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# *DECLARATION*

**I here by declare that the thesis entitled "*Bioactive Molecules From Pomegranate Fruit Waste (Pith and Carpellary Membrane)*" submitted to the University of Mysore, for the award of the Degree of *Doctor of Philosophy* in the Faculty of Biotechnology is the result of work carried out by me under the guidance of Dr. S. M. Aradhya, Scientist, Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Mysore, during the period August 2001-August 2005.**

**I further declare that the results have not been previously submitted for any other degree or fellowship**

**(Anand P. Kulkarni)**

**Date:**

**Place: Mysore**

**Dr. S. M. Aradhya**
Scientist, Research supervisor
Department of Fruit and Vegetable Technology

# *CERTIFICATE*

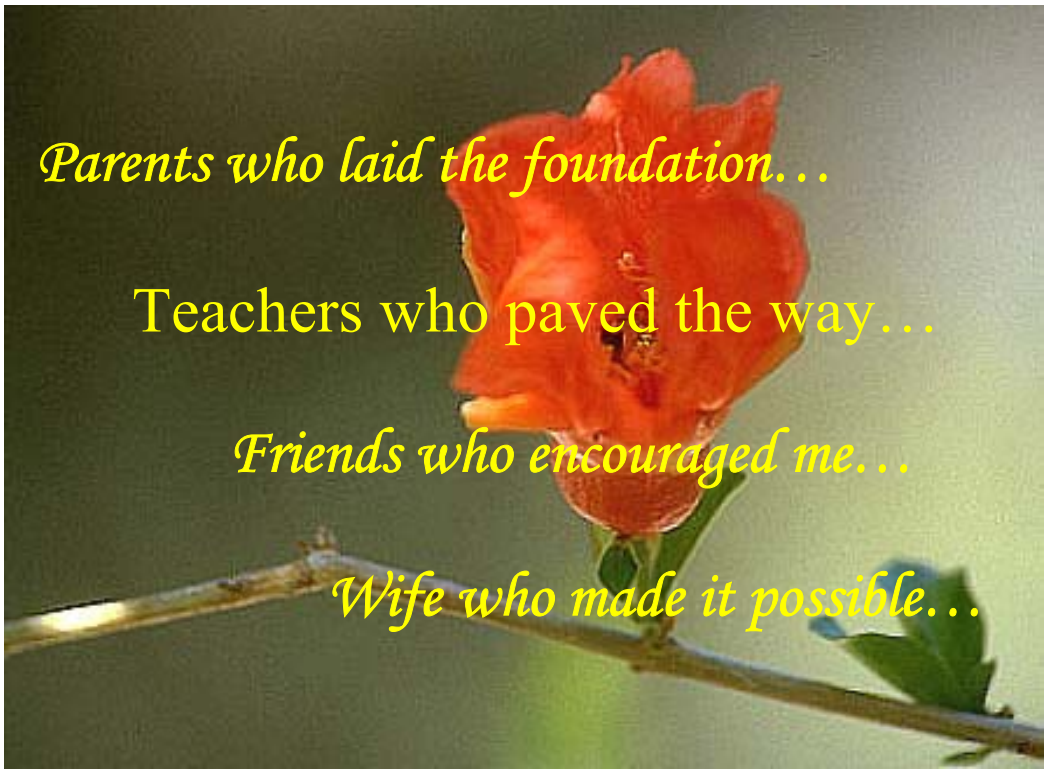
**This is to certify that the thesis entitled "*Bioactive Molecules From Pomegranate Fruit Waste (Pith and Carpellary Membrane)*" submitted to the University of Mysore, Mysore for the award of the Degree of *Doctor of Philosophy* in the Faculty of Biotechnology by Mr. Anand P. Kulkarni is the result of work carried out by him in the Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Mysore, under my guidance during the period August 2001 - August 2005**

**(S.M. Aradhya)**

**Date:**

**Place: Mysore**

*Dedicated to my beloved…*



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*- Anand P. Kulkarni*

# List of Figures

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# *Abbreviations used*

| | |
|---|---|
| °C | Degree Celsius |
| µl | Microliter |
| µM | Micromolar |
| 2D-HMQCT | Two dimensional heteronuclear multiple quantum coherence transfer spectroscopy |
| A-549 | Human small cell lung carcinoma cell line |
| AA | Ascorbic acid |
| $ABTS^{\bullet}$ | 2,2-Azinobis (3-ethylbenzithiazole-6-sulphonate) |
| B.A.R.C | Bhabha atomic research center, Trombay, Mumbai, India |
| BHA | Butylated hydroxy anisole |
| BSA | Bovine serum albumin |
| Cd | Cadmium |
| cfu | Colony forming units |
| CM | Carpellary membrane |
| $CO_2$ | Carbon dioxide |
| $CTC_{50}$ | Cytotoxicity (kills 50% cells) |
| $CuSO_4$ | Copper sulphate |
| CV | Cyclic voltametry |
| DC | Direct current |
| DPPH | 1,1-Diphenyl-2-picrylhydrazyl |
| ESI | Electrospray ionization |
| $FeCl_2$ | |
| g | Gram |
| Hep-2 | Human larynx epithelial cancer cell line |
| HPLC | High performance liquid chromatography |
| HMF | 5-Hydroxymethyl furaldehyde |
| I | Human serum albumin |
| $IC_{50}$ | Inhibitory concentration (inhibits 50%) |
| KCL | Potassium chloride |

| | |
|---|---|
| KSCN | Potassium thiocyanate |
| Kg | Kilogram |
| LC | Liquid chromatography |
| $MIC_{50}$ | Minimum inhibitory concentration (inhibits 50%) |
| min | Minutes |
| ml | Milliliter |
| mm | Millimeter |
| Mol. Wt | Molecular weight |
| MS | Mass spectrometry |
| MTT | 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide |
| N | Normality |
| NMR | Nuclear magnetic resonance spectroscopy |
| NPG | n-Propyl gallate |
| pH | Negative logarithm of hydrogen ion concentation |
| PMT | Photo multiplier tube |
| POD | Peroxidase enzyme |
| PPO | Polyphenol oxidase enzyme |
| SRB | Sulphorhodamine B |
| TBA | Thiobarbituric acid |
| TCA | Trichloroacetic acid |
| TLC | Thin layer chromatography |
| $Trp^{\bullet}$ | Tryptophan radical |
| UV | Ultraviolet |
| Vero | Normal African green monkey kidney cell line |
| i.e. | That is |
| SOD | Superoxide dismutase |
| NBT | Nitroblue tetrazolium |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| No. | Number |
| PMS | Phenazin methosulphate |
| OD | Optical density |
| A | Absorbance |
| Sec | Seconds |
| V | Volt |

| | |
|---|---|
| v/v | Volume/volume |
| w/w | Weight/weight |
| $EC_{50}$ | Effective concentration (50% effect) |
| SCE | Saturated calomel electrode. |
| NHE | Normal hydrogen electrode |
| W | Watt |

# *Contents*

## Chapter II Functional properties of bioactive molecule isolated from pomegranate pith and carpellary membrane

# Synopsis

## Bioactive molecules

Over the ages, human beings were using the plant sources as a source of food and medicine. It formed the basis of sophisticated traditional medicine systems in the name of unani medicine, ayurvedic medicine, tribal medicine, that have been in existence for thousands of years in the countries such as India and China. It has been estimated by the World Health Organization (WHO) that approximately 80% of the world inhabitants rely mainly on traditional medicines as an alternative system of medicine for their primary health care. The medicinal property of these plants has been ascribed to its phytochemicals, which are universally called as bioactive molecules.

Higher plants are 'treasure houses' for a repertoire of bioactive molecules, which are mostly produced as secondary metabolites. Most of these bioactive phytochemicals are reported to have their own function in the normal functioning of the source system where they are produced. Study on these aspects formed a new branch of science called 'teleology'. Teleology is a doctrine, explaining the phenomena by their ends or purposes.

The present research program has been beset towards both these aspects of bioactive molecules, i.e. health benefits to human beings as well as functional role of bioactive molecule in source system.

## Pomegranate:

The pomegranate is tropical fruit, which thrives well under arid and semiarid conditions. It is considered as a holy fruit since antiquity by many religions, and also extensively used in folk medicine of many cultures including Indian, Chinese, Greek, Hebrew and Babylonians. Most of its health beneficial properties were proved in recent

findings by several researchers. A voluminous literature has been accumulated on various and health beneficial properties of different parts of the pomegranate fruit and bioactive molecules responsible.

The pith and carpellary membrane constitutes 13% of the pomegranate fruit and there is an expressed doubt that phylogenetically carpellary membrane might have evolved to provide a structural and functional protection to the arils. Further it appears to play a definite role in maintaining the quality of the arils. does carpellary membrane evolved to impart functional or regulatory role? It is an intrigue question, which has been attempted to answer. Accordingly, the following work plan was proposed initially and carried out during the present Ph.D. program

1. Isolation and characterization of bioactive molecule from pith and carpellary membrane of pomegranate fruit
2. Study on different functional attributes of bioactive molecule towards human health benefits
3. Elucidation of internal browning disorder of pomegranate fruit and role of bioactive molecules in it.

The results obtained were thus consolidated as a Ph.D. thesis. The thesis preceded with ‘General introduction and review of literature’ followed by a brief account on the scope of the present investigation. The general structure of the three chapters that follows consists of a brief introduction, review of literature, materials and methods, results and discussion and summary. The salient feature inferred from the investigation are summarized under the heading of ‘General summary and conclusion’ at the end of the thesis, followed by bibliography.

## General Introduction and review of literature

Introduction addresses relevant literature on bioactive molecules towards human health benefits and a detailed information about pomegranate fruit. The aspects covered regarding pomegranate includes its origin, history of cultivation, taxonomy, vernacular names, varieties, fruit structure, functional attributes of different parts of pomegranate fruit towards human health, including juice, peel and seeds. The literature survey regarding the pomegranate fruit exposed the lacunae on any information concerned to pith and carpellary membrane on functional role as phyto-protective or as health beneficial factors. Finally scope of the present investigations is indicated.

## Chapter I

## Isolation and characterization of bioactive molecule from pith and carpellary membrane of pomegranate fruit

This chapter enumerates the techniques employed for isolation and characterization of the bioactive molecules from the pomegranate fruit pith and carpellary membrane. The initial screening of pith and CM for possible bioactivity by extraction with different solvents and subsequent bioactivity assay demonstrated that methanol extract could be a potential source of bioactive molecule. Methanol extraction of pith and carpellary membrane followed by repeated column chromatography and preparative HPLC proved successful in isolating a bioactive molecule from it. The spectroscopic analysis viz. UV-visible, infrared spectroscopy, nuclear magnetic resonance spectroscopy and mass spectroscopy (GC-MS and MALDI-MS) data reeled the structure of the bioactive molecule as a punicalagin. Which is a high molecular weight polyphenol. The acid hydrolysis followed by HPLC and LC-MS analysis

revealed the presence of gallic acid, ellagic acid, gallagic acid, punicalin and punicalagin. Among these molecules punicalin was found in higher concentration in the pith and carpellary membrane of pomegranate fruit. The antioxidant activity revealed that punicalagin is most potent one among the five molecules mentioned above. The punicalagin was reported for the first time from pomegranate fruit pith and carpellary membrane from our work

## Chapter II

## Functional properties of Punicalagin isolated from pith and carpellary membrane of pomegranate fruit

This chapter encompasses a brief introduction followed by various functional attributes of punicalagin studied in the present work like antioxidant activity, binding with human serum albumin (HSA), DNA, antiplatelet aggregation, low cytotoxicity, and antibacterial activity.

The antioxidant potential of punicalagin was assessed by radical scavenging assays using DPPH, superoxide, and ABTS radicals. The antioxidant activity was also assessed in terms of lipid peroxidation inhibitory activity in liver microsomal system, total reducing power. The kinetics of free radical reactions of punicalagin with various free radicals generated in-vitro using pulse radiolytically was also studied. The reduction potential of punicalagin was also determined by cyclic voltametric and differential voltametric techniques, which measures the electron donating ability of a antioxidant a various pH range. Results indicated that its higher electron donating potential at neutral pH. In addition, the antioxidant potential of the punicalagin was also assessed in terms of its metal ion (Iron and copper) chelating activity. The results proved the potential antioxidant activity of punicalagin in all the methods of assay.

In order to study the bioavailability of punicalagin across the body, a study on interaction of punicalagin with HSA protein was carried out. Since the later is known to act as transport protein for many drug molecules. The results revealed the potential HSA binding ability of punicalagin, which also suggests the possible bioavailability of punicalagin. Punicalagin and DNA interaction study was carried out to find out any possible interaction, which can render it to an anticancer drug by binding with later. The study showed that, punicalagin binds with DNA with low affinity with possible hydrogen bonding.

Further punicalagin showed moderate platelet aggregation inhibitory activity against the adenosine diphosphate and collagen agonists in an in-vitro study. The low cytotoxicity of punicalagin proved in our study on three cell lines viz. Vero (Normal African green monkey kidney cell line), Hep-2 (Human larynx epithelial cancer cell line) and A-549 (Human small cell lung carcinoma cell line) maintained in laboratory conditions added to its functional activity. The higher functional attributes and low cytotoxicity associated with bioavailability rendered the punicalagin in to promising multifunctional molecule.

The possible antibacterial activity of punicalagin was tested against clinically important gram-negative bacteria viz. viz. *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *salmonella typhi* and *Shigella dysenteriae.* Results revealed its broad-spectrum antibacterial activity of punicalagin against the above food borne pathogens. The acetone extract and methanol extract of pith and CM also showed potential antibacterial activity.

## Chapter III

## Elucidation of mechanism of internal browning in pomegranate fruit and role of bioactive molecules in internal browning

Internal browning of pomegranate arils forms the major bottleneck for commercial exploitation for processing and for export trades. For the first time a detailed investigation was undertaken in the present program of work to have a mechanistic insight in to the mechanism of internal browning of arils and also role of bioactive molecules (punicalagin and its derivatives) in it, which was discussed in the present chapter.

The biochemical analysis of arils under different stages of fruit development and browning indicated that incidence of internal browning is associated with ripening/senescence changes and involved with an array of inter linked endogenous biochemical factors like pH, organic acid content, water content, anthocyanin content, phenolic content, calcium, peroxidase (POD) and polyphenol oxidase (PPO) activities. From the results it appears that ripening/senescence changes associated with membrane disintegration, which leads to decreased water content, organic acid content, and increased calcium content resulting in the increase in pH of the arils. On the other hand, disintegration of cellular compartmentation due to the loss of membrane integrity exposes the enzymes to their substrates, in particular polyphenol oxidase and peroxidase enzymes. The increase in pH of the arils has been identified as one of the critical factor, which induces the reversible structural transformation of anthocyanin pigments resulting in discoloration of arils. In addition increase in pH also provides optimum pH conditions for the higher activity of PPO and POD enzymes, which leads to the browning in pomegranate arils.

A gradual decrease in the concentration of punicalagin and its derivatives in the arils observed during fruit development, maturation, ripening and senescence coinciding with increase in anthocyanin content suggests their possible role in the synthesis of anthocyanin pigments. Similarly increase in browning of arils recorded a drastic decrease in the concentration of punicalagin and its derivatives except gallic acid. It appears that there is a delicate balance between internal browning of pomegranate arils with bioactive molecules along with biochemical attributes of arils. The chapter encompasses the detailed discussion on these aspects.

## General summary and conclusion

In this section of the thesis, salient features inferred from this investigation are summarized as follows:

For the first time, pith and carpellary of the pomegranate fruit waste was explored as a source of the potential bioactive molecules

For the first time punicalagin, the high molecular weight polyphenolic bioactive molecule was isolated from pith and carpellary membrane

The punicalagin showed potential antioxidant activity in terms of radical scavenging activity, lipid peroxidation inhibition, metal chelating activity and electron donating ability

Pulse radiolysis studies reveled the kinetics of radical scavenging ability and also its ability to repair tryptophan radicals generated in-vitro

The DNA binding and platelet aggregation inhibition, and antibacterial activity of the punicalagin adds to its fame as a functional properties

Low cytotoxicity and its interaction with HSA rendered it as promising multifunctional and biologically available bioactive molecule

Based on the extensive research data generated through biochemical analysis of arils during fruit development and during browning of arils, for the first time mechanism of internal browning in pomegranate fruit was elucidated and also role of bioactive molecules in it

# *General Introduction and*

# *Review of Literature*

## Introduction

"Let food be thy medicine and medicine be thy food" was the famous dictum proclaimed by Hippocrates about 2500 years ago. Recently many scientific studies supported the above fact. It appears that diets containing some phytochemicals can provide protection against various chronic diseases like cancer, atherosclerosis, thrombosis, etc (Wildman, 2001) and also impart other health benefits. These phytochemicals are termed as bioactive molecules, nutraceuticals, etc. These discoveries have rapidly intensified the search for new bioactive molecules with multifunctional properties.

Fruit and vegetables form the rich source of bioactive molecules. Owing to the botanically diverse families found in dietary fruits and vegetables, over 10,000 bioactive phytochemicals have been identified and are integral part of the human diet (Wise, 2001). Phytochemicals are used as an alternate medicine in ayurveda, the Indian traditional medicine. Major classes of phytonutrients that have potential health beneficial properties include carotenoids, polyphenols, isothiocyanates, sulfides, and phytosterols. This broad range of natural compounds appears to have multiple biological functions, ranging from antioxidant to anticancer. There is an increasing interest in the use of plant derived bioactive molecules for therapeutic purpose. Advancement in technology to assist the process of isolation, identification, and screening of bioactive molecules towards various functional properties has also triggered the research in the field of bioactive molecules.

Another intriguing observation is that, bioactive molecules produced in plant systems are not for the sole purpose of human benefits. Many of these bioactive molecules are reported to have their own function in the normal functioning of the source system where they are produced. Study on these aspects formed a new branch of science

called ‘Teleology’, a ‘doctrine explaining phenomena by their ends or purposes’, which gives information about ‘why it exist? What is its role? Study of teleology of a bioactive molecule furnishes imperative information about the functional truth following its existence in that system.

Pomegranate a fruit of promise for its therapeutic purpose and considered as holy fruit since antiquity. It is used as an alternative medicine in ayurveda, and other traditional medicines through out the world. It is one of the major commercial crops grown in India, but underutilized for fresh as well as for processing due to its unique problem of internal browning of arils.

The present research work entitled ‘Bioactive molecules from pomegranate fruit waste (pith and carpellary membrane)’ is focused on to isolate and characterize the bioactive molecules from pith and CM of pomegranate fruit. An attempt has also been made to enumerate some of their functional properties of human health benefits and teleological role in pomegranate fruit.

# Review of Literature

Review of literature was focused to provide a detailed account of information on bioactive molecules of pomegranate fruit.

# Pomegranate

## Origin and History of Cultivation

Archeobotanical investigations show that pomegranate is probably originated in Iran and Afghanistan, and has been cultivated since ancient times throughout the Mediterranean basin to India, where it is still a fruit of commerce. It was considered as a holy fruit since antiquity by many religions. The plant was used in many ways, including juice, dyes inks, tannins for leather (bark) and a variety of remedies for various ailments. The fruit has a fairly long shelf life at room temperature, and hence was carried on long journeys through desert climates as a source of water and nourishment (Langley, 2000).

## Taxonomy of pomegranate:

| **Engler and Prantl's (1931)** | | **Hutchinson (1959)** | |
|---|---|---|---|
| Division: | **Angiospermae** | Phylum | **Angiospermae** |
| Class: | **Dicotyledonae** | Subphylum: | **Dicotyledonae** |
| Subclass: | **Archichlamydeae** | Division | **Lignosae** |
| Order: | **Myrtiflorae** | Order | **Myrtales** |
| Family: | **Punicacae** | Family: | **Punicacae** |
| Genus: | **Punica** | Genus: | **Punica** |
| Species: | ***P. granatum* (Linn)** | Species: | ***P. granatum* (Linn)** |

## Pomegranate fruit: Botany

Pomegranate fruit is botanically a unique type of fruit classified as balausta. The fruit is many chambered and multi seeded, developed from an inferior ovary and synchronous pistil with two whorls of basal carpels lying within the receptacle. During

the development of ovary, the outer carpels become tilted up and superimposed. The result is that two layers of chambers are formed, with the outer or upper ones occupying a parietal position. The pericarp (peel) of fruit is tough and leathery, and chambers made of thin walls of carpels (carpellary membrane). The seeds are anchored on a soft and fleshy placental (pith) tissue. The testa of the seed (aril) is filled with an acid juice while the tegmen (seed) is horny.

