STUDIES ON ANTIOXIDANTS AND ANTIMETASTATIC COMPONENTS FROM SPICES AGAINST CANCER

A thesis submitted to the

University of Mysore

for the award of the Degree of

Doctor of Philosophy

in

Biochemistry

by

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DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON ANTIOXIDANTS AND ANTIMETASTATIC COMPONENTS FROM SPICES AGAINST CANCER"** submitted to the **University of Mysore** for the award of degree of **Doctor of Philosophy** in **Biochemistry,** is the result of research work carried out by me under the guidance of Dr. Shylaja M Dharmesh, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore - 570020, India, during the period 2000-07. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date:
Place: Mysore

(M.A. Harish Nayaka)

Dr. Shylaja M Dharmesh, PhD
Scientist,
Department of Biochemistry and Nutrition

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON ANTIOXIDANTS AND ANTIMETASTATIC COMPONENTS FROM SPICES AGAINST CANCER"** submitted by Mr. M. A. Harish Nayaka, for the award of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore** is the result of research work carried out by him in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, under my guidance during the period 2000-07.

Date:
Place: Mysore

(Shylaja M Dharmesh)
(Guide)

Dedicated to

all the ***Devoted Scientists*** *across time who have helped us to realize life*

and to my ***beloved teachers.***

Acknowledgements

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Contents

Abbreviations and units

λ	-	Lambda
°C	-	Degree centigrade
µg	-	Microgram
µL	-	Microliter
µM	-	Micromolar
2D NMR	-	Two-dimensional nuclear magnetic resonance
ALP	-	Alkaline phosphatase
AMC	-	Antimetastatic component
ANOVA	-	Analysis of variance
AOA	-	Antioxidant activity
AOX	-	Antioxidant
APAF1	-	Apoptotic protease-activating factor 1
ATP	-	Adenosine triphosphate
AU	-	Absorbance units
B[a]P	-	Benzo[a] pyrene
BC	-	Black cumin
BCAE	-	Black cumin aqueous extract
BCD	-	Black cumin decoction
BCPP	-	Black cumin pectic polysaccharide
BHA	-	Butylated hydroxyl anisole
BHT	-	Butylated hydroxyl toluene
bw	-	Body weight
cAMP	-	Cyclic adenosine monophosphate
CAT	-	Catalase
CDK	-	Cyclin dependent kinase
CPP	-	Citrus pectic polysaccharide
CRD	-	Carbohydrate recognition domain
CRD	-	Carbohydrate recognition domain
CREB	-	cAMP response element binding protein
Crtl	-	Control

Cul3	-	Cullin 3
CVD	-	Cardiovascular diseases
Cyt-c	-	Cytochrome c
Da	-	Dalton
DEAE	-	Diethylaminoethyl
DHG	-	Dehydrogenase
DMEM	-	Dulbecos minimum essential medium
DMRT	-	Duncan's multiple range test
DNA	-	Deoxyribonucleic acid
DPPH	-	1, 1-Diphenyl-2-picryl hydrazyl
DTNB	-	5, 5'-Dithionitrobenzoic acid
ECM	-	Extracellular matrix
EDTA	-	Ethylenediaminetetraacetic acid
EGCG	-	Epigallocatechin
ELISA	-	Enzyme-linked immunosorbent assay
ER	-	Endoplasmic reticulum
ERK	-	Extracellular signal-regulated protein kinase
EtBr	-	Ethidium bromide
ETC	-	Electron transport chain
FAD	-	Flavin adenine dinucleotide
$FADH_2$-Ox	-	1, 5-Dihydro-flavin adenine dinucleotide oxidase
FCS	-	Fetal calf serum
FMN	-	Flavin mononucleotide
g	-	Gram
g	-	g force
GAE	-	Gallic acid equivalent
GLC	-	Gas liquid chromatography
GPx	-	Glutathione peroxidase
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathione
GST	-	Glutathione-S-transferase

h	-	Hour
HBV	-	Hepatitis B virus
HEPES	-	(4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid)
HIV	-	Human immunodeficiency virus
HMBA	-	2-Hydroxy-4-methoxybenazldehyde
HO1	-	Heme oxygenase 1
HPLC	-	High performance liquid chromatography
HPV	-	Human papilloma virus
HSA	-	Human serum albumin
IC_{50}	-	Half maximal inhibitory concentration
IU	-	International unit
JNK	-	c-Jun N-terminal kinase
KEAP1	-	Kelch-like ECH-associating protein 1
Kg	-	Killogram
K_{SV}	-	Stern-Volmer constant
$L^{\bullet}$	-	Lipid radical
LDL	-	Low density lipoprotein
$LOO^{\bullet}$	-	Lipid peroxyl radical
M	-	Molar
MAPK	-	Mitogen-activated protein kinase
MCA	-	20-Methylcholanthrene
MDA	-	Malondialdehyde
MEM	-	Minimum essential medium
mg	-	Milligram
MIC	-	Minimum inhibitory concentration
min	-	Minute
mL	-	Milliliter
mM	-	Millimolar
MMP	-	Matrix metallo proteases
MMP	-	Matrix metalloproteinase
MRP1	-	Multidrug resistance associated protein 1
MTT	-	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium

N	-	Normality
NAD^+	-	Nicotinamide adenine dinucleotide (oxidized)
NADH	-	Nicotinamide adenine dinucleotide (reduced)
NADP	-	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
NADPH-Ox	-	Reduced nicotinamide adenine dinucleotide phosphate oxidase
NBT	-	Nitro blue tetrazolium
NF-kB	-	Nuclear factor Kappa B
nm	-	Nanometer
NMR	-	Nuclear magnetic resonance
NRF2	-	Nuclear factor E2-related factor 2
OD	-	Optical density
Ox	-	Oxidase
PB	-	Phosphate buffer
PBS	-	Phosphate buffer saline
PERK	-	Double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase
PI3K	-	Phosphatidylinositol 3-kinase
PKC	-	Protein kinase C
PKR	-	RNA dependent protein kinase
PNPP	-	Paranitrophenyl phosphate
PUFA	-	Polyunsaturated fatty acid
ROS	-	Reactive oxygen species
ROS	-	Reactive oxygen species
SCC	-	Squamous cell carcinoma
SD	-	Standard deviation
SGOT	-	Serum glutamate oxaloacetate transaminase
SGPT	-	Serum glutamate pyruvate transaminase
SOD	-	Superoxide dismutase
SR	-	Swallow root
SRAE	-	Swallow root aqueous extract

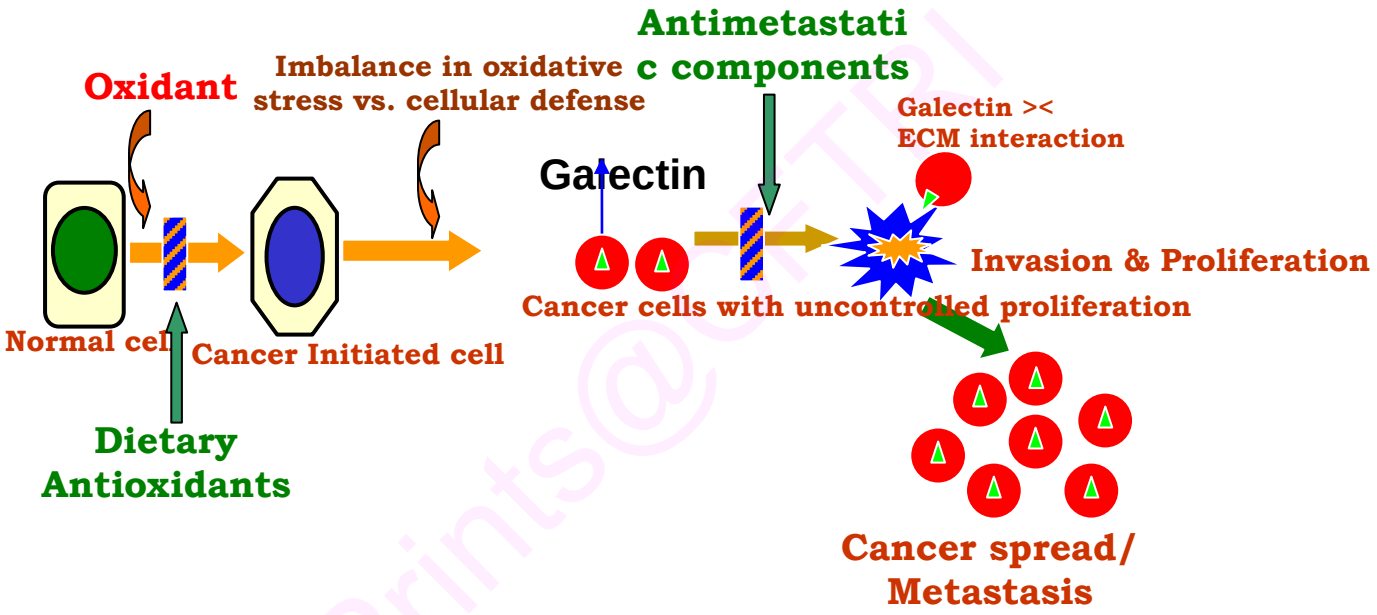
SRBP	-	Swallow root bound phenolic acids
SRFP	-	Swallow root free phenolic acids
SRHP	-	Swallow root hydrolyzed phenolic acids
SRPP	-	Swallow root pectic polysaccharide
TBA	-	2-Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TCA	-	Trichloroacetic acid
TMP	-	1, 1, 3, 3-Tetramethoxy propane
TMS	-	Tetramethylsilane
UV	-	Ultraviolet
v-onc	-	Viral oncogene
w/v	-	Weight/Volume
w/w	-	Weight/Weight

Abstract

Thesis Title: **Studies on Antioxidants and Antimetastatic Components from Spices Against Cancer**

Cancer is a complex multi-step disease, rising constantly in both developing and developed countries leading to mortality. Developing a strategy for cancer therapy has been difficult since cancer cell poses lots of challenges to normal cells by self-regulating their survival and dysregulating normal cellular action. Antioxidants, quenchers of free radicals - that initiates cancer, chemo/radio therapeutics, that inhibit proliferative cancer cell, surgery/immuno therapies that envisage the removal of the affected tissue/cell, have been in practice for cancer therapy; yet cancer cells find their way successful due the limitations encountered in these therapies such as lack of discrimination between cancer cell and a normal cell. Current thesis entitled "**Studies on Antioxidants and Antimetastatic Components from Spices Against Cancer"** explores the possibility of dietary antioxidants and antimetastatic polysaccharides that can effectively bring down oxidative stress and target galectin-3, a key molecule in metastasis causing severity of cancer pathogenicity and death as revealed in scheme - 1. During the study, potential water soluble antioxidants and antimetastatic polysaccharides from Black cumin – BC - *Nigella Sativa* and Swallow root – SR- *Decalepis hamiltonii* have been identified, characterized and determined their efficacy *in vitro* and *in vivo*. Results indicated tannic acid and gentisic acid followed by gallic, protocatechuic, and vanillic acid as major multi-potent antioxidants in BC and SR respectively. SR also found to contain higher levels of 2-Hydroxy-4-methoxybenzaldehyde (HMBA) but contributing little to the activity. A potent galectin-3 inhibitory pectic polysaccharide was also identified in SR - SRPP and structure – function analysis reveal the presence of higher levels of galactose and arabinose and their probable contribution to antimetastatic potency. Antioxidants of BC

and SR and antimetastatic SRPP was effective in inhibiting Reactive oxygen species - mediated hepatotoxicity/cervical cancer and B16F10 melanoma cells induced lung metastasis *in vivo*, suggesting their *in vivo* efficacy against cancer. Overall data thus suggest that selected sources have both antioxidants and antimetastatic components that can effectively inhibit cancer spread/metastasis (Scheme-1).



Scheme-1: Cancer process with initiation, transformation, upregulation of galectin-3, initiation of interaction between cancer cell and normal cell leading to cancer spread / metastasis; Potent blockade by dietary antioxidants and antimetastatic components

Synopsis of the thesis submitted for the award of PhD degree (Biochemistry) of the University of Mysore, India.

Title: **Studies on antioxidants and antimetastatic components from spices against cancer**

Candidate: M. A. Harish Nayaka

Cancer has been identified as a major devastating disease among populations of both developing and developed countries. Diet and tobacco are two major factors contributing 35 % each to the total disease incidence of 70 %. Tobacco induced cancers have been predicted to come down due to awareness in the public. However, the same is not possible in diet causing cancers - i.e., lacunae in appropriate diet, due to socio-economic problems. The exogenous supply of antioxidants has been the choice to manage the disease. However, the dietary supplementation trials with vitamin antioxidant and antioxidants from green tea showed only a partial success. Recently, the use of dietary antimetastatic components has elicited promises in cancer disease management. Although several antimetastatic components have been reported, galactose rich polysaccharides appear to inhibit the metastasis effectively by blocking the initial stage of galectin mediated cancer and normal cell interaction, a crucial event in metastasis and tumor spread. Presence of such components in spices and the role of spices antioxidants, the efficiency of combinational supplementation of antioxidants and antimetastatic components against cancer are to be elucidated.

The main objective of the proposed research is to understand the efficacy of combinational supplementation of antioxidants and antimetastatic components from spices against cancer. Following objectives have been proposed.

Objectives

1. **Isolation and characterization of newer antioxidants from black cumin and swallow root.**

2. **Isolation and characterization of antimetastatic components from spices**

3. **Determination of efficacy and mechanism of action of antioxidants and antimetastatic components using *in vitro* biochemical and cell culture assay model systems**

4. **Efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing *in vivo* cancer animal models**

The research work carried towards achieving these objectives makes the subject matter of the thesis. The thesis is divided in to 5 chapters, and contents provided in each chapter is highlighted as follows:

Chapter I

This chapter begins with a general account on the cancer and cancer incidences as well as the role of dietary agents, in particular spices in cancer prevention. The role of dietary antioxidants, their source and mechanism of anticancer effect has been mentioned in the chapter. A brief introduction to the biochemical aspects starting from free radical generation, their effect on biomolecules, ultimately leading to cancer initiation, progression and metastasis has been depicted. The problem of metastasis and the necessity for dietary antimetastatic compounds also has been highlighted.

Further, various cancer treatments mainly surgery, radiation therapy and chemotherapy and their drawbacks have been emphasized in order to justify for the need for the alternatives which is the main goal of the thesis. The chapter also provides an overview of current literature on black cumin (*Nigella sativa*) and swallow

root (*Decalepis hamiltonii*), the spice sources selected for the proposed investigation. End of this chapter also highlights the needs-aims and scope of the current study.

Subsequent chapters - chapter II to V deals objectives 1 - 4, respectively. These chapters have a uniform format-depicting hypothesis underlying the specific objective, work concept adapted, a brief introduction pertaining to the respective objectives, materials and methods, results, discussion and a relevant summary and conclusions highlighting the important outcome of the chapter. Literatures cited, references for methodologies are provided as references at the end of the thesis.

Chapter II deals with Objective-1 - Isolation and characterization of newer antioxidants from black cumin (*Nigella sativa*) and swallow root (*Decalepis hamiltonii*).

Black cumin (BC) and swallow root (SR) sources were selected to look for newer water soluble antioxidants (AOX). During screening of some of the commonly consumed spices for antioxidant activity, BC and SR were two sources reportedly showing more antioxidant activity in aqueous extract than that of solvent extracts. Plenty of reports are available on lipid soluble antioxidants from spices. The biopotencies against health disorders are also reported. However the current need is to adjunct the lipid soluble with water soluble AOX since majority of the diseases are caused by oxidative stress (OS) and successful inhibition or prevention of the same *in vivo* necessitates both water and lipid soluble antioxidants. BC and SR with higher activity in aqueous extracts were selected. Also these are the two major spices used successfully in Ayurveda medicine particularly in the form of aqueous extracts. Studies were therefore initiated with the above objective - 1.

Results indicated higher antioxidant potency in both black cumin (BCAE) and swallow root (SRAE) water extracts as evident from various antioxidant assays in comparison with known standard antioxidants. Further, swallow root extracts showed 30-100 fold higher antioxidant activity in various assays including cytoprotective assay on NIH 3T3 fibroblasts. Attempts made to investigate potential

bioactive compound by HPLC analysis indicated tannic acid (77 %) and protocatechuic acid (23 %) in BC. In SR, however, since 30-fold higher phenolic content was noticed than BC and since the phenolics were also associated with polysaccharides, efforts were made to isolate free (SRFP), hydrolyzed (SRHP) and bound (SRBP) fractions of SR and activity was determined. Among the phenolic extracts of SR, SRHP exhibited higher activity followed by SRBP and SRFP.

Swallow root phenolic extracts contained both hydroxybenzoic and cinnamic acid derivatives as antioxidant molecules to different extent. Although HMBA was found to be a predominant component in SRAE, the bulk of the activity was contributed mainly from gentisic, gallic, protocatechuic, vanillic and *p*-coumaric acids. Earlier studies have shown the presence of HMBA as a major constituent in methanol extracts and in the volatile oil fraction of swallow root. However, the present investigation demonstrates HMBA also in aqueous extracts, which was further confirmed by NMR spectral analysis.

Health beneficial properties reportedly exhibited upon Ayurveda medications of consuming aqueous extracts of BC and SR could be from phenolic acids-tannic acid in BC, gentisic, gallic, protocatechuic and vanillic acids in SR, respectively. Although, these antioxidants are reported in other sources, it is newer in the selected sources, particularly in understanding their attributes to health beneficial properties.

Chapter III deals with Objective – 2 - Isolation and characterization of antimetastatic components from spices

Majority of cancer mortalities are due to metastasis, a process of cancer spread and secondary tumor formation. Galectin-3, a β-galactoside binding lectin present on the cancer cell surface has been implicated in tumor spread and metastasis, from studies of our laboratory and other investigators. Further, higher levels of galectin-3 correlated with the advancement of the cancer disease and it is believed that galectin-3 of cancer cells bind to normal cells and establishes secondary tumors. Recently the role of pectic polysaccharides has gained importance due to their key

participation in controlling cancer metastasis through the blockade of galectin-3 present on the metastatic cancer cells. In this chapter therefore, efforts have been made to isolate galectin-3 inhibitory pectic polysaccharides from black cumin and swallow root. Galectin-3 agglutinates red blood cells due to cross-linking by binding to galactose residues present on the red blood cells. Components that inhibit this agglutination or galectin-3 inhibitor therefore can be a potential antimetastatic compound. Results indicated 6.2 % yield of a pectic polysaccharide from SR with significant activity. A minimum inhibitory concentration (MIC) of 1.85 µg equivalent carbohydrate/mL with ~ 70 and 30 fold higher activity was observed for swallow root pectic polysaccharide - SRPP when compared to that of black cumin pectic polysaccharide - BCPP (130 µg equivalent carbohydrate/mL) and standard galactose (25 µg/mL). Further, SRPP was subjected to purification and separated into 4 fractions on DEAE cellulose column chromatography, of which 0.15 M ammonium carbonate eluted fraction showed higher antimetastatic activity. Characterization studies of this fraction revealed the presence of arabinose (66 %) and galactose (27 %) in its polysaccharide chain. The molecular weight of this pectic polysaccharide was found to be ~ 250 kDa. Comparison of sugar composition analysis with antimetastatic potency revealed that arabinose and galactose residues are important for galectin-3 blocking effect.

Chapter IV deals with Objective - 3 - Determination of efficacy and mechanism of action of antioxidants and antimetastatic components using *in vitro* biochemical and cell culture assay model systems

Chapter II and III provides potent antioxidant and antimetastatic compounds from black cumin and swallow root. In order for them to act, they need to interact with the host protein (human serum albumin) or DNA, in case of antioxidants and β-galactosides, galactose containing extra cellular matrix components of cells in case of antimetastatic polysaccharides.

Binding of antioxidants to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action.

Results presented in chapter IV suggest the different degree binding of BCAE and SRAE due to their constituted phenolic acids to both protein and DNA indicating their probable mechanism of protection a) by shielding from free radicals *in vivo* ; b) by enhancing their bioavailability and c) by limiting their degradation. Also inhibition of invasion of highly metastatic MDA-MB-231 cells and triggering of apoptosis was observed upon interaction of SRPP with galectin-3. Studies in chapter IV therefore substantiate the results of chapter II and III and provide additional evidence for their potential bioactivity.

Chapter V deals with the Objective - study of the efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing *in vivo* cancer animal models

This chapter deals with the antioxidant extracts of BC and SR and their *in vivo* efficacy using CCL_4 induced hepatotoxicity and 20 - methylcholanthrene induced cervical cancer models in female albino Wistar rats and Swiss albino mice, respectively. Also, the chapter deals with the *in vivo* antimetastatic potency of SRPP in addition to SR - antioxidants.

Results of CCl_4 toxicity studies indicated significant protection by BCAE and SRAE. About 50 - 62 % of antioxidant enzymes were modulated when compared to that of the CCl_4 induced group. No significant increases of liver function enzymes (SGPT, SGOT, ALP) were observed upon treatment with the extracts suggesting non-toxic nature of BC and SR extracts. BC and SR extracts also indicated protection against 20-methylcholanthrene induced cervical cancer.

Results from *in vivo* antimetastatic studies showed significant reduction (88 %) of lung metastases induced by B16F10 melanoma cells, upon treatment with SRPP. SRPP was effective in reducing superficial tumor nodules, implantation percentage, growth index and penetrability of cancer cells into the lung tissue indicating the effective inhibition of metastasis and invasion. Down regulation of galectin-3 levels -

a marker molecule for metastasis, suggest that SRPP is effective in blocking galectin-3 and galectin-3 mediated cell invasion and metastasis. SRPP treated animals also safeguarded antioxidant enzymes that could be attributed to the presence of bound phenolics in them. HMBA an antioxidant of SR did not show antimetastatic activity. Also antioxidant rich SR extract – SRAE did not potentiate the activity of SRPP suggesting their poor efficacy in inhibiting metastasis *in vivo*.

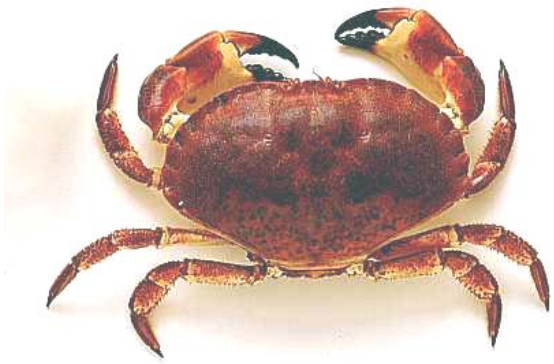
Overall research results of the thesis highlight the importance of antioxidants of BC and SR, more so the SR, in inhibiting oxidant mediated molecular and cellular damages that may lead to cancer. Besides, it also contained a potent antimetastatic polysaccharide that is effective in modulating galectin-3 mediated metastasis *in vitro* and *in vivo*. Less toxic nature of these dietary sources and specific targeting of SRPP to only cancer cell via their binding ability to galectin - 3 further emphasizes their potential role as alternatives in the management of cancer.

Candidate

M.A. Harish Nayaka

Guide

Dr. Shylaja M Dharmesh



Chapter I

General Introduction

General Introduction

Background

Cancer development is a long-term process that appears to proceed by step-by-step carcinogenesis events that ultimately spread from one area of the body to other parts of the body during the late metastasis stage. Current clinical therapies of cancer, which include surgery, radiation and chemotherapy, are limited, particularly during the terminal metastasis phase. However, there is increasing evidence from cancer epidemiological and pathological studies suggesting that many human cancers could be prevented or their progression slowed down [Weinstein, 1991]. Therefore, there might be opportunities for interference and prevention of cancer progression in the early stages of carcinogenesis by dietary phytochemicals. The need for biologically active dietary compounds with low profiles of adverse reactions compared to pharmacological drugs has led to an extensive investigation of dietary sources for phytochemicals and their mechanism of action in the current thesis.

Cancer statistics

Studies on worldwide cancer incidence, mortality and prevalence of cancers in the year 2002 are depicted in detail by compilation of observation from 20 large areas of the world and indicate 10.9 million new cases, 6.7 million deaths, and prevalence of 26 types of cancers. 24.6 million Persons are believed to be alive with cancer (within three years of diagnosis). The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million), and colorectal (1 million); the most common causes of cancer death are lung cancer (1.18 million deaths), stomach cancer (700,000 deaths), and liver cancer (598,000 deaths). Breast cancer appears to be the most prevalent type of cancer in the world (4.4 million survivors up to 5 years following diagnosis). Incidences also vary as per variations in geographical parameters, life style, environmental factors and occupational hazards have been known to be the causative factors for cancer.

The National Cancer Registry Program in Bangalore which used data from 105 hospitals and private clinics in 82 of the 593 districts in India to map the incidence of cancer, as part of a project funded by the World Health Organization, reports (Ganapati, 2005) the world's highest incidence of cancers of gall bladder, mouth, and lower pharynx in India. The atlas, produced by the Indian Council of Medical Research, has also found pockets of stomach and thyroid cancer in the southern parts of the country. The survey included more than 200 000 patients with histopathologically confirmed cancers. The age adjusted incidence of gall bladder cancer in women in New Delhi is 10.6 per 100 000 of the population—the world's highest rate for women for this cancer. Districts in central, south, and northeast India had the world's highest incidence of cancers associated with tobacco, which is chewed as well as smoked in India. Aizawl district in the northeastern state of Mizoram has been shown to be the world's highest incidence of cancers in men of the lower pharynx (11.5 per 100 000 people) and tongue (7.6 per 100 000 people) as per the atlas. This district also had the country's highest rate of stomach cancers among men. The incidence of mouth cancer among men in Pondicherry was 8.9 per 100 000, one of the highest rates in the world for men. Rates of stomach cancer were high among men in Bangalore and Chennai. The survey also detected "a belt of thyroid cancer" in women in coastal districts of Kerala, Karnataka, and Goa. The incidences also confirmed earlier observations that breast cancer has replaced cervical cancer as the leading site of cancer among women in Indian cities and that lung cancer is the most common cancer in men in Calcutta, Mumbai, and New Delhi (Sinha et al., 2003).

Approximately 35 % of all cancers worldwide are due to tobacco use, while many of the skin cancers are related to infectious exposures, such as HBV, HPV, HIV, and *Helicobacter pylori*. Research shows that about 35 to 40 % of all cancers worldwide are due to dietary factors and lack of physical activity. Hence by making suitable changes in the diet in addition to exercise, control of tobacco use etc., the incidence of cancer around the world has been expected to be reduced by 60-70 %.

Cancer – Carcinogenesis and Cancer Pathogenecity

Cell division or cell proliferation is a physiological process that occurs in almost all tissues and under many circumstances. Normally the balance between proliferation and programmed cell death is tightly regulated to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer, disrupt these orderly processes. The uncontrolled and often rapid proliferation of cells can lead to either a benign tumor or a malignant tumor (cancer). Benign tumors do not spread to other parts of the body or invade to other tissues, and they are rarely a threat to life unless they extrinsically compress vital structures. Malignant tumors can invade other organs, spread to distant locations (metastasis) and become life-threatening (Figure 1.1).

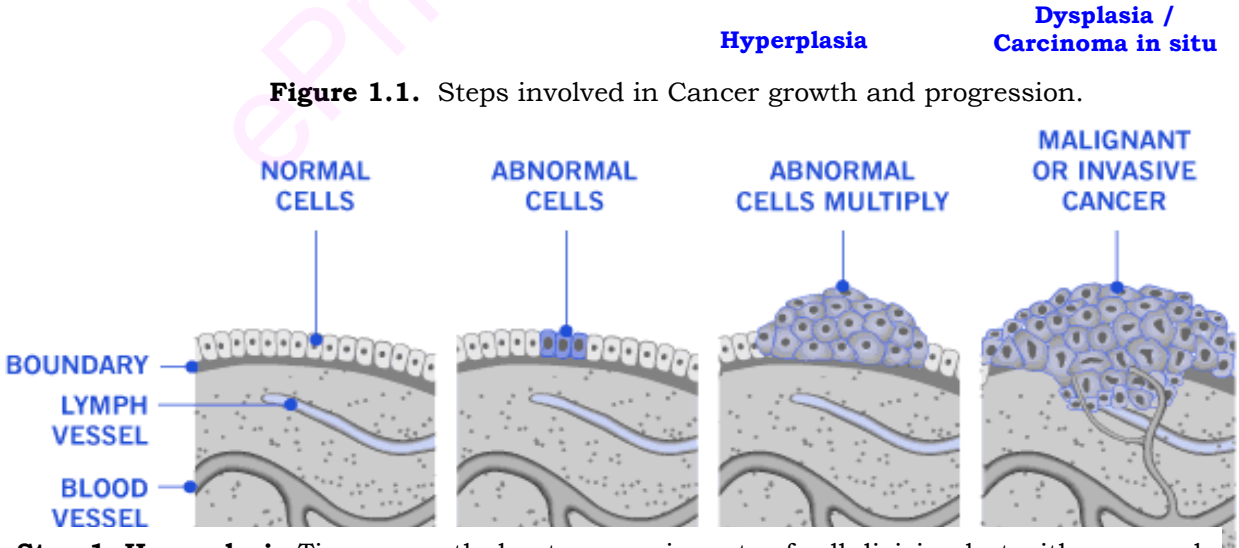


Figure 1.1. Steps involved in Cancer growth and progression.

Step 1: Hyperplasia Tissue growth due to excessive rate of cell division but with a normal orderly arrangement of cells within the tissue; **Step 2: Dysplasia** Abnormal excessive cell proliferation characterized by loss of normal tissue arrangement and cell structure; **Step 3: Carcinoma in situ** Refers to an uncontrolled growth of cells that remain in the original location and shows no propensity to invade other tissues; **Step 4: Invasive Cancer; Step 5: Metastasis**

Factors causing cancer

Multiple factors such as free radicals induced oxidative stress, genetic factors, occupational hazards, socioeconomic status leading to deprivation of appropriate nutrients, infectious disease conditions etc., have been implicated in cancer. Following literature enumerates the role of these factors in contributing to cancer incidence and cancer pathogenecity. Multicellular organisms have developed a system to distribute oxygen in a controlled manner. Most eukaryotic cells have an oxygen gradient, decreasing from the cell membrane to the mitochondria in which oxygen is consumed during aerobic metabolism. Most human body cells are exposed to fairly low oxygen concentration, which may be regarded as an antioxidant defense mechanism (Halliwell & Gutteridge, 1999a).

In Mammals, oxygen is transported to all the cells that need it and about 85-90 % of the oxygen taken up from the plasma by animal cells is utilized by the mitochondria to produce ATP, a biological energy source (Halliwell, 1994). The essence of metabolic energy production involves the oxidation of food molecules, i.e., the loss of electrons that are accepted by electron carriers such as NAD^+, FMN and FAD. These reduced compounds inurn are re-oxidized by oxygen in the mitochondria, producing ATP. The terminal enzyme in this electron transport chain, cytochrome oxidase, adds four electrons to oxygen. It is through a stepwise process that partially reduced oxygen species are generated (1.2).

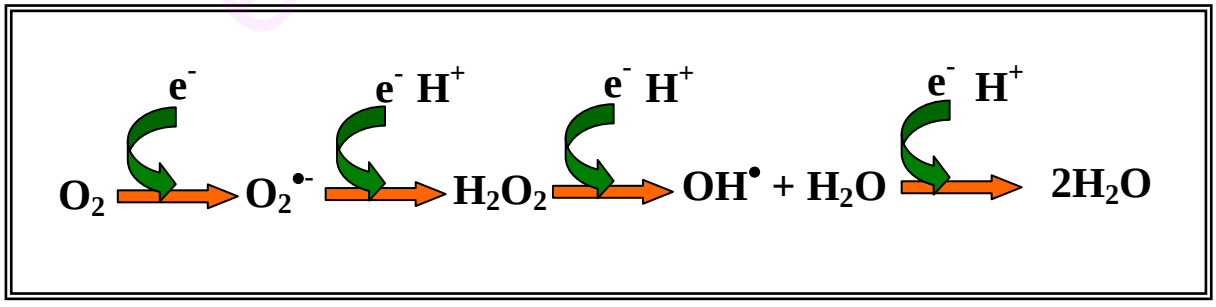


Figure 1.2. Step-wise Generation of Hydroxyl Radical from Oxygen.

$O_2^{\bullet-}$ = Superoxide radical, $OH^{\bullet}$ = Hydroxyl radical

Sources of free radical generation

Free radicals generated can be of endogenous or exogenous origin. Endogenously they are generated during metabolic process, body's defense process and tissue damage.

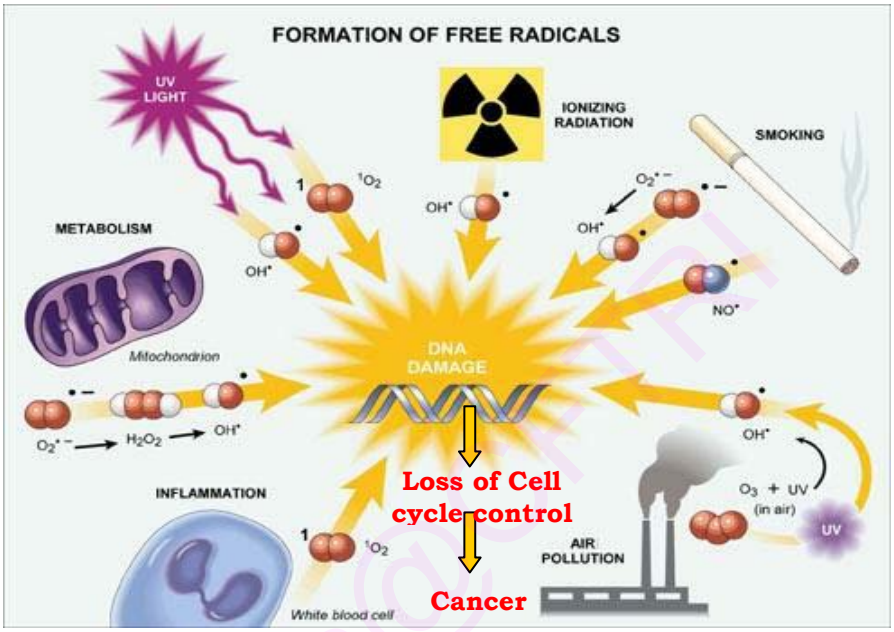


Figure 1.3. Free radical generation and cancer

1) Metabolic process

When a single electron is added to the ground state oxygen molecule, the superoxide radical is produced ($O_2^{\bullet -}$) (Figure 1.2 & 1.3). Several organic molecules oxidize in the presence of oxygen to produce the superoxide radical, including glyceraldehydes, the reduced flavins, adrenaline, L-dopa, dopamine, cysteine etc. These auto oxidations are catalyzed by the presence of metal ions such as iron and copper. The most important source of superoxide radicals *in vivo* are the electron transport chains present in mitochondria, endoplasmic reticulum, and nuclear membranes in eukaryotic cells. Most of these radicals are produced by the incomplete transfer of electrons on oxygen prior to the terminal cytochrome oxidase step. This production of radicals is increased as the oxygen concentration increases. It is estimated that 1-3 % of the oxygen reduced in mitochondria may form the superoxide radical

(Turrens, 1997).

Superoxide production increases when the integrity of the mitochondrial electron transport chain organization is compromised. The production of these oxygen radicals thus result in damage to the proteins, lipids and DNA in the respiring mitochondria, leading to mutations in mitochondrial DNA that has been associated with a wide range of human diseases (Shigenaga et al., 1994). In addition to the electron transport system release of electrons, the liver endoplasmic reticulum may generate superoxide radicals through the desaturase enzyme system. In this system, desaturase introduces C=C bonds into fatty acids. The superoxide radical is relatively innocuous, but at physiological pH approximately 1 % will be protonated to the more reactive peroxyl radical ($HO_2^{\bullet}$) (Borg, 1993). The superoxide may exert its effect in 3 ways (1) by decreasing the activity of certain enzymes including some antioxidant defense enzymes such as catalase, glutathione peroxidase, and several in the energy metabolism scheme as NADH dehydrogenase; (2) by damaging the ribonucleotide reductase that makes the precursors required for DNA synthesis and (3) by damaging calcineurin, a protein involved in signal transduction.

In addition, apart from direct damage, superoxide can be more cytotoxic by generating other reactive species, such as hydrogen peroxide, H_2O_2, by the addition of one more electron. H_2O_2 is not a radical since the additional electron fills the orbital, but it can attack certain enzymes such as glyceraldehydes-3-phosphate dehydrogenase, an enzyme in the glycolytic pathway. It can also oxidize certain keto-acids such as pyruvate. H_2O_2 leads to the depletion of ATP, reduced GSH, and NADPH. It induces a rise in free cytosolic Ca^{2+} and activates a polymerase that leads to cell death. Finally, H_2O_2 can cross cell membranes to react with iron and copper ions to form much more damaging species such as hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($NO^{\bullet}$). (Robertford & Calderon, 1995).

2) *Superoxide generation by body's defense system*

Free radicals can also be generated by activated leukocytes as part of the immune response. Macrophages and neutrophils possess the enzyme NADPH oxidase which can catalyze the one-electron reduction of oxygen to superoxide which in turn generates the other oxygen radicals discussed. Another enzyme found in leukocytes, myeloperoxidase, generates hypochlorous acid (HOCl) from H_2O_2. These reactive oxygen species (ROS) are used in the immune response to kill ingested or extracellular bacteria. Unfortunately, their actions are not limited to their intended purpose and they may contribute to the detrimental effects seen by the free radical-induced oxidative process. (Punchard & Kelly, 1997).

3) *Superoxide generation by tissue damage*

Tissue damage will also uncouple electron transport chains and release compartmentalized reactions to generate free radicals. Ischemia, as occurs during myocardial infarction and organ transplants, can produce free radicals through the action of xanthine oxidase (Punchard & Kelly, 1997). Other environmental factors such as exposure to UV radiation, air pollution, cigarette smoke also generates free radicals (Chow, 1993).

Generation of Oxidative stress by free radicals

Under ideal circumstances the body would be in a steady state despite free radical production because they are equipped with endogenous antioxidants for effective quenching of free radicals. However, it has been observed that this balance is not perfect because when there is overwhelming exposure to free radicals, free radicals mount their effect by initiating against action against biomolecules, particularly oxidation.

Oxidative damage occurs to DNA, proteins, lipids, and small molecules in living systems under ambient oxygen states – oxidative stress. Oxidative stress thus refers to the situation in which there is a significant imbalance between free radicals and the antioxidant defense system. The resulting harm is termed oxidative damage.

Cells normally deal with mild oxidative stress by upregulating the synthesis of antioxidant defense mechanisms through changes in gene expression. However, at higher levels of oxidative stress, cell injury occurs when adaptation is not adequate for the elimination of oxidation products. This leads to oxidative damage to all types of biomolecules including DNA, proteins and lipids that have been associated with many diseases. The target of oxidative damage varies depending on the characteristics of the cell and the type and degree of stress imposed. As a secondary event, however, oxidative stress plays an important role in furthering tissue damage in several diseases. Tissue damage by infection, trauma, toxins, temperature extremes and other causes usually leads to the formation of increased amount of free radicals that contribute to disease pathology. (Halliwell, & Gutteridge, 1999b). The imbalance of reduction-oxidation homeostasis has been shown to be one of the processes that regulate gene expression in many pathological conditions. (Haddad, 2002).

Free radicals and cancer

Molecular oxidation such as lipid peroxidation and DNA oxidation by oxidative stress leads to cancer

Lipid peroxidation

Dietary fats, after being digested and absorbed, are transported through the body via lipoproteins. The more cholesterol rich lipoprotein, LDL, contains approximately 2700 fatty acids/molecules, about half of which are PUFAs, that are very sensitive to

oxidation (Esterbauer et al., 1991). Polyunsaturated fatty acids are found in cell membranes where their side chains determine cell membrane fluidity. Membrane fluidity are affected by oxidation. Biological membrane fluidity is essential for the proper functions of the cell including the action of many important receptors; lipid peroxidation hampers this function (Arora et al., 2000).

Worst part of lipid peroxidation *in vivo* is that it is a non-stop chain reaction (Figure 1.4) in the body unless it is controlled appropriately. The most reactive species, hydroxyl radical ($K = 10^9 M^{-1}S^{-1}$) is capable of interacting with almost every type of molecule found in living cells (Halliwell & Gutteridge, 1992). It reacts by hydrogen abstraction, addition, and electron transfer from non-radical molecules, thus initiating the oxidation of macromolecules such as lipids resulting in a lipid radical ($L^{\bullet}$) that in the presence of oxygen generates a lipid peroxyl radical ($LOO^{\bullet}$). This $LOO^{\bullet}$ can continue the chain of oxidation by attacking another lipid and the oxidation process continues. Another fate of the oxidized fatty acids is the formation of cyclic peroxide that continues to be oxidized to malondialdehyde, F_2-isoprostanes, or other oxidation products. (Roberts & Morrow, 2000).

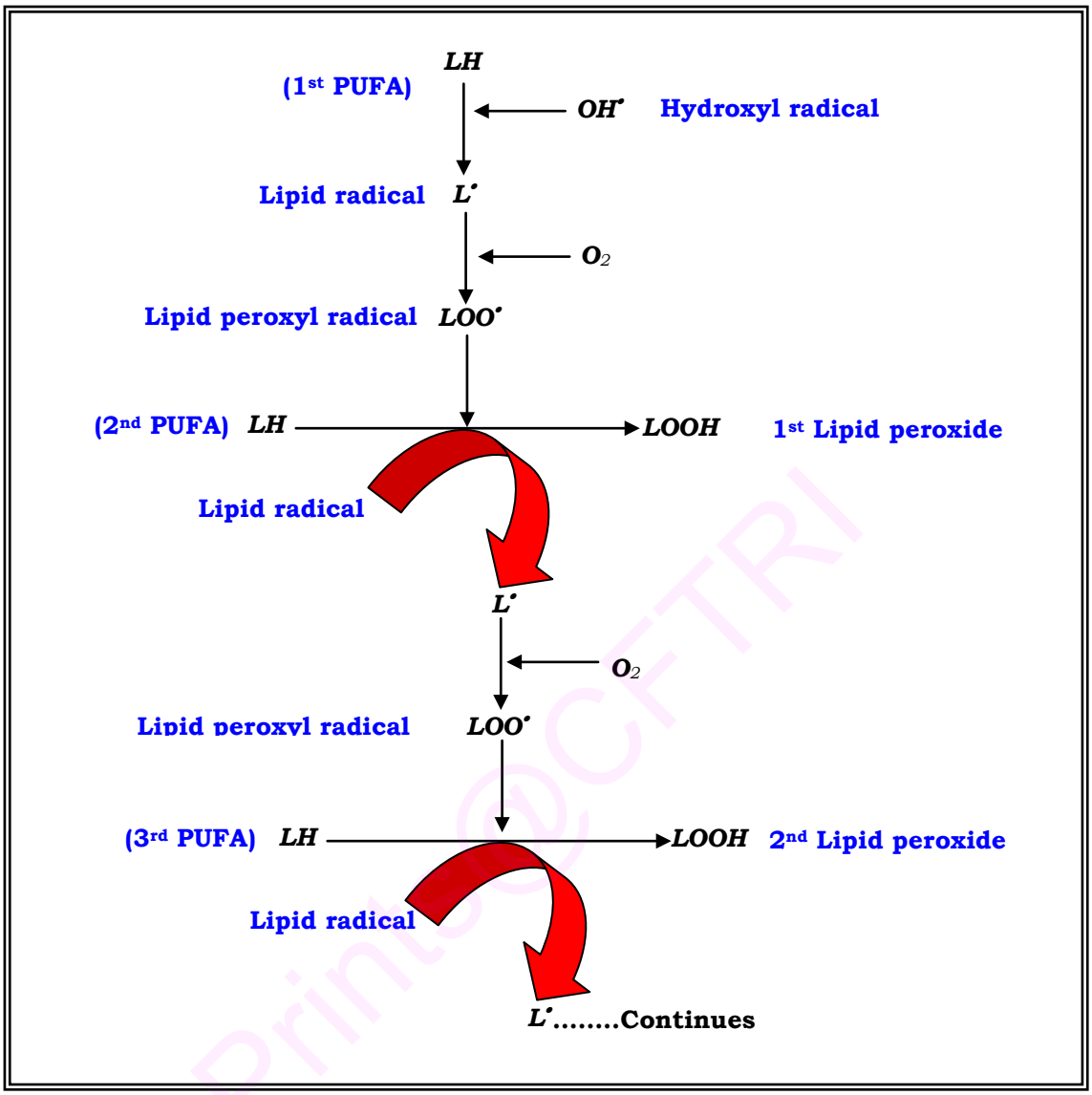


Figure 1.4. The lipid peroxidation chain reaction.

Lipid peroxidation begins when a methylene group (-CH_2-) of the PUFA is attacked by a free radical to abstract a hydrogen atom and an electron. An adjacent double bond weakens the attachment of the hydrogen atoms present on the next carbon, especially if there is a double bond on either side of the methylene group with subsequent rearrangement and reaction with more oxygen, lipid peroxy radical (LOO•) are formed. The lipid peroxyl radicals can oxidize adjacent lipids in the cell membrane or LDL molecule. Through this chain reaction, a single initiating radical can result in the conversion of hundreds of fatty acid chains into lipid peroxides that alter the integrity and biochemical function of cell membranes (Yoshikawa et al., 1997).

DNA Oxidation

It is estimated that 88-90 % of human cancers are environmentally induced. Much experimental data indicate that free radicals have a role in the initiation and promotion of cancer (Cross, 1987), which involves changes in DNA either as a result of an inherited genetic anomaly or damage to the DNA strand by free radicals (Figure 1.5). The most potent free radical, hydroxyl radical ($OH^{\bullet}$), attacks on DNA and generates a series of modified purine and pyrimidine bases. It is estimated that oxidative lesions in DNA in normal cells average 1 per 10^6 bases, a value that is even higher than the levels of adducts of known carcinogens that are detected in carcinogen-exposed cells. This implies that endogenous damage to DNA by free radicals is an important contributor to the age-related development of cancer (Totter, 1980; Loft & Poulsen, 1996).

An important factor in cancer development may be related to mutations in the p53 tumor-suppressing gene, a transcription factor that acts to block cell division. If p53 genes are inactivated, then cells can enter the cell cycle with damaged DNA. Mutations of p53 are found in 50 % of cancer lesions. For example: about 75 % of colorectal cancers and 90 % of squamous cell skin cancers have mutations on the p53 gene. Oxidative damage may account for some of the C to T and G to A changes often seen in the p53 gene in human cancers (Cerutti, 1994).

As indicated earlier DNA oxidation is a crucial step in mutation and cancer. The most prevalent product of oxidative DNA damage is 8-hydroxy-2'-deoxyguanosine (8-OhdG; Figure 1.6). It is considered to be reliable biological marker for oxidative stress (Shigenaga et al., 1990). The role of oxidation in DNA damage has been established by several *ex vivo* and *in vivo* animal and human studies in which 8-OhdG, 5-hydroxymethyluracil and other DNA oxidation products were measured (Djuric et al., 1991; Fisher-Nielsen et al., 1993; Haegele et al., 1994).

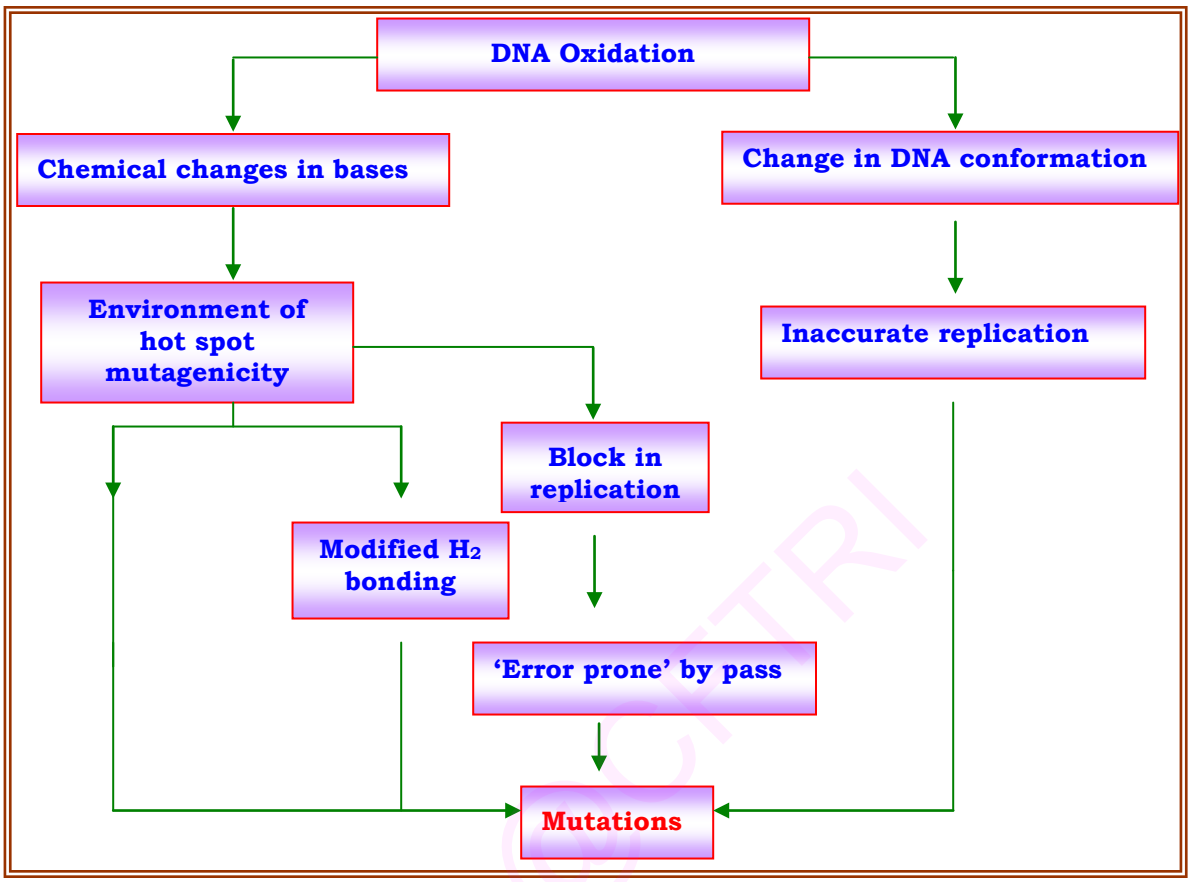


Figure 1.5. How structural changes in DNA cause mutations (Halliwell & Gutteridge, 1999b).

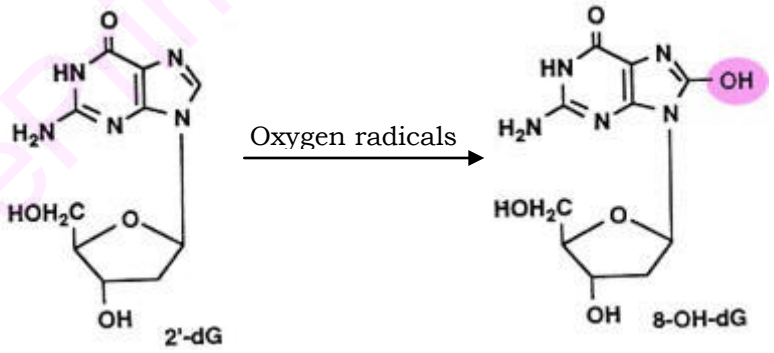


Figure 1.6. 8-hydroxy-2'-deoxyguanosine (8-OhdG) formation by Oxygen radicals

Other than direct damage to DNA by free radicals, oxidative damage to lipids and to proteins such as DNA repair enzymes could also lead to DNA mutations.

Genes and cancer

Cancers are initiated (*Carcinogenesis*) by derangement of the rate of cell division due to damage to DNA. In order for cells to start dividing uncontrollably, genes which regulate cell growth must be damaged. Mutations in genes (Proto-oncogenes) which promote cell growth and mitosis or tumor suppressor genes that discourage cell growth, or temporarily halt cell division in order to carry out DNA repair of damaged DNA are responsible for cancer. In other words imbalance in proliferation and differentiation result in cancer. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell and steps involved in this carcinogenic route is depicted in Figure 1.7.

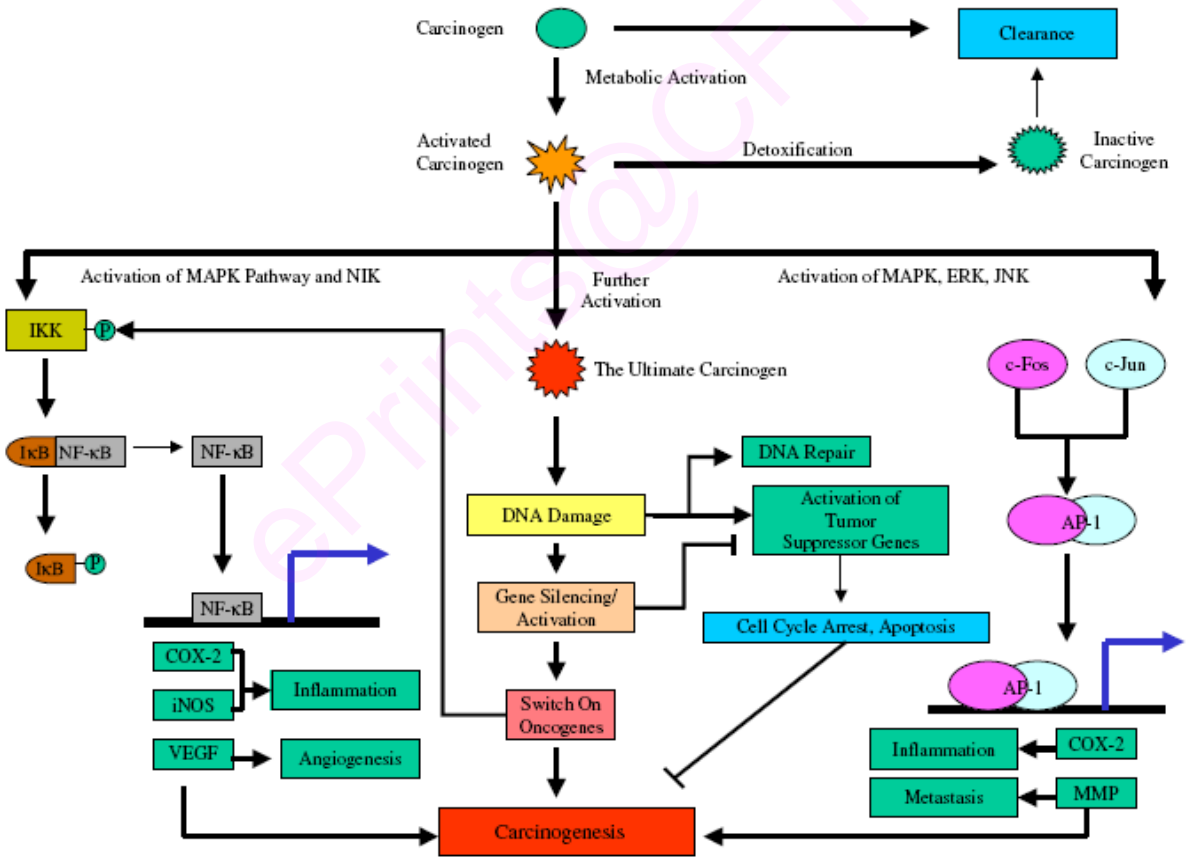


Figure 1.7. Multistep process of chemical induced carcinogenesis

The pathway of inducing carcinogenesis includes activation of MAPK pathway and nuclear factor kappa B inducing kinase (NIK) leading to activation of Inhibitor of Kappa B Kinase (IKK). IKK phosphorylates IkB leading to the release of NF-kB and activation of its target genes. AP-1, which is a heterodimer composed of c-fos and c-jun, shares many target genes with NF-kB. It also induces the expression of matrix metalloproteinases (MMP) which play a crucial role in angiogenesis and metastasis. Dietary chemopreventive agents may interfere with any of the above mentioned pathways (Figure 1.7) leading to inhibition of carcinogenesis.

