not significantly enhance the AOEs in Caco-2 monolayer. In contrast, β-carotene enhanced the production of $H_2O_2$ suggesting its failure to protect high risk individuals against degenerative diseases. However, the chemoprotection offered by β -carotene may be linked to conversion to retinoids, but may also be independent of, though not necessarily exclusive from, retinoid formation and may involve direct antioxidant activity (Krinsky, 1996; Handelman, 1996).

Anthocyanins from berry fruits have been gaining immense importance due to their wide pharmacological benefits. They possess antioxidant, anti-inflammatory, anticancer, antidiaetic and antiobesity properties (Dai *et al.,* 2007; Mulbagal *et al.,* 2009). Anthcyanins were found intact and as metabolites in human plasma and urine after ingestion of low concentrations of anthocyanins from red wine with an associated increase in antioxidant capacity (Garcia-Alonso *et al.,* 2009). Consumption of pigmented (red) rice was found to ameliorate the iron induced lipid peroxidation in rats which was associated with an increase in CAT activity (Toyokuni *et al.,* 2002). Intra-oral application of muco-adhesive freeze dried black raspberry gel showed high levels of anthocyanins in saliva and oral tissues with a distribution in blood suggesting the potential for anthocyanins as local drug delivery targets in chemoprevention (Mallery *et al.,* 2007; Ugalde *et al.,* 2009). Based on the available experimental data it is evident that anthocyanins possess potent chemopreventive actions. No significant amount of data is available on the effect of anthocyanin extract of *S. cumini* on the AOEs. The extract showed protection to Hep3B cells chiefly by modulating CAT and SOD while it did not increase the GPx activity to a significant level. In order to further characterize its effect on AOEs, the stability of the mRNA transcripts of AOEs was studied.

## 4.4 Effect of SCA on antioxidant enzyme mRNA stability

It has been proven time and again that oxidative stress evokes a variety of cellular responses. One such response is the attack on cellular defense system through alteration in activity of the major AOEs CAT, SOD and GPx right from transcription to post-transcriptional modifications. Considering transcription, the genes coding for CAT, SOD and GPx can either be upregulated or downreguated depending on the kind of oxidant the cell is exposed to. Hyperoxia has been associated with an increased activity of these enzymes while exposure to xenobiotics such as $CCl_4$ inhibits their activity. Such changes in antioxidant defense systems have been ascribed to change in the rate of gene transcription and mRNA translation as well as in the stabilities of

mRNA and protein. Oxidant-induced conformational changes of regulatory proteins may influence a spectrum of genes by initiating transcription and/or stabilizing specific mRNAs.

In the present thesis it was earlier documented that SCA exerts its protective effects against oxidative stress induced alterations in AOE activity and transcription. A recent study demonstrated an elevation of hepatic AOE activities in streptozotocin-induced diabetic rats by the aqueous extract of *S. cumini* pulp (Namasivayam *et al.,* 2008). Though several reports exist on the effect on anthocyanins on the activity of AOEs and their transcription, data on their effect on the stability of mRNA transcripts in the presence of oxidative stress is lacking.

The stability of mRNA molecules all along the process of transcription, mRNA processing, translational efficiency and post-translational modifications is an important determinant of protein levels in the cell. Unlike transcriptional control, the molecular mechanisms that govern post-transcriptional RNA processing are poorly understood (Sampath and Perez-Polo, 1997). The enhanced stability of AOE transcripts may play a significant function in cells during conditions of oxidative stress by enhancing the turn over of end proteins available for accomplishing defense functions and protect the other metabolic pathways from oxidative stress. Since *S. cumini* confers protection against TBH-induced oxidative stress mainly by maintaining the levels of CAT and SOD, this study was designed to know if the extract has any effect on the stability of the mRNA of these enzymes. Half lives of mRNA are measured by blocking the mRNA synthesis with transcription inhibitors such as actinomycin D, a DNA intercalating agent which preferentially intercalates into transcriptionally active nucleosomes, isolating the total RNA at different time intervals and monitoring the loss of a particular transcript with a message specific probe.

Anthocaynins from other berry sources have known to abrogate oxidative stress induced elevation in AOE levels and to even induce AOEs. It has been recently reported that flavonoids can form stable complexes with RNA. Flavonoids such as quercetin, kaempferol, apigenin and delphinidin were proved to intercalate RNA duplex resulting in stable adducts, delphinidin being the most stable among others (Nafisi *et al.,* 2009). There are reports on substances increasing the stability of AOE transcripts in cell models. For example, nerve growth factor enhances the stability of CAT and GPx in rat pheochroma cytoma (PC12) cells (Sampth and Perez-polo, 1997). However, no such reports exist on the ability of anthocyanins from natural source to increase the AOE mRNA stability. This study is an initial approach to know if anthocyanins of SCA do exert their action partly by increasing the mRNA stability of AOEs.

### 4.4.1 Effect of actinomycin D on antioxidant enzymes

Effect of transcription inhibitor actinomycin D on the mRNA abundance of CAT, SOD, GPx and β-actin, in control, untreated cells was evaluated. Stability measurements are generally carried out for a period which exceeds the half life of mRNA. However, in the present study actinomycin D was found to be cytotoxic and to reduce the cell number especially in cells treated with TBH leading to reduced RNA yield. Actinomycin D has been found to be cytotoxic in different cell models (Lafuse *et al.,* 2000) limiting its use for short durations of time. Therefore, to maintain uniformity in sampling, treatment was done for a period of 6 h to measure the stability of mRNA transcripts of AOE. Transcript levels of mRNAs of CAT, SOD and β-actin were not significantly affected up to 6 h after treatment with actinomycin D in control cells (**Fig. 4.20, 4.21**). However, significant and visibly obvious reduction in GPx mRNA abundance was observed in control cells (**Fig. 4.22)**. A similar observation was made by Maitre *et al.,* (1993) where they observed no substantial difference in the mRNA transcripts of AOE after 9 h of actinomycin D treatment in human umbilical cord vein epithelial cells.

### 4.4.2 Effect of SCA on mRNA stability of antioxidant enzymes

Reactive species are now known to activate gene expression through modulation of intracellular redox state of nuclear proteins. It was observed that SOD activity as well as its mRNA level was enhanced when Hep3B cells were exposed to TBH while anthocyanin rich extract of *S. cumini* (SCA) abrogated this effect, particularly at 100ppm. Therefore, in the present study two concentrations were selected based on various other tests presented in previous chapters. 100ppm and 250ppm were used to evaluate if SCA offers protection via mechanisms involving stabilization of the mRNA transcripts or enhancing transcriptional turn-over of genes coding AOEs. No changes in mRNA transcripts level were observed in control cells after addition of actinomycin D for a period up to 6 h suggesting that SOD mRNA is stable during this period (**Fig. 4.20**).

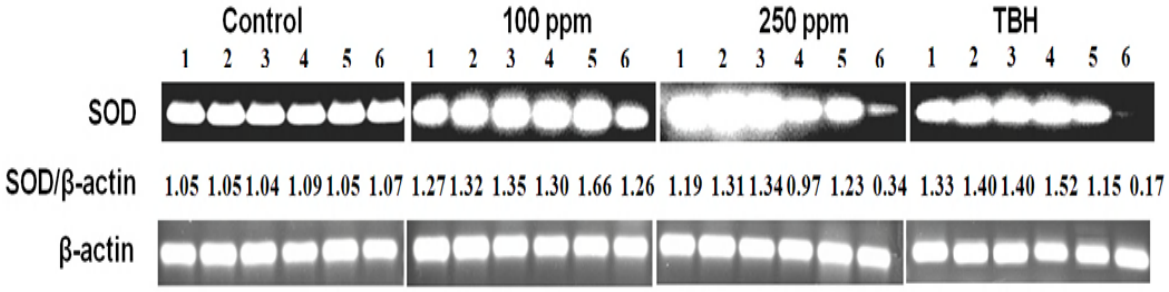



Fig. 4.20. Decay of SOD mRNA in Hep3B cells as a function of time in response to TBH in the presence of SCA. Hep3B cells were treated with 100ppm and 250ppm of SCA followed by incubation with 0.5mM TBH. Untreated cells served as negative control and cells treated with

TBH alone served as positive control. Gels showing decay patterns of SOD. β-actin amplification from the respective cDNA was used for loading normalization and denistometric quanification expressed as the ratio of SOD mRNA to that of β-actin.

The SOD mRNA level was higher in TBH treated cells compared to control, untreated cells which is in accordance with the earlier observation (**Fig. 4.14**). Oxidative stress inducing agents are known to cause induction of AOEs in cells and tissues. However, Hep3B cells treated with TBH also showed high steady state SOD mRNA up to 5 h after actinomycin D suggesting that TBH may not only increase the rate of transcription in response to oxidative stress but also enhance the stability of the mRNA so as to ensure a constant supply of the transcript for translation into its protein. At 5$^{th}$ hour a 15% reduction compared to zero hour was observed after which there was a drastic reduction in the same (87.23%) (**Table 4.9**).

**Table 4.9.** Percent changes in mRNA levels of CAT, CuZnSOD, GPx and β-actin in Hep3B cells 6 h after addition of actinomycin D.

| | **CAT** | **CuZnSOD** | **GPx** | **β-actin** |
|---|---|---|---|---|
| Control | +23.89 | +1.91 | -21.26 | -24.38 |
| SCA 100ppm | -38.17‡ | -0.61‡ | -100.00* | -21.77 |
| SCA 250ppm | +6.61‡ | -70.91* | -90.81* | -28.92 |
| TBH | -79.77* | -87.23* | -100.00* | -2.33* |

Percent changes were calculated with respect to the zero hour samples of respective treatments. *significantly different from control cells. ‡ Significantly different from TBH treated cells.

In treated cells whether with TBH alone or pretreated with SCA, there was a slight increase in steady state SOD mRNA level. Strong expression signals were visualized in the gels at 100ppm SCA with intense early signals at 250ppm. Higher level of SCA produced strong early expression indication abundant trun-over rates of SOD mRNA (**Fig. 4.20**). Pretreatment of Hep3B cells with SCA did not significantly alter the SOD mRNA level, except that at 100ppm the stability was extended up to 6 h while at 250ppm it failed to do so. It was found that SCA abrogates the TBH-induced increase in SOD activity as well as mRNA level. Contrarily, though it reduces the mRNA level enhanced by TBH, it retains its abundance perhaps by enhancing its stability through certain mechanisms which are not understood at this point.

There was a slight increase in the mRNA transcript level of CAT with time which may be due to the insufficient inhibition of transcription in control cells. A similar pattern was observed with SOD mRNA in Hep3B cells treated with TBH and also in those treated with SCA prior to TBH (**Fig. 4.20**). At 6 h after actinomycin D treatment, a 23% increase in CAT mRNA was observed as compared to the zero hour which was in accordance with the results of Maitre *et al.,* (1993) who observed a 8% increase in CAT transcript and 34% CuZnSOD mRNA 9 h after actinomycin

D treatment. As observed earlier, there was no significant difference in the CAT mRNA levels of control and TBH treated cells at the zero hour. Exposure of Hep3B cells to TBH-stress caused a time dependent decay of CAT mRNA with a 79.77% reduction in its abundance at 6$^{th}$ h (**Table 4.9**). Pretreatment of Hep3B cells with SCA inhibited the CAT mRNA decay in a dose dependent manner, completely abolishing the TBH-induced mRNA decay at 250ppm (**Fig. 4.21**). Catalase mRNA transcript level in control cells was constant even after 6 h of actinomycin D treatment. This is attributed to the higher stability and longer half life (about 42 h) of CAT mRNA transcripts with a gradual decay in mRNA levels (Sampath and Perez-Polo, 1997).

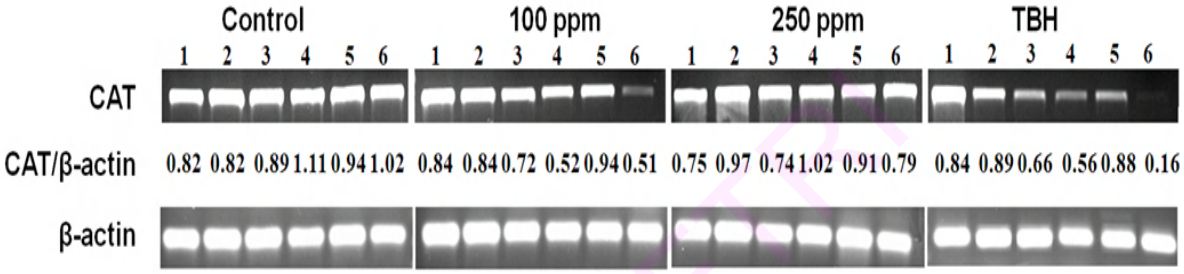



Fig. 4.21. Decay of CAT mRNA in Hep3B as a function of time in response to TBH in the presence of SCA. Hep3B cells were treated with 100ppm and 250ppm of SCA followed by incubation with 0.5mM TBH. Untreated cells served as negative control and cells treated with TBH alone served as positive control. Gels showing decay patterns of CAT. β-actin amplification from the respective cDNA was used for loading normalization and denistometric quanification expressed as the ratio of CAT mRNA to that of β-actin.

The time dependent reduction in CAT mRNA was observed in cells treated with TBH. Catalase gene is relatively stable and has a 3′ flanking region with T-rich clusters and CA repeats known to be susceptible for regulation by destabilization or ubiquination (Sampath and Perez-Polo, 1997). TBH perhaps causes oxidative damage, breaking the bonds between the nucleosides causing destabilization thus causing rapid mRNA decay. Pretreatment of the cells with SCA was found to stabilize the CAT mRNA thus almost completely abolishing the TBH-induced mRNA decay during the period of testing. The mRNA decay is perhaps delayed in the presence of SCA indicating a higher mRNA stability conferred by the anthocyanin extract (**Fig. 4.21**). A number of antioxidant response element binding factors have been associated with the regulation of AOEs in response to ROS (Favreau and Picket, 1993). The elevation of catalase activity in neonatal hyperoxia was found to be post-transcriptionally regulated by enhanced stability of CAT mRNA (Clerch *et al.,* 1991) and this enhanced stability is conferred through the formation of a specific rat lung protein-catalase mRNA complex in a redox sensitive manner (Clerch *et al.*, 1998). Nerve growth factor has been found to stimulate CAT activity associated with an increase in the respective protein and mRNA in PC12 cells (Jackson *et al.,* 1994; Sampath *et al.,* 1994) which

was reported to be due to an increased mRNA stability (Sampath and Perez-Polo, 1997). The present study clearly demonstrates the stabilizing effects of SCA on TBH-induced CAT mRNA decay.

Glutathione peroxidase is another $H_2O_2$ detoxifying enzyme known to have a long half life of ≈ 45 h (Sampath and Perez-Polo, 1997). In the present study GPx mRNA was found to decay in a time dependent manner in control cells with a reduction of 21.26% at the end of 6 h (**Fig. 4.22**). Exposure of Hep3B cells to 0.5mM TBH was found to show a marked degradative effect on GPx mRNA stability. GPx mRNA was not detected after the first hour suggesting complete decay of the same in the presence of oxidative stress which perhaps is the reason for the reduced GPx activity in TBH treated cells as described earlier in this chapter. Pretreatment of SCA substantially increased the stability of the GPx mRNA at both the concentrations. At 250ppm the mRNA abundance was found to be more than that in control cells at zero hour. mRNA transcript level was maintained to that of control level up to 4 h in SCA pretreated cells. SCA at 100ppm was more effective in stabilizing the GPx mRNA with retention of 65% of transcripts as compared to 13% at 250ppm at the end of 4 h. The mRNA abundance in Hep3B cells treated with SCA at 100ppm and 250ppm matched at 4 h and 3 h respectively suggesting that mRNA transcripts were more stable at 100ppm.

Though SCA had no significant reversal of TBH-induced reduction in GPx activity (**Table 4.7**), it does play an important role in maintaining the GPx transcript pool. At 250ppm the GPx mRNA level was found to be higher than that of control or TBH-treated cells. This implies that if this concentration (instead of the lower level) was used in the activity study earlier, it would perhaps show a protective effect against TBH-induced reduction in GPx activity and mRNA level. However, in the absence of concrete data such an assumption cannot be completely justified.

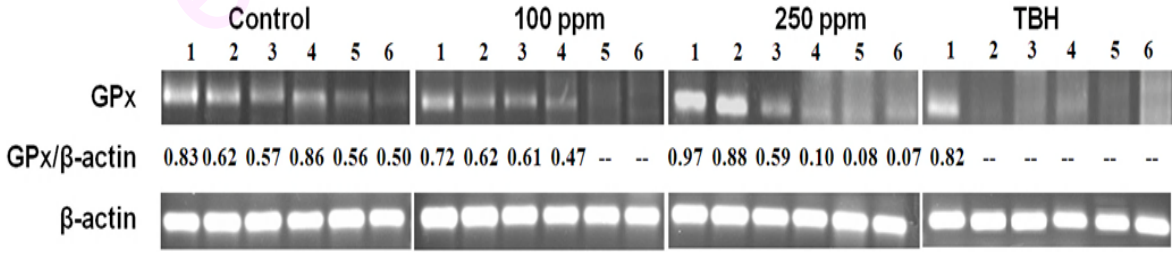



Fig. 4.22. Decay of GPx mRNA in Hep3B as a function of time in response to TBH in the presence of SCA. Hep3B cells were treated with 100ppm and 250ppm of SCA followed by incubation with 0.5mM TBH. Untreated cells served as negative control and cells treated with TBH alone served as positive control. Gels showing decay patterns of GPx. β-actin amplification from the respective cDNA was used for loading normalization and denistometric quanification expressed as the ratio of GPx mRNA to that of β-actin.

Experimental evidence is mounting regarding the involvement of transcription factors in induction of AOEs in response to oxidative stress or by protective phytochemicals. Nrf-2 has been found to be a critical transcription factor that binds to the antioxidant response elements in the promoter region of genes encoding antioxidant and detoxifying enzymes (Kwak *et al.,* 2004; Kobayashi and Yamamoto, 2005). Incubation of Nrf-$2^{+/+}$ cardiac fibroblasts with the natural antioxidant 3H-1,2-dithiole-3-thione (D3T) was found to cause significant induction of AOEs and phase II detoxifying enzymes which was completely abolished in Nrf2-/- cells. This suggests the positive involvement of Nrf-2 in the regulation of basal expression and chemical induction of AOE which in turn confers resistance to reactive species induced cytotoxicity. The transcriptional induction of AOEs in the presence of SCA in Hep3B cells may also be a result of activation of Nrf-2 thus reducing the TBH-induced cytotoxicity although this hypothesis needs to be confirmed through further investigation.

A look at the results of this study suggests that SCA plays a major role in enhancing the stability of AOE mRNAs. It appears that SCA also causes an induction of AOEs in Hep3B cells. Natural antioxidants are credited with their ability to induce AOEs under conditions of oxidative stress as well as in normal cells. Flavonoids found in fruits and vegetables are associated with better health and reduced risk of cancer owing to their ROS scavenging effect and activation of transcription factors causing induction of AOEs and phase II detoxifying enzymes (Mandel *et al.,* 2005). Extensive work has been done on tea polyphenols where they are reported to prevent or attenuate decreases in AOEs activities mediated via oxidative stress induced by UV-B radiation (Agarwal *et al.,* 1993), *Mycobacterium tuberculosis* infection (Guleria *et al.,* 2002), exposure to carcinogens (Das *et al.,* 2002), consumption of alcohol (Skrzydlewska *et al.,* 2002) etc. Anthocyanins being a member of flavonoids family are also efficient antioxidants and stimulate induction of antioxidants. Black rice extract, rich in anthocyanins, inhibited LDL oxidation via induction of AOEs CAT and SOD (Cheng *et al.,* 2006). The overall results suggest that SCA acts via post-transcriptional stabilization of mRNA transcripts of AOEs and perhaps also helps increase the rate of transcription, thereby protecting the cells from oxidative damage.

In the absence of data on effect of anthocyanins alone on the stability of mRNA transcripts of AOEs, this study may be further extended to unravel the intimate mechanisms involved in the enhanced stability and the involvement of transcription factors and antioxidant response elements in conferring stability in the presence of externally supplemented anthocyanins.

Actinomycin D was toxic to Hep3B cells over time. Cells treated with TBH showed marked exacerbation in cell toxicity which was evident by severe morphologic changes followed by detachment of the cells. It was interesting to note that pretreatment of Hep3B cells with SCA

showed no morphologic changes and cells appeared normal with no detached cells in the medium. This suggests that SCA protects the cells against actinomycin D toxicity which perhaps is the reason for high stability of mRNA transcripts of AOEs in SCA pretreated cells.

## 4.5 Antiproliferative activity

There is mounting evidence regarding the anticarcinogenic effects of phytochemicals and the search for novel anticancer drugs from natural sources has continued through the collaboration of scientists worldwide. During the course of this study it was observed that incubation of cells with the extracts for longer than 16 h showed characteristics of cytotoxicity evidenced by morphological changes. There are reports suggesting the biphasic effects of natural antioxidants which can act both as cytoprotective and cytotoxic agents. Therefore, a short experiment was carried out to develop information about the cytotoxic effects of the extracts, particularly in proliferating cells.

### 4.5.1 Cell viability assay

It was interesting to note that anthocyanins of both *S. cumini* and *D. regia* were particularly toxic to Hep3B cells, SCA being more toxic than DRA. Incubation of the Hep3B cells with SCA for 24 h caused a dose dependent increase in cytotoxicity reducing the cell viability by 23%, 34%, 70% and 92% at 50ppm, 100ppm, 250ppm and 500ppm respectively (**Fig. 4.23 A**). Incubation at 50ppm of SCA for 48 h and 72 h revived the cells slightly increasing the cell viability compared to 24 h. However this was not the case with higher concentrations of SCA. The increased viability or reduced cytotoxic effect of low concentrations of SCA may be due to the cells ability to adapt to the effects of anthocyanins over time. Significant reduction in cell viability was observed above 100ppm nearly killing all the cells at 500ppm. The effect of 250ppm SCA was comparable to that of 50µM EGCG at 24 h incubation. Incubation of Hep3B cells with DRA for 24 h had slight stimulatory effects on the cell growth at 50ppm and 100ppm. However, higher concentrations were clearly cytotoxic even at 24 h. The reduction in cell viability was dose dependent showing 35% and 58% decline at 250ppm and 500ppm of DRA respectively (**Fig. 4.23 B**). At 250ppm DRA was more effective in reducing the cell viability compared to 50µM quercetin and was equivalent to 100µM quercetin and this also brought about a 35% reduction in cell viability. After 24 h incubation, quercetin at 10µM significantly increased the viability of Hep3B cells by 20% compared to untreated cells (**Fig. 4.23 D**) while it had no effect at 20µM suggesting a stimulatory effect of the same at lower concentrations. However, higher concentrations of 50µM and 100µM reduced the viability by 20% and 40% respectively. Longer incubation period had no significant difference between either the time of incubation or the doses.