**Fig. G1. Pomegranate fruit and its parts**

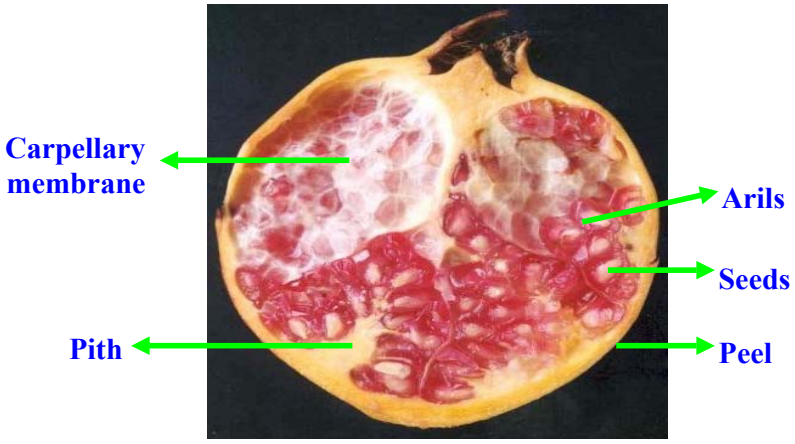



**Table. G1. Vernacular names of pomegranate**

| Language (Indian) | Vernacular name | Language (International) | Vernacular name |
|---|---|---|---|
| Bengali | Dalim | Chinese | Shiliu |
| Gujrati | Dadam | French | Grenade |
| Hindi | Anar | German | Granatapfel |
| Kannada | Dalimbe | Hebrew | Rimon |
| Malayalam | Matalam | Italian | Melogranate |
| Oriya | Dalimba | Japanese | Zakuro |
| Punjabi | Anar | Romanian | Rodie |
| Sanskrit | Darimba | Russian | Granatnik |
| Tamil | Madulam | Spanish | Granada |
| Telugu | Dhanimmapandu | Swedish | Granatäpple |

Ref: Morton (1987)

**Table G2. Commercial varieties of Pomegranate grown in India**

| Varieties / types | Characteristics of fruit | States in which grown |
|---|---|---|
| Alandi or Vadki | Fruit medium in size, fleshy testa blood red or deep pink, with sweet, slightly acidic juice: seeds very hard | Maharashtra |
| Araktha | Fruit medium to large size, moderately thick rind, blood colored fleshy testa, juice, soft seeds | Karnataka Maharashtra |
| Bedana | Fruit medium in size, rind brownish or whitish: fleshy testa pinkish white with sweet juice; seeds soft | Maharashtra |
| Dholka | Fruit large in size, rind greenish white fleshy testa pinkish white or whitish with sweet juice; seeds soft | Maharashtra |
| Ganesh | Prolific yield, medium size fruits soft seeds, pinkish flesh and sweet juice, agreeable taste | Maharashtra Karnataka |
| Kabul | Fruit large in size, rind deep red, mixed with pale yellow, thick, fleshy testa dark red, with slight bitter juice | Maharashtra |
| Kandhari | Fruit large in size, rind deep red, fleshy testa, blood red or deep pink with sweet, slightly acidic juice, seeds hard | Maharashtra |
| Muskat Red | Fruit small or medium in size, rind somewhat thick, fleshy testa with moderately sweet juice, seeds not very hard | Andhra Pradesh & Maharashtra |
| Paper shell | Fruit medium in size, rind thick, fleshy testa reddish to pink with sweet juice, seeds soft | Andhra Pradesh |
| Poona | Fruit large size, rind crimson, dark gray or grayish green, fleshy testa deep scarlet or pink or red | Andhra Pradesh |
| Sindhoor | Prolific yield, medium to large size fruits, soft seeds, red flesh and sweet juice | Karnataka Maharashtra |
| Spanish Ruby | Fruit small to medium size, rind thin, fleshy testa rose colored, seeds soft | Andhra Pradesh |
| Yellodu | Fruit medium to large size, rind moderately thick, fleshy testa, juice, seeds moderately hard | Maharashtra |

Among the different varieties grown all over India, variety Ganesh is more popular because of, prolific yield, medium size fruits soft seeds, pinkish flesh and sweet juice, consumer acceptance. In the present study, variety Ganesh was used as a source pomegranate fruit for experimental purposes.

## Pomegranate: a fruit of promise for bioactive molecules

### An historical perspective

Pomegranate was considered as a holy fruit since antiquity by many religions. The pomegranate tree is said to have flourished in the garden of Eden, has been extensively used in the folk medicine of many cultures including Greek, Hebrew, Babylonians, Chinese and Indian. In ancient Greek mythology, pomegranates were known as the 'fruit of dead', in the Hebrew tradition, pomegranates adorned the vestments of the high priest. The Babylonians regarded pomegranate seeds as an agent of resurrection; the Persians believed the seeds conferred invincibility on the battlefield and for the ancient and for Chinese, the seeds symbolized longevity and immortality. Not only has the pomegranate been revered through the ages for its medicinal properties, it also features in the heraldic crests of several medical institutions including British medical association, London (Langley, 2000).

Ayurveda –an ancient science of Indian medicine has identified pomegranate as a medicinal plant and consumption of pomegranate juice was prescribed as remedy for various ailments (Table G3).

**Table G3. Ayurvedic importance of Pomegranate**

| Ayurvedic prescription | Meaning |
|---|---|
| ***Agnideepak*** | Appetizer |
| ***Pittakarak*** | Enhances *Bile pigments* |
| ***Hridya*** | Strengthens heart |
| ***Grahi*** | *Anti – diarrhea* |
| ***Sheetal*** | Cooling |
| ***Rakta Shodak*** | Blood purifier |

Ref: http://www.vshiva.net/archives/naturefacts

## Current status

A vast literature is available regarding the health beneficial properties of pomegranate fruit. The major functional properties include antioxidant, antibacterial, anticancer, antitumor, antiplatelet aggregation, antimutagenic, estrogenic property and many more as listed below in the table (G4).

**Table G4 Functional properties attributed to phytochemicals/extracts from different parts of pomegranate.**

| Functional property | Phytochemical or extract | Fruit part | Reference |
|---|---|---|---|
| Anthelmintic | Aqueous extract | Pericarp | De Amorim *et al.* 1996 |
| Anti-HIV | Juice | Juice | Neurath *et al.* 2004 |
| Anti-angiogenic | Seed oil | Seeds | Toi *et al*. 2003 |
| Anti-atherosclerotic | Flavonoids | Juice | Aviram *et al*. 2002 |
| Antibacterial | Methanol extract | Peel | Negi & Jayaprakash, 2003 |
| Anti-breast cancer | Fermented juice | Juice | Mehta, 2004 |
| Anti-colon cancer | Seed oil | Seeds | Kohno *et al*.2004 |
| Anti-cholesterol | Juice | Juice | Esmaillzadeh *et al*. 2004 |
| Antifungal | Water extract | Fruit | Vasconcelos *et al*. 2003 |
| Anti-inflammatory | Punicalagin | Fruit | Lin *et al*.1999 |
| Antimutagenic | Acetone extract | Peel | Negi *et al* 2003 |
| Antioxidant | Anthocyanins | juice | Gil *et al.* 2000 |
| Anti-platelet aggregation | Juice | Juice | Aviram *et al*. 2000 |
| Anti- prostate cancer | Supercritical extract | Peel | Albrecht *et al.* 2004 |
| Anti-skin tumor | Seed oils | Seeds | Hora *et al*. 2003 |
| Antitumorigenic | Ellagitannins | Peel | Castonguay *et al.* 1997 |
| Cytotoxic | Trienoic fatty acids | Seed | Suzuki *et al.* 2001 |
| Estrogenic | Seed extract | Seeds | Mori-Okamoto *et al.* 2004 |
| Hepatoprotective | Punicalagin | Fruit | Lin *et al.* 2001 |
| Larvicidal | Acetone extract | Fruit | Morsy *et al.* 1998 |
| Wound healing | Methanol extract | Peel | Murthy *et al*. 2004 |

Interesting to note that most of these properties were attributed to the phenolics present in the pomegranate or extracts rich in these phenolics. Many of these phenolics were isolated, purified and characterized by different workers (Table G5) and these properties were attributed to the functional OH groups.

**Table G5. Bioactive phytochemicals isolated and characterized from different parts of pomegranate fruit**

| Bioactive molecule | Part of fruit | Functional property | Reference |
|---|---|---|---|
| Cyanidin-3,5-Diglucoside | Juice, pericarp | Antioxidant | Noda *et al*. 2002 |
| Cyanidin-3- Diglucoside | Juice, pericarp | Antioxidant | |
| Delphinidin-3,5-diglucoside | Juice, pericarp | Antioxidant | |
| Delphinidin-3 -Diglucoside | Juice, pericarp | Antioxidant | |
| Pelargonidin-3, 5-Diglucoside | Juice | Antioxidant | |
| Pelargonidin-3-Diglucoside | Juice | Antioxidant | |
| Ellagic acid | Juice | Antioxidant, Antitumorigenic | Gil *et al*. 2000 Castonguay *et al*. 1997 |
| Gallic acid | Juice, pericarp | Antioxidant | Gil *et al*. 2000 |
| Gallagic acid | Juice | Antioxidant | |
| Gallocatechin | Peel | Antioxidant | Plumb *et al.* 2002 |
| Chlorogenic acid | Juice | Antioxidant, | Duke, 2001 |
| Granatin-A | Pericarp | Antioxidant | |
| Granatin-B | Pericarp | Antioxidant | |
| Isoquercetin | Pericarp | Antioxidant | |
| Neochlorogenic acid | Pericarp | Antioxidant | |
| Punicalagin | Juice | Antioxidant Antitumorigenic | Gil *et al*. 2000 Castonguay *et al*. 1997 |
| Punicalin | Juice | Antioxidant Antitumorigenic | Gil *et al*. 2000 Castonguay *et al*. 1997 |

The above observations regarding the functional properties of pomegranate fruit revealed that most of the properties are due to the phenolics of the pomegranate fruit. Pomegranate fruit contains variety of phenolic molecules like anthocyanins,

ellagitannins, flavonoids, etc. All the parts of pomegranate reported to have one or the other functional attribute towards human health. A clinical trial on healthy male volunteers by Aviram *et al* (2000) regarding the health beneficial properties of pomegranate fruit juice revealed that, in humans, pomegranate juice consumption decreased LDL susceptibility to aggregation and retention and increased the activity of serum paraoxonase (an esterase that can protect against lipid peroxidation) by 20%.

The vivid functional properties shown by pomegranate fruit can be classified based on the parts of the fruit as follows (Table G6)

**Table G6. Current status of research on functional properties of pomegranate fruit (classified based on the parts of fruit)**

| Pomegranate Fruit part | Functional property | Reference |
|---|---|---|
| **Juice** | Antioxidant | Gil *et al.* 2000 |
| | Antiatherosclerotic | Kaplan *et al.* 2001 |
| | Platelet aggregation inhibitor | Aviram *et al.* 2000 |
| | Anti-cholesterol | Esmaillzadeh *et al.* 2004 |
| | Anticancer | Nam *et al*. 2002 |
| **Peel** | Antioxidant | Singh *et al. 2002* |
| | Antibacterial | Negi and Jayaprakash 2003 |
| | Wound healing | Murthy *et al*. 2004 |
| | Antitumor | Castonguay *et al*. 1997 |
| | Antimutagenic | Negi *et al.* 2003 |
| **Seed** | Antioxidant | Singh *et al. 2002* |
| | Antitumor | Hora *et al*. 2003 |
| | Estrogenic | Mori-Okamoto *et al*. 2004 |
| | Anticancer | Kohno *et al*. 2004 |
| **Pith and carpellary membrane** | **No reports so far** | **No References** |

The extensive review of available literature clearly indicated that pomegranate is a fruit of nutraceutical importance. However, it also distinctly demarked the lack of scientific literature on functional properties of pith and carpellary membrane, which are strategically evolved to anchor and protect the seeds (arils). This has prompted to undertake the present work to study the functional properties of pith and carpellary membrane of pomegranate fruit. The efforts were also made to understand the teleological role of them in health and disease status of arils.

## Scope of the present investigation

Bioactive molecules have the unique structural diversity and multiple functions of human health benefits. In addition they are also having their own function in normal functioning of the source system where they are produced. Bioactive molecules have become a major area of focus in functional foods over the past 10 years. There is an increasing interest in the use of plant derived bioactive molecules as natural preservatives for food. The bioactive molecules have also been linked to reduce risk of major chronic diseases and they are considered as safe alternative medicine. This situation is a consequence of in adequate, safe lead compounds in many therapeutic areas like neuropathy, retinopathy, nephropathy and others.

The ancient science of Indian medicine namely ayurveda has identified pomegranate as a medicinal plant. From various parts of pomegranate fruit, nearly 20 bioactive molecules have been identified each with varied functional properties. Recent clinical trials provide sufficient evidence for beneficial properties from the edible parts of pomegranate fruit, called arils, which originates from pith and is well protected by carpellary membrane. Both tissues together constitute 13% (w/w) of the fruit. Teleologically, such tissues may be rich sources of bioactive molecules, which may be able to prevent discoloration of arils and subsequent browning, a disorder of high economic importance. Literature survey revealed that, so far no attempt has been made to study the chemical composition and bioactive properties of pomegranate fruit pith and carpellary membrane. Are these tissues constituted with bioactive molecules of any health benefit of importance either for pomegranate fruit arils or for human health? This is an intriguing question that prompted us to undertake this research work.

# *Chapter I*

## *Isolation and characterization of bioactive molecule from pith and carpellary membrane of pomegranate fruit*

## 1.1. Introduction and review of literature

Plants have also formed the basis for sophisticated traditional medicine systems that have been in existence for thousands of years in the countries such as China and India (Chang & But, 1986; Kapoor, 1990). These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world inhabitants rely mainly on traditional medicines for their primary health care (Farnswoth *et al*. 1985). Plant products also play an important role in the health care systems of remaining 20% of the population mainly residing in developed countries. The higher plants are 'treasure houses' for a repertoire of phytochemicals also called as bioactive molecules, which serve as valuable drugs that helped to combat several fatal diseases world over. Well known examples of plant derived bioactive molecules include: the antimalarial drug quinine, obtained from the bark of *Cinchona officinalis*; the analgesics, codeine and morphine from *Papaver somniferum;* the antyhypersensitivereserpine from *Rauwolfia serpentina;* and the cardiac glycoside, digitoxin, from *Digitalis purpurea* (Kinghorn, 1994).

### 1.1.1. Importance of isolating the bioactive molecules from plants

The use of herbal and other naturally based medicines has a long history. However, the utilization of pure bioactive molecules as compared to the usage of whole plant or other crude preparations for therapeutic or experimental reasons have several advantages, which include

- Pure bioactive molecules can be administered in reproducible, accurate doses, with obvious benefits from an experimental or therapeutic point of view.
- It can lead to the development of analytical assays for particular compound or for classes of compounds.

- It permits the structural determination of bioactive compounds that may enable the production of synthetic molecules, incorporation of structural modifications, and a rationalization of mechanism of action.

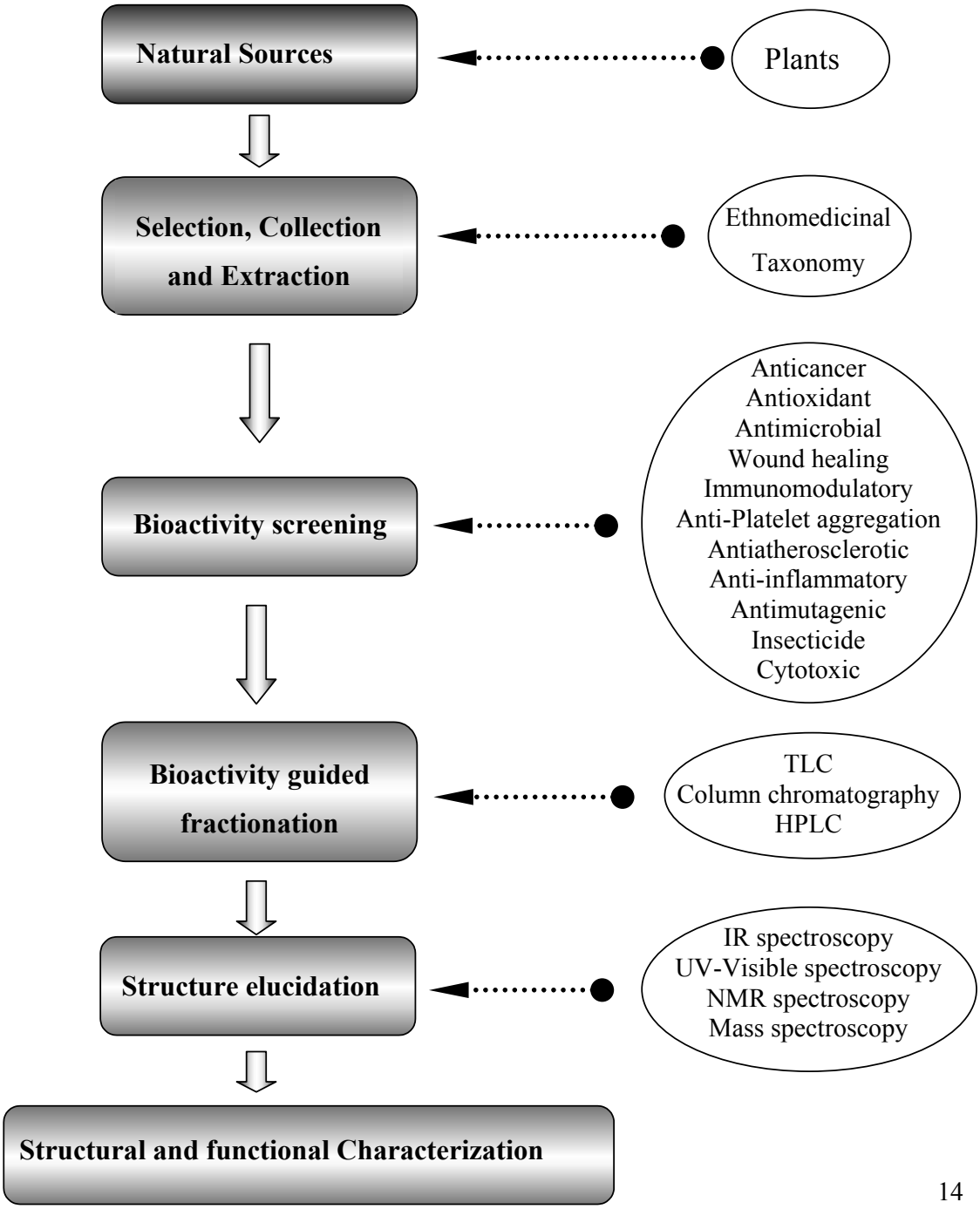
### 1.1.2. Techniques of isolation and purification of bioactive molecules from plants

Research on bioactive molecules has undergone a renaissance in recent years. This renewed interest parallels the development and availability of a range of new bioassays on the one hand, and more sophisticated and precise techniques of isolation, separation, and purification on the other. Primary requirement to search for a bioactive compound is an appropriate assay to screen the source material for bioactivities like antioxidant, antibacterial, or cytotoxicity etc., should be simple, specific, and rapid. Usually an *In-vitro* test is more desirable than *in-vivo* assay, as animal trials are expensive and time consuming and involves ethical controversies.

Due to the vast reservoir of plants, the variation of different parts in a plant and diverse chemical structures and physicochemical properties of the bioactive phytochemicals, it is nearly impossible to have any definitive procedure or protocol for the isolation and characterization of a bioactive molecule. However, isolation and characterization of bioactive phytochemicals follows few definitive steps like selection and collection of plant material based on the ethno-botanical information, followed by extraction using variety of solvents and screening for bioactivity. The crude solvent extracts were subjected for bioassay of interest. The active extract can then be subjected for isolation and purification of the active principle (bioactive molecule) responsible for the bioactivity. Isolation and purification can be achieved by column chromatographic techniques. Modern instruments like preparative HPLC hastens the process of purification of the bioactive molecule. The purified compound can be identified using

data obtained from variety of spectroscopic techniques like UV-Visible, IR, NMR, Mass spectroscopy followed by functional characterization. Basic steps involved in isolation and characterizations of bioactive molecule can be generalized as follows (Flow chart 1.1)

**Flow chart 1.1. Schematic representation of steps involved in isolation and characterization of a bioactive molecule**



### 1.1.2.1. Selection, collection and extraction

Selection of the plant material is one of the important steps before proceeding in to the isolation and characterization of bioactive molecules. Basic criteria for selection of plant material can be given as follows

**Criteria for selection of plant material**

- Usage in traditional medicine
- Unexplored plant or plant parts
- Easily accessible at all times
- Available in bulk quantity
- Teleological importance

Once the plant material is selected, the next step is its collection then drying using suitable methods. It is common to dry the plant material in the shade at room temperature, if the targeted bioactive molecule is thermolabile. Otherwise it can be dried in hot air oven, usually at less than 48°C. Once the dried plant material is ready, in common practice it will be powdered, prior to extraction.