Proto-oncogenes promote cell growth through a variety of ways (Table 1.1) Mutations in proto-oncogenes can modify their expression and function, increasing the amount

Table 1.1. List of selected Proto-oncogenes involved in cancer

Genes for growth factors or their receptors	
PDGF	Codes for platelet derived growth factor. Involved in glioma
erb-B	Codes for the receptor for epidermal growth factor. Involved in glioblastoma and breast cancer
erb-B2	Also called HER-2 or neu. Codes for a growth factor receptor. Involved in breast, salivary gland and ovarian cancers.
RET	Codes for a growth factor receptor. Involved n thyroid cancer.
Genes for cytoplasmic relays in stimulating signaling pathways	
Ki-ras	Involved in lung, ovarian, colon and pancreatic cancers
N-ras	Involved in leukemias
Genes for transcription factors that activate growth-promoting genes	
C-myc	Involved in leukemias and breast, stomach and lung cancers
N-myc	Involved in neuroblastoma and glioblastoma
L-myc	Involved in lung cancer
Genes for other kinds of molecules	
Bcl-2	Codes for a protein that normally blocks cell suicide. Involved in follicular B cell lymphoma
Bcl-1	Also called PRAD1. Codes for cyclin D1, a stimulatory component of the cell cycle clock. Involved in breast, head and neck cancers
MDM2	Codes for an antagonist of the p53 tumor suppressor protein. Involved in sarcomas and other cancers

or activity of the product protein. When this happens, they become oncogenes, and thus cells have a higher chance to divide excessively and uncontrollably.

Tumor suppressor genes code for anti-proliferation signals and proteins that suppress mitosis and cell growth (Table 1.2). Generally tumor suppressors are transcription factors that are activated by cellular stress or DNA damage. The functions of such genes is to arrest the progression of cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells.

Table 1.2. List of selected tumor suppressor genes involved in cancer

Genes for proteins in the cytoplasm	
APC	Involved in colon and stomach cancers
DPC4	Codes for a relay molecule in a signaling pathway that inhibits cell division. Involved in pancreatic cancer.
NF-1	Codes for a protein that inhibits a stimulatory (Ras) protein. Involved in neurofibroma and pheochyrmocytoma (cancers of the peripheral nervous system) and myeloid leukemia
NF-2	Involved in meningioma and ependymoma (brain cancers) and schwannoma (affecting the wrapping around peripheral nerves)
Genes for proteins in the nucleus	
MTS1	Codes for the p16 protein, a braking component of the cell cycle clock. Involved in a wide range of cancers
RB	Codes for pRB protein, a master brake of the cell cycle clock. Involved in retinoblastoma and bone, bladder, small cell lung and breast cancer.
p53	Codes for p53 proteins, which can halt cell division and induce abnormal cells to kill themselves. Involved in a wide range of cancers.
WT1	Involved in Wilms tumor of the kidney

However, a mutation can damage the tumor suppressor gene itself, or the signal pathway which activates it leading to tumor supressor gene switch off. The invariable consequence of this is that DNA repair is hindered or inhibited; DNA damage accumulates without repair leading to abnormalities and pathogenecity. Not all mutations lead to cancers; however specific mutations as mentioned above result in cancer.

Mutations in Cancer

In general, mutations in both protooncogene and tumor suppressor genes are required for cancer to occur. For example, a mutation limited to one oncogene would be suppressed by normal mitosis control and tumor suppressor genes. A mutation to only one tumor suppressor gene would not cause cancer either, due to the presence of many "backup" genes that duplicate its functions. It is only when enough proto-oncogenes have mutated into oncogenes, and enough tumor suppressor genes deactivated or damaged so that the signals for cell growth overwhelm the signals to regulate it leading to cancer. Cancer pathology is ultimately due to the accumulation of DNA mutations that negatively affect expression of tumour suppressor proteins or positivly affect the expression of proteins that drive the cell cycle.

Substances that cause these mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. A carcinogen is often metabolically activated into an activated carcinogen, which may be further activated to produce the ultimate carcinogen. Detoxification may occur for a carcinogen or an activated carcinogen leading to neutralization of the carcinogen and subsequent elimination. Activation of oncogenes and silencing of tumor suppressor genes lead eventually to tumor formation and malignancy.

Table 1.3. Some carcinogens in the workplace		
Carcinogen	**Occupation**	**Type of cancer**
Arsenic	Mining, pesticide workers	Lung, skin, liver
Asbestos	Construction workers	Lung, mesothelioma
Benzene	Petroleum, rubber, chemical workers	Leukemia
Chromium	Metal workers, electroplaters	Lung
Leather dust	Shoe manufacturing	Nasal, bladder
Naphthylamine	Chemical, dye, rubber workers	Bladder
Radon	Underground mining (also in some homes)	Lung
Soots, tars, oils	Coal, gas, petroleum workers	Lung, skin, liver
Vinyl chloride	Rubber workers, polyvinyl chloride manufacturing	Liver
Wood dust	Furniture manufacturing	Nasal

Particular substances have been linked to specific types of cancer (Table 1.3). Tobacco smoking is associated with lung cancer. Prolonged exposure to radiation, particularly ultraviolet radiation from the sun, leads to melanoma and other skin malignancies. Breathing asbestos fibers is associated with mesothelioma. Other types of mutations can be caused by chronic inflammation, as neutrophil granulocytes secrete free radicals that damage DNA. Chromosomal translocations, such as the Philadelphia chromosome, are a special type of mutation that involve exchanges between different chromosomes.

Viral Infections in Cancer

Many cancers originate from a viral infection. Evidence suggests that chronic viral infections are associated with up to one-fifth of all cancers. These include human papilloma virus (HPV) causing cervical cancer, hepatitis B virus (HBV), which can lead to cancer of the liver; the Epstein-Barr virus, a type of herpes virus that causes infectious mononucleosis and has been associated with Hodgkin's disease, non-Hodgkin's lymphomas, and nasopharyngeal cancer; the human immunodeficiency virus (HIV), which is associated with an increased risk of developing several cancers, especially Kaposi's sarcoma and non-Hodgkin's lymphoma.

Virally-induced tumors can be due to two mechanisms, *acutely-transforming* or *slowly-transforming*. In acutely transforming viruses, the viral particles carry a gene that encodes for an overactive oncogene called viral-oncogene (v-onc), and the infected cell is transformed as soon as v-onc is expressed. In contrast, in slowly-transforming viruses, the virus genome is inserted, especially as viral genome insertion is an obligatory part of retroviruses, near a proto-oncogene in the host genome. The viral promoter or other transcription regulation elements in turn cause overexpression of that proto-oncogene, which in turn induces uncontrolled cellular proliferation. Because viral genome insertion is not specific to proto-oncogenes and the chance of insertion near that proto-oncogene is low, slowly-transforming viruses have very long tumor latency compared to acutely-transforming viruses, which already carry the viral-oncogene.

Heredity and cancer

About 5 to 10 percent of cancers are hereditary, in that a faulty gene or damaged DNA that has been inherited predisposes a person to be at a very high risk of developing a particular cancer. Two genes, BRCA1 and BRCA2, have been found to cause breast cancers. Other genes have been discovered that are associated with some cancers that run in families, such as cancers of the colon, rectum, kidney,

ovary, esophagus, lymph nodes, skin melanoma, and pancreas.

Metastasis

Tumor metastasis is a multistep process by which a subset or individual cancer cells disseminate from a primary tumor to distant secondary organs or tissues (Figure 1.8). Tumor cells fulfill their metastatic potential after acquiring those advantageous characteristics, which allow them to escape from the primary tumor, migrate and invade surrounding tissues, enter the vasculature, circulate and reach secondary sites, extravasate, and establish metastatic foci [Hanahan & Weinberg, 2000; Chambers et al., 2002; Pantel & Brakenhoff, 2004; Geho et al., 2005]. All these steps of the metastatic cascade require from tumor cells some survival and communication skills. Failure to overcome challenges imposed by normal physiological barriers often leads to tumor cell death or clearance, thereby making each phase of metastasis apparently a rate-limiting step [Hynes, 2003]. During metastasis, tumor cells are involved in numerous interactions with the extracellular matrix (ECM) itself, and those proteins, growth factors and cytokines associated with the ECM, basement membranes, endothelial cell lining of the vasculature, blood cells in the circulation, and the microenvironment of the secondary site where tumor cells eventually displace the normal tissue as they grow out and form metastatic foci. Several regulatory pathways are either altered or aberrantly expressed to render tumor cells the ability to successfully accomplish each and all steps of the metastatic process. Matrix metalloproteinases (MMPs) have been regarded as major critical molecules assisting tumor cells during metastasis (Sternlicht & Werb, 2001; Egeblad & Werb, 2002; Lynch & Matrisian, 2002; Fingleton, 2006). From the earliest work on MMPs in cancer (Liotta, 1980; Liotta, 1986), there has been a clear connection between MMPs, ECM degradation and cancer cell invasion. Numerous studies linked inhibition of MMPs by synthetic and natural inhibitors (tissue inhibitors of matrix metalloproteinase, or TIMPs) with a corresponding inhibition of cell invasion. Conversely, up-regulation of MMPs by inducers, transcriptional enhancement, or transgene constructs usually led to enhanced tumor cell invasion, monitored *in vivo* by histology, or in model systems by appearance of tumor cells in distant sites and *in*

vitro by Matrigel or ECM invasion. All these studies led to the conclusion that enhanced MMP levels yielded increased tumor cell invasion. Studies in the 1980s and 1990s often linked MMPs with metastasis, but generally cancer invasion, cancer cell migration or cancer cell-mediated tissue remodeling were actually the major positive correlations with MMP levels.

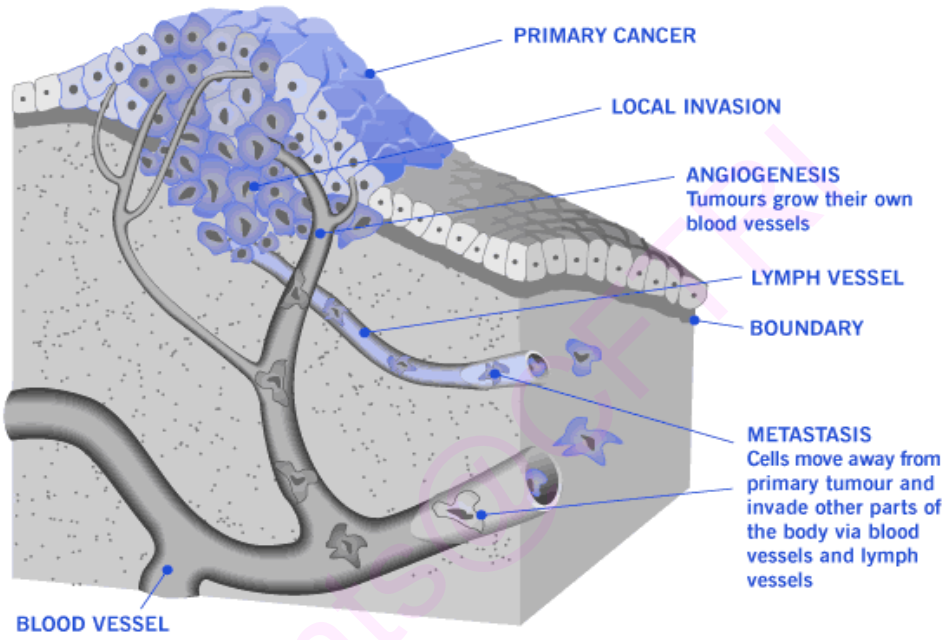


Figure 1.8. Process of metastasis

Galectins are a group of proteins, which are evolutionarily conserved family of mammalian lectins with multifunctional properties (Rabinovich, 1999). Galectins are a family of carbohydrate binding proteins that recognize structural variations among β-galactoside containing glycoconjugates. They are widely distributed in the animal kingdom from lower invertebrates to mammals. Sixteen galectin proteins representing a high degree of sequence identity in their carbohydrate recognition domains (CRD) have been described (Ogden et al., 1998; Stierstorfer et al., 2000; Dunphy et al., 2002; Gray et al., 2004). Galectin-3, an approximately 31–42 kDa protein is one of the β-galactoside binding proteins that bind to the carbohydrate portion of cell surface glycoconjugates (Figure 1.9). Recent studies have indicated that galectin-3 expression is correlated with metastatic potential in certain

malignancies (Bresalier et al., 1997). Results of several investigations have revealed the possibility of galectin-3 as a diagnostic marker in certain cancers and also one of the target proteins for cancer treatment (Konstantinov et al., 1996).

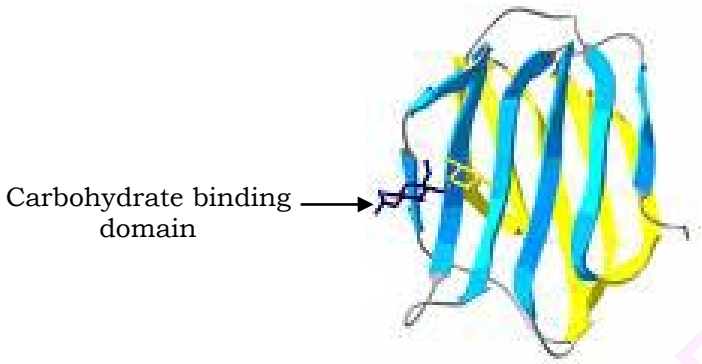


Figure 1.9. Human Galectin-3 molecule

Further, higher levels of galectin-3 have been shown to correlate with the advancement of the cancer disease and it is believed that galectin-3 of cancer cells binds to normal cells and transforms them to cancer cells and hence establishes secondary tumors. Galectin-3 hence has been implicated in tumor spread and metastasis (Takenaka et al., 2004.). Studies have also indicated that oral administration of modified citrus pectin reduced the rate of cancer cell spread and inhibited metastasis in animal models (Pienta et al., 1995). However, despite a promising role of dietary pectins against metastasis, only few reports are available on identification of such components from dietary sources, which can block the binding of galectin-3 to β-D-galactoside residues present on the extra cellular matrix components of normal cell and basement membranes leading to effective management of metastasis.

Spices, Diet and Cancer

Dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public due to their various health promoting effects such as suppression of cancers, prevention of diseases, delay in the onset of diseases, disease cure etc. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of cancer (Temple, 2000; Hashimoto et al., 2002; Gosslau & Chen, 2004). Cancer has been identified as a major devastating disease among populations of both developing and developed countries (Oldham & Bowen, 1998). Diet and tobacco are two major factors contributing 35 % each to the total disease incidence of 70 % (Willett et al., 1996).

Epidemiological data have shown a very strong inverse relationship between consumption of fruits, vegetables and spices and the risk of cancer with an overall risk reduction between 30 and 50 % (Djuric, 1991; Block, 1999). Fruits, vegetables and spices are known to contain numerous substances with antioxidant/anticancer property (Table 1.4) including carotenoids, vitamin C and polyphenols and many studies have examined the association of these nutrients with various cancers. For example, blood levels of β-carotene, vitamin C, vitamin E and selenium showed association with curative effect of lung cancer (Block et al., 1992; Combs, 1997; McLarty, 1997). Individual studies suggest protective association for each, but the totality of the evidence of an association at present is not convincing for any one of these antioxidant micronutrients (Ziegler et al., 1996). A meta-analysis (Howe et al., 1990) of 12 major breast cancer studies found a strong and significant protective association with vitamin C intake.

Table 1.4. Antioxidants/Anticarcinogens in diet

Antioxidant / Anticarcinogenic phytochemicals	Food source	Cancers treated	Reference
Allyl sulfides	Onions, garlic, leeks, chives	Colon, breast	Nakagawa et al., 2001
Carotenoids (e.g., lycopene, lutein, zeaxanthin)	Carrots, squash, broccoli, sweet potatoes, tomatoes, kale, watermelon, collards, cantaloupe, spinach, peaches and apricots	Prostrate	Cook et al., 1999.
Curcumin	Turmeric	Many	Aggarwal et al., 2003
Flavonoids (e.g., anthocyanins, resveratrol, quercitin, catechins)	Grapes, blueberries, strawberries, cherries, apples, grapefruit, cranberries, raspberries, blackberries	Prostrate, Breast etc.	Fink et al., 2007
Glutathione	Green leafy vegetables	---	---
Indoles	Broccoli, cauliflower, cabbage, Brussels sprouts, bok choy	Cervical	Aggarwal & Ichikawa, 2005
Isoflavones (e.g., genistein, daidzeins)	Legumes (peas, soybeans)		Kumar et al., 2007
Isothiocyanates (e.g., sulforaphane)	Broccoli, cauliflower, cabbage, Brussels sprouts, bok choy	Prostrate	Zhang & Tang, 2007
Monoterpenes	Citrus fruit peels, cherries, nuts	Leukemia	Lu et al., 2007
Phytic acid	Whole grains, legumes	Colon	Shamsuddin, 2002

Phenols, polyphenols, phenolic compounds (e.g., ellagic acid, ferulic acid, tannins)	Grapes, blueberries, strawberries, cherries, grapefruit, cranberries, raspberries, blackberries, tea	Many	Rudolf et al., 2007
Saponins	Beans, legumes	Colon	Wang et al., 2007
Lignans	Seeds (flax seeds, sunflower seeds)	Breast	Cotterchio et al., 2007
Vitamin C	Citrus fruits, green peppers, broccoli, green leafy vegetables, strawberries, raw cabbage and potatoes	---	---
Vitamin E	Wheat germ, nuts, seeds, whole grains, green leafy vegetables, vegetable oil and fish-liver oil	Breast	Kline et al., 2004
Selenium	Fish, shellfish, red meat, grains, eggs, chicken and garlic	Colon, lung etc.	Knekt et al., 1998

A strong inverse relationship has been shown between lycopene with reduced risk of several cancers, especially prostrate, lung and stomach (Giovannucci, 1999). However, some data show that β-carotene may act as a co-carcinogen, especially in individuals exposed to cigarette smoke and other carcinogens found in industrial settings (Paolini, 2003). Two studies have demonstrated that free radical-induced DNA damage is greater in cancerous compared to non-cancerous prostrate tissues. Some dietary nutrients with antioxidant properties have been shown to slow down progression of prostrate cancer (Willis & Wians, 2003). In the diet antioxidant components have been shown to play a key role in combating severity of cancer disease since in highly invasive or metastatic cancer cells, oxidative stress were found to imbalances antiproliferation and apoptosis rates. These cancer cells generate large amount of H_2O_2 and function as signal molecules that are involved in

the survival of cancer cells. Antioxidants hence may suppress these hydrogen peroxide signal molecules and thereby inhibit cancer cell proliferation (Loo, 2003). There is growing evidence that antioxidants may be beneficial as an adjunct to certain types of chemotherapy. A randomized clinical trial is currently underway to study this in cases of newly diagnosed ovarian cancer (Drisko et al., 2003).

Dietary customs and habits in India are diverse, owing in part, to the range of religions in the society, many of which provide specific dietary guidance for their followers. Furthermore, there is a tradition of linking vegetarianism with medicine (Sharma et al., 2007). For example, Ayurveda also provides dietary guidance and prescriptions that have been developed over millennia to prevent and treat multiple ailments, including CVD, cancer, and diabetes. It prescribes more than 700 plant-based medicines that contain spices and food additives to encourage good health (Sinha et al., 2003). Many of these foodstuffs have been studied for their disease prevention capabilities, including turmeric (curcumin), cumin, chilies, kalakhar, and various plant seeds (Tapsell et al., 2006). Among them the most studied in recent years is turmeric, an ingredient in the common Indian curry and a spice that has been shown to be a potent antioxidant and anti-inflammatory agent with additional promise as a chemo-preventive agent (Gautam et al., 2007). Micronutrient deficiencies specifically of vitamins A, C and E, may contribute to the high prevalence of oral cancers in India (Tandon et al., 2000). A study carried out in rural India found that the presence of lesions was associated in patients with oral pre-cancerous lesions with low plasma levels of vitamins E and β-carotene (Patel et al., 2001).

A study investigated the anti-carcinogenic effects of nine Indian spices against induction by dietary benzo[a] pyrene (B [a] P) of squamous cell carcinomas (SCC) of the stomach in mice and induction by dietary 3'-methyl-4-dimethylaminoazobenzene of hepatomas in rats. Cumin seeds and basil leaves significantly decreased the incidence of both SCC and hepatomas; poppy seeds significantly inhibited B [a] P-induced SCC; and the other six spices showed no effect (Aruna & Sivaramakrishnan,

1992). Studies on spices and food additives have been conducted *in vitro* and in animal studies. **Intriguing findings from these studies prompted us to explore the potential anticancer compounds from spices.**

Mechanism of Anticancer Effect of Antioxidants

Antioxidant has been defined by Halliwell and Gutteridge (1999a) as **"any substances that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate"**. When free radicals are generated *in vivo* many antioxidants act in defending the organism from oxidative damage (Figure 1.10).

As a first line of defense, the preventive antioxidants such as peroxidases and metal chelating proteins suppress the generation of free radicals. Next, the radical-scavenging antioxidants such as Vitamin C and Vitamin E, scavenge radical to inhibit the oxidation chain initiation and prevent chain propagation as a second line of defense. This may also include the termination of a chain by the reaction of two radicals. The repair and *de novo* enzymes act as the third line of defense by repairing damage and reconstituting membranes. These include lipases, proteases, DNA repair enzymes and endogenous antioxidants (Niki, 1997). These antioxidant defense systems are in built in all living organisms including plants and animals. During disease condition imbalance in oxidative stress warrants exploration of exogenous antioxidants (Rahman, 2007).

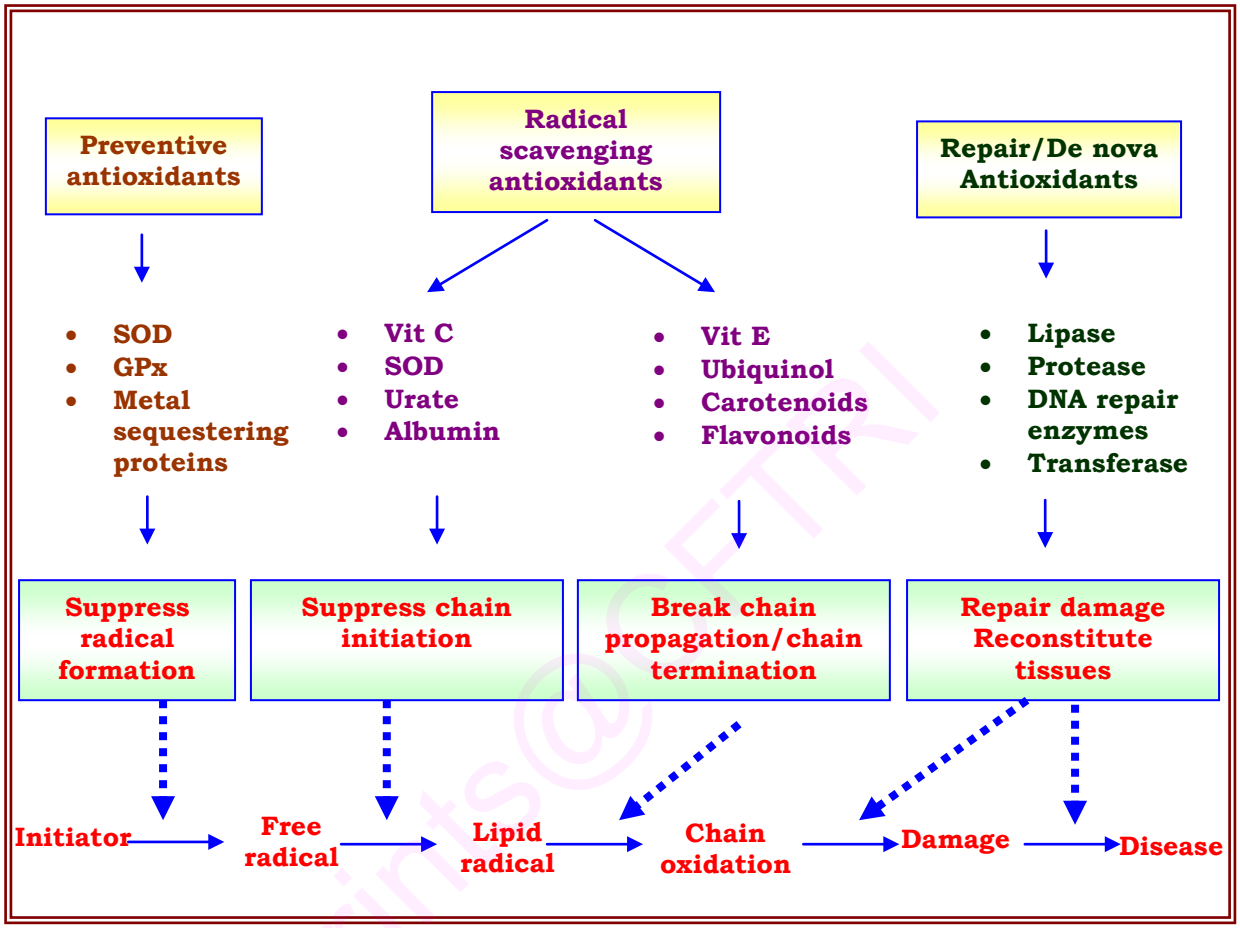


Figure 1.10. Antioxidant groups and actions. SOD = Superoxide dismutase; GPx = Glutathione peroxidase; Dotted lines = suppression.

Endogenous antioxidant

There is a vast net work of intracellular and extracellular antioxidants with diverse roles within each area of defense (Figure 1.11). Catalase converts H_2O_2 to O_2 and H_2O while superoxide dismutase (SOD) converts the superoxide radical ($O_2^{-\bullet}$) to H_2O_2 and O_2. Some of the antioxidant enzymes exist in several forms. For example, membrane, cytosolic and plasma forms of glutathione peroxidase have been isolated and SOD has membrane, cytosolic and extra cellular forms (Johnson & Giulivi, 2005)

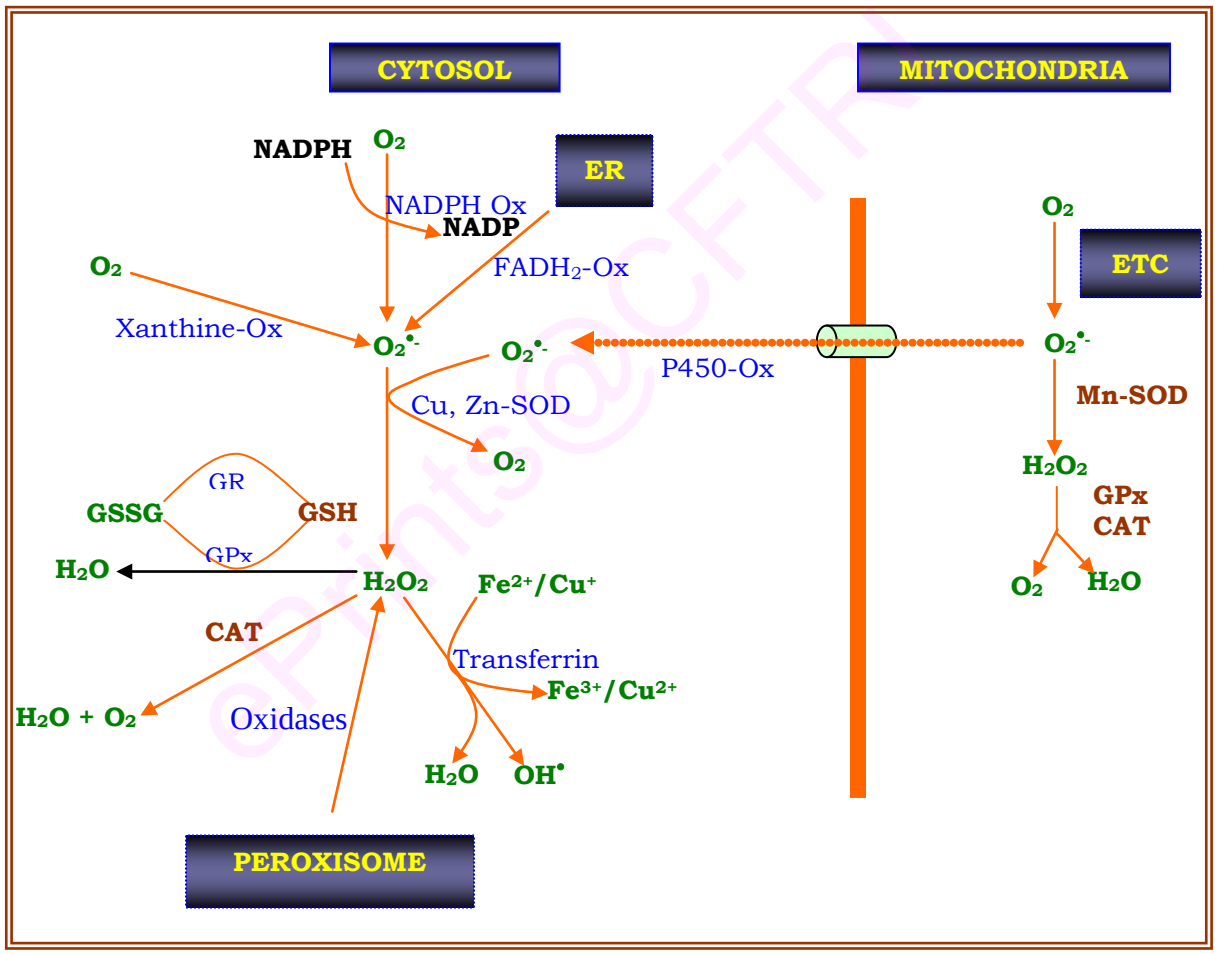
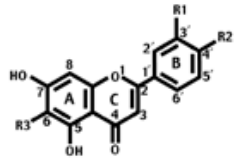


Figure 1.11. Generation of reactive oxygen species and endogenous antioxidant mechanisms. ER: Endoplasmic reticulum; ETC: Electron transport Chain; Ox: Oxidases; GSH: Glutathione; GSSG: Oxidized glutathione; GR: Glutathione reductase; GPx: Glutathione peroxidase and SOD: Superoxide dismutase, Endogenous antioxidants are bolded (Brown in color).

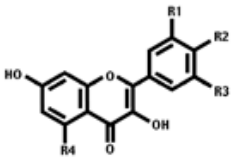
The levels and locations of these antioxidants must be tightly regulated for cell survival. The antioxidant enzymes, SOD, glutathione peroxidase (GPx) and catalase (CAT), work within the cells to remove most superoxides and peroxides before they react with metal ions to form more reactive free radicals. **Peroxidative chain reactions initiated by free radicals that escaped the antioxidant defenses are terminated by chain-breaking water or lipid soluble antioxidants (Mates et al., 1999).**

Exogenous antioxidants

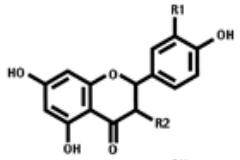
Diet plays a vital role in the production of the antioxidant defense system by providing essential nutrient antioxidants such as vitamin E, C and β-carotene, other antioxidant plant phenols including flavonoids (Figure 1.12 and 1.13), and essential minerals that form important antioxidant enzymes. For example, SOD contains zinc and glutathione peroxidase contains selenium (Punchard & Kelly, 1997).



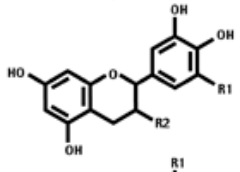
Flavone	R1	R2	R3
Apigenin	H	OH	H
Baicalein	H	H	OH
Luteolin	OH	OH	H



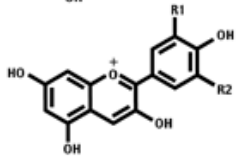
Flavonol	R1	R2	R3	R4
Fisetin	OH	OH	H	H
Galangin	H	H	H	OH
Quercetin	OH	OH	H	OH
Kaempferol	H	OH	H	OH
Myricetin	OH	OH	OH	OH
Isorhamnetin	H	OH	OMe	OH



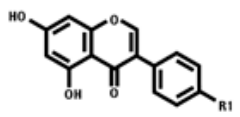
Flavanone	R1	R2
Eriodictyol	OH	H
Naringenin	H	H
Taxifolin	OH	OH



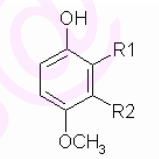
Flavanol	R1	R2
Catechin	H	OH
Epicatechin	H	OH
EGCG	OH	Gallate

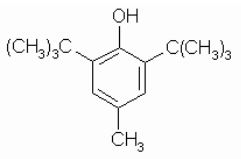


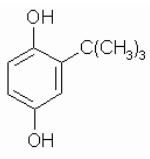
Anthocyanidin	R1	R2
Cyanidin	OH	H
Malvidin	OMe	OMe
Peonidin	OMe	H

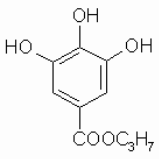


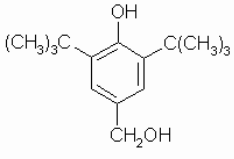
Isoflavone	R1
Genistein	OH

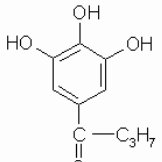


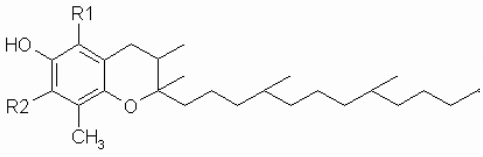












alpha R1-CH_3 R2-CH_3 gamma R1-H R2-CH_3

beta R1-CH_3 R3-H delta R1-H R2-H

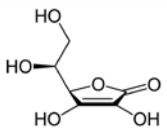
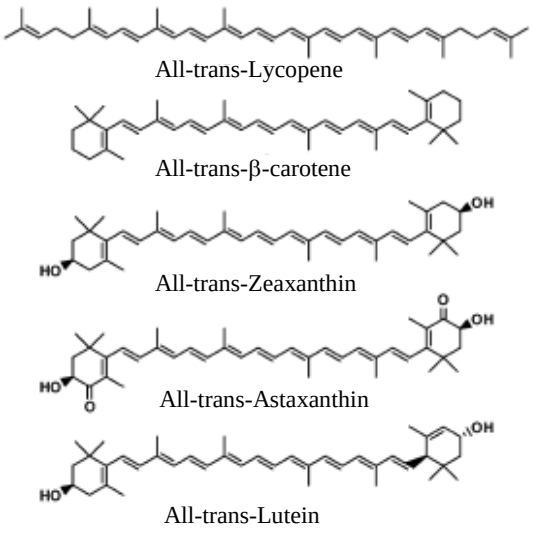
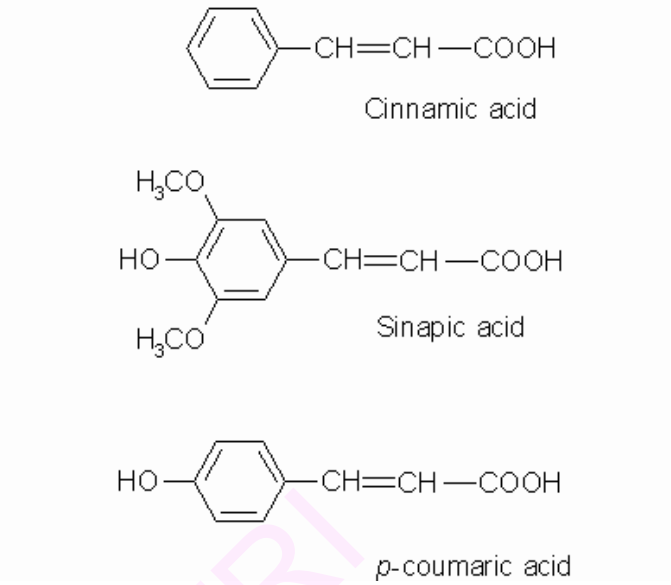
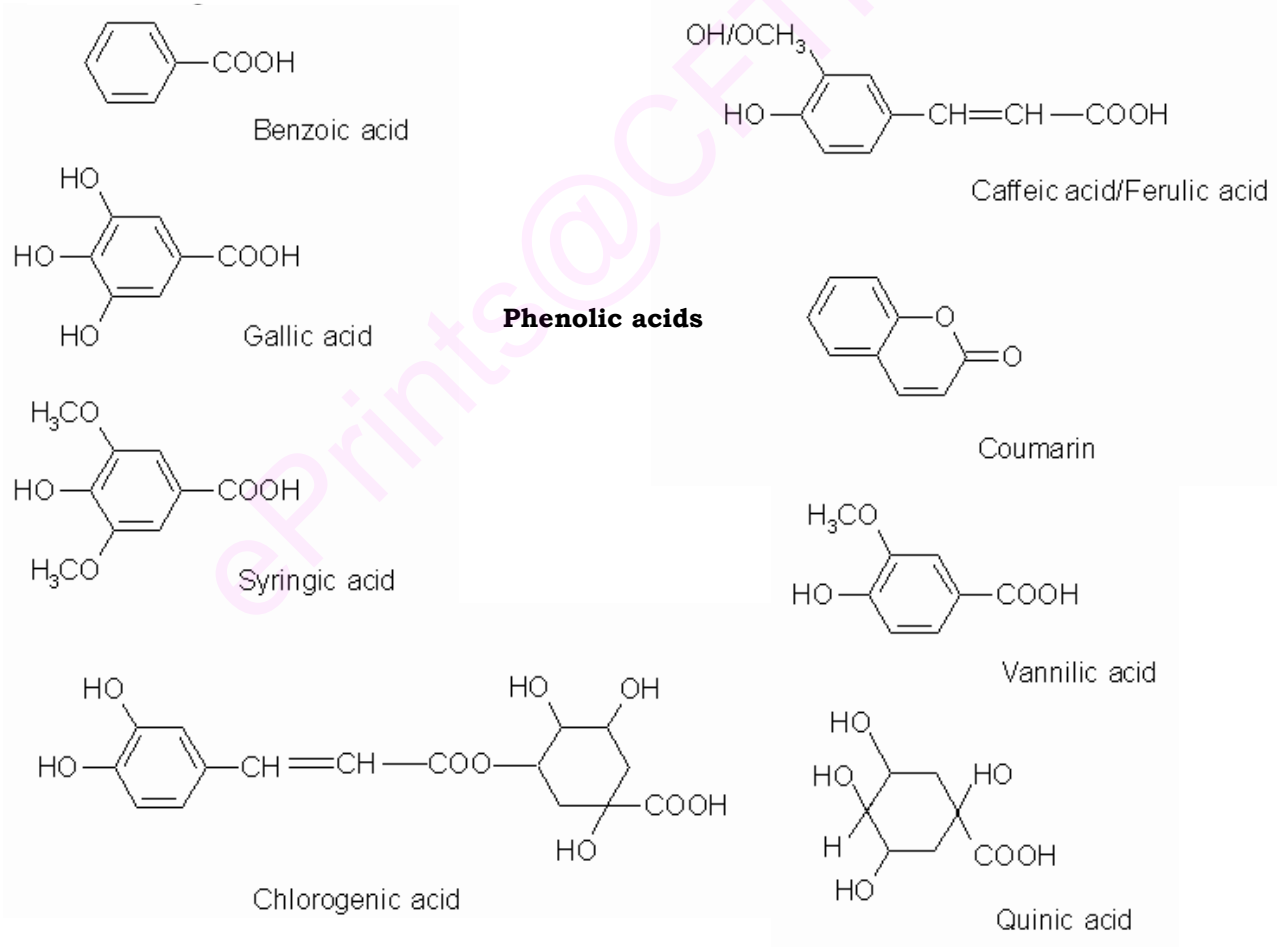


Figure 1.12. Chemical structures of major antioxidant groups and representative individual antioxidant phytochemicals



Carotenoids





Phenolic acids

Figure 1.13. Chemical structures of major antioxidant groups and representative individual antioxidant phytochemicals

Antioxidants Play an Essential Role against Oxidative Stress and Cancer

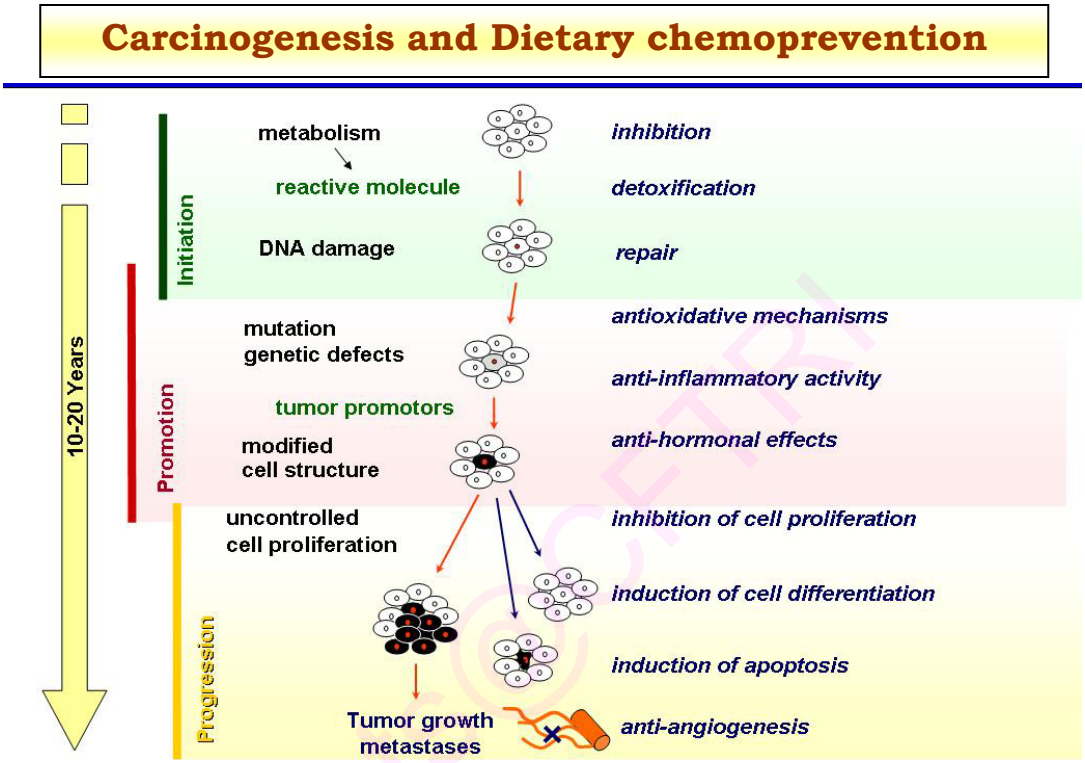


Figure 1.14. Different stages of carcinogenesis and their inhibition at various steps by dietary anticancer components

Carcinogenesis comprises three different stages, initiation, promotion and progression, many potential dietary chemopreventive agents can be categorized broadly as blocking agents (antioxidants), which impede the initiation stage, or suppressing agents, which arrest or reverse the promotion and progression of cancer, presumably by affecting or perturbing crucial factors that control cell proliferation, differentiation, senescence or apoptosis [Figure 1.14; Chemoprevention Working Group, 1999]. Many natural products from our daily consumption of fruits, vegetables and tea beverages, such as sulforaphane from broccoli, resveratrol from grapes, genistein from soy, curcumin from tumeric powdered food preparations, and

epigallocatechin-3-gallate (EGCG) from green tea, can be considered blocking or suppressing agents [Manson, 2003]. The mechanisms that are likely to underlie the effectiveness of these dietary chemopreventive compounds are summarized in Figure 1.15 and 1.16.

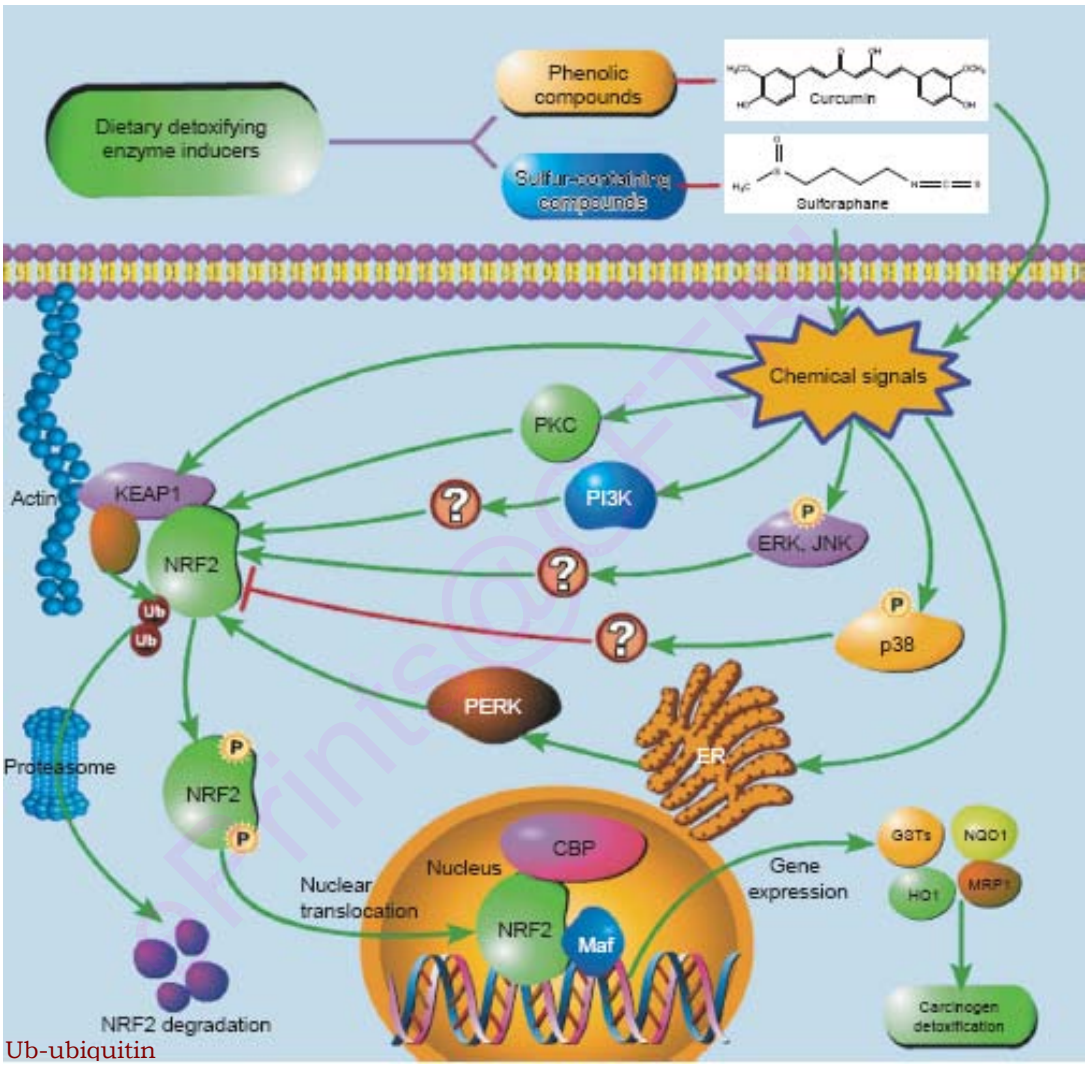


Figure 1.15. Mechanism of action of dietary anticarcinogens (detoxifying enzyme inducers)

Dietary anticarcinogens regulate the expression of genes encoding detoxifying enzymes through the transcription factor NRF2 (nuclear factor E2-related factor 2). Under basal conditions, NRF2 protein is retained in the cytosol by Kelch-like ECH-associating protein 1 (KEAP1). KEAP1 also facilitates the proteasome mediated

degradation of NRF2 protein by facilitating as an adaptor for Cullin 3 (Cul3)-based E3 ligase. The function and localization of NRF2 is regulated by multiple upstream kinases that are activated by inducers of detoxifying enzymes. Modification of multiple cysteine residues in KEAP1 following treatment with these enzyme inducers leads to disassociation of NRF2 from KEAP1. Among three mitogen-activated protein kinase (MAPK) kinases, activated extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) might promote translocation of NRF2 to the nucleus but p38 might inhibit NRF2 translocation in some cell types. Phosphorylation of Ser40 in NRF2 protein by protein kinase C (PKC) also leads to the release of NRF2 from KEAP1. Similarly, phosphatidylinositol 3-kinase (PI3K), and stress-related RNA-dependent protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) derived from the endoplasmic reticulum (ER), might also elicit positive influences on NRF2 and subsequent induction of cellular defense enzymes. Once in the nucleus, NRF2 interacts with small Maf proteins, such as MafG and MafK, forming heterodimers, and binds to the antioxidant response element (ARE) sequence in the promoter regions of many genes encoding cytoprotective enzymes. Transcription of these genes is also enhanced by interactions between NRF2 and coactivators, such as cAMP response element binding protein (CREB)-binding protein (CBP) or p300. **Finally, elevated levels of detoxifying enzymes [e.g. glutathione-S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1) and multidrug resistance associated protein 1 (MRP1)] facilitate the detoxification and elimination of carcinogens and reactive intermediates, and protect the cells and the body against chemical carcinogenesis.**

There appear to be several different cellular and molecular mechanisms that underlie the blocking and suppressing effects of chemopreventive compounds, and many of these compounds appear to possess both antioxidant effects and suppressing effects. **Thus, the anti-carcinogenic function of these compounds might be attributed to a combination of their cytoprotective effect on normal cells and their cytotoxic effect on pre-neoplastic and/or neoplastic cells.**

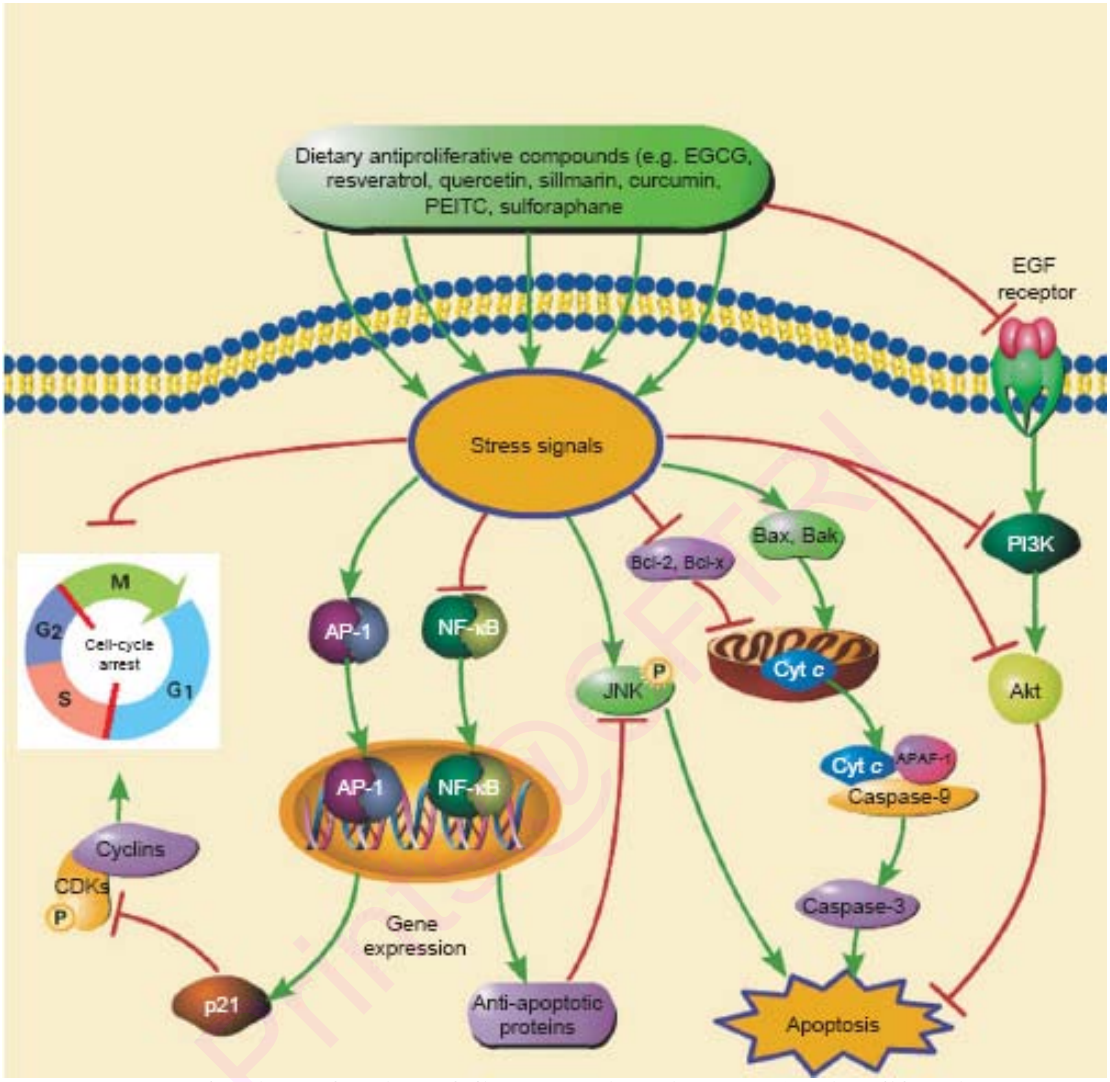


Figure 1.16. Mechanism of action of dietary anticarcinogens (antiproliferative compounds)

Apoptosis and cell-cycle arrest are two important goals of dietary antiproliferative compounds against the promotion and progression of pre-neoplastic or neoplastic cells. Contrasting with death receptor-mediated cell death, chemopreventive compounds generally induce oxidative stress, which downregulates anti-apoptotic molecules such as Bcl-2 or Bcl-x and upregulates pro-apoptotic molecules such as Bax or Bak. The imbalance between anti-apoptotic and pro-apoptotic proteins elicits the release of cytochrome c (Cyt c) from the mitochondrial inner membrane; cytochrome c then forms an 'apoptosome' with apoptotic protease-activating factor 1

(APAF-1) and caspase-9, with the subsequent activation of caspases-3, -6 and -7. The activated caspases catalyze the dissolution of intracellular structure, which leads to apoptotic cell death. **To enhance this mitochondria-mediated apoptosis, dietary cytotoxic compounds also activate pro-apoptotic c-Jun N-terminal kinase (JNK) and inhibit anti-apoptotic NF-kB signaling pathways. Furthermore, dietary chemopreventive compounds can also block growth factor-mediated anti-apoptotic signals through the direct inhibition of the binding of growth factors to the receptor, or inhibition of the downstream phosphatidylinositol 3-kinase (PI3K)–Akt pathway.** At relatively lower concentrations, these same dietary chemopreventive compounds can achieve cell-cycle arrest by changing the balance between the cell-cycle regulators [cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors]. One potential mechanism is through the induction of the transcription factor activator protein 1 (AP-1) by dietary chemopreventive compounds, leading to the increased expression of p21, a CDK inhibitor, which subsequently arrests cells in G1–S or G2–M interphases.