Cells lost 60% viability at 100µM of quercetin when incubated above 48 h (**Fig. 4.23 D**). EGCG too showed a slight stimulatory effect at 10µM when incubated for 24 h which further increased the viability at 48 h. However, the viability was drastically reduced after 72 h (34% loss). Doses higher than 10µM were toxic to cells exhibiting 50% toxicity at 20µM after 24 h incubation. EGCG was found to be highly toxic among the compounds tested with a comparable effect with SCA at 250 ppm and above.

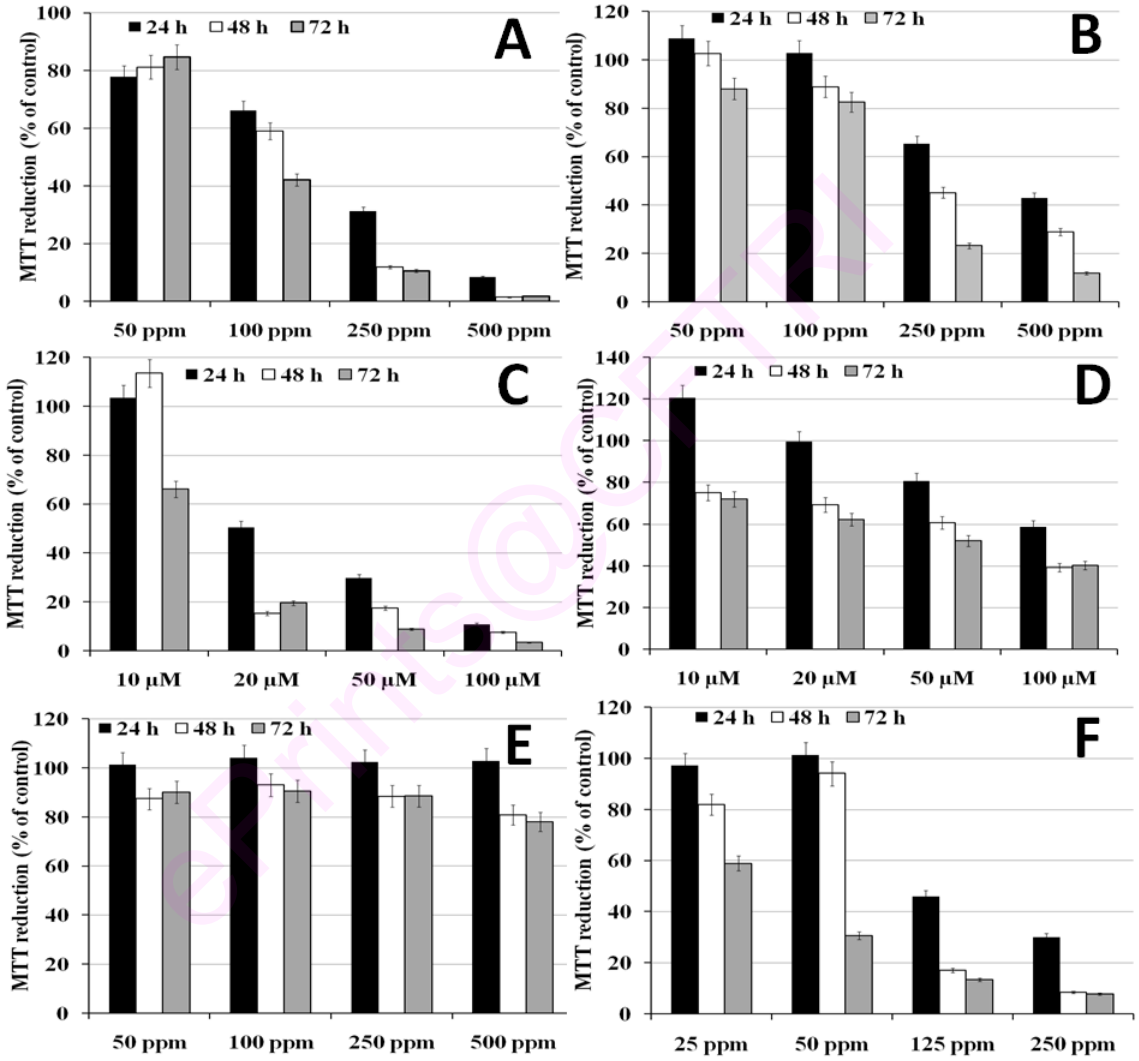



Fig. 4.23. Antiproliferative activity of extracts and standards on Hep3B cells. Cells were incubated with different concentrations of (A) SCA, (B) DRA, (C) EGCG, (D) quercetin, (E) CF and (F) XF for 24 h, 48 h and 72 h in serum free medium. Cell viability was assessed by MTT assay calculated as percentage reduction in MTT with respect to untreated cells.

The carotenoinds of *D. regia* were not found to be as effective as the anthocyanins or the standards. A 24 h incubation of Hep3B cells with CF had no effect on the viability of cells at the tested concentrations. However longer incubation of cells with CF caused a significant reduction

in viability compared to untreated cells with no significant difference between the incubation time (**Fig. 4.23 E**). Viability of cells treated with XF was not altered at lower concentrations (25ppm and 50ppm) however, was drastically reduced at 125ppm (55% loss) which further reduced at 250ppm (70% loss). Effect of 125ppm XF was comparable to 20µM EGCG (**Fig. 4.23 F**). Incubation of cells with XF for 48 h and 72 h caused a drastic reduction in viable cells nearly killing all the cells at 250ppm.

### 4.5.2 Effect of extracts on morphology of cells

In order to further confirm the antiproliferative effect of extracts on cancer cell line, Hep3B cells were incubated with the extracts for 24 h and visualized under fluorescence microscope following staining with propidium idode (PI). PI is a fluorochrome which specifically binds to the DNA showing bright red fluorescence. Hep3B cells treated with SCA, DRA, EGCG and quercetin showed clear apoptotic characteristics such as cell shrinkage, membrane blebbing, condensed chromatin, fragmented nuclei and apoptotic bodies as described in an earlier similar study (Chodon et al 2007). The morphological features varied with different extracts suggesting different modes of action. Control cells showed uniform fluorescence. The apoptotic features were most obvious in cells treated with DRA, EGCG and quercetin. DRA showed features similar to that of EGCG suggesting an involvement of a similar apoptotic mechanism by the two. However, SCA showed characteristic vacuole formation which was not evident in any other treatment (**Fig. 4.24 A**). SCA treated cells showed very few cells with condensed chromatin and fragmented nuclei at 250 ppm while at 500ppm the cells lost structure suggesting a necrotic mode of cell death. Treatment with 100ppm DRA had no effect on morphology of cells and this result is in agreement with the viability assay where no cell death was observed (**Fig. 4.23 B**). At 250 ppm, DRA showed typical apoptotic features. Similar features were observed with EGCG where number of apoptotic cells increased with dose. Fragmentation of nuclei was highest in cells treated with 20µM EGCG followed by 50µM quercetin.

Quercetin was not toxic at 20µM and the cells exhibited normal morphology except for a few showing signs early apoptosis (**Fig. 4.24 F**). The cytotoxicity increased in a dose dependent manner increasing the number of apoptotic cells and reducing the cell number. The carotenoids of *D. regia* were not as effective as the anthocyanins or the standards under the testing conditions. As was obvious in the MTT assay, CF showed no significant cytotoxicity up to 500ppm with only a few cells showing bright fluorescence (**Fig. 4.24 C**). XF showed cytotoxicity with an increase in the dose, the increase however was not significant with doses (**Fig. 4.24 D**). Increase in the dose of SCA, DRA and the standards resulted in decline in the number of apoptotic cells and most

cells appeared to have lost cellular structure with a single bright spot in the centre with no cell membrane. It appears that the cell death is mainly effected via apoptotic machinery followed by necrosis. Necrosis may also be the consequence of a lack of phagocytic machinery in *in vitro* cell culture conditions.

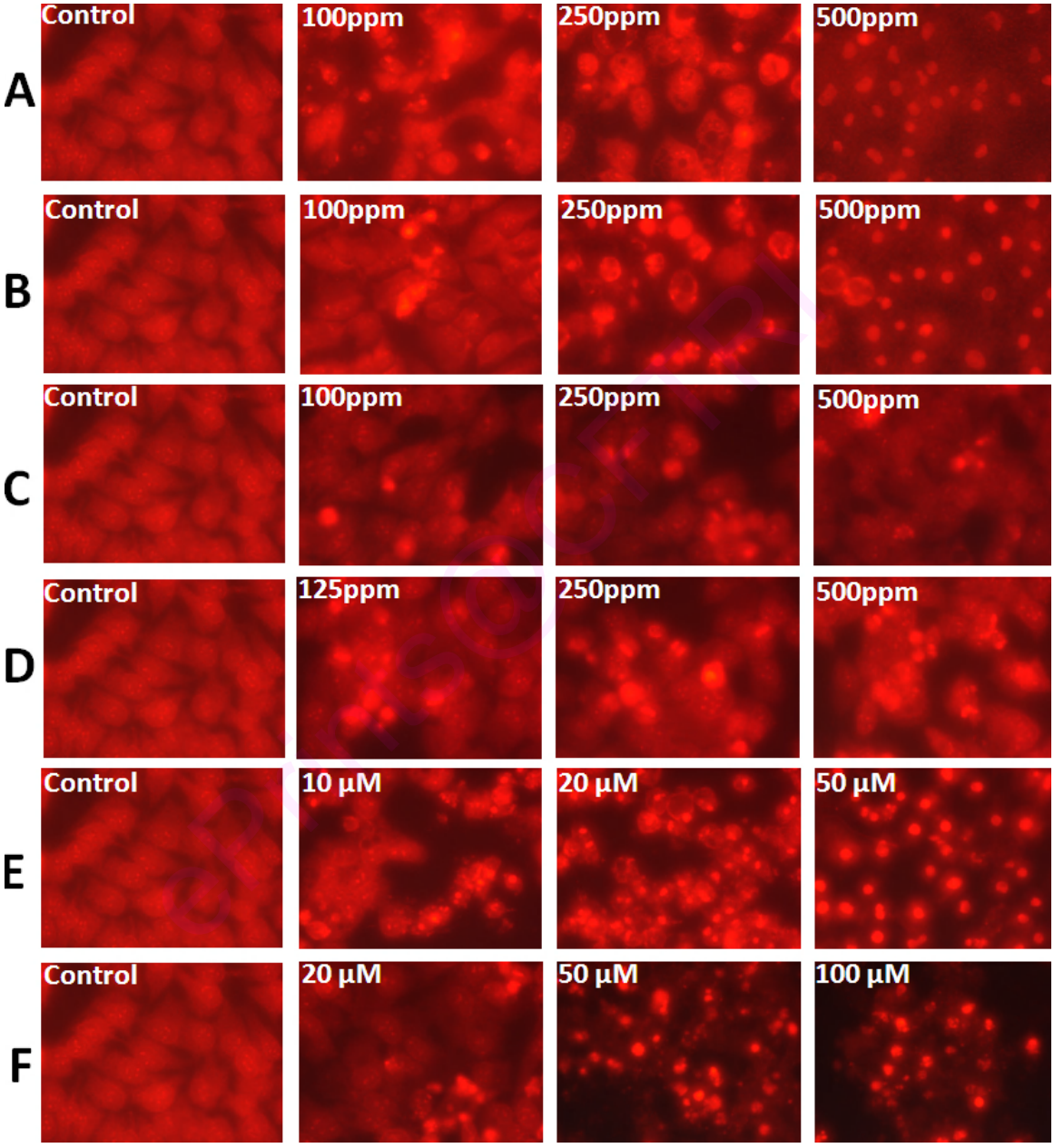



Fig. 4.24. Morphological changes in Hep3B cells incubated with different concentrations of (A) SCA, (B) DRA, (C), CF (D), XF (E) EGCG and (F) quercetin for 24 h in serum free medium. The figures shown are representative of three independent experiments.

The loss of plasma membrane at the highest concentrations of SCA, DRA and standards is obvious while the carotenoids treated cells still retained plasma membrane integrity suggesting

early apoptotic stage and that the carotenoids may be needed at still higher concentrations to cause complete apoptosis. It is interesting to observe the dual effect of a single extract/compound here. Short term exposure to extracts is cytoprotect ive against an ensuing oxidative stress while long term exposure actually causes inhibition of cell proliferation and stimulates cell death in fast growing cells. Another interesting point noted here is the extracts can be growth stimulatory at low concentrations. Such observations are not new in literature. For example anthocyanins protect against CD-40 induced inflammation in atherosclerosis (Xia *et al.,* 2009) while anthocyanins from red potato induce apoptotic cell death in prostate cancer cells (Reddivari *et al.,* 2007). Quercetin whose antioxidant effect has been extensively studied, also has a putative role in numerous prooxidant consequences. Significant amount of literature is available on the biphasic effect of quercetin in different cell models (van der Woude *et al.,* 2003; Dihal *et al.,* 2006). The mechanisms underlying the shift in activity is chiefly attributed to the oxidative degradation of quercetin to *o*-semiquinone and *o*-quinone leading to the formation of superoxide and depletion of GSH (Metodiewa *et al.,* 1999). Similarly, EGCG has been found to exert cytotoxic effects by spontaneous generation of $H_2O_2$ and consequent DNA damage which may be an important mechanism of its anticancer property (Azam *et al.,* 2004; Elbling *et al.,* 2005). Overwhelming evidence exists on the beneficial effects of berry fruits which extend beyond antioxidation. They are known to regulate carcinogen and xenobiotic metabolizing enzymes, various transcription and growth factors, inflammatory cytokines and subcellular signaling pathways of cancer cell proliferation, apoptosis and tumor angiogenesis. Berry phytochemicals may also potentially sensitise tumor cells to chemotherapeutic agents by inhibiting pathways that lead to treatment resistance and consumption of fruits rich in anthocyanins and polyphenols may provide protection from therapy-associated toxicities (Seeram 2008). Epidemiological studies indicate an inverse relationship between carotenoids consumption and risk of cancer. However, controversy surrounds the role of β-carotene in relation to health and disease. While many a study suggest the protective role of carotenoids rich diet in the prevention of cancer, cardiovascular diseases and other degenerative diseases (Mayne 1996), β-carotene is associated with an increased incidence of lung cancer especially in high risk populations such as smokers and asbestos workers (ATBC 1994).

From the results it was obvious that the anthocyanins derived from *S. cumini* and *D. regia* possess strong cell inhibitory properties in vitro. While the effect of SCA is solely due to the anthocyanins, the effect of DRA may be a concerted effect of other unidentified phenolic compounds present in the extract in addition to the anthocyanins. It appears that cytotoxicity of these compounds is not necessarily an out come of direct prooxidant effect but may be mediated

via signaling pathways involving transcription factors, activator proteins, caspases etc., that are probably more relevant to cancer associated pathways. However, an elaboration of the cytotoxic effects/anticancer potentials of the extracts and their mechanisms is beyond the scope of this thesis and needs further investigation.

## 4.6 Conclusions

The results presented in this chapter reveal a large body of information regarding the antioxidant activity of natural colors derived from *S. cumini* and *D. regia*. Apart from the direct antioxidant effects demonstrated in the previous chapters, this study indicates that the carotenoids and anthocyanins confer protection against oxidative stress mediated cellular injury via mechanisms not only dependent on pathways involving antioxidant enzymes, but also other cell survival mechanisms including caspases and apoptotic and antiapoptotic family of proteins. Anthocyanins of *S. cumini* were also found to offer protection through increased stability of transcripts of antioxidant enzymes. Among the extracts tested anthocyanins were most effective and the effect was on par with the standard and a powerful antioxidant EGCG. Xanthophylls of *D.regia* offered more protection against TBH-stress compared to the carotenes. Apart from a powerful antioxidant activity against oxidative stress induced cell damage, the extracts, particularly anthocyanins and xanthophylls, also exhibited antiproliferative activity in Hep3B cells on long term exposure suggesting a cancer chemopreventive property.

# *Summary and Conclusions*

## Summary

The beneficial effects of consumption of fruits and vegetables and decrease in the incidence of degenerative diseases mediated by ROS is well known. Consequences of oxidative stress are serious and in many cases are manifested by alterations in activities of enzymes involved in detoxification. The beneficial effects of fruits and vegetables are ascribed to their high content of antioxidant biomolecules such as flavonoids, carotenoids, polyphenols etc. The present study dealt with the identification of two rich sources of anthocyanins and carotenoids, fruits of *S. cumini* and flowers of *D. regia* respectively, and the elucidation of their antioxidant activity *in vitro* using chemical and cell models.

*S. cumini* fruit peel was found to be a rich source of anthocyanins. The anthocyanins were identified to be diglucosides of malvidin, petunidin and delphinidin. The purified anthocyanin rich extract at low concentrations demonstrated high antioxidant activity in chemical based *in vitro* tests. Flower petals of *D. regia,* a source with unique combination of hydrophilic (anthocyanins) and lipophilic (carotenoids) pigments were chosen where major carotenoids were identified as β-carotene and its isomers as well as astaxanthin, lutein, zeazanthin and β-cryptoxanthin. β-carotene was found to comprise 76% of the total carotenoids. β-carotene content in freshly harvested flowers after oven drying was found to be approximately 660 mg/kg of dry weight. However, it was noticed that the β-carotene content nearly doubled (1.3 g/kg) in flowers which fall naturally. Therefore it is suggested that the flowers that fall from the tree may be collected for extraction of pigments. This bypasses the laborious process of plucking the flowers while avoiding any damage to the trees as well. The anthocyanins of *S. cumini* were found to be stable when stored at low pH and low temperatures protected from light. Incorporation in a formulation retains most of the pigment when stored at temperatures up to 20°C protected from light. This suggests that it can be a very good source of natural color in food as well as pharmaceutical formulations with short shelf lives.

Further study was carried out to extrapolate the antioxidant potentials of the pigment rich extracts of *S. cumini* and *D. regia* in the presence of oxidative stress in a biological system involving isolated rat primary hepatocytes which retains most of the *in vivo* characteristics. Carbon tetrachloride and *tert*-butyl hydroperoxide were chosen as the oxidative models. Isolated rat hepatocytes showed extensive cellular damage in the presence of these two agents as evidenced by alterations in cell survival, cellular GSH pool, lipid peroxidation and antioxidant enzyme status. Anthocyanins of *S. cumini* effectively reverse the $CCl_4$- and TBH-induced toxicity in terms of enhancing the viability and increasing cellular GSH, lipid peroxidation and antioxidant

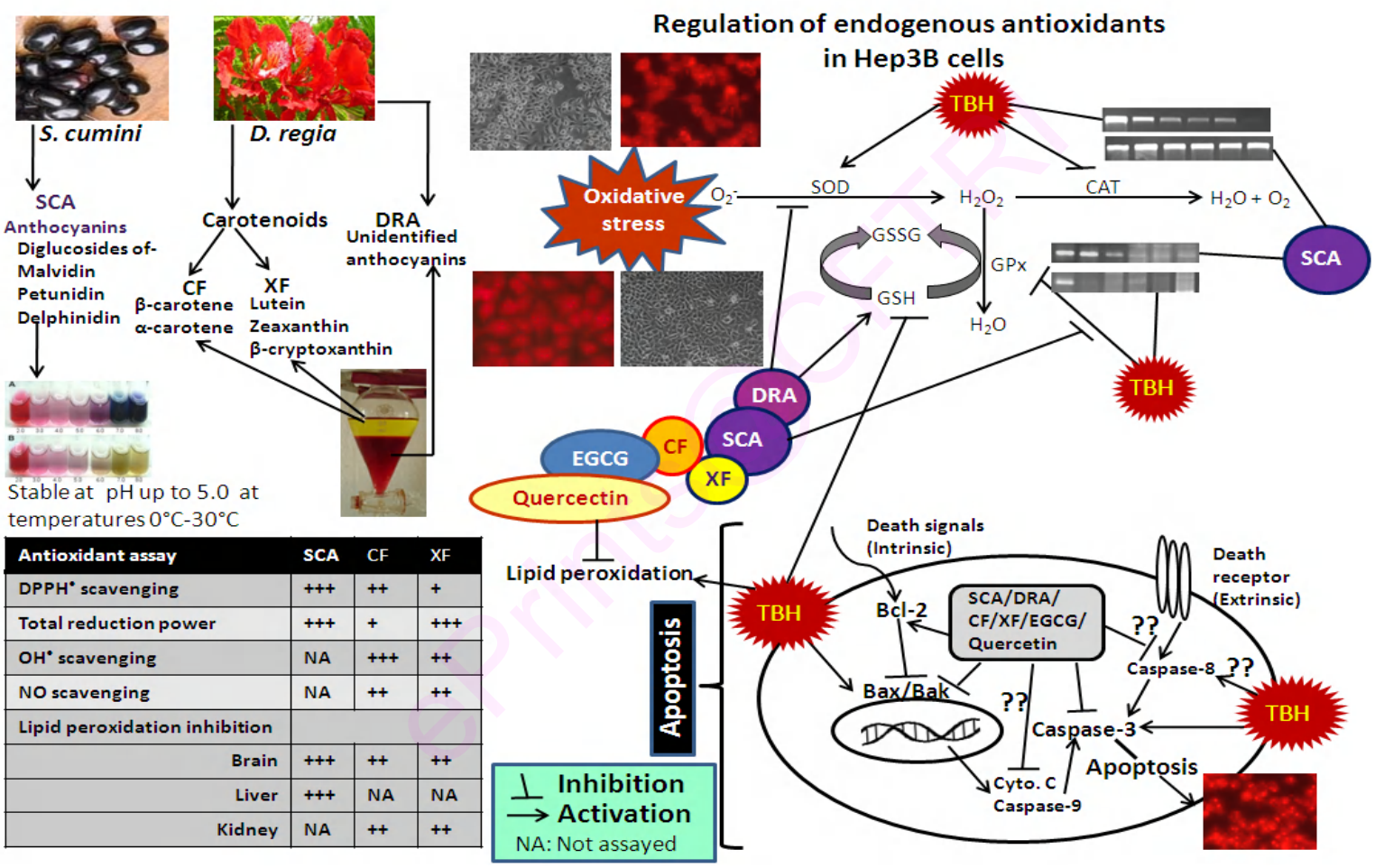
activity both in models. The effects of SCA were comparable with that of EGCG and quercetin in terms cell viability and lipid peroxidation but not with respect to their response to antioxidant enzymes. SCA effectively reversed the TBH-induced alterations in enzyme activities in while its activity was chiefly mediated by the glutathione redox system in $CCl_4$-induced toxicity. The carotenoids of *D. regia* did not alleviate the toxin-induced alterations in enzyme activities but reversed the TBH-induced increase in mRNA expression suggesting a posttranscriptional regulation. Overall results using isolated rat hepatocytes reveal that the extracts and standards offer protection against oxidative stress mechanisms involving or independent of antioxidant enzymes.