### 1.1.2.2. Solvent extraction

Extraction from the plant is a trial-and-error exercise in which different solvents are tried under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process is monitored by the most appropriate bioactivity assay. There are various extraction methods for screening the plants for bioactivity like cold extraction and soxhlet extraction. Selection of extraction methods depends upon the thermal stability of the targeted molecule. Several organic solvents

with varying elution effects find common usage in phytochemical research for extracting phytochemicals (Table 1.1)

**Table 1.1. Eluotropic series of some commonly used solvents for extraction**

| Solvent | Dielectric constant (at 20 $^{o}$C) | Boiling point (at 760 mm) $^{o}$C |
|---|---|---|
| n-Hexane | 1.89 | 68.7 |
| Heptane | 1.92 | 98.4 |
| Cyclohexane | 2.02 | 81.4 |
| Carbon tetrachloride | 2.24 | 76.8 |
| Benzene | 2.28 | 80.1 |
| Chloroform | 4.81 | 61.3 |
| Diethyl ether | 4.34 | 34.6 |
| Ethyl acetate | 6.02 | 77.1 |
| Pyridine | 12.30 | 115.3 |
| Acetone | 20.70 | 56.5 |
| Ethanol | 24.30 | 78.5 |
| Methanol | 33.62 | 64.6 |
| Water | 80.37 | 100.0 |

Ref: George *et al*. 2001

Usually dried powder of plant material is preferred for extraction to avoid interference of water. For isolating an unknown bioactive molecule from a plant material, the samples are normally extracted with a variety of solvents, sequentially from low polarity to high polarity. In sequential extraction the molecules will be eluted based on their solubility in respective solvents. Since same material is used for extraction with all solvents sequentially with increasing polarity, extracting the same chemicals in more than one solvent system can be avoided. Most preferred solvent sequence used for extracting the plant material with unknown composition is as follows, which is based on the polarity of solvents (George *et al*., 2001).

Hexane<Chloroform<Ethyl acetate<Acetone<Methanol<Water

Once the extracts are ready, they will be subjected to different *in vitro* bioactivity assays. The active extract can then be fractionated and purified employing bioassay-guided fractionation.

**1.1.2.3. Purification of the bioactive molecule**

Paper, thin layer, column chromatographic methods have been used for the separation and purification of many bioactive molecules. However, due to lack of recovery of sample, and difficulties in quantification thin layer and paper chromatography were used as an analytical tool in comparing the different fractions. Column chromatography and TLC are still widely used because of convenience, economy, availability of variety of stationary phases for separation of phytochemicals with varying nature and availability of solvents. Silica, alumina, cellulose, polyamide, etc., are still widely used for separating the phytochemicals. Plant materials contain highly complex profiles of phytochemicals; isocratic separation cannot achieve satisfactory separation. Multiple mobile phases with increasing polarity are therefore useful for good separation. In majority, TLC was used for analytical purpose for analyzing the fractions that were fractionated by column chromatography. In the present study, silica gel column chromatography technique has been employed for separation of bioactive molecule and thin layer chromatography (TLC) as an analytical tool.

**1.1.3. Structural elucidation of the bioactive molecules**

The process of structural determination involves accumulating data from a wide range of spectroscopic techniques, such as UV-visible, Infra Red (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy each of which gives some basic clue regarding the structure of the molecule.

The principle of the spectroscopy is that, if electromagnetic radiations passed through an organic molecule, some of the radiations are absorbed and others are virtually unaffected. By plotting the changes in absorption against electromagnetic radiation passed, spectrum can be produced. This spectrum is unique for a bond in a molecule / for a molecule / for a class of molecule. Based on these spectra, structures of organic molecules can be elucidated.

Although almost all parts of the electromagnetic spectrum are used for studying matter in organic chemistry, here we are mainly concerned with energy absorption from three or four regions- Ultraviolet (UV), Visible, Infrared (IR), radio frequency, and electron beam (Kemp, 1991), which are most often used for structural elucidation.

**1.1.3.1. UV-Visible spectroscopy**

Qualitative analysis may be performed in the UV-visible regions to identify certain classes of compound both in the pure and in biological mixtures. However, it is used preferentially for quantitative analysis by making use of the fact that aromatic molecules are powerful chromophores in the UV range. Nature of the compound can be determined making use of UV-visible spectroscopy (Kemp, 1991a).

**1.1.3.2. Infra Red Spectroscopy**

When Infra Red light is passed through a sample of an organic compound, some of the frequencies are absorbed, while other frequencies are transmitted through the sample without being absorbed. A plot of absorbance or transmittance against frequency is called infrared spectrum. Infrared absorptions are associated with vibrational changes within the molecule. Infra red spectroscopy is therefore basically vibrational spectroscopy. Different bonds (C-C, C=C, C≡C, C-O, C=O, O-H, N-H etc) have different vibrational frequencies, and the presence of these bonds in an organic molecule

can be detected by identifying the characteristic frequency as an absorption band in the infrared spectrum (Kemp, 1991b).

#### 1.1.3.3. Nuclear magnetic resonance spectroscopy (NMR)

NMR is concerned with the magnetic properties of certain atomic nuclei, notably the nucleus of the hydrogen atom-the proton-and that of the carbon-13, an isotope of carbon. Studying a molecule by NMR spectroscopy enables us to record differences in the magnetic properties of the various magnetic nuclei present, and to deduce in large measure what the positions of these nuclei are within the molecule, and also which atoms are present in neighboring groups. It can measure how many atoms are present in each of these environments (Kemp, 1991c).

#### 1.1.3.4. Mass spectrometry

In the simplest mass spectrometer, organic molecules are bombarded with electrons or by some other means such as laser disorption, converted to highly energetic positively charged ions (molecular ions or parent ions), which can break up into smaller ions (fragment ions, or daughter ions). The molecular ions, the fragment ions and the fragment radical ions are separated by deflection in a variable magnetic field according to their mass and charge, and generate a current (ion current) at the collector in proportion to their relative abundance. A mass spectrum is a plot of relative abundance against the ratio of mass/charge. Using mass spectrometry, relative molecular mass (molecular weight) with high accuracy can be determined and exact molecular formulae with the help of places where molecule preferred to fragment (Kemp, 1991d).

In the present work, bioactive molecule from pith and CM was isolated and purified by bioactivity guided solvent extraction, column chromatography, and HPLC. The techniques viz. UV-Visible, IR, NMR and Mass spectroscopy were employed to

characterize the structure of the bioactive molecule. Further, it was hydrolyzed and its derivatives were characterized.

# 1.2. Materials and methods

## 1.2.1. Isolation of bioactive compound from pith and carpellary membrane of pomegranate fruit

### 1.2.1.1. Chemicals

All the organic solvents used for extraction and column chromatography were of AR grade from E. Merck (Mumbai, India). Methanol, Acetonitrile, used for HPLC was HPLC grade from Ranbaxy fine chemicals limited (India). Tris–HCl was from Sisco Research Laboratories (India). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Butylated hydroxy anisole (BHA), Ellagic acid, Gallic acid were from Sigma Chemical, USA. Acetic anhydride was from S.d. Fine Chemicals (India), and Silica gel (60-120 mesh) for column chromatography, Silica Gel for TLC, trichloroacetic acid and pyridine from Qualigens Fine Chemicals (India).

### 1.2.1.2. Plant material

Mature pomegranate (var. Ganesh) fruits ≈ 100 Kg were harvested from orchards in the Bagalkot district, India, and brought immediately to the Central Food Technological Research Institute, Mysore. Pith and CM were separated from the fruit manually, dried in a hot air oven at 40° C for 24 h and powdered to 60 mesh in an apex grinder.

### 1.2.1.3. Extraction of pith and carpellary membrane

About 50 g of dry powder of pith and CM was extracted with a series of solvents of increasing polarity like hexane, chloroform, ethyl acetate and methanol in a soxhlet apparatus. Solvent was removed by distillation and assayed for antioxidant activity using DPPH radical scavenging method. Since methanol extract showed maximum antioxidant activity (Table 1.2), it was selected as a source extract for isolation of bioactive molecule.

**1.2.1.4. Fractionation of methanol extract of pith and CM**

The methanol extract (≈20g) was subjected to column (450 x 40 mm) chromatography using silica gel (60 –120 mesh) and eluted stepwise with hexane, chloroform followed by linear gradient of chloroform: ethyl acetate: methanol (100:0:0 to 30:20:50 v/v/v). About 97 fractions measuring 250 ml were collected, concentrated by distillation and after analysis by TLC pooled into 6 groups, labeled as fraction No. 1–6 (Flow chart 1.2) and assayed for antioxidant activity using DPPH free radical scavenging assay.

**1.2.1.5. Thin layer chromatography (TLC)**

An aliquot of column fraction was spotted on to a silica gel TLC plate (20x20 cm). The plates were developed in ascending direction from 18 to 19 cm of height with different proportions of chloroform and methanol as mobile phase. After air-drying, the spots on the plate were located by exposure to iodine in an iodine chamber.

**1.2.1.6. Purification of active fraction**

The active fraction (fraction No. 6), which showed maximum antioxidant activity (Fig. 1.1) was further subjected to silica gel column (450x40 mm) chromatography and eluted first with linear gradient of chloroform and ethyl acetate (100:0 to 0:100 v/v) and then with linear gradient of ethyl acetate and methanol (100: 0 to 50:50 v/v). About 88 fractions measuring 250 ml were collected, concentrated by distillation and after analysis by TLC, pooled into five groups, labeled as fraction Nos. 1′–5′ (Flow chart 1.2).

**1.2.1.7. Purification of bioactive molecule by preparative high performance liquid chromatography**

Partially purified active fraction (Fraction No. 3′ from the above procedure) was further purified by preparative HPLC, using a Shimadzu Preparative Liquid Chromatograph LC-8A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 100 μl sample loop and a 250x20 mm, i.d. 5 μm, Shimpack

C-18 column (Kyoto, Japan). The fraction was eluted with an isocratic solvent mixture comprising water: methanol: ethyl acetate (70:25:05) with a flow rate at 5 ml/min. The UV detection was carried out at 260 nm with a Shimadzu diode array detector, series SPDM10 Avp, Shimadzu (Singapore).

**1.2.1.8. Antioxidant activity**

The antioxidant activity of pomegranate pith and CM extracts, column chromatography fractions and purified bioactive molecule was measured using DPPH radical scavenging assay according to Moon and Terao (1998). About 100 µl of test sample was mixed with 0.9 ml Tris HCL buffer (pH 7.4), to which 1 ml of DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV-Visible spectrophotometer (UV-160A, Shimadzu co. Japan). All measurements were made in triplicates with BHA as positive control. Antioxidant activity was calculated with the following formula.

$$\text{Antioxidant activity}(\%) = \left(1 - \frac{A_{\text{sample}\,(517nm)}}{A_{\text{control}\;\;(517nm)}}\right) x100$$

Results expressed as $EC_{50}$, which represents the concentration of test sample that scavenged 50% radicals.

**1.2.1.9. Acetylation of purified compound**

Acetylation was carried out according to the method of Markham (1982). About 20 mg of bioactive molecule was dissolved in 0.5 ml pyridine to which 0.5 ml acetic anhydride was added and stirred in a stoppered conical flask overnight at room temperature. Reaction mixture was then poured into ice-cold water (≈50 ml) with constant stirring and left for 1 h. The insoluble acetylated molecule was separated by filtration, using a Whatman filter paper No. 1.

## 1.2.2. Identification of bioactive compound

### 1.2.2.1. UV-Visible spectrophotometry

UV–visible spectra of purified compound was recorded on a *Shimadzu UV- 160A* UV-Visible spectrophotometer at room temperature. The region from 200–800 nm was employed for scanning. About 2 mg sample dissolved in 20 ml methanol was used for recording the spectrum.

### 1.2.2.2. Infra red spectroscopy

IR spectrum of purified compound was recorded on a Perkin–Elmer FT-IR Spectrometer (Spectrum 2000) at room temperature. A region from 400 to 4000 $cm^{-1}$ was scanned. Sample was dissolved in DMSO-d6 and spectrum recorded along with solvent blank for correction.

### 1.2.2.3. Matrix-assisted laser disorption ionization mass spectrometry (MALDI-MS)

MALDI-MS analysis was carried out on a Kompact Seq, Kratos Analytical spectrometer with time of flight detector at room temperature and the matrix used was α-cyano-4-hydroxy cinnamic acid.

### 1.2.2.4. Gas chromatography–mass spectrometry (GC– MS)

GC–MS analysis of the acetylated compound was carried out using an Agilent 6890 GC instrument equipped with a 5973 N mass selective detector. An HP-5 MS capillary column (length 30 m, internal diameter 0.25 mm, .lm thickness 0.25 lm) was used. Helium was the carrier gas employed with 1.2 ml/min constant flow mode. The oven temperature was maintained at 50° C for 2 min and ramped to 300° C at 10° C/min and held there for 5 min. Inlet temperature was 300° C, interface temperature was 280° C, ion source temperature was 230° C and quadrupole temperature was 150° C.

#### 1.2.2.5. Nuclear Magnetic Resonance Spectroscopy

Two-dimensional heteronuclear multiple quantum coherence transfer (2D HMQCT-NMR) spectra were recorded on a Bruker DRX500 NMR instrument operating at 500 MHz, for 6 hours at room temperature. The region from 0 to 12 ppm for $^{1}H$ and 0–200 ppm for $^{13}C$ was employed for scanning. Signals were referred to tetramethylsilane to within ± 0.01 ppm. About 30 mg sample dissolved in 0.5 ml DMSO-d6 was used for recording the spectra.

### 1.2.3. Acid hydrolysis of purified compound

The purified compound was identified as punicalagin, a high molecular weight polyphenol based on spectroscopic data, which was. This molecule was hydrolyzed in acidic conditions as follows: About 1 g of purified molecule was dissolved in 10 ml of triple distilled water; Solution was raised to pH 1 by adding concentrated HCl and kept stirring overnight on a magnetic stirrer. After about 18 hours of stirring, the solution was diluted to 25 ml using triple distilled water and about 10 ml methanol was added. The solution was filtered through Whatman filter paper No. 42 (ash less) to remove any precipitate formed. The filtrate was then concentrated by flash evaporation and then lyophilized to a powder.

#### 1.2.3.1. Analysis of hydrolyzed products of purified compound by analytical HPLC

The hydrolyzed punicalagin (1 mg/ml triple distilled water) was analyzed in analytical HPLC, using a Shimadzu Analytical Liquid Chromatograph LC-10A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 20 µl sample loop and a 250 x 4.6 mm, i.d. 5 µm, Shimpack C-18 column (Kyoto, Japan). The fraction was eluted with a binary mobile phase comprising: (A) 2% formic acid in HPLC grade water; (B) Acetonitrile. The elution profile was 0-5 min, 95-90 % A (linear

gradient); 5-10 min, 90-85 % A (linear gradient); 10-15 min, 85-80% A (linear gradient); 15-20 min, 80-40% A (linear gradient); 20-25 min, 40-10% A (linear gradient); 25-28 min, 10 % (isocratic); 29 min stop. The injection volume was 10 µl. Flow rate 1 ml/min. The UV detection was carried out at 280 nm with a Shimadzu diode array detector, series SPDM10 Avp, Shimadzu (Singapore). Gallic acid, ellagic acid and punicalagin were used as internal standards under same conditions of HPLC and data was used to identify the respective hydrolyzed fractions of punicalagin.

**1.2.3.2. Separation of hydrolyzed products from punicalagin by preparative HPLC**

Hydrolyzed punicalagin was fractionated by preparative HPLC, using a Shimadzu Preparative Liquid Chromatograph LC-8A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 100 µl sample loop and a 250x20 mm, i.d. 5 µm, Shimpack C-18 column (Kyoto, Japan). The fraction was eluted with a binary mobile phase comprising: (A) Water (HPLC grade); (B) Acetonitrile (HPLC grade). The elution profile was 0-5 min, 95-90 % A (linear gradient); 5-10 min, 90-85 % A (linear gradient); 10-15 min, 85-80% A (linear gradient); 15-20 min, 80-40% A (linear gradient); 20-25 min, 40-10% A (linear gradient); 25-28 min, 10 % (isocratic); 29 min stop. Flow rate 5 ml/min. The UV detection was carried out at 280 nm with a Shimadzu diode array detector, series SPDM10 Avp, Shimadzu (Singapore). Each individual peaks collected separately, concentrated and lyophilized.

**1.2.3.3. Liquid chromatography–electro spray ionization-mass spectrometry (LC–ESI-MS) analysis of hydrolyzed products from punicalagin**

LC–ESI-MS was conducted on Shimadzu LCMS-2010A system. Chromatographic separation of hydrolyzed fractions from punicalagin was conducted using a C-18, reversed-phase (5 µm) column (250 x 4.6 mm I.D.; Shimpack) and isocratic mobile phase comprising acetonitrile: water (90-10 v/v) with a flow rate of 0.5

ml/min. HPLC detection of fractions was by means of an ultra violet (UV) detector set at an absorbance of 260 nm at room temperature. The mass spectra in the negative ion mode were generated on a quadrapole LC–ESI-MSD under the following conditions: probe temperature 200°C; CDL temperature 250°C; Block temperature 200°C; Detector voltage 1.5 kv, Nebulizer gas flow 1.5 L/min; Drying gas 2.0 L/min; mass range = 50–1500 D. The mobile phase was utilized in isocratic over a total run time of 50 min.

## 1.3 Results and Discussion

### 1.3.1. Screening of pith and carpellary membrane extracts for antioxidant potential

The pomegranate fruits variety Ganesha, about 100 KG were freshly harvested from orchards of Kaladagi village, Bagalkot District, India, brought to Central Food Technological Research Institute, Mysore. Fruit parts were separated manually; pith and carpellary membrane were collected (5.98 KG) and dried in a hot air oven for 24 hours at 40°C. Completely dried material was powdered in an apex grinder to 60 –120 mesh; yield of the dry powder of pith and CM was about (23.1%).

About 50 g of pith and CM powder was extracted sequentially with organic solvents of increasing polarity in soxhlet apparatus. The dry powder was first extracted with hexane in a soxhlet apparatus in order to separate non-polar compounds, the yield of which was negligible (0.2% w/w). Then it was extracted with chloroform followed by ethyl acetate, which are known to extract low molecular weight phenolics; yields of the extract for these solvents was also negligible (0.6% and 1.2% w/w, respectively). Finally it was extracted with methanol, which is known to extract both low and high molecular weight phenolics (Hagerman *et al*., 1998). Yield of methanol extract was highest (49% w/w). The extracts were collected separately, concentrated by distillation followed by lyophilization. The extracts were subjected for antioxidant assay by DPPH method (Table. 1.2). Antioxidant activity of both ethyl acetate extract (92.1±0.32 %/100µg) and methanol extract (94.5±0.02 %/100µg) was high compared to hexane extract (no activity) and chloroform extract (3.35±0.04 %/100µg/ml). Since the antioxidant activity and yield of methanol extract was higher than the ethyl acetate extract (Table 1.2), it was selected as a source extract for isolating a bioactive molecule.

**Table 1.2 Yield and antioxidant activity of different solvent extracts of pomegranate pith and CM of pomegranate fruit.**

| Extract | Yield (% W/W) | Antioxidant activity (%/100 μg/ml) |
|---|---|---|
| Hexane extract | 0.3 | NA |
| Chloroform extract | 0.12 | 3.35±0.04 |
| Ethyl acetate extract | 1.3 | 92.1±0.32 |
| Methanol extract | 49.6 | 94.5±0.02 |

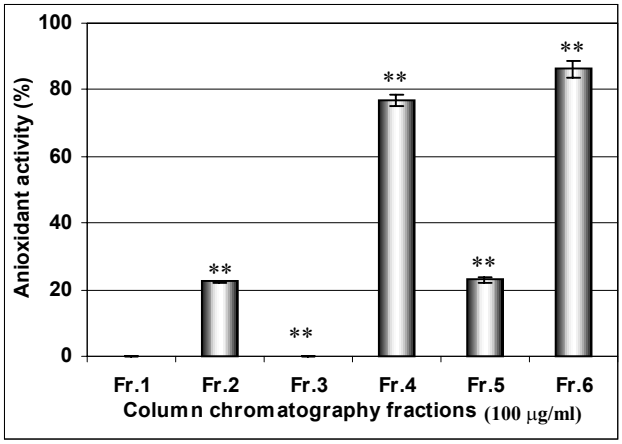
**NA = No activity**

- Values are mean ± standard error of four replicates

### 1.3.2. Isolation and purification of antioxidant molecule

About 20 g of dried powder of methanol extract of pith and CM obtained by lyophilization was coated on activated silica gel by mixing and loaded on a column (length 45 cm; diameter 4 cm), packed with 120 g of pre-activated silica gel (60-120 mesh). Methanol extract was eluted with chloroform: ethyl acetate: methanol in a linear gradient from 100:0:0, to 30:20:50 as solvent system and elute was collected. About 97 fractions were collected of 250 ml each; solvent was removed by distillation and subjected for thin layer chromatographic analysis. All the 97 fractions were pooled into 6 major groups based on thin layer chromatography analysis and labeled as fraction 1 – 6 (Flow chart 1.2). All the six fractions were tested for antioxidant potential by DPPH method. The fraction No. 6 showed highest antioxidant activity (86.3±2.6 %/100μg) with an $EC_{50}$ of 15.74 μg/ml reaction mixture (Fig 1.1).