Cancer development is a long-term process that appears to proceed by step-by-step carcinogenesis events that ultimately spread from one area of the body to other parts of the body during the late metastasis stage. Current clinical therapies of cancer, which include surgery, radiation and chemotherapy, are limited, particularly during the terminal metastasis phase. However, there is increasing evidence from cancer epidemiological and pathological studies suggesting that many human cancers could be prevented or their progression slowed down [Weinstein, 1991]. Therefore, there might be opportunities for interference and prevention of cancer progression in the early stages of carcinogenesis by dietary phytochemicals. The need for biologically active dietary compounds with low profiles of adverse reactions compared to pharmacological drugs has led to an extensive investigation of dietary sources for phytochemicals and their mechanism of action in the current thesis.

Cancer Treatments

Surgery

The major cancer treatments available are surgery, radiation and chemotherapy. The earliest therapy established for cancer and still the most widely used approach is surgery. Surgical excision of a tumor is both quick and effective, and it accounts for the largest number of cures. Surgery is also the one method of therapy that offers the opportunity to confirm that a tumor has been fully excised, because a pathologist can examine the specimen removed (which should contain a layer of unaltered cells fully surrounding the cancerous ones).

Radiation therapy

Radiation therapy is preferable to surgery in many instances. With this method, powerful X-rays or gamma rays (delivered by using an externally applied beam or, in some instances, by implanting tiny radioactive sources) irradiate the region of the patient's cancerous tumor. Radiation treatments act either by inflicting genetic damage sufficient to kill cells directly or by inducing cellular suicide, a process called apoptosis, which is deeply ingrained in mammalian cells (Apoptosis is especially important during the embryonic development of mammals, when structures, such as gills, arise but then are lost as the cells constituting them undergo programmed cell death).

Because healthy tissue can recover from radiation exposure more readily than cancerous cells, radiation therapy can preserve the anatomical structures that surround a cancerous growth, thus curing the cancer without sacrificing the patient's ability to function. Cancer of the uterine cervix and the early stages of both prostrate cancer and Hodgkin's disease are well treated with radiation therapy. This technique is also especially important for treating cancer of the larynx (voice box), which can be cured without impairing the patient's ability to speak. In addition to preserving normal tissue, radiation therapy has another advantage over surgical

removal of a tumor. Radiation can, for instance, destroy microscopic extensions of cancerous tissue around a tumor that a scalpel might miss. Radiation is a safer option for older, frailer patients who might have difficulty recovering from surgery.

Chemotherapy

The century of the surgeon had begun with the discovery of anesthesia in 1846. Fifty years later, in 1896, Roentgen presented his famous paper on the X-ray. During World War II, naval personnel who were exposed to mustard gas as a result of a military action were found to have severe bone marrow depression. During that same period, the U.S Army was studying a number of agents related to mustard gas in order to develop more effective agents and to develop protective measures. In the course of that work, compound called nitrogen mustard was studied and found to have substantial activity against a cancer of the lymph nodes called lymphoma. This agent served as the model for a long series of similar but more effective agents (called "alkylating" agents) that killed rapidly proliferating cancer cells by damaging their DNA.

Table 1.5. Chemotherapeutic agents/Antineoplastic agents

Alkylating agents	***Nitrogen mustards*:** Chlorambucil, chlormethine, Cyclophosphamide, ifosfamide, Melphalan. ***Nitrosoureas*:** carmustine, fotemustine, lomustine, streptozocin. ***Plantinum*:** carboplatin, cisplatin, oxaliplatin, BBR3464 Busulfan, Dacarbazine, Mechlorethamine, procarbazine, temozolomide, ThioTEPA, Uramustine

Antimetabolites	***Folic acid*:** Aminopterin, methotrexate, pemetrexed, rattitrexed ***Purine*:** Cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, thioguanine. ***Pyrimidine*:** Capecitabine, cytarabine, fluorouracil, floxuridine, gemcitabine.
Spindle poison plant alkaloids	***Taxane:*** docetaxel, paclitaxel. ***Vinca*:** Vinblastine, vincristine, vindesine, vinorelbine.
Cytotoxic/antitumor antibiotics	***Anthra cycline family*:** Daunorubicin, doxorubicin, epirubicin, idarubicin, mitoxantrone, valrubicin. Bleomycin, hydroxyurea, mitomycin, actinomycin.
Topoisomerase inhibitors	***Camptotheca*:** Camptothecin, topotecan, irinotecan. ***Podophyllum*:** Etoposide, teniposide.
Cancer immunotherapy monoclonal antibodies	Alemtuzumab, bevacizumab, cetuximab, gemtuzumab, panitumumab, rituximab, tositumomab, trastuzumab.
Photosensitizers	Aminolevulinic acid, methyl aminolevulinic acid, porfimer sodium, verteporfin.
Kinase ihibitors	Dasatinib, erlotinib, gefitinib, imatinib, lapatinib, nilotinib, sorafenib, sunitinib, vandetanib.
Others	Alitretinoin, altretamine, amsacrine, anagrelide, arsenic trioxide, asparaginase, bexarotene, bortezomib, denileukin diftitox, estramustine, hydroxy carbamide, masoprocal, mitotane, pegaspargase.

Not long after the discovery of nitrogen mustard, Sidney Farber of Boston demonstrated that aminopterin, a compound related to the vitamin, folic acid, produced remission in acute leukemia in children. Aminopterin blocked a critical chemical reaction needed for DNA replication. That drug was the predecessor of methotrexate, a commonly used cancer treatment drug today. Since then, other researchers have discovered drugs that block different functions involved in cell growth and replication (Table 1.5). The era of chemotherapy had begun. The first cure of metastatic cancer was obtained in 1956 when methotrexate was used to treat a rare tumor called choriocarcinoma. Over the years, the development and use of chemotherapy drugs have resulted in the successful treatment of many people with cancer.

Drawbacks of available cancer treatments

Unfortunately, the available cancer treatments have several critical shortcomings. In surgery, removal of the tumor mass visible to the surgeon does not in itself guarantee elimination of the microscopic extensions that so often characterize cancer. To fully encompass this invasive edge around a tumor, a surgeon may be forced to cut out a large amount of healthy tissue and in doing so may severely damage the patients functioning or appearance. Sometimes cancer grips vital structures that cannot be surgically removed. Even when surgery is possible, major operations (and the general anesthesia required for them) invariably traumatize patients. Perhaps the most crucial limitations of surgery is that it cannot treat cancer that has metastasized widely throughout the body.

Radiation therapy at times also proves inadequate, because it like surgery, radiation cannot treat widespread metastasis that will eventually form full fledged tumors at numerous sites (whole body radiation exposure sufficient to kill widely dispersed cancer cells would destroy some delicate tissues that are vital). In such cases, a patient must make use of chemotherapy, the systematic administration of anticancer drugs that travel through out the body via the blood circulatory system.

The majority of the most common cancers (breast, lung, colorectal and prostrate cancer) are not yet curable with chemotherapy alone. For these conditions, chemotherapy can serve only as one component in an overall program of care that may also involve surgery and radiation. The available chemotherapeutic drugs often fail patients because they kill many healthy cells and thus bring on serious side effects that limit the doses physicians can administer. Damage to the rapidly growing cells of the bone marrow, for instance, causes anemia, an inability to fight infection and a propensity for internal bleeding, because the patient cannot produce an adequate number of red blood cells, white blood cells and platelets (the cells responsible for clotting). Other side effects of chemotherapy include diarrhea, nausea, vomiting and hair loss. Less commonly, these drugs may damage the nervous system.

Although strategies for ameliorating many of these unwanted side effects are quickly evolving, chemotherapy as currently offered retains another fundamental weakness. Like bacteria resistant to antibiotics, some tumors are able to survive the anticancer drugs used to treat them. Certain tumors prove to be drug resistant from the outset, whereas others develop resistance with repeated treatment. The problem of drug resistance in chemotherapy in particular is serious because tumors can develop a resistance to multiple drugs after only one drug has been administered to the patient.

Side effects

The treatment for cancer can be physically exhausting for the patient. Current chemotheraputic techniques have a range of side effects mainly affecting the fast-dividing cells of the body. Important common side-effects include (dependant on the agent):

(1) hair loss; (2) nausea and vomiting; (3) diarrhea or constipation; (4) anemia; (5) depression of the immune system and hence potentially lethal infections and sepsis; (6) hemorrhage; (7) secondary neoplasms; (8) cardiotoxicity; (9) hepatotoxicity; (10)

nephrotoxicity etc.

Virtually all chemotherapeutic regimens can cause depression of the immune system, often by paralyzing the bone marrow and leading to a decrease of white blood cells, red blood cells and platelets. Chemotherapy may cause an increased risk of cardio-vascular disease.

Problem in therapeutics for Metastasis

A primary cancer is a tumor mass present at the site of initial conversion of a normal cell to a tumor cell. If all cells remained in the primary tumor, cancer would be of little clinical importance. Growth of the tumor would produce pressure on surrounding tissue, which would ultimately become deleterious to the host, but the well-defined tumor mass could be excised by surgery in a straightforward and permanent way. However, tumor cells do not always remain at the primary site, but move away by one of two processes:

(1) Invasion, or the movement of cells into neighboring space occupied by other tissues and

(2) Metastasis, the spread of cells to distant sites, usually via the bloodstream, lymphatic system, or through body spaces.

Substantial invasion usually occurs before any metastasis takes place (Willis, 1973; Oppenheimer, 2004). Invasion in its simplest form is merely the expansion of tumor cells into surrounding tissues as a result of continuous cell division. However, active cell movement may also occur. Cells in cancers tend to adhere poorly to each other, break away from the tumor mass, and then move away from each other. Such movement does not occur in normal tissue. Normal cells do indeed move both in culture and in the course of embryological development, but when normal adult cells in culture touch each other, they usually stop both growth and movement. However, cancer cells, at least in culture, are not controlled by cell contact, and in the body they continue to grow and move into surrounding tissues. Also, most cancer cells

release proteases, which probably help them digest away surrounding tissue materials, thereby facilitating invasion (Oppenheimer, 2004; Mazzocca et al., 2005; Wilson et al., 2005). Metastasis is the spread of tumor cells to areas not directly adjacent to the primary tumor. Such spread can occur by invasion of blood or lymph vessels or by penetration of the cancer cells into the body cavity or spaces surrounding organs.

Metastasis generally occurs in several stages (Wood, 1958; Wood et al., 1966; Willis, 1973; Oppenheimer, 2004; Gao et al., 2005; Mazzocca et al., 2005; Wilson et al., 2005)

1. Detachment of cells from the primary tumor.
2. Migration of tumor cells, penetration (invasion) of these cells into lymph vessels or blood vessels, and dissemination of the cells or cell clusters to distant areas.
3. Lodging of tumor cells in blood vessels of distant organs.
4. Invasion of tumor cells through the vessel walls and into the tissue of secondary sites.
5. Growth of secondary tumors at the secondary sites.

The major drawback of majority of the cancer therapies is that they cannot treat cancer that has metastasized widely throughout the body. Cancer is a deadly disease because of its nature to spread. Also, most of the patients when they approach the doctors will be in stage IV condition (metastatic) and hence it is difficult to find a cure and often the patient dies. The only treatment practiced under such circumstances is by giving analgesic drugs until death.

In addition, the available chemotherapeutic drugs often kill many healthy cells, impair the immune system and thus bring on serious side effects that limit the doses physicians can administer. The patient has to compromise with lot of side effects and becomes weak more from the side effects of the drugs rather than from the tumor alone. Also, the problem of drug resistance in chemotherapy in particular

is serious because of the ability of tumors to develop resistance to multiple drugs. A drug that targets only cancer cells with little or no side effects has been the choice in cancer therapy research.

Alternative Therapy

Animals, including humans and most microorganisms depend directly or indirectly on plants as a source of food. It is reasonable to assume that through evolution plants have developed defense strategies like the ability for regeneration of damaged or eaten parts (leaves); mechanical protection (i.e., thorns, spikes, stinging hairs etc.,); thick bark in roots and stems, or the presence of hydrophobic cuticular layers; latex or resins which deter chewing insects; indigestible cell wall; and the production of secondary plant metabolites (Fritz & Simms, 1992). The latter may be the most important for plant defense. Plants produce numerous chemicals for defense and communication, but also plants can elicit their own form of offensive chemical warfare targeting cell proliferation of pathogens. These chemicals may have general or specific activity against key target sites in bacteria, fungi, viruses, or neoplastic diseases. Throughout recorded time humans have knowingly and unknowingly utilized plant metabolites as source of agrochemicals and pharmaceuticals. Discovery of vincristine and vinblastine (Svoboda, 1961; Neuss & Neuss, 1990) and its successful use initiated the search for natural product leads for the treatment of various cancers. Recent natural product discovery and development of avermectin (antihelmintic), cyclosporin and FK-506 (immunosuppressive), mevinolin and compactin (cholesterol – lowering), and taxol and camptothecin (anticancer) have revolutionized therapeutic areas in medicine (Kirst et al., 1992). Similar successful development of bioactive compounds has created a renewed interest in natural product agrochemical discovery. Because biologically derived chemicals are perceived by consumers as having less environmental toxicity and lower mammalian toxicity, there is a great desire to discover and develop natural product based nutraceuticals.

Furthermore, natural products had certain inherent desirable features. They tended

to be target specific, had high specific activity and most important, they were biodegradable. The last point should be emphasized because while some biologically active organic natural products can be quite toxic, they are, nevertheless, very biodegradable. Hence in the present investigation we selected two spices black cumin (***Nigella sativa***) and (***Decalepis hamiltonii***) which are widely used in Ayurveda for studying their antioxidant and antimetastatic activity.

Nigella sativa

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ranunculales
Family	:	Ranunculaceae
Genus	:	Nigella
Species	:	sativa

Black cumin (*Nigella sativa*) is an annual flowering plant belonging to the family Ranunculaceae, native to southwest Asia. It grows to 20-30 cm tall, with finely divided, linear (but not thread-like) leaves. The flowers are delicate, and usually coloured pale blue and white, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds. The seed is used as a spice since antiquity by Asian herbalist and pharmacist. Black cumin is used in India and the Middle East as a spice and condiment and occasionally in Europe as both a pepper substitute and a spice. It is widely used in Indian cuisines, particularly in mildly braised lamb dishes such as korma. It is also added to vegetable and dhal dishes as well as in chutneys. The seeds are sprinkled on to naan bread before baking. Black cumin is an ingredient of some garam masalas and is one of the fine spices in panch phoran. In the Middle East black cumin is added to bread dough. Black cumin is used in Indian medicine as a carminative and stimulant and is used against indigestion and bowel complaints. In India, it is used to induce post-natal uterine contraction and promote

lactation. Several studies on black cumin have shown its anticancer (Medenica et al., 1997), antimicrobial (Morsi, 2000), anti-ulcer (El-Dakhakhny et al., 2000), anti-inflammatory (Al-Ghamdi, 2001), diuretic and hypotensive activity (Zaoui, 2000).

***Decalepis hamiltonii* Wight & Arn.**

Swallow root (*Decalepis hamiltonii*, which belongs to the Asclepiadaceae family commonly called, as makali beru (in Karnataka), maredu kommulu or barre sugandhi or maradu gaddalu by the Yanadi tribe of Andhra Pradesh in India is an endangered species. The plant is indigenous to Andhra Pradesh and extends throughout the Eastern Ghats of South India and it can also be seen in the hilly and forest areas of the Western Ghats. The Yanadi tribals habitually carry the roots with them and chew the same to seek relief from indigestion and other intestinal disorders. It is largely used in making pickles along with lime. Swallow roots are being used in Ayurveda, the ancient Indian system of medicine to stimulate appetite, relieve flatulence and as a general tonic. The roots are also used as a substitute for *Hemidesmus indicus* in Ayurvedic preparations [Nayar et al., 1978].

Need for the current Study

Epidemiological studies show that a high intake of antioxidant rich foods is inversely related to cancer risk. Animal and cell cultures techniques confirm the anticancer effects of antioxidants. Intervention trials undertaken to determine their ability to reduce cancer risk in humans however, have been inconclusive, although selenium and vitamin E reported to reduce the risk of some forms of cancer such as prostate and colon cancers. Carotenoids have been shown to help in reducing breast cancer risk. In some cases the incidence of cancer in β-carotene treated groups were

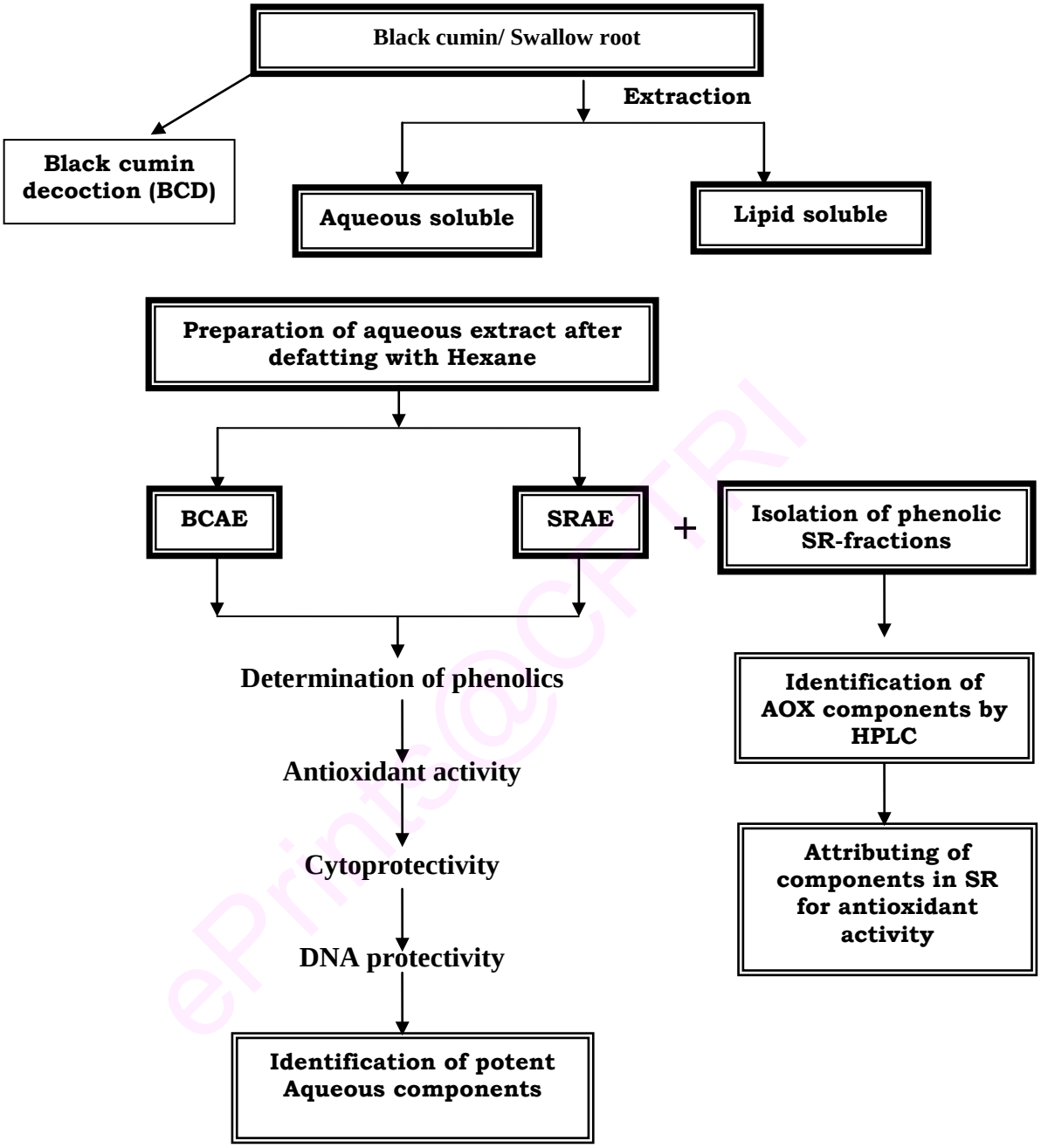
reported to be higher. Cancer treatment by radiation and anticancer drugs reduces inherent antioxidants and induces oxidative stress, which increases with disease progression. Vitamins E and C have been shown to ameliorate adverse side effects associated with free radical damage to normal cells in cancer therapy, such as mucositis and fibrosis, and to reduce the recurrence of breast cancer (Borek, 2004). While clinical studies on the effect of antioxidants in modulating cancer treatment are limited in number and size, experimental studies show that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells, suggesting a potential role for antioxidants as adjuvants in cancer therapy. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water or in lipids. water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. In general, cells are exposed to oxidative stress both at the cytosol as well as outside the cell membrane. Hence both water soluble and lipid soluble antioxidants are needed to effectively counteract oxidative stress. However, majority of the antioxidant molecules identified are lipid soluble and hence warrants the search for newer water soluble antioxidants. In addition, majority of cancers are incurable at the stage of metastasis because of cancer cells ability to spread to other tissues and organs. The available dietary antioxidants work at the initiation and promotion stages of the disease and are ineffective at the metastatic stages. These findings prompted us to investigate for newer water soluble antioxidant molecules and dietary components which can supress oxidative stress and prevent the spread of cancers, respectively, from selected spice sources namely black cumin (*Nigella sativa*)and swallow root (*Decalepis hamiltonii*).

Chapter II

Isolation and characterization of newer antioxidants from black cumin and swallow root

2.1. Hypothesis

Spices are known for good antioxidant potency and they have been implicated against varieties of biological disorders. However majority of the studies have reported the presence of lipid soluble antioxidant. In human diseases major diseases including cancer are initiated by oxidative stress and oxidative stress mediated membrane damage, cellular/DNA damage, tissue damage etc. For efficient quenching of these oxidative stress induced damage in cells and molecules, both water soluble and lipid soluble antioxidants are essential. Current study thus focuses on the identification of any newer antioxidants and their potency in selected spices-black cumin (*Nigella sativa*) and swallow root (*Decalepis hamiltonii*). Studies also aimed at aqueous extracts since these extracts were found effective in Ayurveda and traditional medicinal formulations



2.2. Work concept

2.3. Introduction

Plant and plant products are being recognized in recent days as safer and potential health promoting (Khan and Mukhtar, 2007), disease curing (Rathore et al., 2005), immune system boosting (Bin-Hafeez et al., 2003), detoxifying (Kaviarasan and Anuradha, 2007) and nutritive (Bovell-Benjamin, 2007) sources. Phenolic compounds constitute a group of substances that are widely distributed in the plant kingdom with different structures and biological activities. Among the phytochemicals, phenolic acids especially hydroxy cinnamates and hydroxy benzoates, the secondary metabolites, are commonly found in fruits, vegetables, seeds and other plant derived food sources (Herrmann, 1989). They have been reported to possess pharmacological properties such as antioxidant, antibacterial, and anticancer (Breinholt, 1999; Shahidi & Naczk, 1995).

The role of natural products are gaining more popularity in both developed and developing countries and much appreciated towards their applications as "alternatives" against chronic diseases such as diabetes, ulcer, cancer etc; particularly those diseases that require a long-term treatment, mainly due to complexities in their disease pathogenicity pattern. Although synthetic drugs are required for immediate relief, long term use of these drugs not only cause side effects such as nausea, allergy, immnosuppression etc., but leading themselves to be a causative factor for several disorders.

Cells and tissues of living system may become an easy target for exposure to free radicals generated during drug metabolism, in addition to the exogenous sources like ozone, exposure to UV radiations, cigarette smoke that result in free radical induced biomolecular changes, such as DNA damage, protein oxidation and generation of lipid peroxides leading to severity of the chronic diseases. Antioxidants are the choice to prevent oxidative stress induced diseases. Antioxidants are molecules which can donate electrons to free radicals and gets converted to a stable molecule

without becoming a free radical itself. The basic mechanism underlying the phenolic antioxidant action is depicted in Figure 2.1. In the present investigation two spice sources namely black cumin (*Nigella sativa*) and swallow root (*Decalepis hamiltonii*) were investigated for their biologically active components with an emphasis on antioxidant activity.

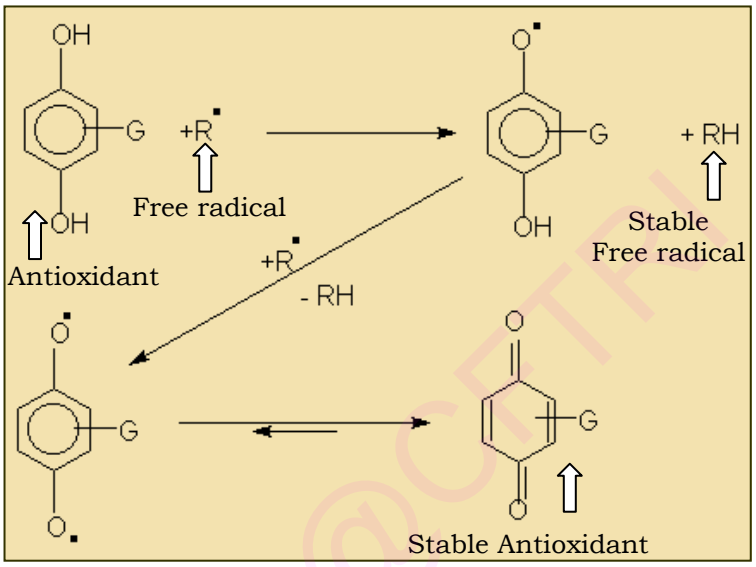


Figure 2.1. Mechanism of action of phenolic antioxidants

Black cumin (*Nigella sativa*)

Black cumin (*Nigella sativa*) is an annual herb belonging to the family Ranunculaceae. Its seeds have been used since antiquity by Asian herbalist and pharmacist for its therapeutic effects against oxidative stress induced diseases such as cancer (Medenica et al., 1997), immune disorders, as analgesic and antipyretic activity (Al-Ghamdi, 2001). Recent studies have indicated that a number of plant products (Oldham & Bowen, 1998), medicinal herbs (Andrea, 1997; Craig, 1999) and spices (Shobana & Naidu, 2000; Reddy & Lokesh, 1994) exhibit potent antioxidant activity. Black cumin has been reported to posses various biological activities (Morsi, 2000; El-Dakhakhny, Barakat, El-Halim & Aly SM, 2000; Zaoui, 2000; Burits & Bucar, 2000) mainly in its seed oil.

Figure 2.2. *Nigella sativa* (Black cumin) plant (a) and its seeds (b)

Black cumin is used in India and the Middle East as a spice and condiment and occasionally in Europe as both a pepper substitute and a spice. It is widely used in Indian cuisines, particularly in mildly braised lamb dishes such as korma. It is added to vegetable and dhal dishes as well as in chutneys. Black cumin is an ingredient of some garam masalas and is one of the five spices in panch phoran. Black cumin seeds are used to make tea by simply pouring hot water over the seeds and letting the brew steep for 10 min. In the Middle East Nigella is added to bread dough. The seeds are also added to casseroles or breads, used in canning, or extracted in wine or vinegar. Some people grind the seeds and mix them with honey or sprinkle them on salads (Schleicher & Saleh, 2000).

Black cumin seed oil is applied eventually for eruptions of skin. Seeds are useful in the form of tincture in indigestion, loss of appetite, fever, diarrhea, dropsy and puerperal fevers. Water decoction of seeds is given to recently delivered females. In combination with few other medicines, it stimulates uttering contraction. In doses of 10-20 g they are useful in amenorrhea and dysmenorrheal and in large doses cause abortions. Decoctions are also found to be beneficial against gastric disorders. Roasted seeds also have antibilious property and are administered internally in intermittent fevers and to arrest vomiting (Kapoor, 1990).

In cumin (*Cuminum cyminum*), the major components cuminaldehyde and eugenol have been identified along with trace amounts of ascorbic acid, β-carotene, gamma-terpinene, luteolin-7-glucoside, myrcene, tannin and terpinen-4-ol in the seeds (Duke, 2005). In Black cumin, the seeds (oil fraction) contain numerous esters of structurally unusual unsaturated fatty acids with terpene alcohols (7 %); furthermore, traces of alkaloids are found which belong to two different types: isochinoline alkaloids are represented by nigellimin and nigellimin-N-oxide, and pyrazol alkaloids include nigellidin and nigellicin. In the essential oil (avr. 0.5 %, max. 1.5 %), thymoquinone was identified as the main component (up to 50 %) besides *p*-cymene (40 %), á-pinene (up to 15 %), dithymoquinone and thymohydroquinone. Other terpene derivatives were found only in trace amounts: Carvacrol, carvone, limonene, 4-terpineol, citronellol. Furthermore, the essential oil contains significant (10 %) amounts of fatty acid ethyl esters. On storage, thymoquinone yields dithymoquinonene and higher oligocondensation products (nigellone). The seeds also contain a fatty oil rich in unsaturated fatty acids, mainly linoleic acid (50 – 60 %), oleic acid (20 %), eicodadienoic acid (3 %) and dihomolinoleic acid (10%) which is characteristic for the genus. Saturated fatty acids (palmitic, stearic acid) amount to about 30 % or less. Commercial Nigella oil may also contain parts of the essential oil, mostly thymoquinone, by which it acquires an aromatic flavour (Katzer, 2007). Even though the presence of various phytochemicals have been noted in black cumin (Duke, 2005), they were all lipid soluble components associated with the oil fraction. There have been no reports on the role of water soluble components towards biological activities including antioxidant activity in black cumin.

Since attention has been given mostly on lipid soluble antioxidants, current study was undertaken to evaluate the antioxidant potency in the water extract of black cumin. Black cumin is also consumed as boiled water extract or decoction and in soups and occasionally it is taken in ghee. The present investigation has therefore been simulated under these conditions to study the multi-potent antioxidant

activities in aqueous extract of black cumin and compared its efficacy with that of methanol extract. Attempts were also made to identify the probable active antioxidant molecules in aqueous extracts of black cumin (BCAE).

Swallow root (*Decalepis hamiltonii*)

Figure 2.3. Swallow root plant (a) and its roots (b)

Swallow root (*Decalepis hamiltonii*), also called as swallow root, belongs to the Asclepiadaceae family. It is one of the most potent sources with varieties of bioactivities (Georgea et al., 1999; Anup and Shivanandappa, 2006; Naik et al., 2007), and the roots are being used in Ayurveda, the ancient Indian system of medicine to stimulate appetite, relieve flatulence and as a general tonic. The roots are also used as a substitute for *Hemidesmus indicus* in Ayurvedic preparations (Nayar et al., 1978). Various laboratories have also put sustainable efforts in exploring the biochemical constituents present in swallow root and to understand their role as health beneficial sources (ref). Studies could provide not only the scientific basis for the previous observations made by traditional practioners, but also indicate the presence of phytochemicals that can contribute to the activity. Aldehydes, inositol, saponins, amyrins and lupeols (Murti and Seshadri, 1941) as well as volatile compounds such as 2-hydroxy-4-methoxybenzaldehyde (HMBA), vanillin, 2-phenyl ethyl alcohol and benzaldehyde (Nagarajan et al., 2001) have been

reported in swallow root. 4-hydroxyisophthalic acid, 14 - aminotetradecanoic acid, 4 - (1-hydroxy - 1 - methylethyl) – 1 – methyl - 1, 2-cyclohexanediol, 2 - (hydroxymethyl) – 3 - methoxybenzaldehyde, 2, 4, 8-trihydroxybicyclo[3.2.1]octan-3-one, bis-2, 3, 4, 6-galloyl-α/β-D-glucopyranoside, borneol and ellagic acid has been identified in swallow root (Srivastava et al., 2006; Srivastava et al., 2007) and also HMBA have been attributed to various bioactivities including the major component HMBA. HMBA has also been extracted from the tissue culture source of swallow root (ref).

Extensive work carried out in our laboratory revealed that phenolic acids play an important role against swim/ethanol stress induced ulcers, inhibition of H+K+-ATPase, DNA protective, inhibition of *Helicobacter pylori* (Srikanta et al., 2007; Siddaraju & Dharmesh, 2007, 2007a), in addition to antimetastatic property exhibited by pectic polysaccharide (Sathisha U.V et al., 2007). Characterization of bioactive pectic polysaccharide indeed showed the presence of 0.12 mg GAE phenolics/g of pectic polysaccharide.

In the current study therefore we report fractionation of phytoconstituents from aqueous extract of swallow root, since our previous studies revealed at least 2-4 fold increase in activity in aqueous extract of swallow root (SRAE) than that of methanol extract. Further fractionation of SRAE to SRAE-phenolics and other fractions and determination of free radical scavenging activity revealed that SRAE-phenolic fraction had ~ 4-fold increased activity with an IC_{50} of 0.046 µg/mL as opposed to that of crude. SRAE had an IC_{50} of 0.17 µg/mL in the same assay. In the current study therefore emphasis has been made towards isolation of free, hydrolyzed and bound phenolics of SRAE and determination of their antioxidant phenolics in comparison with already reported component in swallow root. In addition, nature of phenolic constituents present in these fractions and their precise contribution to antioxidant and antiproliferation activity has also been investigated This is the first report in swallow root highlighting the bioactive potentials of phenolic acids and understanding on their overall contribution to the bioactivity.

2.4. Materials and Methods

2.4.1. Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2-thiobarbituric acid (TBA), Folin-Ciocalteau reagent, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), gallic acid, tannic acid, ferulic acid, vanillic acid, ascorbic acid, linoleic acid, agarose, Tris HCl, tris barbitol, ethidium bromide, sudan black, tryphan blue, 1,1,3,3–tetramethoxy propane (TMP), soybean lipoxygenase, cell culture media (RPMI 1640), fetal bovine serum, L-glutamine, penicillin, streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and tert-butyl hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). λ Phage DNA was procured from GENEI, Bangalore, India. NIH 3T3 Fibroblast cells were purchased from National Center for Cell Sciences, Pune, India. The other chemicals such as ferric chloride, trichloro acetic acid n-butanol, copper sulphate, hydrogen peroxide, sodium carbonate, potassium ferricyanide, and solvents used in this experiment were of the highest quality purchased from local chemical company.

2.4.2. Plant material

(1) Black cumin (BC) seeds and fresh swallow roots (SR) were purchased from three different vendors (n = 3) from a local market (Devaraja market, Mysore, Karnataka, India), sun dried for 3 days, and powdered in a mixer (Gopi, C. Lal Electrical and Mechanicals Co. Ambala, India) and preserved in dry condition at 4 °C until further extraction.

2.4.3. Preparation of water extract

One gram of defatted (refluxed with hexane) powdered sample was mixed with 10 mL of distilled water and boiled for 5 min, cooled and centrifuged at 5000 *g* for 10 min. The supernatant was collected, stored at 4 °C till the completion of the experiment and referred as aqueous extract.

Black Cumin decoction was prepared by boiling 1 g of sample (black cumin seeds) in

100 mL of water for 20 min, cooled, filtered and concentrated to 10 mL, stored at 4 °C till the completion of the experiment. The extract was referred as black cumin decoction (BCD)

2.4.4. Preparation of methanol extract

One gram of powdered sample was mixed with 10 mL of methanol and stirred for 20 min and centrifuged at 5000 *g* for 10 min. The supernatant was collected, stored at 4 °C till the completion of the experiment and referred as methanol extract.

2.4.5. Determination of total phenol content

The total phenolic content of the samples were determined colorimetrically using the Folin-Ciocalteau method (Singleton and Rossi, 1965). A sample aliquot of 100 μL was added to 900 μL of water, 5 mL of 0.2 N Folin-Ciocalteau reagent and 4 mL of saturated sodium carbonate solution (100 g/L). The absorbance was measured at 765 nm in a spectrophotometer (Shimadzu UV-160 spectrophotometer, Tokyo, Japan) after incubation for 2 h at room temperature. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram of dry sample.

2.4.6. Determination of antioxidant activity

Antioxidant activity on human Low-Density lipoprotein (LDL) oxidation

Oxidative modification of low-density lipoprotein (LDL) in the arterial wall plays a key role in the pathogenesis of atherosclerosis. Hence, LDL is a good substrate for oxidation and to study the antioxidant activity of phytochemicals from spice sources (Aviram et al., 2005).

Plasma was collected from blood of human volunteers and stored at 4 °C. The LDL was prepared from the plasma using a differential ultra centrifugation method (Havel et al., 1995). LDL (100 μg protein/mL) with 16-80 μg GAE phenol/mL of sample (BC) was taken in a reaction tube and the reaction was initiated with the addition of 40 μL

of copper sulfate (2 mM) and the volume was made up to 1.5 mL in all tubes with phosphate buffer (50 mM, pH 7.4) (Akhilender Naidu and Thippeswamy, 2002). A tube without extract and copper sulfate served as negative control and another tube without extract served as positive control. All tubes were incubated at 37 °C for 3 h. The reaction was terminated by the addition of 0.25 mL TBA (1 % in 50 mM NaOH) followed by 0.25 mL of TCA (2.8 %). Tubes were vortexed and kept in boiling water bath for 45 min. After cooling to room temperature, the pink chromogen developed was extracted into n-butanol (2.0 mL) and its absorbance was measured spectrofluorimetrically (Shimadzu, Tokyo, Japan) at 515 nm excitation and 553 nm emission wavelengths. Thiobarbituric acid reactive substances (TBARS) were calculated from a calibrated standard graph of 1,1,3,3-tetramethoxypropane (0.05 – 0.25 nmoles/mL). The amount of protein was estimated by using Folin-phenol method (Lowry et al., 1951) and the results were expressed as amount of malondialdehyde (MDA) formed in nmoles/mg LDL protein in presence and absence of different concentrations of samples.

Measurement of LDL protection in gel shift assay

Electrophoretic mobility of LDL before and after oxidation and in presence and absence of samples (BC) were examined by agarose gel electrophoresis according to the method of Nobel (1968). 10 μL of native LDL (200 μg) was preincubated with (BC, 7.5 μg GAE phenol) for 30 min in 50 mM phosphate buffer saline (PBS), pH 7.4 in a total volume of 50 μL and oxidation reaction was initiated with 10 μM of $CuSO_4$. After 12 h the samples were applied on to 1 % agarose gel and electrophoresis was performed in tris-barbitol buffer pH 8.6 for 60 min at 100 v. The gels were fixed for 30 min in fixing solution (5 % acetic acid and 75 % ethanol), then washed and stained with Sudan black B.

Inhibition of lipoxygenase dependent lipid peroxidation

Lipoxygenases are iron-containing enzymes that catalyse the dioxygenation (Oxidation) of polyunsaturated fatty acids (PUFA).

Fatty acid (PUFA) + O_2 → Fatty acid hydroperoxide

To study the inhibition of enzymatic lipid peroxidation by spice antioxidants, lipoxygenases are the best enzyme system. Lipoxygenases are found in plants, animals and fungi. In this study lipoxygenase from soybean has been employed.

Enzymatic lipid peroxidation was measured spectrophotometrically by following an increase in absorbance due to the formation of lipid hydroperoxides according to the method of Narayan et. al (1999). Briefly the reaction mixture (3.0 mL final volume) in the sample cuvette contained 20 μM linoleic acid, 0.1 mL soybean lipoxygenase enzyme in 50 mM tris-HCl buffer pH 7.4 with and without sample (BC, 3.2 – 16 μg GAE phenol) as an enzyme inhibitor. A substrate blank with and without extracts was used to check the non-enzymatic lipid peroxidation. The formation of lipid hydroperoxide was measured at 234 nm in a UV spectrophotometer. An extinction coefficient of 25 $mM^{-1}cm^{-1}$ was used for quantification of lipid hydroperoxides. The enzyme activity was expressed as μmole of hydroperoxide formed/min/mg protein (IU).

Scavenging effect of extracts on DPPH radical

1,1-Diphenyl-2-picrylhydrazyl is a stable free radical that accepts an electron or hydrogen to become a stable 1,1, diphenyl-2-picrylhydrazine molecule. The reduction in DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally employed as a substrate to evaluate antioxidant activity of plant extracts (Lai et al., 2001).

An aliquot (200 μL) of sample (BC & SR) extract and standard antioxidants of various concentrations were mixed with 100 mM tris-HCl buffer (800 μL, pH 7.4) and then added to 1 mL of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Scavenging effect (\%)} = \frac{\text{(Absorbance of control - Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

Measurement of reducing power

The presence of reductants (i.e. antioxidants) in the sample causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of the Perl's Prussian blue colour at 700 nm. The $FeCl_3/K_3Fe(CN)_6$ system offers a sensitive method for the semi-quanitative determination of dilute concentrations of antioxidants, which participate in the redox reaction.

The reducing power of samples and standard antioxidants were determined according to the method of Yen and Chen (1995). The sample (BC & SR) extracts and standard antioxidants of various concentrations were mixed with equal volume of 0.2 M phosphate buffer, pH 6.6 and 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10 % trichloroacetic acid was added to the mixture and centrifuged at 1000 g for 10 min. The supernatant was mixed with distilled water and 0.1 % $FeCl_3$ at a ratio of 1 : 1 : 2 (v/v/v) and the absorbance were measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4.7. Cytoprotective assay of BC & SR extract on cultured NIH 3T3 fibroblast cells exposed to tert – butyl hydroperoxide.

A cytoprotective test on NIH 3T3 cells was conducted as previously reported (Nardini et al., 1998). NIH 3T3 is a murine fibroblast taken from the embryonic tissue with characteristic suitable for studying contact inhibition, transformation, transfection in cell biology. It is susceptible to murine leukemia virus, murine sarcoma virus, vesicular stomatitis, vaccinia, herpes simplex and N-tropic oncornaviruses C. NIH 3T3 fibroblast cells procured from National Centre for Cell Sciences (NCCS), Pune,

India. (1 X 10^6 cells/mL) were maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum, 2 mM l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5 % CO_2 and 95 % RH in complete medium.

Suspensions of NIH 3T3 fibroblast cells (2.8 X 10^4 cells/mL) were incubated with or without sample extract at various concentrations in a 96 well microplate (180 μL suspension/well). After 30 min of incubation, cells were treated with 500 μM tert – butyl hydroperoxide for another 3 h. Cell viability was assessed as described by Hansen et al., 1989). The reaction was terminated by adding 25 μL of MTT solution (5 mg/mL) to each well, and the cells were incubated at 37 °C for 4 h. Then, 100 μL of lysis buffer was added to each well and the cells were again incubated at 37 °C for overnight (about 16 h) to dissolve the dark blue crystals. Each well was completely pipetted and then the absorption of formazan solution at 570 nm was measured using a microplate reader.

2.4.8. DNA protection assay

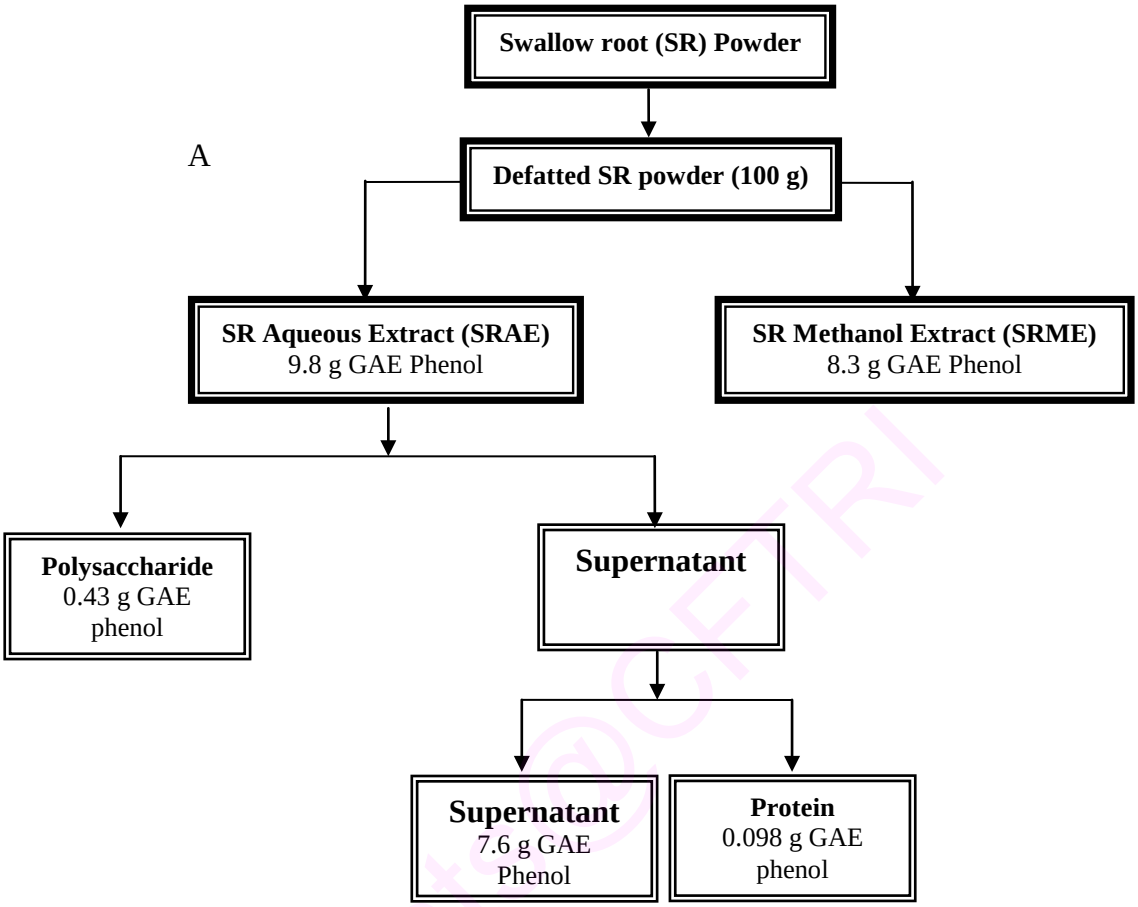
Fenton's reagent generates hydroxyl radical, and when this reagent is added to DNA, it induces strand breaks due to oxidation reaction. When the DNA is electrophoresed on an agarose gel it will migrate faster compared to that of native DNA. Comparing the electrophoretic mobilities of oxidized, native and antioxidants treated DNA, hydroxyl radical scavenging abilities of antioxidants were evaluated.

DNA protection activity was performed using λ phage DNA according to the method of Henry and Steven (1998) with little modification. Briefly λ phage DNA (0.5 μg) was added to Fenton's reagent (0.3 mM H_2O_2, 0.5 μM ascorbic acid and 0.8 μM $FeCl_3$) containing 0.16 – 0.64 μg GAE phenol of BC and 0.6 μg each of SR phenolic acid extracts per reaction mixture. The final volume of the mixture was brought up to 20 μL and then incubated for 30 min at 37 °C and the DNA was analyzed on a 1 % agarose gel followed by ethidium bromide staining.

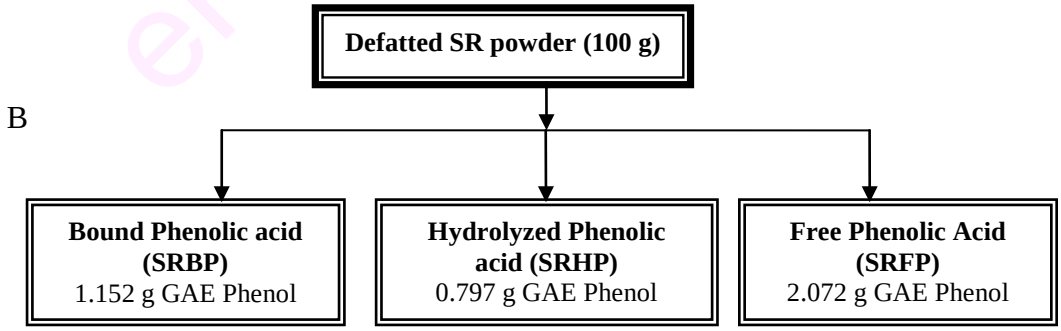
2.4.9. Extraction of free, hydrolyzed and bound phenolic acids of swallow root

The free, hydrolyzed and bound phenolic fractions in swallow roots were isolated according to the method followed by Liyana-Pathirana and Shahidi (2006) with slight modification. Two grams of defatted swallow root powder (in triplicates, n = 3) was extracted six times with 40 mL of methanol-acetone-water (7:7:6 v/v) at room temperature (25 ± 2 °C) for 2 h with constant stirring. The mixtures were then centrifuged at 4000 g for 20 min (Sigma 3-16K, USA) and supernatants were collected and combined. The solvent was evaporated at 30 °C under vacuum in a flash evaporator (Buchi 011, Switzerland) to approximately 40 mL. Concentrated supernatant was acidified to pH 2 with 6 M hydrochloric acid, extracted six times with diethyl ether and phenolic acids so extracted were labeled as free phenolic acids (SRFP). The supernatants with hydrolyzed phenolic acids were then treated with 30 mL of 4 M sodium hydroxide for 4 h at room temperature under nitrogen atmosphere. The resultant hydrolysate was acidified to pH 2 using 6 M hydrochloric acid and extracted six times with diethyl ether. The ether extracts were then combined and evaporated to dryness at 30 °C under vacuum. The phenolic acids extracted were those liberated from their esters and labeled as hydrolyzed phenolics (SRHP). The leftover residue after extractions was treated with 20 mL of 4 M sodium hydroxide for 4 h at room temperature under nitrogen atmosphere and then acidified to pH 2 with 6 M hydrochloric acid followed by centrifugation (4000 g, 20 min). The mixture was extracted six times with diethyl ether. The ether extracts were combined and evaporated to dryness under vacuum at 30 °C. The phenolics so extracted were labeled as bound phenolics (SRBP). Free, hydrolyzed and bound phenolic acids were dissolved separately in 2 mL of methanol and stored at –20 °C until used within 1 week.

A. Fractionation of Swallow root



B. Isolation of Phenolic Fractions from Swallow root



Scheme 2.1 Fractionation of Swallow root

2.4.10. Identification of phenolic acids by HPLC

The potential active components in sample (BC & SR) extracts were characterized by HPLC (model LC-10A. Shimadzu Corporation, Tokyo, Japan) analysis on a reverse phase Shimpak C_{18} column (4.6 × 250 mm) using a diode array UV-detector (operating at 280 nm). A solvent system consisting of water : acetic acid : methanol (isocratic; 80 : 5 : 15, v/v/v) was used as mobile phase at a flow rate of 1 mL/min (Subba Rao and Murlikrishna, 2002). Standard phenolic acids such as tannic, caffeic, *p*-coumaric, *p*-hydroxybenzoic, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were used for identification of phenolic components present in the sample extract. In addition vanillin and 2-hydroxy-4-methoxybenzaldehyde (HMBA) was also used as standards.

2.4.11. Spectroscopic measurements of antioxidant molecules

The purified phytochemicals from the samples were characterized by UV absorption, infrared, mass and NMR studies. Mass were obtained with Finnigan MAT 95 mass spectrometer by injecting the sample dissolved in millipore water. Infrared spectroscopy of bioactive antioxidant isolated from the samples (~1.0 mg) were made by blending the sample thoroughly with potassium bromide and made into a thin disc. Its spectra was obtained with Perkin-Elmer (2000 system GC-IR) operating at 4 cm^{-1} resolution. ^{13}C and ^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. The samples (50 mg) were dissolved in DMSO (1 mL) for recording the spectra. The spectral data are reported as ppm downfield from tetramethylsilane (TMS) (δ=0).

2.4.12. Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation (n=3). The significance of difference was calculated by Student's *t* test, and values *p < 0.05 and **p < 0.01 were considered to be significant. One way ANOVA followed by Duncan's multiple range test was also used to determine the difference in mean values between sample groups.

2.3. Discussion

Generally phenolic compounds found in plants are secondary metabolites, and have been reported to posses' bioactivity including antioxidant activity (Sato et al., 1996). Since majority of the studies have reported lipid soluble antioxidants, there is a growing need for water soluble antioxidants which are helpful in bring down oxidative stress at the cytosolic level. In the present investigation, detailed efforts have been put to identify major antioxidant components from black cumin (BC) and swallow root (SR). The current chapter addresses the role of phenolics of BC and SR in exhibiting cytoprotective/DNA protective and antioxidant potency.

In the current study, our results indicate 3 - 4 fold higher phenolic content in BCAE compared to BCD and BCME. However, in comparison with SRAE, BCAE has 31 fold lesser phenolic content. In swallow root also, the aqueous extract showed higher (1.2 fold) phenolic content than methanol extract.

Lipid peroxidation caused by oxidative stress is detrimental to the cell both at membrane and genetic level, and has been attributed in the diseases such as cancer, cardiovascular diseases and diabetic complications (Cai and Harrison, 2000; Ames, 1987; Perry et al., 2000; Phull et al., 1995; Hannon-Fletcher et al., 1999). The hypothesis that the oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in the progression of atherosclerosis has been widely accepted (Niki, 2004). This prevalent oxidation hypothesis implies that the antioxidants, which inhibit oxidation of LDL should be effective for suppressing atherosclerosis. In the present study we have studied the effect of BCAE and BCD as they were prescribed in the Ayurveda medicine for many ailments and also of BCME to compare the efficiency of extraction of active components using water and methanol as solvents.

Figure 2.4A shows the antioxidant activity of various fractions of black cumin. It is evident from the results that BCAE had a higher LDL protective ability than that of BCD and BCME. The antioxidant activity in black cumin decoction may be due to

the leaching of water soluble components during boiling which supports the use of BC decoction for health promoting effects as prescribed by the Ayurveda practitioners.

In addition, the ability of BCAE, BCME and BCD to protect LDL against Cu^{++} induced oxidative damage was also studied and the results showed higher activity in BCAE compared to BCME and BCD. These results indicated that lipoprotein peroxidation could be inhibited effectively by BCAE.

The oxidation of low density lipoprotein (LDL) by lipoxygenase has been implicated in the pathogenesis of atherosclerosis. It has been known that lipoxygenase mediated lipid peroxidation proceeds in general via regio, stereo and enantiospecific mechanisms, but that it is sometimes accompanied by a share of random hydroperoxides as side reaction products (Kuhn et al., 1994). In this study we investigated the inhibitory effect of BCAE and BCME on soybean lipoxygenase mediated oxidation of linoleic acid. From figure 2.5 it is evident that the enzyme inhibitory activity of BCME increases gradually and it proceeds in a stoichiometric manner. Results indicated higher inhibitory activity in BCAE at similar concentrations compared to BCME. These results clearly showed that the water extract of black cumin was a potent inhibitor of lipoxygenase mediated lipid peroxidation, at least 10 fold better than BCME in *in vitro* inhibition of LDL oxidation.