Numerous studies in the past have shown that beyond their well established antioxidant activities, flavonoids and carotenoids appear to regulate various signaling pathways involved in cellular survival, growth and differentiation. Therefore, apart from studying the antioxidative effects, the role of anthocyanins and carotenoids in modulating the apoptotic pathways were partly studied. A well established, continuously growing hepatocellular carcinoma cell line of human origin (Hep3B) was chosen for the study where the extracts from *S. cumini* and *D. regia* were evaluated for their protective effects in the presence oxidative stress and also for antiproliferative activity. The anthocyanin extracts of *S. cumini* and *D. regia* offered maximum protection against TBH-induced toxicity by enhancing the cell viability, cellular GSH pool, reducing lipid peroxidation, inhibiting Fenton-mediated oxidative DNA damage and transcriptional regulation of CAT and SOD and increasing the stability of CAT and GPx mRNA making it available for transcription. Among the carotenoids of *D. regia*, xanthophylls fraction was found to be more effective in offering protection against cytotoxicity. All the extracts significantly enhanced cellular GSH content suggesting a possible involvement of the stimulation of its synthesis *in vivo* by these extracts. It was also noted that the extracts prevented TBH-induced apoptotic cell death by inhibiting caspase-3 activation and increasing the Bcl-2/Bax ratio. This finding was supported by morphological analysis of cells pretreated with the extracts. Here again, xanthophylls fraction was more effective compared to the carotenoids fraction. The effect is mainly attributed to the higher bioavailability of xanthophylls such as lutein and zeaxanthin which are known to be pharmacologically active. Further findings revealed that the anthocyanin extracts and xanthophylls fraction had growth inhibitory activity in Hep3B cells comparable to that of EGCG and the inhibition of growth was found to be mediated by apoptosis, which however needs further investigation. A pictorial representation of the mechanism of action of anthocyanins and carotenoids is summarized in Fig. S.1.

## Conclusions

- The fruits of *S. cumini* are a rich source of anthocyanins the chief anthocyanins being diglucosides of malvidin, petunidin and delphinidin having antioxidant properties.
- Flowers of *D. regia* are rich in both carotenoids and anthocyanins and possess excellent antioxidant activities. *D. regia* is a rich source of β-carotene (0.6%) which goes up to 1.3% when the extraction is done from flower petals that fall off naturally from the trees. It is also a rich source of lutein, zeaxanthin and β-cryptoxanthin, the pharmacologically important xanthophylls.
- The anthocyanin pigment of *S. cumini* is a relatively stable pigment particularly when present in solutions of low pH up to 5.0 and stored at low temperatures protected from light. It can be an excellent source of natural color for incorporation in food and pharmaceutical formulations due to its additional antioxidant properties.
- The protective activity of *S. cumini* anthocyanins is mediated by GPx redox regulation in $CCl_4$- and TBH-induced toxicity in rat hepatocytes where as in Hep3B cells it acts by modulating CAT and SOD suggesting different modes of action depending on the tissue of origin. Antioxidant action was attributed to the increased stability of CAT and GPx mRNA by SCA.
- Carotenoids of *D. regia* offer protection against oxidative stress mainly via mechanisms independent of antioxidant enzymes and via pathways involving GSH synthesis.
- Additionally the protection by anthocyanins and carotenoids against TBH-induced toxicity in Hep3B cells was mediated by regulation of antiapoptotic and proapoptotic genes (up regulation of Bcl-2/bax ratio) and inhibition of caspase-3 activation by TBH.
- Anthocyanins of *S. cumini* and *D. regia* and xanthophylls of *D. regia* also possess growth inhibitory properties mediated by apoptosis.

The overall results suggest that the extracts of *S. cumini* and *D. regia* offer protection to cells by improving cellular defense systems such as the antioxidant enzymes either directly or by enhancing their turn over by extending their mRNA half life; and also by directly quenching free radicals and probably reversing the damage by prooxidants and other toxins through regulation of transcription factors and signaling cascades involved in cell survival.




| Antioxidant assay | SCA | CF | XF |
|---|---|---|---|
| DPPH• scavenging | +++ | ++ | + |
| Total reduction power | +++ | + | +++ |
| OH• scavenging | NA | +++ | ++ |
| NO scavenging | NA | ++ | ++ |
| Lipid peroxidation inhibition | | | |
| Brain | +++ | ++ | ++ |
| Liver | +++ | NA | NA |
| Kidney | NA | ++ | ++ |

Fig. S.1. Schematic representation of the work carried out and regulation of endogenous antioxidants and apoptosis is representative of Hep3B cells.

# *References*

**APPENDIX A**

**List of amplicons, accession numbers and sequences of antioxidant enzyme genes from isolated rat hepatocytes**

| Gene | Acc. No. | Sequence (5'-3') |
|---|---|---|
| β-actin | V01217 | CTGACCGAGCGTGGCTACAGCTTCACCACCACAGCTGAGAGGGAAATC<br>GTGCGTGACATTAAAGAGAAGCTGTGCTATGTTGCCCTAGACTTCGAG<br>CAAGAGATGGCCACTGCCGCATCCTCTTCCTCCCTGGAGAAGAGCTAT<br>GAGCTGCCTGACGGTCAGGTCATCACTATCGGCAATGAGCGGTTCCGA<br>TGCCCCGAGGCTCTCTTCCAGCCTTCCTTCCTGGGTATGGAATCCTGT<br>GGCATCCATGAAACTACATTCAATTCCATCATGAAGTGTGACGTTGAC<br>ATCCGTAAAGACCTCTATGCCAACACAGTGCTGTCTGGTGGCACCACC<br>ATGTACCCAGGCATTGCTGACAGGATGCAGAAGGAGATTACTGCCCTG<br>GCTCCTAGCACCATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAG<br>TACTCTGTGTGGATTGGTGGCTCTATCCTGGCCTCACTGTCCACCTTC<br>CAGCAGATGTGGATCAGCAAGCAGGA |
| CAT | M25669 | ACTCAGCGGGCCTGACTGACGCGATTGCCTACCCCGGGTGGAGACCGT<br>GCTCGTCCGGCCCTCTTGCCTCACGTTCTGCAGCTCTGCAGCTCCGCA<br>ATCCTACACCATGGCGGACAGCCGGGACCCAGCCAGCGACCAGATGAA<br>GCAGTGGAAGGAGCAGCGGGCCCCTCAGGTATCCAGTGTCCCTCAGAG<br>CCTAAACTAAATGCATTCGCGAGGTACCGAGCTCT |
| CuZnSOD | X05634 | CACCTGTCTGTAGGTGAGTGACGGACTGACTGTCGGCCAGGGATATGT<br>TCCATGTCCATAAGTGTGCCGTCCCTCTACGACGTGTGTACGTCAGCA<br>GGACCTCATTTTAATCCTCACTCTAAGTAACATGGTCGGTCCAGCGGA<br>TGAAGAGAGGCATGTTGCAGACCTGGGCAATGAGGCTGCTGGAAAGGA<br>CGGTGTGGCCAATGTGTCCATTGAAGATCGTGTGATCTCACTCTCAGG<br>AGAGCATTCCATCATTGGCCGTACTATGGTGGTCCACGAGAAACAAGA<br>TGACTTGGGCAAAGGTGGAAATGAAGAAAGTACAAAGACTGGAAATGC<br>TGGAAGCCGCTGTTTGGTGTGGGGTGA |
| GPx | M21210 | CTCTCCGCGGTGGCACAGTCCACCGTGTATGCCTTCTCCGCGCGCCCG<br>CTGGCGGGCGGGGAGCCCGTGAGCCTGGGCTCCCTGCGGGGCAAGGTG<br>CTGCTCATTGAGAATGTCGCGTCCCTCTGAGGCACCACGACCCGGGAC<br>TACACCGAAATGAATGATCTGCAGAAGCGTCTGGGGCCTCGTGGCCTG<br>GTGGTGCTCGGTTTCCCGTGCAATCAGTTCGGACATCAGGAGAATGGC<br>AAGAATGAAGAGATTCCGAATCCCCTCAAGTATGTCCGACCCGGTGGT<br>GGA |

**APPENDIX B**

**List of amplicons, accession numbers and sequences of genes from Hep3B cell line**

| Gene | Acc. No. | Sequence (5'-3') |
|---|---|---|
| β-actin | NM001101 | GCCGATCCACACGGAGTACTTGCGCTCAGGAGGAGCAATGATCTTG<br>ATCTTCATTGTGCTGGGTGCCAGGGCAGTGATCTCCTTCTGCATCC<br>TGTCGGCAATGCCAGGGTACATGGTGGTGCCGCCAGACAGCACTGT<br>GTTGGCGTACAGGTCTTTGCGGATGTCC |
| CAT | NM001752 | GATACGAGGATTCATCCGACTTGGCTTGGGCTTGGGCGACAAATCT<br>GTGGGCTATCAGCCTTGCAACACAGCTGTTCGTGCGCACCCATCAC<br>CTGGAGCTTCTGTCACGTTGGAAATTTTCTTCTACAGGAAAGTAAT<br>GCGATAGAACTCGATGTGATCTTCAGTTGTGTGCAGATACACGCAC<br>CAGCACCTTTTCCTCTCTAGCCAAGTGCATCCCATATTGTTTCGAT<br>CTTTTATCCACAGCCAAAAGAGAAATCCTCAGACACATCTGAAAGA<br>TCCGGACATGGTCTGGGACTTCTGGAGCCTACGTCCTGAGTCTCTG<br>CATCAGGTTTCTTTCTTGTTCAGTATCGGGGGATTCCAGATGGACA<br>TCGCCACATGAATGGATATGGATCACATACTTTCAAGCTGGTTAAT<br>GCAAATGGGGAGGCAGTTTATTGCAAATTCCATTATAAGACTGACC<br>AGGGCATCAAAAACCTTTCTGTTGAAGATGCGGCGAGACTTTCCCA<br>GGAAGATCCTGACTATGGCATCCGGGATCTTTTTAACGCCGTTGCC<br>ACAGGAAAGTACCCCTCCCGGACTTTTTACATCCCGGTCATGACAT<br>TTAATCAGGCAGAAACTTTTCCATTTAATCCATTCGATCTCACC |
| CuZnSOD | NM000454 | GAAGGTGTGGGGAAGCATTAAAGGACTGACTGAAGGCCTGCATGGA<br>TTCCATGTTCATGAGTTTGGAGATAATACAGCAGGCTGTACCAGTG<br>CAGGTCCTCACTTTAATCCTCTATCCAGAGAACACGGTGGGCCAAA<br>GGATGAAGAGAGGCATATTGGAGACTTGGGCAATGTGACTGCTGAC<br>AAAGATGGTGTGGCCGATGTGTCTATTGAAGATTCTGTGATCTCAC<br>TCTCAGGAGACCATTGCATCATTGGCCGCACACTGGTGGTCCATGA<br>AAAAGCAGATGACTTGGGCAAAGGTGGAAATGAAGAAAGTACAAAG<br>ACAGGAAACGCTGGAAGTCGTTTGGCTTGTGGTGTAATTGGGATCG<br>CCCAATAAACATTCCCTTGGATGTAGTCTGAGGCCC |
| GPx | NM002084 | TTGGAGACACGGATTTCTTCCTGAACAAAGTCCCTCCCCTACATGG<br>TGGACATGGGATGAGACGGCCTTCAGTTACTTCCTCTTGACCCCCA<br>GGGCTGCCTGCCGCCTCATGTAGGACAGGATGTCCATCTTGACGTT<br>GCTGACCGTGGTCCGGTGGTGCCAGCGCATGATGGGTATACCATCT<br>GGCCCCACCAGGAACTTCTCAAAGTTCCAGCGGATGTCGTGAACCT<br>TCATGGGTTCCCAGAAGAGGCGGTCAGATGTACCCAGGAGCTCCGA<br>GGTGGGAGGACAGGAGTTCTTTAGGAAAGTGTAGAATTTCTGCTCT<br>TTCTCTCCATTGACATCCCCTTTCTCAAAGAGCTGGAAATTAGGGA<br>CAAAGCCTCCACCTGGTCGGACATACTTGAGGGTAGGAAGGATCTC<br>TGAGTTCTCTCCTGGTTCCTGTTTTCCAAATTGGTTGCAGGGAAAG<br>CCCAGAATGACCAGACCGAATGGTGCAAGCTCTTCCTGTAGTGCAT<br>TCAGTTCAATGTACTGGCCCGTCAGGCCTCAGTAGCTGGCCACGTT<br>GACAAAGAGGACGTATTTGCCAGCATA |
| Bcl-2 | NM000657 | CTCTCTCGCGTATTACTACGCGACTATCCCCCAGATGGAATGACTG<br>CAGTACCTGTACCGGCACCTGCACACCTGGATCCAGGTATAACGGA<br>GGCTGGGTAGGTGCACTTGGTGATGTGAGGTCTGGGCTGAGAGTAT<br>CTGGCTGAGAA |

| | | |
|---|---|---|
| Bax | NM138765 | TGGGATATGCATGCGAATGGGGGGGGAGGCACCCGAGCTGGCCCTG<br>GACCCGGTGCCTCAGGATGCGTCCACCAAGAAGCTGAGCGAGTGTC<br>TCAAGCGCATCGGGGACGAACTGGACAGTAACATGGAGCTGCAGAG<br>GATGATTGCCGCCGTGGACACAGACTCCCCCCGAGAGGTCTTTTTC<br>CGAGTGGCAGCTGACATGTTTTCTGACGGCAACTTCAACTGGGGCC<br>GGGTTGTCGCCCTTTTCTACTTTGCCAGCAAACTGGTGCTCAAGGC<br>CCTGTGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGG<br>ACATTGGACTTCCTCCGGGAGCGGCTGTTGGGCTGGATCCAAGACC<br>AGGGTGGTTGGGACGGCCTCCTCTCCTACTTTGGGACGCCCACGTG<br>GCAGACCGTGACCATCTTTGTGGCGGGAGTGCTCACCGCCTCACTC<br>ACCATCTGGAA |

## BIBLIOGRAPHY


Abajo C, Boffill MA, Campo JD, Mendez MA, Gonzalez Y, Mitjans M, Vinardell MP. *In vitro* study of the antioxidant and immunomodulatory activity of aqueous infusion of Bidens pilosa. J Ethnopharmacol 93: 319-323, 2004.

Abdel-Aal EM, Young JC, Rabalski I. Anthocyanin composition in black, blue, pink, purple, and red cereal grains. J Agric Food Chem 54: 4696- 4704, 2006.

Abraham S, Soundararajan CC, Vivekanandhan S, Behari M. Erythrocyte antioxidant enzymes in Parkinson's disease. Ind J Med Res 121: 111-115, 2005.

Achrekar S, Kakliji GS, Pote MS, Kelkar SM. Hypoglycemic activity of *Eugenia jambolana* and *Ficus bengalensis*: mechanism of action. In vivo 5: 143-147, 1991.

Adams E, Spencer H, Rowe V, McCollister D, and Irish D. Vapor toxicity of carbon tetrachloride determined by experiments on laboratory animals. Arch Indust Hyg Occup Med 6(1): 50-66, 1952.

Adamson DC, Wildemann B, Sasaki M, Glass JD, McArthur JC, Christov VI, Dawson TM, Dawson VL. Science 274: 1917–1919, 1996.

Aden DP, Vogel A, Plotkin S, Damjanov I, Knowles BB. 1979. Controlled synthesis of HBS Ag in a differentiated human liver carcinoma-derived cell line. Nature 282: 615–616.

Adje F, Lozano YF, Meudec E, Lozano P, Adima A, N'zi GA, Gaydou EM. Anthocyanin characterization of pilot plant water extracts of *Delonix regia* flowers. Molecules 13: 1238-1245, 2008.

Adzet T, Camarasa J, Laguna JC. Hepatoprotective activity of polyphenolic compounds in isolated rat hepatocytes from *Cynara scolymus* against $CCl_4$ toxicity. J Nat Prod 50: 612-617, 1987.

Aebi HE, Catalase *in vitro*. Methods Enzymol 105: 121-126, 1984.

Agarwal R, Katiyar SK, Khan SG, Mukhtar H. Protection against ultraviolet B radiation-induced effects in the skin of SKH-1 hairless mice by a polyphenolic fraction isolated from green tea. Photochem Photobiol 58: 695-700, 1993.

Alamaas R, Rootwelt T, Oyasaeter S, Saugstad OD. Ascorbic acid enhances hydroxyl radical formation in iron-fortified infant cereals and infant formulas. Eur J Paed 156: 488-492, 1997.

Alia M, Mateos R, Ramos S, Lecumberri E, Bravo L, Goya L. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). Eur J Nutr 45: 19-28, 2006a.

Alia M, Ramos S, Mateos R, Bravo L, Goya L. Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). J Biochem Mol Toxicol 19: 119-128, 2005.

Alía M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L, Goya L. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide.Toxicol Appl Pharmacol 212: 110-118, 2006b.

Alija AJ, Bresgen N, Sommerburg O, Laghans C-D, Siems W, Eckl PM. β-carotene breakdown products enhance genotoxic effects of oxidative stress in primary rat hepatocytes. Carcinogenesis 27: 1128-1133, 2006.

Alison MR, Sarraf CE. Apoptosis: Regulation and relevance to toxicology. Hum Exp Toxicol 14: 234–247, 1995.

Amstad P, Moret R, Cerruti P. Glutathione peroxidase compensates for the hypersensitivity of Cu,Zn-superoxide dismutase overproducers to oxidant stress. J Biol Chem 269: 1606–1609, 1994.

Amstad P, Peskin A, Shah G, Mirault M-E, Moret R, Zbinden I, Cerutti P. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. Biochem 30: 9305–9313, 1991.

Andersen OM, Viksund RI, Pedersen AT. Malvidin 3-(6-acetylglucoside)-5-glucoside and other anthocyanins from flowers of *Geranium sylvaticum*. Phytochem 38: 1513-1517, 1995.

Anjum S, Mohy-ud-Din G, Imran-ul-Haq. Application of jambolana (*Eugenia jambolana*) color and aroma in manufacture of value added ice-cream. Pak J Arid Agric 3: 21-25, 2000.

Anonymous. *Wealth of India: Raw materials*. Vol. 3: New Delhi, India: Publication and information directorate: CSIR, 1952.

Anonymous. Medicinal plants of India. Vol. 1: New Delhi, India: Publication and Information Directorate, Indian Council for Medical Research, 1956.

Anonymous. *Syzygium cumini*. In: Chadha YR, Ed. The Wealth of India, Raw materials - A dictionary of Indian raw materials and Industrial products, New Delhi, India: Publication and Information Directorate, Council for Scientific and Industrial Research, Vol. X: p100, 1976.

AOAC International. Methods of analysis of nutrition labeling. In: Sullivan DM and Carpenter DE, Eds. Virginia, USA: AOAC International, p149-150. 1993.

Aqil F, Ahmad I, Mehmood Z. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk J Biol 30: 177-183, 2006.

Arita Y, Harkness SH, Kazzaz JA, Koo HC, Joseph A, Melendez JA, Davis JM, Chander A, Li Y. Mitochondrial localization of catalase provides optimal protection from $H_2O_2$-induced cell death in lung epithelial cells. Am J Physiol 290: L978 –L986, 2005.

Aruoma OI, Halliwell B, Gajewski E, Dizdaroglu M. Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. J Biol Chem 264: 20509-20512, 1989.

Aruoma OI, Halliwell B. DNA and free radicals. Techniques, principles and applications. OICA International, London. 1998.

Aruoma OI. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Res 523-524: 9-20, 2003.

ATBC. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group [no authors listed]. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. New Engl J Med 330: 1029-1035, 1994.

Augustyniak A, Waszkiewicz E, Skrzydlewska E. Preventive action of green tea from changes in the liver antioxidant abilities of different aged rats intoxicated with ethanol. Nutrition 21: 925-932, 2005.

Awe SO, Adeagbo ASO. Analysis of *tert*-butyl hydroperoxide induced constrictions of perfused vascular beds *in vitro*. Life Sci 71: 1255-1266, 2002.

Awe SO, Tsakadze NL, D'Souza SE, Adeagbo ASO. *tert*-Butyl hydroperoxide-mediated vascular responses in DOCA-salt hypertensive rats. Vasc Pharmacol 40: 51-57, 2003.

Azam S, Hadi N, Khan NU, Hadi SM. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicol In Vitro 18: 555-561, 2004.

Azevedo-Meleiro CH, Rodriguez-Amaya DB. Confirmation of the identity of the carotenoids of tropical fruits by HPLC-DAD and HPLC-MS. J Food Comp Anal 17: 385-396, 2004.

Babior BM. Respiratory burst oxidase. Adv. Enzymol. Relat. Areas Mol Biol 65: 49-95, 1992.

Bai J, Cederbaum AI. Mitochondrial catalase and oxidative injury. Biol Signals Recept 10: 189-199, 2001.

Baker M, He S. Elaboration of cellular DNA breaks by hydroperoxides. Free Radic Biol Med 11, 563–572, 1991.

Baker RD, Baker SS, LaRosa K, Whitney C, Newburger PE. Selenium regulation of glutathione peroxidase in human hepatoma cell line Hep3B. Arch Biochem Biophys 304: 53-57, 1993.

Bakowska A, Kucharska AZ, Oszmianski J. The effects of heating, UV irradiation and storage on stability of the anthocyanin–polyphenol copigment complex. Food Chem 81: 349–355, 2003.

Balasubramanian S, Efimova T, Eckert RL. Green tea polyphenol stimulates a Ras, MEKK1, MEK3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. J Biol Chem 277: 1828-1836, 2002.

Balasuramanian B, Pogozelski WK, Tullius TD. DNA strand breaking by the hydroxyl radical is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone. Proc Natl Acad Sci USA 95: 9738-9743, 1998.

Baldwin WS, Barrett JC. Melatonin attenuates hydrogen peroxide toxicity in MCF7 cells only at pharmacological concentrations. Biochem Biophys Res Communic 250: 602-605. 1998.

Banerjee A, Dasgupta N, De B. *In vitro* study of antioxidant activity of Syzygium cumini fruit. Food Chem 90: 727-733, 2005.

Batt AM, Ferrari L, Abid A, Sabolovic N. Human cell lines in pharmcotoxicology. An introduction to a panel discussion. Cell Biol Toxicol 11: 179-185, 1995.

Beaver JP, Waring P. A decrease in intracellular glutathione concentration precedes the oneset of apoptosis in murine thymocytes. Eur J Cell Biol 68: 47-54, 1995.

Beck MA, Esworthy RS, Ho YS, Chu FF. Glutathione peroxidase protects mice from viral-induced myocarditis. FASEB J 12: 1143–1149, 1998.

Bell DR, Gochenaur K. Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. J Appl Physiol 100(4): 1164-1170, 2006.

Bellacosa A and Moss EG. RNA repair: Damage control. Curr Biol 13: R482-R484, 2003.

Bellomo G, Jewell SA, Thor H, Orrenius S. Regulation of intracellular calcium compartmentation: Studies with isolated hepatocytes and *t*-butyl hydroperoxide. Proc Nat Acad Sci USA 79: 6842-6846, 1982.

Bellomo G, Orrenius S. Altered thiol and calcium homeostasis in oxidative hepatocellular injury. Hepatol 5: 876-882, 1985.

Ben-Amotz A, Levy Y. Bioavailabitlity of a natural isomer mixture compared to synthetic all-*trans* ß-carotene in human serum. Am J Clin Nutr 63: 729-734, 1996.