**Fig 1.1 Antioxidant activity of column chromatographic fractions No. 1-6 of methanol extract of pith and CM of pomegranate fruit**

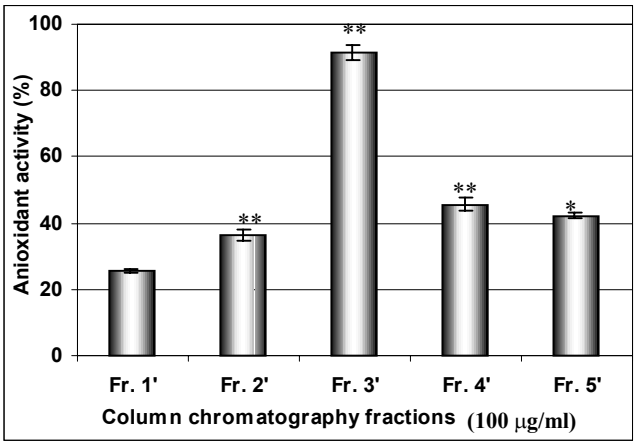



- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

Thin layer chromatographic analysis of fraction No. 6 exhibited five distinguished spots, hence it was further subjected to silica gel column chromatography to obtain pure active molecule. The sample was eluted with a gradient of chloroform: ethyl acetate solvent system in a proportion of 100:0 to 0:100 followed by ethyl acetate: methanol solvent system in a proportion of 100:0 to 50:50. Eighty eight fractions were collected each measuring about 250 ml. Solvent was removed from the fractions by distillation and pooled in to five different groups based on TLC analysis and labeled as fraction 1′ to 5′. The pooled fractions were subjected for antioxidant activity assay (Fig 1.2). Among the five fractions, fraction 3′ showed highest antioxidant activity (61.3±1.3 %/100 µg/ml) with an $EC_{50}$ of 10.86 µg/ml.

Thin layer chromatographic analysis of fraction 3′ revealed presence of slight impurities in low quantity; hence it was further subjected for fractional crystallization using solvents like isopropyl alcohol, acetone, chloroform and ethyl acetate. Since fractional crystallization technique did not purify the active principle completely, the reverse phase preparative HPLC technique was employed to achieve the purity.

**Fig 1.2 Antioxidant activity of column chromatographic fractions 1′- 5′ obtained from the of 6th column chromatographic fraction of the methanol extract of pith and carpellary membrane**

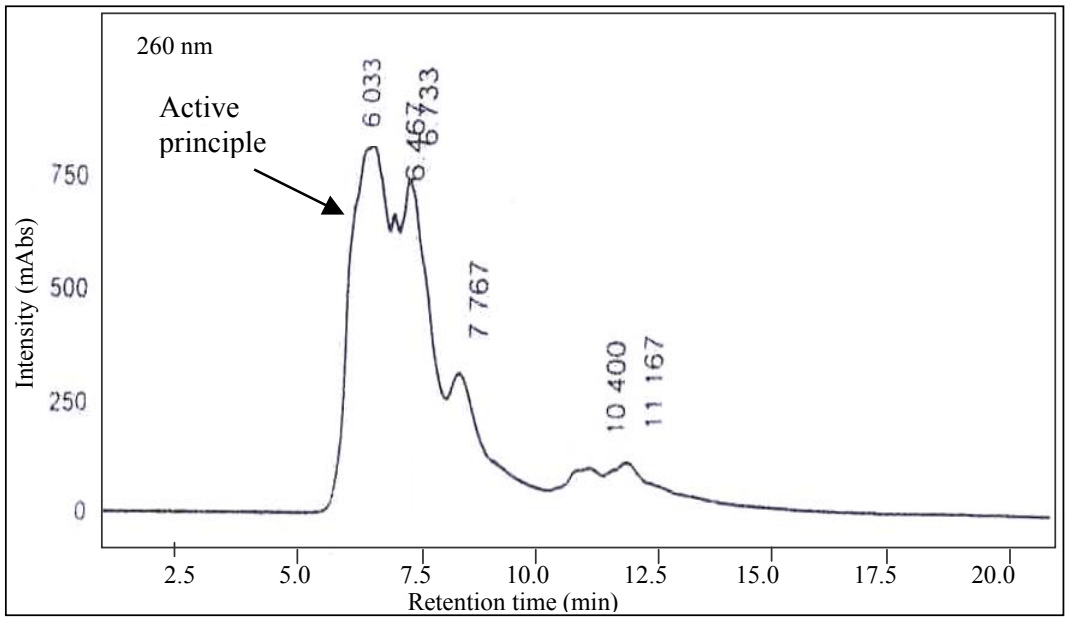



- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Purification of active molecule by preparative HPLC**

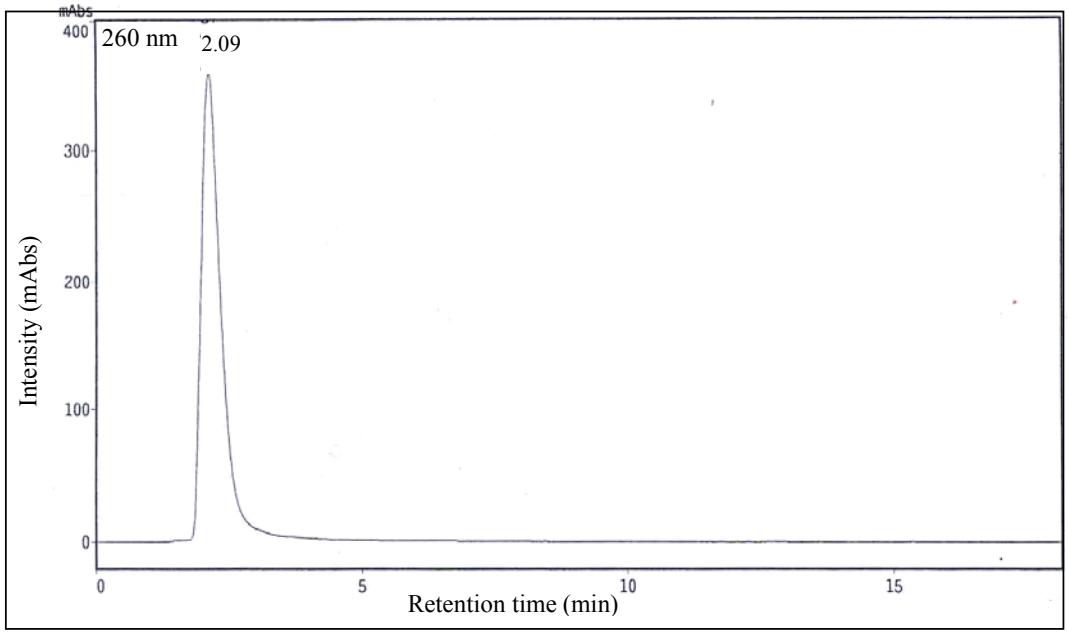
Using reverse phase preparative HPLC technique, six fractions were collected by preparative HPLC of the fraction No. 3′ (Fig 1.3).

**Fig. 1.3 Preparative HPLC chromatogram of fraction 3″ from pith and CM of pomegranate fruit**

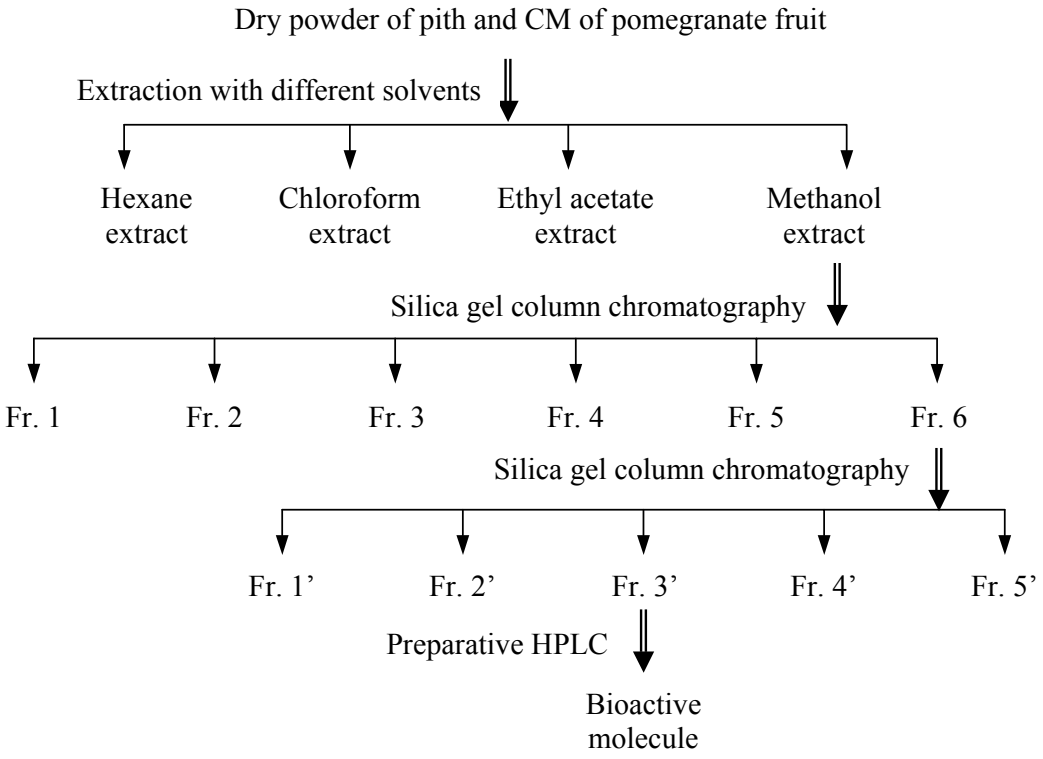


Solvent was removed from the preparative HPLC fractions by vacuum distillation and lyophilized. Five fractions (Retention time 6.47; 6.73; 7.76; 10.40; 11.16) among the six are of negligible quantity. The purity of the active principle (Retention time 6.033) obtained from preparative HPLC was ascertained by analytical HPLC analysis, which showed single peak with a retention time of 2.05 (Fig 1.4). The pure antioxidant molecule obtained by preparative HPLC, showed antioxidant potential with $EC_{50}$ of 16.66 μg/ml reaction mixture. The yield of which was about 2.3% w/w.

**Fig. 1.4 Analytical HPLC chromatogram of purified bioactive molecule from methanol extract of pith and CM of pomegranate fruit**



**Flow chart 1.2. General scheme for the isolation and purification of antioxidant compound from pith and CM of pomegranate fruit.**



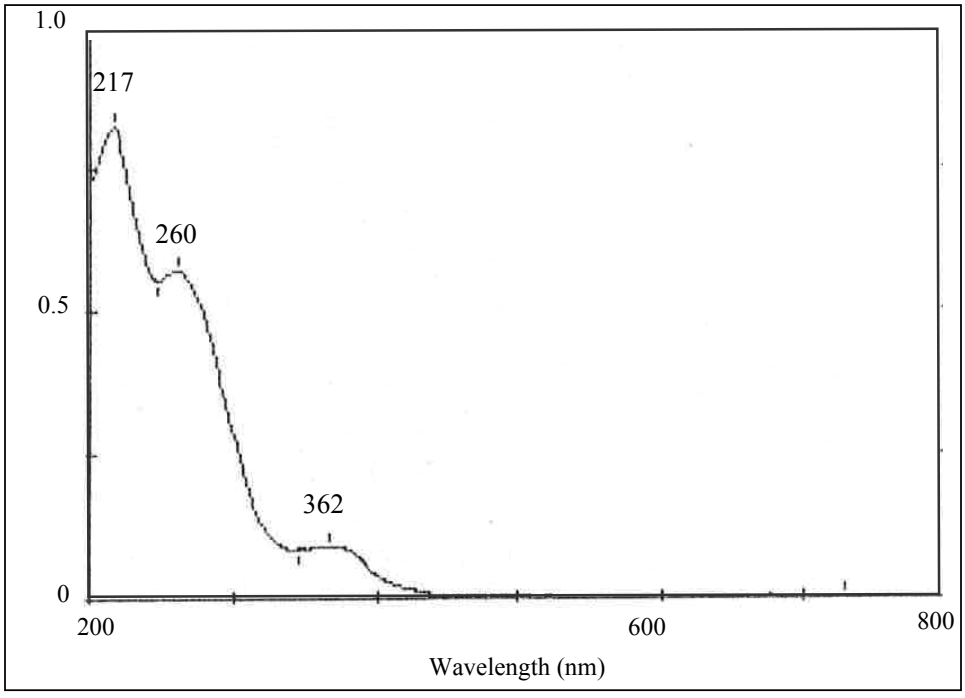
### 1.3.3. Identification of the bioactive molecule

The structure of the bioactive compound was determined by various spectroscopic techniques viz. UV-Visible spectroscopy, IR spectroscopy, NMR spectroscopy, and mass spectrometry.

#### 1.3.3.1. UV-Visible spectroscopy

The UV-Visible spectrum of the purified bioactive molecule dissolved in methanol showed characteristic absorption maxima at 217 nm, 260 nm and 362 nm (Fig. 1.5). The absorption maxima at 260 nm suggests the presence of aromatic molecule like phenolics and absorption maxima at 362 nm suggests the presence of sugar moiety

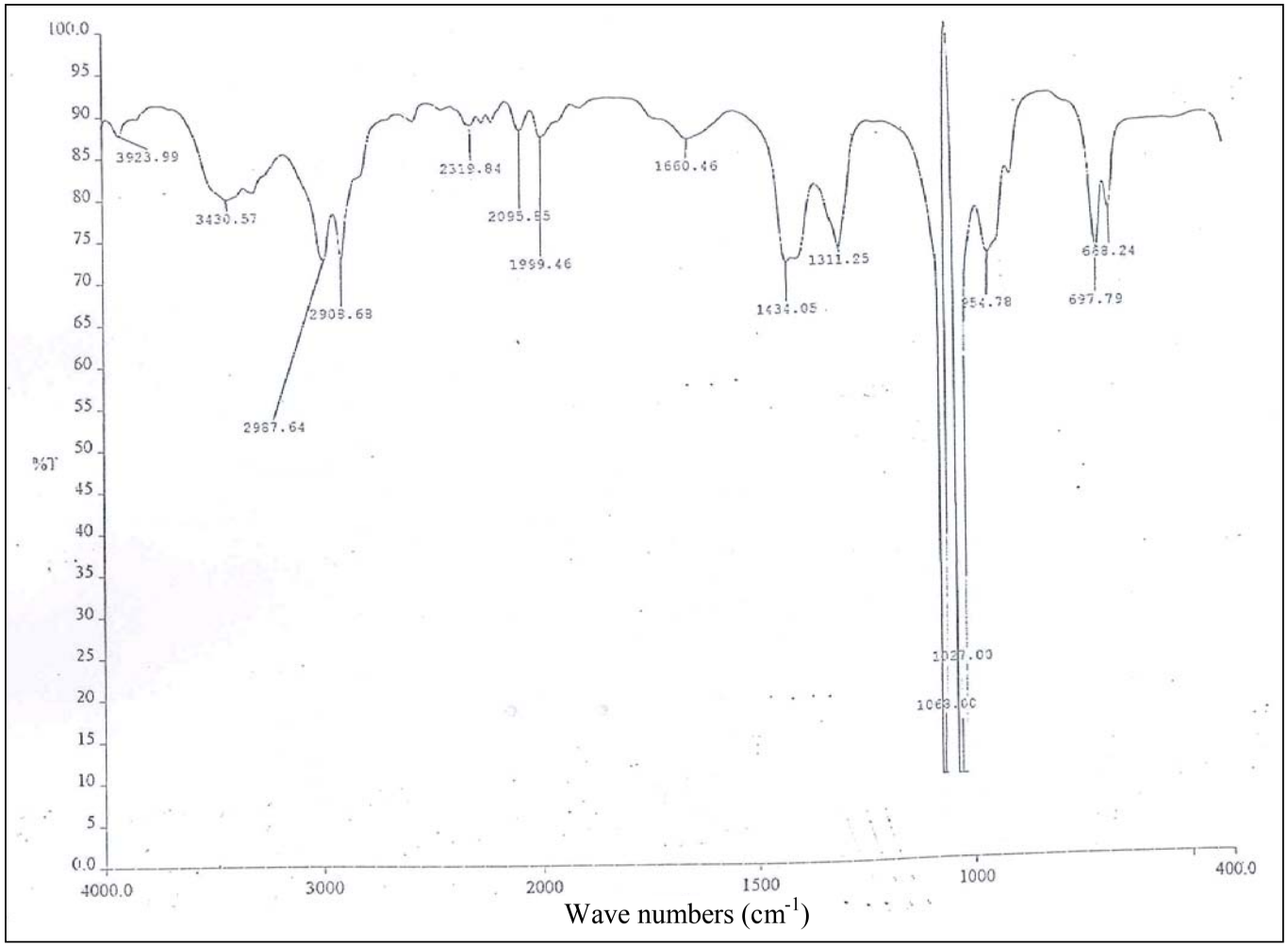
**Fig 1.5 UV-Visible spectra of bioactive molecule isolated from pith and CM of pomegranate fruit.**




#### 1.3.3.2. IR spectroscopy

Further, IR data exhibited OH stretching (3430 $cm^{-1}$) and carbonyl stretching (1660 $cm^{-1}$) besides other stretching at 2988 and 1027 $cm^{-1}$ (Fig. 1.6).

**Fig 1.6 IR spectra of bioactive molecule isolated from pith and CM of pomegranate fruit.**



#### 1.3.3.3. NMR Spectroscopy

The $^{1}H$ and $^{13}C$ NMR data were obtained from the detailed $^{1}H$ NMR, $^{13}C$ NMR and 2D HMQCT (two dimensional heteronuclear multiple quantum coherence transfer) NMR spectrum (Fig. 1.7, 1.8, 1.9). The NMR spectrum (Table 1.3) showed signals corresponding to glucose and gallagyl units. The C1 signal observed at 97.6 ppm corresponded to the β-anomer of glucose. The other glucose signals are $C_2$ – 75.7, $C_3$ – 72.7, $C_4$ – 74.0, $C_5$ – 70.7 and $C_6$ – 62.2 ppm. The corresponding proton signals were observed in the range 2.89–4.28 ppm. Most of the glucose signals were shifted up-field due to esterification at the $C_6$ and $C_4$ OH groups and $C_2$ and $C_3$ OH groups. The aromatic carbon signals from gallagyl units were observed between 105.7 and 110.7 ppm. Broad phenolic OH signals were observed in the proton region between 6.15 and 9.60 ppm. The aromatic region showed only four protons in the $^{1}H$ NMR spectrum, at 6.30, 6.44, 6.43 and 6.74 ppm consistent with gallagyl and ellagyl units, which have only two protons each, respectively, attached to the phenyl rings. The other protons were from phenolic OH groups and there are a large number of these in the molecule, as gallagyl and ellagyl units possess ten and six phenolic OH groups, respectively. This, coupled with above-mentioned spectral data, clearly indicated the structure is one as proposed in Fig. 1.12.