DPPH is a stable free radical that accepts electron or hydrogen to become a stable diamagnetic molecule. The reduction in DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally employed as a substrate to evaluate antioxidant activity of plant extracts (Hu and Kitts, 2000). We studied the antioxidant activity of BC and SR using this free radical. The extracts of BC, SR and the individual phenolic compounds isolated from these sources in addition to standard antioxidants were evaluated for DPPH radical scavenging activity (Table 2.1) In comparison with the standard antioxidants

BCAE exhibited an IC_{50} of 1.36 mg/mL, which was nearly equal to the IC_{50}'s of tannic and ascorbic acid. Also, 2-fold reduction in scavenging activity was observed in BCME in comparison with BCAE. In swallow root extract, the IC_{50}'s of SRFP, SRHP and SRBP for DPPH radical scavenging activity were in the range 0.04-0.13 µg GAE phenol/mL, which are nearly 34-98 folds more active than black cumin extract. These results indicate the presence of antioxidant components which acts as free radical inhibitors both in BC and SR, but the activity is substantially low in BC compared to SR. The higher antioxidant activity of SR extracts over BC extracts is evident in all the antioxidant assays.

The antioxidant components present in these extracts have effective activities as hydrogen donors and hence may stabilize the free radicals avoiding its reaction with cellular components.

The reducing power ability at 6.4 µg GAE of BCAE was compared with BCME and standard antioxidants. The reducing power of BCAE compared to the standard antioxidants indicated higher reducing power than that of ferulic, BHA, BHT and vanillic acid. However the activity was almost similar to that of tannic acid while the BCAE activity was much lesser (1.5 – 2.0 fold) than gallic acid and ascorbic acid. In comparison with BCME, the reducing power of water extract was more pronounced with 4-fold higher activity. Among the SR extracts tested for reducing power, SRHP had the highest reducing power followed by SRBP and SRFP. At 0.15 µg GAE phenol/mL of SRFP the reducing power is almost equal to that of BHA (0.125 µg/mL)

The reducing power of a compound is related to its electron transfer ability and may, therefore, serve as an indicator of its potential antioxidant activity (Yildirim et al., 2003). Moreover, extracts with phenolic substance-mediated antioxidant activity were shown to be concomitant with the development of reducing power (Siddhuraju and Becker, 2003) thus, BC and SR extracts can act as electron donors and can react with free radicals and convert them to more stable products and

terminate radical chain reaction (Chung et al., 2002).

To examine whether the BC and SR extracts could also inhibit the oxidative stress in normal NIH 3T3 fibroblast cells they were preincubated with various concentrations of the extracts followed by treatment with tert-butylhydroperoxide. The viable cells were determined based on the MTT assay in which, MTT gets converted to an insoluble dark blue formazan crystals in the actively respiring cells. The absorption value of this complex gives an estimation of the viable cells. Our results indicated a higher cytoprotection by BCAE in a dose dependent response. The maximum cell protection (65 %) against oxidative stress was observed at a concentration of 32 μg GAE phenol/mL for BCAE and an equal percent protection was offered by SR phenolic acid extracts in the concentration range of 0.12-0.15 μg GAE phenol/mL. These results indicate the presence of antioxidant components in the water-soluble extract of BC and the phenolic acid fractions SR. Therefore it is possible that the intracellular reactive oxygen species in NIH 3T3 cells may also be reduced after treating cells with BC and SR extracts. BCD and BCME also protect cells but to a lesser extent (data not shown).

In biochemical systems the superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently generate extremely reactive hydroxyl radicals in the presence of transition metal ions especially iron (Halliwell & Gutteridge, 1981). Hydroxyl radicals can damage DNA. To examine the DNA protective effect of BC and SR extracts, λ phage DNA was damaged using Fenton's reagent (hydrogen peroxide, ferric chloride and ascorbic acid). Figure 2.11 shows the extent of DNA damage induced by Fenton's reagent and the protection offered by BCAE, SRFP, SRHP and SRBP as revealed by the relative electrophoretic mobility of the oxidized and BCAE treated DNA compared to that of undamaged DNA (lane 1). Addition of Fenton's reagent caused the fragmentation of DNA (lane 2) and hence increased the electrophoretic mobility. However, BCAE showed an increased DNA protection (lane 3-6) with an increase in the dose (0.16 - 0.64 μg GAE phenol) of the extract. A maximum DNA protection was observed at 0.64 μg GAE phenol of the

extract (lane 6). In SR extracts treated study, SRHP showed higher DNA protection followed by SRBP and SRFP at 0.6 μg dosage. These results indicate that BC and SR extracts protects DNA from damage by quenching the hydroxyl radicals generated by the Fenton's reagent.

To assess the active molecule (s) exhibiting antioxidant activity in BCAE, the extract was fractionated by HPLC. A major component with retention time of 6.33 min constituting 1.49 mg/g and a minor component with a retention time of 8.63 min constituting 0.51 mg/g were isolated. The major component bound effectively to a tannin specific affinity matrix, and was confirmed as tannic acid by 2D NMR analysis. The other component matched with that of protocatechuic acid. Of the total activity in BCAE 77 % contribution is from tannic acid and the remaining is from protocatechuic acid (23%).

Further, the presence of tannins has been reported in various other sources exhibiting biological activities (Seeram et al., 2005; Cai et al., 2002). However, the present investigation provides a novel source of water-soluble antioxidants in *N. sativa* seeds, which are helpful to counteract the oxidative stress induced conditions in the body. Several reports are available in literature on organic solvent soluble antioxidants and but very little information on water soluble antioxidant in spices are available. BCAE thus finds greater significance in providing water soluble antioxidants to the body. Black cumin decoction envisaging health beneficial properties in Ayurveda/traditional system may thus be working via water soluble antioxidant – tannic acid.

In swallow root, the study addresses whether the bioactivity is attributed to HMBA, a predominant component as reported earlier (Nagarajan et al., 2001) or to some other component. We determined the bioactive capacities employing various antioxidant assays along with standards and calculated the precise contribution of these components responsible for antioxidant activities of various fractions of swallow root. However, there are no reports available on the presence of phenolic

acids and their contribution towards cytoprotective and antioxidant activity in swallow root. SRFP, SRHP and SRBP were isolated from swallow root by differential extraction procedure. Since phenolic acids were known to possess biological activity (Hsu et al., 2006), the isolated phenolic acid extracts were evaluated for cytoprotective effect, antioxidant activity and identification of their constituent phenolic acids. The demonstrated cytoprotective and antioxidant ability may be involved in the health benefits that are attributed to swallow root phenolics and may work synergistically with other cytoprotective (Srivastava et al., 2007) and bioactive molecules reported earlier (Harish et al., 2005; Srivastava et al., 2006). A correlation coefficient was established between the phenolic content and their activity in each assay. Results indicated no significant correlation co-efficient suggesting that not only phenolic content but different phenolic constituent present in each fraction may be responsible for the activity. Even though the total phenolic content is higher in SRFP extract (Table 2.2), at equal GAE phenolic concentration SRHP (1.2-fold) and SRBP (1.3-fold) showed higher antioxidant and cytoprotective activity than SRFP probably because of the presence of gallic acid, a very good antioxidant molecule up to 75 and 58 %, respectively. SRFP and SRBP indicated the presence of abundant poor antioxidant molecules like vanillin, HMBA, and *p*-hydroxy benzoic acid compared to SRHP. The differences in antioxidant activity may be attributed to the presence of different phenolic acids with different antioxidative potential and their synergistic effects. These results may strengthen the view of use of swallow root for their medicinal properties in Ayurveda and folklore medicine.

The presence of phenolic acids in bound form particularly in association with polysaccharides/lignin has been reported earlier (Iiyama et al., 1990; Lapierre et al., 2001). Cinnamic acid derivatives are usually seen bound to polysaccharides (Shyama Prasad Rao and Muralikrishna, 2004). In swallow root however (Table 2) the presence of derivatives of hydroxybenzoate (gallic aicd) and hydroxybenzaldehyde (vanillin and HMBA) were found in the bound form. Vanillin, HMBA and *p*-hydroxybenzoic acid being poor antioxidant molecules, the presence of these compounds had little contribution towards total antioxidant activity evidently as in

the case of SRFP and SRBP. Hence, the cytoprotective and antioxidant properties can be attributed to the phenolic acids like gallic, protocatechuic, gentisic, and vanillic acid in SRFP; gallic and gentisic in SRHP and gallic and *p*-coumaric acid in SRBP extracts. These phenolic acids were reported to possess good antioxidant activities (Miller & Rice-Evans, 1997). The presence of phenolic acids both in free and bound form attached to various polysaccharides is of significant interest in preventing oxidative stress induced diseases. The free phenolic acids are easily absorbed into the circulation while the phenolic acids bound to the polysaccharides are released by the intestinal enzymes as well as by the colonic microflora and can be absorbed into the circulatory system (Andreasen et al., 2001; Andreasen et al., 2001a). This may have a significant role in the reduction of oxidative stress in lower alimentary canal also.

Earlier studies have shown the presence of HMBA as a major constituent in water/methanol extracts and in the volatile oil fraction of swallow root (Nagarajan & Rao, 2003; Harish et al., 2005). The presence of HMBA in the SRFP extract is therefore obvious. Hence, the presence of HMBA in the SRBP extract is an additional finding and the precise way in which HMBA is associated with the macromolecules especially polysaccharides needs to be addressed. The relative percent contribution of HMBA in SRBP extract was 2-fold higher than in the SRFP extract.

In conclusion, Black cumin and swallow root phenolic acid extracts showed antioxidant activity including cytoprotectivity, reducing power, radical scavenging ability and protection to DNA damage induced by hydroxyl radical. Black cumin aqueous extract had significant activity than their methanolic extact and decoction. While, in swallow root, hydrolyzed phenolic acid extract (SRHP) showed good antioxidant activity followed by bound (SRBP) and free (SRFP) phenolic acid extracts. Black cumin aqueous extract showed the presence of tannic acid as the major water soluble antioxidant in addition to protocatechuic acid. Swallow root phenolic acid extracts contained both hydroxybenzoic and cinnamic acid derivative as antioxidant molecules to different extent and the bulk of the activity is contributed mainly from

gentisic, gallic, protocatechuic, vanillic and *p*-coumaric acid. Studies thus support the use of black cumin and swallow root in Ayurveda and folklore medicine and as a nutraceutical in the preparation of food and health drinks.

Summary and conclusions

- Aqueous extracts of Black cumin – BCAE and Swallow root – SRAE showed multi-potent antioxidant properties including free radical scavenging, reducing power, inhibition of lipid peroxidation and cyto/DNA protectivity.

- BCAE and SRAE showed 1.9-2.5 fold increase in antioxidant potency as evaluated by free radical scavenging effect than their respective solvent (methanol) extracts – BCME and SRME.

- Antioxidant activity was attributed to tannic acid in BCAE; while gentisic acid, gallic acid, protocatechuic acid and vanillic acid in SRAE. HMBA although found in abundant quantity, contributed much less than phenolic acids.

2.5. Results

2.5.1 Total Phenolic content in Black Cumin and Swallow root extracts

The total phenolic contents of various extracts of black cumin as estimated by the Folin-Ciocalteau method are shown in Table 2.1. Our results indicated 3 - 4 fold higher phenolic content in BCAE (3.2 mg/g) compared to BCD (1.02 mg/g) and BCME (0.82 mg/g). Comparison of antioxidant activity by various assays are also consolidated in this Table and results are discussed at later part.

Table 2.1. DPPH radical scavenging activity, total antioxidant activity, reducing power and phenolic content, of black cumin extracts and known standard antioxidants.

Sample / Standard Antioxidants	DPPH radical Scavenging activity (IC_{50} in µg/mL)	Total reducing power in Units*	Total phenol (in mg/g GAE phenol)	Total AOA in Units** (% contribution)
Ascorbic acid	1.30 ± 0.02^{b}	8.56 ± 0.11^{f}	Standard	-
Tannic acid	1.15 ± 0.03^{a}	4.56 ± 0.12^{d}	1.49 ± 0.02^{c}	1296^{a} (77%)
Protocatechuic acid	1.35 ± 0.08^{b}	3.88 ± 0.15^{c}	0.51 ± 0.01^{a}	378^{b} (23%)
Gallic acid	1.1 ± 0.04^{a}	6.41 ± 0.17^{e}	Standard	-
Ferulic acid	2.55 ± 0.05^{c}	2.8 ± 0.07^{b}	Standard	-
Vanillic acid	40.00 ± 0.7^{f}	0.089 ± 0.02^{a}	Standard	-
BHT	8.5 ± 0.08^{e}	0.85 ± 0.05^{a}	Standard	-
BHA	3.9 ± 0.05^{d}	1.36 ± 0.05^{a}	Standard	-
BCAE	1.36 ± 0.06^{b}	4.16 ± 0.05^{cd}	3.18 ± 0.16^{d}	-
BCME	2.55 ± 0.09^{c}	1.06 ± 0.05^{a}	0.82 ± 0.08^{ab}	-
BCD	1.77 ± 0.04^{b}	2.89 ± 0.09^{b}	1.02 ± 0.06^{b}	-

* 1 Unit = 0.1 OD at 700 nm; ** 1 Unit = IC_{50}. Values are expressed as mean ± SD (n=3). Mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$).

Swallow root was fractionated into polysaccharide (SRAE-PS), protein (SRAE-protein), phenolic (SRAE-phenolic) fractions as per the scheme 2.2A and free, hydrolyzed and bound phenolic acid fractions and estimated the percent of phenolics found in each fraction. Data presented in Scheme 2.1 and Table 2.2 indicate that of the total of ~ 98 mg/g of phenolics in SRAE, 75.7 mg/g has been recovered in SRAE-phenolic fraction. Remaining ~ 4 % has been found associated with polysaccharide and negligible amount (~1 %) in protein fraction of swallow root. Infact total yield of protein in itself is less ~ 3 % in case of aqueous extract which is ~ 7-fold lesser than carbohydrates. SRME – SR methanolic extract indicated 83.6 mg/g of total phenolic content.

Swallow root fraction	Total phenol (in mg/g GAE phenol)	Total protein (in mg/g)	Total Carbohydrate (in mg/g)
SRAE	98.1 ± 1.01^{h}	3.76 ± 0.08^{c}	31.68 ± 1.34^{c}
SRME	83.6 ± 1.53^{fg}	--ND--	--ND--
SRAE-PS	3.9 ± 0.09^{b}	0.063 ± 0.002^{a}	12.79 ± 0.19^{b}
SRAE-Protein	0.94 ± 0.02^{a}	2.9 ± 0.06^{b}	0.53 ± 0.02^{a}
SRAE-Phenolic	75.7 ± 3.59^{f}	0.06 ± 0.001^{a}	12.7 ± 0.21^{b}
SRFP	20.72 ± 0.77^{e}	--ND--	--ND--
SRHP	7.97 ± 0.50^{c}	--ND--	--ND--
SRBP	11.52 ± 0.54^{d}	--ND--	--ND--

Table 2.2 Total phenol, protein and carbohydrate content of various fractions of swallow root SRAE – swallow root aqueous extract; SRME – swallow root methanol extract; SRAE-PS – polysaccharide fraction of swallow root aqueous extract; SRAE-Protein - protein fraction of swallow root aqueous extract; SRAE-Phenolic – Phenolic fraction of swallow root aqueous extract; SRFP, SRHP and SRBP are swallow root free , hydrolyzed and bound phenolic acid fractions, respectively. Values are mean ± SD (n = 3). Mean value followed by different letters (a, b, c, d, e, f, g, h) in the same column are significantly different ($p \leq 0.05$); ND – Not determined.

The total phenolic content of phenolic acid fractions as per Table 2.2 indicated higher amount of total phenolics in the SRFP extract (20.72 ± 0.77 mg GAE/g) followed by SRBP (11.52 ± 0.54 mg GAE/g) and SRHP extract (7.97 ± 0.5 mg GAE/g). In comparison with SRFP extract, 1.8-fold and 2.6-fold reduction in total phenolic content was observed in SRBP and SRHP extracts, respectively.

2.5.2 Effect of Black Cumin extracts in inhibiting LDL oxidation.

Figure 2.4A shows the antioxidant activity of various fractions of black cumin. It is evident from the results that BCAE had a higher LDL protective ability than that of BCD and BCME. Even though all the extracts showed activity with a significance level of $p < 0.05$, 3 and 4 fold higher activity was observed in BCAE compared to BCD and BCME at equal concentration of 7.5 μg GAE phenol/mL respectively.

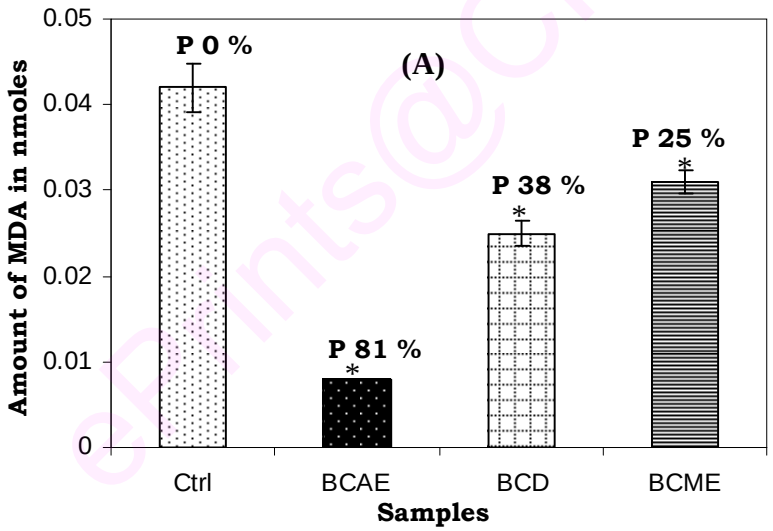
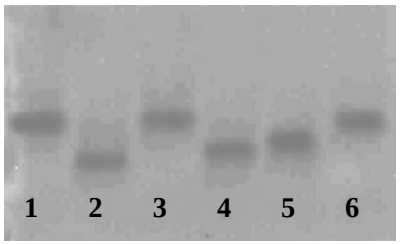


Figure 2.4. (A) Effects of BCAE, BCME and BCD on Cu^{++} induced LDL oxidation. Equivalent amounts of GAE phenol from each extract were used for inhibition. Results are expressed as mean ± SD (n = 3). * Represent $p < 0.05$ compared with control. % protection is indicated on top of the respective bar.

(B)



(B) Electrophoretic analysis of LDL oxidation induced by Cu^{++} and its protection by BC extracts and ascorbic acid. Native LDL - lane 1; oxidized LDL - lane 2. Antioxidant and black cumin extract treated - ascorbic acid 2 μg - lane 3; 2 μg GAE phenol of BCME – lane 4; 2 μg GAE phenol of BCD – lane 5; 2 μg GAE phenol of BCAE – lane 6.

Figure 2.4B illustrates the protective effect of BCAE, BCD and BCME on LDL oxidation as evidenced by agarose gel shift assay also. Oxidized LDL (lane 2) showed an increased anodic mobility relative to that of unoxidized native LDL (lane 1). At equal concentrations (2 μg GAE phenol/mL), BCME and BCD showed lesser protective effect while BCAE had a higher LDL protective ability, which was nearly equal to that of the standard antioxidant ascorbic acid, tested at 2 μg/mL concentration.

2.5.3 Effect of Black Cumin extracts on soybean lipoxygenase induced linoleic acid peroxidation.

Soybean lipoxygenase enzyme inhibitory activity of BCME increased gradually in a stoichiometric manner (Figure 2.5). In the case of BCAE there is a higher inhibitory activity at similar concentrations compared to BCME and gradually reached a saturation point showing 100 % inhibition at 120 μg GAE phenol/mL concentration. An IC_{50} of 60 μg GAE phenol/mL was there fore observed for BCAE extract compared to BCME, which showed an IC_{50} of 316 μg GAE phenol/mL and BCD which showed an IC_{50} of μg GAE phenol/mL.

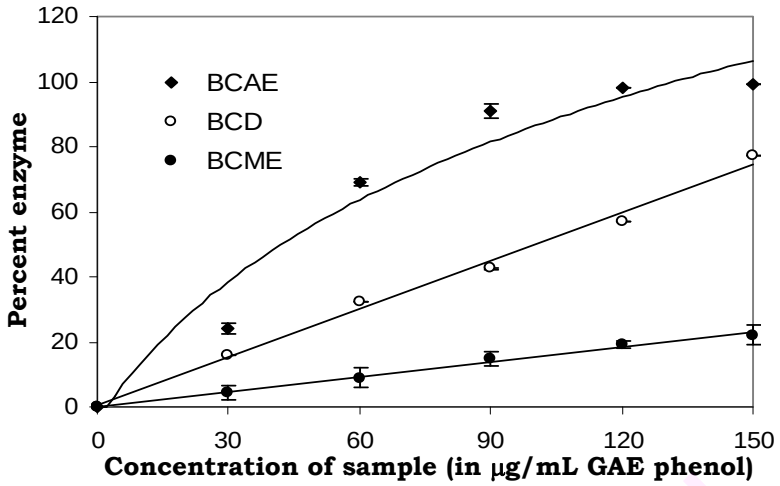


Figure 2.5. Effect of BCAE, BCD and BCME on soybean lipoxygenase induced linoleic acid peroxidation. Results are expressed as mean ± SD (n =3).

2.5.4. DPPH radical Scavenging ability

DPPH radical scavenging activity of the spice extracts (SR) showed significant scavenging activity. In comparison with the standard antioxidants, BCAE exhibited an IC_{50} of 1.36 mg/mL, which was nearly equal to the IC_{50}'s of tannic and ascorbic acid (Table 2.1). In addition, ferulic acid, BHA and BHT showed lesser (2 – 6 fold) activity while vanillic acid indicated a very poor radical scavenging activity compared to BCAE with an IC_{50} of 40 μg/mL. Further a 2-fold reduction in scavenging activity was observed in BCME in comparison with BCAE.

Figure 2.6 shows percent DPPH radical scavenging activity of swallow root phenolic extracts in addition to standard BHA. All the phenolic acid extracts showed free radical scavenging ability with an IC_{50} of 0.046, 0.128, 0.06 μg GAE/mL and 0.08 μg/mL for SRHP, SRFP, SRBP and BHA, respectively, suggesting that SRHP SRBP are more potent in free radical scavenging activity equivalent to that of BHA. These results from both BC and SR indicate the presence of antioxidant components, which acts as free radical inhibitors.

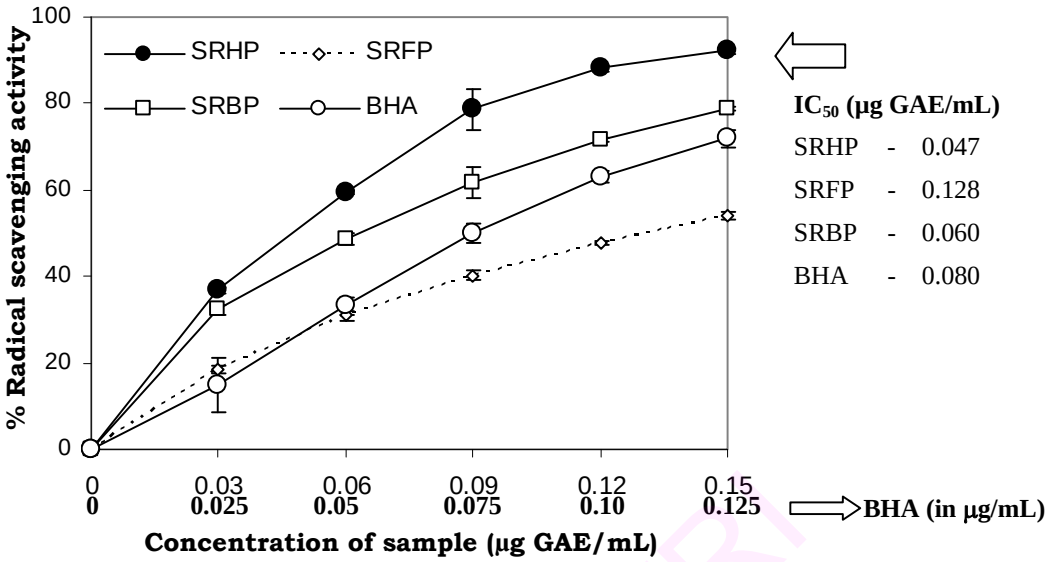


Figure 2.6. DPPH radical scavenging activity of swallow root extracts and standard antioxidant BHA. Values are expressed as mean ± SD

2.5.5. Reducing power ability.

The reducing power ability of BCAE was compared with BCME, BCD and standard antioxidants. Reducing power of the phenolic extracts of swallow root (Figure 2.8) indicated a dose dependent increase in activity of phenolic acid fractions of swallow root. The increased absorbance at 700 nm due to the reduction of potassium ferricyanide/ferric chloride complex indicates the presence of reducing power in all the phenolic acid extracts tested including the standard antioxidant BHA. The reducing power of BCAE indicated 1.4 and 3.9-fold higher activity compared to BCD and BCME, respectively (Figure 2.7). The standard antioxidants indicated higher reducing power than that of ferulic, BHA, BHT and vanillic acid (Table 2.1). However the activity was almost similar to that of tannic acid while the BCAE activity was much lesser (1.5 – 2.0 fold) than gallic acid and ascorbic acid.

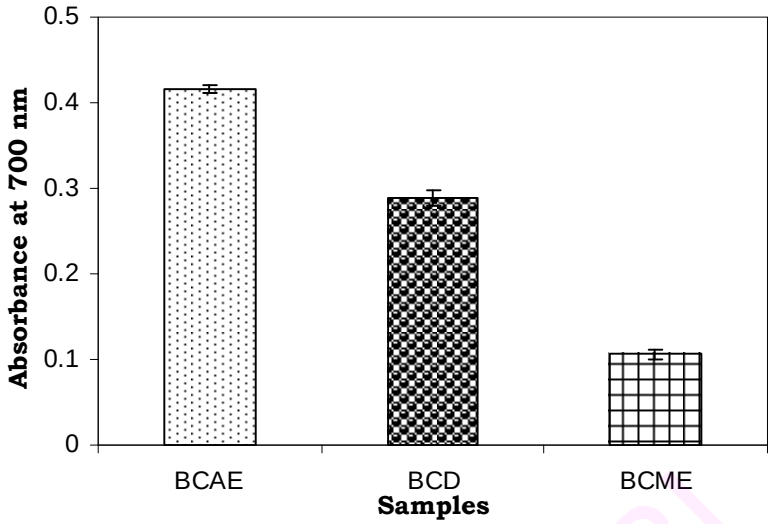


Figure 2.7. Reducing power of black cumin extracts. BCAE-black cumin aqueous extract; BCD-black cumin decoction and BCME-black cumin methanol extract. Values are expressed as mean ± SD.

At 12 μg GAE/mL concentration, although 2-fold higher activity was observed in SRHP, when absolute activity was calculated a total reducing power of 41440 absorbance units (AU) followed by 23040 AU and 15940 AU were observed for SRFP, SRBP and SRHP, respectively. An ~ 1.7 and 2.6-fold higher activity was observed in SRFP than SRBP and SRHP, respectively. This increase in absolute activity is due to abundance of phenolics in them. The reducing power of phenolic acid extracts tested correlates (r = 0.9122) well with their total phenolic content. However, the difference in the reducing power between the phenolic acid extracts may be due to their constituent phenolic acids.

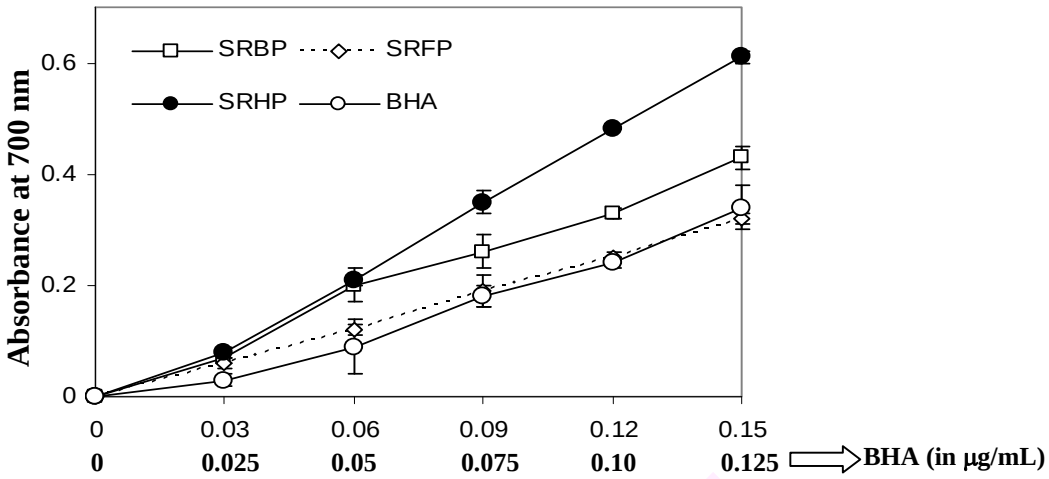


Figure 2.8. Reducing power of swallow root phenolic acid extracts. SRFP-swallow root free phenolic acids; SRHP-swallow root hydrolyzed phenolic acids; SRBP-swallow root bound phenolic acids and BHA-butylated hydroxyl anisole. Results are expressed as mean ± SD (n =3).

2.5.6. Cytoprotective effect

Cytoprotective effect of BCAE

Since BCAE showed potent antioxidant activity in all assays cytoprotective ability was therefore examined only in BCAE. The cytoprotective effect of BCAE at various concentrations on the NIH 3T3 fibroblast cells treated with tert-butylhydroperoxide is shown in Figure 2.9. The results indicated an increased cell protection by BCAE and it followed a dose dependent response. The maximum cell protection of 65 % against oxidative stress was observed at a dosage of 32 µg GAE phenol/mL extract indicating the presence of antioxidant components in the water-soluble extract of black cumin.

Cytoprotective effect of SR-Fractions

From figure 2.10 it is evident that phenolic acid extracts of SR showed dose dependent protection from 0.03 to 0.15 µg/mL concentrations. Among the phenolic acid extracts tested, at equal concentration of 0.12 µg/mL, SRHP extract showed highest cytoprotectivity with ~ 87 % protection. SRFP and SRBP extract protected cells up to 47 and 65 %, respectively. Standard antioxidant BHA could show ~ 67 %

cytoprotectivity at 0.125 µg/mL concentration

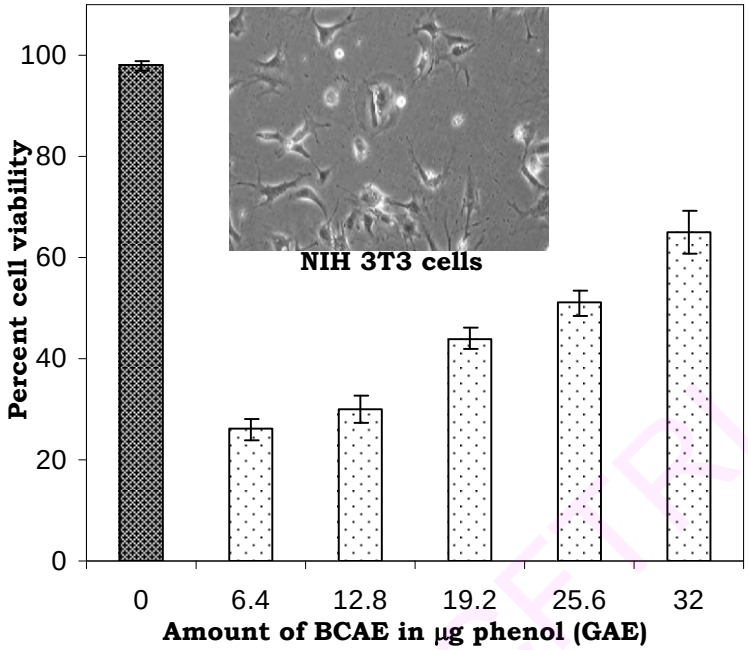


Figure 2.9 Cytoprotective effect of black cumin extracts on tert-butyl hydroperoxide induced cell damage of NIH 3T3 fibroblast cells. Results are mean ± SD (n = 3). ** Represent $p < 0.01$ compared with control.

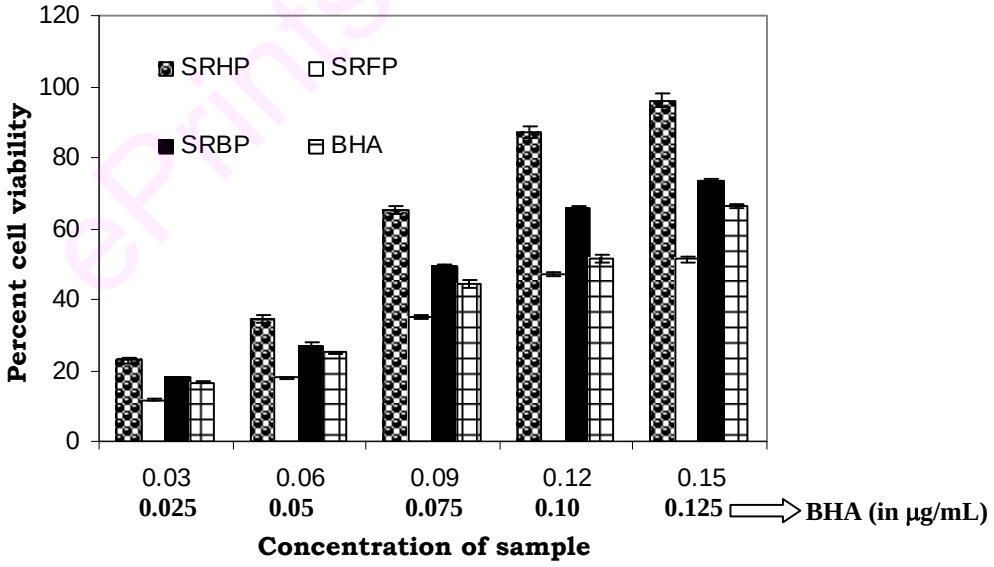
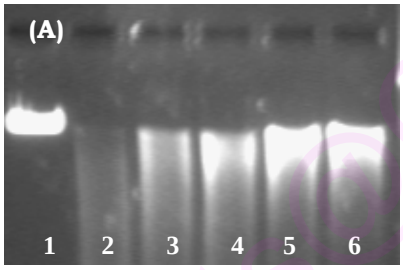


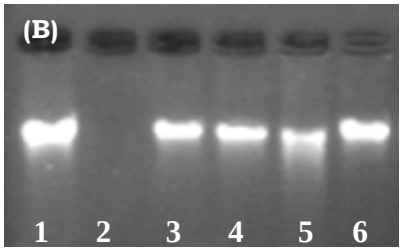
Figure 2.10. Cytoprotective effect of swallow root extracts on tert-butyl hydroperoxide induced cell damage of NIH 3T3 fibroblast cells. Results are mean ± SD (n = 3). ** Represent $p < 0.01$ compared with control.

2.5.7. DNA Protective effect

To examine the DNA protective effect of BC & SR extracts, λ phage DNA was subjected to oxidation using Fenton's reagent (hydrogen peroxide, ferric chloride and ascorbic acid). Figure 2.11 shows the extent of DNA damage induced by Fenton's reagent and the protection offered by BC & SR extracts as revealed by the relative electrophoretic mobility of the oxidized and extracts treated DNA compared to that of undamaged DNA (lane 1). Addition of Fenton's reagent caused the fragmentation of DNA (lane 2) and hence increased the electrophoretic mobility. However, BCAE showed an increased DNA protection (lane 3-6, Figure 2.11A) with an increase in the dose (0.16 - 0.64 µg GAE phenol) of the extract. A maximum DNA protection was observed at 0.64 µg GAE phenol of the extract (lane 6 of Figure 2.11A).



Native DNA	+	+	+	+	+	+
Fenton's Reagent	-	+	+	+	+	+
BCAE – 0.16 µg	-	-	+	-	-	-
BCAE – 0.32 µg	-	-	-	+	-	-
BCAE – 0.48 µg	-	-	-	-	+	-
BCAE – 0.64 µg	-	-	-	-	-	+



Native DNA	+	+	+	+	+	+
Fenton's Reagent	-	+	+	+	+	+
SRHP	-	-	+	-	-	-
SRBP	-	-	-	+	-	-
SRFP	-	-	-	-	+	-
BHA	-	-	-	-	-	+

Figure 2.11. Electrophoretic analysis of DNA damage and its protection by BC (A) and SR (B) extracts. (A) Lane 1 – Native DNA; Lane 2 – Oxidized DNA; Lane 3 – 6, BCAE treated (0.16-0.64 µg GAE phenol). (B) Electrophoretic analysis of DNA protection by free (SRFP), bound (SRBP) and hydrolyzed phenolic acid (SRHP) extracts (0.6 µg GAE each) in addition to standard antioxidant BHA (1 µg). Lane 1- native DNA; lane 2-oxidised DNA; lane 3-SRHP treated DNA; lane 4-SRBP treated DNA; lane 5-SRFP treated DNA and lane 6-BHA treated DNA

Figure 2.11B shows the DNA protective activity of SRFP, SRHP and SRBP extracts

including BHA. Results indicated higher protection (82 %) in BHA treated (1 μg) while 80, 67 and 42 % protection were observed for SRHP, SRBP and SRFP extracts at each 0.6 μg GAE phenol concentration, respectively.

2.5.8. Identification of phenolic acids in BC and SR extracts

We have standardized previously a HPLC column chromatographic conditions for the maximum resolution, identification of phenolic acids in water extracts of some dietary sources (Siddaraju & Dharmesh, 2007). Figure 2.12 presents HPLC profile of BCAE. BCAE profile in Figure 2.12a indicated a major component with retention time of 6.33 min (Fig 2.12a) similar to tannic acid (Figure 2.12c) and 8.63 min peak of protocatechuic acid was identified. Although other peaks with retention time of 5.88, 10.15 and 11.75 min were observed, only 6.33 min fraction exhibited activity as evaluated by the isolation of each fraction and determining antioxidant activity in them. In order to confirm that there are no overlapping peaks, different conditions of HPLC was performed and compared the peaks with standard tannic acid. As indicated in figure 2.12b, a peak coinciding with retention time equivalent to that of tannic acid was observed (in BCD) indicating that tannic acid is a major antioxidant. The HPLC profile of water, methanol and phenolic acid fractions-SRFP, SRHP and SRBP are shown in figure 2.13. In addition, the constituent phenolic acids in SRFP, SRHP and SRBP extracts are listed in Table 2.3. Water and methanolic extracts of swallow root indicated the presence of phenolic acids (Figure 2.13, inlaid).

In phenolic acids extracts, a total of 12 phenolic compounds were detected of which five were hydroxybenzoate derivatives and four were cinnamate derivatives. Also, two hydroxybenzaldehyde derivatives were identified. In total 12, 9, and 9 phenolic compounds were identified in SRFP, SRHP and SRBP extracts of swallow root, respectively. In SRFP extract, gentisic acid, 2-hydroxy-4-methoxybenzaldehyde (HMBA), vanillin, vanillic acid and *p*-hydroxybenzoic acid were the major phenolic compounds as evaluated by their retention time with standards. In SRHP extract, gallic acid and gentisic acid were the abundant phenolic acids, while the SRBP extract was constituted by HMBA, vanillic acid and *p*-hydroxybenzoic acid

contributing to more than 70 % of phenolic acid content.

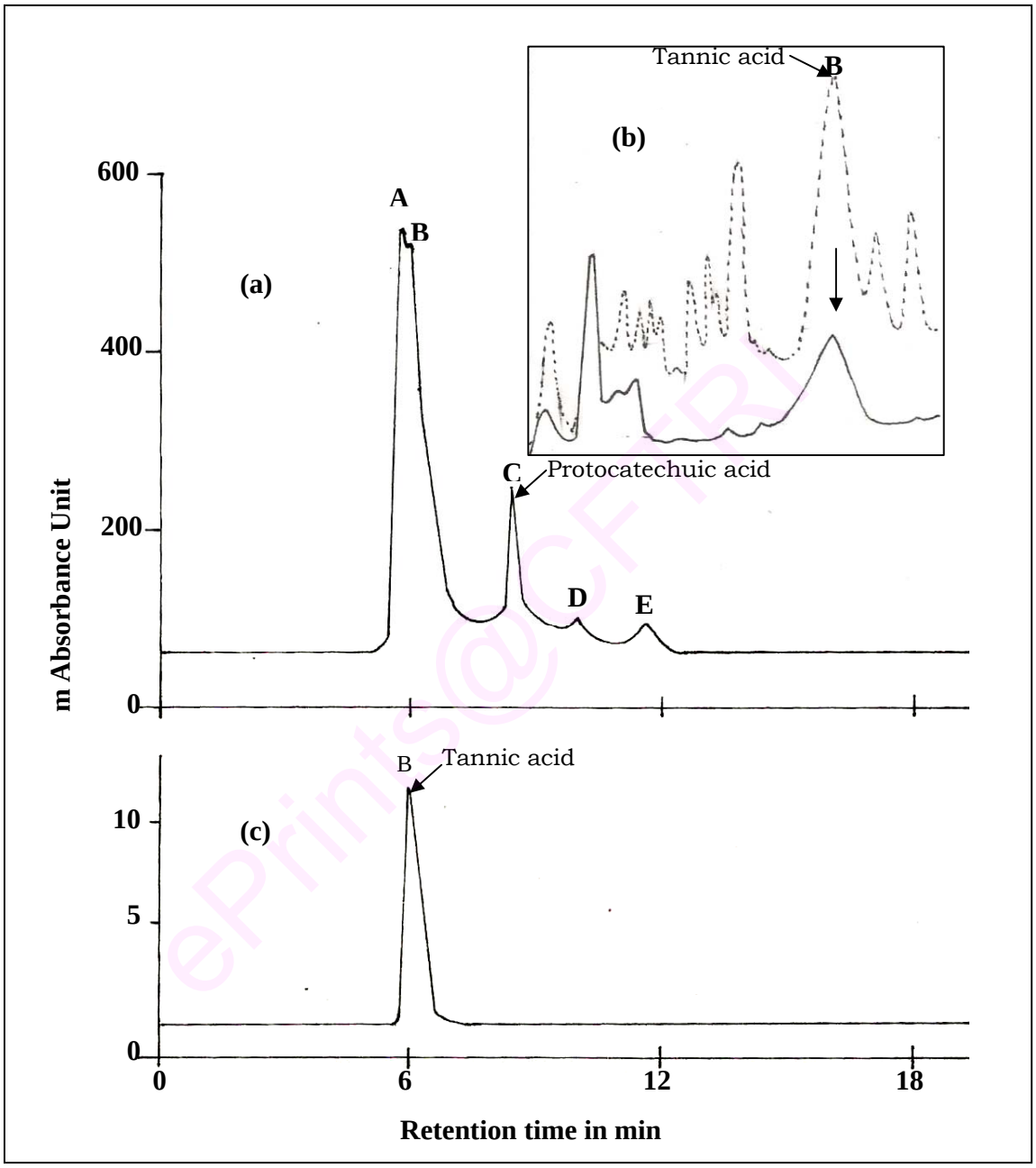


Figure 2.12. HPLC profile of black cumin water extract, decoction and standard tannic acid on C-$_{18}$ column. (a) A – 5.88 min; B – 6.33 min (active); C – 8.63 min; D – 10.15 min; E – 11.75 min. (b) HPLC profile of BCD and standard tannic acid (STA) on an amino column (inlaid). (c) HPLC profile of standard tannic acid on C18 column with RT – 6.33.

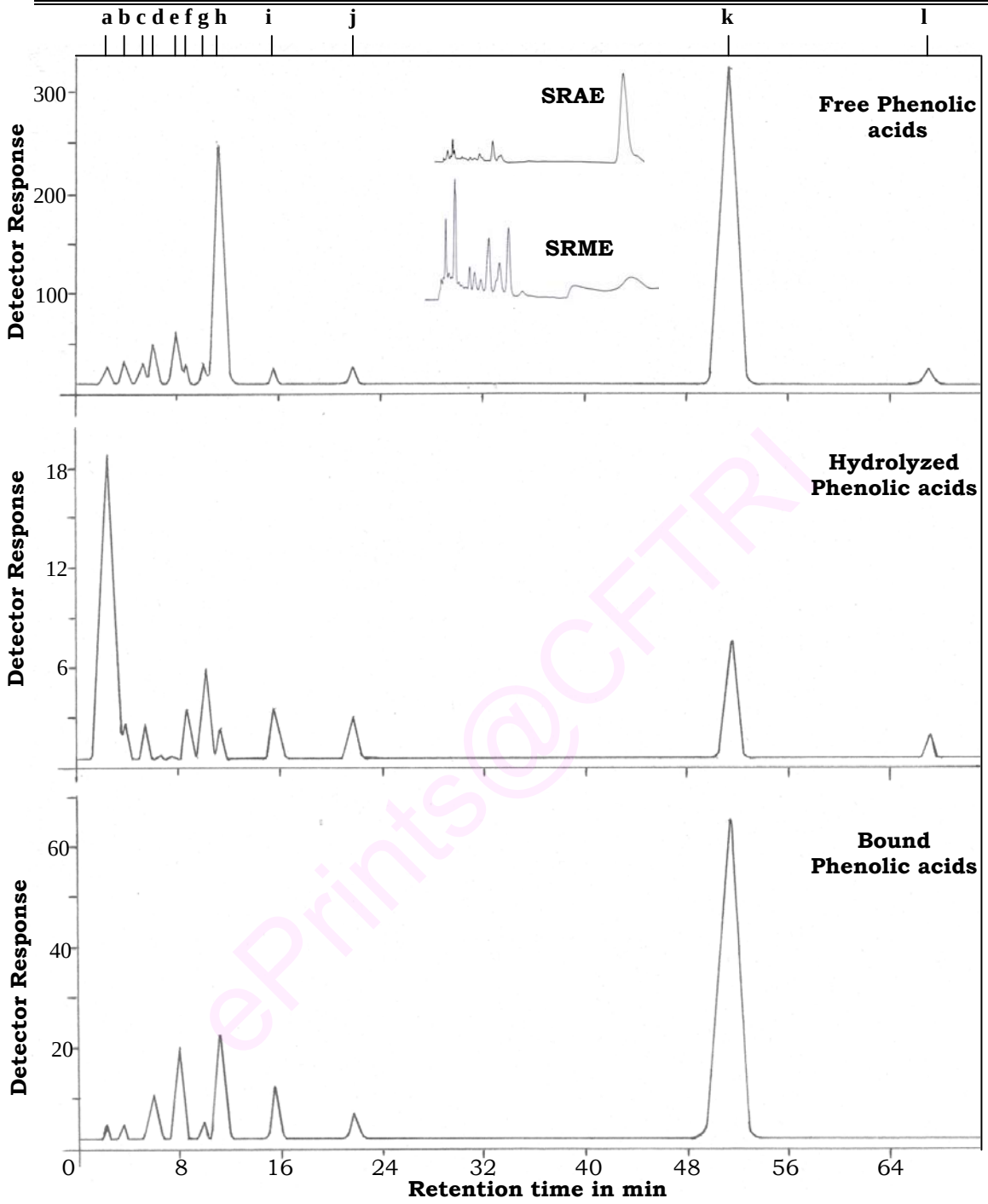


Figure 2.13. HPLC profile of phenolic extracts of swallow root. **a** – Gallic acid (2.37 min); **b** – protocatechuic acid (3.66 min); **c** – gentisic acid (5.30 min); **d** – *p*-hydroxy benzoic acid (6.04 min); **e** – vanillic acid (8.01 min); **f** – Caffeic acid (8.43 min); **g** – syringic acid (10.00 min); **h** – vanillin (11.18 min); **i** - *p*-coumaric acid (15.86 min); **j** – ferulic acid (21.70 min); **k** – 2-hydroxy-4-methoxybenzaldehyde (51.44 min) and **l** – cinnamic acid (66.88 min). (Inlaid HPLC profile: SRAE – swallow root aqueous extract; SRME – swallow root methanol extract)

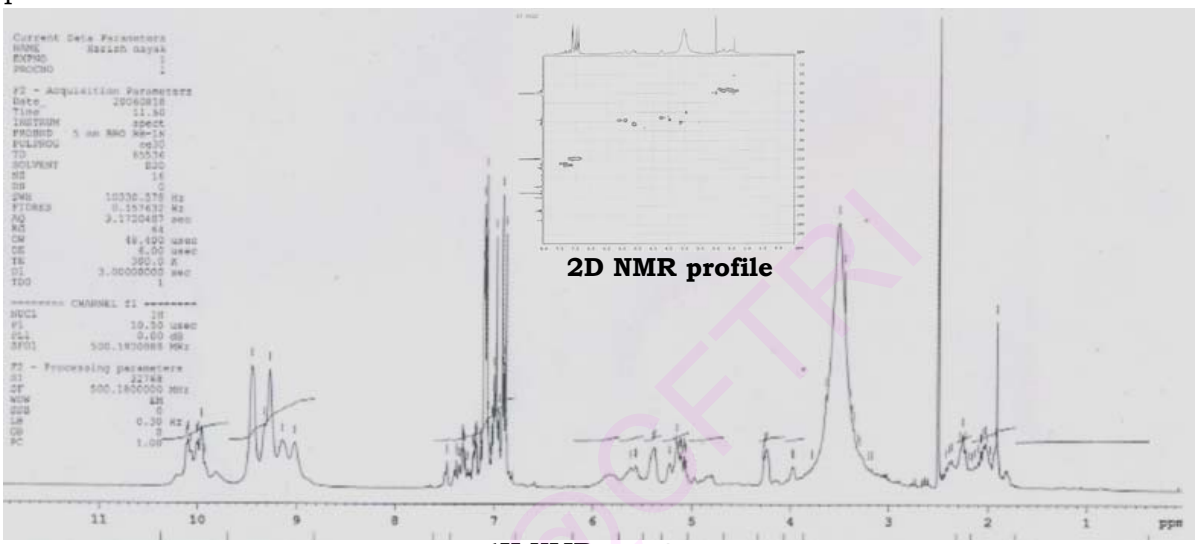
2.5.9. Characterization of the major antioxidant compounds of BC and SR extracts

BCAE as well as BCD showed the presence of tannic acid as a major phenolic acid component. 6.33 min peak was isolated under similar conditions of HPLC and subjected to mass spectral analysis. In order to confirm that these fragments are from BCAE alone, we subjected this fraction to bind to hide powder, an affinity matrix that is specific for tannic acid. Results indicated that a BCAE active peak, which constituted 31.9 mg/100 g, yielded three tannin components with a molecular mass of 774, 926 and 1078 Da. All these components were bound effectively to a tannin specific affinity matrix (Hide powder), with proportionate reduction in antioxidant activity suggesting that tannins are the important antioxidant components in BCAE. BCD also showed the presence of major component constituting 0.04 % (w/w) as tannic acid (Fig. 2.12 [b]).

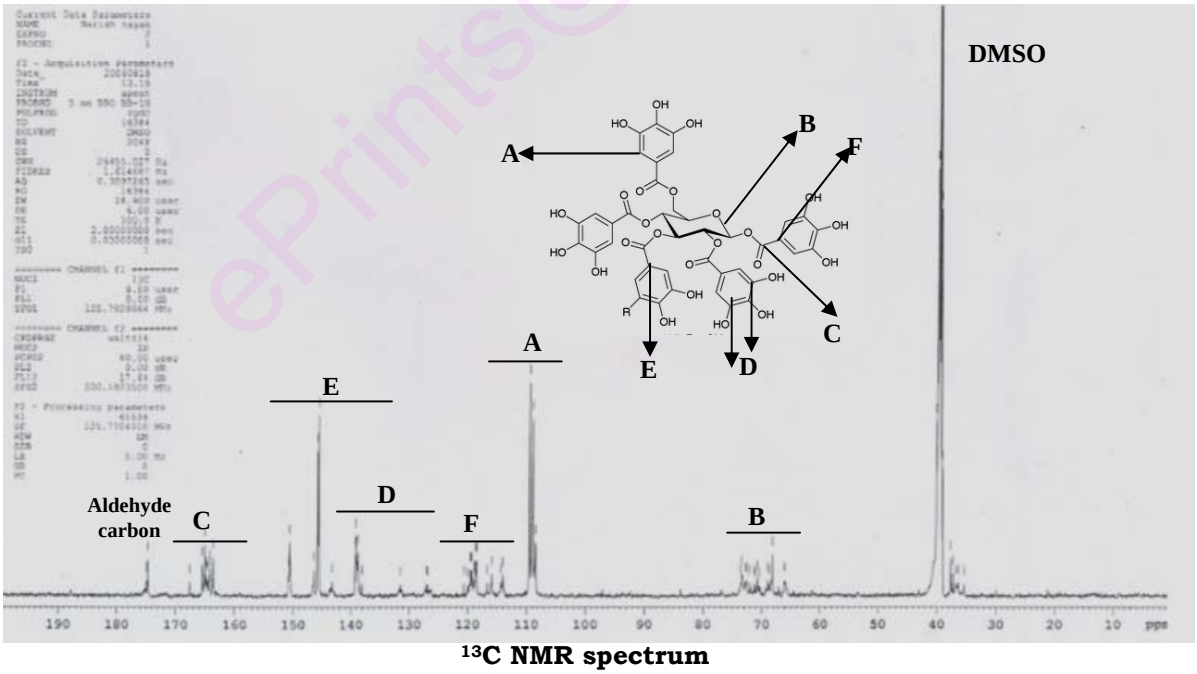
Further, the tannic acid component was isolated using a preparative C_{18} column using the same conditions described but with a flow rate of 10 mL/min and 1 mL sample injection. The isolated sample was subjected to 2D NMR analysis and it confirmed the presence of tannic acid. The NMR spectrum (Figure 2.14) showed finer details of the presence of glucose with shifts in the region of delta 60-75 ppm and the presence of aromatic carbon signals in the region of 110 to 130 ppm. The presence of hydroxyl groups was confirmed by the shifts in the region of 125-145 ppm. The presence of aldehyde group of the glucose is indicated by shifts between 170-180 ppm. Carbonyl carbon showed signals at 160-170 ppm. These results were further substantiated by ^{1}H NMR and 2D NMR profile. Results thus suggest a greater contribution from tannic acid for the antioxidant potency of BCAE.

In swallow root, SRAE and SRBP showed predominant levels of HMBA, which was reported earlier as a major component in methanol extract in the free form only, we subjected this peak to biophysical analysis after isolating pure compound from preparative HPLC. Results (Figure 2.15) indicated the presence of an aromatic compound with a mass of 152 and a λ_{max} of 213, 279 and 305 nm and the infrared

measurements showed the presence of aldehydic, methoxy and hydroxyl functional group. From two-dimensional NMR (^{1}H and 13C) studies the compound was identified and confirmed as 2-hydroxy-4-methoxybenzaldehyde. Data thus provides evidence for the presence of HMBA in bound phenolic fraction. In addition other phenolic acids were also found in varied levels.