Ben-Dor A, Steiner M, Gheber L, Danilenko M, Dubi N, Linnewiel K, Zick A, Sharoni Y, Levy J. Carotenoids activate the antioxidant response element transcription system. Mol Cancer Therap 4: 177-186, 1995.

Benherlal PS, Arumughan C. Chemical composition and *in vitro* antioxidant studies on *Syzygium cumini* fruits. J Sci Food Agric 87: 2560-2569, 2007.

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239: 70-76, 1996.

Bestwick CS, Milne L. Effects of β-carotene on antioxidant enzyme activity, intracellular reactive oxygen and membrane integrity within post confluent Caco-2 intestinal cells. Biochim Biophys Acta 1474: 47-55, 1999.

Bharti A, Kraeft SK, Gounder M, Pandey P, Jin S, Yuan ZM, LeesMiller SP, Weichselbaum R, Weaver D, Chen LB, Kufe D, Kharbanda S. Inactivation of DNA-dependent protein kinase by protein kinase Cδ: Implications for apoptosis. Mol Cell Biol 18: 6719-6728, 1998.

Bhattacharjee R, Sil PC. Protein isolate from the herb, *Phyllanthus niruri* L. (Euphorbiaceae), plays hepatoprotective role against carbon tetrachloride induced liver damage via its antioxidant properties. Food Chem Toxicol 45: 817-826, 2007.

Bhor VM, Raghuram N, Sivakami S. Oxidative damage and altered antioxidant enzyme activities in the small intestine of streptozotocin-induced diabetic rats. Int J Biochem Cell Biol 36: 89-97, 2004.

Binda D, Lasserre-Bigot D, Bonet A, Thomassin M, Come MP, Guinchard C, Bars R, Jacqueson A, Richert L. Time course of cytochromes P450 decline during rat hepatocyte isolation and culture: Effect of l-NAME. Toxicol In Vitro 17: 59-67, 2003.

Block G, Patterson B, Subar A. Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. Nutr Cancer 18: 1-29, 1992.

Blois MS. Antioxidant determination by the use of a stable free radical. Nature 181: 1199-1200, 1958.

Blum J, Fridovich I. Inactivation of glutathione peroxidase by superoxide radical. Arch Biochem Biophys 240: 500-508, 1985.

Bohm F, Haley J, Truscott TG, Schalch W. Cellular bound β-carotene quenches singlet oxygen in man. J Photochem Photobiol B: Biol 21: 219-221, 1993.

Böhm V, Schlesier K. Methods to evaluate the antioxidant activity. In: Dris R, Jain MS, Eds. Production practices and quality assessment of food crops quality handling and evaluation, Netherlands: Springer, p55-71. 2004.

Bomser J, Madhavi D, Singletary K, Smith MA. *In vitro* anticancer activity of fruit extracts from Vaccinium species. Planta Med 62: 212-216, 1996.

Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free Radic Biol Med 34: 546-552, 2003.

Bose KSC, Agrawal BK. Effect of lycopene from cooked tomatoes on serum antioxidant enzymes, lipid peroxidation rate and lipid profile in coronary heart disease. Singapore Med J 48: 415-420, 2007.

Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, Smulson M. Role of poly (ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase-3 resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274: 22932-22940, 1999.

Brattin WJ, Glende EA, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. J Free Rad Biol Med 1: 27-38, 1985.

Bray RC, Cockle SH, Fielden EM, Roberts PB, Rotilio G, Calabrese L. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. Biochem J 139: 43-48, 1974.

Bridle P, Timberlake CF. Anthocyanins as natural food colors- Selected aspects. Food Chem 58: 103-109, 1997.

Britton G. Structure and properties of carotenoids in relation to function. FASEB J 9: 1551-1558, 1995.

Brouillard R. Flavonoids and flower colour. In: Harborne JB, Ed.The flavonoids: Advances in research since 1980, London: Chapman and Hall, p525. 1988.

Bruchelt G, Schraufstatter IU, Niethammer D, Cochrane CG. Ascorbic acid enhances the effects of 6-hydroxydopamine and $H_2O_2$ on iron-dependent DNA strand breaks and related processes in the neuroblastoma cell line SK-N-SH. Can Res 51: 6066-6072, 1991.

Buc-Calderon P, Latour I, Roberfroid M. Biochemical changes in isolated hepatocytes exposed to *tert*-butyl hydroperoxide. Implication for its cytotoxicity. Cell Biol Toxicol 7: 129-143, 1991.

Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 302-310, 1978.

Bursch W, Oberhammer F, Schulte-Hermann R. Cell death by apoptosis and its protective role against diseases. Trends Pharmacol Sci 13: 245-251, 1992.

Burton GW, Ingold KU. β-Carotene: An unusual type of lipid antioxidant. Science 224: 569-573, 1984.

Burton GW. Antioxidant action of carotenoids. J Nutr 119: 109-111, 1989.

Cabrita L, Fossen T, Andersen OM. Colour and stability of the six common anthocyanidin 3-glucosides in aqueous solutions. Food Chem 68: 101-107, 2000.

Camhi SL, Lee P, Choi AM. The oxidative stress response. New Horiz 3: 170-182, 1995.

Cao C, Leng Y, Huang W, Liu X, Kufe D. Glutathione peroxidase 1 is regulated by the c-Abl and Arg tyrosine kinases. J Biol Chem 278: 39609–39614, 2003.

Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 14: 303-311, 1993.

Cao G, Muccitelli HU, Sanchez-Moreno C, Prior RL. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr 73: 920-926, 2001.

Cao G, Russell RM, Lischner N, Prior RL. Serum antioxidant capacity is increased by consumption of strawberries, spinach, red wine or vitamin C in elderly women. J Nutr 128: 2383 – 2390, 1998.

Cao L, Leers-Sucheta S, Azhar S. Aging alters the functional expression of enzymatic and non-enzymatic antioxidant defense systems in testicular rat Leydig cells. J Steroid Biochem Mol Biol 88: 61-67, 2004.

Casey SA, Brewster D, Viau C, Acosta D. Effect of glutathione depletion and oxidative stress on the in vitro cytotoxicity of velnacrine maleate. Toxicol Lett 76: 257-265, 1995.

Castro JA, De Ferreyra EC, De Castro CR, Fenoes OM, Sasame H, Gillette JR. Prevention of carbon tetrachloride induced necrosis by inhibitors of drug metabolism- further studies on their mechanism of action. Biochem Pharmacol 23: 295-302, 1974.

Ceballos-Picot I, Nicole A, Sinet PM. Cellular clones and transgenic mice overexpressing copper-zinc superoxide dismutase: models for the sutdy of free radical metabolism of

aging. In: Emerit I, Chance B, eds. Free radicals and aging. Basel: Birkhauser Verlag, pp. 89-98, 1992.

Cevallos-Casals BA, Cisneros-Zevallos L. Stability of anthocyanin-based aqueous extracts of Andean purple corn and redfleshed sweet potato compared to synthetic and natural colorants. Food Chem 86: 69–77, 2004.

Chakraborty S, Roy M, Bhattacharya RK. Prevention and repair of DNA damage by selected phytochemicals as measured by single cell gel electrophoresis. J. Environ. Pathol Toxicol Oncol 23: 215-226, 2004.

Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 59: 527-605, 1979.

Chang YC, Huang KX, Huang AC, Ho YC, Wang CJ. Hibiscus anthocyanins-rich extract inhibited LDL oxidation and oxLDL-mediated macrophages apoptosis. Food Chem Toxicol 44: 1015-23, 2006.

Cheeseman KH. Free radicals and liver injury. In: Blake D, Winyard PG, Eds. Immunopharmacology of Free Radical Species, Elsevier, p233-245. 1995.

Chen C, Shen G, Hebbar V, Hu R, Owuor ED, Kong AN. Epigallocatechin3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. Carcinogenesis 8: 1369-1378, 2003.

Chen J, Fannery JG, La Vail MM, Steinberg RH, Xu J, Simon MI. Bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. Proc Nat Acad Sci USA 93: 7042-7047, 1996.

Chen L, Yang X, Jiao H, Zhao B. Effect of tea catechins on the change of glutathione levels caused by $Pb^{++}$ in PC12 Cells. Chem Res Toxicol 17: 922-928, 2004.

Chen X, Ahn DU. Antioxidant activities of six natural phenolics against lipid oxidation induced by $Fe^{2+}$ or ultraviolet light. J Am Oil Chem Soc 75: 1717-1721, 1998.

Chen Y, Wang M, Rosen RT, Ho CT. DPPH radical scavenging active components from *Polygonum multiflorum* Thunb.J Agri. Food Chem 47: 2226–2228, 1999.

Cheng W-H, Ho YS, Valentine BA, Ross DA, Combs GF Jr., Lei XG. Glutathione peroxidase is the mediator of the body selenium to protect against paraquat lethality in transgenic mice. J Nutr 128: 1070-1076, 1998.

Cheng Z, Li Y, Chang W. Kinetic deoxyribose degradation assay and its application in assessing the antioxidant activities of phenolic compounds in a Fenton-type reaction system. Anal. Chem. Acta 478(1): 129-137, 2003.

Cheng Z, Moore J, Yu L. High-throughput relative DPPH radical scavenging capacity assay. J Agric Food Chem 54(20): 7429–7436, 2006.

Chern C-L. Role of differentiation status and total intracellular redox reserves in the modulation of metastatic propensity of human hepatocellular carcinoma cells. Doctoral dissertation. http://etd.lib.nsysu.edu.tw/ETD-db/ETD-search/view_etd?URN=etd-0205102-145228, 2002.

Chew BP, Brown CM, Park JS, Mixter PF. Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis. Anticancer Res 2394: 3333-3339, 2003.

Chiang A-N, Wu H-L, Yeh H-I, Chu C-S, Lin H-C, Lee W-C. Antioxidant effects of black rice extract through the induction of superoxide dismutase and catalase activities. Lipids 41(8): 797-803, 2006.

Chigurupati N, Saiki L, Gayser Jr C, Dash AK. Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. Int J Pharm 241: 293–299, 2002.

Chitchumroonchokchai C, Bomser JA, Glamm JE, Failla ML. Xanthophylls and alpha tocopherol decrease UVB-induced lipid peroxidation and stress signaling in human lens epithelial cells. J Nutr 134: 3225-3232, 2004.

Chodon D, Ramamurty N, Sakthisekaran D. Preliminary studies on induction of apoptosis by genistein on HepG2 cell line. Toxicol In Vitro 21: 887–891, 2007.

Choi EH, Chang H-J, ChoJY, Chun HS. Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. Food Chem Toxicol 45: 1873-1881, 2007.

Chung SK, Osawa T, Kawakishi S. Hydroxyl radical scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). Biosci Biotech Biochem 61: 118-123, 1997.

Chung W, Miranda CL, Maier CS. Epigallocatechin gallate (EGCG) potentiates the cytotoxicity of rotenone in neuroblastoma SH-SY5Y cells. Brain Res 1176: 133-142, 2007.

Clerch LB, Iqbal J, Massaro D. Perinatal rat lung catalase gene expression: influence of corticosteroid and hyperoxia. Am J Physiol Lung Cell Mol Physiol 260: L428-L433, 1991.

Clerch LB, Massaro D, Berkovich A. Molecular mechanisms of antioxidant enzyme expression in lung during exposure to and recovery from hyperoxia. Am J Physiol 274: L313–L319, 1998.

Cleveland DW, Rothstein JD. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci 2: 806-819, 2001.

Codoñer-Franch P, Muñiz P, Gasco E, Domingo JV, Valls-Belles V. Effect of a diet supplemented with α-Tocopherol and β-Carotene on ATP and antioxidant levels after hepatic ischemia-reperfusion. J Clin Biochem Nutr 43: 13–18, 2008.

Collins AR, Olmedilla B, Southon S, Granado F, Duthie SJ. Serum carotenoids and oxidative DNA damage in human lymphocytes. Carcinogenesis 19: 2159–2162, 1998.

Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J. Histochem. Cytochem 45: 923-934, 1997.

Collins QF, Liu H-Y, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. J Biol Chem 282(41): 30143–30149, 2007.

Cooke D, Steward WP, Gescher AJ, Marczylo T. Anthocyans from fruits and vegetables – Does bright colour signal cancer chemo preventive activity? Eur J Cancer 41: 1931-1940, 2005.

Cooke MS, Olinski R, Evans MD. Does measurement of oxidative damage to DNA have clinical significance? Clin Chim Acta 365: 30-49, 2006.

Cooper DA, Eldridge AL, Peters JC. Dietary carotenoids and lung cancer: A review of recent research. Nutr Rev 57: 133–145, 1999.

Coustan-Smith E, Kitanaka A, Pui CH, McNinch L, Evans WE, Raimondi SC, Behm FG, Arico M, Campana D. Clinical relevance of Bcl-2 over expression in childhood acute lymphoblastic leukemia. Blood 87: 1140-1146, 1996.

Crack PJ, Taylor JM, Flentjar NJ, de HJ, Hertzog P, Iannello RC, Kola I. Increased infarct size and exacerbated apoptosis in the glutathione peroxidase-1 (Gpx-1) knockout mouse brain in response to ischemia/reperfusion injury. J Neurochem 78: 1389-1399, 2001.

Craig W, Beck L. Phytochemicals: health protective effects. Can J Diet Pract Res 60: 78-84, 1999.

Crapo JD, Oury T, Rabouille C, Slot JW, Chang LY. Copper, zinc superoxide dismutase is primarily a cytosolic protein in human cells. Proc Nat Acad Sci USA 89: 10405–10409, 1992.

Cregan SP, Smith BP, Brown DL, Mitchel REJ. Two pathways for the induction of apoptosis in human lymphocytes. Int J Rad Biol 75: 1069-1086, 1999.

Cryns V, Yuan J. Proteases to die for. Genes Develop 12: 1551-1570, 1998.

Cuello S, Ramos S, Mateos R, Martín MA, Madrid Y, Cámara C, Bravo L, Goya L. Selenium methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide. Anal Bioanal Chem 389: 2167-2178, 2007.

Cutler RG. Oxidative stress and aging: Catalase is a longevity determinant enzyme. Rejuv Res 8: 138-140, 2005.

Cyrne L, us Martins L, Fernandes L, Marinho HS. Regulation of antioxidant enzymes gene expression in the yeast *Saccharomyces cerevisiae* during stationary phase. Free Radic Biol Med 34: 385-393, 2003.

Dai J, Patel JD, Mumper RJ. Characterisation of blackberry extract and its antiproliferative and anti-inflammatory properties. J Med Food 10: 258-265, 2007.

Dai Y, Cederbaum AI. Cytotoxicity of acetaminophen in human cytochrome P4502E1-transfected HepG2 cells. J Pharmacol Exp Ther 273: 1497–1505, 1995.

Das M, Sur P, Gomes A, Vedasiromoni JR, Ganguly DK. Inhibition of tumour growth and inflammation by consumption of tea. Phytother Res 16: S40-S44, 2002.

Davies KJ. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp 61: 1-31, 1995.

de Brito ES, de Araújo MCD, Alves RE, Carkeet C, Clevidence BA, Novotny JA. Anthocyanins present in selected tropical fruits: Acerola, Jambolão, Jussara, and Guajiru. J Agric Food Chem 55: 9389-9394, 2007.

de Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, GPX1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem 273: 22528–22536, 1998.

de Haan JB, Crack PJ, Flentjar N, Iannello RC, Hertzog PJ, Kola I. An imbalance in antioxidant defense affects cellular function: the pathophysiological consequences of a reduction in antioxidant defense in the glutathione peroxidase-1 (Gpx1) knockout mouse. Redox Rep 8: 69-79, 2003.

de Haan JB, Cristiano F, Iannello R, Bladier C, Kelner MJ, Kola MJ. Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide. Hum Mol Genet 5: 283–292, 1996.

de Lorgeril M, Salen P, Stone, WL, Krishnaswamy G, Yang H, Rasmussen SE. Diet and heart disease prevention. Fun Foods Nutra 32-38, 2006.

de Rosso VV, Mercadante AZ. The high ascorbic acid content is the main cause of the low stability of anthocyanin extracts from acerola. Food Chem 103: 935–943, 2007.

Decker T, LohmannMatthes ML. quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 15: 61-69, 1988.

Del Pozo-Insfran D, Del Follo-Martinez A, Talcott ST, Brenes CH. Stability of copigmented anthocyanins and ascorbic acid in muscadine grape juice processed by high hydrostatic pressure. J Food Sci 72: S247-S253, 2007.

Di Mascio P, Kaiser S. and Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Archs Biochem Biophys 274: 532-538, 1989.

Dieterich S, Bieligk U, Beulich K, Hasenfuss G, Prestle J. Gene expression of antioxidative enzymes in the human heart: Increased expression of catalase in the end-stage failing heart. Circulation 101: 33–39, 2000.

Dihal AA, Woutersen RA, van Ommen B, Rietjens IMCM, Stierum RH. Modulatory effects of quercetin on proliferation and differentiation of the human colorectal cell line Caco-2. Cancer Lett 238: 248–259, 2006.

Dilsiz N, Sahaboglu A, Yıldız MZ, Reichenbach A. Protective effects of various antioxidants during ischemia-reperfusion in the rat retina. Graefe's Arch Clin Exp Ophthalmol 244: 627-633, 2006.

Diplock A. The safety of β-carotene and the antioxidant vitamins C and E. In: Garewal HS, Ed. Antioxidants and Disease Prevention, USA: CRC Press, p3-17. 1997.

Dixon ZR, Burri BJ, Clifford A, Frankel EN, Schneeman BO, Parks E, Keim NL, Barbieri T, Wu MM, Fong AK. Effects of a carotene-deficient iet on measures of oxidative susceptibility and superoxide dismutase activity in adult women. Free Radic Biol Med 17: 537-544, 1994.

Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 32: 1102-1115, 2002.

Dominguez M, Nieto A, Marin JC, Keck AS, Jeffery E, Cespedes C. Antioxidant activities of extracts from *Barkleyanthus salicifolius* (Asteraceae) and *Penstemon gentianoides* (Scrophulariaceae). J. Agric. Food Chem 53: 5889-5895, 2005.

Dorgan JF, Sowell A, Swanson CA, Potischman N, Miller R, Schussler N., and Stephenson, H. E., Jr. Relationships of serum carotenoids, retinol, alpha-tocopherol, and selenium with breast cancer risk: results from a prospective study in Columbia, Missouri (United States). Cancer Causes Control 9: 89-97, 1998.

Dragsted LO, Pedersen A, HermetterA, Basu S, Hansen M, Haren GR, Kall M, Breinholt V, Castenmiller JJM, Stagsted J, Jakobsen J, Skibsted L, Rasmussen SE, Loft S, Sandström B. The 6-a-day study: effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. Am J Clin Nutr 79: 1060-1072, 2004.

Driscoll DM, Copeland PR. Mechanism and regulation of selenoprotein synthesis. Annu Rev Nutr 23: 17-40, 2003.

Eaton JW. Actalasemia. The Metabolic Basis of Inherited Diseases. In: Scriver CR, Beaudet AL, and Sly WS, Eds. New York: McGraw-Hill, p1551-1561. 1989.

Edmondson JM, Armstrong LS, Martinez AO. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. Methods in Cell Sci 11: 15-17, 1988.

Elbling L, Weiss R-M, Teufelhofer O, Uhl M, Knasmueller S, Schulte-Hermann R, Berger W, Micksche M. Green tea extract and (–)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. FASEB J 19: 807-809, 2005.

Elchuri S, Oberley TD, Qi W, Eisenstein RS, Roberts LJ, Van Remmen H, Epstein CJ, Huang T-T. CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepato-carcinogenesis later in life. Oncogene 24:367-380, 2005.

Elisia I, Kitts DD. Anthocyanins inhibit peroxyl radical-induced apoptosis in Caco-2 cells. Mol Cell Biochem 312: 139-145, 2008.

Elroy-Stein O, Bernstein Y, Groner Y. Overproduction of human Cu/Zn-superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation. EMBO J 5: 615– 622, 1986.

Erdman JW, Thatcher AJ, Hofmann NE, Lederman JD, Block SS, Lee CM, Mokady S. All-*trans* ß-carotene is absorbed preferentially to 9-*cis*-ß-carotene, but the latter accumulates in the tissues of domestic ferrets (*Mustela putorius puro*). J Nutr 128: 2009-2013, 1998.

Erzurum SC, Lemarchand P, Rosenfeld MA, Yoo J-H, Crystal RG. Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA. Nucleic Acids Res 21: 1607-1612, 1993.

Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11:** 81-128, 1991.

Evans RW, Shaten BJ, Day BW, Kuller LH. Prospective association between lipid soluble antioxidants and coronary heart disease in men: the Multiple Risk Factor Intervention Trial. *Am J Epidemiol* 147: 180-186, 1998.

Fariss MW, Bryson KF, Hylton EE, Lippman HR, Stubin CH, Zhao XG. Protection against carbon tetrachloride-induced hepatotoxicity by pretreating rats with the hemisuccinate esters of tocopherol and cholesterol. Environ Health Perspect 101: 528-536, 1993.

Farombi EO, Hansen M, Ravn-Haren G, Møller P, Dragsted LO. Commonly consumed and naturally occurring dietary substances affect biomarkers of oxidative stress and DNA damage in healthy rats. Food Chem. Toxicol. 42: 1315-1322, 2004.

Favreau LV, Pickett CB. Transcriptional regulation of the rat NAD(P)H:quinone reductase gene. Characterization of a DNA-protein interaction at the antioxidant responsive element and induction by 12-O-tetradecanoylphorbol 13-acetate. J Biol Chem 268: 19875–19881, 1993.

Fee J, Bergamini R, Brigg R. Observations on the mechanism of the oxygen dialuric acid induced hemolysis of vitamin-E- deficient rat blood cells and the protective roles of catalase and superoxide dismutase. Arch Biochem Biophys 169: 160-167, 1975.

Feng R, Ni H, Wang SY, Tourkova IL, Shurin MR, Harada H, Yin X. Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. J Biol Chem 282: 13468-13476, 2007.

Flohe A, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 105: 114-120, 1984.

Floris G, Medda R, Padigalia A, Musci G. The physiopathological significance of ceruloplasmin: a possible therapeutic approach. Biochem Pharmacol 60: 1735-1741, 2000.

Foote CS, Chang YC, Denny RW. Chemistry of singlet oxygen X. Carotenoid quenching parallels biological protection. J Am Oil Chem Soc 92: 5216-5218, 1970.

Forstrom JW, Zakowski JJ, Tappel AL. Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochem 17: 2639-2644, 1978.

Fossen T, Cabrita L, Andersen OM. Colour and stability of pure anthocyanins influenced by pH including the alkaline region. Food Chem 63: 435-440, 1998.

Foy CJ, Passmore AP, Vahidassr MD, Young IS, Lawson JT. Plasma chain-breaking antioxidants in Alzheimer's disease, vascular dementia and Parkinson's disease. Q J Med 92: 39-45, 1999.

Francis FJ. Analysis of anthocyanins. In: Markakis P, Ed. Anthocyanins as food colors. New York: Academic press, p181-207. 1986.

Franco AA, Odom RS, Rando TA. Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. Free Radic Biol Med 27: 1122–1132, 1999.