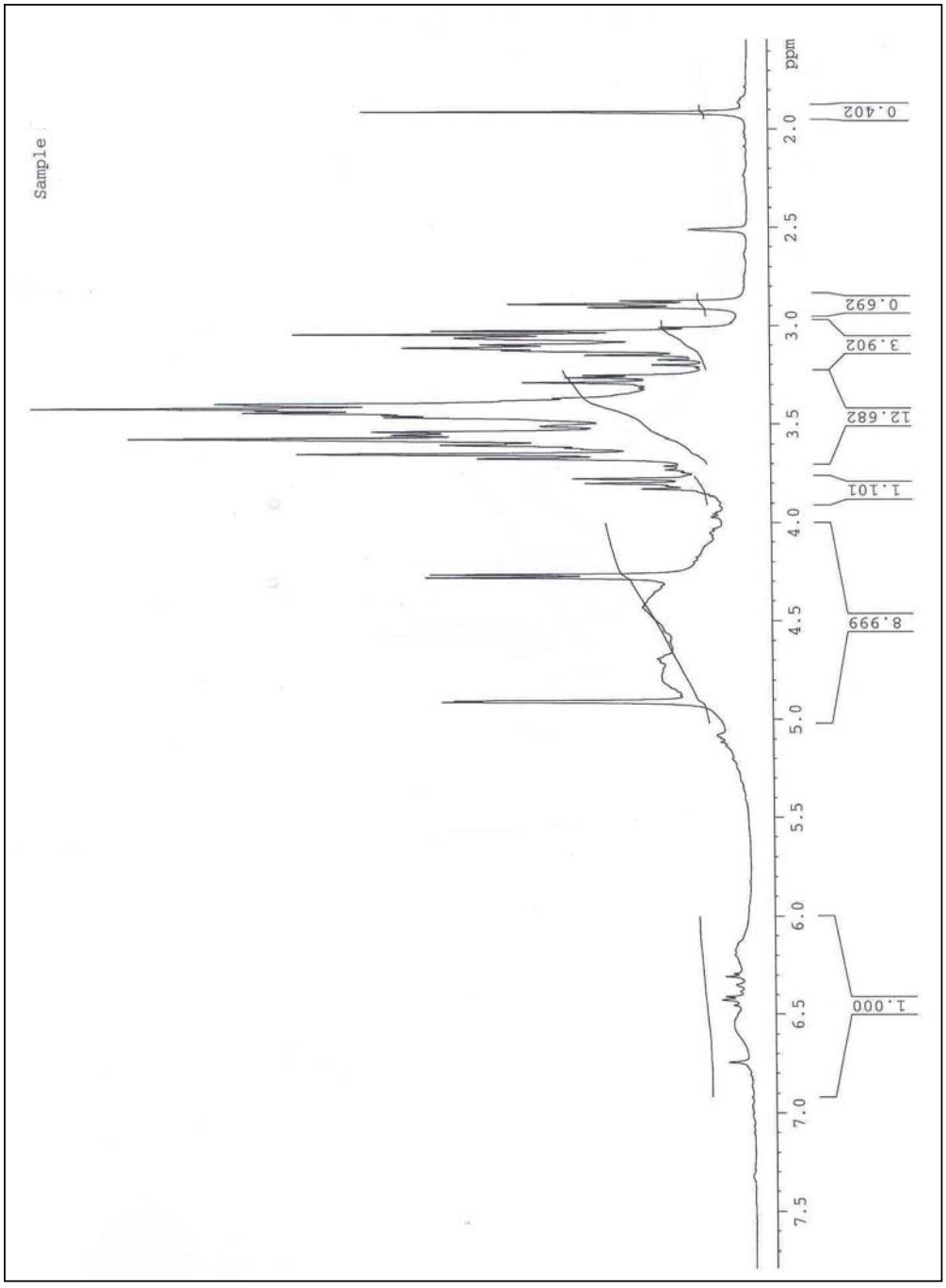



**Fig 1.7. $^{1}$H NMR spectra of bioactive molecule isolated from pith and CM of pomegranate fruit.**

**Fig 1.8.** $^{13}$C NMR spectra of bioactive molecule isolated from pith and CM of pomegranate fruit.

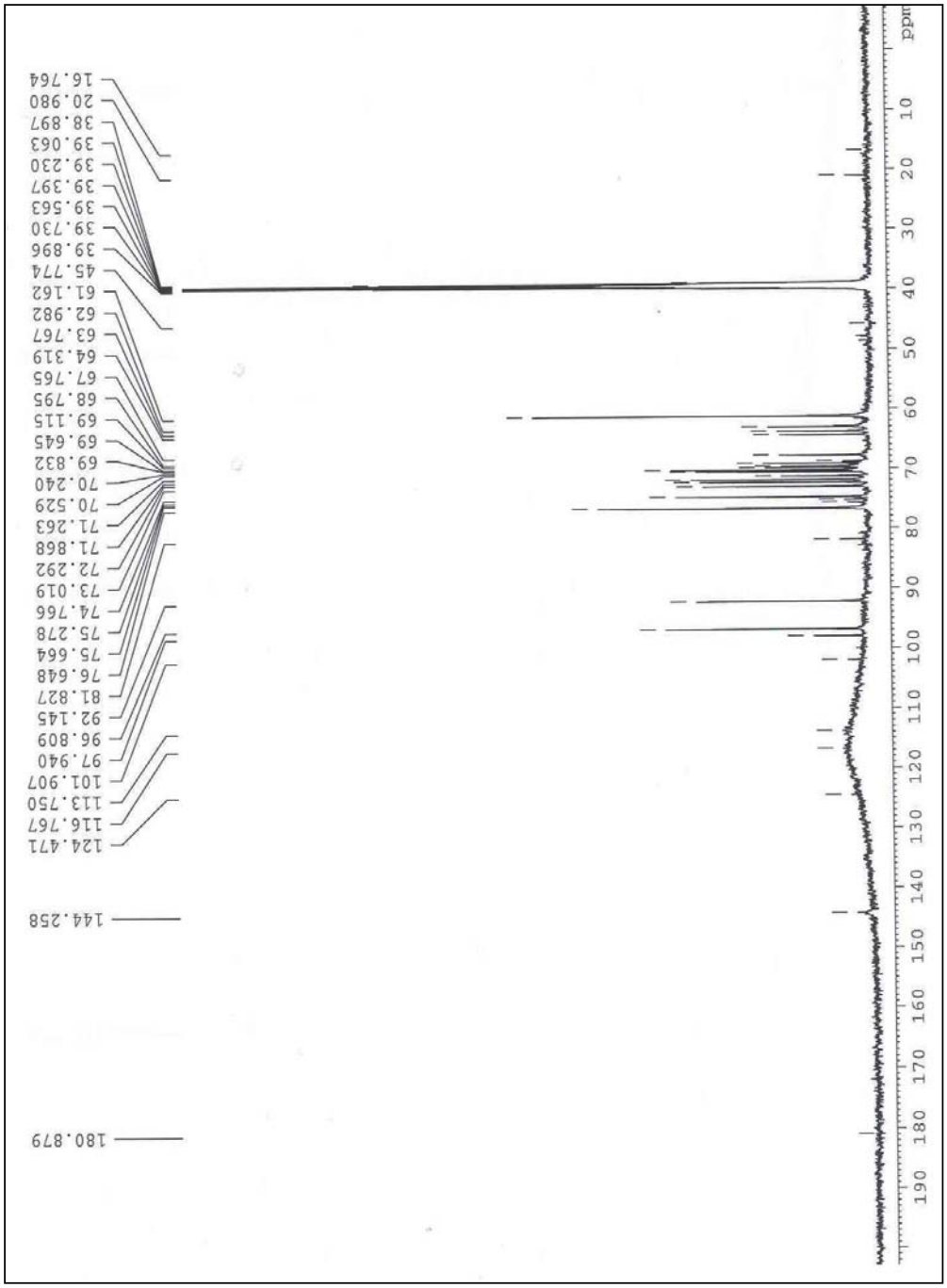


**Fig 1.9. Two-dimensional heteronuclear multiple quantum coherence transfer NMR spectrum (2D-HMQCT) of bioactive molecule isolated from pith and CM of pomegranate fruit.**

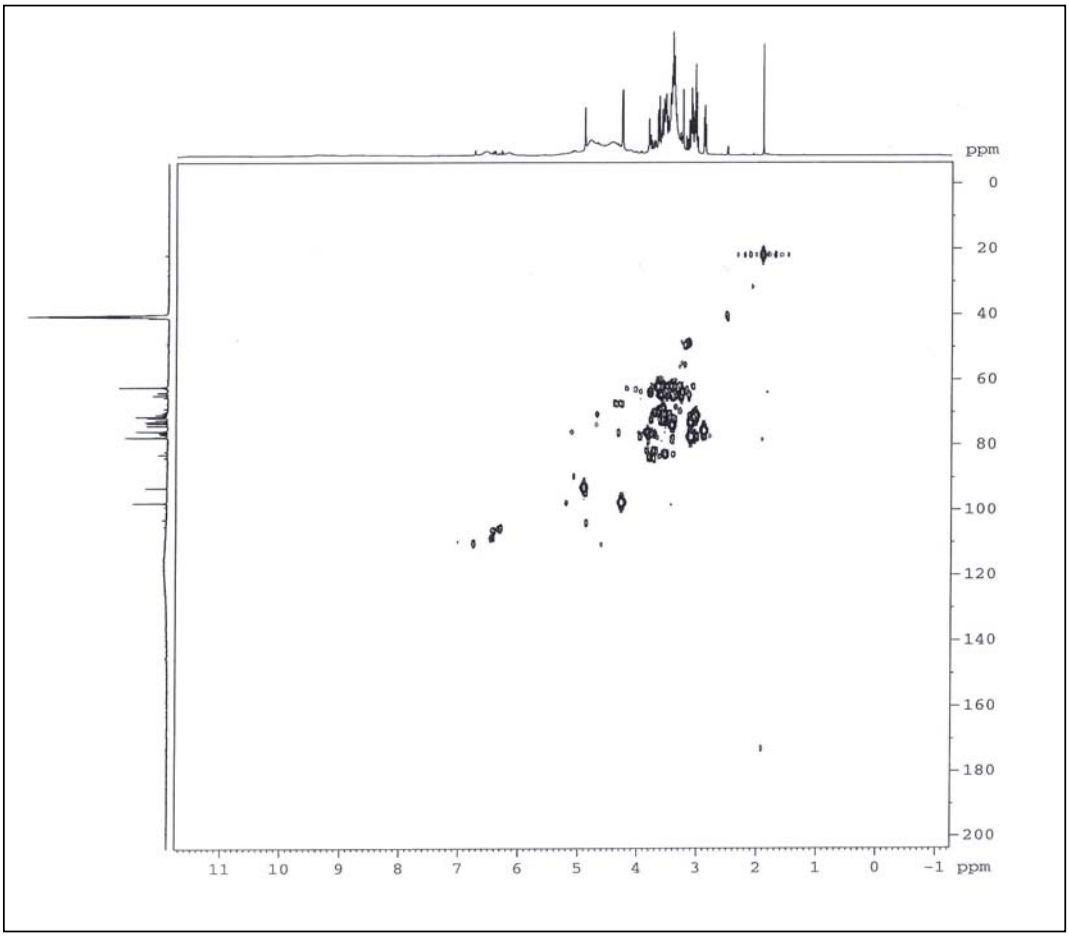


**Table 1.3. $^{1}H$ and $^{13}C$ data ($\delta$ in ppm) of bioactive molecule obtained from 2D HMQCT NMR spectroscopy**

| $^{13}C$ NMR | Assignment | $^{1}H$ NMR | Assignment |
|---|---|---|---|
| 62.2 | $^{a}C_6$ | 3.65 | $^{a}H_{6a}$ |
| | | 3.43 | $^{a}H_{6b}$ |
| 70.7 | $^{a}C_5$ | 3.54 | $^{a}H_5$ |
| 72.7 | $^{a}C_3$ | 3.53 | $^{a}H_3$ |
| 74.0 | $C_4$ | 3.42 | $H_4$ |
| 75.7 | $C_2$ | 2.89 | $H_2$ |
| 97.6 | $C_1$ | 4.28 | $H_1$ |
| 105.7 | C-Ar | 6.30 | Ar-H |
| 106.3 | C-Ar | 6.44 | Ar-H |
| 108.1 | C-Ar | 6.43 | Ar-H |
| 110.7 | C-Ar | 6.74 | Ar-H |
| | | 6.15 | Phenolic OH (Broad) |
| | | 6.56 | Phenolic OH (Broad) |
| | | 7.9-9.6 | Phenolic OH (Broad) |

[a]Assignments are interchangeable

### 1.3.3.4. Mass spectroscopy

**The MALDI-MS** spectrum (Fig 1.10) of the compound showed the mass of molecular ion as 1109 (hydrated parent ion, punicalagin).

**The GC–MS data** (Fig 1.11) of the peracylated compound showed 100% abundance of acyl group ($CH_3$–CO–). The m/z peak at 98 indicated a glucopyranoside ring in the molecule. Other m/z peaks at 115, 127, 157, 169 indicated galloyl groups in the molecule.

**Fig 1.10. MALDI-Mass chromatogram of bioactive molecule isolated from pith and CM of pomegranate fruit.**

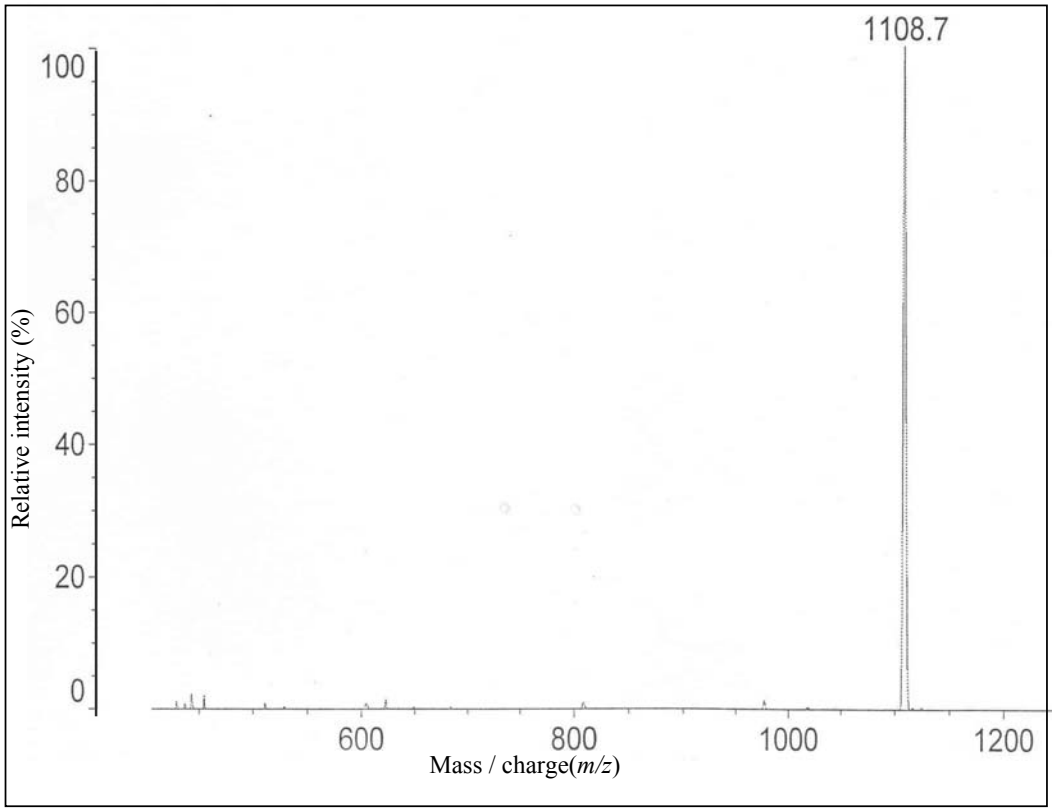
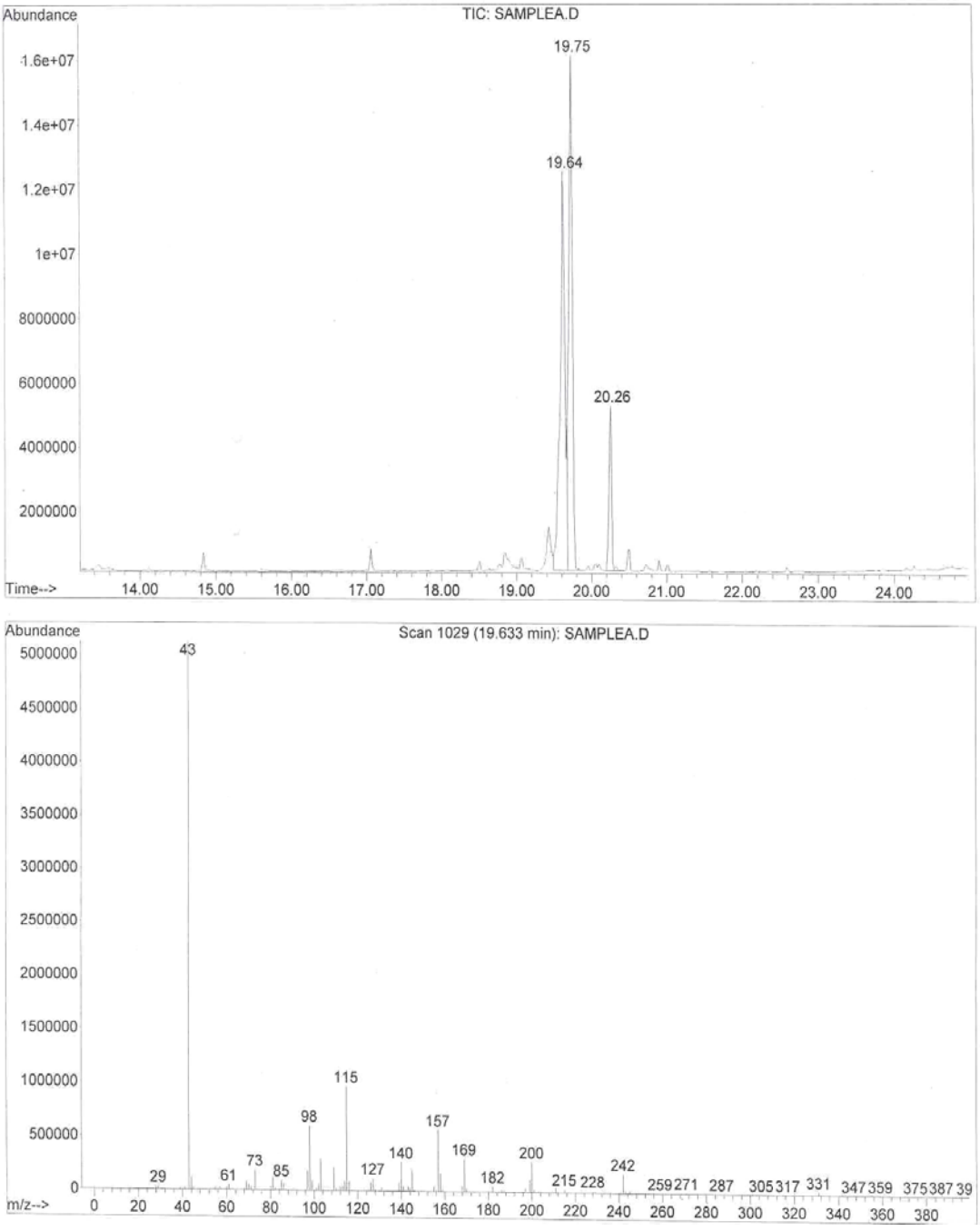


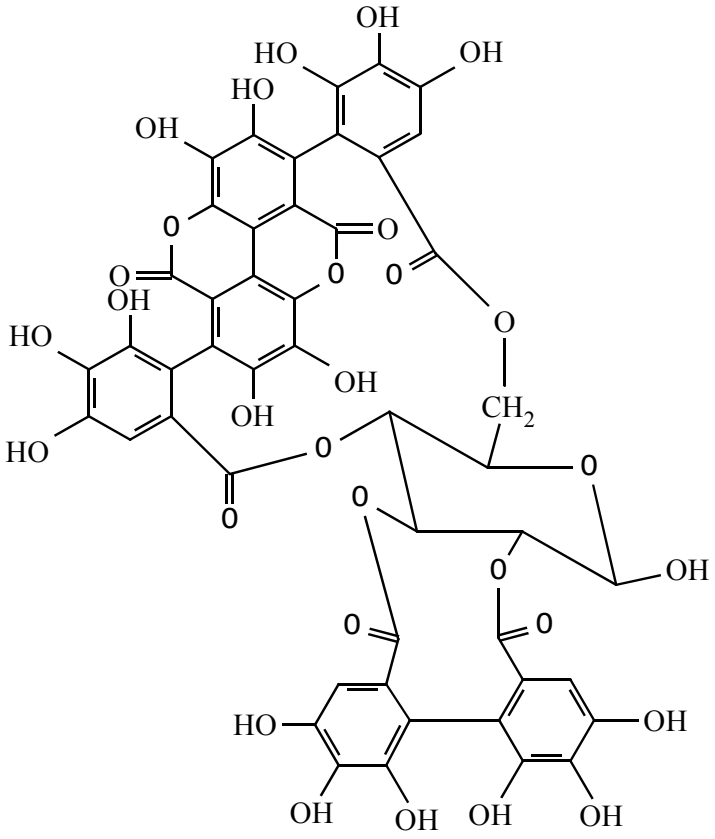
**Fig 1.11. GC-Mass chromatogram of bioactive molecule isolated from pith and CM of pomegranate fruit.**




Based on the data obtained from the UV-Visible, IR, NMR, MALDI-Mass and GC Mass, the molecular structure of the bioactive molecule was deduced as punicalagin (Fig

1.12). The molecule is polyphenolic in nature and contains gallagyl and sugar units. The molecular weight of the molecule was 1081

**Fig 1.12. Structure of the punicalagin isolated from pith and CM of pomegranate fruit.**




**Molecular weight 1081**

### 1.3.3. Characterization of hydrolyzed products obtained from punicalagin

Analytical HPLC analysis of hydrolyzed punicalagin showed five peaks with retention time 4.12; 6.01; 8.71; 15.30; 18.32 (Fig. 1.13). HPLC analysis of respective standards under same analytical conditions revealed that peak 1 (Rt 4.12) corresponds to gallic acid; peak 3 (Rt 8.71) corresponds to the punicalagin and peak 5 (Rt. 18.32) corresponds to the ellagic acid moiety (Fig. 1.13; Fig. 1.14).