^{1}H NMR spectrum



^{13}C NMR spectrum

Figure 2.14. NMR profile of isolated active fraction from balck cumin water extract

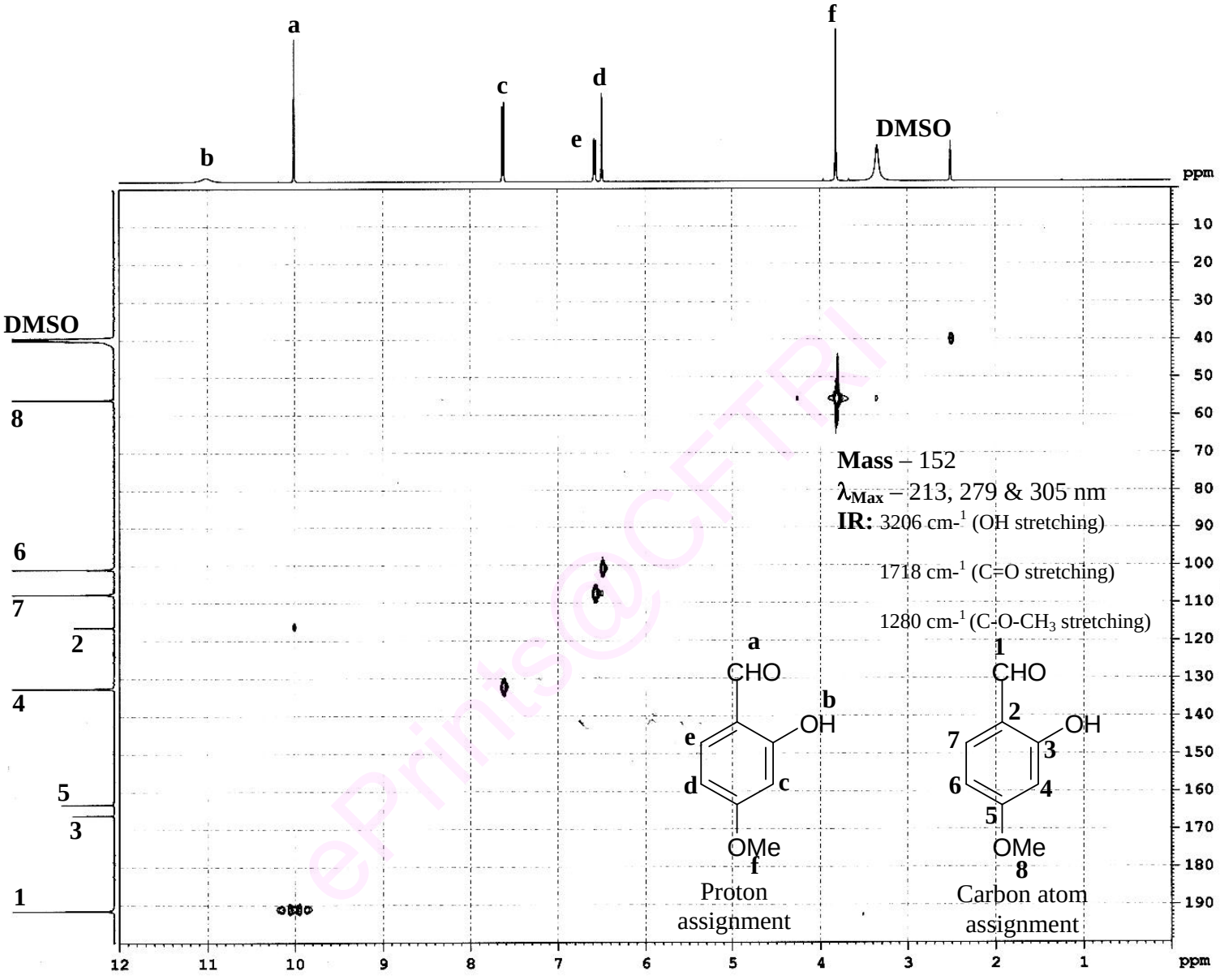


Figure 2.15 Two-dimensional NMR profile of the major component of bound phenolic acid extract of swallow root in dimethyl sulphoxide, and spectroscopic details (Mass, Infrared radiation and Ultraviolet absorption measurements).

2.5.10. Contribution from phenolic acids in BC and SR antioxidant activity

In BCAE, tannic acid and protocatechuic acids are the major antioxidant components contributing to the antioxidant activity. The percentage contribution to antioxidant (Table 2.1) using reducing power of the antioxidant molecules indicated 77 % contribution by tannic acid and 23 % by protocatechuic acid. Although, few other peaks were observed in the HPLC, they did not exhibit any activity.

Swallow root aqueous extract has been found to be one of the most potent source possessing antioxidant activity and the profile of phenolic acids are being reported for the first time in swallow root. Calculation has been made towards the antioxidant potency. For this, antioxidant capacity of individual phenolic acids including HMBA was recorded. Data as indicated in Table 2.3 suggest that gentisic acid contributes significantly in terms of phenolic content. In SRHP, gallic acid contributes significantly (54 %), while 18 and 28 % contribution was made by HMBA in SRFP and SRBP, respectively. However, when we consider the antioxidant potency, which is represented in Figure 2.16, only around 5 % of activity is contributed from HMBA, *p*-hydroxybenzoic acid and vanillin, while major contributors are gentisic acid (55 %), protocatechuic acid (22 %) and gallic acid in SRFP; gallic acid (75 %) and gentisic acid (14 %) in SRHP and gallic acid (58 %) and *p*-coumaric acid (22 %) in SRBP, respectively.

The fact that SRHP showed better antioxidant activity than SRFP and SRBP in all assays could be due to the presence of higher levels of gallic acid and gentisic acid, which are potent antioxidants (Table 2.3). It should be noted here that despite the presence of a major amount of HMBA both in SRFP and SRBP, the contribution towards antioxidant is very negligible due to the poor antioxidant potency of HMBA. The poor antioxidant potency of HMBA was also substantiated by other workers (Harish et al., 2005).

Table 2.3. Phenolic acid composition of free, hydrolyzed and bound phenolic acid extracts of swallow root and their contribution to antioxidant activity

Compound	DPPH Radical scavenging activity (IC_{50} in µg/mL)	Amt. phe. Acids (mg/g)	Total activity in Units	Amt. phe. Acids (mg/g)	Total activity (Units)	Amt. phe. Acids (mg/g)	Total activity (Units)
		Free phenolic acids		Hydrolyzed phenolic acids		Bound phenolic acids	
Gallic acid	1.1 ± 0.04	0.54 ± 0.052	491 (12 %)	1.49 ± 0.11	1354 (75 %)	0.38 ± 0.055	345 (58 %)
Protocatechuic acid	1.35 ± 0.08	1.16 ± 0.045	859 (22.8 %)	0.03 ± 0.004	22 (1 %)	0.03 ± 0.008	22 (4 %)
Gentisic acid	3.0 ± 0.11	6.54 ± 0.242	2180 (55 %)	0.76 ± 0.036	253 (14 %)	-	-
Vanillic acid	49.5 ± 2.4	4.72 ± 0.104	95 (2 %)	-	-	1.04 ± 0.05	21 (4 %)
Caffeic acid	1.80 ± 0.05	0.15 ± 0.008	83 (2 %)	0.08 ± 0.008	44 (2 %)	-	-
Syringic acid	64.9 ± 5.42	0.21 ± 0.007	3 (<1 %)	0.15 ± 0.010	76 (4 %)	0.05 ± 0.008	25 (4.2 %)
p-Coumaric acid	1.90 ± 0.04	0.08 ± 0.01	42 (1 %)	0.08 ± 0.01	42 (2 %)	0.25 ± 0.005	132 (22 %)
Ferulic acid	6.60 ± 0.21	0.08 ± 0.017	12 (<1 %)	0.07 ± 0.003	11 (<1 %)	0.10 ± 0.017	15 (3 %)
t-Cinnamic acid	4.60 ± 0.34	0.02 ± 0.001	4 (<1 %)	-	-	-	-
p-Hydroxy benzoic acid	33.75 ± 1.7	3.91 ± 0.141	116 (3 %)	-	-	0.83 ± 0.043	25 (4 %)
HMBA *	213.2 ± 5.12	5.06 ± 0.239	24 (<1 %)	0.10 ± 0.003	0.5 (<1 %)	1.14 ± 0.06	5.3 (<1 %)
Vanillin	80.0 ± 2.01	4.61 ± 0.101	58 (2 %)	0.02 ± 0.002	0.3 (<1 %)	0.25 ± 0.016	3 (<1 %)

Values are expressed as mean ± standard deviation. Total activity: IC_{50} = 1Unit

Tannic acid

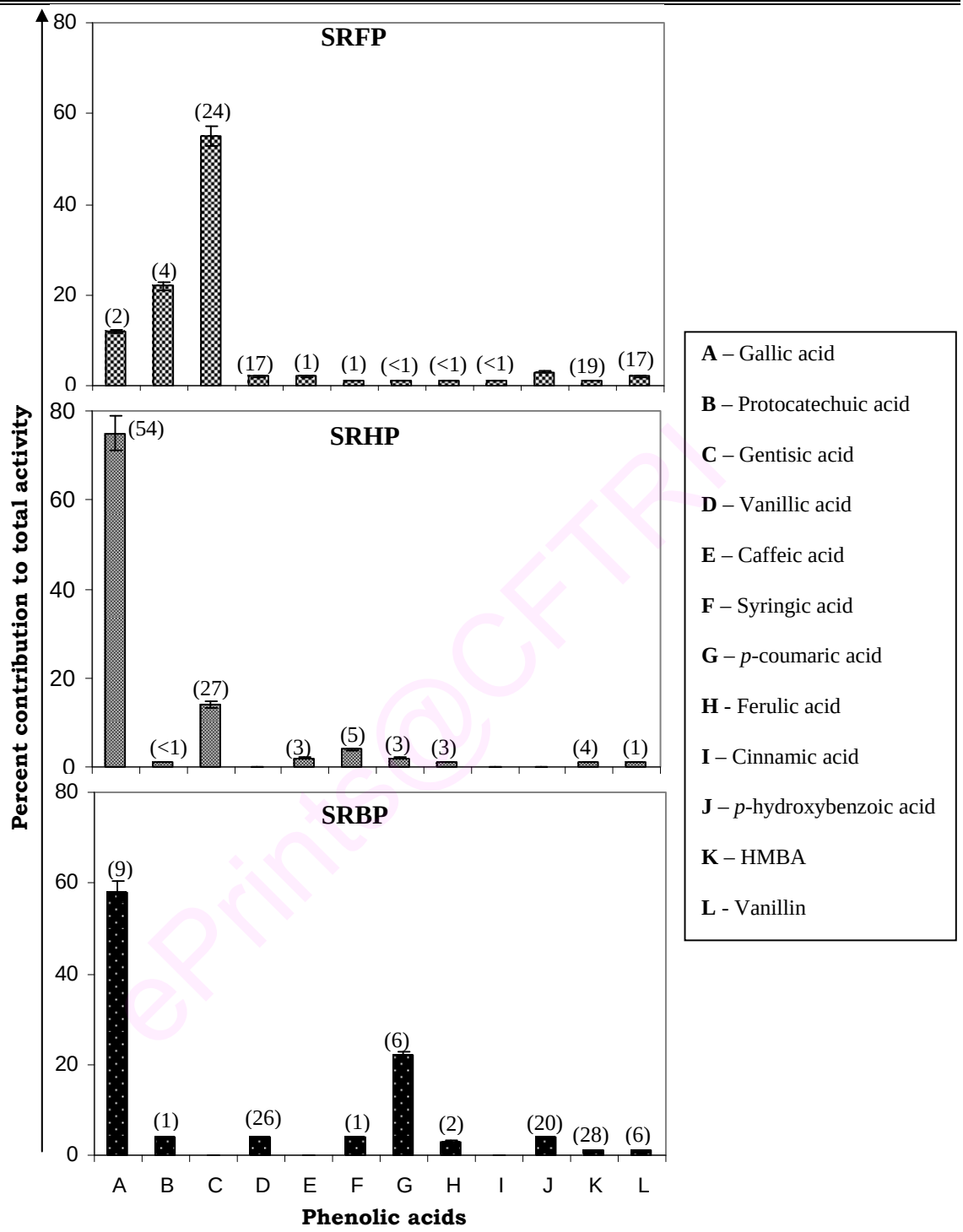
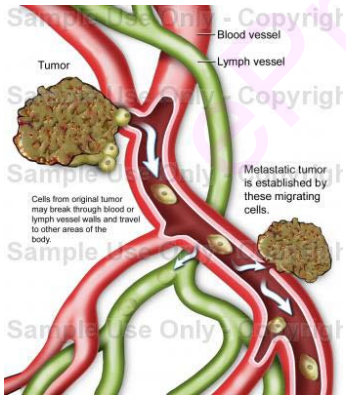


Figure 2.16. Percent contribution of various phenolic components to total antioxidant activity of swallow root free (SRFP), hydrolyzed (SRHP) and bound (SRBP) phenolic acid fractions. Numbers in the parenthesis indicate percent phenolic content. Values are expressed as mean ± SD (n=3).

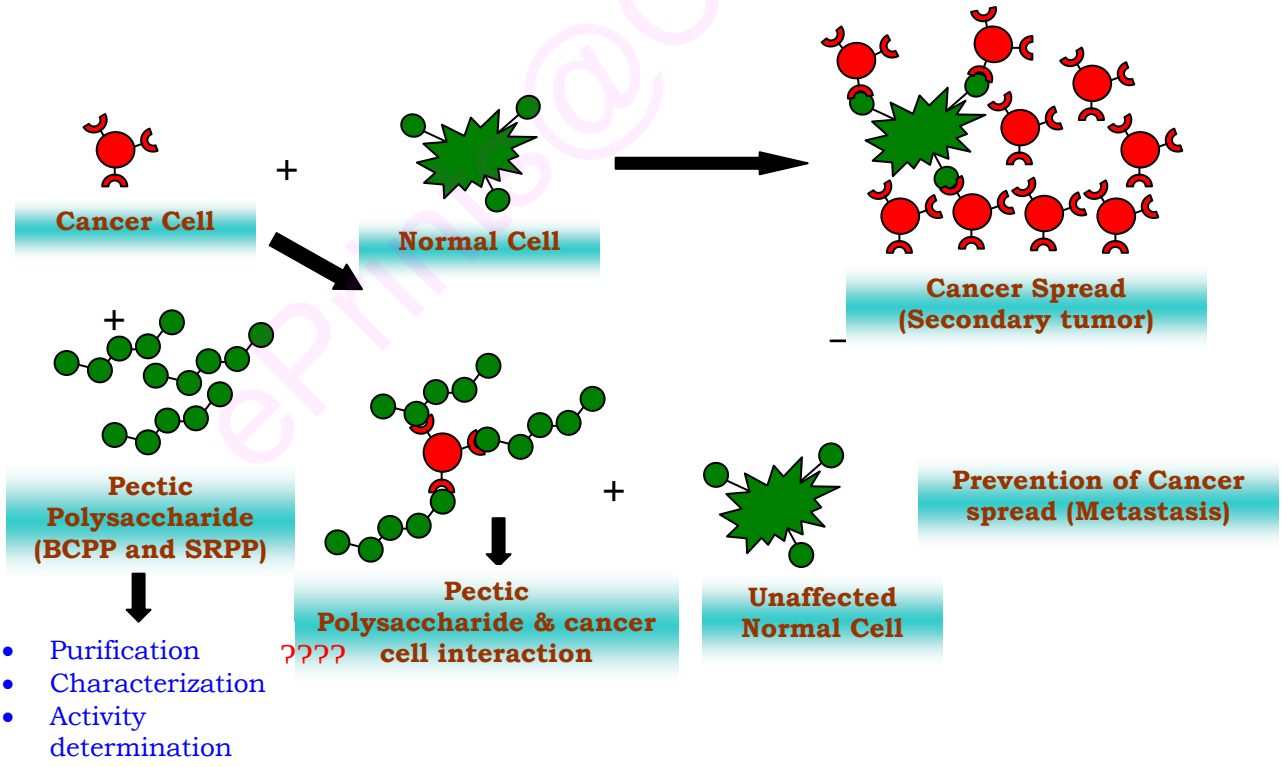
Chapter III

Isolation and characterization of antimetastatic components from spices



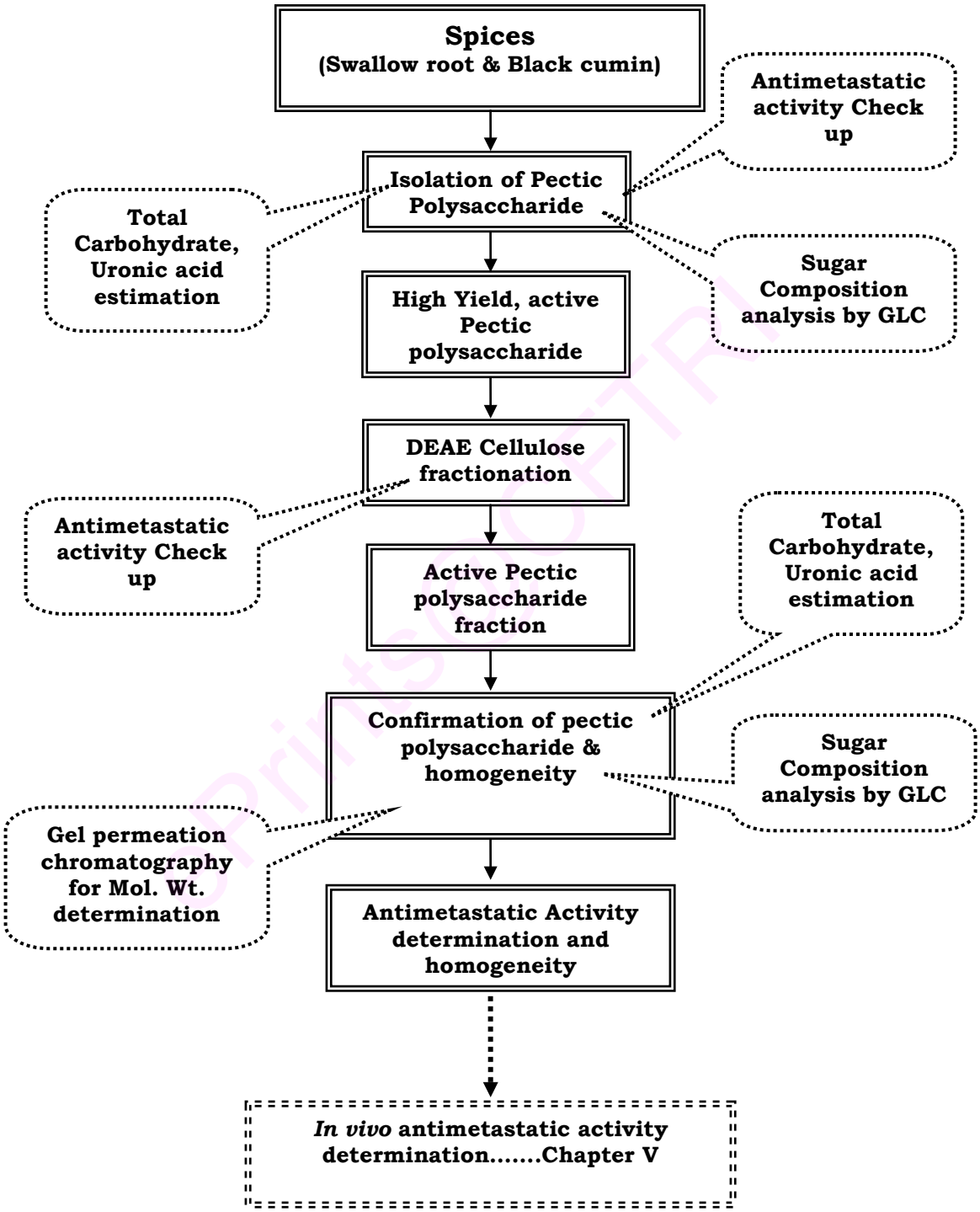
3.1. Hypothesis

Spices are known to contain antioxidants and health beneficial compounds including anticancer and antimetastatic. Anticancer activity has been attributed to these compounds. Current chapter however, addresses whether any molecule which blocks a key molecule of metastasis i.e., galectin-3. Galectin-3 appear to trigger the metastatic spread by interacting with β-galactosides of normal cell. Study therefore attempts to isolate pectic polysaccharides (PP) from selected spices - Black Cumin (BCPP) and Swallow Root (SRPP) and to examine their probable galectin blocking ability as per Scheme 3.1. In order to understand the interaction and the binding of galectin inhibitors, isolation, fractionation and characterization including composition analysis, molecular size determination and its relation to bioactivity has been investigated.



Scheme 3.1

3.2. Work Concept



3.3. Introduction

Diet contains carbohydrates of various size, concentrations and chemical sequences starting from simple monosaccharides to complex polysaccharides. Among them pectic polysaccharides in particular has been shown to play critical therapeutic roles against cancer (Olano-Martin et al., 2003), immunomodulation (Wong et al., 1994), ulcer (Kiyohara et al., 1994) etc.

Pectic polysaccharide also called pectin is a linear chain of α-(1-4)-linked D-galacturonic acid that forms the pectin-backbone, a homogalacturonan. Into this backbone, there are regions where galacturonic acid is replaced by (1-2)-linked L-rhamnose. From rhamnose, sidechains of various neutral sugars branch off. This type of pectin is called rhamnogalacturonan I. Over all, up to every 25th galacturonic acid; in the main chain is exchanged with rhamnose. Some stretches consisting of alternating galacturonic acid and rhamnose – "hairy regions", others with lower density of rhamnose – "smooth regions". The neutral sugars are mainly D-galactose, L-arabinose and D-xylose; the types and proportions of neutral sugars vary with the origin of pectin. Often sugars are also found derivatized as - methylated (a), amidated (b) etc. General structure of pectin is depicted in Figure. 3.1.1.

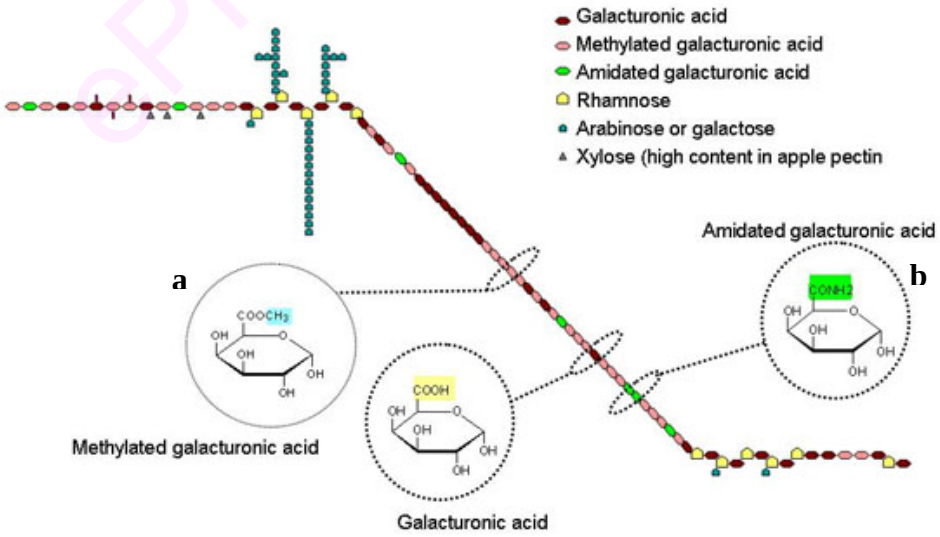


Figure 3.1

Since the literature enumerates the presence of galactose with multiple branching points and associated sugars, it is expected that these polysaccharides may have the ability to compete with β-galactosides of normal cells for which galectin-3 is known to bind to complete the process of metastasis successfully.

Recently the role of pectic polysaccharides has gained importance due to their role played in controlling cancer metastasis through the blockade of galectin present on the metastatic cancer cells (Inohara & Raz, 1994) as galectin-3 expression is correlated with metastatic potential in certain malignancies (Bresalier et al., 1997). Results of several investigations and our studies (Kruthika et al.,) have revealed the possibility of galectin-3 as a diagnostic marker in certain cancers and also one of the target proteins for cancer treatment (Konstantinov et al., 1996). Further higher levels of galectin-3 have been shown to correlate with the advancement of the cancer disease and it is believed that galectin-3 of cancer cells bind to normal cells and establishes secondary tumors. Galectin-3 hence has been implicated in tumor spread and metastasis (Takenaka et al., 2004). Studies have also indicated that oral administration of modified citrus pectin reduced the rate of cancer cell spread and inhibited metastasis in animal models (Pienta et al., 1995). Despite promising role of dietary pectins against metastasis, hardly few reports are available on identification of such components from dietary sources, which can potentially inhibit the binding of galectins to β-D-galactoside residues present on the extracellular matrix components of normal cell surface as well as to the basement membranes, thereby preventing cancer spread. We designate such components as antimetastatic components (AMC). In the present study efforts were made to isolate pectic polysaccharides from swallow root (SRPP) and black cumin (BCPP) and studied their antimetastatic potential. Purification and characterization studies were also undertaken for swallow root pectic polysaccharide which was significantly active. Sugar composition analysis however was compared between BCPP and SRPP to understand the probable composition required for the activity.

3.4. Materials and Methods

3.4.1 Chemicals

Minimum essential medium (MEM), Dulbecco's minimum essential medium (DMEM), glutamine, sodium bicarbonate, penicillin, kanamycin, F12 mixture and heat inactivated fetal calf serum (FCS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sucrose, ethylenediaminetetraacetic acid (EDTA), triton X 100, tween 20, skimmed milk powder, paranitrophenyl phosphate (PNPP), diethanolamine, Alsever's medium, trypsin, hematoxylin, eosin, acridine orange, carbohydrate standards such as rhamnose, arabinose, xylose, mannose, galactose and glucose, protease, thermoamylase, glucoamylase, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sepharose CL-4B (4 % cross-linked, fractionation range for dextrans 30,000-50,000 Da), DEAE-cellulose (0.99 meq/g), Amberlite IR-120-P (8% cross-linked, 16-50 mesh), Dextran standards, T-Series viz., T-10, T-20, T-40, T-70, T-150, T-500, T-2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Alkaline phosphatase conjugated – rabbit anti mouse IgG secondary antibody was procured from GENEI, Bangalore, India. HPLC column (Shimpak C_{18}) was obtained from Shimadzu Corp. Tokyo, Japan. 3 % OV-225 (1/8"×6') on Chromosorb W (80-100 mesh) was from Pierce Chemical Company, Rockford, USA. Other chemicals such as hexane, ammonium oxalate, iodine solution, sodium phosphate buffer, perchloric acid ($HClO_4$), acetic acid, sodium acetate glutaraldehyde, glycine, sodium chloride, sulphuric acid and solvents used were of the analytical grade purchased from local chemical company. All chemicals and solvents used for HPLC and GLC were HPLC grade.

3.4.2. Isolation of pectic polysaccharides

Fresh swallow root and black cumin, were purchased from a local market (Devaraja Market, Mysore, Karnataka, India), while citrus pectin was procured from Sigma Chemical Co, USA. Fresh swallow roots were chopped into small pieces and air dried in the dark in a ventilated hood. The air dried samples of swallow root and black cumin were ground and defatted in a soxhlet apparatus using hexane (200 mL/g, w/v). The defatted powder was air dried and preserved in dry condition until further extraction of pectic polysaccharides.

Pectic polysaccharides were isolated following the ammonium oxalate extraction method (Phatak et al., 1988). Briefly 100 g defatted sample was treated with 100 mg of protease in 500 mL of 0.1 M sodium phosphate buffer (PB) of pH-7.5 and incubated overnight at 37 °C with stirring. Contents were filtered and the filtrate was discarded after successive washing-3 times with PB. The residue was suspended in 500 mL of PB along with thermoamylase (100 mg) and boiled for 15 min. After ensuring complete degradation of starch with iodine solution, the contents were filtered and supernatant was discarded. The residue was resuspended in acetate buffer (0.1 M, pH-4.5) along with glucoamylase (100 mg) and incubated overnight. Further, the contents were filtered and the residue was taken in 200 mL ammonium oxalate (0.25 % w/v, pH-3.5) solution and boiled for 1 h at 70 °C with occasional shaking. The contents were filtered and the supernatant was precipitated with 4 volumes of absolute ethanol and kept in cold for 1 h. Contents were centrifuged at 5000 *g* at RT for 20 min and the pellet was washed twice with 50 mL ethanol (45 %). Finally, the pellet was resuspended in 100 mL of water, dialyzed extensively against water and lyophilized to get pectic polysaccharide. In order to confirm that the isolated fraction is a pectic polysaccharide sugar composition analysis and uronic acid contents were determined since it is the characteristic sugar component of pectic polysaccharide.

3.4.3. Total carbohydrate estimation

Total carbohydrate content was estimated (Dubois et al., 1956) in 0.5 mL of the sample (10 mg/100 mL of water) in a test tube. To samples, 0.3 mL of phenol (5 %) and 1.8 mL of concentrated sulphuric acid were added and the contents were mixed thoroughly. After cooling the tubes at room temperature (~20 min), the absorbance was read at 480 nm against a reagent blank. Sugar content was determined against the calibration graph, prepared by using D-glucose (4-20 µg/mL).

3.4.4. Uronic acid estimation

Uronic acid was estimated (Bitter & Muir, 1962) in 0.5 mL of the sample solution (10 mg/100 mL) in a test tube and kept in ice cold water bath for 10 min. To this was added concentrated sulphuric acid (3 mL) slowly, contents were mixed thoroughly and kept in boiling water bath for 20 min. Contents were cooled to which carbazole solution (0.1 mL, 0.1 % prepared by dissolving re-crystallized carbazole in alcohol) was added. The tubes were kept in dark for 2 h and the absorbance was recorded at 530 nm. Uronic acid content was determined against the calibration graph prepared by using D-galacturonic acid (10-50 µg/mL).

3.4.5. Fractionation of Pectic polysaccharides

DEAE cellulose column chromatography

DEAE-cellulose was washed with water to remove fine particles. It was then regenerated successively with HCl (0.5 N) and NaOH (0.5 M). After each treatment, the pH was adjusted to neutrality by washing thoroughly with water. The regenerated exchanger was suspended in ammonium carbonate (0.5 M, pH 9.3), packed in a column (3.5 cm x 26 cm) and excess carbonate was washed off with water.

The pectic polysaccharide (1 g) was dissolved in 2.0 mL of water and loaded on to

DEAE-cellulose column and the elution was carried out with water, followed by ammonium carbonate (0.05 to 0.20 M) and sodium hydroxide (0.1 and 0.2 M) solutions. The flow rate was maintained at 60 mL/h and fractions (10 mL) were collected, assayed for total sugar by phenol-sulfuric acid method as described earlier. Carbohydrate positive fractions were pooled, dialyzed and lyophilized. Fractions – 0.01 M, 0.1 M, 0.15 M, and 0.2 M were examined for galectin-3 inhibitory property in composition with the crude SRPP and active fraction was further characterized. Fractions were designated as SRPP - 0.05 M, SRPP – 0.1M, SRPP – 0.15 M and SRPP - 0.2M respectively.

3.4.6. Growth and maintenance of MDA-MB-231 cells

MDA-MB-231 is a metastatic human breast adenocarcinoma was procured from National Centre for Cell Sciences, Pune, India and maintained in the animal cell culture laboratory at CFTRI. Cells were grown in DMEM-high glucose (4.5 g/L) with 4 mM glutamine, 1.5 g/L sodium bicarbonate, penicillin (100 units/mL) and kanamycin (0.1 mg/mL) with 10 % fetal calf serum at 37 °C in a humidified chamber with 95 % air and 5 % CO_2. Cultured cell growth and survival was monitored by MTT assay (Hansen et al., 1989). Cells (2.8×10^4 cells/mL) were cultured with the specified medium in a 96 well microplate and after 72 h, 25 µL of MTT solution (5 mg/mL) was added, incubated at 37 °C for 4 h. One hundred micro litre of lysis buffer was added and cells were continued to incubate at 37 °C overnight (about 16 h) to dissolve the dark blue crystals and absorption of formazan solution was measured at 570 nm in a microplate reader (Spectra Max-340, Molecular Devices, Germany). Presence of galectin-3 was measured employing monoclonal antibody specific to galectin-3 by ELISA as described by us (Sathisha et al., 2007). Of the several cell lines screened MDA-MB-231 was expressing the highest level with 15-fold higher over a Normal - NIH-3T3- a Mouse embryonic fibroblast cells. Media of MDA-MB-231 cells after 48 h of growth, also showed higher levels of galectin and this has been used in *in vitro* to study galectin-3 mediated agglutination of red blood cells, while evaluating the

galectin- inhibitory pectic polysaccharides as a measure of antimetastatic property.

3.4.7. Agglutination inhibition assay

Microplate agglutination assay was performed for the evaluation of potential dietary galectin inhibitors (antimetastatic components) employing the protocol of Nowak et al. (1976). Briefly, human erythrocytes were prepared from 10 mL of fresh blood (collected in Alsever's medium), washed four times with five volumes of 0.15 M NaCl. A 4 % erythrocyte suspension in 0.02 M PBS, pH 7.4 containing 1 mg/mL trypsin was incubated for 1 h at 37 °C. The trypsin treated cells were washed with five volumes of 0.15 M NaCl and fixed in five volumes of 0.02 M PBS pH 7.4 containing 1 % glutaraldehyde for 1 h at room temperature. Glutaraldehyde fixation was terminated by the addition of five volumes of 0.1 M glycine in PBS, pH 7.4 at 4 °C and the fixed erythrocytes were employed for the hemagglutination assay.

Hemagglutination assays were done in microtitre agglutination assay plate using MDA-MB-231 media in presence of serially diluted pectic polysaccharide in 0.15 M NaCl. The reaction mixture contained 150 µL of 4 % erythrocyte suspension with or without serially diluted pectic polysaccharides. Minimum Inhibitory Concentration (MIC) of the substances was determined.

3.4.8. Determination of purity of active SRPP fraction – 0.15 M by HPLC.

Of the two SRPP fractions, 0.15 M fraction showed higher activity. In order to ascertain the purity 10 mg of this was dissolved in deionized water (1 mL) and loaded on to E-linear and E-1000 column (4.0 x 250 cm) connected to a Shimadzu LC-10A

HPLC system (Shimadzu Corp. Kyoto. Japan) Elution was performed with 100 % Milli Q water as a mobile phase at room temperature at a flow rate of 0.8 mL/min and using RI detector. Emergence of peaks was monitored.

3.4.9. Sugar composition analysis

3.4.9.1 Sulphuric acid hydrolysis

SRPP and SRPP fractions - 10 mg each was suspended in water and was hydrolyzed by prior solubilization with 72 % sulphuric acid at ice cold temperature followed by dilution to 8 % acid and heating in a boiling water bath at 100 °C for 10-12 h. The above mixture was neutralized with barium carbonate (solid), filtered, deionized with Amberlite IR 120 H^+ resin and concentrated using a flash evaporator.

3.4.9.2 Regeneration of Amberlite IR-120 H^+ resin

The Amberlite resin was washed with water to remove the fines, colour and other impurities. The water was drained by filtering it through a nylon cloth. The resin was then regenerated by suspending in HCl (2 N) for 1 h at room temperature with intermittent shaking. The resin was then filtered through nylon cloth and washed thoroughly with water till the filtrate gave neutral pH.

3.4.9.3. Preparation of alditol acetates

The neutralized and deionised sample was concentrated to about 0.5 mL. Sodium carbonate was added to a concentration of about 0.07 M to decompose uronic acids. Sodium borohydride (20 - 30 mg) was added and the test tubes were stoppered and taped with adhesive plaster around to hold the stoppers. They were left overnight. Next day, excess borohydride was destroyed with acetic acid (2N). The excess borate

and other salts were removed by co-distilling with methanol (1mL, x4) and then evaporated to dryness. Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept in an oven at 100 °C for 2 h after tightly stoppering the tubes. Excess reagents were removed by co-distilling with water (1 mL, x3) and toluene (1 mL, x3). After thorough drying, the contents were taken in chloroform and filtered through glass wool and dried by passing nitrogen gas. They were taken in chloroform for Gas liquid chromatographic (GLC) analysis.

3.4.9.4. Gas liquid chromatography Operating conditions

Shimadzu GLC (Model-CR4A) fitted with flame ionization detector was used for analysis. OV-225 (1/8" x 6') was the column used with column, injector and detector block temperatures maintained at 200, 250 and 250 °C, respectively. Nitrogen with the flow rate of 40 mL/min was used as the carrier gas.

3.4.10. Determination of molecular weight of the active antimetastatic component.

Gel permeation chromatography was performed on Sepharose CL-4B (1.6 cm x 92 cm). The active pectic polysaccharide fractions (10 mg) were dissolved in distilled water, centrifuged at 6000 *g* for 10 min at RT and the supernatant was loaded (1 mL) on to the column. The elution was carried out by using NaCl (0.1 M) containing sodium azide (0.05 %) at a constant flow rate of 16 mL/h. Fractions (3 mL) were collected and analyzed for the presence of total sugar and appropriate fractions were pooled. Dextran series standards (T-40, T-70, T-150, T-500, T-2000) were used to determine molecular weight. Blue dextran was used to determine void volume. A calibration curve was prepared by plotting V_e/V_0 versus log molecular weight. Where, V_e = void volume, V_0 = elution volume. Molecular weight of the unknown polysaccharide was determined from this graph.

3.4.11. NMR spectral analysis of 0.15 M fraction

The active fraction of SRPP - 0.15 M fraction was dissolved in 1 mL of D_2O. After ensuring complete dissolution, the spectra were recorded with a Bruker amx 400 spectrometer at 500/700 mHz. IR spectral studies were performed to understand the extent of carboxymethyl reduction.

3.5. Results

3.5.1. Extraction of pectic polysaccharide from Black cumin and Swallow root

Pectic polysaccharides were isolated from black cumin and swallow root using ammonium oxalate extraction method. Swallow root had the higher yield of pectic polysaccharide (6.2 %), as it was a fleshy tuberous root, while the yield of pectic polysaccharide was very low in case of black cumin (0.8 %) which had high amount of oils stored in it.

3.5.2. Antimetastatic property of pectic polysaccharides of black cumin (BCPP) and swallow root (SRPP)

Since our studies in the laboratory and results revealed the role of galectin-3 in inhibiting metastasis by enabling cancer cell to interact with normal cells via galectin-3 of cancer cell and beta-galactosides of extra cellular matrix of normal cells, galectin-3 inhibitory components were therefore considered as antimetastatic compounds. Antimetastatic activity thus was studied in different sources by galectin-3 interaction with red blood cells which resulted in agglutination. Agglutination inhibition therefore was measured to understand the antimetastatic potency of the selected polysaccharide. In order to understand the relative efficiency of the sources in inhibiting galectin-3 mediated agglutination, minimum concentration required to inhibit agglutination was studied by serial dilution technique in the agglutination plate. A source which inhibits at lowest concentration was considered as the most potent source, MIC was therefore determined.

Pectic polysaccharides isolated from different dietary sources were evaluated for their galectin inhibitory activity based on hemagglutination assay. Minimum Inhibitory Concentration of the polysaccharide (MIC) in inhibiting the galectin mediated agglutination of red blood cells was determined and results were compared with

standard galectin specific sugars - galactose and lactose. Results presented in Table 3.1 revealed that SRPP showed a potent agglutination inhibition with MIC of 1.85 µg/mL. MIC of 27.1 and 4.16 µg/mL was observed for galactose and lactose that are specific for galectin-3 respectively. Citrus pectin had higher inhibition with MIC of 25 µg/mL than, BCPP (130 µg/mL). Among the standards, lactose showed a good inhibitory activity with MIC of 4.16 µg/mL than galactose (MIC-27 µg/mL). Data thus suggested that at least 15 and 2 fold increase in the activity was exhibited by SRPP over galactose and lactose respectively. Also, SRPP gave ~14 fold increased activity when compared to that of another reported source - citrus pectin which showed an MIC of 25 µg/mL.

Samples	**Agglutination inhibition (MIC in µg eq. carbohydrate/mL)**
Galactose	27.10
Lactose	4.16
SRPP	1.85
CPP	25
BCPP	130

Table 3.1. Agglutination inhibitory activity of various dietary polysaccharides, SRPP fractions. Lactose and Galactose against MDA-MB-231-galectin-3 induced hemagglutination of human erythrocytes.

3.5.3. Purification of SRPP

Since SRPP showed potent activity it was further fractionated on DEAE cellulose ion exchange chromatography. Upon purification, SRPP was fractionated in to four fractions namely 0.05, 0.10, 0.15 and 0.20 M ammonium carbonate eluted fraction (Figure 3.1).

Initially, a neutral fraction was also collected by water elution. The percentage yield of 0.15 M fraction was higher (62 %) followed by neutral (17 %), 0.10 M (15 %) and 0.05 M (4 %) fractions. These fractions were examined for antimetastatic activity. Results presented in Table 3.2 suggest that neutral, 0.1 M and 0.2 M fractions did not show any activity. While, 0.05 M and 0.15 M fraction showed potent antimetastatic activity with an MIC of 0.025 and 0.004 µg/mL as opposed to that of the crude SRPP which showed an MIC of 1.86 µg/mL. Increase of ~ 74 - 400 times in the activity in 0.05 and 0.15 M fraction indicate that it is due to increase in specific activity probably due to the elimination of inactive components during purification. Approximately 15-fold higher yield and 6-fold higher activity was observed in 0.15 M fraction when compared to that of 0.05 M fraction. Science 0.15M fraction showed the better activity than all other fractions, the purity of the fractions examined on HPLC (Figure 3.2) provides the HPLC profile with the homogeneous peak.

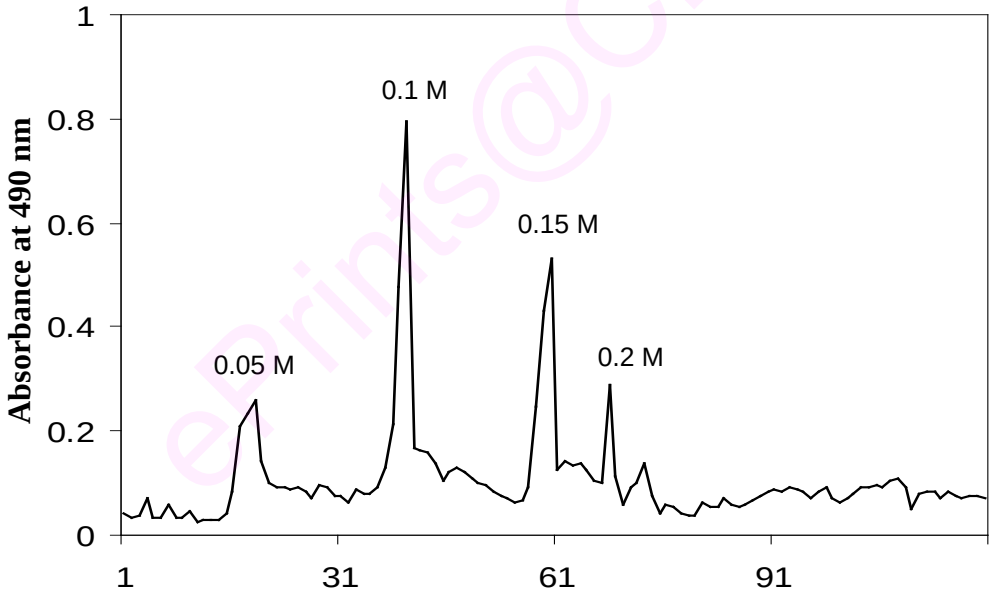


Figure 3.1. DEAE cellulose column chromatography profile of SRPP.

3.5.4. Determination of purity of active SRPP 0.15 M fraction

The active SRPP fraction - 0.15 M fraction by HPLC was shown to be homogenous on gel permeation chromatography on sepharose CL-4B column chromatography and HPLC analysis (Figure 3.2).

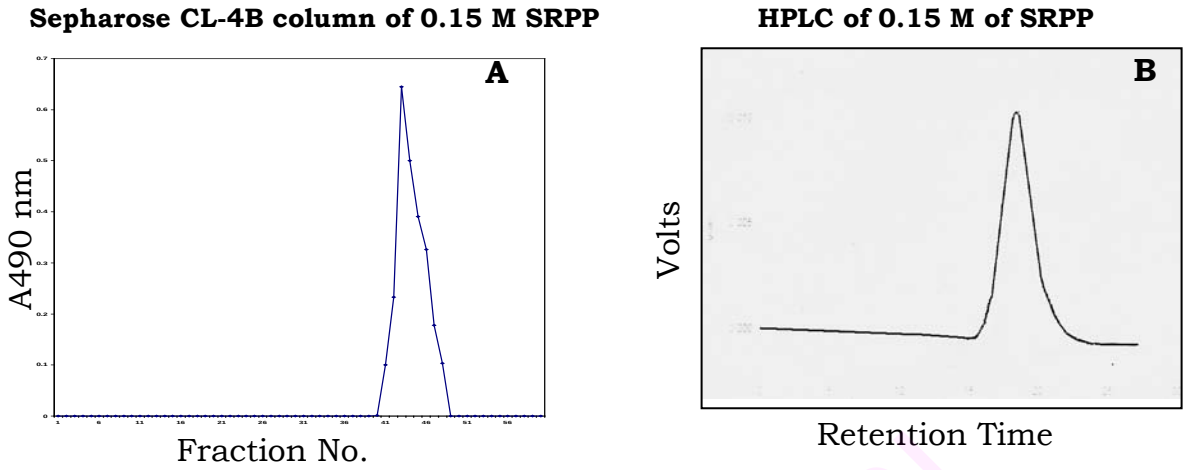


Figure 3.2. Determination of purity of active SRPP fraction – 0.15 M by gel permeation chromatography on sepharose CL-4B column chromatography (A) and HPLC analysis (B).

3.5.5. Sugar composition analysis of SRPP and DEAE fractions of SRPP

Sugar composition analysis of galectin-3 inhibitory polysaccharide from swallow root, black cumin and citrus pectin were compared by GLC analysis. Data presented in Table 3.2 indicated the presence of rhamnose (16 %), arabinose (50 %), xylose (02 %), galactose (32 %) in SRPP; rhamnose (05 %), arabinose (30 %), xylose (03 %), galactose (19 %), glucose (42 %) in CPP and rhamnose (49 %) arabinose (42 %), xylose (02 %), galactose (04 %) and glucose (03 %) in BCPP. In addition, uronic acid content of 141, 295 and 30 mg/g were observed for SRPP, CPP and BCPP, respectively suggesting the pectin nature.

As observed previously with different sources, differential activity was observed. Sugar composition analysis was determined in order to verify whether arabinose and galactose are present in these fractions. Swallow root pectic polysaccharide of 0.05 M and 0.15 M fractions which exhibited antimetastatic activity with an MIC of 0.025 µg/mL and 0.004 µg/mL did not contain significant content of rhamnose, mannose and glucose although 7 % of rhamnose was found in 0.15 M fraction indicating that these sugars are not involved in the activity. Also the potent fraction (0.15 M fraction)

was more active than 0.05 M fraction, did not contain xylose indicating that xylose also may not be involved in the activity. Arabinose was absent in 0.05 M SRPP fraction suggesting that arabinose may not be crucial for the activity. Also 0.1 M and 0.2 M fractions which contained higher levels of arabinose 83 and 100 %, respectively did not show activity supporting the fact that arabinose may not be a crucial sugar for the activity. Data thus points to the fact that galactose may be a crucial sugar component responsible for the activity. Further, 0.05 M fraction contained ~ 2.7 fold higher levels of galactose (76 %) as opposed to only 27 % in 0.15 M fraction. If only galactose is important for the activity, 0.05 M should have showed better activity than 0.15 M fraction. Together data may suggest that both arabinose and galactose may be important for the activity. A definite proportion of arabinose and galactose hence may be important for the activity. However this alone do not explain 6-fold increase in activity in 0.15 M over 0.05 M, since ~ 37 fold difference in uronic acid content were observed in 0.15 M fraction. Uronic acid may also be important for the activity. Precise structure - function analysis is required to completely understand the requirement for pectic polysaccharide to be antimetastatic, in addition to the presence of galactose as a basic requirement.

Sugars	**SRPP**	**CPP**	**BCPP**
Rhamnose	16	05	49
Arabinose	50	30	42
Xylose	02	03	02
Mannose	-	-	-
Galactose	32	19	04
Glucose	-	43	03
Uronic acid (mg/g)	141	295	30

Table 3.2. Relative percent sugar composition analysis and uronic acid contents of pectic polysaccharides from various sources. SRPP – swallow root pectic polysaccharide; CPP - citrus pectic polysaccharide and BCPP – black cumin pectic polysaccharide.

DEAE Fractionated SRPP Fraction (% Yield)	Agglutination inhibition (MIC in μg)
Neutral (16.7)	-
0.05 M (4.26)	0.025
0.10 M (14.89)	-
0.15 M (62.31)	0.004
0.20 M (1.84)	-

Table 3.3. Agglutination inhibitory activity of DEAE fractionated swallow root pectic polysaccharides against MDA-MB-231 cell extracts containing galectin-3 induced hemagglutination of human erythrocytes

Sugars	SRPP	0.05 M	0.10 M	0.15 M	0.20 M
Rhamnose	16	-	17	7	-
Arabinose	50	-	83	66	100
Xylose	02	24	-	-	-
Mannose	-	-	-	-	-
Galactose (Free Gal)	32	76	-	27	-
Glucose	-	-	-	-	-
Uronic acid (mg/g)	141	3.0	21	113	1.0

Table 3.4. Relative percent sugar composition and uronic acid content of DEAE fractionated swallow root pectic polysaccharide.

3.5.6. Determination of molecular size of active 0.15 M pectic polysaccharide fraction of SRPP

Molecular weight of the active pectic polysaccharide 0.15 M was determined through gel permeation chromatography on sepharose CL-4B using dextran standards. The molecular weight was found to be 250 kDa (Figure. 3.3).

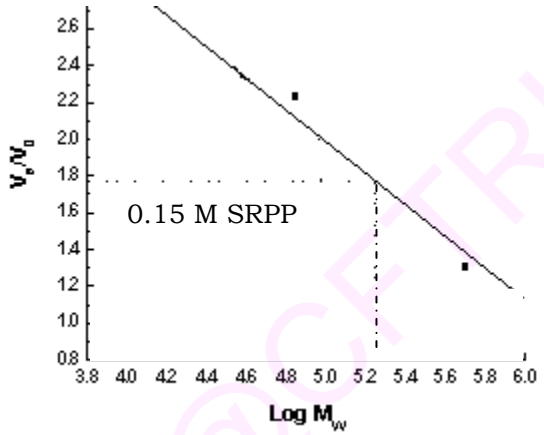


Figure 3.3. Determination of molecular weight analysis by using Sepharose CL-4B column chromatography.

3.5.7. Determination of NMR analysis of active 0.15 M pectic polysaccharide fraction of SRPP

Although the inactive fractions do not also contain higher levels of uronic acids, there is no direct evidence for correlation between uronic acid content and the activity. The nature of uronic acids present in them may be responsible for the same. Hence detailed structural studies were undertaken for SRPP. The ^{13}C NMR spectral profile obtained for 0.15 M fraction of SRPP showed (Figure 3.4) the presence of characteristic pectic type polysaccharide. The galacturonic acid main chain occurring as a back bone of the polysaccharide has been observed. The six signals at 100.41, 69.54, 70.42, 79.44, 72.86 and 176.76 ppm corresponds to the ring carbon C-1, C-2, C-3, C-4, C-5 & C-6 respectively of 1,4 ά linked D-galactopyranosyluronic

acid units. The signal at 62.54 is assigned to C-6 of β-Galactose residue linked to ά – arabinose by 1,4 linkage. The signal at 22.23 is possibly due to the presence of methyl groups. This suggests that the pectic polysaccharide is methylated.

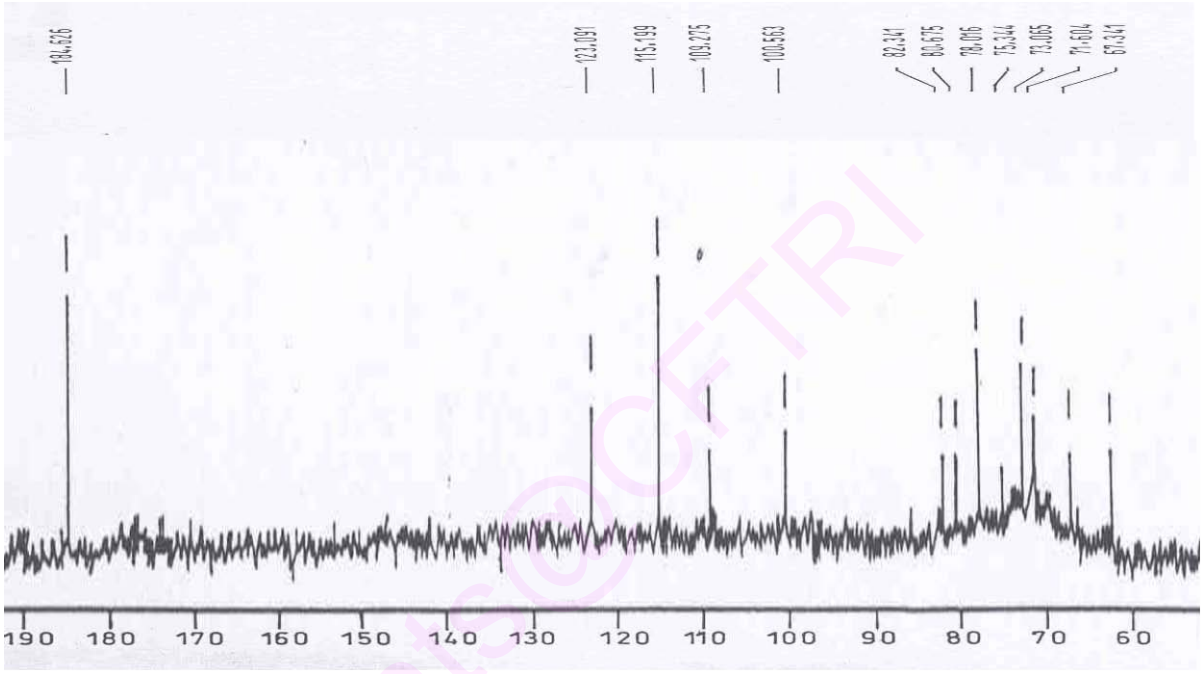


Figure 3.4. NMR analysis of active fraction of SRPP - 0.15 M fraction.

3.6. Discussion

In the present study, we isolated pectic polysaccharides from swallow root and black cumin to examine their ability to block galectin-3 molecule reported to be involved in metastasis progression of various types of cancers (Song et al., 2002).

Pectic polysaccharides are present in plant cell walls with D-galacturonic acid back bone linked via α-1-4 linkage containing variable amount of neutral sugars - arabinose, galactose, xylose present as side-chains. Rhamnose may also be present in the backbone. The main applications for pectic polysaccharides generally isolated from citrus or apple as food additives and as gelling and thickening agents in many food products. Due to their gelling nature, these, soluble polysaccharides may decrease the rate of gastric emptying and influence small intestinal transit time, which explains their hypoglycemic properties (Jenkins et al., 1978) and reported to decrease serum cholesterol (Kay & Truswell, 1977). Various human studies show that pectins are fermented to a large extent in the colon (Cummings et al., 1979). In addition, pectic polysaccharides are known to possess galectin-binding activity (Kidd, 1996).