Fridovich I. Fundamental aspects of reactive oxygen species (ROS) or What's the matter with oxygen. Ann N Y Acad Sci 893: 13-18, 1999.

Fry JR, Hammond AH. Assessment of functional integrity of hepatocytes: a brief review. Alternatives to live animals 21: 324-329, 1993.

Fu Y, Cheng W-H, Ross DA, Lei XG. Cellular glutathione peroxidase protects mice against lethal oxidative stress induced by various doses of Diquat. Proc Soc Exp Biol Med 222: 164-169, 1999.

Fu Y, Sies H, Lei XG. Opposite roles of selenium-dependent glutathione peroxidase-1 in superoxide generator diquat- and peroxynitrite-induced apoptosis and signaling. J Biol Chem 276: 43004-43009, 2001.

Galati G, Lin A, Sultan A, O'Brien PJ. Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. Free Radic Biol Med 40: 570-580, 2006.

Garcia-Alonso M, Minihane A-M, Rimbach G, Rivas-Gonzalo JC, de Pascual S. Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. J Nutr Biochem (In Press: 2009)

Garcia-Cohen E, Marin J, Diez-Picazo LD, Baena AB, Salaices M, Rodriguez-Martinez MA. Oxidative stress induced by tert-butyl hydroperoxide causes vasoconstriction in the aorta from hypertensive and aged rats: role of cyclooxygenase-2 isoform. J Toxicol Exp Therap 293: 75-81, 2000.

Garcia-Vigeura C, Bridle P. Influence of structure on color stability of anthocyanins and flavylium salts with ascorbic acid. Food Chem 64: 21-26, 1999.

Gardner R, Salvador A, Moradas-Ferreira P. Why does SOD overexpression sometimes enhance, sometimes decrease, hydrogen peroxide production? A minimalist explanation. Free Radic Biol Med 32(12) 1351-1357, 2002.

Gartner C, Stahl W, Sies H. Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to ß-carotene in the human. Int J Vitam Nutr Res 66: 119-125, 1996.

Gelderman KA, Hultqvist M, Olsson LM, Bauer K, Pizzolla A, Olofsson P, Holmdahl R. Rheumatoid arthritis: the role of reactive oxygen species in disease development and therapeutic strategies. Antiox Redox Signaling 9: 1541-1567, 2007.

Gergel D, Misik V, Ondrias K, Cederbaum AI. Increased Cytotoxicity of 3-Morpholino sydnonimine to HepG2 Cells in the Presence of Superoxide Dismutase. Role of hydrogen peroxide and iron. J Biol Chem 270: 20922-20929, 1995.

Ghiselli A, Nardini M, Baldi A, Scaccini C. Antioxidant activity of different phenolics fractions separated from Italian red wine. J Agric Food Chem 46: 361-367, 1998.

Gill JH, Dive C. Apoptosis: Basic mechanisms and relevance to toxicology. In: Roberts R, Ed. Apoptosis in toxicology. New York: Taylor & Francis, p1-20. 2000.

Giovannocci E, Ascherio A, Rimm EB, Stampfer MJ, Colditz GA, Willet WC. Intake of carotenoids and retinol in relation to risk of prostate cancer. J Natl Cancer Inst 87: 1767-1776, 1995.

Giovannucci E. Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J Natl Cancer Inst 91: 317-331, 1999.

Giudice A, Montella M. Activation of the Nrf2-ARE signaling pathway: A promising strategy in cancer prevention. Bioessays 28: 169-181, 2006.

Giusti MM, Wrolstad RE. Characterization of red radish anthocyanins. J Agric Food Chem 61: 322-326, 1996.

Gomez-Lechon MJ, O'Connor E, Castell JV, Jover R. Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. Toxicol Sci 65: 299-308, 2002.

Gopalakrishnan A, Kong AT. Anticarcinogenesis by dietary phytochemicals: Cytoprotection by Nrf2 in normal cells and cytotoxicity by modulation of transcription factors NF-κB and AP-1 in abnormal cancer cells. Food Chem Toxicol 46: 1257-1270, 2008.

Góth L, Tóth Z, Tarnai I, Bérces M, Török P, Bigler WN. Blood catalase activity in gestational diabetes is decreased but not associated with pregnancy complications. Clin Chem 51: 2401-2404, 2005.

Goya L, Mateos R, Bravo L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by *tert*-butylhydroperoxide. Eur J Nutr 46: 70-78, 2007.

Grant MH, Melvin MAL, Shaw P, Melvin WT, Burke MD. Studies on the maintenance of cytochromes P-450 and $b_5$ monooxygenases and cytochrome reductases in primary cultures of rat hepatocytes. FEBS 190: 99-103, 1985.

Greenberg ER, Baron JA, Tosteson TD, Freeman DH Jr., Beck GJ, Bond JH, Colacchio TA, Coller JA, Frankl HD, Haile RW. A clinical trial of antioxidant vitamins to prevent colorectal adenoma. Polyp Prevention Study Group. N Engl J Med 331: 141-147, 1994.

Grivell AR, Berry MN. The effects of phosphate and substrate-free incubation conditions on glycolysis in Ehrlich ascites tumour cells. Biochim Biophys Acta 1291: 83-88, 1996.

Grommeck R and Markakis P. The effect of peroxidase on anthocyanin pigments. J Food Sci 29: 53-57, 1964.

Guerra MC, Galvano F, Bonsi L, Speroni E, Costa S, Renzulli C, Cervellati R. Cyanidin-3-O-β-glucopyranoside, a natural free radical scavenger against aflatoxin B1- and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). Brit J Nutr 94: 211-220, 2005.

Gulcin İ, Berashvili D, Gepdiremen A. Antiradical and antioxidant activity of total anthocyanins from Perilla pankinensis decne. J Ethnopharmacol 101: 287-293, 2005.

Gulcin İ. Antioxidant activity of caffeic acid (3, 4-dihydroxycinnamic acid). Toxicol 217: 213, 2006.

Guleria RS, Jain A, Tiwari V, Misra MK. Protective effect of green tea extract against the erythrocytic oxidative stress injury during mycobacterium tuberculosis infection in mice. Mol Cell Biochem 236: 173-181, 2002.

Gumpricht E, Dahl R, Devereaux MW, Sokol RJ. Beta-carotene prevents bile acid-induced cytotoxicity in the rat hepatocyte: Evidence for an antioxidant and anti-apoptotic role of beta-carotene *in vitro*. Pediatr Res 55: 814-821, 2004.

Gupta S, Jyothilakshmi A, Manjunath MN, Prakash J. Analysis of nutrient and antinutrient content of underutilized green leafy vegetables. LWT 38: 339-345, 2005.

Gurgul E, Lortz S, Tiedge M, Jorns A, Lenzen S. Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. Diabetes 53: 2271-2280, 2004.

Haegele AD, Gillette C, O'Neill C, Wolfe P, Heimendinger J, Sedlacek S, Thompson HJ. Plasma xanthophyll carotenoids correlate inversely with indices of oxidative DNA damage and lipid peroxidation. Cancer Epidemiol Biomark Preven 9: 421-425, 2000.

Haidara K, Moffat P, Denizeau F. Metallothionein induction attenuates the effects of glutathione depletors in rat hepatocytes. Toxicol Sci 49(2): 297-305, 1999.

Halliwell B, Aruoma OI, Wasil M, Gutteridge JMC. The resistance of transferrin, lactoferrin and caeruloplasmin to oxidative damage. Biochem J 250(2): 613-616, 1988.

Halliwell B, Gutteridge J. Free radicals in biology and medicine. 2nd Ed, Oxford, UK: Clarendon Press, p543. 1995.

Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine, 2nd Ed, Oxford: Oxford University Press, 1989.

Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple ''test-tube'' assay for determination of rate constants for reaction of hydroxyl groups. Anal Biochem 165: 215-219, 1987.

Halliwell B, Gutteridge JMC. Formation of thiobarbituric acid reactive substance from deoxyribose in the presence of iron salts. FEBS Lett 128: 347-352, 1981.

Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd Ed,Oxford: Oxford University Press. 1999.

Halliwell B. How to characterize a biological antioxidant. Free Rad Res Commun 9: 1-32, 1990.

Halliwell B. Antioxidant defense mechanisms: From the beginning to the end. Free Radic Res, 31: 261-272, 1999.

Halliwell B. Oxidative stress in cell culture: an under-appreciated problem? FEBS Lett 540: 3-6, 2003.

Halvorsen BL, Holte K, Myhrstad MCW, Bariko I, Hvattum E, Remberg SF, Wold A-B, Haffner K, Baugerod H, Andersen LF, Moskaug JO, Jacobs Jr. DR, Blomhoff R. A systematic screening of total antioxidants in dietary plants. J Nutr 132: 461-471, 2002.

Han K-H, Sekikawa M, Shimada K-I, Hashimoto M, Hashimoto N, Noda T, Tanaka H, Fukushima M. Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats. Br J Nutr 96: 1125-1133, 2006.

Handelman GJ. Carotenoids as scavengers of active oxygen species, In: Cadenas E, Packer L, Eds. Handbook of Antioxidants, New York: Marcel Dekker, p259-313. 1996.

Hara Y. Prophylacti functions of tea polyphenols: In Food phytochemicals for cancer prevention II. In: Ho CT, Osawa T, Huang MT, Rosen RT, Eds. Washington DC: American Chemical Society, 547: p34-50. 1994.

Harman D. Free radical theory of aging: an update: increasing the functional life span. Ann N Y Acad Sci 1067: 10-21, 2006.

Hartmann A, Michel PP, Troadec JD, Mouatt-Prigent A, Faucheux BA, Ruberg M, Agid Y, Hirsch EC. Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? J Neurochem 76: 1785-1793, 2001.

Heber D. Phytochemicals beyond antioxidation. J Nutr 134: 3175S-3176S, 2004.

Heller B, Wang ZQ, Wagner EF, Randons J, Burkle A, Fehsel K, Burkart V, Kolb H. Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. J Biol Chem 270: 11176-11180, 1995.

Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, Belanger C, LaMotte F, Gaziano JM, Ridker PM, Willett W, Peto R. Lack of effect of long-term supplementation with β-carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 334: 1145-1149, 1996.

Hercberg S, Galan P, Preziosi P, Alfarez M- J, Vazquez C. The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers. Nutr 14: 513-520, 1998.

Hertog MGL, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Glampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkainen M, Simic BS, Toshima H, Fesens EJM, Hollman PCH, Katan MB. Flavonoid intake and longterm risk of coronary heart disease and cancer in the seven countries study. Arch Int Med 55: 381-386, 1995.

Herzenberg LA, de Rosa SC, Dubs JG, Roederer M, Anderson MT, Ela SW, Seresinski SC, Herzenberg LA. Glutathione deficiency is associated with impaired survival in HIV disease. Proc Natl Acad Sci USA 94: 1967-1972, 1997.

Higuchi A, Yonemitsu K, Koreeda A, Tsunenari S. Inhibitory activity of epigallocatechin gallate (EGCg) in paraquat-induced microsomal lipid peroxidation-/a mechanism of protective effects of EGCg against paraquat toxicity. Toxicol 183: 143-149, 2003.

Hill KE, Burk RF. Toxicity studies in isolated hepatocytes from selenium-deficient and vitamin E-deficient rats. Toxicol Appl Pharmacol 72: 32-39, 1984.

Ho Y-S, Xiong Y, Ma W, Spector A, Ho DS. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. J Biol Chem 279: 32804-32812, 2004.

Ho YS. Transgenic and knockout models for studying the role of lung antioxidant enzymes in defense against hyperoxia. *Am J Resp Crit Care Med* 166: S51-S56, 2002.

Hofer T, Seo AY, Prudencio M, Leeuwenburgh C. A method to determine RNA and DNA oxidation simultaneously by HPLC-ECD: greater RNA than DNA oxidation in rat liver after doxorubicin administration. Biol Chem 387: 103-111, 2006.

Hogeboom GH. In: Colowick SP, Kaplan NO, Eds. Methods Enzymology, New York: Academic Press, Vol. I: p16-19, 1955.

Holownia A, Mapoles J, Menez JF, Braszko JJ. Acetaminophen metabolism and cytotoxicity in PC12 cells transfected with cytochrome P4502E1. J Mol Med 75: 522-527, 1997.

Hosokawa N, Hirayoshi K, Nakai A, Hosokawa Y, Marui N, Yoshida M, Sakai T, Nishino H, Aoike A, Kawai K, Nagata K. Flavonoids inhibit the expression of heat shock proteins. Cell Struct Funct 15: 393-401, 1990.

Hou D-X, Kai K, Li J-J, Lin S, Terahara N, Wakamatsu M, Fujii M, Young MR, Colburn N. Anthocyanidins inhibit protein-1 activity and cell transformation: structure-activity relationship and molecular mechanisms. Carcinogenesis 25: 29-36, 2004.

Hou D-X. Potential mechanisms of cancer chemoprevention by anthocyanins. Curr Mol Med 3: 149-159, 2003.

Hou R, Chen J, Chen , Kang X, Li M, Wang B. Neuroprotective effects of (−)-epigallocatechin-3-gallate (EGCG) on paraquat-induced apoptosis in PC12 cells. Cell Biol Int 32: 22-30, 2007.

Howell BF, McCune B, Schaffer R. Lactate-to-pyruvate or pyruvate-to-lactate assay for lactate dehydrogenase: a re-examination. Clin Chem 25: 269-272, 1979.

Hozawa A, Jacobs Jr. DR, Steffes MW, Gross MD, Steffen LM, Lee D-H. Relationships of circulating carotenoid concentrations with several markers of inflammation, oxidative stress, and endothelial dysfunction: The Coronary Artery Risk Development in Young Adults (CARDIA)/Young Adult Longitudinal Trends in Antioxidants (YALTA) Study. Clin Chem 53: 447-455, 2007.

Hsu YW. Tsai CF, Chang WH, Ho YC, Chen WK, Lu FJ. Protective effects of *Dunaliella salina*-a carotenoids-rich alga, against carbon tetrachloride-induced hepatotoxicity in mice. Food Chem Toxicol 46: 3311-3317, 2008.

Hu ML, Shih MK. Ascorbic acid inhibits lipid peroxidation but enhances DNA damage in rat liver nuclei incubated with iron ions. Free Radic Res 26: 585-592, 1997.

Huang Y-L, Shen CKF, Luh T-Y, Yang HC, Hwang KC, Chou C-K. Blockage of apoptotic signaling of transforming growth factor-β in human hepatoma cells by carboxyfullerene. Eur J Biochem 254: 38-43, 1998.

Huang ZZ, Chen C, Zeng Z, Yang H, Oh J, Chen L, Lu SC. Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. FASEB J 15: 19-21, 2001.

Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Hsu JD, Chu CY. Protective effect of baicalin on tert-butyl hydroperoxide-induced rat hepatotoxicity. Arch Toxicol 79: 102-109, 2005.

Igarashi K, Kimura Y, Takenaka A. Preventive effects of dietary cabbage acylated anthocyanins on paraquat-induced oxidative stress in rats. Biosci Biotechnol Biochem 64: 1600-1607, 2000.

Igarashi K, Suzuki O, Hara Y. Comparison of the preventive effects of epigallocatechin gallate, epicatechin gallate and epicatechin on paraquat-induced oxidative stress in rats. Food Sci Technol Res 5: 69-73, 1999.

Ilavarasan R, Mallika M, Venkataraman S. Anti-inflammatory and antioxidant activities of *Cassia fistula* bark extracts. Afr J Trad CAM. 2: 70-85, 2005.

Ip C, Hayes C, Budnick RM, Ganther HE. Chemical form of selenium, critical metabolites, and cancer prevention. Cancer Res 51: 595-600, 1991.

Ishige K, Schubert D, Sagara Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. Free Rad Biol Med 30: 433-446, 2001.

Jackson JH, Schraufstätter IU, Hyslop PA Vosbeck K, Sauerheber R, Weitzman SA, Cochrane CG. Role of oxidants in DNA damage. Hydroxyl radical mediates the synergistic DNA damaging effects of asbestos and cigarette smoke. J Clin Invest 80: 1090-1095, 1987.

Jaeschke H, Ho YS, Fisher MA, Lawson JA, Farhood A. Glutathione peroxidase-deficient mice are more susceptible to neutrophil-mediated hepatic parenchymal cell injury during endotoxemia: importance of an intracellular oxidant stress. Hepatol 29: 443-450, 1999.

Jagetia GC, Baliga MS. *Syzygium cumini* (Jamun) reduces the radiation-induced DNA damage in the cultured human peripheral blood lymphocytes: a preliminary study. Toxicol Lett 132: 19-25, 2002.

Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9: 515-540, 1990.

Ji L, Liu T, Liu J, Chen Y, Wang Z. Andrographolide Inhibits Human Hepatoma-Derived Hep3B Cell Growth through the Activation of c-Jun N-Terminal Kinase. Planta Med 73: 1397-1401, 2007.

Johansson I, Ingelman-Sundberg M. Carbon tetrachloride induced lipid peroxidation dependent on an ethanol-inducible form of rabbit liver microsomal cytochrome P-450. FEBS Lett 183: 265-269, 1985.

Johnson IT. Antioxidants and antitumour properties. In: Pokorny J, Yanishlieva N, Gordon M, Eds. Antioxidants in foods, London, U.K: CRC Woodhead publishing Ltd, p101-123. 2001.

Johnson MK, Loo G. Effects of epigallocatechin and quercetin on oxidative damage to cellular DNA. Mutat Res 459: 211-218, 2000.

Johnston DE, Kroening C. Mechanism of early carbon tetrachloride toxicity in cultured rat hepatocytes. Pharmacol. Toxicol 83: 231-239, 1998.

Jornot L, Junod AF. Response of human endothelial cell antioxidant enzymes to hypeorxia. Am. J. Respir. Cell Mol Biol 6: 107-115, 1992.

Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG. Flavonoids as antioxidants. J Am Chem Soc 116: 4846-4851, 1994.

Juarez JC, Manuia M, Burnett ME, Betancourt O, Boivin B, Shaw DE, Tonks NK. Mazar AP, Donate F. Superoxide dismutase 1 (SOD1) is essential for $H_2O_2$-mediated oxidation and inactivation of phosphatases in growth factor signaling. Proc Nat Acad Sci USA 105: 7147-7152, 2008.

Jungalwala FB, Cama HR. Carotenoids of *Delonix regia* (Gul Mohr) flower. Biochem J 85: 1-8, 1962.

Kakkar R, Kalra J, Mantha SV, Prasad K. Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. Mol Cell Biochem 151: 113-119, 1995.

Kalt W, Blumberg JB, Vinqvist-Tymchuk MR, Graf BA, McDonald JE, Fillmore SAE, O'leary JM, Milbury PE. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. J Agric Food Chem 56: 705-712, 2008.

Kamendulis LM, Jiang J, Xu Y, Klaunig JE. Induction of oxidative stress and oxidative damage in rat glial cells by the acrylonitrile. Carcinogenesis 20: 1555-1560, 1999.

Kamiya H. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides. Nucleic Acid Res 31: 517-531, 2003.

Kanakis CD, Tarantilis PA, Polissiou MG, Diamantoglou S, Tajmir-Riahi HA. An overview of DNA and RNA bindings to antioxidant flavonoids. Cell Biochem Biophys 49: 29-36, 2007.

Kandil F, Song L, Pezzuto J, Seigler D, Smith MA. Isolation of oligomeric proanthocyanidins from flavonoid-producing cell cultures. In Vitro Cell Dev Biol Plant 36: 492-500, 2000.

Kang J, Zhang D, Chen J, Liu Q, Lin C. Antioxidants and trichostatin A synergistically protect against in vitro cytotoxicity of Ni in human hepatoma cells. Toxicol In Vitro 19: 173-182, 2005.

*Kang J-O, Kim S-J, Kim H.* **Effect of astaxanthin on the hepatotoxicity, lipid peroxidation and antioxidative enzymes in the liver of $CCl_4$-treated rats.** Methods Find Exp Clin Pharmacol 23(2): 79-84, *2001.*

Kang S, Seeram N, Nair M, Bourquin L. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. Cancer Lett 194(1): 13-19, 2003.

Kapoor VP. Food colours: Concern regarding their safety and toxicity. EnviroNews, News Letter of International Society of Environmental Botanists, 12: 2006. http://www.isebindia.com/05_08/06-04-3.html

Kappus H, Diplock AT. Tolerance and safety of vitamin E: a toxicological position report. Free Radic Biol Med 13: 55-74, 1992.

Katunuma N, Ohashi A, Sano E, Ishimaru N, Hayashi Y, Murata E. Catechin derivatives: Specific inhibitor for caspases-3, 7 and 2, and the prevention of apoptosis at the cell and animal levels. FEBS Lett 580: 741-746, 2006.

Kaur G, Rao LVM, Agrawal A, Pendurthi UR. Effect of wine phenolics on cytokine-induced C-reactive protein expression. J Thromb Haemost 5: 1309-1317, 2007.

Kaur P, Kaur G, Bansal MP. *Tertiary*-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: Role of transcription factor NF-κB and testicular antioxidant enzymes. Reprod. Toxicol 22: 479-484, 2006.

Kazantzis G, Bomford RR, and Oxon DM. Dyspepsia due to inhalation of carbon tetrachloride vapour. Lancet 1: 360-362, 1960.

Kedziora J, Bartosz G. Down's Syndrome: a pathology involving the lack of balance of reactive oxygen species. Free Radic Biol Med 4: 317-330, 1988.

Kehrer JP. Haber-Weiss reaction and mechanisms of toxicity. Toxicol. 149: 43-50, 2000.

Keilin D, Hartree EF. Properties of catalase. Catalysis of coupled oxidation of alcohols. Biochem J 39: 293-301, 1945.

Kelloff GJ, Crowell JA, STteele VE, Lubet RA, Boone CW, MaloneWA, Hawk ET, Lieberman R, Lawrence JA, Kopelovich L, Ali I, Viner JL, Sigman CC. Progress in cancer chemoprevention. Ann NY Acad Sci 889: 1-13, 1999.

Kennedy TA, Lieber DC. Peroxyl radical scavenging of β-carotene in lipid bilayers. Effect of oxygen partial pressure. J Biol Chem 267: 4658-4663, 1992.

Kerr JFR, Harmon BV. Current communications on cell and molecular biology. In: Apoptosis: The Molecular basis of cell death. NY: Cold Spring Harbor Lab. Press, Plainview, Vol.3: p5, 1991.

Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239-357, 1972.

Kim H. Carotenoids protect cultured rat hepatocytes from injury caused by carbon tetrachloride. 1995. Int J Biochem Cell Biol 27: 1303-1309, 1995.

Kim J, Sharma RP. Cadmium-induced apoptosis in murine macrophages is antagonized by antioxidants and caspase inhibitors. J Toxicol Env Health, Part A. 69: 1181-1201, 2006.

Kim JA, Kang YS, Kim YO, Lee SH, Lee YS. Role of $Ca^{++}$ influx in the *tert*-butyl hydroperoxide-induced apoptosis of HepG2 human hepatoblastoma cells. Exp Mol Med 30: 137-144, 1998.

Kim J-A, Lee YS, Huh K. The mechanism of t-butylhydrperoxide-induced apoptosis in IMR-32 human neuroblastoma cells. Kor J Physol Pharmacol 3: 19-27, 1999.