Based on UV-Visible absorption spectra and LC-ESI-MS spectra (Table 1.4) other two peaks were identified as punicalin (peak 2, Rt. 6.01) and gallagic acid (peak 4; Rt. 15.30)

**Table 1.4. UV-visible and LC-ESI-Mass spectroscopic data of hydrolyzed fractions of punicalagin**

| Peak No | HPLC retention time (min) | UV absorption maxima $\lambda_{max}$ (nm) | Mass | Assigned |
|---|---|---|---|---|
| 1 | 4.12 | 260 | 171 | Gallic acid |
| 2 | 6.01 | 260 | 781 | Punicalin |
| 3 | 8.71 | 260 | 1081 | Punicalagin |
| 4 | 15.30 | 254 | 601 | Gallagic acid |
| 5 | 18.32 | 254 | 301 | Ellagic acid |

**Fig. 1.13 Analytical HPLC chromatogram of hydrolyzed punicalagin (A); Standard gallic acid (B); Punicalagin (C) and Ellagic acid (D)**

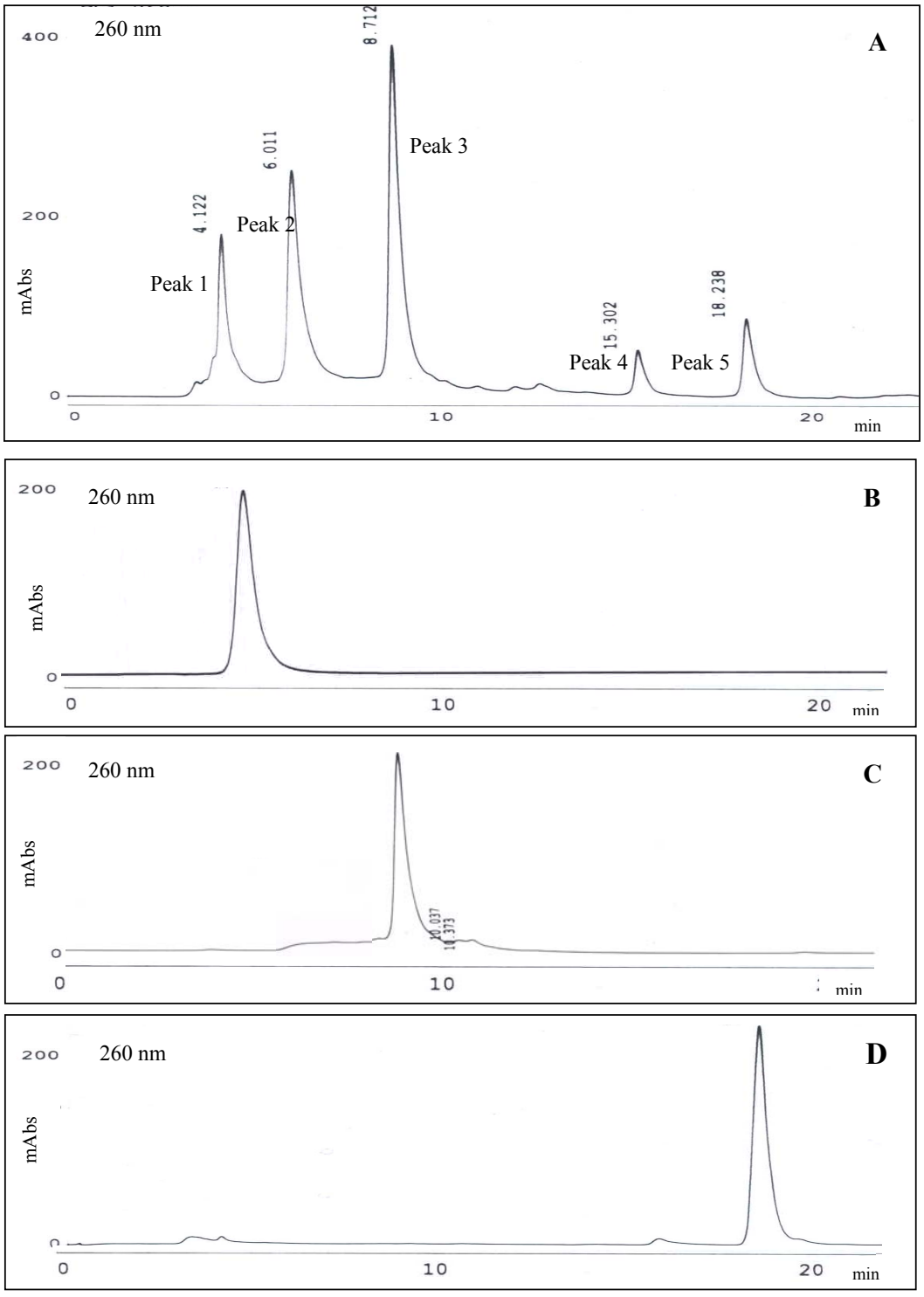
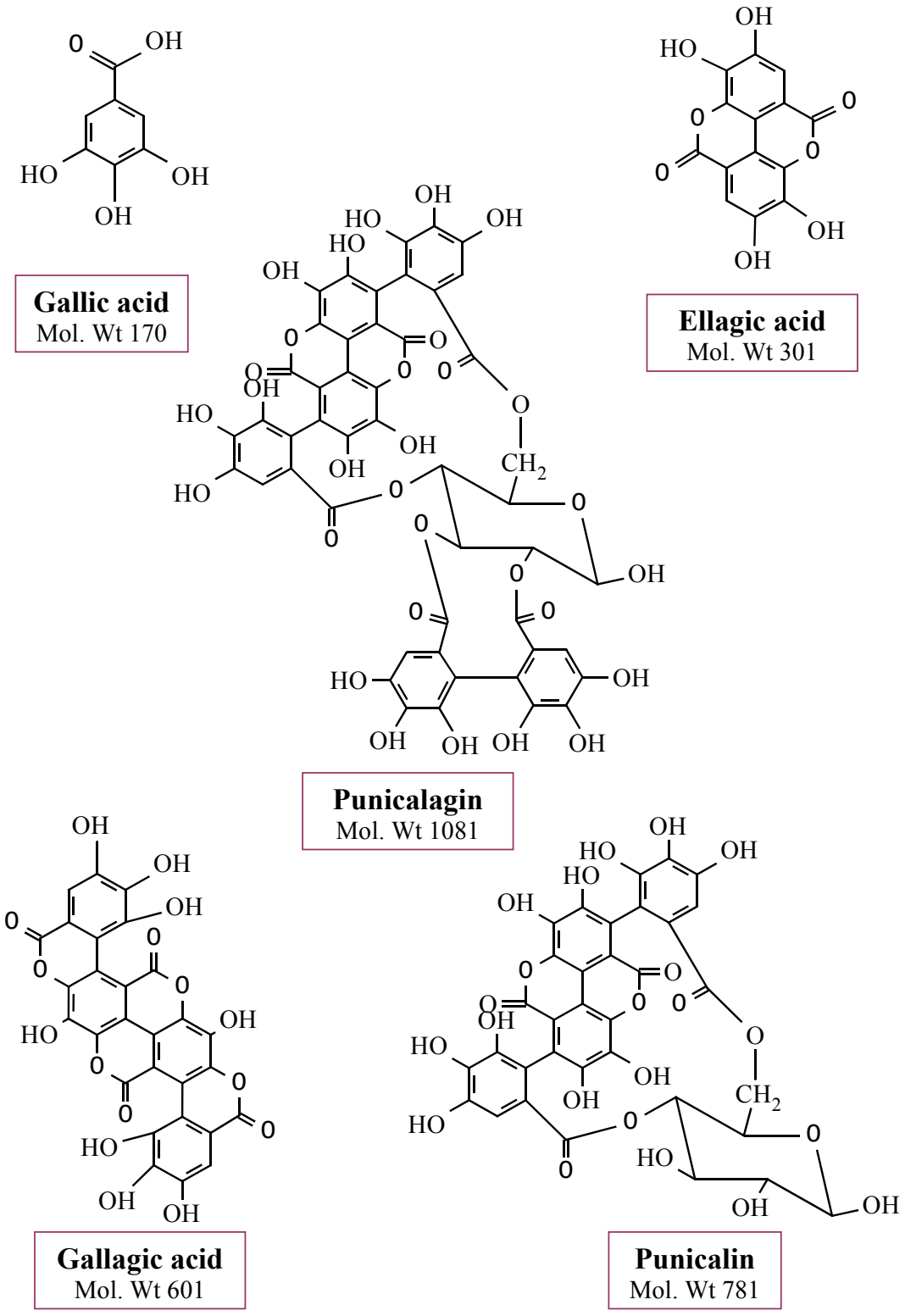


**Fig. 1.14 Punicalagin and its derivatives obtained by hydrolysis**



### 1.3.3.1. Antioxidant activity of hydrolyzed fractions of punicalagin

The hydrolyzed fractions were subjected for antioxidant assay using DPPH radical scavenging method. The results show that, punicalagin is more potent antioxidant in terms of its radical scavenging potential with an $EC_{50}$ of 15.54 μM, followed by punicalin, gallagic acid, ellagic acid and gallic acid (Table 1.5). Though, increase in the number of functional OH groups, the $EC_{50}$ value recorded from all the five bioactive molecules was not significantly different. It may be attributed to the increase in the molecular weight with corresponding increase in the number of OH groups.

**Table 1.5. Antioxidant potential of punicalagin and its derivatives**

| Bioactive molecule | Functional OH groups | Antioxidant potential ($EC_{50}$ μM) |
|---|---|---|
| Gallic acid | **3** | **16.61 ± 0.03** |
| **Ellagic acid** | **4** | **16.40 ± 0.04** |
| **Gallagic acid** | **8** | **15.82 ± 0.01** |
| **Punicalin** | **10** | **15.53 ± 0.01** |
| **Punicalagin** | **16** | **15.44 ± 0.01** |

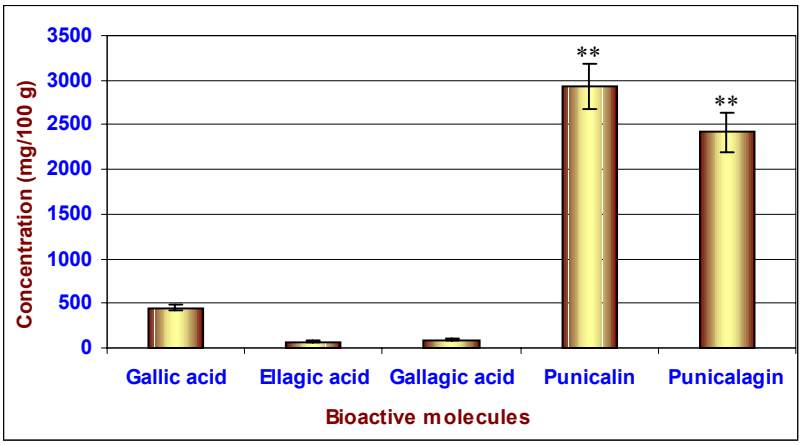
- Values are mean ± standard error of four replicates

### 1.3.3.2. Quantification of punicalagin and its derivatives in the pith and carpellary membrane of pomegranate fruit

The bioactive molecules isolated from pith and CM from mature pomegranate fruit were quantified by analytical HPLC. Among the five bioactive molecules, punicalin found to present in highest concentration (2937 ± 256 mg/100g) in the pith and CM (Fig

1.15) followed by punicalagin (2420 ± 225 mg/100g), gallic acid (450 ± 36 mg/100g), gallagic acid (90±9 mg/100 g), and ellagic acid (70 ± 6 mg/100g).

**Fig. 1.15 Concentration of punicalagin and its derivatives in pith and CM of mature pomegranate fruit**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

## 1.4. Summary and conclusion

A maiden attempt was made to search for bioactive molecule from pith and carpellary membrane of pomegranate fruit. Pith and carpellary membrane were collected from freshly harvested mature pomegranate fruits, dried in a hot air oven and powdered to 60 mesh in apex grinder. The dry powder was extracted sequentially with a series of solvents like hexane, chloroform, ethyl acetate, and methanol and subjected for antioxidant assay. The yield and antioxidant activity of methanol extract was highest; hence it was selected for isolation and characterization of bioactive molecule. The methanol extract was fractionated over a silica gel column by eluting stepwise with hexane, chloroform and a linear gradient of chloroform: ethyl acetate: methanol (100:0:0 to 30:20:50 v/v/v). Ninety-seven fractions, measuring 250 ml each, were collected, concentrated by distillation. Based on TLC analysis the fractions were pooled into 6 groups (fraction No. 1–6) and subjected for antioxidant activity using DPPH free radical scavenging assay. The fraction No 6 showed maximum DPPH radical scavenging activity. It was subjected to further silica gel column (450x40 mm) chromatography and eluted first with linear gradient of chloroform and ethyl acetate (100:0 to 0:100 v/v) and then with linear gradient of ethyl acetate and methanol (100: 0 to 50:50 v/v). Eighty-eight fractions, measuring 250 ml each were collected, concentrated by distillation. Based on TLC analysis the fractions were pooled into five groups (fraction No. 1′–5′) and subjected for antioxidant assay. The fraction 3′ exhibited highest antioxidant activity. Since TLC analysis revealed slight impurities in fraction 3′, it was further purified by preparative HPLC. The yield of active molecule was about 2.3% w/w.

The UV data exhibited the presence of aromatic molecule. The IR data showed signals corresponding to carbonyl stretching and OH stretching. The 2D-HMQCT NMR

spectra revealed the presence of gallagyl, ellagyl moieties and also a glucopyranosyl molecule. The GC-mass spectrum exhibited the presence of gallagyl moieties and MALDI-mass spectrum revealed that the hydrated molecular ion mass as 1081. Based on all the above data, the structure of the compound was identified as punicalagin.

Punicalagin is a high molecular weight (1081) polyphenol. This high molecular weight polyphenol was acid hydrolyzed and the fractions obtained from it were characterized as gallic acid, ellagic acid, gallagic acid, and punicalin. Among the five, punicalin was observed in highest quantity in pith and carpellary membrane of mature pomegranate fruit. The punicalagin molecule was isolated and reported for the first time in pith and CM of pomegranate fruit. An extensive work on the functional properties of this molecule towards human health benefits as well as its role in health and disease status of the arils of pomegranate fruit was studied and discussed in the following chapters in detail.

# *Chapter II*

## *Functional properties of Punicalagin isolated from pith and carpellary membrane of pomegranate fruit*

## 2.1. Introduction and review of literature

### 2.1.1. Polyphenols

The polyphenols are a series of phytochemicals synthesized by plants as secondary metabolites and present in most plants, concentrating in seeds, fruit pulp, peel, bark and flowers (Middleton & Kandaswami, 1994). The base for this very diverse family of molecules is a phenol structure, characterized by multiple functional hydroxyl groups on an aromatic ring. From this structure, larger and interesting molecules such as anthocyanins, coumarins, flavonoids, tannins and lignins are formed. Phenolic compounds perform a variety of functions for plants including defending against herbivores and pathogens, absorbing light, attracting pollinators, reducing the growth of competitive plants, and promoting symbiotic relationship with nitrogen fixing bacteria (Wildman, 2001).

Recently the health beneficial properties of phenolics were identified and since then vast number of researchers have proved that phenolics are the miracle molecules, with vivid functional properties like antioxidant, anticancer, antiatherosclerotic, antibacterial, anti-inflammatory, anti allergic, antimutagenic, antiviral, antineoplastic, antithrombotic, and vasodilator activity (Chantal *et al.*, 1996; Hollman & Katan, 1999; Polissero *et al.*, 1996).

The ability of phenolics in scavenging hydroxyl radicals, superoxide anions and lipid peroxyl radicals may be one of the most important properties, which underlies in the above mentioned multifunctional properties (Leighton *et al.*, 1992; Hollman *et al.,* 1996; Havsteen, 1983). Since oxidative damage is implicated in most disease and epidemiological processes, research reports on phenolics suggest their use in the

prevention and treatment of the above diseases (Van Acker *et al.*, 1996; Kono *et al.*, 1997). The antioxidant activity of the phenolics seems to be primary function of their functional OH groups, which have ability to act as free radical acceptors (Bors *et al.*, 1990).

### 2.1.2 Punicalagin

Punicalagin (Fig. 1.12) is a high molecular weight (Mr. Wt 1081) polyphenolic bioactive molecule isolated from pomegranate fruit waste viz. pith and carpellary membrane. This molecule has been reported from pomegranate juice (Gil *et al.*, 2000) and also from *Terminalia catappa* **(**Chen *et al.*, 2000**)**.

Punicalagin has shown remarkable pharmacological activities including antioxidant (Lin *et al.*, 2001; Lin *et al.*, 1998), anti-inflammatory (Lin *et al.*, 1999), hepatoprotective (Lin *et al.*, 2001; Lin *et al.*, 1998), and antigenotoxic (Chen *et al.*, 2000) activity. A study on toxicity of punicalagin revealed that repeated oral administration of high doses of the punicalagin to rats for 37 days was found to be non-toxic (Cerda *et al.*, 2003). A study on bioavailability of punicalagin in the rats reported that punicalagin and its metabolites were observed in faeces and urine and also in plasma (Cerda *et al.*, 2003), but the mechanism of intestinal uptake of punicalagin is unknown.

There were no detailed studies on many important functional properties like antioxidant activity and actual free radical reactions of punicalagin, interaction with metal ions, proteins and DNA, platelet aggregation inhibitory activity, antibacterial activity, toxicity, and teleology. Keeping in view of the above, a detailed study was undertaken regarding different functional properties of punicalagin. The antioxidant activity in terms of scavenging DPPH radical, superoxide radical, $ABTS^{\bullet}$ radical, lipid peroxyl radicals, and total reducing power was studied in detail. Pulse radiolysis studies on the reactions of

punicalagin with various radiolytically produced oxidizing and reducing radicals at near physiological pH in aqueous media was carried out and the resultant transient intermediates were characterized. In addition antioxidant ability of the punicalagin has also been assessed in terms of reaction ratio for the repair of radiolytically produced tryptophan radicals. Further, the interaction with metal ions viz. $Fe^{2+}$ and $Cu^{2+}$ was also studied since these metal ions were reported to involve in formation of free radicals leading to oxidative damages.

Though the bioavailability of punicalagin was reported, the mechanism of its transportation through blood across the body system was not studied so far; hence a spectroscopic study of the interaction of punicalagin with human serum albumin (HSA) has been carried out. Since HSA acts as carrier protein for drugs in the body, it was selected for the study. Apart from this, interaction of punicalagin with DNA was studied. The interaction of drugs with DNA is considered as one of the possible mode of actions of anticancer drugs. Other important functional properties viz. antibacterial activity against six clinically important food borne human pathogenic bacteria, and platelet aggregation inhibitory activities were also carried out. A critical module in the characterization of a functional molecule is to assess the safety of the candidate in a series of acute and sub acute toxicity. Hence toxicity of punicalagin was studied against the different cell lines maintained *in vitro*.

## 2.2. Materials and Methods

### Chemicals:

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxy anisole (BHA), Di-ammonium salt of 2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) and Tryptophan, Human serum albumin (fatty acid free, 99%), N-acetyl tryptophan amide, Trizma base Collagen, and Adenosine diphosphate (disodium salt, ADP), were purchased from Sigma Chemical Co., (St Louis, MI, USA). Nicotinamide adenine dinucleotide (reduced form, NADH), Nitroblue tetrazolium (NBT), Phenazin methosulphate (PMS), L-Ascorbic acid, Ferrozine and Tris–HCl were from Sisco Research Laboratories (India). 2-Thiobarbituric acid was from ICN Biomedicals Inc. Ohio, and Trichloroacetic acid from Qualigens Fine Chemicals India. Ferrous ammonium sulphate, Cupric chloride, Sodium nitrite and Perchloric acid GR grade were from Merck, India. DNA sodium salt from Herring's sperm, Dulbecco's Modified Eagles Medium (DMEM) and Nutrient agar were purchased from HiMedia lab. pvt Ltd Bombay, India. All the solutions were freshly prepared prior to experiment using this water. Sodium hydroxide was further purified by passing through thermolyne's nanopure water filtering assembly (Sp. conductivity < 0.1 $\mu s\ cm^{-1}$). Sample solutions for pulse radiolysis experiments were bubbled by either $N_2O$, or $N_2$ or $O_2$ gas (Indian oxygen, IOLAR grade) using syringe bubbling technique prior to pulse irradiation. All reagents were used as supplied without further purification. Other chemicals were of extra pure analytical grade purchased locally.