Galectin binding activity of pectic polysaccharide is of importance because galectin is a key molecule in metastasis which functions by binding to β-galactosides on the normal cell. As presented in Scheme 3.1, ability of pectic polysaccharide to bind to galectin may inhibit or hinder the interaction of galectin from cancer cell to normal cells which triggers metastasis. Pectic polysaccharide hence may block metastasis as indicated by Dr. Raz's group (Inohara & Raz, 1994).

Galectin-3 inhibitory activity of pectic polysaccharides isolated form black cumin and swallow root was examined in agglutination assay to evaluate galectin-3 binding activity. Among the sources studied swallow root yielded higher amount of pectic polysaccharide. Also, swallow root pectic polysaccharide showed comparatively higher (70-fold) galectin-3 inhibitory activity than black cumin pectic polysaccharide. Hence, SRPP was taken for further characterization.

Results of the study indicated the association between arabinose and galactose with activity. Pectic polysaccharide from different sources showed different activity and also differences in sugar composition. In order to understand the relation between type of sugars in selected pectic polysaccharides and antimetastatic activity, correlation coefficient-R was determined. Results indicated a correlation coefficient of 0.06851, 0.6341, -0.1678, 0.695 and -0.3922 for rhamnose, arabinose, xylose, galactose and glucose respectively. A strong correlation was observed between arabinose/galactose and antimetastatic activity (higher arabinose/galactose - higher antimetastatic activity). Significant inverse relationship between rhamnose and antimetastatic activity was also observed (higher rhamnose content – low activity); while no significant correlation existed between the activity and other sugars. Results thus suggest that the presence of galactose and arabinose appear to be crucial for the activity of the sources studied. Further, ~6.25-fold higher active SRPP fraction (0.15 M) contained 2.8-fold lesser galactose than that found in 0.05 M fraction, but contained additionally 66 % of arabinose indicating that both arabinose and galactose may be important for the activity. Further, to confirm the fact that arabinose and galactose are important for the galectin-inhibitory property, purified larch wood arabinogalactan that contained 98 % (w/w) of arabinogalactan was examined for galectin inhibitory property. Galectin inhibitory activity with an MIC of ~200 µg/mL was obtained, indicating that not only the presence of arabinose and galactose may be essential for the activity; while a precise arabinose-galactose structure may be involved.

It was reported that polysaccharides containing arabinogalactan residues inhibits metastasis (Hagmar et al., 1991; Uhlenbruck et al., 1986). Modified citrus pectin (MCP) containing galactose residues have been reported as a possible anti-metastatic agent (Nangia-Makker et al., 2002). A systematic treatment with D-galactose and arabinogalactan as well as cell pretreatment with arabinogalactan and two other glycoconjugates has been carried out on animals in a syngenic tumor-host system using tumor, which primarily colonizes the liver upon intravenous injection. Host

treatment with arabinogalactan significantly reduced the amount of liver metastasis and prolonged the survival times of the animals indicating the blockade of potential liver receptors by covering galactose-specific binding sites (Beuth et al., 1987). In the present study, we showed that by the sugar composition analysis of swallow root and citrus pectin the presence of arabinose and galactose as the major sugars correlating to their higher galectin-3 inhibitory activity compared to black cumin pectic polysaccharide. Further, galectin-3 inhibitory activity needs to be substantiated by further *in vivo* studies. Chapter IV and V deals with the effect of galectin-3 blockade by the selected SRPP fraction on further events of metastasis such as cell invasion (Chapter IV) and metastasis *in vivo* (Chapter V) has been highlighted.

Current study has a greater implication in that it is supporting a workable concept of probable prevention of metastasis. This is the subsequent 2nd report apart from citrus pectin and 1st report in spices with antimetastatic potency. Nevertheless as indicated in results section, SRPP had higher potency since it showed ~20-fold higher activity than citrus pectin. Besides this SRPP also contain bound phenolics (Srikanta et al., 2007) which may also contribute significantly to anticancer property. Details have been described along with the results in Chapter V.

Summary and Conclusions

- Among the sources studied – Black cumin (BC) and Swallow root (SR), SR had higher yield of ~ 6.2 % of pectic polysaccharide [SRPP] as opposed to 0.8 % in Black cumin pectic polysaccharide (BCPP).

- SRPP also had ~ 14.6 and 70 fold higher galectin-3 inhibitory activity when compared to galectin specific sugar - galactose and BCPP, respectively, indicating the increased antimetastatic potency of SRPP.

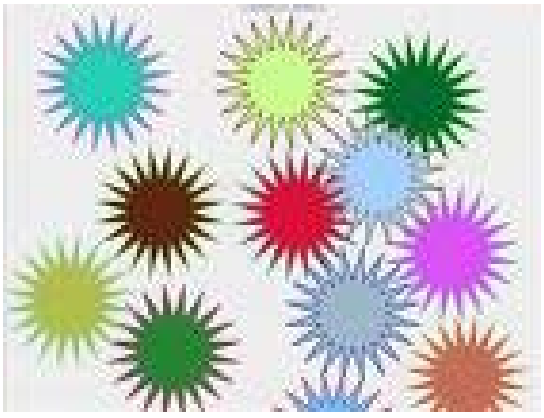
- Purification of SRPP followed by activity determination indicated that SRPP – 0.15 M fraction was more active with ~ 462 fold increase in activity than that of the crude SRPP; Active fraction had a molecular size of 250 kDa.

- Sugar composition analysis and correlation studies suggested that precise arrangement of arabinose and galactose in an arabinogalactan pectic polysaccharide is important in offering potent galectin-3 inhibitory effect.

- Galectin-3 inhibitory effect could be attributed to antimetastatic activity.

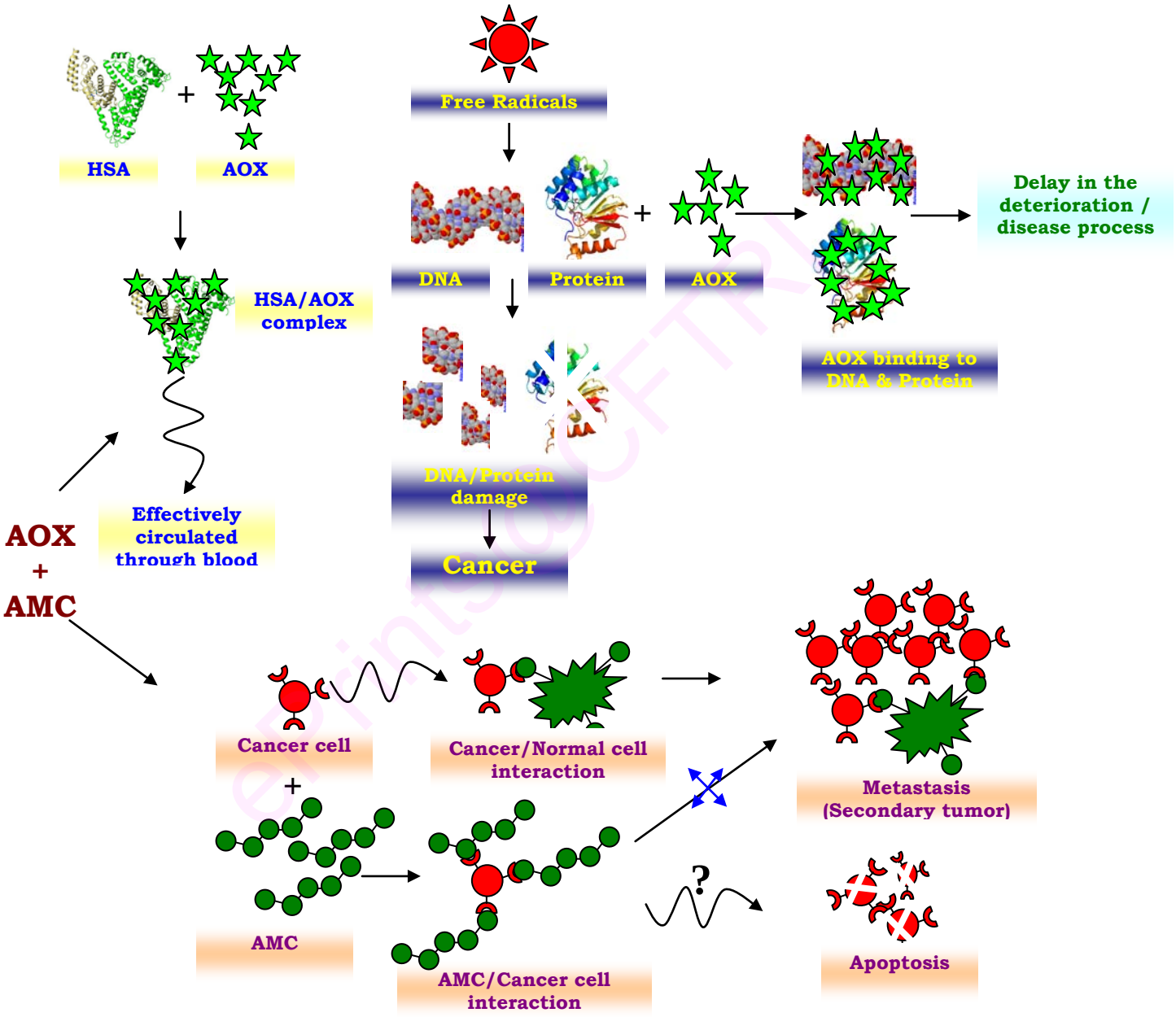
Chapter IV

Determination of efficacy and mechanism of action of antioxidants and antimetastatic components using *in vitro* biochemical and cell culture assay model systems

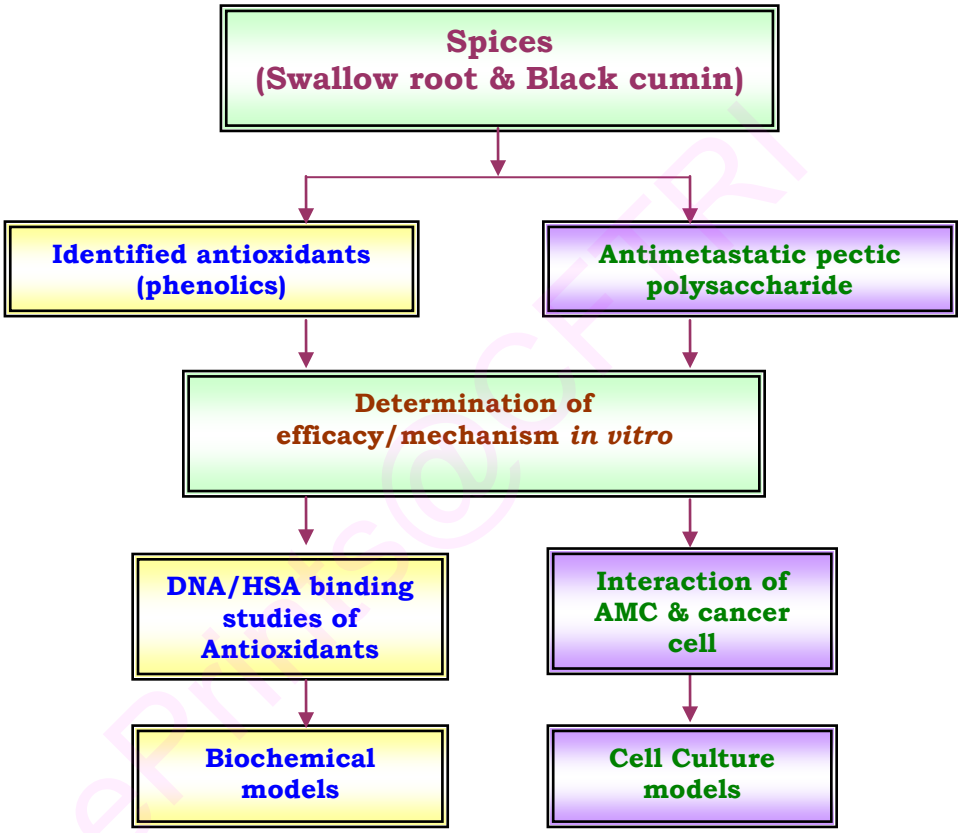


4.1. Hypothesis

Chapter 1 and 2 provides potent antioxidant (AOX) and antimetastatic pectic polysaccharide compounds (AMC) from black cumin and swallow root. In order for them to act, they need to interact with the host protein or DNA in case of antioxidants and β-galactosides/galactose containing extra cellular matrix components of cells in case of antimetastatic pectic polysaccharides. Study in this chapter addresses do antioxidant of black cumin bind to protein and DNA? What may be its implications? Similarly if antimetastatic compounds interact with galectin-3 containing cancer cells, what is the fate of such cells?



Scheme 4.1



4.2. Work concept

4.3. Introduction

Binding of phenolics including antioxidants to plasma proteins is an important pharmacological parameter, since it frequently affects the distribution and elimination of these compounds, which dictates the duration and intensity of physiological action. [Kragh-Hansen et al., 2002; Lin et al., 1987; Fichtl et al., 1991]. The effect is especially significant for highly protein bound drugs, where only a small alteration in bound fraction can produce a profound change in the pharmacodynamically active free antioxidant concentration [Levy & Moreland, 1984; Rowland, 1980]. Generally the bound antioxidant component to serum albumin serves as a storehouse and the unbound may be pharmacologically active (Seedher & Bhatia, 2006). Thus the nature and magnitude of their interaction with serum albumin has important pharmacokinetic and pharmacodynamic implications. Fluorescence spectroscopy has been the most widely used spectroscopic technique for monitoring drug/antioxidant binding to plasma albumin because of its sensitivity, accuracy, rapidity and ease of use [Oravkova et al., 1996; Epps et al., 1999]. In the present investigation interaction of the phenolics of black cumin-BCAE and swallow root-SRAE with human serum albumin has been studied using fluorescence spectroscopic technique. In addition, to understand the probable structure-function relationship interaction of pure phenolic acids with protein and DNA were also studied. Results have been discussed in terms of the binding parameter.

Also, phenolics bind to DNA leading to various biochemical changes (Labieniec & Gabryelak, 2006) including DNA-adduct formation, strand breaks etc. Nucleic acid bound to ethidium bromide - a polycyclic aromatic dye, exhibits marked increase in fluorescence and this phenomenon is used in the current study to study the interaction between DNA and isolated phenolics from black cumin and swallow root.

Further, antimetastatic component isolated from swallow root has shown galectin-3 inhibitory activity *in vitro* which reflects inhibition of interaction between galectin-3 and red blood cells (Chapter III). However, the effect of binding of these components on to the cell surface and the changes induced by this interaction is important to

understand the biopotency of these molecules. Cell-cell interaction and cell invasion being the role played by galectin-3 of cancer cell via β-galactoside containing normal cell, alteration in interaction by pectic polysaccharide resulting in inhibition of cell-cell interaction and cell invasion – important events of metastasis has been studied in this chapter.

4.4. Materials and methods

4.2.1. Chemicals

Phenolic acids such tannic, gallic, protocatechuic, syringic, procoumaric, vanillic, cinnamic, ferulic, gentisic, caffeic and *p*-hydroxy benzoic acid, and aldehydes such as vanillin and 2-hydroxy-4-methoxybenzaldehyde, human serum albumin, calf thymus DNA, ethidium bromide, acridine orange, N-acetyl-ASP-Glu-Val-ASP-P-nitroanilide (Ac-DEVDpNA), hematoxylin and eosin stain, Dulbecco's minimum essential medium (DMEM), citrus pectin, glucose, glutamine, sodium bicarbonate, penicillin and kanamycin, fetal calf serum, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), paranitrophenyl phosphate (PNPP), diethanolamine, Alsever's medium, trypsin, hematoxylin, eosin, acridine orange, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel invasion chamber with pore size (0.8 µm) were purchased from BD Biosciences, USA). Other chemicals and solvents used were of analytical grade procured from a local chemical company, Mysore, India.

4.2.2. HSA - Phenolics interaction studies

Stock solution of human serum albumin was prepared to a concentration of 1.0×10^{-4} M in Tris-HCl buffer of pH 7.4 containing 100 mM sodium chloride. All the phenolic compounds were prepared to a concentration of 10 mg/100 mL in ethanol (95 %) because ethanol has no fluorescence and does not affect the determinations. Crude water extracts of black cumin and swallow root were prepared by extracting 1 g sample in 20 mL of distilled water. The concentrations of crude extracts, free, hydrolyzed and bound phenolics of swallow root were expressed in terms of gallic acid equivalents of phenol. All the stock solutions were then diluted with the buffer to obtain the actual assay concentration. All fluorescence measurements were made in a Shimadzu RF-5301PC Spectrofluorophotometer equipped with a xenon lamp source and 1.0 cm x 1.0 cm x 4.0 cm quartz cells.

A series of assay solutions were prepared by adding 10 µL of stock solution of HSA and varied concentrations of phenolics (0.5-2.5 µg/mL) into each mark tube, respectively, and diluted to the mark of 1.0 ml with Tris–HCl buffer of pH 7.4. In

each tube therefore, the total concentration of HSA was constant and the total concentration of phenolics were different. Tubes were mixed thoroughly and placed in the thermostat water-bath at 37 °C for 5 min, and transferred to the quartz cuvette and fluorescence emission spectra were recorded in the wavelength range 290–500 nm by exciting HSA at 280 nm using a slit width of 5/5 nm. Wavelength nearer to shift observed was recorded to understand the involvement of tryptophan/tyrosine residue in HSA.

4.2.3. DNA - Phenolics interaction studies

Calf thymus DNA sufficiently free of protein was used. A_{260}/A_{280} of 1.8-1.9 were considered for the assay. DNA concentrations were determined spectrophotometrically with an extinction coefficient of 6600 M^{-1} at 260 nm (Song et al., 2002). All the experiments were carried out in 5 mM Tris-HCl buffer pH 7.0 containing 50 mM NaCl. DNA and ethidium bromide were dissolved in buffer at a concentration of 3 and 1 µg/mL, respectively. Phenolics concentration ranged was between 0.5-2.5 µg/mL. The isolated phenolics were added to ethidium bromide bound to calf thymus DNA and the intensity of fluorescence of ethidium bromide was measured. Fluorescence spectra was recorded using an excitation wavelength of 478 nm and the emission range set between 480 and 850 nm using a slit width of 5/5 nm.

4.2.4. Interaction studies with swallow root pectic polysaccharides (SRPP)

Pectic polysaccharides from black cumin and swallow root have been shown to inhibit galectin mediated agglutination of red blood cells, indicating the efficacy of inhibition of interaction by the selected pectic polysaccharide. In the current chapter therefore the implications of inhibition of SRPP has been studied. Generally in cancer cell spread state galectin-3 mediated interaction triggers invasion of cancer cell to normal cell. Inhibition of invasion hence results in the lack of ability to invade and cell death. Current chapter therefore addresses the effect of SRPP on cell death.

4.2.4.1. Effect of galectin-3 blockade by SRPP; Inhibition of cell invasion

Matrigel invasion chamber with pore size (0.8 µm, BD Biosciences, USA) was used to measure cell invasion in *vitro* [Zoltan-Jones et al., 2003]. SRPP at 50 & 100 µg/mL were added to the metastatic MDA-MB-231 cell suspension in 0.5 ml medium without serum. The control and SRPP treated cell suspensions (0.5 ml of 3×10^4 cells) were added to each Matrigel insert, the bottom chamber contained growth medium with 5 % FBS. After 24 h chambers were removed, cells that remained in the upper chamber were counted under the inverted microscope. Percent of cells invaded were calculated as 1. (No. of cells invaded into the bottom chamber) (BC)/(Total No. of cells)×100, and 2. (No. of cells in upper chamber (UC) at 0 h)-(No. of cells in UC at 24 h)/(Total No. of cells)×100.

4.2.4.2. Apoptosis assay

Apoptosis assay was performed using ethidium bromide and acridine orange dye method [Powell et al., 2001] as well as observing characteristic features of cells by microscopy. Briefly, MDA-MB-231 and Buccal (1×10^4 cells/well) cells were treated with SRPP at 50 and 100 µg/mL for 72 h and 1 h respectively. Twenty five microliters of cell suspension of both treated and untreated cells were mixed with 1 µl of dye mix containing 100 µg/mL each acridine orange and ethidium bromide and observed under the microscope at 40X. Viable cell nuclei stained green with acridine orange and apoptotic cell nuclei stained red with ethidium bromide were counted. Percent apoptosis was measured and compared. In order to understand the probable route of apoptosis, levels of Caspase activity was measured [Chanana et al., 2007] using caspase-3 specific peptide-substrate N-acetyl-ASP-Glu-Val-ASP-P-nitroanilide (Ac-DEVDpNA). The release of P-nitroaniline moiety from the substrate was measured at 405 nm in microplate reader (SpectraMAX plus, Molecular Devices).

4.5. Results

4.3.1. HSA-phenolics interaction studies

Results from HSA interaction studies indicate that the changes occurred in the environment of tryptophan residues which is dependent on the applied phenolic compounds. As shown in Figure 4.1A-C nearly all the phenolic compounds and extracts tested showed HSA binding except but to varying extent with a variable range of 30-200 fold or over the maximum quencher – tannic acid with a K_{SV} of 3.178 x 10^6 M^{-1}. Next best phenolic acid for binding was ferulic acid with a K_{SV} of 0.084 x 10^6 M^{-1} followed by HMBA (0.068 x 10^6 M^{-1}), syringic (0.059 x 10^6 $M^{-1)}$, vanillic (0.053 x 10^6 M^{-1}) caffeic (0.050 x 10^6 M^{-1}), p-hydroxybenzoic (0.043 x 10^6 M^{-1}), procoumaric (0.039 x 10^6 M^{-1}), gentisic (0.035 x 10^6 M^{-1}), cinnamic (0.024 x 10^6 M^{-1}) vanillin (0.019 x 10^6 M^{-1}) and gallic acid (0.016 x 10^6 M^{-1}). However regression coefficient was although moderate to some phenolic acids as reported in Table 4.1, they were quite good for some indicating dose dependent interaction. Stern-Volmer constants of the qenching of tryptophan fluorescence by phenolics and spice crude extracts is shown in Table 4.1. It is evident from the results that, HSA fluorescence was strongly quenched by tannic acid (K_{SV} – 3.178 x 10^6 M^{-1}) and moderate quenching was observed in BCAE (K_{SV} – 0.249 x 10^6 M^{-1}), and swallow root bound phenolic acid extract (SRBP, K_{SV} – 0.203 x 10^6 M^{-1}). Low quenching was observed for SRAE (K_{SV} – 0.008 x 10^6 M^{-1}), vanillin (K_{SV} – 0.0.019 x 10^6 M^{-1}) and gallic acid (K_{SV} – 0.016 x 10^6 M^{-1}). However, vanillic acid even though indicated fluorescence quenching initially, but increased the fluorescence of HSA molecule at higher concentrations.

Interestingly, BCAE is constituted by 77 % tannic acid and ~ 23 % by protocatechuic acid. BCAE showed the second highest binding next to standard tannic acid (Table 4.1), suggesting that BCAE - phenolics particularly tannic acid may contribute to the binding. The same may therefore circulated effectively *in vivo* via binding to HSA. Generally HSA being the carrier protein, increased binding efficiency also indicate the increased bioavailability which may potentiate the effect *in vivo*.

In order to understand the probable binding of SR phenolics in SRAE, SRAE-characteristic compounds such HMBA, hydroxyl benzoic acid, vanillin and vanillic acids were also examined for their binding ability with HSA. Figure 4.1C provides the fluorescence emission spectra of different phenolic fractions of SR, which are constituted by different phenolic acids in a definite proportion (Table 4.2). Data together with the calculation of Stern-Volmer constant indicate that SRBP showed binding more or less equivalent to that of BCAE with a K_{SV} of 0.203 X $10^6 M^{-1}$ with a good correlation coefficient (r) of ~ 0.998 suggesting a stronger binding ability to HSA. SRHP and SRFP showed ~ 35 - 38 fold less binding than SRBP. SRFP appear to be less effective in binding than SRHP since the r value for SRHP is 0.977 which is acceptable than SRFP which showed an r value of ~ 0.67. Reduction in regression coefficient could also be due to saturation effect and the threshold of which may be decided by the nature of interacting molecules, their size, functional group and ionic nature in them. Indeed interaction studies thus reflect the probable bioavailability and biodistribution. Phenolic acid action may be better upon binding to HSA because 1) it can be distributed widely making the compound bioavailable to each tissue; 2) it can influence delayed clearance from the circulation which obviously potentiate the bioavailability and 3) limit the degradation rate and 4) the potency to exhibit prooxidant nature may also be reduced.

In order to understand the interaction with the constituents present in the extract, in the current study attempts were made to determine the binding efficiency based on the Stern-Volmer constant of individual constituent in pure form and relative percent contribution was calculated for BC and SR extracts. Data is presented in Table 4.2. Figure 4.2 has been plotted to depict the expected ability of these extracts in comparison with standards.

At equal phenolic concentration, BCAE showed more binding (80 %) considering tannic acid as 100 %, followed by SRFP (79 %), SRAE (60 %), SRBP (42 %) and SRHP (30 %). Increased efficiency in SRFP and SRAE could be due to the presence of HMBA, p-HBA and vanillic acid. Although similar levels and slightly higher levels of

HMBA and vanillic acid was found in SRBP, ~ 2-fold reduction in activity could be due to associated gallic acid which is a poorer quencher than gentisic acid which was found in SRFP (Table 4.2). It should be noted that expected binding efficiency depicted in figure 4.2 varies with the actual experimental binding as depicted in Table 4.1. SRFP which was one of the best binder to HSA is almost equivalent to that of tannic acid (79 %) when compared to SRBP (42 %), SRBP showed stronger binding – 38 fold better than SRFP the extract as per table 4.1. However, there was no alteration in BCAE. Data thus may suggest that phenolic constituents may interact themselves and may exhibit profound synergistic or antagonistic effects. Therefore biopotency of the antioxidant extract depends not only on the efficacy of antioxidant activity as estimated *in vitro*, but also on its bioavailability and biodistribution via binding to a carrier protein like HSA and their synergistic or antagonistic effect within themselves.

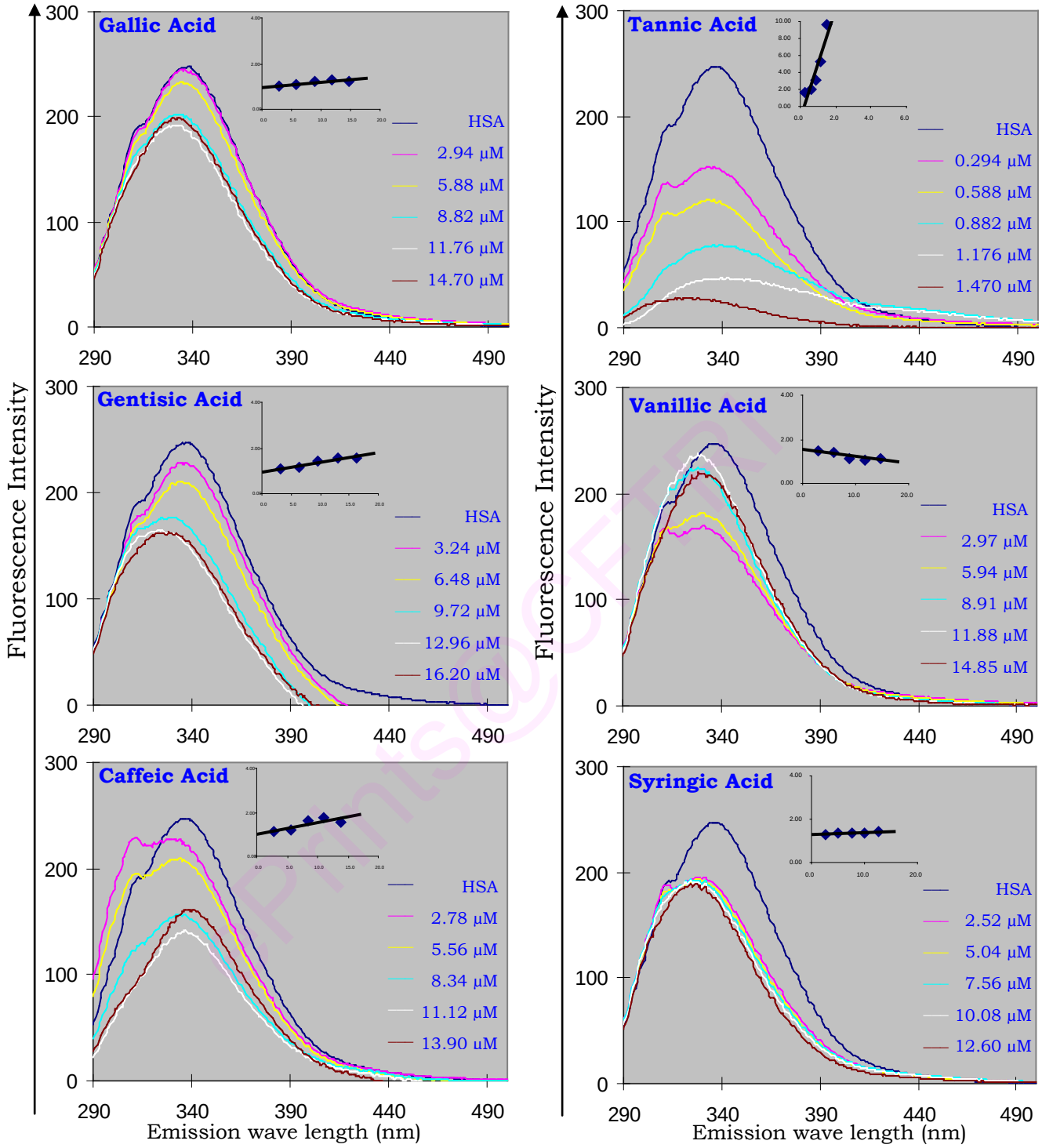


Figure 4.1A. Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F°/F)

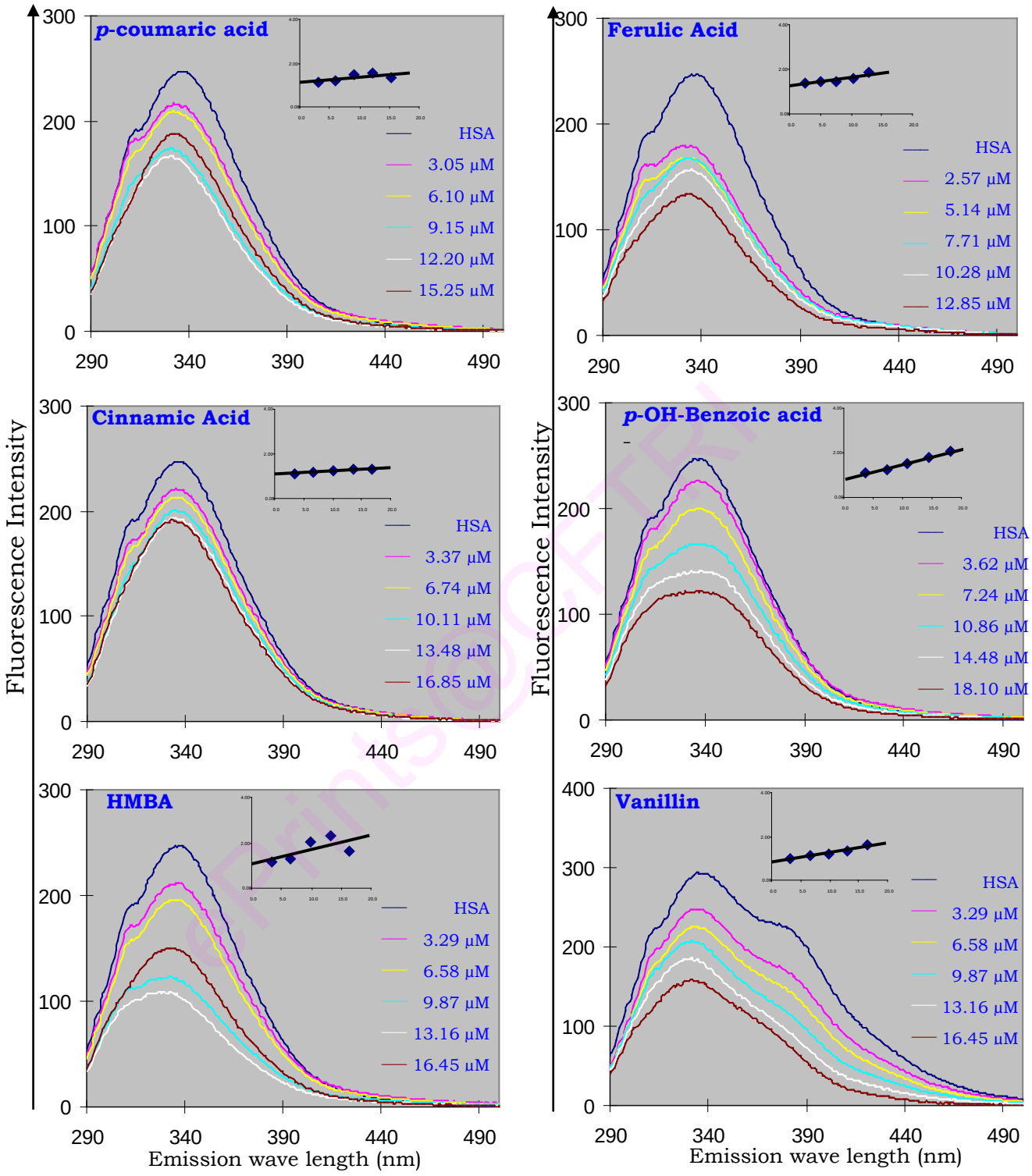


Figure 4.1B. Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F°/F)

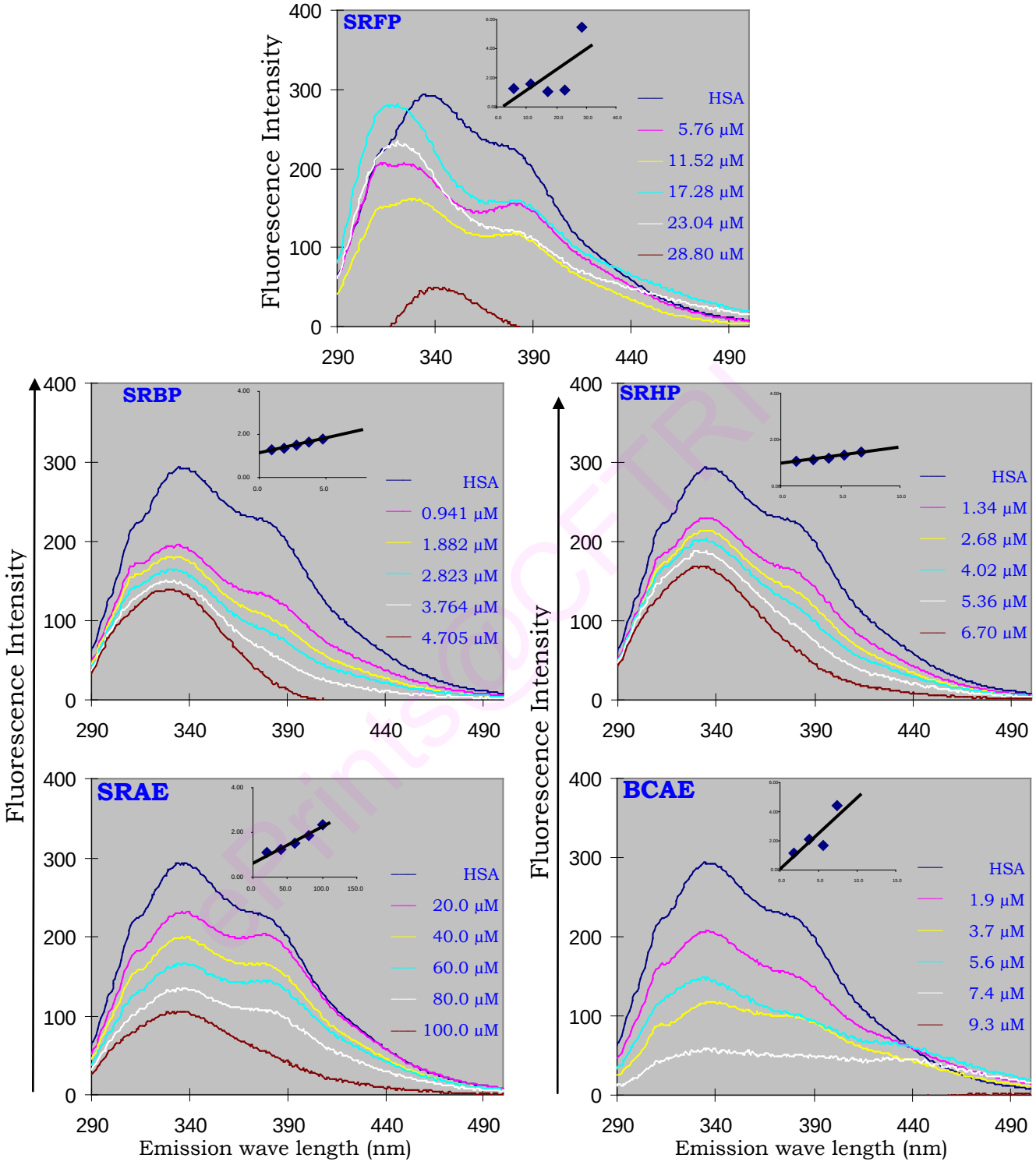


Figure 4.1C. Fluorescence emission spectra of HSA in the presence of various phenolics isolated from swallow root at different concentrations. For all solutions, the concentrations of HSA were constant: 0.5 µM. The excitation wave length was 280 nm. Both excitation and emission slits widths were 5 nm. Inlaid figure - Stern-Volmer plots (X- axis: Concentration in µM; Y-axis: F°/F)

Table 4.1. Stern-Volmer constants of the quenching of tryptophan fluorescence by phenolics and spice extracts

Sample	Stern-Volmer Constant, Ksv (M^{-1})	Regression coefficient (r)
Tannic acid	3.178 x 10^6	0.931
BCAE*	0.249 x 10^6	0.836
SRBP*	0.203 x 10^6	0.998
Ferulic acid	0.084 x 10^6	0.909
HMBA	0.068 x 10^6	0.642
Syringic acid	0.059 x 10^6	0.988
SRHP*	0.058 x 10^6	0.977
Vanillic acid	0.053 x 10^6	0.894
SRFP*	0.053 x 10^6	0.670
Caffeic acid	0.050 x 10^6	0.828
p-HBA	0.043 x 10^6	0.994
p-coumaric acid	0.039 x 10^6	0.689
Gentisic acid	0.035 x 10^6	0.945
Cinnamic acid	0.024 x 10^6	0.983
Vanillin	0.019 x 10^6	0.981
Gallic acid	0.016 x 10^6	0.912
SRAE*	0.008 x 10^6	0.981

* Concentrations are expressed as gallic acid equivalent (GAE) phenol.

Table 4.2. Contribution of different phenolic components to total HSA fluorescence quenching in black cumin and swallow root extracts

Sample	Contribution of phenolic components to the total fluorescence quenching (binding) capacity (in Units)				
	BCAE	SRAE	SRFP	SRHP	SRBP
Tannic acid	1341 (92%)	4940 (39%)	-	-	-
Ferulic acid	-	-	37 (>1%)	32 (3%)	46 (4%)
HMBA	-	7491 (58%)	1973 (22%)	490 (41%)	445 (34%)
Syringic acid	-	-	59 (>1%)	42 (4%)	14 (1%)
Protocatechuic acid	122 (8%)	-	278 (3%)	7 (>1%)	7 (>1%)
Vanillic acid	-	330 (3%)	566 (6%)	-	125 (10%)
Caffeic acid	-	-	53 (>1%)	28 (2%)	-
p-HBA	-	-	1994 (22%)	-	423 (33%)
p-coumaric acid	-	-	20 (>1%)	20 (2%)	63 (5%)
Gentisic acid	-	-	2289 (25%)	266 (22%)	-
Cinnamic acid	-	-	4 (>1%)	-	-
Vanillin	-	-	1706 (19%)	7 (>1%)	93 (7%)
Gallic acid	-	-	108 (1%)	298 (25 %)	76 (6%)

Numbers in the brackets indicate percent contribution to total fluorescence quenching

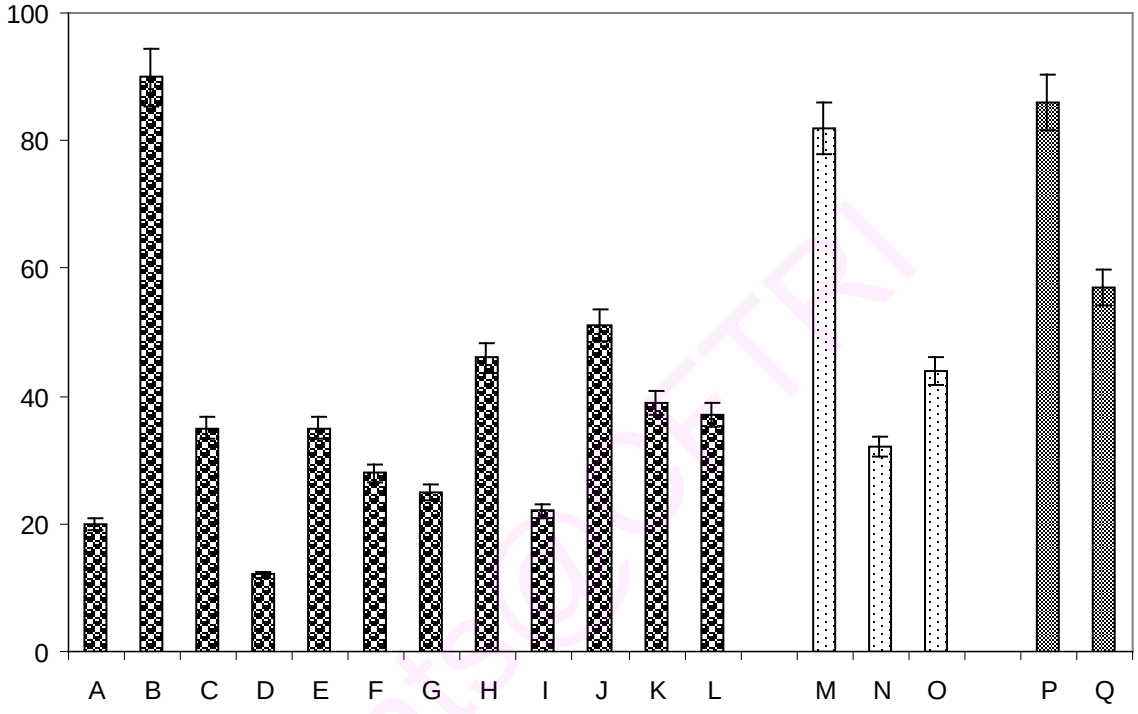


Figure 4.2. Percent fluorescence quenching (binding) of different phenolics at 2.5 µg/mL concentration

A- Gallic acid; **B**- Tannic acid, **C**-Gentisic acid; **D**-Vanillic acid; **E**-Caffeic acid; **F**-Syringic acid; **G**-*P*-coumaric acid; **H**-Ferulic acid; **I**-Cinnamic acid; **J**-p-Hydroxybenzoic acid; **K**-HMBA; **L**-Vanillin; **M**- SRFP; **N-**SRHP; **O-**SRBP; **P-**BCAE; **Q**-SRAE. Values are mean ± SD.

4.3.2. DNA – phenolics interaction studies

Ethidium bromide emits intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs. It is reported that the enhanced fluorescence can be quenched by the addition of a DNA binding molecule. The quenching extent of fluorescence of ethidium bromide bound to DNA is used to determine the extent of binding between the second molecule and DNA (Liu et al., 2002). The emission spectra of ethidium bromide bound to DNA in the absence and presence of various isolated phenolics from black cumin and swallow root, free, hydrolyzed and bound phenolic acid extracts of swallow root and water extract of both the sources are given in Figure 3A-C. The addition of these phenolics to DNA being in complex with ethidium bromide caused an appreciable reduction in emission intensity, indicating that the phenolics compete with ethidium bromide in binding to DNA.

At minimal phenolic concentration tannic acid had the highest binding capacity (24 %) followed by *p*-coumaric acid (17 %), caffeic acid (17 %), gallic acid (15 %), ferulic and cinnamic acid (14 %). Hydrolyzed phenolic acid had stronger affinity for DNA with 14 % followed by bound phenolic acid (13 %) and free phenolic acid extract. The binding ability of SRWE, BCWE and *p*-HBA were almost negligible.

As noticed in protein-phenolic binding studies, tannic acid bound strongly to both protein and DNA. The degree of binding in one way may inhibit the molecule from getting oxidized and in that case it may protect. On the other hand its role in inhibiting the function of the bound component may not be ruled out. Further, the order of binding ability of BC extracts differ depending on the size of molecule either protein or DNA. The effect of phenolic acid in vivo in a given condition of disease may depend on the nature of the biomolecule circulating. This may to some extent responsible for contradictory effects of antioxidants *in vivo* when compared to that of *in vitro*.

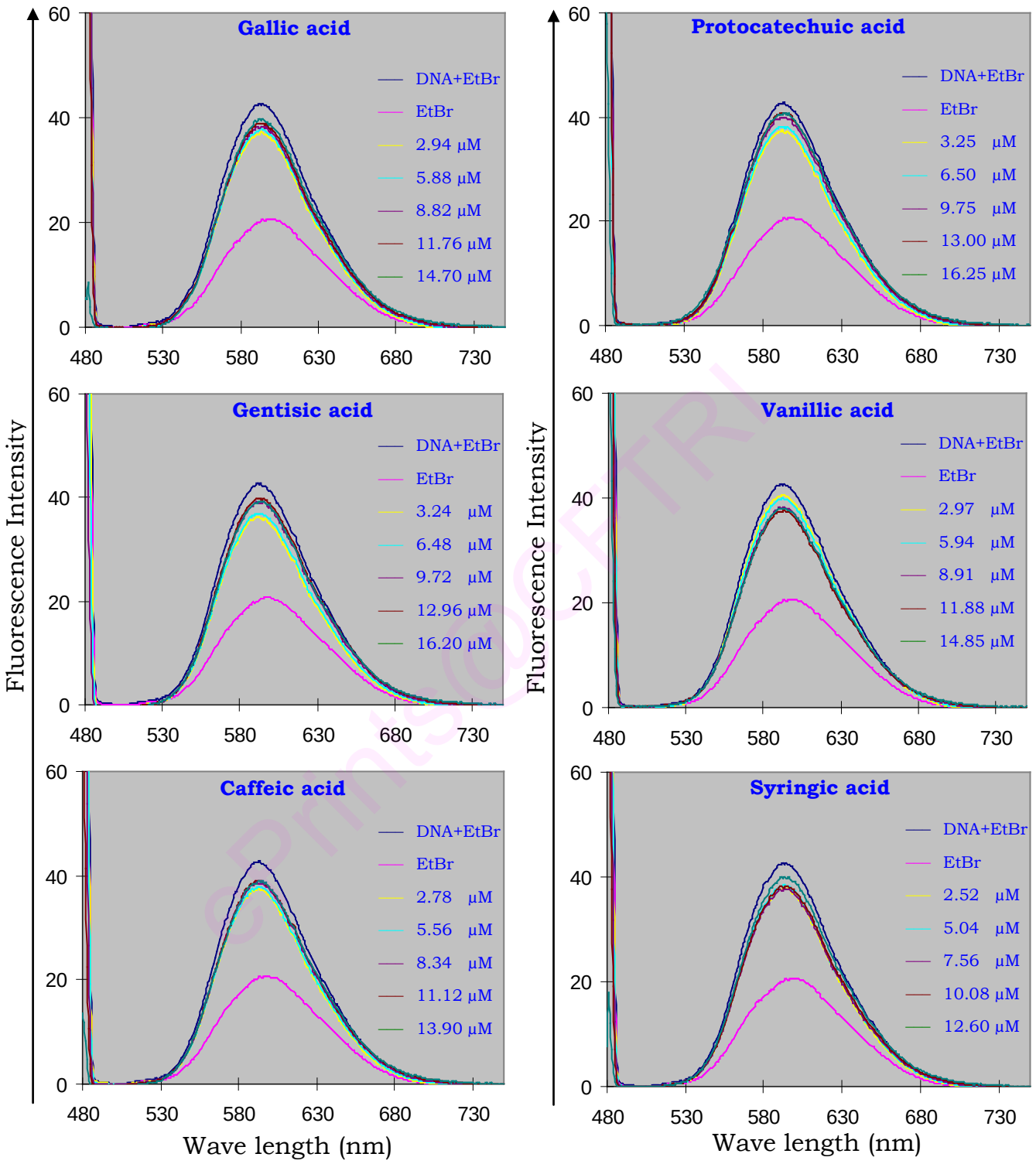


Figure 4.3A. Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics, λ_{exc}= 478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml

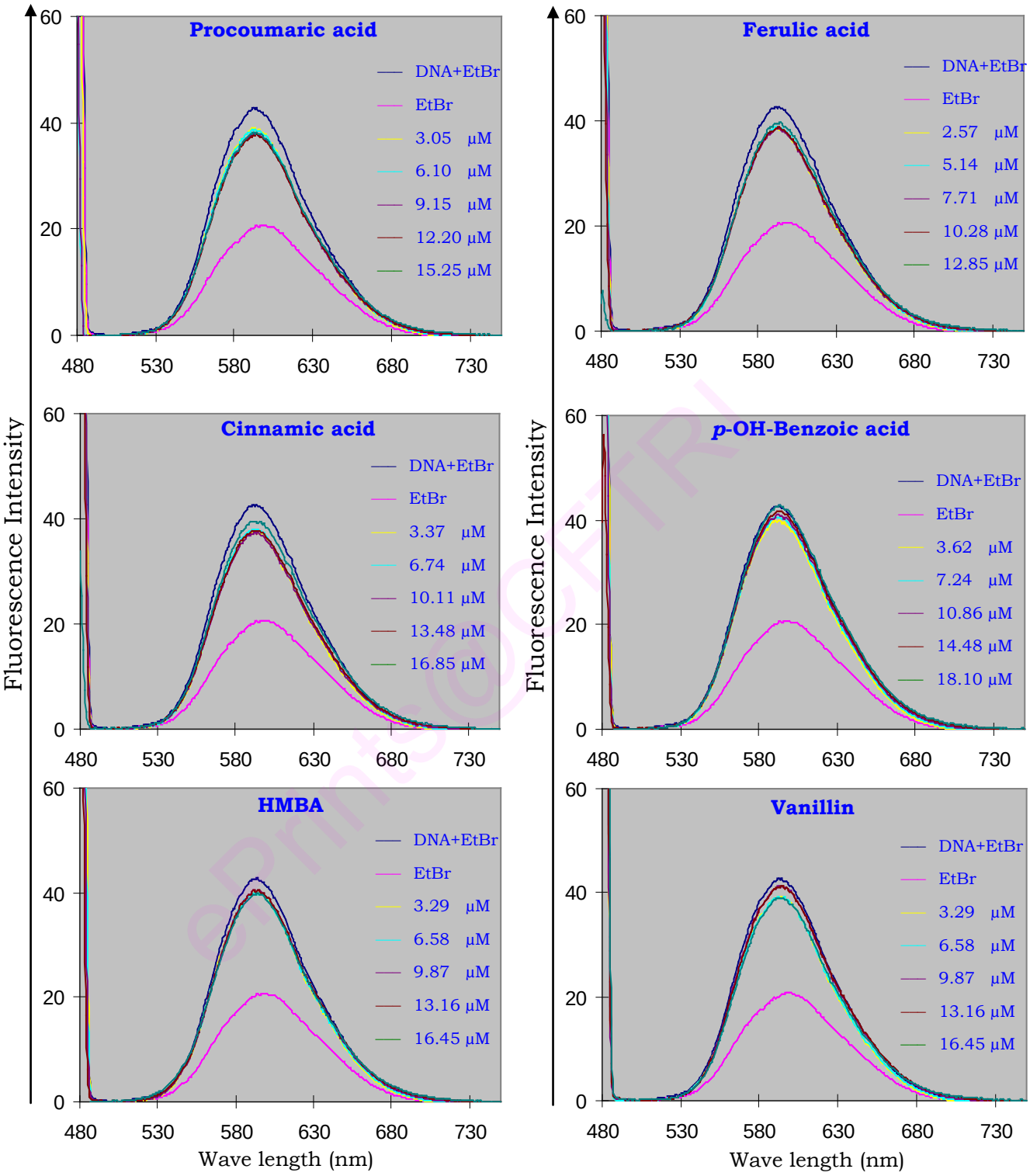


Figure 4.3B. Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics and spice extracts, λ_{exc}=478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml

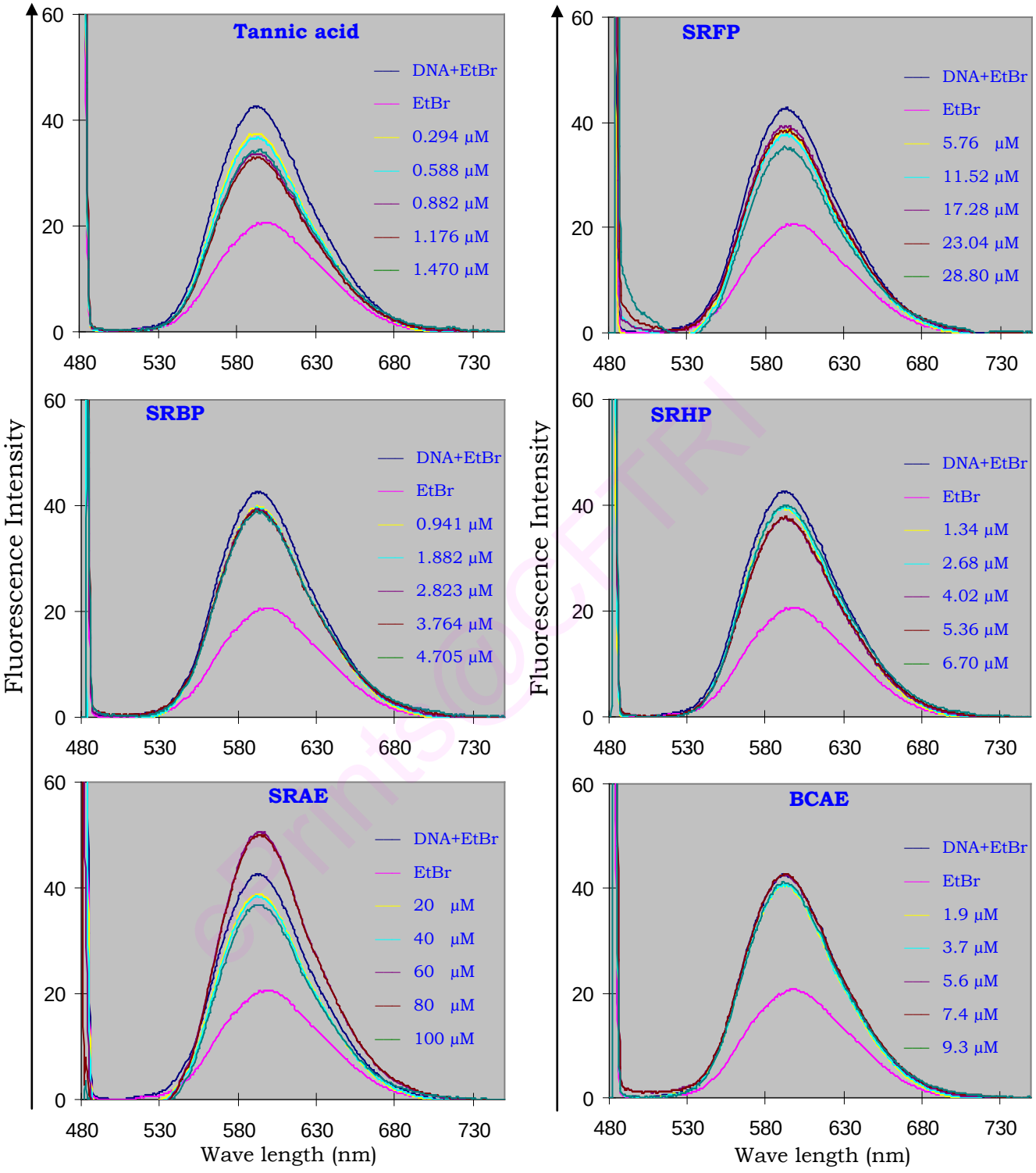


Figure 4.3C. Fluorescence emission spectra of ethidium bromide (Etbr) bounded to DNA in the absence and presence of various phenolics, λ_{exc}= 478 nm. EtBr at a concentration of 3 µg/ml, DNA at a concentration of 5 µg/ml

Table 4.3. Percent DNA binding of phenolic compounds and extracts of black cumin and swallow root.