Kirca A, Ozkan M, Cemeroglu B. Stability of black carrot anthocyanins in various fruit juices and nectars. Food Chem 97: 598-605, 2006.

Kirca A, Ozkan M, Cemeroglu B. Effects of temperature, solid content and pH on the stability of black carrot anthocyanins. Food Chem 101: 212-218, 2007.

Klivenyi P, Andreassen OA, Ferrante RJ, Dedeoglu A, Mueller G, Lancelot E, Bogdanov M, Andersen JK, Jiang D, Beal MF. Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. J Neurosci 20: 1-7, 2000.

Knasmüller S, Mersch-Sundermann V, Kevekordes S, Darroudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants: current state of knowledge. Toxicol 198: 315-328, 2004.

Knasmüller S, Parzefall W, Sanyal R, Ecker S, Schwab C, Uhl M, Mersch-undermann V, Williamson G, Hietsch G, Langer T, Darroudi F, Natarajan AT. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat Res 402, 185-202, 1998.

Knekt P, Järvinen R, Seppännen R, Heliövaara M, Teppo L, Pukkala E, Aromaa A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 146: 223-230, 1997.

Knight JA. Reactive oxygen species and the neurodegenerative disorders. Ann Clin Lab Sci 27(1): 11-25, 1997.

Knowles BB, Aden DP. Human hepatoma derived cell line, process for preparation thereof, and uses therefor. United States Patent. 1983. 4393133. http://www.freepatentsonline.com/4393133.html

Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209: 497-499, 1980.

Kobayashi M, Yamamoto M. Molecular mechanisms activating the nrf2-keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal 7: 385-394, 2005.

Kobayashi S, Yoshida K, Ueda K, Sakai H, Komano T. DNA damage caused by ascorbate in the presence of $Cu^{2+}$ induces mutations. Nucleic Acids Symp Ser 19: 29-32, 1988.

Koh SH, Kim SH, Kwon H, Song CW, Kim JH, Kim J, Chung B, Kim MH, Gong G, Yu HJ, Jung HK. Cytoprotective Effect of Epigallocatechin gallate (EGCG) in oxidative-stressed PC12 cells following $H_2O_2$ exposure-effect of EGCG on phosphoinositide 3-kinase/Akt and glycogen synthase kinase-3 pathway. *J Korean Neurol Assoc* 21: 392-400, 2003.

Kohli DPS, Shah DH. Drug formulation manual. 2nd Ed. Eastern Publishers, New Delhi, India, p272. 1990.

Koide T, Hashimoto Y, Kamei H , Kojima T, Hasegawa M, Terabe K. Antitumor effect of anthocyanin fractions extracted from red soy beans and red beans in vitro and in vivo. Cancer Biother. Radiopharm 12: 277-280, 1997.

Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovascular Research 43: 562-571, 1999.

Kong AN, Yu R, Lei W, Mandlekar S, Tan TH, Ucker DS. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis: the role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. Restor Neurol Neurosci 12: 63-70, 1998.

Kong J, Chia L, Goh N, Chia T, Brouillard R. Analysis and biological activities of anthocyanins. Phytochem 64: 923-933, 2003.

Kono Y, Fridovich I. Superoxide radical inhibits catalase. J Biol Chem 257: 5751-5754, 1982.

Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med 26: 436-471, 1999.

Krinsky NI, Johnson EJ. Carotenoid actions and their relation to health and disease. Mol Asp Med 26: 459-516, 2005.

Krinsky NI. Cellular aspects of carotenoid actions. In: Cadenas E, Packer L, Eds. Handbook of Antioxidants. New York: Marcel Dekker, p315-336. 1996.

Ku HH, Brunk UT, Sohal RS. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. Free Radic Biol Med 15: 621-627, 1993.

Kuhlman PA. Characterization of the actin filament capping state in human erythrocyte ghost and cytoskeletal preparations Biochem J 349: 105-111, 2000.

Kuhnau J. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr Diet 24: 117-191, 1976.

Kurz C, Carle R, Schieber A. HPLC-DAD-$MS^n$ characterisation of carotenoids from apricots and pumpkins for the evaluation of fruit product authenticity. Food Chem 110: 522–530, 2008.

Kwak MK, Wakabayashi N, Kensler TW. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. Mutat Res 555: 133-148, 2004.

Lafuse WP, Alvarez GR, Zwilling BS. Regulation of Nramp1 mRNA stability by oxidants and protein kinase C in RAW264.7 macrophages expressing $Nramp1Gly^{169}$. Biochem J 351: 687-696, 2000.

Lago ES, Gomes E, da Silva R. Extraction and anthocyanic pigment quantification of the Jamun fruit (*Syzygium cumini* Lamark). 2004. http://www.iqsc.usp.br/outros/eventos/2004/bmcfb/trabalhos/docs/trab-76.pdf

Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P. Alice in caspase land. A phylogenetic analysis of caspases from worm to man. Cell Death Differ 9: 358-361, 2002.

Langley RE, Palayoor ST, Coleman CN, Bump EA. Modifiers of radiation-induced apoptosis. Radiat Res 136: 320-326, 1993.

Langseth L. Oxidants, antioxidants and disease prevention, ILSI Europe concise monograph series, Belgium: ILSI Press, 1995.

Larini A, Bianchi L, Bocci V. Effect of 4-hydroxynonenal on antioxidant capacity and apoptosis induction in Jurkat T cells. Free Radic Res 38: 509-516, 2004.

Lazzé MC, Pizzala R, Savio M, Stivala LA, Prosperi E, Bianchi L. Anthocyanins protect against DNA damage induced by *tert*-butyl hydroperoxide in rat smooth muscle and hepatoma cells. Mut Res 535: 103-115, 2003.

Lebovitz RM, Zhang H, Vogel H Cartwright Jr. J, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Nat Acad Sci USA 93: 9782-9787, 1996.

Lee AU, Farrell GC. Mechanism of azathioprine-induced injury to heaptoytes: roles of glutathione depletion and mitochondrial injury. J Hepatol 35: 756-764, 2001.

Lee H-S, Won NH, Kim KH, Lee H, Jun W, Lee K-W. Antioxidant effects of aqueous extract of *Terminalia chebula in vivo* and *in vitro*. Biol Pharm Bull 28: 1639-1644, 2005a.

Lee J, Durst RW, Wrolstad RE. Determination of total monomeric anthocyanins pigment content of fruit juices, beverages, natural colorants and wines by the pH differential method: collaborative study. J AOAC Int 88: 1269-1278, 2005b.

Lee J, Koo N, Min DB. Reactive oxygen species, aging and antioxidative nutraceuticals. Comprehensive Reviews in Food Science and Food Safety 3: 21-33, 2006.

Lee L-S, Rhim J-W, Kim S-J, Chung B-C. Study on the stability of anthocyanin pigment extracted from purple sweet potato. Kor. J Food Sci Technol 28: 352-359, 1996.

Leifert WR, Abeywardena MY. Cardioprotective actions of grape polyphenols. Nutr Res 28: 729-737, 2008.

Lemarie A, Lagadic-Gossmann D, Morzadec C, Allain N, Fardel O, Vernhet L. Cadmium induces caspase-independent apoptosis in liver Hep3B cells: role for calcium in signaling oxidative stress-related impairment of mitochondria and relocation of endonuclease G and apoptosis inducing factor. Free Radic Biol Med 36: 1517-1531, 2004.

Lepage G, Champagen J, Ronco N, Lamarre A, Osberg I, Sokol RJ, Roy CC. Supplementation with carotenoids corrects increased lipid peroxidation in children with cystic fibrosis. Am J Clin Nutrition 64: 87-93, 1996.

Leung HW-C, Lin C-J, Hour M-J, Yang W-H, Wang M-Y, Lee H-Z. Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. Food Chem Toxicol 45: 2005-2013, 2007.

Levy P, Viljoen M. Lactoferrin: a general review. Hematologica 80: 252-267, 1995.

Lewerenz V, Hanelt S, Nastevska C, El-Bahay C, Röhrdanz E, Kahl R. Antioxidants protect primary rat hepatocyte cultures against acetaminophen-induced DNA strand breaks but not against acetaminophen-induced cytotoxicity. Toxicol 191: 179-187, 2003.

Lewis P, Stefanovic N, Pete J, Calkin AC, Giunti S, Thallas-Bonke V, Jandeleit-Dahm KA, Allen TTJ, Kola I, Cooper ME, de Haan JB. Lack of the antioxidant enzyme glutathione peroxidase-1 accelerates atherosclerosis in diabetic apolipoprotein e–deficient mice. Circulation 115: 2178-2187, 2007.

Li L, Adams LS, Chen S, Killian C, Ahmed A, Seeram NP. *Eugenia jambolana* Lam. berry extract inhibits growth and induces apoptosis of human breast cancer but not non-tumorigenic breast cells. J Agric Food Chem *57*(3): 826-831, 2009a.

Li S-Y, Fu Z-J, Ma H, Jang W-C, So K-F, Wong D, Lo ACY. Effect of lutein on retinal neurons and oxidative stress in a model of acute retinal ischemia/reperfusion. Invest. Ophthalmol. Visual Sci 50: 836-843, 2009b.

Lila MA. Anthocyanins and human health: An in vitro investigative approach. J Biomed Biotechnol 5: 306-313, 2004.

Lin WL, Wang CJ, Tsai YY, Liu CL, Hwang JM, Tseng TH.. Inhibitory effect of esculetin on oxidative damage induced by *t*-butyl hydroperoxide in rat liver. Arch. Toxicol. 74(8): 467-472, 2000.

Liochev SI, Fridovich I. Effects of overproduction of superoxide dismutase on the toxicity of paraquat toward *Escherichia coli*. J Biol Chem 266(14): 8747-8750, 1991.

Liochev SI, Fridovich I. The role of $O_2^{\cdot-}$ in the production of $HO^{\cdot}$ *in vitro* and *in vivo*. Free Radic Biol Med 16: 29-33, 1994.

Lipikin M, Reddy B, Newmark H, Lamprecht SA. Dietary factors in human colorectal cancer. Ann Rev Nutr 19: 545-586, 1999.

Liu C, Russell RM, Wang XD. Low dose β-carotene supplementation of ferrets attenuates smoke-induced lung phosphorylation of JNK, p38 MAPK, and p53 proteins. *J Nutr* 134: 2705-2710, 2004.

Liu J, Chen C, Wang W, Hsu J, Yang M, Wang C. The protective effects of *Hibiscus sabdariffa* extract on $CCl_4$-induced liver fibrosis in rats. Food Chem Toxicol 44: 336-343, 2006.

Liu RH, Finley J. Potential cell culture models for antioxidant research. J Agric Food Chem 53: 4311-4314, 2005.

Liu SS. Generating, partitioning, targeting and functioning of superoxide in mitochondria. Biosci Rep 17: 259-272, 1997.

Ljubuncic P, Portnaya I, Cogan U, Azaizeh H, Bomzon A. Antioxidant activity of *Crataegus aronia* extract used in traditional Arab medicine in Israel. J Ethnopharmacol 101: 153-161, 2005.

Lohr JB. Oxygen radicals and neuropsychiatric illness. Some speculations. Arch Gen Psychiatry 48: 1097-1106, 1991.

Long LH, Lan AN, Hsuan FT, and Halliwell B. Generation of hydrogen peroxide by "antioxidant" beverages and the effect of milk addition. Is cocoa the best beverage? Free Radic Res Communic 31: 67-71, 1999.

Lötscher HR, Winterhalter KH, Carafoli E, Richter C. Hydroperoxides can modulate the redox state of pyridine nucleotides and the calcium balance in rat liver mitochondria. Proc Nat Acad Sci USA 76: 4340-4344, 1979.

Lowry OH, Rosenberg NJ, Farr AL, Randall RJ. Protein measurement with the Folinphenol reagent. J Biol Chem 193, 265-267, 1951.

Luo D, Rando TA. The regulation of catalase gene expression in mouse muscle cells is dependent on the CCAAT binding factor NF-Y. Biochem Biophys Res Commun 303, 609-618, 2003.

Luo D, Rando TA. The regulation of catalase gene expression in mouse muscle cells is dependent on the CCAAT binding factor NF-Y. Biochem Biophys Res Commun 303: 609-618, 2003.

Luqman S, Rizvi SI. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. Phytother Res 20: 303-306, 2006.

Maitre B, Jornot L, Junod AF. Effects of inhibition of catalase and superoxide dismutase activity on antioxidant enzyme mRNA levels. Am J Physiol Lung Cell Mol Physiol 9: 636-643, 1993.

Majer BJ, Mersch-Sundermann V, Darroudi, Laky B, deWit K, Knasmüller S. Genotoxic effects of dietary and lifestyle related carcinogens in human derived hepatoma (HepG2, Hep3B) cells. Mutat Res 551: 153-166, 2004.

Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 146: 3-15, 1995.

Mallery S, Stoner G, Larsen P, Fields H, Rodrigo K, Schwartz S, Tian Q, Dai J, Mumper R. Formulation and *in vitro* and *in vivo* evaluation of a mucoadhesive gel containing freeze dried blak raspberries: Implications for oral cancer chemoprevention. Pharm Res 24: 728-737, 2007.

Mandel S, Packer L, Youdin MBH, Weinreb O. Proceedings from the "Third International Conference on Mechanism of Action of Nutraceuticals". J Nutr Biochem 16: 513-520, 2005.

Manov I, Hirsh M, Iancu TC. Acetaminophen hepatotoxicity and mechanisms of its protection by N-aetylcysteine: a study of Hep3B cells. Exp Toxicol Pathol 53: 489-500, 2002.

Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. The nitric oxide scavenging properties of Ginkgo biloba extract EGb 761. Biochem Biophys Res Communic 15: 748-755, 1994.

Markakis P, Livingston GE, Fellers RC. Quantitative aspects of strawberry pigment degradation. Food Res 22: 117-130, 1957.

Markakis P. Stability of anthocyanins in foods. In: Markakis P, Ed. Anthocyanins as food colors. New York: Academic Press, p163-180. 1982.

Martin-Aragon S, De Las Heras B, Sanchez-Reus MI, Benedi J. Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. Exp Toxicol Pathol 53: 199-206, 2001.

Martinet W, De Meyer GRY, Herman AG, Kockx MM. Reactive oxygen species induce RNA damage in human atherosclerosis. Eur J Clin Invest 34: 323-327, 2004.

Matés JM. Antioxidant enzymes and molecular control of reactive oxygen species. Toxicol 153: 83-104, 2000.

Matsumoto H, NakamuraY, Tachibanaki S, Kawamura S, Hirayama M. Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. J Agric Food Chem 2003: 51: 3560-3563, 2003.

Mayne ST. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J 10: 690-701, 1996.

Mazlan M, Mian TS, Top GM, Ngah WZW. Comparative effects of α-tocopherol and γ-tocopherol against hydrogen peroxide induced apoptosis on primary astrocytes. J. Neurol Sci 243: 5-12, 2006.

Mazza G, Brouillard R. Recent developments in the stabilization o f anthocyanins in food products. Food Chem 25: 207-225, 1987.

Mazza G, Brouillard R. The mechanism of co-pigmentation of anthocyanins in aqueous solutions. Phytochem 29: 1097-1102., 1990.

Mazza G, Kay CD, Cottrell T, Holub BJ. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. J Agric Food Chem 50: 7731-7737, 2002.

Mazza G, Miniati E. Anthocyanins in fruits, vegetables and grains. Boca Raton: CRC Press, p120-145, 1993.

McQueen CA, Williams GM. The hepatocyte primary culture/DNA repair test using hepatocytes from several species. Cell Biol Toxicol 3: 209-218, 1987.

Mecocci P, Polidori MC, Troiano L, Cherubini A, Cecchetti R, Pini G, Straatman M, Monti D, Stahl W, Sies H, Franceschi C, Senin U. Plasma antioxidants and longevity: A study on healthy centenarians. Free Rad Biol Med 28: 1243-1248, 2000.

Medvedev AI, Leschenko VV. Generators of reactive oxygen forms gamma irradiation and ascorbic acid-cobalt metallo complexes induced large-scale fragmentation and reparation of DNA in tumor cells. Bull Exp Biol Med 145: 600-603, 2008.

Meiers S, Kemeny M, Weyand U, Gastpar R, von Angerer E, Marko D. The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. J Agric Food Chem 49: 958-962, 2001.

Merante F, Altamentova SM, Mickle DA, Weisel RD, Thatcher BJ, Martin BM, Marshall JG, Tumiati LC, Cowan DB, Li RK. The characterization and purification of a human transcription factor modulating the glutathione peroxidase gene in response to oxygen tension. Mol Cell Biochem 229: 73-83, 2002.

Mersch-Sundermann V, Knasmüller S, Wu X-J, Darroudi F, Kassie F. Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. Toxicol 198: 329-340, 2004.

Metodiewa D, Jaiswal AK, Cenas N, Segura-Aguilar J, Dickancaite E. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 26: 107-116, 1999.

Miccadei S, Kyle ME, Gilfor D, Farber JL. Toxic consequence of the abrupt depletion of glutathione in cultured rat hepatocytes. Arch Biochem Biophys 265: 311-320, 1988.

Migliato KF. Standardization of the extract of. *Syzygium cumini* (l.) skeels fruits. through the antimicrobial activity. Caderno de Farmácia 21: 55-56, 2005.

Miliauskas G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem 85: 231-237, 2004.

Mills GC. Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J Biol Chem 229: 189-197, 1957.

Miyashita T, Kitada S, Krajewski S, Horne WA, Delia D, Reed JC. Over expression of the Bcl-2 protein increases the half-life of $p21^{Bax}$. J Biol Chem 270: 26049-26052, 1995.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene 9: 1799-1805, 1994.

Moldeus P, Hogberg J, Orrenius S. Isolation and use of liver cells. Methods Enzymol 52: 60-71, 1978.

Mollov P, Mihalev K, Shikov V, Yoncheva N, Karagyozov V. Colour stability improvement of strawberry beverage by fortification with polyphenolic copigments naturally occurring in rose petals. Innovative Food Sci Emerging Technologies. 8: 318-321, 2007.

Moolgavkar SH. The multistage theory of carcinogenesis and the age distribution of cancer in man. J National Cancer Institute 61: 49-52, 1978.

Moon YJ, Wang X, Morris ME. Dietary flavonoids: Effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro 20: 187-210, 2006.

Moriarty P, Reddy CC, Maquat LE. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. Mol Cell Biol 18: 2932-2939, 1998.

Morton J. Fruits of warm climates. Miami, FL, p 375. 1987.

Moskaug JO, Carlsen H, Myhrstad MC, Blomhoff R. Am. J Clin Nutrition 81: 277S-283S, 2005.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.

Moyer RA, Hummer KE, Finn CE, Frei B, Wrolstad RE. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. J Agric Food Chem 50: 519-525, 2002.

Mulabagal V, Lang GA, DeWitt DL, Dalavoy SS, Nair MG. Anthocyanin content, lipid peroxidation and cyclooxygenase enzyme inhibitory activities of sweet and sour cherries. J Agric Food Chem 57: 1239-1246, 2009.

Munkres KD, Colvin HJ. Ageing of *Neurospora crassa*. II. Organic hydroperoxide toxicity and the protective role of antioxidant and the antioxygenic enzymes. Mech Ageing Dev 5: 99-107, 1976.

Murthy KNC, Singh RP, Jayaprakasha GK. Antioxidant activities of Grape (*Vitis vinifera*) pomace extracts. J Agric Food Chem 50: 5909-5914, 2002.

Muthukumaran S, Sudheer AR, Menon VP, Nalini N. Protective effect of quercetin on nicotine-induced prooxidant and antioxidant imbalance and DNA damage in Wistar rats. Toxicol 243: 207-215, 2008.

Myhrstad MC, Carlsen H, Nordstrom O, Blomhoff R, Moskaug JO. Flavonoids increase the intracellular glutathione level by transactivation of the gamma-glutamylcysteine synthetase catalytical subunit promoter. Free Radic Biol Med 32: 386-393, 2002.

Mytilineou C, Kramer BC, Yabu JA. Glutathione depletion and oxidative stress. Parkinsonism and Related Disorders 8: 385-387, 2002.

Na H-K, Surh Y-J. Modulation of Nrf2-mediated antioxidant and detoxigying enzyme induction by the green tea polyphenols EGCG. Food Chem Toxicol 46: 1271-1278, 2008.

Nadkarni KM. Indian Materia Medica. India: Dhootapapeshwar Prakashan Ltd. Vol. 1: p516. 1954.

Nafisi S, Shadaloi A, Feizbakhsh A, Tajmir-Riahi HA. RNA binding to antioxidant flavonoids. J Photochem Photobiol B: Biol 94: 1-7, 2009.

Nakaishi H, Matsumoto H, Tominaga S, Hirayama M. Effects of black currant anthocyanoside intake on dark adaptation and VDT work-induced transient refractive alteration in healthy humans. Altern Med Rev 5: 553-562, 2000.

Nakajima JI, Tanaka I, Seo S, Yamazaki M, Saito K. LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. J Biomed Biotechnol 2004: 241-247, 2004.

Nakane T, Asayama K, Kodera K, Hayashibe H, Uchida N, Nakazawa S. Effect of selenium deficiency on cellular and extracellular glutathione peroxidases: immunochemical detection and mRNA analysis in rat kidney and serum. Free Radic Biol Med 25: 504-511, 1998.

Namasivayam R, Munuswamy D, Ramachandran B. Effect of aqueous extract of *Syzygium cumini* pulp on antioxidant defense system in streptozotocin induced diabetic rats. Iran. J Pharmacol Therap (in Press: 7, 2008)

Negre-Salvayre A, Salvayre R. Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cell lines. Free Radic Biol Med 12: 101-106, 1992.

Nelson SK, Bose SK, Grunwald GK, Myhill P, McCord JM. The induction of human superoxide dismutase and catalase in vivo: A fundamentally new approach to antioxidant therapy. Free Radic Biol Med 40: 341-347, 2006.

Nenoi M, Ichimura S, Mita K, Yukawa O, Cartwright IL. Regulation of the catalase gene promoter by Spl, CCAAT-recognizing factors, and a WTl/Egr-related factor in hydrogen peroxide-resistant HP100 cells. Cancer Res 61: 5885-5894, 2001.

Neuman MG, Koren G, Tiribelli C. *In vitro* assessment of the ethanol induced hepatotoxicity on HepG2 cell line. Biochem Biophys Res Communic 197: 932-941, 1993.

Nicod L, Viollon C, Regnier A, Jacqueson A, Richert L. Rifampicin and isoniazid increase acetaminophen and isoniazid cytotoxicity in human HepG2 hepatoma cells. Hum Exp Toxicol 16: 28-34, 1997.

Nielsen IL, Dragsted LO, Ravn-Haren G, Freese R, Rasmussen SE. Absorption and excretion of black currant anthocyanins in humans and watanabe heritable hyperlipidemic rabbits. J Agric Food Chem 51: 2813-2820, 2003.

Noda Y, Kaneyuki T, Mori A, Packer L. Antioxidant activities of pomegranate fruit extract and its anthocyanidins: delphinidin, cyanidin and pelargonidin. J Agric Food Chem 50: 166-171, 2002.