### 2.2.1. Antioxidant activity of punicalagin

#### 2.2.1.1. Radical scavenging activity

##### 2.2.1.1.1. DPPH Radical scavenging activity:

The antioxidant activity of pomegranate pith and CM extracts on DPPH radical was measured according to the method of Moon and Terao (1998). About 0.2 ml of test sample, of different concentrations, was mixed with 0.8 ml of Tris–HCl buffer (pH 7.4) to which 1 ml DPPH (500 μM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV–visible spectrophotometer (UV-160A, Shimadzu co. Japan). DPPH radical scavenging potential was calculated with the following formula.

$$\text{DPPH radical scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample }(517\,nm)}}{A_{\text{control }(517\,nm)}}\right) x\,100$$

DPPH radical scavenging potential was expressed as $EC_{50}$ value, which represents the concentration of sample, which scavenged 50% of the DPPH radicals.

##### 2.2.1.1.2. Superoxide radical scavenging activity:

The superoxide radical scavenging assay was carried out according to the method of Liu *et al.* (1997). Superoxide radicals were generated in 1 ml of Tris–HCl buffer (0.02 M, pH 8.3) containing 0.1 mM NADH, 0.1 mM NBT, 10 mM PMS and crude extract of pith and CM of pomegranate and punicalagin. The colour reaction of superoxide radicals and NBT was detected at 560 nm using a Cintra 10 UV–Visible spectrophotometer. Superoxide radical scavenging potential was calculated with the following formula.

$$\text{Superoxide radical scavenging activity } (\%) = \left(1 - \frac{A_{\text{sample}(560\,nm)}}{A_{\text{control}(560\,nm)}}\right) x100$$

Results expressed as $EC_{50}$ i.e. the concentration required for scavenging 50% of the radicals.

**2.2.1.1.3. ABTS radical scavenging activity**

The diammonium salt of ABTS exists in the anionic form as ($ABTS^{2-}$) at neutral pH it undergoes one-electron oxidation to form $ABTS^{\bullet-}$ radical. Scavenging of this radical by punicalagin resulting in discoloration, was determined according to Pellegrini *et al.* (1999). Briefly, to a 50 ml aqueous solution of of $ABTS^{2-}$ (2 x $10^{-3}$ mol $dm^{-3}$), containing phosphate ($10^{-3}$ mol $dm^{-3}$) was added 0.2 ml of of potassium persulphate (7 x $10^{-2}$ mol $dm^{-3}$) and the solution kept in the dark for 24 hours, (27$^{o}$ C) which resulted in the formation of $ABTS^{\bullet-}$ radical. This solution was appropriately diluted to yield an initial absorbance of ~ 0.8 at 734 nm. The radical scavenging activity of punicalagin was assessed by adding 0.05 or 0.1 ml of punicalagin (5 x $10^{-4}$ mol $dm^{-3}$) to 2 ml of appropriately diluted buffered $ABTS^{\bullet-}$ solution. The resulting change in the absorbance of $ABTS^{\bullet-}$ solution due to discoloration after 6 min of addition of antioxidant was monitored and the results compared with that of n-propyl gallate and Trolox-c using equimolar concentrations of antioxidants under similar experimental conditions. All determinations were run in triplicate. An appropriate solvent blank was run with each assay. The antioxidant capacity was expressed as percentage inhibition of absorbance of $ABTS^{\bullet-}$ radical at 734 nm.

#### 2.2.1.1.4. Lipid peroxidation inhibitory activity in liver microsomal model system

Rat liver (1 g) was homogenized in 5 ml Tris-buffer (2 x $10^{-3}$ mol $dm^{-3}$, pH 7.4) and the microsomes were isolated by the calcium aggregation method (Vijayalakshmi, 1989). The pellet was re-suspended in phosphate buffer ($10^{-1}$ mol $dm^{-3}$). Microsomal lipid peroxidation was assayed by the thiobarbituric acid method (Buege, & Aust, 1978). Peroxidation was initiated by the addition of known aliquots of ferrous sulphate ($10^{-4}$ mol $dm^{-3}$), and ascorbic acid ($10^{-4}$ mol $dm^{-3}$) to the 100 µl of microsomes, in the absence and presence of punicalagin (0.1 – 2 x $10^{-4}$ mol $dm^{-3}$ /ml) in 0.1 M phosphate buffer (pH 7.4) and incubated at 37° C for 1 h. This was followed by the addition of 20% trichloroacetic acid (2 ml) and 1% thiobarbituric acid (2 ml). The mixture was heated in a boiling water bath for 10 min, cooled, centrifuged for 20 min. The absorbance of the supernatant was measured spectrophotometerically at 535 nm, and the $EC_{50}$ values were calculated for BHA a reference antioxidant and punicalagin.

$$\text{Lipid peroxidation inhibitory activity (\%)} = \left(1 - \frac{A_{\text{sample (532 nm)}}}{A_{\text{control (532 nm)}}}\right) x100$$

Lipid peroxidation inhibitory potential was expressed as $IC_{50}$ value, which is the concentration of test sample inhibited 50% of lipid peroxidation.

#### 2.2.1.1.5. Total Reducing power assay

The reducing power of the methanol extract and punicalagin was quantified by the method described earlier by Yen & Chen (1995) with minor modifications. Reaction mixture containing test samples at different concentrations (10-100 µl) in phosphate

buffer (0.2 M, pH 6.6) was incubated with potassium ferricyanide (1% w/v) at 50ºC for 20 min. The reaction was terminated by the addition of TCA solution (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

### 2.2.1.2. Pulse radiolysis experiments:

Pulse radiolysis studies were carried out at Chemical Dynamics and Radiation Chemistry Division, Bhabha Atomic Research Institute, Trombay, Mumbai. The technique of pulse radiolysis utilizes a pulsed electron source together with a suitable detection system. In the present study, a linear electron accelerator coupled with an optical detection system was used.

#### 2.2.1.2.1. Instrumentation

**Linear accelerator**

***Principle:*** An accelerator is a sophisticated device for increasing the kinetic energy of atomic or subatomic particles such as electrons, protons and heavy ions by employing electromagnetic field developed across a series of circular irises spaced at well-defined intervals in an evacuated wave-guide. The charged particles injected into the traveling wave-guide are made to ride on the crest of the wave in an electrical field. In the process, they are accelerated in the direction of the field. Thus the kinetic energy as well as the velocity of the particle increases the latter approaching close to the velocity of light.

**Instrument:** The accelerator used in the present study is a linear electron accelerator (LINAC), consisting of a magnetron, an electron gun, main modulator, and a wave-guide. The accelerated electrons are focused by employing static electric and magnetic fields so as to produce a sufficiently high concentration of product transients in a reaction cell. Detection of these species is done by optical absorption.

**Detection system**

***Kinetic spectrophotometry:*** Optical absorption method is one of the most widely used fast reaction technique for studying short-lived species. It consists of analyzing light source, a monochromator, a detector, a recorder, besides other optical systems comprising of lenses and mirrors.

***Analyzing the light source*:** In the present set up a 450 W Osram Xenon arc lamp was used as a source for analyzing light. A stabilized current regulated DC power supply (90 V) with a ripple of ~5% was used to strike the arc lamp. A pulser power supply fabricated at B.A.R.C. is used to boost the steady-state power of the arc lamp for ~3 ms duration so as to get a peak current of ~ 400 milliamperes.

***Monochromator:*** The analyzing light after passing through the sample cell (Quartz cuvettes of 1 cm path length, Suprasil) is made to focus on the entrance slit of the monochromator (Kratos Model, GM-252A, having a dispersion of 3.3 nm/mm) using glass optics comprising of lenses and a set of mirrors whose front surfaces are coated with aluminum.

***Detector*:** The light beam from a Xenon arc lamp after passing through the exit slit of the monochromator falls onto a photo multiplier tube (Hamamatsu model R-995)

placed in a light tight housing immediately after the monochromator where it is converted into an electrical signal and amplified

***Transient digitizer:*** The Photo multiplier tube (PMT) out put voltage is fed to an L & T digital oscilloscope (model 4072). The time base of the oscilloscope is triggered externally by signal derived from the SDPG synchronous with the electron beam pulse. A delay sweep facility on the X-Y screen of the storage scope permits recording of signals.

***Data analysis:*** Variation in the intensity of the transmitted light dependent on the light absorbing transient species formed and is given by the relation

$$I_t = I_0\ 10^{-\varepsilon cl} \quad \text{--------} \ (1)$$

Where, $I_0$ is the intensity of the incident light falling on an absorbing medium

$I_t$ is the intensity of the transmitted light

ε is the molar extinction coefficient

c is the molar concentration of the species

l is the cell path length.

Equation 1 can be rewritten in the logarithmic form as

$$\text{Log } I_0/I_t = \varepsilon\ c\ l = OD$$

Where, OD is optical density

The measured OD can be used to obtain first order rate constant from the plot of log OD versus time. or second order rate constant by plotting 1/OD versus time.

**2.2.1.2.2. Generation of radicals**

**Generation of $^{\bullet}$OH radicals**

Water, when subjected to ionizing radiations from linear electron accelerator generates $e^-_{aq,}$ $^{\bullet}$OH and $^{\bullet}$H as the three main primary species besides small amounts of

molecular products**.** The yield of these primary species being $G(^{\bullet}OH^{-}) \cong G(e_{aq}^{-}) = 2.9 \times 10^{-7}$ mol $J^{-1}$, $G(H^{\bullet}) = 0.6 \times 10^{-7}$ mol $J^{-1}$) **(**Pan, *et al* 1993) The yield of hydroxyl radicals was doubled by irradiating a nitrous oxide ($N_2O$) saturated aqueous solution, where $e_{aq}$ was quantitatively converted to the $^{\bullet}OH$ radical. Generation of different oxidizing radicals has been extensively covered in the literature (Spinks, *et al* 1990) and hence only a brief mention is made here.

$$H_2O \rightsquigarrow e^{-}_{aq,}\ {}^{\bullet}OH,\ {}^{\bullet}H,\ H_3O^{+},\ H_2 \text{ and } H_2O_2 \quad \text{------} \quad (2.1)$$

$$e^{-}_{aq} + N2O + H_2O \longrightarrow {}^{\bullet}OH + OH^{-} + N_2 \text{----------} (2.2)$$

**Generation of azide radicals**

Azide radicals ($N^{\bullet}_3$) were generated by irradiating an $N_2O$ saturated aqueous solution containing 1 X $10^{-2}$ M sodium azide, where all of the $^{\bullet}OH$ radicals were exclusively converted to $N^{\bullet}_3$ with the reaction chemical yield of 0.56 μ mol $J^{-1}$.

$$^{\bullet}OH + N_3^{-} \longrightarrow N^{\bullet}_3 + OH^{-} \quad \text{----------} \quad (2.3)$$

**Generation of Nitrogen dioxide radicals**

Nitrogen dioxide radicals ($NO^{\bullet}_2$) were generated by the reaction of $^{\bullet}OH$ radicals with nitrite ions,

$$^{\bullet}OH + NO_2^{-} \longrightarrow NO^{\bullet}_2 + OH^{-} \quad \text{---------} \quad (2.4)$$

**Generation of tryptophanyl radicals**

Tryptophanyl radicals were generated using azide radicals since they are known to generate tryptophanyl radicals specifically

$$^{\bullet}OH + N_3^{-} \longrightarrow N^{\bullet}_3 + OH^{-} \text{----------} (2.3)$$

$$N^{\bullet}_3 + TrpH \longrightarrow Trp^{\bullet} + N_3 + H^{+} \quad \text{----------} \quad (2.5)$$

**Generation of ABTS radicals**

ABTS radicals were generated using azide radicals since they are known to generate ABTS radicals specifically

$$^{\bullet}OH + N_3^- \longrightarrow N^{\bullet}_3 + OH^- \quad \text{----------} \quad (2.3)$$

$$N^{\bullet}_3 + ABTS^{2-} \longrightarrow ABTS^{\bullet-} + N_3 + H^+ \quad \text{--------} \quad (2.6)$$

**2.2.1.3. Cyclic voltametric studies of punicalagin**

Reduction potential of punicalagin was determined in aqueous solutions by cyclic voltammetric and differential pulse voltammetric technique. The experiments were carried out using an Eco Chemie make Potentiostat / Galvanostat (Autolab 100). Data acquisition and analysis were made by Autolab-GPES software. Aqueous buffered solutions of samples were placed in a glass cell containing a glassy carbon electrode, a saturated calomel reference electrode and a platinum auxiliary electrode. The experiments were carried out at 28°C. Pre-conditioning of the glassy carbon electrode was carried out prior to every measurement by polishing the surface of the electrode using very fine alumina powder and then rinsing it thoroughly before use. Aqueous solutions containing 5 x $10^{-4}$ mol $dm^{-3}$ of punicalagin, 0.1 mol $dm^{-3}$ KCl and $10^{-3}$ mol $dm^{-3}$ of phosphate buffer, was bubbled with pure $N_2$ prior to measurement. The sweep rate was 20 mV / s. All the experimental potentials values were then converted with respect to the NHE. The electrochemical system was calibrated by doing the cyclic voltammetry scan of Cd (II) and a standard potassium ferricyanide solution. All the potential values were measured with respect to the SCE and converted to NHE by suitable corrections.

#### 2.2.1.4 Interaction of punicalagin with metal ions

Chelation of iron and copper ions with punicalagin was evaluated at room temperature by adding 5 μl aliquots of ($10^{-2}$ mol $dm^{-3}$) of Fe (II) / Cu (II) to 2 ml of (5 x $10^{-4}$ mol $dm^{-3}$) punicalagin, containing ($10^{-3}$ mol $dm^{-3}$) phosphate buffer, pH 7. The UV-visible spectra were recorded between 300 – 900 nm on a Chemito spectrascan UV-2600 spectrophotometer after a lag time of 5 min. The Metal /Ligand (M/L) ratio was determined when the intensity of absorbance reached saturation at the wavelength of maximum absorption.

### 2.2.2. Interactions of punicalagin with human serum albumin (HSA)

The interaction of punicalagin with HSA was measured by the fluorescence quenching of the HSA by punicalagin on Shimadzu RF-5000 fluorescence spectrometer with inbuilt stirrer and thermostat. To a freshly prepared aqueous Tris-HCl buffer solution ($10^{-3}$ mol $dm^{-3}$, pH = 7.4), HSA (1.3 x $10^{-6}$ mol $dm^{-3}$) was added. A 2.5 ml aliquot of the above solution was taken in a quartz cuvette, maintained at 27° C. Titration was carried out by a stepwise addition of (2.5 μl) aqueous Tris-HCl buffered punicalagin solution of (1.1 x $10^{-3}$ mol $dm^{-3}$) in the concentration range (0 – 25 x $10^{-5}$ mol $dm^{-3}$). A constant lag time was maintained between two additions. Keeping the excitation wavelength at 280 nm, the fluorescence emission spectra were recorded from 300 to 400 nm.

### 2.2.3. Interaction of punicalagin with DNA

Absorption titrations and absorption measurements for UV-Visible spectroscopy were performed on a Cintra 10 UV-Visible spectrophotometer. All measurements were

made in 1 cm quartz cells at 27° C. Titrations were performed using 1ml aliquots, containing 50 µl of 1mg /ml of DNA and 950 µl of Tris buffer-HCl at pH 7.4. To this 1 ml solution, was added (10 µl) aliquots of punicalagin (5 x $10^{-4}$ mol $dm^{-3}$) and the spectral changes recorded after each addition, keeping the time interval constant. In the reverse titration, 10 µl of (1 mg / ml DNA) solution was added to 1 ml of punicalagin (5 X $10^{-5}$ mol $dm^{-3}$) solution containing Tris-HCl buffer (pH 7.4) and the spectral changes recorded.

### 2.2.4. Platelet aggregation inhibitory activity of punicalagin

#### 2.2.4.1 Platelet preparation

Blood samples were taken from healthy volunteers who assured not to have taken any drugs during the 2 weeks prior to the blood sampling. Blood was collected into buffered sodium citrate (3.8 % w/v) pH 6.5 as the anticoagulant at a ratio of 9:1 v/v and used within 3 hr of collection. Platelet-rich plasma (PRP) was obtained by centrifugation of the citrated blood at 1100 rpm for 20 min. the residual blood was again centrifuged at 2500 rpm for 20 min to obtain the homologous Platelet Poor plasma (PPP). Platelet count was adjusted to 1.6 X $10^7$ platelet per µl of PRP.

#### 2.2.4.2. Platelet aggregation assay

Aggregation was measured turbidimetrically at 37° C with constant stirring at 1000 rpm in a Chronolog Dual Channel Aggregometer. About 0.45 ml of PRP was kept stirred at 1200 rpm at 37°C, and aggregation was induced by different agonist's viz. collagen (10 µM) and ADP (2.5 mM). The change in turbidity was recorded with reference to PPP

using an omniscribe recorder for at least 5 min. The slope was calculated for each of the agonists and they were used as control.

Similarly, 100-500 μM of the punicalagin was added to PRP, incubated for five min after which agonists viz. collagen (10 μM), ADP (2.5 mM) were added. Platelet aggregation was recorded using an omniscribe recorder for 5 min. The slope was calculated. The difference in the slope between the control and the treated was expressed as percent inhibition of platelet aggregation by punicalagin.

### 2.2.5. *In- vitro* assay for cytotoxicity of punicalagin

#### 2.2.5.1. Cell lines and culture medium

The cell lines viz. Vero (Normal African green monkey kidney cell line), Hep-2 (Human larynx epithelial cancer cell line) and A-549 (Human small cell lung carcinoma cell line) were obtained from Pasteur Institute of India, Coonoor, India, were cultured with Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% heat-inactivated new born calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin maintained at 37º C in a 5% $CO_2$ atmosphere with 95% humidity.

#### 2.2.5.2. Microculture tetrazolium assay

Cellular viability in the presence and absence of punicalagin was determined using the 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Alley, *et al* 1988)**.** Principle involved is the cleavage of yellow colored MTT to a blue formazan by mitochondrial enzyme succinate dehydrogenase. The formation of formazan is found to be proportional to the number of viable cells. The assay in brief, monolayer cell culture was trypsinised and the cell count was adjusted to 1.0 x $10^5$ cells/ml using medium

containing 10% newborn calf serum. To each well of the 96 well microtiter plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, the supernatant was flicked off; washed and 0.1 ml of different concentration of test sample was added to the cell in microtiter plate. The plates were then incubated at 37ºC for 3 days in 5% $CO_2$ atmosphere. After 72 hours, the drug solutions in the wells were discarded and 50µl of MTT was added to each well. The plates were gently shaken and incubated for 3 hours at 37ºC in 5% atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 540 nm. Absorbance, recorded from cells grown in the absence of punicalagin was taken as 100% cell viable (control). The viability was plotted against punicalagin concentration and the $IC_{50}$ (Concentration required to reduce viability by 50%) value against the cell lines was calculated.

**2.2.5.3. Sulphorhodamine B (SRB) assay**

The SRB assay (Skehan, *et al*, 1990) was performed to assess growth inhibition, which measures cell number indirectly by staining total cellular protein with dye SRB. In brief, treated and control cells were fixed by layering 0.1 ml of ice-cold 40% trichloroacetic acid (TCA) on top of the growth medium. Cells were incubated at 4ºC for 1 hr, after which plates were washed five times with cold water, the excess water was drained off and the plates left to dry in air. SRB stain (0.1 ml, 0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min, after which they were washed with 50 ml 1% acetic acid, rinsed four times until only dye adhering to the cells was left. The plates were dried. 0.1 ml of 10mM Tris base was then added to the wells to

solubilise the dye. The plates were shaken vigorously for 5 min. the absorbance was measured using micro plate reader at a wavelength of 492 nm. $IC_{50}$ was calculated as in MTT assay. $CTC_{50}$ value is the average of the $IC_{50}$ of MTT assay and SRB assay.

**2.2.5.4. Cell viability assay by Trypan Blue Dye – Exclusion assay**

The Trypan blue dye has the ability to penetrate in to dead cells and gives it blue colour. This method gives exact number of dead and viable cells. The treated and control cells were trypsinised and incubated for 15 min. Then 0.1 ml of growth medium was added to all the wells. The cells were dispersed and cell suspension was transferred from the wells to eppendorf tubes. Aliquot of trypan blue dye was added and the cell count was taken using haemocytometer.

## 2.2.6. Antibacterial assay

## 2.2.6.1. Bacterial strains and culture conditions

To determine the antibacterial activity of pith and CM extracts and punicalagin following clinical isolates viz. *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *salmonella typhi* and *Shigella dysenteriae* were obtained from the stock culture center of Department of Microbiology, Mysore Medical College, Mysore, India. The bacterial stock cultures were maintained on nutrient agar slants at 37±1°C.