Sample	**% DNA binding**
Tannic acid	46.72 ± 2.11
SRFP	35.28 ± 1.56
Gentisic acid	29.93 ± 0.98
Gallic acid	26.86 ± 2.08
SRAE	26.13 ± 2.03
Cinnamic acid	25.64 ± 2.01
Protocatechuic acid	24.18 ± 0.79
Caffeic acid	24.09 ± 1.58
SRHP	23.80 ± 1.66
p-coumaric acid	23.55 ± 1.02
Vanillic acid	23.21 ± 1.11
Syringic acid	22.77 ± 1.74
SRBP	20.00 ± 1.74
Ferulic acid	19.95 ± 1.02
Vanillin	17.18 ± 0.79
HMBA	13.92 ± 0.35
P-HBA	12.70 ± 0.68
BCAE	10.71 ± 0.19

Values are expressed as mean ± SD (n = 3)

4.3.3. Antimetastatic components-cell interaction studies

4.3.3.1. Cell invasion assay

Filtration of metastatic MDA-MB-231 cells treated with and without SRPP/CPP through matrigel coated invasive chamber indicated more number of cells equivalent to the absorbance of ~0.8 as per MTT assay in treated cells in the upper chamber, while an absorbance of only 0.296 was observed in untreated controls. The cells invading through matrigel were also counted. Data suggested concentration dependence and ~73 % inhibition of cell invasion at 100 µg/mL of SRPP/CPP (Figure 4.4). Results thus suggest that the isolated SRPP has anti-invasive property similar to that of CPP [Nangia-Makker et al., 2002].

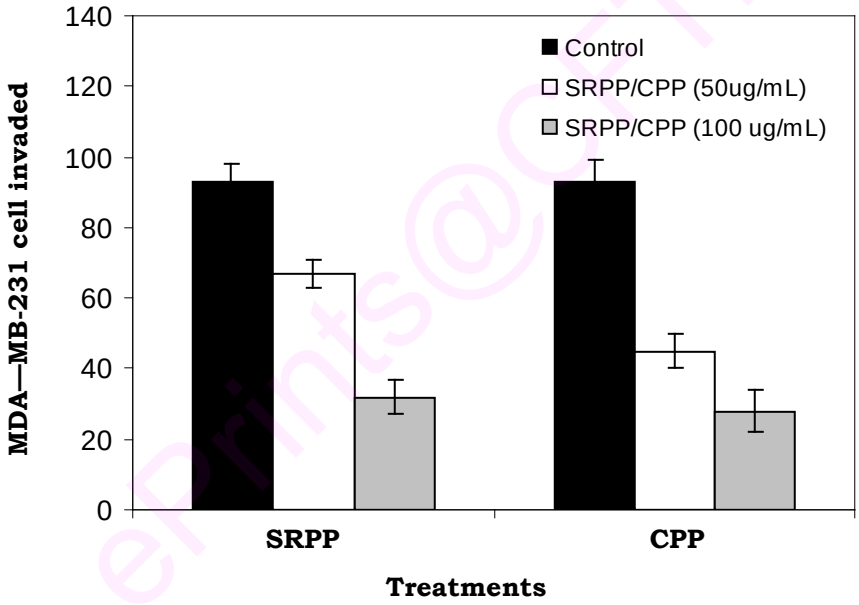


Figure 4.4. Inhibition of MDA-MB-231 cell invasion *in vitro* by SRPP/CPP. Values are expressed as mean ± SD (n=)

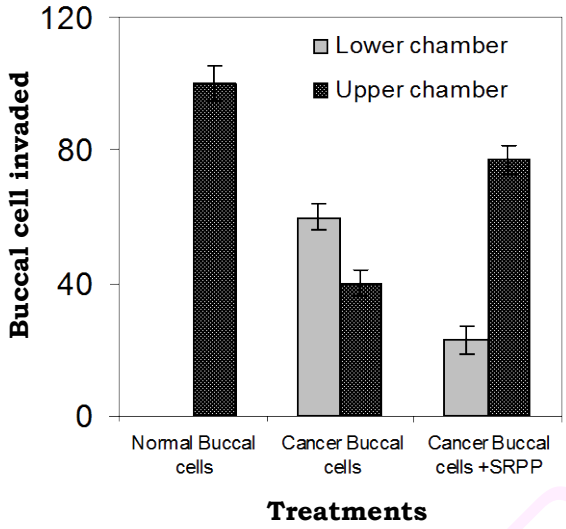


Figure 4.5. Inhibition of buccal cell invasion *in vitro* by SRPP/CPP. Results on % invasion were compared with normal buccal cells (NBC). Values are expressed as mean ± SD (n=3).

4.3.3.2. Apoptotic effect of SRPP on MDA-MB-231/buccal cells

In the control cells (Figure 4.6a), predominant granular pattern was observed upon immunostaining. Galectin was found both in the nucleus and the cytoplasm. Upon treatment of control cells with SRPP, cells showed distinct morphological changes as evident from nuclear/chromatin structures, oozing out of cytoplasmic contents and formation of apoptotic bodies (Figure 4.6b), increase in cell volume and membrane disruption, cell membrane blebbing and intracellular bridges typical of apoptosis was observed. Results were substantiated by the appearance of 40–60% of apoptotic cells as evaluated by acridine orange and ethidium bromide staining methods (Figure 4.6c) in metastatic cancer buccal cells.

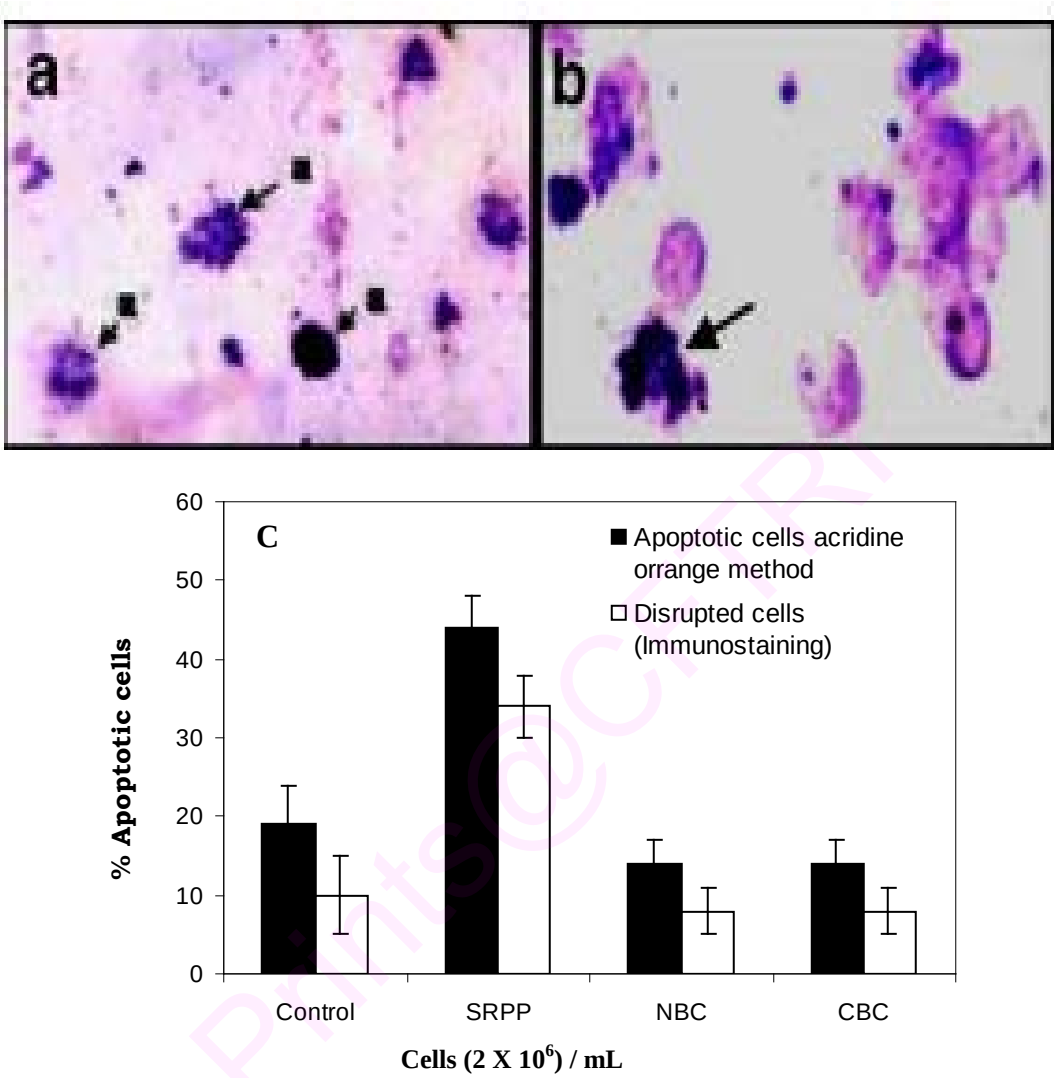


Figure 4.6. Measurement of apoptosis in metastatic-MDA-MB-231 and non-metastatic cells: cells were treated with SRPP at 100 µg/mL for 24 h and subjected to immunostaining using anti-galectin-3 antibody. Control MDA-MB-231 cells showing granular structure (a) was disrupted by SRPP treatment. Cell shrinkage and oozing of cellular contents are evident (b). Figure C shows bar graph with percent apoptotic cells during the treatment with galectin inhibitory polysaccharide. Number of cells lysed as per acridine method/immunostaining method were counted and percent apoptosis was calculated.

4.6. Discussion

4.6.1. HSA-phenolics interaction studies

Human serum albumin has been accepted widely as a principal transport protein for various drugs and phytonutrients including antioxidants and hence responsible for distribution of antioxidants through the circulation.

Phenolic antioxidants have been known for their therapeutic effects and have become an important component of nutraceuticals. One of the important mechanisms through which phenolic antioxidants exhibit bioactivity is via influencing longer half life in the circulation and by avoiding themselves from degradation by drug degrading enzymes. Enhancement of bioactivity often has been attributed to its binding to human serum albumin. The most unique feature of serum albumin is its ability to reversibly bind to a wide variety of endogenous and exogenous bioactive compounds.

Human serum albumin (HSA) is a single-chain protein containing 585 amino acids and has an isoelectric point of 4.7. The protein has three homologous domains (I–III) and each of these is comprised of two sub domains (A and B). It contains a single tryptophan residue at position 214 in sub domain IIA [Kragh-Hansen et al., 2002]. The conformational changes of HSA were evaluated by the measurement of intrinsic fluorescence intensity of protein tryptophan residues at 296 nm before and after the addition of various phenolic compounds. Fluorescence measurements give information about the molecular environment in a vicinity of the chromophore molecules. Changes in the emission spectra of tryptophan are common in response to protein conformational transitions, subunit association, substrate binding or denaturation. Thus, the intrinsic fluorescence of proteins can provide considerable information about their structure and dynamics, and their probable association reactions with phytonutrients (Tian et al., 2004).

The effect of phenolic compounds on HSA fluorescence intensity is shown in Figure 1A-C. Increasing concentrations of the isolated phenolic compounds, and crude

extracts caused a linear reduction in the fluorescence of the tryptophan residues. The effect was strongest for tannic acid followed by bound phenolic acid extract and black cumin water extract. All phenolic acids studied decreased the fluorescence intensity significantly that their behavior could be compared to the quenchers and hence the data was analyzed by Stern-Volmer equation (Klajnert et al., 2003).

$$F_O/F = 1 + K_{SV}(Q)$$

Where F_O and F are, respectively, fluorescence intensities in the absence and presence of quencher, K_{SV} is the Stern-Volmer dynamic quenching constant and (Q) is the concentration of the quencher. The equation assumes a linear plot of F_O/F versus (Q) and the slope equals to K_{SV} (Klajnert et al., 2003). The Stern-Volmer constants for quenching of tryptophan fluorescence by various phenolic compounds and extracts hence are presented in Table 4.1. K_{SV} were calculated from plots shown in figure 4.1A-C (inlaid graphs). These results indicate that the changes of the environment of tryptophan residues depend on the applied phenolic compounds.

Plant phenols are known to precipitate proteins and it is a well known phenomena. The mechanism of this precipitation has been of interest for a long time, and hydrogen bonding and hydrophobic interactions have been reported as major binding modes. Hydrogen bonding between the phenolic hydroxyl group of plant phenols and the amide group of protein has been emphasized by the fact that phenol can be absorbed on the protein surface. They may interact with protein in reversible and irreversible ways leading to its conformational changes (Kawamoto et al., 1997). Thus tannic acid possessing the highest affinity to HSA is more effective in causing conformational modification of human serum albumin. Our data also indicated that although binding ability of BCWE is 31 fold higher than SRAE, increased abundance of phenolics 98 mg/g in SRAE as opposed to 3.2 mg/g in BCAE is evident. On ingestion of BCAE/SRAE extracts as used in traditional medicine the conformational changes are higher with SRAE. These changes influence the clearance rate and the bioavailability. During diseases more of binding of these antioxidants may inhibit

may inhibit oxidative damage to protein and DNA. This property hence may be useful in employing antioxidant as adjunct therapy during chemotherapy in cancer patients.

4.6.2. DNA – phenolics interaction studies

The interaction between DNA and phenolics isolated form the spice sources indicated higher binding ability for tannic acid compared to all other components used for the study. It is evident from the results that phenolic compounds which were simple in structure and aldehydes exhibited weak binding. The phenolic compounds which had almost similar structures at the aromatic ring showed similar binding ranging between 20-30 %. These results suggest the possible role of hydroxyl groups in the process of intercalation. Earlier studies (Magdalena and Teresa, 2006; Whitley et al., 2003) have revealed the binding ability of tannic, gallic and ellagic acid to DNA. Tannic acid has been demonstrated to modify DNA bases by strongly binding to nucleic acid (Khan et al., 2000). In our investigation, the ability of various other phenolic compounds especially hydroxybenzoic and cinnamic acid derivatives have been studied including the crude water extracts of *N. sativa* and *D. hamiltonii*. As seen in Figure 3A-C and Table 2, the ability of majority of phenolic compounds to bind DNA is 50 % lesser in comparison with tannic acid except for free phenolic acid extract which showed 25 % lesser binding. The property of intercalation of these phenolic compounds appears to be related to the number of hydroxyls on the molecule (Ahmad et al., 1992), this fact can probably explain why phenolic compounds containing 1-3 hydroxyl groups demonstrates lesser affinity to DNA than tannic acid which has 21 hydroxyl groups. Thus the structural features of the used compound are important for its effect on a biomolecule such as DNA under appropriate conditions. The other possibility could be that binding to DNA is facilitated by the higher molecular size of tannic acid, which possibly gives rise to a greater hydrophobic character. Higher ionic strength does not inhibit the binding of tannic acid to DNA, suggesting that electrostatic interaction is not predominantly involved and possibly the binding is the result of a hydrophobic interaction (Goppelt et al., 1981). Knowing that DNA is a highly organized macromolecular complex and

its double helix provide a unique hydrophobic core of stacked bases (Jin et al., 1997), the last explanation concerning the differences in affinity to DNA base pair between chosen chemicals seems quite probable. Moreover, the obtained results prove that the direct intercalation between phenolic compounds and DNA must be taken into account when evaluating the mechanism underlying the observed biological effects of these plant phenolic compounds

4.6.3. Antimetastatic components-cell interaction studies

SRPP which showed galectin-3 inhibitory activity as evaluated by inhibition of agglutination of red blood cells, also inhibited invasion and induced apoptosis in vitro. Data that inhibition of galectin-3 mediated interaction inhibits invasion and induced apoptosis suggest that galectin-3 is a key molecule for successful metastasis. Unpublished data from our laboratory provided evidence for the effect of galectin-3 inhibitory polysaccharide in inhibiting the subsequent signaling cascade of metastasis. For the first time we showed that SRPP can block significantly the activation of matrix metalloproteinase which are involved in favoring invasion of cancer cells and establishment.

Evidently as per Figures 4.4 & 4.6, inhibition of invasion and induction of apoptosis were observed. Interestingly SRPP also exhibited differential effects such as induction of apoptosis in only metastatic cells- MDA-MB-231 and not in normal cells. No SRPP induced toxicity was also observed. SRPP therefore may be considered as a potent-galectin blocker which can be employed in the arrest of metastasis. Marked reduction in cell invasion (Figure 4.4 and 4.5), may further emphasize the importance of dietary carbohydrate as potential cancer-preventive and therapeutic agents as already highlighted in case of citrus pectin [Beuth et al., 1987]. The complex nature of carbohydrate may enunciate the development of new antagonists for galectin-3. The identified polysaccharide has additional advantage of being non-toxic and inexpensive. It should be highlighted here that galectin inhibitors may also have a greater impact in inhibiting matrix metalloproteinase activity. Matrix

metalloproteinases of metastatic cells help invading into normal cell via acting on galectin-3 itself on extracellular matrix, since galectin-3 is the substrate for matrix metalloproteinases [Ochieng et al., 1994]. The binding of pectic polysaccharide to galectin-3 of cancer cell may also abolish the invasive action of metalloproteinases. These observations taken together suggest that SRPP can reduce tumor cell invasiveness by suppressing galectin-3 mediated cell adhesion to extracellular matrix proteins in the basement membrane of normal cells and hence may subject such cells to apoptosis and hence may become a potential cancer therapeutic agent.

Summary and Conclusions

- Determination of binding ability of BCAE and SRAE indicated that all phenolic acids have the ability to bind to protein and DNA at varied levels. Tannic acid followed gentisic and gallic acid appear to bind strongly to both - a carrier protein – Human Serum Albumin and DNA.

- Binding of these components to target protein is implicated in enhancement of bioavailability and hence bioactivity. Interaction of phenolic acids thus is an important tool to predict the antioxidant potency *in vivo*.

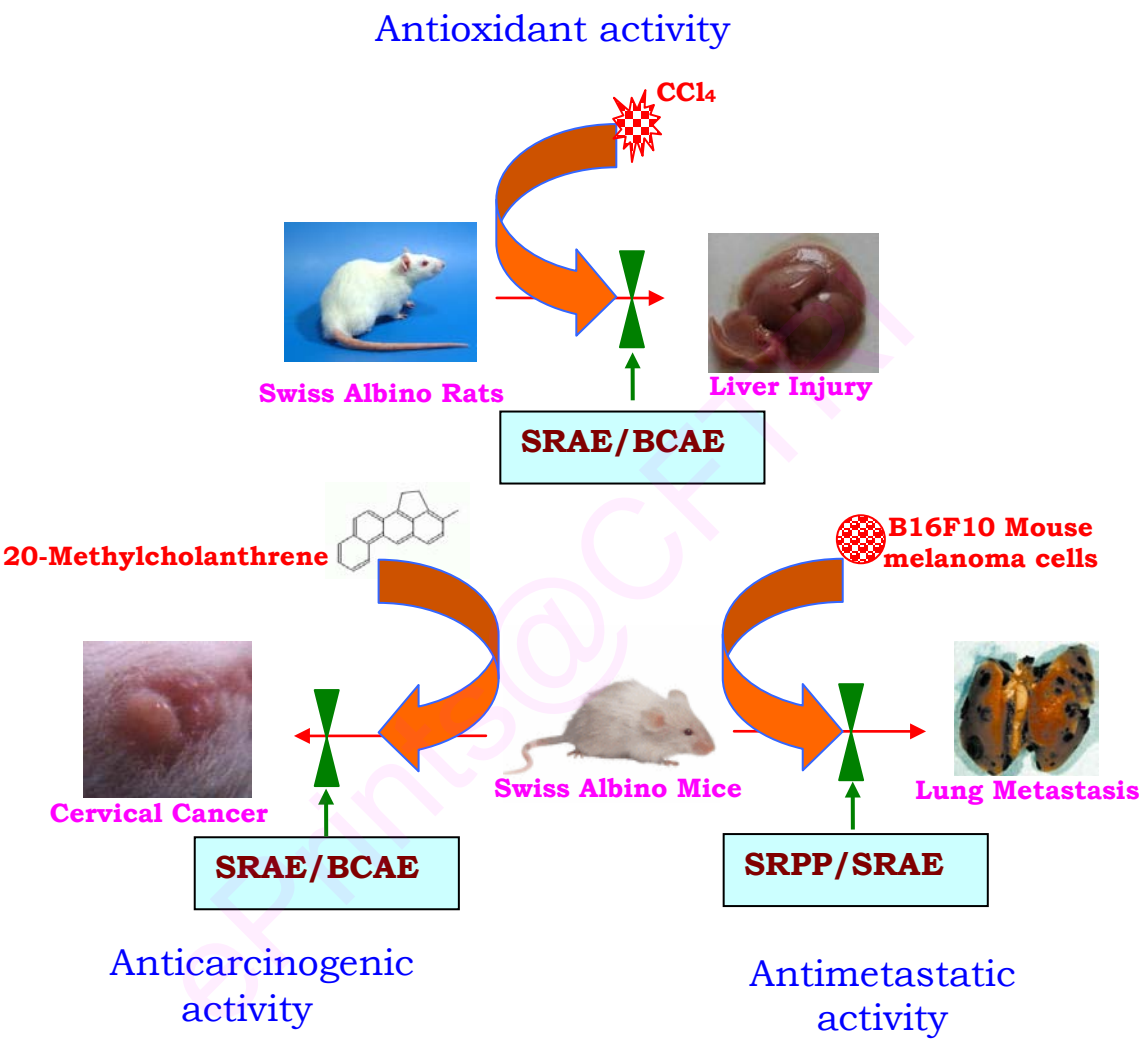
- Potent galectin-3 inhibitory polysaccharide – SRPP also inhibited cell invasion *in vitro* and induced apoptosis of a highly metastatic MDA-MB-231 cells.

Chapter V

Efficacy of combinational supplementation of antioxidants and antimetastatic components against cancer employing *in vivo* cancer animal model

5.1. Hypothesis

Chapter 2 and 3 provides a multi-potent antioxidant components in aqueous extracts of black cumin (*Nigella sativa*)-BCAE, swallow root (*Decalepis hamiltonii*)-SRAE, and a potent galectin-3 blocker from swallow root (SRPP), which is 14 fold better than the reported only source of galectin-3 blocker-citrus pectin. Chapter 4 provides their probable in vitro effect as they showed effective interaction with human serum albumin and DNA which potentially depicts the protection ability and bioavailability. Effect of galectin-3 blockade on inhibition of invasion and triggering of apoptosis by SRPP also has been highlighted. Current chapter addresses where such biochemically potent, *in vitro* active antioxidants and antimetastatic polysaccharide can work effectively also *in vitro* in appropriate model systems



SRAE – Swallow root aqueous extract; **BCAE** – Black cumin aqueous extract;
SRPP – Swallow root pectic polysaccharide

5.2. Work concept

5.3. Introduction

Reactive oxygen species generated by various sources are known to damage cells, initiate cancer including various other chronic diseases. Cancer however as highlighted earlier appear to be one of the most complex disease to devise a strategy for cure and hence is life threatening. As a result there is a constant raise in cancer incidence as per cancer statistics. Our earlier chapters enabled us to explore the anticancer potential in selected spices – Black cumin and Swallow root since they showed multi-potent antioxidant activities with effective inhibition of lipid peroxidation, cellular oxidation, DNA oxidation/damage, protein oxidation etc (Chapter 2). Results indicated that aqueous extracts – BCAE and SRAE possessed potent phenolic acids contributing to the multi-potent antioxidant activity. Despite several knowledge and application of antioxidants against cancer, due to their diverse effect, search is constantly on by various investigators for potent antioxidants particularly from dietary/spice sources. Innumerous amount of literature provides the cancer preventive role of flavonoids and hydrophobic antioxidant principles such as quercetin, curcumin, capsaicin etc. Our laboratory since continuously showed major contribution of antioxidant activity particularly in aqueous extracts from phenolic acids, and showed that these can attribute to health beneficial properties as highlighted in chapter – 2, in the current chapter to substantiate their further application, we examined the *in vivo* efficacy using accepted animal models. Role of spices / dietary antioxidants are still the choice of today since they are non-toxic and is partly supported with epidemiological data. In addition, we have found a novel pectic polysaccharide in black cumin and swallow root, but later being the best in inhibiting one of the most deleterious step in cancer – cancer spread / metastasis as dealt in chapter 3. Current chapter also envisages the determination of *in vivo* efficacy of the potent swallow root pectic polysaccharide (SRPP) in delaying or preventing metastasis. In order to study the *in vivo* efficacy of both antioxidants and anti-metastatic compounds that are essential to be anticancer, following models were selected –Effect of 1. antioxidants against lipid peroxidation caused hepatotoxicity by CCl_4; 2. against oxidative stress induced by 20 - methyl cholanthrene, a carcinogen that causes cervix cancer and 3. against B16F10 mouse melanoma cell induced lung

metastasis in Wistar rats and albino mice respectively. Macroscopic, microscopic, immuno/biochemical/enzyme assays and disease apoptotic markers and histopathological analysis were performed to understand the potential *in vivo* effect of antioxidants and antimetastatic compounds from selected sources in selected models.

5.4. Materials and Methods

5.4.1. Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), Folin-Ciocalteau reagent, 2-thiobarbituric acid (TBA), hydroxylamine hydrochloride, hydrogen peroxide, Nitro blue tetrazolium (NBT), 2-thiobarbituric acid (TBA), hydroxylamine hydrochloride, hydrogen peroxide, glutathione reductase, NADPH, reduced glutathione (GSH), 5, 5'- dithionitrobenzoic acid (DTNB), butylated hydroxy toluene (BHT), 1,1,3,3 tetramethoxypropane (TMP), and Tris-HCl, 20-methylcholanthrene, gallic acid, Dulbecco's modified eagle's medium (DMEM), glutamine, sodium bicarbonate, penicillin, kanamycin, FBS (fetal bovine serum), MTT (3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2-H-tetrazolium bromide), skimmed milk powder, paranitrophenyl phosphate, were purchased from Sigma Chemical Co. (St. Louis, MO). Twenty six and half gauge insulin syringe and monoclonal anti human galectin-3 antibody were purchased from Becton Dickinson Co., USA. Alkaline phosphatase conjugated-rabbit anti mouse IgG secondary antibodies was procured from GENEI, Bangalore, India. Other chemicals such as carbon tetrachloride, copper sulphate, sodium potassium tartarate, sodium carbonate, hexane, hydrochloric acid, trichloro acetic acid (TCA), phosphate buffer, sucrose, ethylenediaminetetraacetic acid (EDTA) and solvents used were of the analytical grade purchased from local chemical company, Sisco Research Laboratories, Mumbai, India.

5.4.2. Collection of sources and preparation

Antioxidant – rich fraction from black cumin – **BCAE**, swallow root – **SRAE**, and potent antimetastatic pectic polysaccharide from swallow root - **SRPP** were selected for *in vivo* studies. The total phenolic content in BCAE and SRAE were determined colorimetrically using Folin-Ciocalteau method (Singleton and Rossi, 1965), since antioxidants found were phenolics in nature. 2-hydroxy-4-methoxybenzaldehyde - **HMBA** was isolated from swallow root as pure crystals as per the protocol (Nagarajan et al., 2001), purity and identification of the compound was confirmed by NMR

technique as revealed in Chapter – 2. Total carbohydrate content in **SRPP** was measured by phenol sulphuric acid method, as revealed in Chapter – 3.

5.4.3. Study of the effect of BCAE and SRAE on Oxidative Stress/CCl_4 induced - Hepatotoxicity in Albino wistar Rats

5.4.3.1. Animals and treatments

Female albino rats of Wistar strain weighing 90-150 g, were selected for the study. Animal experiments were carried out upon the clearance from the CFTRI Ethics committee, at the animal house facility of CFTRI, which has been registered with CPCSEA (Reg. No. 49, 1999), Government of India, New Delhi, India. Animals were obtained from the animal house of Central Food Technological Research Institute, India and were acclimatized to the laboratory conditions in a light and temperature controlled room for 15 days before starting the experiments in the same animal house. Animals received standard diet and water throughout the study. Animals were divided into four main groups of six animals in each group.

Group I: Control, normal healthy rats - **H**; **Group II:** CCl_4 induced where animals were challenged with CCl_4 - orally (2 mL/Kg b.w.) on 16^{th} day of the experimental schedule – **CCl_4I**; **Group III :** BCAE pretreatment for 15 days followed by CCl_4 challenge (similar concentration as mentioned in Group II. Rats administered with BCAE orally 200 mg GAE phenol/Kg b.w.) for 15 days and challenged with CCl_4 on 16^{th} day – **BC**; **Group IV:** SRAE pretreatment (200 mg GAE phenol/Kg b.w.) for 15 days followed by CCl_4 treatment (2 mL/kg b.w.] - **SR**. Rats administered with SRAE orally for 15 days and challenged with CCl_4 on 16^{th} day. At the end of the experimental schedule, the rats were fasted for 24 h and all the animals were sacrificed as per the procedures approved by the Institutional Ethics Committee of CFTRI, Mysore, India.

5.4.3.2. Assessment of Hepatotoxicity by CCl_4; protection by BCAE/SRAE.

5.4.3.2.1. Preparation of serum and liver homogenates

All the animals after the treatement, were anaesthesized with diethyl ether. Serum was collected from blood samples. Liver was removed and five percent liver homogenate was prepared using cold 0.15 M potassium chloride and centrifuged at 5000 *g* for 20 min at 4 °C. The supernatants obtained were collected and used for the estimation of thiobarbituric acid reactive substances (TBARS), peroxidase (POX), catalase (CAT) and total protein (Lowry et al., 1951). Five percent liver homogenate using 0.25 % sucrose in phosphate buffer (pH 7.4, 20 mM) was employed for the estimation of superoxide dismutase (SOD).

5.4.3.2.2. Assessment of hepatomarkers/antioxidant and antioxidant enzymes

To find out the antioxidant or hepatoprotective effect of the sample, the following serum marker enzymes for liver function such as serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), and alkaline phosphatases (ALP) were analyzed as per the standard protocols. In addition, the total protein content, TBARS and radical scavenging activity in the serum were also analyzed. Further, antioxidant enzymes like SOD, CAT and POX were analyzed in addition to total protein and TBARS content in liver homogenate also.

Estimation of Superoxide dismutase (SOD, EC 1.15.1.1)

The activity of SOD was assayed using nitroblue tetrazolium (NBT) as the substrate (Flohe and Otting, 1984). Briefly 0.1 mL of 5 % liver homogenate in 0.2 M sucrose in phosphate buffer (pH 7.4) or serum was taken in Beckman quartz cuvette of 1 cm path length. To this, a mixture containing 1 mL of sodium carbonate (50 mM), 0.4 mL of NBT (24 μM) and 0.2 mL of EDTA (0.1 mM) was added and the zero min reading was taken at 560 nm. The reaction was initiated by the addition of 0.4 mL of

1 mM hydroxylamine hydrochloride. The reaction mixture was then incubated at 25 °C for 5 min and the reduction of NBT was read at 560 nm. A parallel control without homogenate or serum was also run and was considered as 100 % autoxidation. The enzyme activity was expressed as unit/mg protein.

Estimation of Catalase (CAT, EC 1.11.1.6)

The activity of catalase was assayed according to the method described previously (Aebi, 1984). Briefly, 0.1 mL of liver homogenate or serum was added to 1.9 mL of phosphate buffer, pH 7.0 and absorbance was measured at 240 nm. To this 1 mL of hydrogen peroxide was added and the absorbance was measured after 1 min at 240 nm using phosphate buffer as blank solution. The activity of catalase was expressed as units/mg protein (1 unit is the amount of enzyme that utilizes 1 μ moles of hydrogen peroxide/min).

Glutathione peroxidase (POX, EC.1.11.1.9)

The activity of glutathione peroxidase was determined according to the method described (Flohe and Gunzler, 1984). The mixture containing 0.1 mL liver homogenate or serum, 0.1 mL of 10 mM glutathione reductase (0.24 U) and 0.1 mL of 10 mM GSH was preincubated for 10 min at 37 °C and, thereafter 0.1 mL of NADPH solution was added. The hydroperoxide independent consumption of NADPH was monitored for 3 min. Overall reaction was started by adding 0.1 mL of prewarmed hydroperoxide solution and the decrease in absorption at 340 nm was monitored for 3 min and the activity was expressed as n moles of NADPH oxidized/min/mg protein.

Measurement of Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (as malondialdehyde) in serum and liver homogenate were analyzed according to the method of Buge and Aust (1978). Briefly, 0.25 mL of liver homogenate or serum was mixed with 2 mL of TCA- TBA-HCl reagent (15 % TCA, 0.375 % TBA in 0.25 N HCl) containing 0.05 % BHT and heated for 15 min in boiling water bath. The solution was cooled to room temperature. The

precipitate was removed by centrifugation at 1000 *g* for 10 min at RT and the absorbance of the supernatant was measured at 532 nm. The amount of malondialdehyde was quantified using 1,1,3,3 tetramethoxypropane as standard. Antioxidant status in the serum was measured in terms of DPPH radical scavenging effect in serum samples.

5.4.3.3. Statistical analysis

All Experimental values are mean ± standard deviation (n = 6). One way ANOVA was carried out to find out the significant difference between the control and treatment groups followed by Dennett's test. A value of $p < 0.05$, 0.01 and 0.001 was considered significant (*), very significant (**) and highly significant (***). p value > 0.05 was considered non significant (^{NS}).

5.4.4. Assessment of carcinogenicity by 20-Methylcholanthrene, protection by BCAE/SRAE.

5.4.4.1. Animals/Animal treatments

Swiss albino virgin female mice, in the age group of 5-6 weeks, weighing 25-30 g were maintained in the Animal House, CFTRI (CPCSEA, Reg. No. 49, 1999). The mice were categorized into six groups, each containing six animals caged in groups of 3 per cage and kept in alternating periods of light and dark conditions of 12 h each. The animals were allowed to acclimatize to the animal house conditions for 1 week and were fed with standard rat pellet feed and water was provided *ad libitum*. Animal body weight was checked regularly and dosage of extracts were calculated based on the body weight of animals and administered orally at indicated concentration for different groups of animals as per the following experimental design. **Group I** (n = 6) - Healthy control group **(H)**: All animals were administered with normal saline once daily for 45 days; **Group II** (n = 6) – MC-induced **(MC)** where animals received MC only into the uterine cervix through the vaginal opening, at a dose of 10 mg/kg b.w. for 45 days. **Group III/Group IV** (n = 6) – BCAE - treated groups - **BC1/BC2** where animals were administered with BCAE at 100/200 mg gallic acid equivalent (GAE) phenol/kg b.w., for BC1 and BC2 respectively, once daily for 45 days along with 20-methylcholanthrene treatment; **Group V/Group V1** (n = 6) – SRAE treated groups - **SR1/SR2**: where animals were administered with SRAE at 100/200 mg GAE phenol/kg b.w., for SR1 and SR2 respectively, once daily for 45 days along with 20-methylcholanthrene treatment. Tolerance of BCAE and SRAE by the mice was determined using three groups of six mice each. The first group formed the control and the second group was administered with 100 mg GAE phenol/Kg b.w. of BCAE and SRAE extract and the third group received 200 mg GAE phenol/Kg b.w. of BCAE and SRAE orally once a day for 45 days. The body weights of the animals were recorded at 3-day intervals. During each experiment, food and water consumption was checked daily. The mice were observed for behavioral responses and mortality. At the end of the study, the animals were sacrificed and the weight of the liver, lungs, heart, thymus, spleen and kidney were determined.

5.4.4.2. Assessment of chemopreventive action of BCAE and SRAE

At the end of 45th day, all the animals were sacrificed by cervical dislocation following the procedures approved by the Institutional Ethics Committee of CFTRI, Mysore, India. Sacrificing was done swiftly to ensure minimal and negligible stress to the animals. The serum and liver samples were collected and the protein concentration was determined according to the method of Lowry et al, (1951) and assayed for antioxidant enzymes. Further, the uterus was removed, sectioned and stained with hematoxylin and eosin to detect the occurrence of neoplasia.

The liver was weighed and homogenized in chilled Tris-HCl buffer [10 mM, pH 7.4) at a concentration of 5 % (w/v), centrifuged at 5000 *g* at 4 °C for 20 min using high speed cooling centrifuge (Remi C 24, India). The clear supernatant was used for the estimation of lipid peroxidation products (TBARS as malondialdehyde - MDA), reduced glutathione (GSH), peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD)

5.4.4.2.1 Measurement of antioxidant and antioxidant enzyme

Glutathione (GSH) content of liver homogenate and serum was determined as described elsewhere (Das et al., 1997; Sedlak and Lindsay, 1968). One milliliter aliquot of homogenate was mixed with equal volume of 10 % TCA and protein precipitate was removed by centrifugation at 10,000 *g* for 20 min at RT. The supernatant was added to equal volume of 0.5 M Tris-HCl, pH 9 containing 20 mM DTNB to yield yellow chromophore of thionitrobenzoic acid (TNB), which was measured at 412 nm. GSH was used as a reference standard. Antioxidant enzymes namely, CAT, SOD, POX and lipid peroxidation products-TBARS were analyzed as per the methods described earlier (Section 5.4.3.2).

5.4.5. Assessment of Antimetastatic activity of SRPP using B16F10 mouse melanoma cells on Swiss albino mice

5.4.5.1. Growth and maintenance of B16F10

B16F10, a highly metastatic sub-line, of the murine B16 melanoma was procured from National Center for Cell Sciences, Pune, India. Cells were cultured with DMEM—high glucose (4.5 g/L) with 4 mM glutamine, buffered to pH 7.2–7.4 and supplemented with 1.5 g/L sodium bicarbonate, penicillin (100 Units/mL), kanamycin (0.1 mg/mL) and 10 % fetal calf serum at 37 °C, in a humidified chamber with 95 % air and 5 % CO_2. Cultured cell growth and survival was monitored by MTT assay [21]. Cells (2.8×10^4 cells/mL) were cultured with the specified medium in a 96 well microplate and after 72 h, 25 µL of MTT solution (5 mg/mL) was added, incubated at 37°C for 4 h. Hundred microliters of lysis buffer was added and cells were continued to incubate at 37 °C overnight (about 16 h) to dissolve the dark blue crystals and absorption of formazan solution was measured at 570 nm in a microplate reader (Spectra Max-340, Molecular Devices, Germany).

5.4.5.2. Animals and Animal treatments

Animal experiments were carried out upon the clearance from the Central Food Technological Research Institute Ethics committee, at the animal house facility of CFTRI, Mysore 570 020, India, which has been registered with CPCSEA (Reg. No. 49, 1999), Government of India, New Delhi, India. Forty eight female Swiss albino mice, 10–12 weeks old weighing between 28–36 g at the beginning were used for the experiment. Food and water was administered *ad libitum* during the study.

Each animal was injected with 5 x 10^5 cells/200 µL of phosphate buffer saline (PBS) in the lateral vein of the tail. Treatment with SRPP, SRPP+SRAE, HMBA and PBS as control solution was provided for twenty days before inoculation and for another 21 days following inoculation with B16F10 cells. Groups are as follows; **Group I:** Healthy - PBS treatment-**H**; **Group II:** Metastasis induced with B16F10 cells-**MI**;

Group III: SRPP Control - 200 mg/Kg b.w. of SRPP alone; **Group IV:** SRPP treated - 200 mg/Kg b.w, challenged with B16F10 cells-**SRPP**; **Group V:** SRPP+SRAE Control - 200 mg kg-1 b.w. SRPP + 40 mg kg-1 b.w. SRAE (Antioxidant rich) alone; **Group VI:** SRPP+SRAE – treated - 200 mg/Kg b.w. SRPP + 40 mg/Kg b.w. SRAE challenged with cells-**SRPP+SRAE**; **Group VII:** HMBA control, 10 mg/Kg b.w. HMBA alone; **Group VIII:** HMBA- treated - 10 mg/Kg b.w. HMBA treatment challenged with cells-**HMBA**. Control groups - Group III, V and VII were used to study the toxicity effect of compounds under investigation. At the end of the experiments animals were sacrificed by cervical dislocation; serum was collected and analyzed for biochemical parameters. Lungs were excised and the metastatic nodules were quantitatively evaluated by two observers using the following models: In addition tumors if appeared in tail, the site of injection or elsewhere in the body are noted since it indicates the extent of metastatic spread.

Model 1: macroscopic study by stereoscopic magnifying glass (Olympus), counting the metastatic nodules of the pleural surface of the five lobules; **Model 2**: quantitative analysis at microscopic level of five sections of each lobule. Images at described magnification were captured by inverted microscope attached to an image analyzer (Leica DMLS model, Germany), with which the study regions were interactively selected. Following Martınez et al. [2005], the initial parameters evaluated were: the area of each complete lobule was obtained by capturing the image at 21x magnifications. The area of the metastases within each lobule and their maximum and minimum diameters were obtained by capturing the image at 55x magnifications and the following parameters were calculated:

(1) **Percentage of implantation** (area of metastasis per lobule/total area X 100)

(2) **Growth index** (mean area of metastasis/total area) and

(3) **Invasion index** (area of metastasis per lobule/mean area of metastasis).

5.4.5.3. Biochemical analysis in the serum and lung tissue

Serum of all test groups were analyzed for total protein (Lowry et al., 1951), antioxidant enzymes such as SOD (*EC 1.15.1.1*) CAT (*EC 1.11.1.6*) and antioxidant GSH employing protocols described earlier (Section 5.4.3.2). Enzyme activity for SOD and CAT are expressed as units U/mg protein. To evaluate the toxic effects of SRPP components, serum of control groups were analyzed for total protein and liver function enzymes like serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) as per the standard protocols.

5.4.5.4. Preparation of lung homogenates

Lung tissue was excised from the mice and five percent liver homogenate was prepared using cold 0.15 M potassium chloride and centrifuged at 5000 *g* for 20 min at 4 °C. The supernatants obtained were collected and used for the estimation of galectin-3 and melanin content.

5.4.5.5. Determination of galectin-3/and melanin in Lung homogenate

Galectin-3 has been reported earlier that it is a metastatic marker (Nangia-Makker et al., 2000; Sathisha et al., 2007). ELISA was performed for the detection of galectin-3 levels in the lung tissue homogenate employing the protocol standardized previously (Rajeshwari et al., 1998). Monoclonal anti human galectin-3 antibody was employed at dilutions 1: 1,000 as primary antibody. Alkaline phosphatase conjugated rabbit anti mouse IgG at 1:5,000 dilution followed by paranitrophenylphosphate were used as secondary antibody and substrate, respectively. The absorbance was measured at 405 nm in a microplate ELISA reader (Molecular Devices, Spectramax 340, Germany).

The melanin content of lung tissue homogenates were measured according to the method of Oka et al. (1996) with slight modification. The liver homogenate is solubilized in boiling 1 M NaOH for 10 min and the spectrophotometric analysis of melanin content was performed at an absorbance of 400 nm. The entire experiment was performed in triplicate, and results were confirmed by three independent

experiments.

5.4.5.6. Histopathology

The lung tissue of mice were removed carefully, the weights recorded, fixed in 10 % formalin in PBS, and then embedded in paraffin. The paraffin embedded tissue blocks were sectioned using a microtome and the thickness were set between 6-8 microns, the tissue sections were fixed on to the microscopic slide and stained with hematoxylin and eosin as per the standard protocol (Thompson and Samuel, 1966)

5.4.5.7. Statistical analysis

All results are expressed as mean ± standard deviation (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control mean. A value of $p < 0.05$, $p < 0.01$, $p < 0.001$ and was considered significant, very significant, and highly significant, respectively. Statistical evaluation of the data was done using SPSS version 10.0 for Windows.

5.5. Results

5.5.1 Effect of antioxidants in aqueous extracts of black cumin and swallow root on oxidative stress induced hepatotoxicity by CCl_4 in albino rat model.

Carbon tetrachloride (CCl_4) induced liver damage, and its protection by swallow root and black cumin-aqueous extract were carried out on experimental Albino Wistar rats. The serum analysis for total protein content indicated no significant difference between healthy, CCl_4 induced and spices extract treated groups (Figure 5.1D).

Effect on Liver function enzymes

The liver function enzymes namely SGPT, SGOT and ALP were analyzed in serum. ---- to ---- fold increase in the activity observed in CCl_4 induced – stressed rats when compared with those of healthy rats. Both marginal and significantly altered levels of SGOT (1.4 fold increase) and SGPT (4.1 fold increase) [Figure 5.1A & C] were observed in CCl_4 treated group respectively. BCAE and SRAE could protect up to ~39 % and 72 % respectively indicating that SRAE at equal phenol concentration yielded ~ 2 fold better activity than BCAE. ALP levels among all the animal groups remained unaffected.

Biochemical changes in the serum and liver

TBARS levels (Figure 5.1F) in CCl_4 induced rats indicated 2.1-fold increase compared to healthy rats. TBARS levels although not improved upon treatment with BCAE, 62 % protection was observed with SRAE. Free radical scavenging activity (Figure 5.1E) in serum indicated 2.7-fold deprivation in CCl_4 treated groups while improved with > 80 % upon treatment with our selected sources - BCAE and SRAE.

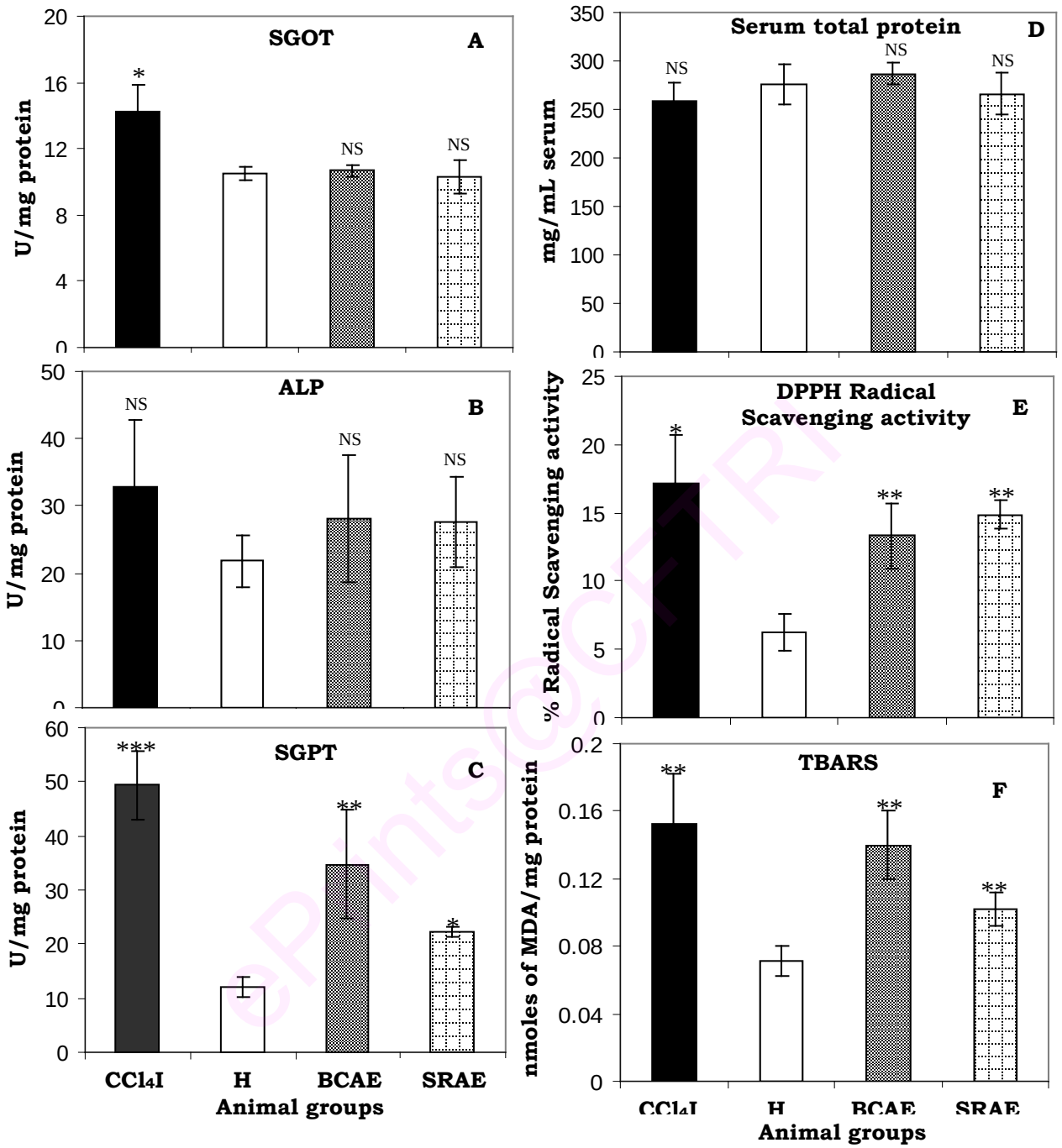


Figure 5.1. Serum analysis for liver function enzymes, protein, DPPH radical scavenging activity and TBARS levels. Values are expressed as mean ± SD (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control (healthy) mean.* Significant ($p< 0.05$); ** very Significant ($p< 0.01$); *** Highly Significant ($p< 0.001$) and ^{NS} – Non Significant.

In the liver although there were no significant alterations in total protein in the tissue, 2 fold increase of TBARS levels observed was normalized with the extracts (Figure 5.2A & B). All the antioxidant enzymes namely SOD, CAT and POX were significantly depleted in CCl_4 induced rats compared to healthy controls (Figure 5.3A-C). The spice extracts treated groups showed significant recovery of these enzymes to varying extent. BCAE treated groups showed 58, 50 and 58 % recovery of CAT, SOD and POX enzymes, respectively. SRAE treated groups showed 59, 62 and 58 % recovery of CAT, SOD and POX enzymes, respectively.

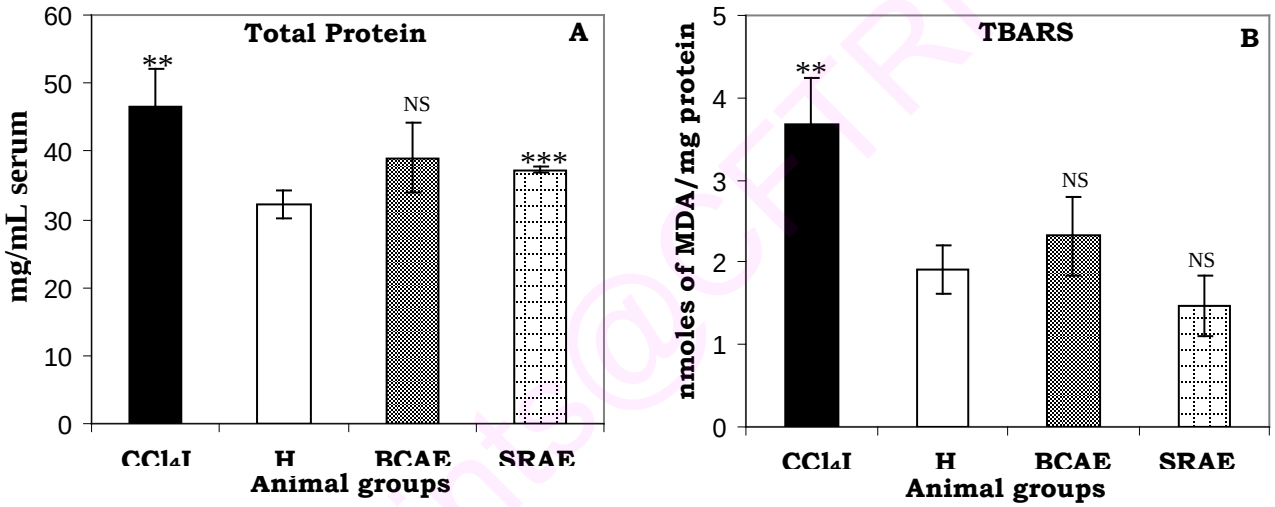


Figure 5.2. Biochemical analysis of liver homogenate. Values are expressed as mean ± SD (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control (healthy) mean * Significant ($p< 0.05$); ** very Significant ($p< 0.01$); *** Highly Significant ($p< 0.001$) and ^{NS} – Non Significant.