Noguchi T, Fong KL, Lai EK, Alexander SS, King MM, Olson L, Poyer JL, McCay PB. Specificity of a phenobarbital-induced cytochrome P-450 for metabolism of carbon tetrachloride to the trichloromethyl radical. Biochem Pharmacol 31: 615-624, 1982.

Novo E, Parola M. Redox mechanisms in chronic hepatic wound healing and fibrinogenesis. Fibrinogen Tissue Rep 1: 5, 2008.

O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267: 5421-5426, 2000.

O'Sullivan L, Ryan L, O'Brien N. Comparison of the uptake and secretion of carotene and xanthophylls carotenoids by Caco-2 intestinal cells. Brit J Nutr 98: 38-44, 2007.

Obi FO, Usenu IA, Osayande JO. Prevention of carbon tetrachloride-induced hepatotoxicity in the rat by *H. rosasinensis* anthocyanin extract administered in ethanol. Toxicol 131: 93-98, 1998.

Ochi T, Miyaura S. Cytotoxicity of an organic hydroperoxide and cellular antioxidant defense against hydroperoxides in cultured mammalian cells. Toxicol 55: 69-82, 1989.

Ochi T. Effects of glutathione depletion and induction of metallothioneins on the cytotoxicity of an organic hydroperoxide in cultured mammalian cells. Toxicol 50: 257-268, 1988.

Ochi T. Effects of an organic hydroperoxide on the activity of antioxidant enzymes in cultured mammalian cells. Toxicol 61: 229-239, 1990.

Offer T, Russo A, Samuni A. The pro-oxidative activity of SOD and nitroxide SOD mimics. FASEB J 14: 1215–1223, 2000.

Ohta Y, Sasaki E, Nishida K, Kobayashi T, Nagata M, Ishigur I. Preventive effect of dia-saiko-to (da-chai-hutang) extract on disrupted hepatic active oxygen metabolism in rats with carbon tetrachloride induced liver injury. Am J Chin Med 23: 53-64, 1995.

Okinaga T, Kasai H, Tsujisawa T, Nishihara T. Role of caspases in cleavage of lamin A/C and PARP during apoptosis in macrophages infected with a periodontopathic bacterium. J Med Microbiol 56: 1399-1404, 2007.

Oliveira MC, Esperanca P, Almoster Ferreira MA. Characterisation of anthocyanidins by electrospray inonisaiton and collision-induced dissociation tandem mass spectrometry. Rapid Commun Mass Spectrom 15: 1525-1532, 2001.

Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435: 677-681, 2005.

Omenn GS, Goodman G, Thornquist M, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a

combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. New Engl J Med 34: 1150-1155, 1996.

Omenn GS. Chemoprevention of lung cancer: the rise and demise of β-carotene. Annu Rev Public Health 19: 73-99, 1998.

Orr WC, Sohal RS. Extension of life-span by over expression of superoxide dismutase and catalase in *Drosophila melanogaster*. Science 263: 1128-1130, 1994.

Oshino N, Oshino R, Chance B. The characteristic of the 'peroxidatic' reaction of catalase in ethanol. Biochem J 131: 555-563, 1973.

Paiva SAR, Russell RM. β-Carotene and other carotenoids as antioxidants. J Am Coll Nutr 18: 426-433, 1999.

Palozza MD. Pro-oxidant actions of carotenoids in biological systems. Nutr Rev 56: 257-265, 1998.

Palozza P, Krinsky NI. Antioxidant effects of carotenoids *in vivo* and *in vitro*: An overview Methods Enzymol 213: 403-420, 1992.

Palozza P, Luberto C, Calviello G, Ricci P, Bartoli GM. Antioxidant and prooxidant role of β-carotene in murine normal and tumor thymocytes. Effects of oxygen partial pressure. Free Radic Biol Med 22: 1065-1073, 1997.

Palozza P, Serini S, Torsello A, Di Nicuolo F, Piccioni E, Ubaldi V, Pioli C, Wolf FI, Calviello G. β-carotene regulates NF- B DNA-binding activity by a redox mechanism in human leukemia and colon adenocarcinoma cells. J Nutr 133: 381-388, 2003.

Park EY, Rho HM. The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. Mol Cell Biochem 240: 47-55, 2002.

Park YO, Hwang ES, Moon TW. 2005. The effect of lycopene on cell growth and oxidative DNA damage of Hep3B human hepatoma cells. Biofactors 23: 129-139.

Parola M, Leonarduzzi G, Biasi F, Albano M, Biocca E, Poli G, Dianzani U. Vitamin E dietary supplementation protects against $CCl_4$-induced chronic liver damage and cirrhosis. Hepatol 16: 1014-1021, 1992.

Pazos M, Lois S, Torres JL, Medina I. Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics**.** J Agric Food Chem 54: 4417-4423, 2006.

Peltz SW, Brewer G, Bernstein P, Hart PA, Ross J. Regulation of mRNA turnover in eukaryotic cells. Crit Rev Eukaryot Gene Expr 1: 99-126, 1991.

Peto R, Doll R, Buckley J, Spom MB. Can dietary beta-carotene materially reduce human cancer rates? Nature 290: 201-208, 1981.

Pias EK, Ekshyyan OY, Rhoads CA, Fuseler J, Harrison L, Aw TY. Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells. J Biol Chem 278: 13294-13301, 2003.

Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary MD, Remacle J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. Mech Ageing Dev 51: 283-297, 1990.

Pi-Jen T, Yu-Yuan H, Tzou-Chi H. Effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system using $^{17}O$ NMR. J Agric Food Chem 52: 3097-3099, 2004.

Pinto RE, Bartley W. Changes in glutathione reductase and glutathione peroxidase activities in rat liver related to age and sex. Biochem J 109: 34P, 1968.

Piper JT, Singhal SS, Salameh MS, Torman RT, Awasthi YC, Awasthi S. Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. Int J Biochem Cell Biol 30: 445-56, 1998.

Piret J-P, Arnould T, Fuks B, Chatelain P, Remacle J, Michiels C. Mitochondria permeability transition-dependent tert-butyl hydroperoxide-induced apoptosis in hepatoma HepG2 cells. Biochem Pharmacol 67: 611-620, 2004.

Pocernich CB, Cardin AL, Racine CL, Lauderback CM, Butterfield DA. Glutathione elevation and its protective role in acrolein induced protein damage in synaptosomal membranes. Neurochem Int 39: 141-149, 2001.

Poei-Langston MS, Wrolstad RE. Color degradation in an ascorbic acid–anthocyanin–flavonol model system. J Food Sci 46: 1218-1222, 1981.

Poot M, Teubert H, Rabinovitch PS, Kavanagh TJ. De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. J Cell Physiol 163: 555-560, 1995.

Popat A, Shear NH, Malkiewicz I, Thomson S, Neuman MG. Mechanism of Impila (*Callilepsis laureola*)-induced cytotoxicity in HepG2 cells. Clin Biochem 35: 57-64, 2002.

Popatovich AI, Kostyuk VA. Comparative study of antioxidant properties and cytoprotective activity of flavonoids. Biochem 68: 514-519, 2003.

Prasad KD, Sai Ram M, Sawhney RC, Ilavazhagan G, Banerjee PK.. Mechanism of tert - butylhydroperoxide induced cytotoxicity in U-937 macrophages by alteration of mitochondrial function and generation of ROS. Toxicol In Vitro 21: 846-854, 2007.

Prendergrast J, Jones R, Jenkins L, and Siegel J. Effects on experimental animals of longterm inhalation of trichloroethylene, carbon tetrachloride, 1,1,1-trichloroethane, dichlorodifluoromethane, and 1,1-dichloroethylene. Toxicol Appl Pharmacol 10: 270-289, 1967.

Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53: 4290-302, 2005.

Pryor WA, Stahl W, Rock CL. Beta carotene: from biochemistry to clinical trials. Nutr Rev 58: 39-53, 2000.

Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological Fenton reagent? Biochem J 249: 185-190, 1988.

Pushpakiran G, Mahalakshmi K, Anuradha CV. Taurine restores ethanol-induced depletion of antioxidants and attenuates oxidative stress in rat tissues. Amino Acids 27: 91-96, 2004.

Radziszewska A, Schroer SA, Choi D, Tajmir P, Radulovich N, Ho JC, Wang L, Liadis N, Hakem R, Tsao M-S, Penn LZ, Evan GI, Woo M. Absence of Caspase-3 protects pancreatic β-cells from c-Myc-induced apoptosis without leading to tumor formation. J Biol Chem 284: 10947-10956, 2009.

Raffray M, Cohen GM, Apoptosis and necrosis in toxicology: A continuum or distinct modes of cell death? Pharmacol Ther 75: 153-177, 1997.

Rajesh MG, Latha MS. Protective activity of *Glycyrrhiza glabra* Linn. on carbon tetrachloride-induced peroxidative damage. Ind J Pharmacol 36: 284-287, 2004.

Ramassamy C. Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: A review of their intracellular targets. Eur J Pharmacol 545: 51-64, 2006.

Ramirez-Mares MV, de Mejia EG. Comparative study of the antioxidant effect of ardisin and epigallocatechin gallate in rat hepatocytes exposed to benomyl and 1-nitropyrene. Food Chem Toxicol 41: 1527-1535, 2003.

Ramirez-Tortosa C, Andersen OM, Gardner PT, Morrice PC, Wood SG, Duthie SJ, Collins AR, Duthie GG. Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. Free Radic Biol Med 31: 1033-1037, 2001.

Rao MN, Ghosh P, Lakshman MR. Purification and partial characterization of a cellular carotenoid- binding protein from ferret liver. J Biol Chem 272: 24455-24460, 1997.

Rao PVL, Jayaraj R, Bhaskar ASB, Om kumar, Bhattacharya R, Saxena P, Dash PK, Vijayaraghavan R. Mechanism of ricininduced apoptosis in human cervical cancer cells. Biochem Pharmacol 69: 855-865, 2005.

Reddivari L, Vanamala J, Chintharlapalli S, Safe SH, Miller Jr JC. Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways. Carcinogenesis 28: 2227-2235, 2007.

Reddy MB, Xu Q, Zhou Y, Kanthasamy AG. Neuroprotective effect of green tea polyphenol, EGCG in animal model of Parkinson's disease. FASEB J 22: lb677, 2008.

Reed DJ. Glutathione: toxicological implications. Annu Rev Pharmcol Toxicol 30: 603-631, 1990.

Reinke LA, Lai EK, McCay PB. Ethanol feeding stimulates trichloromethyl radical formation from carbon tetrachloride in liver. Xenobiotica 18: 1311-1318, 1988.

Remacle J, Lambert D, Raes M, Pigeolet E, Michiels C, Toussaint O. Importance of various antioxidant enzymes for cell stability. Confrontation between theoretical and experimental data. Biochem J 286: 41-46, 1992.

Repine JE. Oxidant–antioxidant balance: some observations from studies of ischemia-reperfusion in isolated perfused rat hearts. Am J Med 91: 45S–53S, 1991.

Ribaya-Mercado JD, Blumberg JB. Lutein and zeaxanthin and their potential roles in disease prevention. J Am Coll Nutr 23: 567S-587S, 2004.

Rice-Evans C, Bruckdorfer KR. Free radicals, lipoproteins and cardiovascular dysfunction. Mol Aspects Med 13, 1-111, 1992.

Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, Bars R, Coassolo P, LeCluyse E. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of culture rat hepatocytes. Toxicol In Vitro 16: 89-99, 2002.

Rieth AG, Baader SL, Lode HN, Bruchelt G, Niethammer D. Ferritin enhances production of DNA strand breaks by 6-hydroxydopamine, ascorbic acid and $H_2O_2$ in CCC PM2 bacteriophage DNA. BioMetals 5: 223-227, 1992.

Rimbach G, Gohil K, Matsugo S, Moini H, Saliou C, Virgili F, Weber SU, Packer L. Induction of glutathione synthesis in human keratinocytes by *Ginkgo biloba* extract (EGb761). Biofactors 15: 39-52, 2001.

Roberts LR, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. Semin Liver Dis 25: 212-225, 2005.

Rodeiro I, Donato MT, Lahoz A, González-Lavaut JA, Laguna A, Castell JV, Delgado R, Gómez-Lechón MJ. Modulation of P450 enzymes by Cuban natural products rich in polyphenolic compounds in rat hepatocytes. Chem Biol Interact 172: 1-10, 2008.

Rodrigo R, Rivera G, Orellana M, Araya J, Bosco C. Rat kidney antioxidant response to long-term exposure to flavonol rich red wine. Life Sci 71: 2882-2895, 2002.

Rodriguez-Amaya. DBA guide to carotenoids analysis. Washington DC, USA: ILSI Press, 2001.

Rohrdanz E, Bittner A, Tran-Thi QH, Kahl R. The effect of quercetin on the mRNA expression of different antioxidant enzymes in hepatoma cells. Arch Toxicol 77: 506-510, 2003.

Rohrdanz E, Kahl R. Alterations in antioxidant enzyme expression in response to hydrogen peroxide. Free Rad Biol Med 24: 27-38, 1998.

Röhrdanz E, Obertrifter B, Ohler S, Kahl R. Influence of adriamycin and paraquat on antioxidant enzyme expression in primary rat hepatocytes. Arch Toxicol 74: 231-237, 2000.

Rohrdanz E, Schmuck G, Ohler S, Kahl R. The influence of oxidative stress on catalase and MnSOD gene transcription in astrocytes. Brain Res 900: 128-136, 2001.

Ross J. mRNA stability in mammalian cells. Microbiol Rev 59: 423-450, 1995.

Rotilio G, Calabrese L, Bossa F, Barra D, Agro AF, Mondovi B. Properties of the apoprotein and role of copper and zinc in protein conformation and enzyme activity of bovine superoxide dismutase. Biochem 11: 2182–2187, 1972.

Rubiolo JA, Mithieux G, Vega FV. Reseveratrol protects primary rat hepatocytes against oxidative stress damage: activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes. Eur J Pharmacol 591: 66-72, 2008.

Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 10: 1003-1008, 1989.

Ruch RJ, Klaunig JE, Schultz NE, Askari AB, Lacher DA, Pereira MA, Goldblatt PJ. Mechanisms of chloroform and carbon tetrachloride toxicity in primary cultured mouse hepatocytes. Environ. Health Perspect 69: 301-305, 1986.

Rukmini MS, D'Souza B, D'Souza V. Superoxide dismutase and catalase activities and their correlation with malondialdehyde in schizophrenic patients. Ind J Clin Biochem 19: 114-118, 2004.

Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol. Appl Pharmacol 78: 473-483, 1985.

Russell RM. The enigma of β-carotene in carcinogenesis: What can be learned from animal studies. J Nutr 134: 262S-268S, 2004.

Saeki K, Kobayashi N, Inazawa Y, Zhang H, Nishitoh H, Ichijo H, Isemura M, Yuo A. Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): A distinct pathway from those of chemically induced and receptor-mediated apoptosis. Biochem J 368: 705-720, 2002.

Saito M, Korsmeyer SJ, Schlesinger PH. BAX-dependent transport of cytochrome c reconstituted in pure liposomes. Nat Cell Biol 2: 553-555, 2000.

Sakai M, Okabe M, Tachibana H, Yamada K. Apoptosis induction by γ-tocotrienol in human hepatoma Hep3B cells. J Nutr Biochem 17: 672-676, 2006.

Sakr SA. Ameliorative Effect of Ginger (*Zingiber officinale*) on mancozeb fungicide induced liver injury in albino rats. Austr J Basic Appl Sci 1: 650-656, 2007.

Saleh NAM, Ishak MS. Anthocyanins of some leguminosae flowers and their effect on color variation. Phytochem 15: 835-836, 1976.

Salvi M, Battaglia V, Brunati AM, La Rocca N, Tibaldi E, Pietrangeli P, Marcocci L, Mondovi B, Rossi CA, Toninello A. Catalase takes part in rat liver mitochondria oxidative stress defense. J Biol Chem 282: 24407–24415, 2007.

Salvi M, Fiore C, Armanini D, Toninello A. Glycyrrhetinic acid-induced permeability transition in rat liver mitochondria. Biochem Pharmacol 66: 2375-2379, 2003.

Sampath D, Perez-Polo R. Regulation of antioxidant enzyme expression by NGF. Nucleosom Res 22: 351-362, 1997.

Sanchez-Moreno C, Larrauri JA, Saura-Calixto F. Free-radical scavenging capacity of selected red, rose and white wines. J Sci Food Agri 79: 1301-1304, 1999.

Sánchez-Reus MI, Gómez del Rio MA, Iglesias I, Elorza M, Slowing K, Benedí J. Standardized *Hypericum perforatum* reduces oxidative stress and increases gene expression of antioxidant enzymes on rotenone-exposed rats. Neuropharmacol 52: 606-616, 2007.

Sandstrom BE. Induction and rejoining of DNA single-strand breaks in relation to cellular growth in human cells exposed to three hydroperoxides at 0 degrees C and 37 degrees C. Free Radic Res Commun 15: 79-89, 1991.

SanGiovanni JP, Chew EY, Clemons TE, Ferris III FL, Gensler G, Lindblad AS, Milton RC, Seddon JM, Sperduto RD. The relationship of dietary carotenoid and Vitamin A, E, and C intake with age-related macular degeneration in a case-control study. *Arch Ophthalmol* 125: 1225-1232, 2007.

Sarma AD, Sharma R. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. Phytochem 52: 1313-1318, 1999.

Sarma AD, Sreelakshmi Y, Sharma R. Antioxidant ability of anthocyanins against ascorbic acid oxidation. Phytochem 45: 671-674, 1997.

Sartippour MR, Pietras R, Marquez-Garban DC, Chen -W, Heber D, Henning SM, Sartippour G, Zhang L, Lu M, Weinberg O, Rao JY, Brooks MN. The combination of green tea and tamoxifen is effective against breast cancer. Carcinogenesis 27: 2424-2433, 2006.

Sastre J, Millan A, Garcia de la Asuncion J, Pla R, Juan G, Pallardo FV, O'Connor E, Martin JA, Droy-Lefaix MT, Vina J. A *Ginkgo biloba* extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. Free Radic Biol Med 24: 298-304, 1998.

Schindowski K, Leutner S, Kressmann S, Eckert A, Muller WE. Age-related increase of oxidative stress-induced apoptosis in mice prevention by *Ginkgo biloba* extract (EGb761). J Neural Transm 108: 969-978, 2001.

Schlesier K, Harwat M, Böhm V, Bitsch R. Assessment of antioxidant activity by using different in vitro methods. Free Radic Res 36: 177-187, 2002.

Schmidt-Dannert C, Umeno D, Arnold FH. Molecular breeding of carotenoids biosynthetic pathways. Nature Biotech 18: 750-753, 2000.

Schrauzer GN. Selenium: mechanistic aspects of anticarcinogenic action. Biol Trace Element Res 33: 51–62, 1992.

Schriner SE, Linford NJ, Martin GM, Treuting PM, Ogburn CE, Emond MJ, Coskun PE, Ladiges WC, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS. Extension of murine lifespan by overexpression of catalase targeted to mitochondria. Science 308: 1909-1911, 2005.

Schwartz JL. The dual roles of nutrients as antioxidants and prooxidants: Their effects on tumor cell growth. J Nutr 126: 1221S-1227S, 1996.

Scopa CD, Vagianos C, Kardamakis D, Kourelis TG, Kalofonos HP, Tsamandas AC. bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with rectal cancer. Appl. Immunohistochem. Mol Morphol 9: 329-334, 2001.

Scott JA, Fischman AJ, Khaw BA, Homcy CJ, Rabito CA. Free radical-mediated membrane depolarization in renal and cardiac cells. Biochim Biophys Acta 899: 76-82, 1987.

Seeram NP. Berry fruits for cancer prevention: Current status and future prospects. J Agric Food Chem 56: 630-635, 2008.

Seetharam YN, Vijay G, Sharanabasappa N, Murthy S, Sangamma VR. Antimicrobial and analgesic ativities of *Delonix elata* Gamble and *Delonix regia*. Aryavaidyan 16: 51-53, 2002.

Semiz A, Sen A. Antioxidant and chemoprotective properties of *Momordica charantia* L. (bitter melon) fruit extract. Afr J Biotechnol 6: 273-277, 2007.

Sen A, Sahin B, Agus HH, Bayav M, Sevim H, Semiz A. Prevention of carbon tetrachloride-induced hepatotoxicity by urtica urens in rats. J Appl Biologic Sci 1: 29-32, 2007.

Sen CK. Cellular thiols and redox-regulated signal transduction. Curr Top Cell Regul 36: 1-30, 2000.

Sen P, Chakraborty PK, Raha S. p38 mitogen-activated protein kinase (p38MAPK) upregulates catalase levels in response to low dose $H_2O_2$ treatment through enhancement of mRNA stability. FEBS Lett 579: 4402-4406, 2005.

Serrano J, Goni I, Saura-Calixto F. Determination of beta carotene and lutein available from green leafy vegetables by an in vitro digestion and colonic fermentation method. J Agric Food Chem 53: 2936-2940, 2005.

Sestili P, Diamantini G, Bedini A, Cerioni L, Tommasini I, Tarzia G, Cantoni O. Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by *tert*-butylhydroperoxide via an iron-chelating mechanism. Biochem J 364: 121-128, 2002.

Sestili P, Guidarelli A, Dacha M, Cantoni O. Quercetin prevents DNA single strand breakage and cytotoxicity caused by *tert*-butylhydroperoxide: free radical scavenging versus iron chelating mechanism. Free Radic Biol Med 25: 196-200, 1998.

Sethuraman MG, Sulochana N. The anti-inflammatory activity of *Delonix elata* Gamble. Curr Sci 55: 343-344, 1986.

Shan X, Aw TY, Jones DP. Glutathione-dependent protection against oxidative injury. Pharmacol Ther 47: 61–71, 1990.

Sharma JN, Sheshadri TR. Survey of anthocyanins from Indian sources: Part II. J Sci Ind Res 14: 211-214, 1955.

Sharma OP, Bhat TK. DPPH antioxidant assay revisited. Food Chem 113: 1202-1205, 2009.

Sharma SB, Nasir A, Prabhu KM, Murthy PS. Antihyperglycemic effect of the fruit-pulp of *Eugenia jambolana* in experimental diabetes mellitus. J Ethnopharmacol 104: 367-373, 2006.

Shi X, Ye J, Leonard S, Ding M, Vallyathan V, Castranova V, Rojanasakul Y, Dong Z. Antioxidant properties of (-)-epigallocatechin-3-gallate and its inhibition of Cr(VI)-induced DNA damage and Cr(IV)-or TPA-stimulated NF-κB activation. Mol Cell Biochem 206: 125-132, 2001.

Shih PH, Yeh CT, Yen GC. Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. J Agric Food Chem *55*: 9427-9435, 2007

Shimada KK, Fujikawa KY, Nakamura T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J Agric Food Chem 40: 945-948, 1992.

Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 99: 483-487, 1999.

Shinoura N, Yoshida Y, Nishimura M, Muramatsu Y, Asai A, Kirino T, Hamada H. Expression level of Bcl-2 determines anti- or proapoptotic function. Cancer Res 59: 4119-4128, 1999.

Short S, Merkel BJ, Caffrey R, McCoy KL. Defective antigen processing correlates with a low level of intracellular glutathione. Eur J Immunol 26: 3015-3020, 1996.

Shull S, Heintz NH, Periasamy M, ManoharM, Janssen YMW, Marsh JP, Mossman BT. Differential regulation of antioxidant enzymes in response to oxidants. J Biol Chem 266: 24398-24403, 1991.

Siems W, Sommerburg O, Schild L, Augustin W, Laghans C-D, Wiswedel I. β-carotene cleavage products induce oxidative stress in vitro by impairing mitochondrial respiration. FASEB J 16: 1289-1291, 2002.

Sies H, Sharov VS, Klotz L, Briviba K. Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. J Biol Chem 272: 27812-27817, 1997.

Sies H, Stahl W, Sundquist AR. Antioxidant functions of vitamins E and C, β-carotene and other carotenoids. Ann NY Acad Sci 368: 7-19, 1992.

Sies H. Strategies of antioxidant defence. Eur J Biochem 215: 213-219, 1993.

Singh RP, Murthy KNC, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J Agric Food Chem 50: 81-86, 2002.

Sipes IG, Krishna G, Gillette JR. Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: role of cytochrome P450. Life Sci 20: 1541-1548, 1977.

Skrzydlewska E, Ostrowska J, Stankiewicz A, Farbiszewski R. Green tea as a potent antioxidant in alcohol intoxication. Addict Biol 7: 307-314, 2002.

Slater TF. Free-radical mechanisms in tissue injury. Biochem J 222: 1-15, 1984.

Smith CV, Jones DP, Guenthner TM, Lash LH, Lauterburg BH. Compartmentation of glutathione: implications for the study of toxicity and disease. Toxicol Appli Pharmacol 140: 1-12.

Smith M, Marley K, Seigler D, Singletary K, Meline B. Bioactive properties of wild blueberry fruits. J Food Sci 65: 352-356, 2000.

Somaatmadja D, Powers JJ, Hamdy MK. Anthocyanins.VI. Chelation studies on anthocyanins and other related compounds J Food Sci 29: 655-660, 1964.

Southon S, Faulks R. Predicting the bioavailability of antioxidants in food: the case of carotenoids. In: Pokorny J, Yanishlieva N, Gordon M, Eds. *Antioxidants in foods,* London, U.K: CRC Woodhead publishing Ltd, p125. 2001.

Spencer CC, Howell CE, Wright AR, Promislow DE. Testing an 'aging gene' in long-lived Drosophila strains: Increased longevity depends on sex and genetic background. Aging Cell 2: 123-130, 2003.

Spormann TM, Albert FW, Rath T, Dietrich H, Will F, Stockis J-P, Eisenbrand G, Janzowski C. Anthocyanin/polyphenolic–rich fruit juice reduces oxidative cell damage in an intervention study with patients on hemodialysis. *Can Epidemiol Biomarkers Preven* 17: 3372-3380, 2008.

Sridhar SB, Sheetal UD, Pai MRSM, Shastri MS. Preclinical evaluation of the antidiabetic effect of *Eugenia jambolana* seed powder in streptozotocin-diabetic rats. Brz J Med Biol Res 38: 463-468, 2005.

Sriram N, Kalayarasan S, Sudhandiran G. Epigallocatechin-3-gallate augments antioxidant activities and inhibits inflammation during bleomycin-induced experimental pulmonary fibrosis through Nrf2–Keap1 signaling. Pulm Pharmacol Therap (In press, 2009).

Stahl W, Sies H. Lycopene: a biologically important carotenoid for humans? Arch Biochem Biophys 336: 1-9. 1996.

Stapleton E, Walbot V. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiol 105: 881-889, 1994.

Steele VE, Kelloff GJ, Balentine D, Boone CW, Mehta R, Bagheri D, Sigman CC, Zhu S, Sharma S. Comparative chemopreventive mechanisms of green tea, black tea and selected polyphenol extracts measured by in vitro bioassays. Carcinogenesis 21: 63-67, 2000.

Steller H. Mechanisms and genes of cellular suicide. Science 267: 1445–1449, 1995.

Stintzing FC, Stintzing AS, Carle R, Frei B, Wrolstad RE. Color and antioxidant properties of cyaniding-based anthocyanin pigments. J. Agric. Food Chem. 50: 6172-6181.

Strasser A, O'Connor L, Dixit VM. 2000. Apoptosis signaling. Annu Rev Biochem 69: 217-245, 2002.

Strober W. Trypan blue exclusion test of cell viability. Curr Proto Immunol **A**ppendix 3: Appendix 3B, 2001.

Su J, Fan C. Antioxidant activity and mechanism of isolated components from flowers of *Delonix regia*. In: Whitaker JR, Haard NF, Shoemaker CF, Singh RP, Eds. Texas, USA: Food for Health in the Pacific Rim, Culinary and Hospitality Industry Publication Services, p243-252. 1997.

Subramaniam R, Roediger F, Jordan B, Mattson MP, Keller JN, Waeg G, Butterfield DA. The lipid peroxidation product, 4-hydroxy-2-trans-nonenal, alters the conformation of cortical synaptosomal membrane proteins. J Neurochem 69: 1161-1169, 1997.

Sugisawa A, Umegaki K. Physiological concentrations of (-)-epigallocatechin-3-O-gallate (EGCg) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. J Nutr 132:1836-1839, 2002.

Sumantran VN, Zhang R, Lee DS, Wicha MS. Differential regulation of apoptosis in normal versus transformed mammary epithelium by lutein and retinoic acid. Cancer Epidemiol Biomarkers Prev 9: 257-263, 2000.

Sun J, Tower J. FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend thelifespanof adult Drosophila melanogaster flies. Mol Cell Biol 19: 216–228, 1999.

Sun X, Majumder P, Shioya H, Wu F, Kumar S, Weichselbaum R, Kharbanda S, Kufe D. Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species. J Biol Chem 275: 17237–17240, 2000.

Swartz LM, Smith SW, Jones MEE, Osborne BA. Do all cell deaths occur via apoptosis? Proc Natl Acad Sci USA 90: 980-984, 1993.

Symons AM, King LJ. Inflammation, reactive oxygen species and cytochrome P450. Inflammopharmacol 11: 75-86, 2003.

Takahashi K, Newburger PE, Cohen HJ. Glutathione peroxidase protein. Absence in selenium deficiency states and correlation with enzymatic activity. J Clin Invest 77: 1402-1404, 1986.

Tate Jr. DJ, Miceli MV, Newsome DA. Phagocytosis and $H_2O_2$ induce catalase and metallothionein gene expression in human retinal pigment epithelial cells. Invest Ophtalmol Vis Sci 36: 2856-2864, 1990.

Teixeira CC, Pinto LP, Kessler FH, Knijnik L, Pinto CP, Gastaldo GJ, Fuchs FD. The effect of *Syzygium cumini* (L.) skeels on post-prandial blood glucose levels in non-diabetic rats and rats with streptozotocin-induced diabetes mellitus. J Ethnopharmacol 56: 209-213, 1997.

Teixeira HD, Schumacher RI, Meneghini R. Lower intracellular hydrogen peroxide levels in cells overexpressing CuZnsuperoxide dismutase. Proc Natl Acad Sci USA 95: 7872-7875, 1998.

Terao J, Piskula M, Yao Q. Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch Biochem Biophys 308: 278-284, 1994.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. Cell 81: 801-809, 1995.

Thomas SH. 1993. Paracetamol (acetaminophen) poisoning. Pharmacol Ther 60: 91–120.

Thomson JA, Schullek KM, Turnipseed SB, Ross D. 1995. Role of cytochrome P450 in the metabolism and toxicity of hydroperoxides in isolated rat hepatocytes. Arch Biochem Biophys 323: 463-470.

Thor H, Hartzell P, and Orrendjs, S. Potentiation of oxidative cell injury in hepatocytes which have accumulated calcium. J Biol Chem 259: 6612- 6615, 1984.

Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 281: 1312-1316, 1998.

Toth I, Rogers JT, McPhee JA, Elliott SM, Abramson SL, Bridges KR. Ascorbic acid enhances iron-induced ferritin translation in human leukemia and hepatoma cells. J Biol Chem 270: 2846-2852, 1995.

Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells-implications in cardiovascular disease. Braz J Med Biol Res 37: 1263-1273, 2004.

Toyokuni S, Itani T, Morimitsu Y, Okada K, Ozeki M, Kondo S, Uchida K, Osawa T, Hiai H, Tashiro T. Protective effect of colored rice over white rice on Fenton reaction based renal lipid peroxidation in rats. Free Radic Res 36: 583-592, 2002.

Treuting PM, Linford NJ, Knoblaugh SE, Emond MJ, Morton JF, Martin GM, Rabinovitch PS, Ladiges WC. Reduction of age-associated pathology in old mice by overexpression of catalase in mitochondria. J Gerontol A Biol Sci Med Sci 63: 813-824, 2008.

Trudeau VL. Drouin CE, Panahi M, Arnason, J. T. Foster, BC, Budzinski JW. Modulation of human cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp) in Caco-2 cell monolayers by selected commercial-source milk thistle and goldenseal products. Can J Physiol Pharmacol 85: 966-978, 2007.

Trumbo PR, Ellwood KC. Lutein and zeaxanthin intakes and risk of age-related macular degeneration and cataracts: an evaluation using the Food and Drug Administration's evidence-based review system for health claims. Am J Clin Nutr 84: 971-974, 2006.

Tsai P-J, Hsieh Y-Y, Huang T-C. Effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system using $^{17}O$ NMR. J Agric Food Chem 52: 3097-3099, 2004.

Tsuchihashi H, Kigoshi M, Iwatsuku M, Niki E. Action of β-carotene as an antioxidant against lipid peroxidation. Arch. Biochem. Biophys 323: 137-147, 1995.

Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, Iigo M, Takasuka N, Moore MA. Cancer prevention and natural compounds. Drug Metabol Pharmacokinet 19: 245-263, 2004.

Tsuda T, Horio F, Osawa T. Role of anthocyanins as an antioxidant under oxidative stress in rats. Biofactors 13: 133-139, 2000.

Tsuda T, Watanabe M, Ohshima K, Norinobu S, Choi SW, Kawakishi S, Osawa T. Antioxidative activity of the anthocyanin pigments cyanidin 3-O-β-D-glucoside and cyanidin. J Agric Food Chem 42: 2407-2410, 1994.

Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. FEBS Lett 466: 6-10, 2000.

Turkmen N, Sari F, Velioglu YS. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. Food Chem 99: 835-841, 2006.

Tyurina YY, Tyurin VA, Carta G, Quinn PJ, Schor NF, Kagan VE. Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells. Arch Biochem Biophys 344: 413-423, 1997.

Ugalde C, Liu Z, Ren C, Chan K, Rodrigo K, Ling Y, Larsen P, Chacon G, Stoner G, Mumper R, Fields H, Mallery S. Bioadhesive black raspberry gel following topical intraoral application in normal healthy volunteers. Pharm Res 26: 977-986, 2009.

Uwe W, Angelika H, Sabine K, Hannelore D. 2004. Protein expression profiling identifies molecular targets of quercetin as a major dietary flavonoids in human colon cancer cells. Proteomics 4: 2160-2174.

Valcheva-Kuzmanova S, Borisova P, Galunska B, Krasnaliev I, Belcheva A. Hepatoprotective effect of the natural fruit juice from *Aronia melanocarpa* on carbontetrachloride-induced acute liver damage in rats. Experimental and Toxicologic Pathol 56: 195–201, 2004.

Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266: 37-56, 2004.

van Acker S, van den Berg DJ, Tromp MN, Griffionen DH, van Bennekom WP, van der Vugh WJF, Bast A. Structural aspects of antioxidant activity of flavonoids. Free Rad Biol Med 20: 331-342, 1996.

van Breeman RB. Liquid chromatography/mass spectrometry of carotenoids. Pure Appl Chem 69: 2061-2066, 1997.

van der Woude H, Gliszczyńska-Swigło A, Struijs K, Smeets A, Alink GM, Rietjens IM. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett 200: 41-47, 2003.

van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31: 1-9, 1998.

van Erk MJ, Roepman P, van der Lende TR, Stierum RH, Aarts JMMJG, van Bladeren PJ, van Ommen B. Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer–related mechanisms in colon cancer cells *in vitro*. Eur J Nutr 44: 143-156, 2005.

Vaux DL, Strasser A. The molecular biology of apoptosis. Proc Natl Acad Sci USA 93: 2239-2244, 1996.

Veigas JM, Narayan MS, Laxman PM, Neelwarne B. Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *Syzygium cumini* Skeels. Food Chem 105: 619-627, 2007.

Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J Agric Food Chem 46: 4113-4117, 1998.

Venkateswarlu G. On the nature of the clouring matter of the jambul fruit (*Eugenia jambolana*). Journal of Indian Chem Soci 29: 434-437, 1952.

Vijayaraghavan R, Gautam A, Sharma M, Satish HT, Pant SC, Ganesan K. Comparative evaluation of some flavonoids and tocopherol acetate against the systemic toxicity induced by sulphur mustard. Ind J Pharmacol 40: 114-120, 2008.

Vina J, Borras C, Gomez-Cabrera M-C, Orr WC. Part of the Series: From dietary antioxidants to regulators in cellular signalling and gene expression. Role of reactive oxygen species and (phyto)oestrogens in the modulation of adaptive response to stress. Free Radic Res 40: 111–119, 2006.

Vitaglione P, Morisco F, Caporaso N, Fogliano V. Dietary antioxidant compounds and liver health. Crit Rev Food Sci Nutr 44: 575–586, 2004.

Walling C. Fenton's reagent revisited. Acc Chem Res 8: 125-131, 1975.

Wang C-J, Wang J-M, Lin W-L, Chu C-Y, Chou F-P, Tseng T-H. Protective effect of Hibiscus anthocyanins against *tert* -butyl hydroperoxide-induced hepatic toxicity in rats. Food Chem Toxicol 38: 411-416, 2000.

Wang L-S, Hecht SS, Carmella SG, Yu N, Larue B, Henry C, McIntyre C, Rocha C, Lechner JF, Stoner GD. Anthocyanins in black raspberries prevent esophageal tumors in rats. *Cancer Prevention Res* 2: 84. 2009. doi: 10.1158/1940-6207.CAPR-08-0155

Wang SY, Ballington JR. 2007. Free radical scavenging capacity and antioxidant enzyme activity in deerberry (*Vaccinium stamineum* L.). LWT 40: 1352-1361.

Wang XD, Liu C, Bronson RT, Smith DE, Krinsky NI, Russell RM. 1999. Retinoid signaling and activator protein-1 expression in ferrets given β-carotene supplements and exposed to tobacco smoke. *J Natl Cancer Inst* 91: 60–66.

Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinogenesis 5: 14, 2006.

Wayner DD, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. FEBS Lett 187: 33-37, 1985.

Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev Toxicol 33: 105-136, 2003.

Weisel T, Baum M, Eisenbrand G, Dietrich H, Will F, Stockis J, Kulling S, Rufer C, Johannes C, Janzowski C. An anthocyanin/polyphenol-rich fruit juice reduces oxidative DNA damage and increases glutathione level in healthy probands. Biotechnol J 1: 388-397, 2006.

Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metabol Disp 31: 1035-1042, 2003.

Willett WC. Diet and health: what should we eat? Science 254: 532-537, 1994.

Willett WC. Balancing life-style and genomics research for disease prevention. Science 296: 695-698, 2002.

Williams GM, Laspia MF, Dunkel VC. Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens. Mutat Res 97: 359-370, 1982.

Williams GT. Programmed cell death: apoptosis and oncogenesis. Cell 65: 1097-1098, 1991.

Willingham MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem 47: 1101-1109, 1999.

Wolf CR, Harrelson WG, Nastainczyk WM, Philpot RM, Kalyanaraman B, Mason RP. Metabolism of carbontetra chloride in hepatic microsomes and reconstituted monooxygenase systems and its relationship to lipid peroxidation. Mol Pharmacol 18: 553-558, 1980.

Wong SP, Leong LP, William KJH. Antioxidant activities of aqueous extracts of selected plants. Food Chem 99: 775-783, 2006.

Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J, Blaauboer BJ. The isoenzyme pattern of cytochrome P450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and in intact monolayers. Biochem Pharmacol 42: 381-390, 1991.

Wrolstad RE, Skrede G, Lea P, Enersen G. Influence of sugar on anthocyanin pigment stability in frozen strawberries. J Food Sci 55: 1064-1066, 1990.

Wrolstad RE. 2003. Anthocyanin pigments-Bioactivity and coloring properties. In: Symposium 12-Interaction of natural color with other ingredients. Presented at the 12th World Congress of Food Science and Technology, July 16-20, Chicago, USA.

Wu G, Fang Y, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 134: 489-492, 2004.

Wu X, Beecher G, Holden JM, Haytowitz D, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J Agric Food Chem 54: 4069-4075, 2006.

Xia M, Ling W, Zhu H, Ma J, Wang Q, Hou M, Tang Z, Guo H, Liu C, Ye Q. Anthocyanin attenuates CD40-mediated endothelial cell activation and apoptosis by inhibiting CD40-induced MAPK activation. Atherosclerosis 202: 41-47, 2009.

Yang B, El Nahas AM, Fisher M, Wagner B, Huang L, Storie I, Barnett D, Barratt J, Smith AC, Johnson TS. Inhibitors directed towards caspase-1 and -3 are less effective than pan caspase inhibition in preventing renal proximal tubular cell apoptosis**.** Nephron Exp Nephrol 96: e39-e51, 2004.

Yang GY, Liao J, Li C, Chung J, Yurkow EJ, Ho CT, Yang CS. Effect of black and green tea polyphenols on c-jun phosphorylation and $H_2O_2$ production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. Carcinogenesis 21: 2035-2039, 2000.

Yao K, Ye P, Zhang L, Tan J, Tang Z, Zhang Y. Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells. Mol Vis 14: 217-223, 2008.

Yawadio R, Morita N. Color enhancing effect of carboxylic acids on anthocyanins. Food Chem 105: 421-427, 2007.

Yeh C-T, Yen G-C. Induction of apoptosis by the anthocyanidins through regulation of Bcl-2 gene and activation of c-Jun N-Terminal Kinase cascade in hepatoma cells. J Agric Food Chem *53*: 1740-1749, 2005.

Yen G, Duh P, Su H, Yeh C, Wu C. Scavenging effects of lotus seed extracts on reactive nitrogen species. Food Chem 94: 596-602, 2006.

Yildiz L, Baris S, Aydin O, Kefeli M, Kandemir B. Bcl-2 positivity in B and C hepatitis and hepatocellular carcinoma. Hepatogastroenterol 55: 2207-2210, 2008.

Ying-Jie W, Meng-Dong L, Yu-Min W, Jian D, Qing-He N. Simplified isolation and spheroidal aggregate culture of rat hepatocytes. World J Gastroenterol 4: 74-76, 1998.

Yokoo T, Kitamura M. Unexpected protection of glomerular mesangial cells from oxidant-triggered apoptosis by bioflavonoid quercetin. Am J Physiol 273: F206-F212, 1997.

Yoo JH, Erzurum SC, Hay JG, Lemarchand P, Crystal RG. Vulnerability of the human airway epithelium to hyperoxia. Constitutive expression of the catalase gene in human bronchial epithelial cells despite oxidant stress. J Clin Invest 93: 297-302, 1994.

Yoshida T, Maulik N, Engelman RM, Ho YS, Magnenat JL, Rousou JA, Flack JE, III, Deaton D, Das DK. Glutathione peroxidase knockout mice are susceptible to myocardial ischemia reperfusion injury. Circulation 96: II-20, 1997.

Yoshimoto M, Okuno S, Yamaguchi M, Yamakawa O. Antimutagenicity of deacetylated anthocyanins in purple-fleshed sweetpotato. Biosci Biotechnol Biochem 65: 165-1655, 2001.

Yoshimura S, Banno Y, Nakashima S, Hayashi K, Yamakawa H, Sawada M, Sakai N, Nozawa Y. Inhibition of neutral sphingomyelinase activation and ceramide formation by glutathione in hypoxic PC12 cell death. J Neurochem 73: 675-683, 1999.

Youdim KA, Joseph JA. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. Free Radic Biol Med 30: 583-594, 2001.

Youdim KA, Martin A, Joseph JA. Incorporation of the elderberry anthocyanins by endothelial cells increases protection against oxidative stress. Free Radic Biol Med 29: 51-60, 2000.

Yu C, Wang F, Jin C, Wu X, Chan W, McKeehan WL. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFR4-deficient mice. Am J Pathol 161: 2003-2010, 2002.

Yuan J-M, Stram DO, Arakawa K, Lee H-P, Yu MC. Dietary cryptoxanthin and reduced risk of lung cancer. The Singapore Chinese Health Study. Cancer Epidemiol Biomark Preven 12: 890-898, 2003.

Yumei F, Zhou Y, Zheng S, Chen A. The antifibrogenic effect of (-)-epigallocatechin gallate results from the induction of de novo synthesis of glutathione in passaged rat hepatic stellate cells. Lab Invest 86: 697-709, 2006.

Zamai L, Canonico B, Luchetti F, Ferri P, Melloni E, Guidotti L, Cappellini A, Cutroneo G, Vitale M, Papa S. Supravital exposure to propidium iodide identifies apoptosis on adherent cells. Cytometry 44: 57-64, 2001.

Zapolska-Downar D, Nowicka G, Sygitowicz G, Jarosz M. Anthocyanin-rich Aronox extract from Aroniamelanocarpa E protects against 7β-hydroxycholesterol-induced apoptosis of endothelial cells. Ann Nutr Metab 53: 283-294, 2008.

Zhang L, Jie G, Zhang J, Zhao B. Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress. Free Radic Biol Med 46: 414-421, 2009.

Zhang S-Z, Lipsky MM, Trump BF, Hsu I-C. Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. Cell Biol Toxicol 6: 219-234, 1990.

Zhao X, Aldini G, Johnson EJ, Rasmussen H, Kraemer K, Woolf H, Musaeus N, Krinsky NI, Russell RM, Yeum K-J. Modification of lymphocyte DNA damage by carotenoid supplementation in postmenopausal women. Am J Clin Nutr 83: 163-169, 2006.

Zhou LZ, Johnson AP, Rando TA. NFκB and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radic Biol Med 31: 1405-1416, 2001.

Zhou LZ, Johnson AP, Rando TA. NFκB and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radic Biol Med 31: 1405-1416, 2001.

Zhu YZ, Huang SH, Tan BKH, Sun J, Whiteman M, Zhu YC. Antioxidants in Chinese herbal medicines: a biochemical perspective. Nat Prod Rep 21: 478-489, 2004.

Zimmerman HJ, Maddrey WC. Acetaminophen (paracetamol) hepatotoxicity with regular intake of alcohol: analysis of instances of therapeutic misadventure, Hepatol 22: 767-773, 1995.

Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates caspase-9. J Biol Chem 274: 11549-11556, 1999.