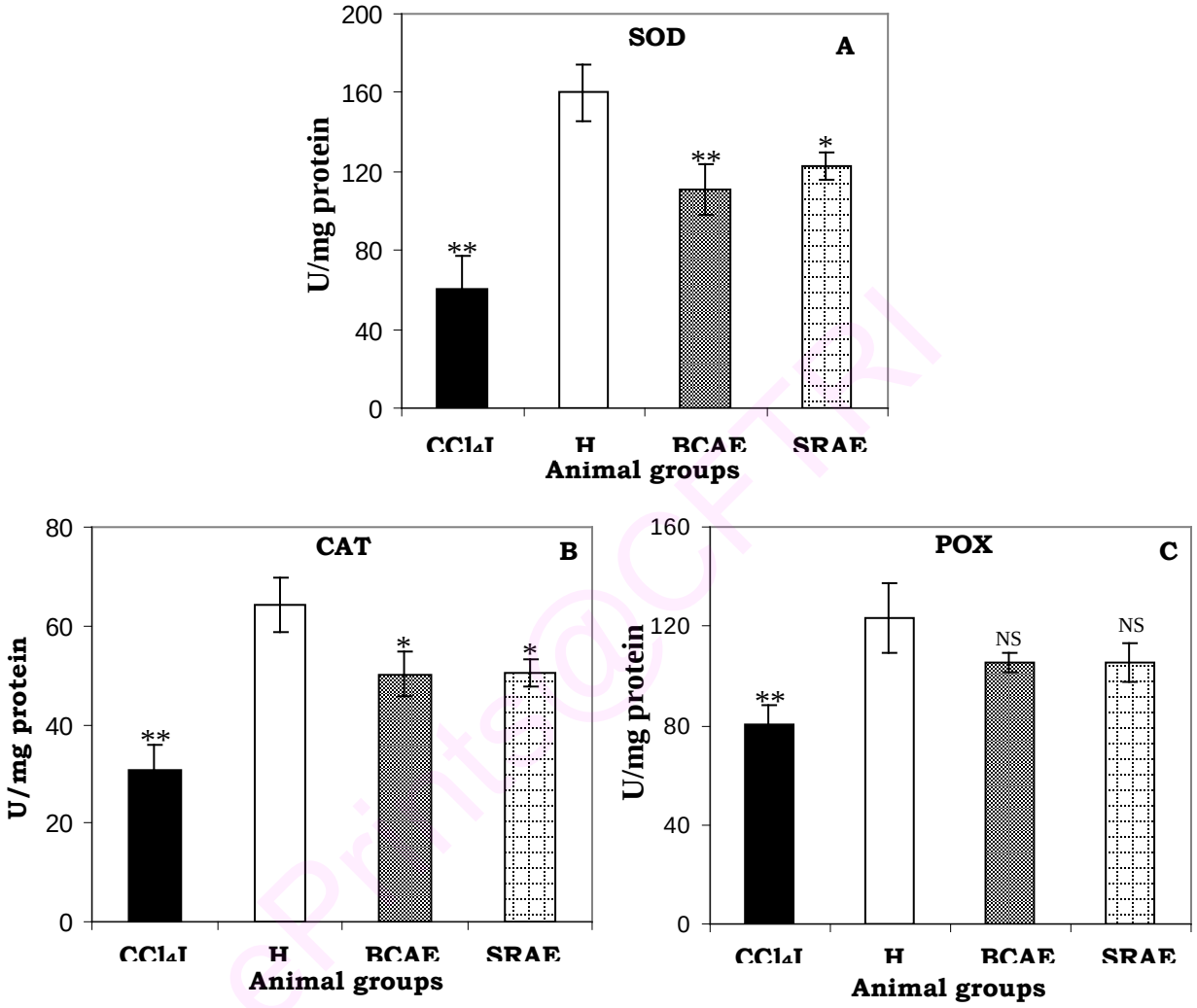
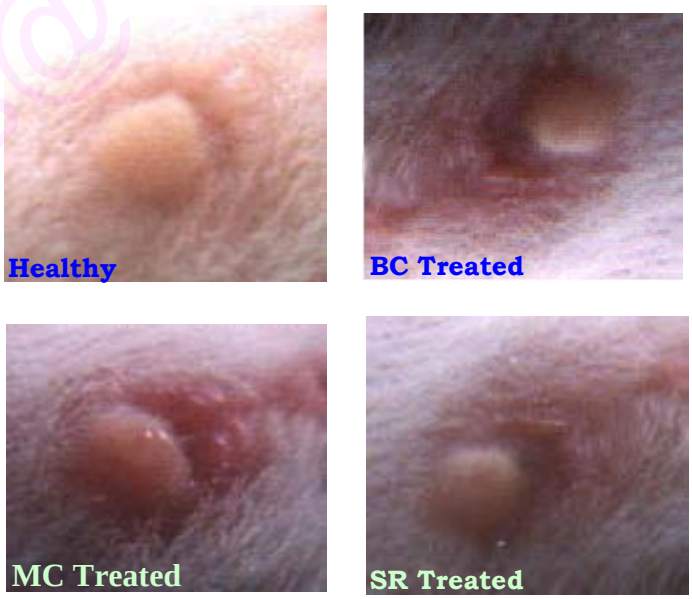


Figure 5.3. Biochemical analysis of liver homogenate. Values are expressed as mean ± SD (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control (healthy) mean * Significant ($p< 0.05$); ** very Significant ($p< 0.01$); *** Highly Significant ($p< 0.001$) and ^{NS} – Non Significant.

5.5.2. Effect of Antioxidants in aqueous extracts of black cumin and swallow root on 20-methylcholanthrene induced cervical cancer in albino mice model.

Severe inflammation and swelling due to cancer induction was observed near the vaginal surface of MC treated animals (Figure 5.4B]; while upon treatment with extracts normalization was observed, similar to that of the untreated control upon treatment with SRAE. BACE showed only a marginal effect. Histological results in figure 5.4A of uterine cervix showed moderate to severe dysplasia in MC administered group. Following treatment with 100 mg GAE phenol/Kg b.w, of BCAE/SRAE, mild dysplasia was observed in only 20 % of the population, while there was no evidence of dysplasia in the remaining 80 % of animals. Administration of extract at 200 mg GAE phenol/Kg b.w showed changes which were comparable to that of normal healthy mice suggesting the dose dependent anticancer effect by antioxidants of BC and SR.

Groups	Histopathological observation
Healthy	Normal
Positive control	Dysplasia
SR1 treatment	Mild Dysplasia
SR2 treatment	Normal
BC1 treatment	Mild Dysplasia
BC2 treatment	Normal



(A) **(B)**

Figure 5.4. (A) Histopathological observation of cervix of mice induced for cervical carcinogenesis. **(B)** Morphological changes of vaginal opening of experimental mice after 45th day of treatment with BC & SR aqueous extract (BC2 and SR2; 200 mg GAE phenol/Kg b.w.

5.5.2.1. Antioxidant and Antioxidant enzyme status in the liver and serum

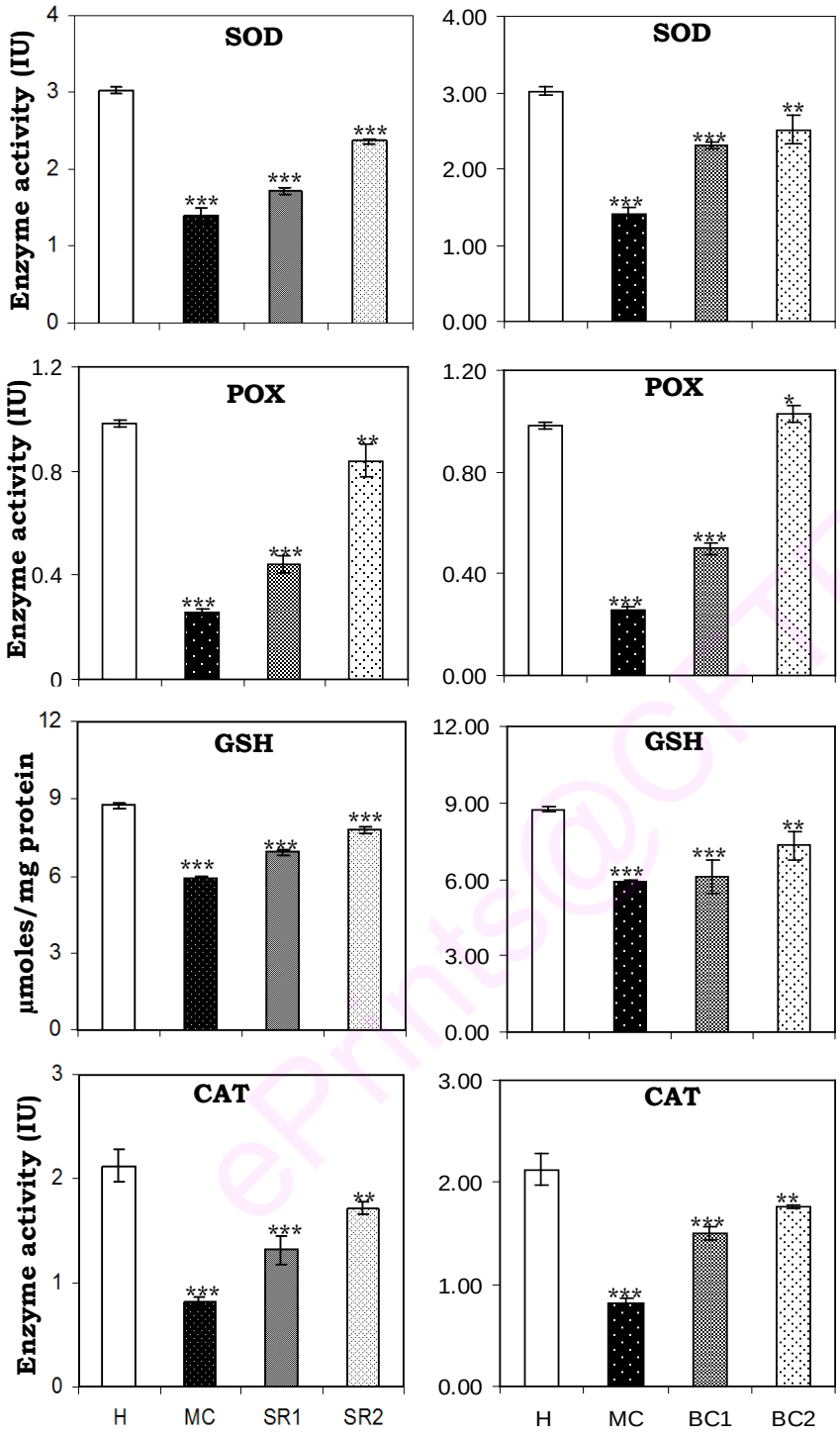
Antioxidant – GSH and Antioxidant enzymes like superoxide dismutase, catalase and peroxidase levels were measured in both liver homogenate and serum of healthy, MC-induced cancerous animals and also groups of animals treated with BCAE and SRAE in different concentrations – 100 and 200 mg GAE phenol/Kg b.w. Data presented in Table 5.1 reveal that there was no significant alteration in SOD, while 2.1 to 3.4 fold depletion was observed in catalase and peroxidase in cancerous animals when compared to those of healthy animals. However upon treatment with SRAE/BCAE ~ 50 – 80 %protection was observed in a dose dependent manner, both in the liver homogenate (Table 5.1] and Serum [Figure 5.5]. Approximately 2 fold reduction in GSH levels in the MC treated animal groups was also normalized upon treatment with the selected extracts – BCAE and SRAE.

Data thus may suggest that despite differences in phenolic content and phenolic acid composition, and although *in vitro* efficacy of SRAE may be 4 fold higher than BCAE as revealed in Chapter – 1, *in vivo* efficiency of antioxidant rich aqueous extracts of black cumin and swallow root are more or less the same. This could be due to the presence of a major amount of tannic acid in BCAE, while HMBA in SRAE where TA is a potent antioxidant - fold better than HMBA with 50 % inhibition of free radical scavenging activity [IC_{50} of 1.15 µg/mL as apposed to that of HMBA, which has an IC_{50} of 213 µg/mL. However other phenolic acids such as gentisic and gallic acid which are good antioxidants with an IC_{50} of free radical scavenging activity of 3 and 1.1 µg/mL respectively, also contribute to SRAE activity.

Table 5.1. Effect of black cumin and swallow root aqueous extract on liver antioxidant enzymes in 20-methylcholanthrene induced carcinogenesis in the uterine cervix of Swiss albino mice.

Groups	**CAT**	**SOD**	**POX**	**GSH**	**TBARS**
I Healthy	3.05 ± 0.21	42.18 ± 0.391	1.17 ± 0.24	22.73 ± 2.73	0.794 ± 0.031
II MC-Induced	1.39 ± 0.16***	39.84 ± 0.78^{NS}	0.34 ± 0.03***	11.82 ± 0.61***	0.988 ± 0.099**
III SR1 treated	1.72 ± 0.12***	44.53 ± 3.52 ^{NS}	0.8 ± 0.10***	13.18 ± 0.76***	0.914 ± 0.049*
IV SR2 treated	2.39 ± 0.16***	51.95 ± 0.78***	1.39 ± 0.19**	18.79 ±1.36***	0.875 ± 0.036**
V BC1 treated	2.07 ± 0.48***	35.05 ± 0.38**	0.50 ± 0.04***	16.26 ± 0.16***	0.851 ± 0.025**
VI BC2 treated	2.39 ± 0.42***	38.08 ± 0.31*	0.77 ± 0.02***	18.44 ± 0.17***	0.843 ± 0.048**

Values are means ± SEM (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control (healthy) mean * Significant (p< 0.05); ** very Significant (p< 0.01); *** Highly Significant (p< 0.01) and ^{NS} – Non Significant.



H: Healthy

MC: Methylcholanthrene treated group;

BC1: Treated with BCAE, 100 mg GAE phenol/kg b.w.

BC2: Treated with BCAE, 200 mg GAE phenol/kg bw;

SR1: Treated with SRAE, 100 mg GAE phenol/kg bw;

SR2: Treated with SRAE, 200 mg GAE phenol/kg bw;

CAT: Catalase;

POX: Peroxidase;

SOD: Superoxide dismutase

GSH: Reduced glutathione.

Figure 5.5. Effect of black cumin and swallow root aqueous extract on serum antioxidant enzymes in 20-methylcholanthrene induced carcinogenesis in the uterine cervix of Swiss albino mice. Values are means ± SEM (n = 6). The analysis utilized student's *t*-test to test each treatment group mean against control (healthy) mean * Significant ($p< 0.05$); ** very Significant ($p< 0.01$); *** Highly Significant ($p< 0.01$) and ^{NS} – Non Significant.

5.5.3. Effect of antioxidants of swallow root-HMBA and galectin-3 inhibitory polysaccharides-SRPP on B16F10 mouse melanoma cells induced metastasis

5.5.3.1. Macroscopic study

Inoculation of B16F10 cells in the lateral tail vein resulted in the formation of metastatic nodules in the lungs (Figure 5.6A). In addition, there were local tumors at the site of injection on the tail (Figure 5.6B). Marked differences in the tumor growth were observed on the tail of SRPP and SRPP+SRAE treated mice compared to the metastasis induced mice which were injected with only metastatic B16F10 melanoma cells without any treatment. There was no observable difference in the tumor growth on the tail in HMBA treated mice. When compared to that of metastasis induced mice. Further, we measured the quantifiable metastatic nodules in the lungs that indicate the successful harboring of metastatic cells in the lung. A nodular structure with blackish coloration that could be distinguished on the lung surface and which were sufficiently separated from each other to be counted individually, and present in several numbers in metastatic mouse and totally absent in healthy lungs of healthy group of animals also suggested the extent of tumor harboring in the lung for survival, to seek more oxygen. The count made of these superficial metastatic nodules is depicted in Figure 5.7. The group (II) injected with only B16F10 cells which served as a metastasis induced group showed metastatic nodules between 176 and 438 randomly distributed over the lung surface with a mean of 328.17 ± 86.04. Group IV treated with SRPP at 200 mg/kg b.w. showed a mean of 40.67 ± 6.05, which represents a significant ($p = 0.0004$) reduction of 88% metastatic nodules compared to that of metastasis induced group. Group VI treated with a combination of SRPP and SRAE at a dosage of 200 mg/kg b.w. of SRPP and 40 mg/kg b.w. of SRAE showed a mean of 108.17 ± 7.44 indicating significant ($p = 0.0011$) reduction of 67% than that of positive control. However, Group VIII treated with HMBA at 10 mg/kg b.w. showed no significant ($p = 0.8229$) difference in the superficial metastatic nodules (315.17 ± 91.12) formation compared to that of metastasis induced group, group - II.

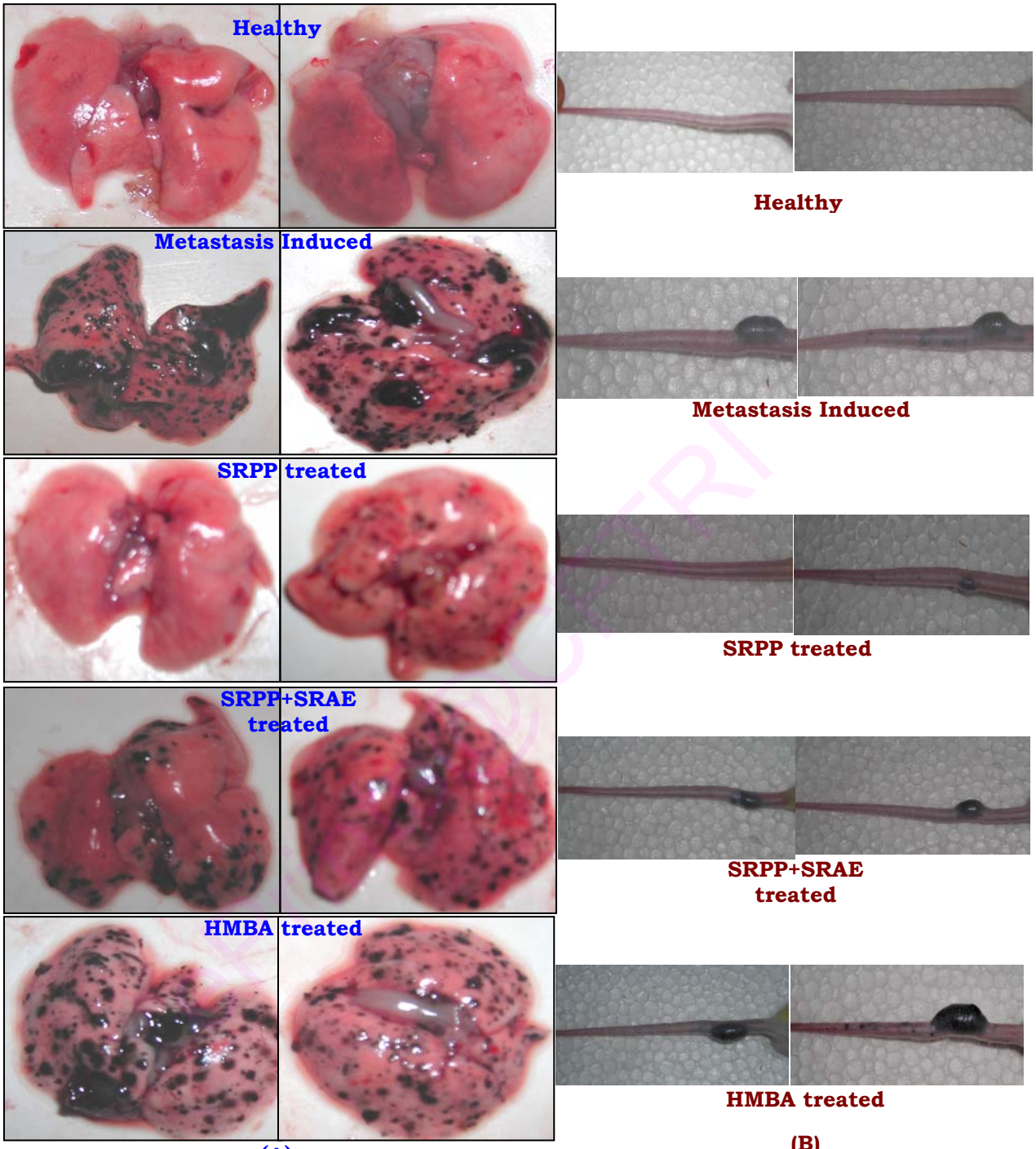


Figure 5.6. Macroscopic characteristics of the pulmonary metastatic nodules (A) and local tumor growth on the tail at the site of B16F10 cells injection (B).

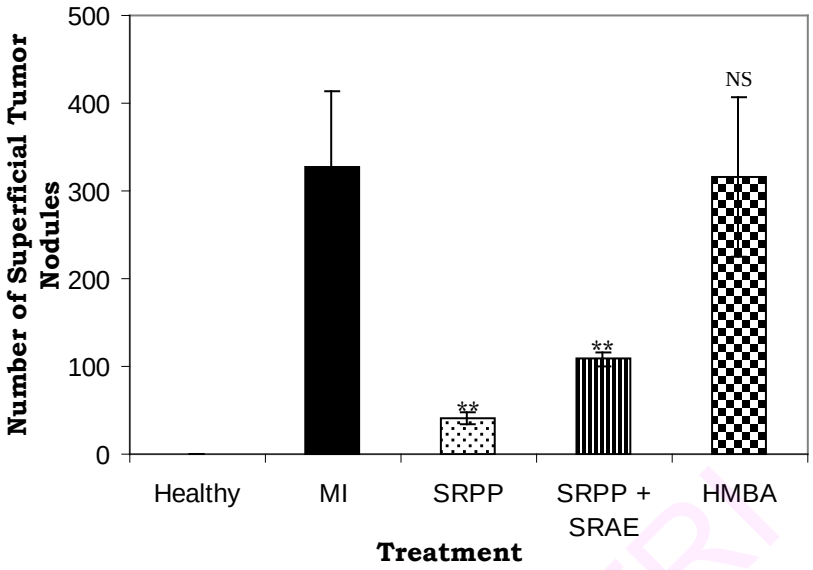


Figure 5.7. Frequency of the pulmonary metastatic nodules of experimental animal groups. MI – metastasis induced; SRPP- swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA – 2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{NS} Non-significant, represent significant changes relative to positive control.

5.5.3.2. Microscopic study

The localization of the metastastic colonies varied widely and was greater in number at the subpleural level Figure 5.8. In the intra-parenchymatose region, they occurred basically around the capillary vessels and veins of the bronchioles and bronchi and were of greater volume than their subpleural metastatic nodules. Morphologically, they were constituted by solid accumulations of neoplastic melanocytes, and in the largest nodules frequently showed small areas of necrosis localized in the central areas with frequent inflammatory infiltrates around the periphery. The melanin pigment was observed as a blackish-brown colored accumulation.

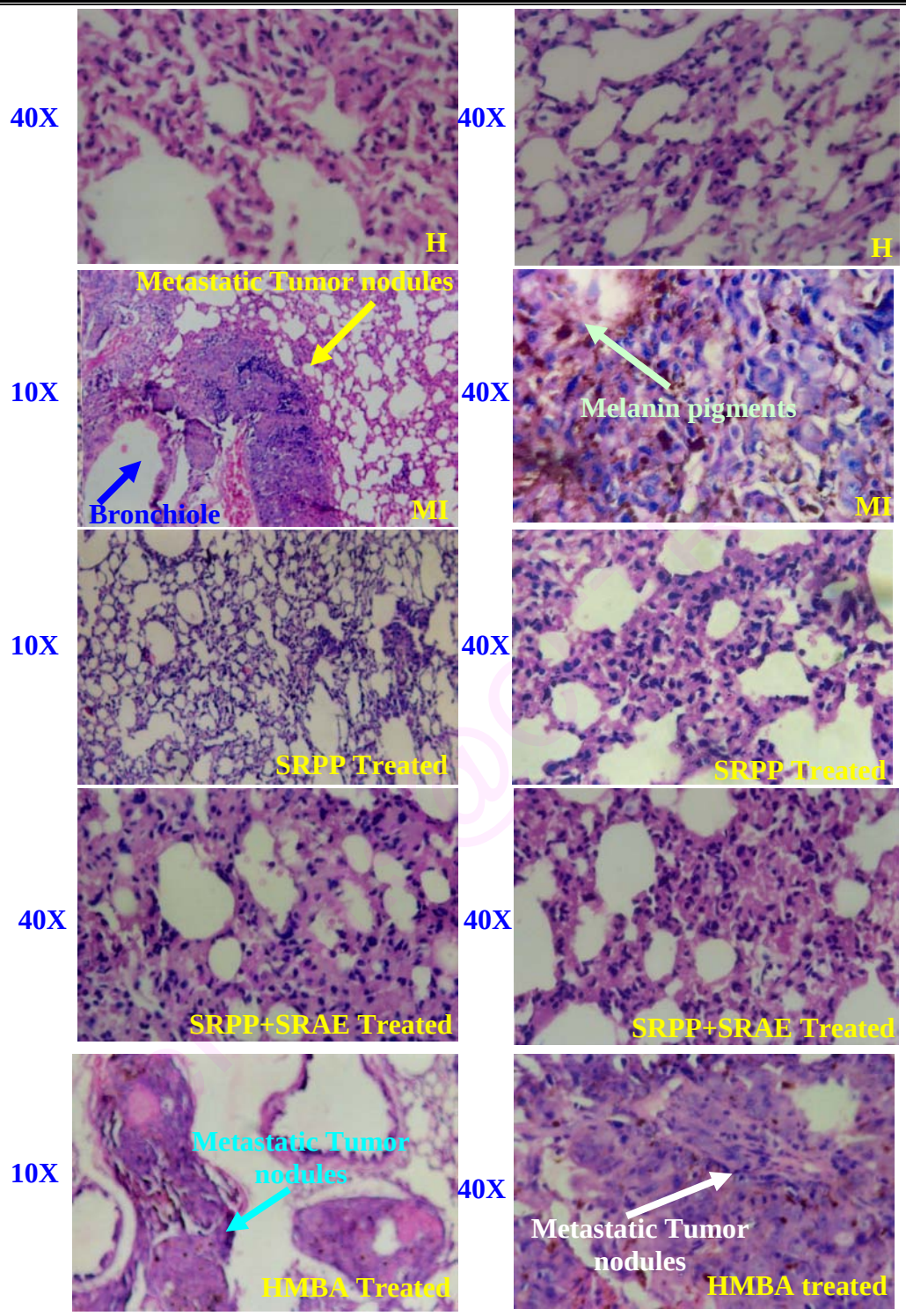


Figure 5.8. Histopathological photographs of healthy (H), and metastasis induced (MI), SRPP, SRPP + SRAE treated and HMBA treated lungs (Swiss Albino Mice)

5.5.3.3. Percentage of implantation

The results (Figure 5.9A) indicated an implantation percentage of 24.54 ± 7.08 for metastasis induced group (group II). Group IV showed 87 % reduction in the number of implants in the lung parenchyma, while 79 % reduction was observed in-group VI. A mean implantation percentage of 3.08 ± 1.38 and 4.98 ± 1.18 were observed for group IV and VI, respectively. The mean implantation percentage of both group IV and VI were significantly (IV – p = 0.0011, VI – p = 0.0013) different compared to metastasis induced group. Group VIII showed a mean invasion level of 20 % with statistically insignificant (p = 0.4599) reduction of implants (15.2 %).

5.5.3.4. Growth index

Figure 5.9B shows the growth index of various groups of mice studied for antimetastatic activity. Group II (metastasis induced group) showed a growth index of 0.0025 ± 0.0007, and Groups IV and VI had a growth index of 0.0009 ± 0.0004, 0.001 ± 0.0003, respectively. A statistically significant reduction in the growth index was also evident for groups IV (p = 0.0062) and VI (p = 0.0043). In HMBA treated group (VIII), there was no significant (0.0611) reduction in growth index (0.0015 ± 0.0004) observed.

5.5.3.5. Invasion index

The metastasis induced group (II) showed a mean invasion index of 97.45 ± 8.44, while Group IV and VI showed significantly (p < 0.001) reduced invasive index of 14.65 ± 3.13 and 32.72 ± 2.75, respectively (Figure 5.9C). Group VIII also showed a significant difference in the invasion index compared to the positive control with a higher invasion index value of 136.06 ± 10.40 indicating increased invasion of B16F10 melanoma cells to the lungs.

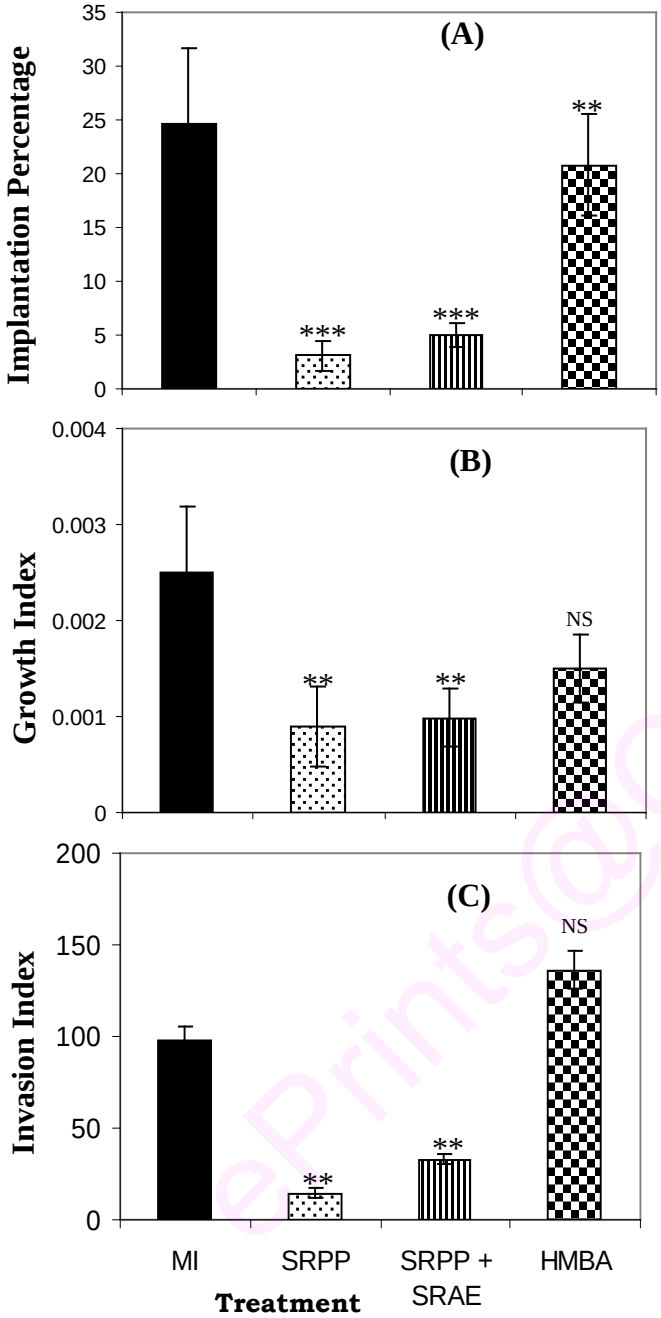


Figure 5.9. Implantation percentage (A), growth index (B) invasion index (C) and melanin levels (D) of pulmonary metastasis induced experimental mice. MI – metastasis induced; SRPP - swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA–2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{NS} Non-significant, represent significant changes relative to MI.

5.5.3.6. Galectin-3 levels in lung tissue

Galectin-3 as indicated earlier is a metastatic marker. When levels were determined by galectin-3 specific monoclonal antibody 2.5 fold increase in galectin-3 was observed (Figure 5.10A) in metastasis induced animals when compared to that of healthy lungs. Data thus may substantiate the results of tumor/invasion index. HMBA which did not reduce tumor index showed no alteration in the galectin-3 levels. Results thus support the role of galectin-3 in metastasis.

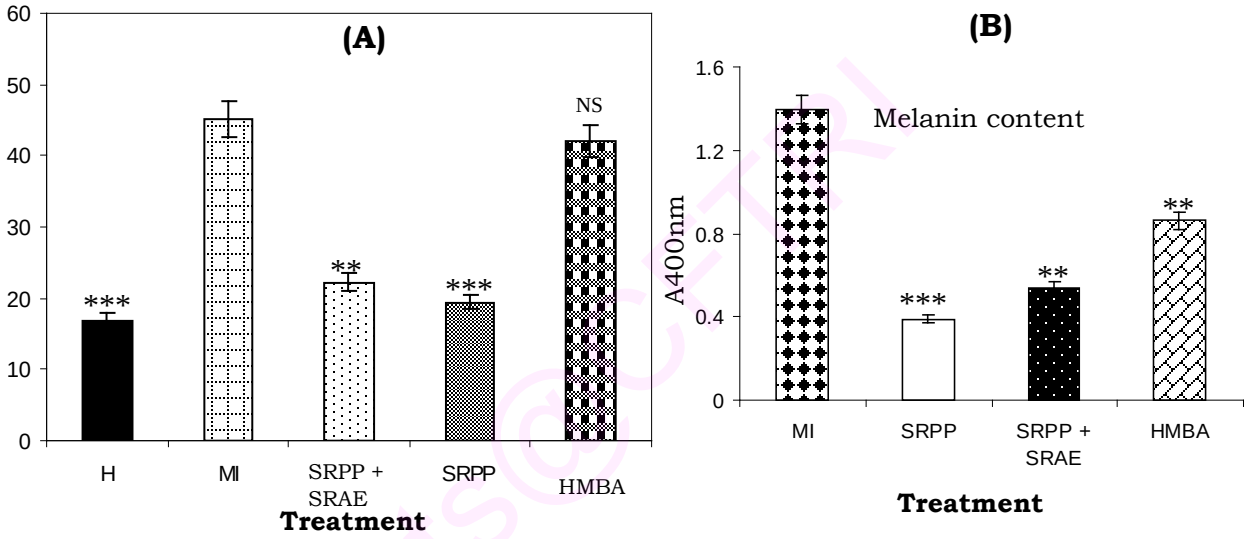


Figure 5.10. Lung galectin-3 (A) and melanin (B) levels in pulmonary metastasis induced experimental mice. MI – metastasis induced; SRPP – swallow root pectic polysaccharide treated group; SRPP + SRAE - swallow root pectic polysaccharide and aqueous extract treated group; HMBA – 2-hydroxy-4-methoxybenzaldehyde treated group. Values are expressed as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{NS} Non-significant, represent significant changes relative to MI.

5.5.3.7. Melanin content in lungs

Melanin is a chromogenic molecule produced specifically by melanoma cells. In this case it is from B16F10 mouse melanoma cells which were harbored in the metastatic lung, at very high levels. Healthy animal notably did not show melanin levels. Melanin content hence indicates the concentration of melanoma cells established in the lung. Data substantiates the results observed in histopathology analysis (Figure

5.10B).

5.5.3.6. Serum analysis for protein, antioxidant and antioxidant enzymes

Serum analysis (Table 5.2) indicated a total protein of 12.11 mg/mL in positive control group (II). Group IV and VIII showed significantly higher total protein content of 21.48 ± 2.18 and 17.45 ± 1.64 mg/mL, respectively. The total protein content in group I and group VI was not significantly different from that of positive control group.

Antioxidant enzyme SOD was depleted in metastasis induced group (1.86 ± 0.334) compared to healthy animal group (3.32 ± 0.129). Significant recovery of enzyme activity was observed in group IV (60 %), group VI (37 %) mice, while no recovery of enzyme activity (non-significant) was observed in HMBA treated animals (VIII). There was no significant difference observed in CAT level in healthy, metastasis induced group and SRPP+SRAE treated animals. However, increased (32.78 ± 2.72) level of enzyme activity was observed in group IV animals, while significant reduction (7.78 ± 2.52) was observed in group VIII animals. Around 18 % reduction was observed in GSH antioxidant in the metastasis induced group, while no difference was observed in group VI and VIII animals. The GSH level in group IV was almost similar to that of healthy group.

Results of toxicity studies indicated a gradual increase in the body weight of mice in control group III, V and VII similar to healthy animals (Fig. 5.11A). In addition, higher amounts of total protein (non-significant) compared to healthy group (I) was also observed in all the control groups. Liver function enzyme SGPT in all the three groups (III, V and VII) were almost similar to healthy animals. The SGOT levels were significantly ($p < 0.05$) lowered in all the three control groups.

Table 5.2. Analysis of serum protein, antioxidant and antioxidant enzymes.

	Healthy	**Metastasis induced**	**SRPP**	**SRPP+ SRAE**	**HMBA**
Total Protein (mg/mL)	13.96 ± 4.14 ^{NS}	12.11 ± 1.35	21.48 ± 2.18 ***	15.2 ± 3.49 ^{NS}	17.45 ± 1.64 ***
SOD (U/mg protein)	3.32 ± 0.129***	1.86 ± 0.33	2.73 ± 0.38**	2.40 ± 0.23*	2.01 ± 0.40^{NS}
CAT (U/mg protein)	16.35 ± 3.64 ^{NS}	13.72 ± 4.89	32.78 ± 2.72***	10.87 ± 3.46 ^{NS}	7.78 ± 2.52**
GSH (mg/mg protein)	2.20 ± 0.16*	1.81 ± 0.23	2.23 ± 0.26*	1.73 ± 0.55^{NS}	1.60 ± 0.49^{NS}

Values are expressed as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{NS} Non-significant, represent significant changes relative to metastasis induced group

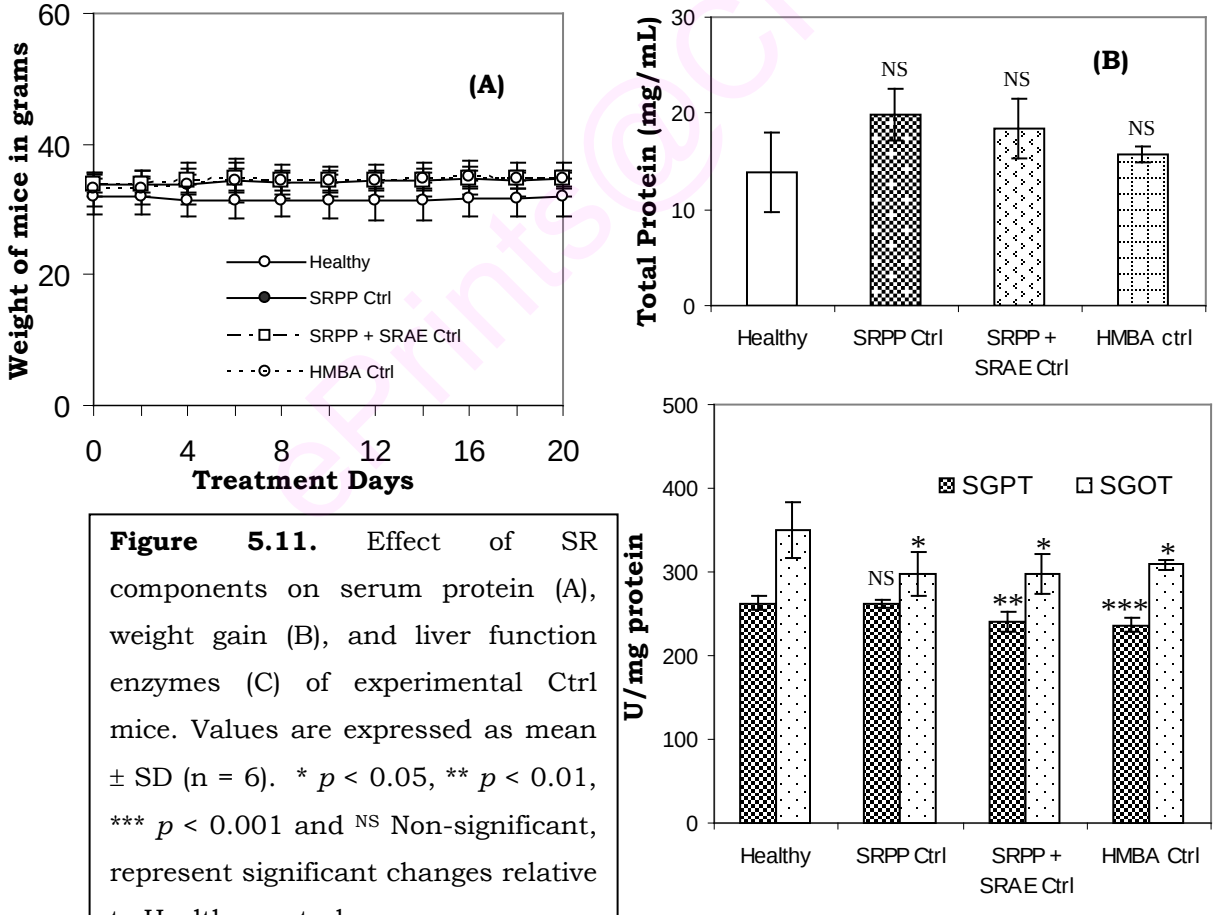


Figure 5.11. Effect of SR components on serum protein (A), weight gain (B), and liver function enzymes (C) of experimental Ctrl mice. Values are expressed as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ^{NS} Non-significant, represent significant changes relative to Healthy control.

5.6. Discussion

Cancer is a complex and multi-step mediated disease as revealed in earlier chapters and poses challenge in the management and cure. Despite several chemotherapeutic drugs available, since it causes toxicity also to the normal cells, it has its own limitations. Unfortunately due to immortal nature of cancer cells and their destined committments to retain in the body resulted in execution of several metabolic pathways of the host leading to uncontrolled all physiological functions in the body irrespective of the type of cancer and finally causing difficulty for the survival of the affected person. Alternatives are hence essential to manage cancer patients, particularly in the advanced stages to overcome their sufferings. In this direction although several antioxidants have been shown to work potentially, they had their limitations in offering protection to host cell. Accumulated countless literature and our own results indicate that dietary/spices antioxidants can be good preventive agents than curative in nature. Mechanism of understanding of cancer spread from various workers and our laboratory is throwing some of ray of hopes to design metastatic blockers so that the life and regular lifestyle of the patient can be maintained. Extensive and commendable work from Dr. Raz's group on identification of citrus pectin as a potential blocker for metastasis as evidenced by the inhibition of prostate cancer upon oral feeding of citrus pectin. Since dietary sources are potentially rich in antioxidants and pectic polysaccharides, in thesis, we examined the role of potent phenolic antioxidants and galectin-3 inhibitory polysaccharides from black cumin and swallow root. In this chapter we evaluated the *in vivo* efficacy of antioxidants in CCl_4-induced hepatotoxicity and 20-methylcholanthrene induced cervix models, where in both the cases the effect is due to the generation of oxidative stress. *In vivo* efficacy of the potential antimetastatic component from swallow root has been examined using widely accepted – B16F10 melanoma cells induced lung metastasis in Swiss mice.

5.6.1. In vivo effect of BCAE and SRAE against oxidative stress – mediated CCl_4 induced hepatotoxicity

Carbon tetrachloride – CCl_4 is known to produce free radicals, which damages hepatocytes. Damage of the liver can be due to 1. increased production of ROS that are generated to eliminate affected liver cells; 2. depletion of antioxidant – GSH and antioxidant enzymes. This imbalance in the antioxidant and oxidative stress status result in oxidation of lipids hence lipid peroxidation of the affected tissue – hepatocytes causing liver injury. The damage to liver can be measured by the elevated levels of SGPT, SGOT and ALP enzymes. In our experimental study we did notice elevated levels of SGPT and SGOT with the exception of ALP indicating liver injury. The spice extracts treated groups had significantly lowered SGPT and SGOT levels compared to CCl_4 induced groups indicating their protective effect. The antioxidants present in the serum were also evaluated using a stable free radical 1, 1, diphenyl-2-picryl hydrazyl. Compared to the healthy rats, the antioxidant activity in the serum of CCl_4 induced and spice extract treated groups were higher with no significant difference between them. Thus CCl_4 may induce the system to synthesize more antioxidant molecules to combat free radicals generated by CCl_4. In liver, CCl_4 treatment depleted the antioxidant enzymes namely CAT, SOD and POX leading to liver damage evidenced by higher TBARS levels. The depletion may be due to inhibition of enzyme synthesis and also damaging the enzyme itself making them inactive. The spice extract treatment has significantly reduced the deleterious effects of free radicals generated by CCl_4 treatment. Almost similar levels of antioxidant enzymes were seen in spice extract treated groups. Further, TBARS levels were almost equal to that of healthy animals indicating the beneficial role of BCAE and SRAE against CCl_4 induced liver toxicity.

In this study, CCl_4 caused an obvious decrease of GSH and antioxidant enzymes activity in liver homogenates, but the enzyme activity was higher in animals pretreated with BCAE and SRAE which are known to contain abundant phenolic acids with multi-potent antioxidant potency (Chapter – 2). Data thus may suggest that BC and SR extracts can ameliorate GSH and antioxidant enzymes or enhance its

activity by participating in biosynthesis in animals with CCl_4-induced liver injury.

SOD is one of the potent antioxidant enzymes in cells and catalyzes the conversion of superoxide ions into oxygen and hydrogen peroxide. In our study, SOD activity was dramatically decreased by CCl_4, but the effect was rescued in a concentration dependent manner by both BCAE and SRAE. SOD is an important antioxidant enzyme of antioxidant defense system for stabilizing oxidative reactions. Decreased activity and content of these enzymes cause the descent of antioxidant ability in the body. Therefore, antioxidant rich BCAE/SRAE may prevent experimental liver injury by modulating or enhancing GSH and SOD activities. Results are substantiated by the hepatoprotectivity by black cumin seed and swallow root, where the protectivity has been attributed to several compounds including thymoquinone, a major (27.8%–57.0%), component in black cumin seed oil and a cocktail of compounds like 2-hydroxy 4 methoxybenzaldehyde, vanillin, decalepin etc., in swallow root. In the current study however we attribute the hepatoprotective ability to mainly phenolic acids which are contributing significantly to antioxidant activity in addition to the reported compounds in SRAE. In black cumin however, since Ayurveda practioners and traditional users use the water decoction, phenolics may contribute significantly to hepatoprotectivity *in vivo*. Increased binding ability to serum albumin may enhance the biopotency of these compounds since the rate of clearance of these phenolics may be delayed due to its binding ability to serum albumin and this may enhance the bioavailability of these compounds (Chapter – 4) and may be responsible for the distribution of these compounds to various tissues since HSA acts as a potent carrier protein which is known to carry such essential small molecular components.

It is concluded that both BCAE and SRAE may decrease the liver function enzymes and increase the antioxidant defence system activity in the CCl_4-treated rats. They may be therefore used in CCl_4-induced hepatotoxicity rats to prevent lipid peroxidation, increase anti-oxidant defense system activity and also to prevent liver damage.

5.6.2 In vivo effect of BACE and SRAE against MC – induced cervix cancer

In view of the medicinal value attributed to black cumin and swallow root, the present study was an attempt to evaluate the effect of black cumin and swallow root aqueous extract on chemical induced carcinogenesis of uterine cervix in mice. As there are no reports on the chemopreventive effect of these spice extracts on chemical (20-methylcholanthrene) induced carcinogenesis on any organ or tissue so far, our study is perhaps the first report to demonstrate the anticarcinogenic activity of these spice extracts. In many studies the carcinogen is withdrawn after initiation, but in the present investigation we studied the effect during chronic carcinogenic exposure and therefore is of more significance with respect to assessment of the protective effect of black cumin and swallow root extract.

As a defense system against oxidative stress caused by reactive oxygen species, cells possess many endogenous non enzymatic antioxidant molecules including reduced glutathione as well as antioxidant enzymes such as superoxide dismutase, catalase and peroxidase (Somani et al., 1999; Lee et al., 2003). Hence, a large number of studies have focused on the pathogenic significance of oxidative stress in liver injury as well as on therapeutic intervention with oxidative scavengers

Glutathione, the major endogenous antioxidant present in all animal cells, participate in diverse biological processes, including detoxification of xenobiotics for their elimination from the system and protection from oxidative stress (Sciuto, 1999). An observation on the association of GSH during carcinogenesis and in cancer is often contradictory and conflicting. A decreased level of GSH was reported in cervical neoplasia and invasive carcinoma (Kumar et al, 1995; Basu et al., 1991). Low plasma GSH was also observed in malignancies of breast, lung, liver, prostate and in lymphoma (Beuyer & Gilbert, 1985). In contrast to the GSH levels in serum, Meister and Griffith (1979) reports increased GSH content in the liver following carcinogen administration, which was in proportion to the carcinogenic potency. Our results showed a reduced serum and liver GSH level after 45 days of MCA exposure. It is

possible that initially the host's defense system provides cellular protection by increasing the level of GSH activity, which helps in neutralizing the action of 20-methylcholanthrene. However, chronic exposure to MCA may derange the defense mechanism by significantly depleting the GSH level. Interestingly, oral administration of spice extracts elevated the GSH level resulting in delayed action of the carcinogen. The present observations suggests that the spice extracts induced increase in the level of GSH activity helps to eliminate the carcinogen and adjourn the onset of cervical neoplasia and its progression.

Several antioxidant enzymes like CAT, SOD and POX can protect cell and cellular DNA from oxidative damage. Cellular damages from radical and non-radical reactive oxygen species including peroxides and superoxides are inactivated enzymatically by CAT, SOD and POX (Vang et al., 1997). We observed a significant decrease in POX and CAT in both serum and liver samples in MCA treated group compared to normal. The reduction in SOD was significant in serum where as it was marginal in the liver. Decreased cellular activities of POX, CAT and SOD causes accumulation of reactive species in the body, which leads to oxidative damage and progression of carcinogenesis. Spice extracts treatment significantly increased the POX, CAT and SOD activity (except in liver treated with BC1 and BC2 for SOD) in addition to the inductive effect on the production of SOD enzyme in the liver. This increased antioxidant enzymes facilitate the removal of peroxides and superoxides produced in large amounts during carcinogen metabolism, thereby preventing the oxidative damage and cancer initiation.

Generation of reactive species following increased lipid peroxidation and consequent tissue injury and cellular damage increases the risk for cancer (Wiseman and Halliwell, 1996; Swierczynski et al., 1997). A number of chemical carcinogens are known to increase formation of lipid peroxides. We observed elevated levels of lipid peroxides during MCA induced carcinogenesis in the uterine cervix. Decrease in lipid peroxides by black cumin and swallow root extracts in this study implies their chemopreventive action which is substantiated by observing the potent DNA

protective and cytoprotective activity of these extracts as revealed in Chapter - 2 which enables the prevention or arrest of DNA and cellular damage by the extracts. Wide array of protective activity may envisage the anticancer effect against MCA induced carcinogenesis and this may be attributed to potent phenolic antioxidants present BC and SR extracts.

These results were further substantiated by the morphological observation of the vagina and histological observation of the uterine cervix which, showed severe dysplasia in the MCA treated group, while black cumin and swallow root treated group (SR2) were found to be normal. The results of this study suggest that the potential chemopreventive activity of the black cumin and swallow root aqueous extract is partly attributable to their antioxidant properties.

5.6.3. Assessment of Antimetastatic activity of SRPP using B16F10 mouse melanoma cells on Swiss albino mice

The present investigation reports the role of swallow root pectic polysaccharide in the inhibition of metastasis in Swiss albino mouse model using B16Fl0 melanoma cells. B16F10 is a highly metastatic melanoma cell line reported by other investigators and our own analysis, and has often been used to investigate the efficacy of chemical agents in inhibiting metastasis or to understand the mechanism involved in the process (Fidler, 1973). Although the literature contains several references to the use of C57BL6J mice for inducing pulmonary metastases (Han et al., 2006; Kakuta et al., 2002; Menon et al., 1995; Banerji et al., 1998), we followed the method of Martınez et al., (2005) using Swiss albino mice, which were available in our laboratory.

Galectin-3 has been implicated in metastasis (Nangia-Makker et al., 2000) and the dietary intake of pectins rich in galactose have been reported to inhibit carbohydrate-mediated tumor growth, angiogenesis and metastasis *in vivo* (Nangia-Makker et al., 2002). Previously, we have investigated galectin inhibitory activity and inhibition of cell invasion by black cumin and swallow root pectic polysaccharides (SRPP) *in vitro* (Chapter – 2, Sathisha et al., 2007). Results indicated good activity for SRPP and

hence used for further studies on antimetastatic activity *in vivo*. SRPP contained high galactose and hence we compared with galactose-mediated inhibition of metastasis *in vivo*. Further, the role of polyphenols as antimetastatic and anticancer agent is available in the literature (Menon et al., 1995) and hence we included swallow root aqueous extract (SRAE), which contained higher polyphenolic content (Naik et al., 2007) to see its possible synergistic effect with SRPP. Swallow root contained 2-hydroxy-4-methoxybenzaldehyde as the major compound (Nagarajan et al., 2001), which is an isomer of methoxy, hydroxy benzaldehyde. Vanillin is also a similar type of isomer whose antimetastatic activity has been reported in the literature (Lirdprapamongkol et al., 2005). Also our own studies indicated in chapter – 2 suggest that HMBA is present in major amounts. Hence, the antimetastatic activity of this compound was also investigated in addition to SRPP and SRPP+SRAE.

Results from the present investigation shows significant reduction in lung metastases and also reduction in local tumor growth at the site of injection in mice treated with SRPP and SRPP+SRAE. HMBA did not show any correlation against lung metastasis. SRPP showed higher activity than the combinational supplementation of SRPP and SRAE. SRPP showed significant reduction of superficial tumor nodules, implantation percentage, growth index, and galectin levels indicating its antimetastatic ability. Although, SRPP+SRAE showed significant antimetastatic activity, it was comparatively less than SRPP alone. This may be due to the interaction of phenolic compounds with small oligomers of digested pectic polysaccharides resulting in lesser binding to galectin receptors. The binding ability of polyphenols to proteins may also have an effect on the reduced antimetastatic activity of combinational supplementation (SRPP+SRAE). Results also indicated significant increase in the serum antioxidant enzymes SOD, CAT and antioxidant GSH in SRPP treated animals indicating their stimulatory effect on the serum defense mechanism. The increase in serum antioxidant enzymes could be due to the presence of free phenolic antioxidants in SRAE and in the bound form with the polysaccharide. Our earlier studies on IR analysis of SRPP and other biochemical studies (srikanta et al., 2007) indicated the presence of 0.12 µg GAE phenol/g of

SRPP with potent antioxidant potency. Further, the increased levels of galectin in the positive control animals and reduction in animals treated with antimetastatic components correlated well with the severity of metastasis. These results further strengthen the view of galectin-3 involved in metastatic process as reported earlier (Inufusa et al., 2001). Toxicity studies indicated no significant damage to the vital organ liver as evidenced by no increase in the liver function enzymes (SGPT and SGOT) and also there is normal weight gain in sample treated groups as compared to healthy mice fed with standard mice feed. The decreased SGPT level is an added beneficial effect of the swallow root components in addition to their antimetastatic activity. There was no inhibition of metastasis by HMBA except that it had low toxic effects on the mice at the dosage used in the experimental study. These results may suggest the use of SRPP in inhibiting metastasis. Since swallow roots are consumed by people in South India in the form of pickles and as health drinks, SRPP may find use as a nutraceutical in controlling metastasis as it had no toxic effects. Further, the effectiveness of SRPP needs to be evaluated in higher animal models and finally in clinical trials.

Summary and conclusions

- Antioxidant in aqueous extracts of BC - BCAE and SR – SRAE inhibited oxidative stress mediated CCl_4 induced hepatotoxicity.

- BCAE and SRAE also inhibited 20-methylcholanthrene induced cervix cancer.

- Pectic polysaccharide fraction of SR - SRPP showed potent (88 %) inhibition of lung metastasis induced by B16F10 melanoma cells in Swiss albino mice.

- Supplementation antioxidants from SRAE however were not as effective in inhibiting metastasis as that of SRPP alone.

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- **Harish Nayaka M. A**., Sathisha U.V & Shylaja M Dharmesh**.** Cytoprotective and antioxidant activity of free, hydrolyzed and bound phenolic acids from swallow root (*Decalepis hamiltonii*). **(to be communicated)**
- **Harish Nayaka M. A**., Lokesh B. R & Shylaja M Dharmesh. Potent water soluble component from black cumin (*Nigella sativa*): contribution to health beneficial properties. **(to be communicated)**