**2.2.6.2. *In- vitro* screening for antibacterial activity**

**2.2.6.2.1. Agar-well diffusion assay**

Antibacterial activity of punicalagin and solvent extracts of pith and CM were assayed separately using agar-well diffusion method modified from Perez *et al.* (1990).

About $10^6$ cfu/ml bacteria were seeded in to the molten nutrient agar (42-43°C) and plated immediately in to sterilized petriplates, allowed to set for 30 min. About 7 mm diameter cup-lets were bored in the plate at equidistance using a sterile cork borer. To each cuplet 60 µl of test sample (5 mg/ml in ethanol) was added. Absolute ethanol was used as control. Amoxycillin was used as standard. After 3 hours of chilling treatment at 4°C in upright position, petriplates were inverted and transferred to bacterial incubator maintained at 37°±1° C and incubated for 48 hours. After 48 hours of incubation, plates were examined for inhibitory zones formed around the well, which were measured and expressed as zone of inhibition. Three replicates were maintained for each treatment.

**2.2.6.2.2. Bacterial growth inhibitory assay**

Bacterial growth inhibitory activity of promising antibacterials viz. acetone, methanol extract and punicalagin from pith and CM of pomegranate fruit were tested according to the method of Willix *et al.* (1992). Cultures were grown in nutrient broth in 100 ml conical flask at 37 °C. Bacterial culture (24 hr old) to be tested was diluted with sterilized nutrient broth to a level of 1 x $10^6$ cfu/ml. About 1 ml of inoculum ($10^6$ cfu/ml) was seeded in to a test tube containing sterilized nutrient broth with different concentration of test sample ranging from 0-800 ppm in triplicates. About 0.5 ml of culture was taken from each culture tube at different time intervals into 0.5 ml of 10% w/v formaldehyde and absorbance was measured at 600 nm to determine the density of the culture. The culture tubes were incubated at 37±1°C up to 72 hours and optical density was measured at 12 hour interval. The culture without any test sample was used as control. The $MIC_{50}$ was determined based on the % bacterial growth inhibition for 72 hours.

## 2.3. Results and Discussion

### 2.3.1. Antioxidant activity of punicalagin

Free radicals are molecules possessing unpaired electron, which are highly reactive, and involved in variety of degenerative diseases. Normal physiological processes such as respiration and cell-mediated immune responses continuously produce the radical species (Hamilton *et al.*, 1997). In healthy living system there is a dynamic balance between the free radicals produced and their quenching by antioxidants. Excess free radicals in the body leads to oxidative stress, which leads to various degenerative diseases in human beings. There is an increasing evidence to support the involvement of free radicals in various degenerative diseases like atherosclerosis, hypertension, ischaemic diseases, Alzheimer's disease, Parkinson's disease, cancer and inflammations are being caused primarily due to the imbalance between pro-oxidants and antioxidant homeostasis (Ames, 1983; Diplock, 1994). Antioxidant principles from natural resources possess multifacetedness in their multitude and magnitude of bioactivities and provide opportunities in correcting the imbalance.

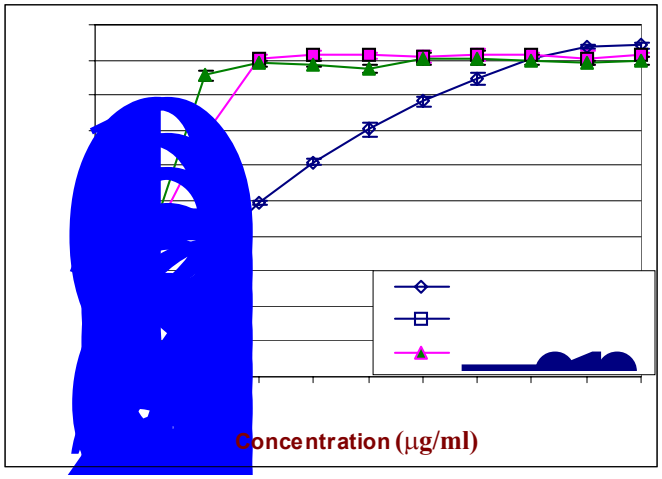
In the present study the antioxidant activity of punicalagin has been studied by various well-established *in-vitro* radical scavenging assays Further bi-molecular kinetics of radical scavenging ability of punicalagin against radiolytically produced radical species was studied using pulse radiolysis techniques. The electron donation potential of the punicalagin at different pH range was studied using cyclic voltametric technique. Further, metal chelating activity against iron and copper was also studied.

### 2.3.1.1. Radical scavenging activity

#### 2.3.1.1.1. DPPH radical scavenging activity

The test samples viz. punicalagin, methanol extract and BHA showed concentration dependent radical scavenging activity (Fig. 2.1). The methanol extract showed very high DPPH radical scavenging activity as compared to that of punicalagin. The $EC_{50}$ value of methanol extract was found to be 8.33 ± 1.6 µg/ ml whereas that of the punicalagin was found to be 16.7 ± 2.3 µg/ml. The higher DPPH radical scavenging potential of methanol extract may be due to the synergistic effect of other phenolics present in it. The BHA showed highest DPPH radical scavenging activity with an $EC_{50}$ value of 5.33 ± 0.6 µg/ml.

**Fig. 2.1 DPPH radical scavenging potential of punicalagin and methanol extract from pith and CM of pomegranate fruit and BHA**




- Values are mean ± standard error of four replicates

**Structure and activity relationship**

Punicalagin is a polyphenol with sixteen functional OH groups; each OH group can reduce a DPPH molecule. The mechanism of radical scavenging and stabilization of DPPH molecule is shown in the fig 2.2.

**Fig. 2.2 Hypothetical model of mechanism of DPPH radical scavenging activity by punicalagin**

**2.2. (a). Reduction of a DPPH molecule by punicalagin**

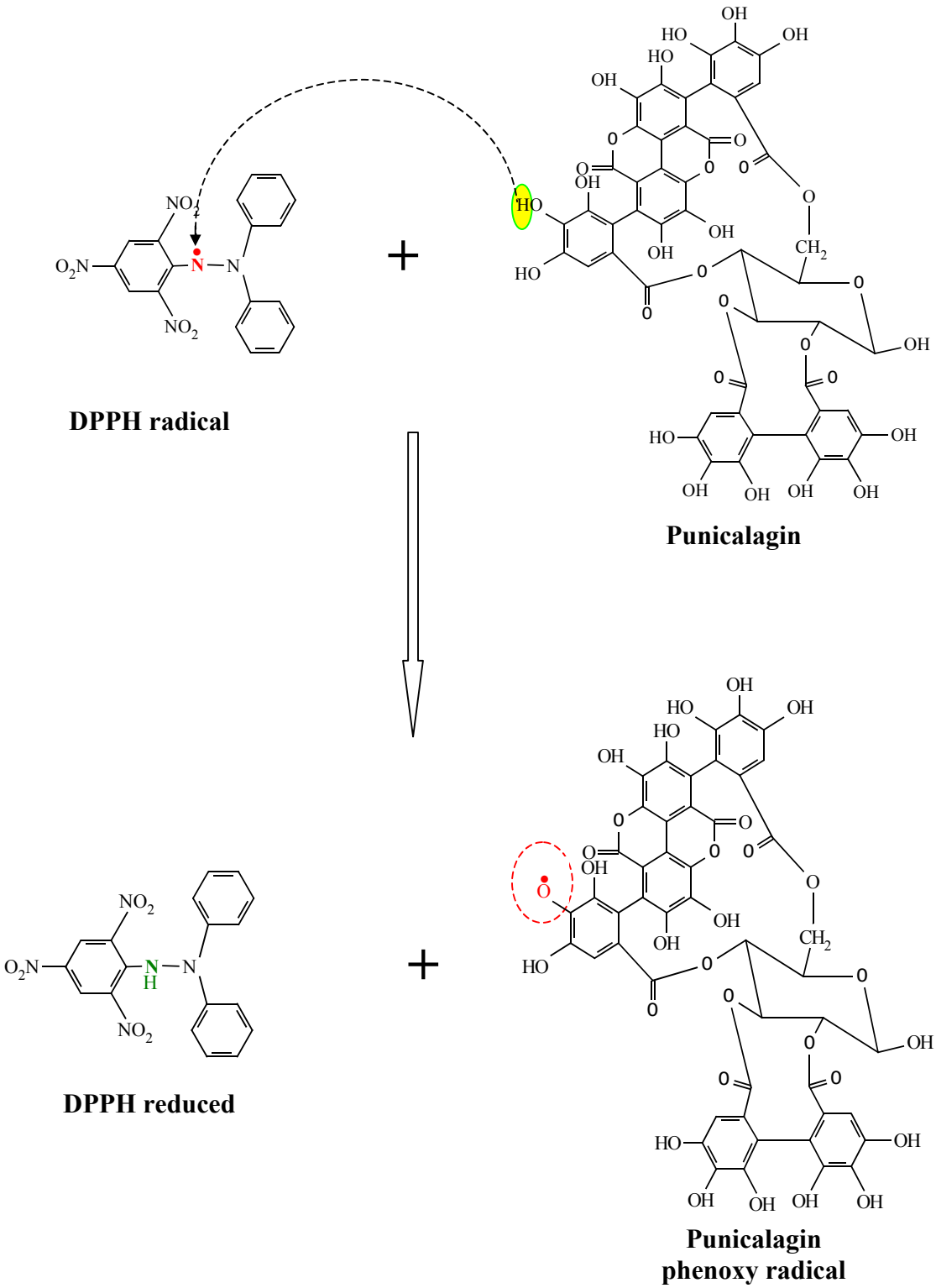


**Fig. 2.2. (b). Stabilization of punicalagin phenoxy radical by reducing another DPPH radical**

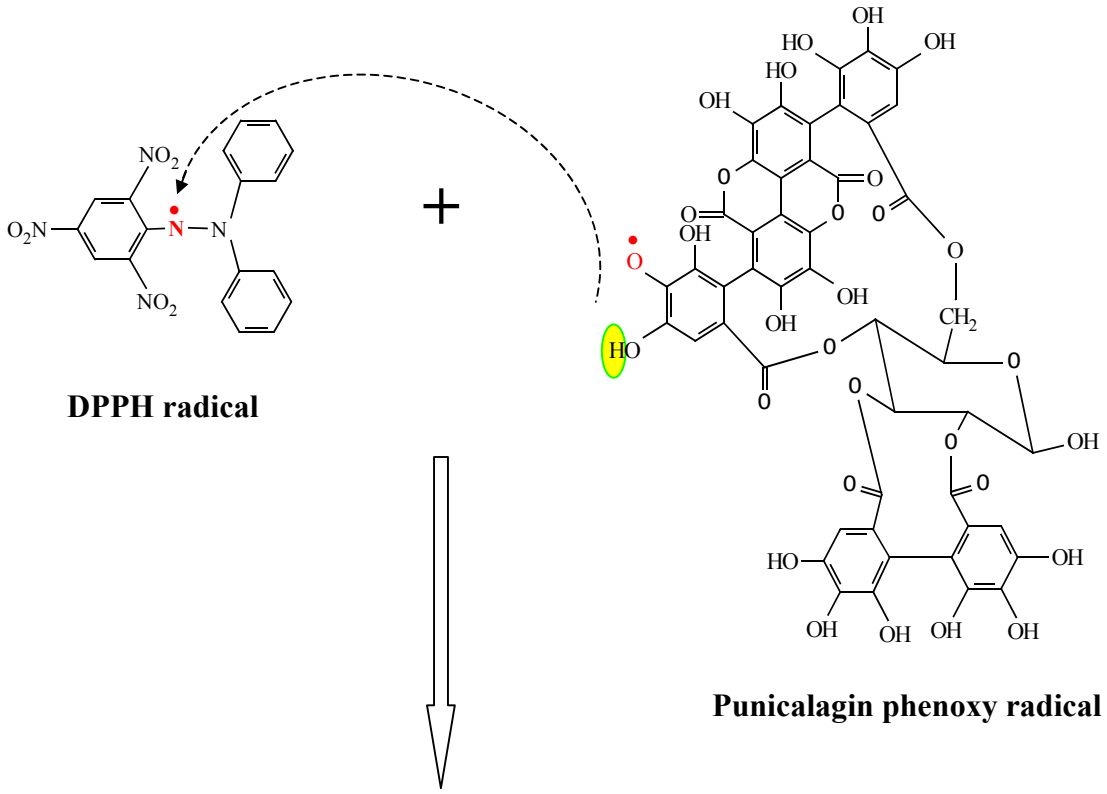
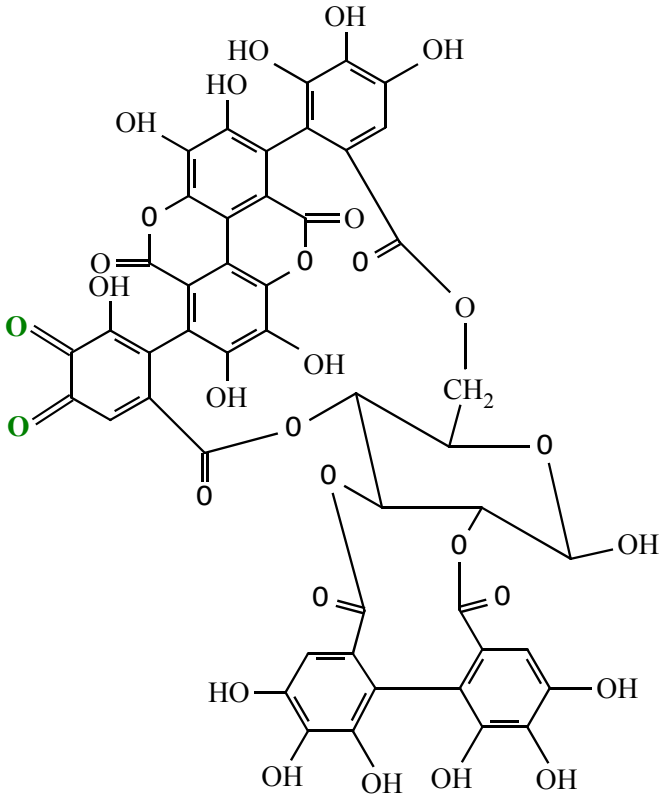


Fig. 2.2. (c). Stabilized punicalagin quinone



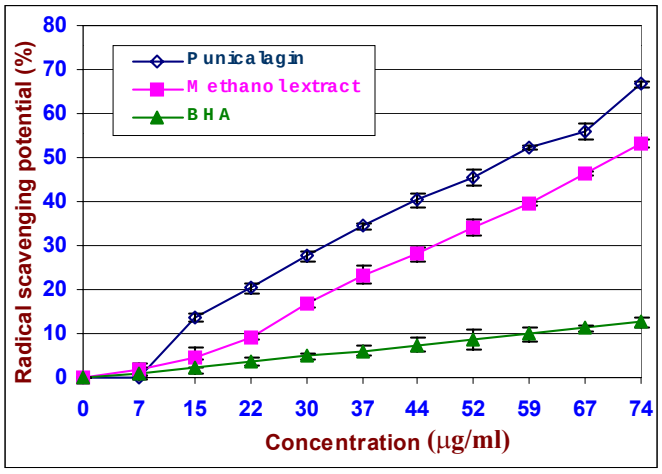
**Punicalagin quinone**

In summary, the punicalagin reduces a DPPH radical by donating hydrogen, resulting in the generation of punicalagin phenoxy radical (Fig 2.2. a). Prior to stabilization of this phenoxy radical, adjacent hydrogen is also abstracted for reducing another DPPH radical (Fig 2.2. b). The phenoxy radical formed by oxidation of punicalagin with two molecules of DPPH radical is stabilized by delocalization of unpaired electrons around the aromatic ring (Fig 2.2. c) resulting in the formation of punicalagin quinine.

### 2.3.1.1.2. Superoxide radical scavenging activity

Although superoxide ($O^{\bullet -}_2$) is relatively weak oxidant, it decomposes to form stronger reactive oxygen species, such as singlet oxygen and hydroxyl radicals, which initiates peroxidation of lipids (Dahl & Richardson, 1978) and many diseases status. Usually in a biological system, its toxic effect was eliminated by superoxide dismutase (SOD). Recently there is an increasing interest in natural and edible sources as external supplements, which exhibit activity similar to SOD.

**Fig. 2.3. Superoxide radical scavenging potential of Punicalagin, methanol extract from pith and CM of pomegranate fruit and BHA**




- Values are mean ± standard error of four replicates

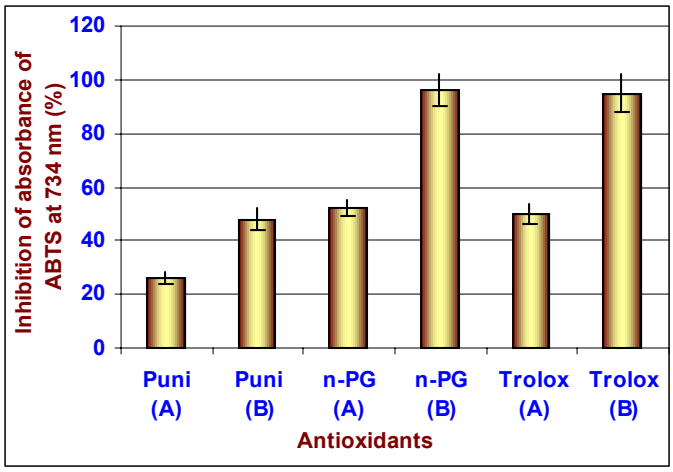
In the present study, the superoxide radicals were generated *in-vitro* in a PMS-NADH system and assayed by the reduction of NBT. The superoxide radical scavenging ability of punicalagin, methanol extract of pith and CM and the synthetic antioxidant BHA has been shown in Fig. 2.3. The punicalagin showed concentration dependent superoxide radical scavenging potential with an $EC_{50}$ value of 58.2 ± 4.6 µg/ml. The $EC_{50}$ value of methanol extract was found to be 71.0 ± 3.2 µg/ml. Synthetic

antioxidant BHA showed very low superoxide scavenging activity with an $EC_{50}$ value of 264 ± 1.6 µg/ml.

**2.3.1.1.3. ABTS assay**

The ABTS method is useful for determining the total radical scavenging activity of antioxidants especially from plant extracts. The results are expressed as percentage decrease in absorbance at 734 nm relative to that of the blank. The present set of experiments carried out at pH 7, and results are expressed as percent inhibition of absorbance of $ABTS^{\bullet-}$ radical on addition of punicalagin as compared against n-propyl gallate (nPG) and that for a reference antioxidant viz. Trolox-c. Fig. 2.4 shows the percent decrease in the absorbance of $ABTS^{\bullet-}$ radical on addition of 50 and 100 µl of either, punicalagin or n-PG, or Trolox-c. It can be seen that both n-PG and Trolox-c have almost identical radical scavenging ability while that of punicalagin is nearly half to that of n-PG and trolox-c.

**Fig. 2.4. ABTS radical scavenging potential of punicalagin compared with n-propyl gallate and Trolox (Puni = Punicalagin, n-PG = n-Propyl gallate; (A)=50 µg dose, (B)=100 µg dose)**

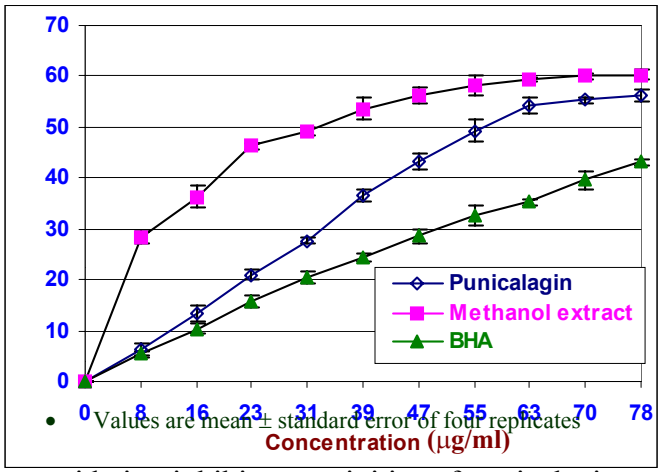



- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

### 2.3.1.1.4. Lipid peroxidation inhibitory activity

Lipid peroxidation is a free radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals. Its termination occurs in biological system through enzymatic means or by radical scavenging activity by antioxidants (Heim *et al.*, 2002). To evaluate the potential of punicalagin and methanol extract of pith and CM to inhibit the lipid peroxidation, the liver microsomal model system was used. In the present study iron –ascorbate system was used to initiate the lipid peroxidation. Malondialdehyde (MDA), the major product of lipid peroxidation reacts with thiobarbituric acid (TBA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. The intensity of pink color was used as a marker for the extent of lipid peroxidation.

**Fig. 2.5. Lipid peroxidation inhibitory activity of punicalagin and methanol extract from pith and CM**




- Values are mean ± standard error of four replicates

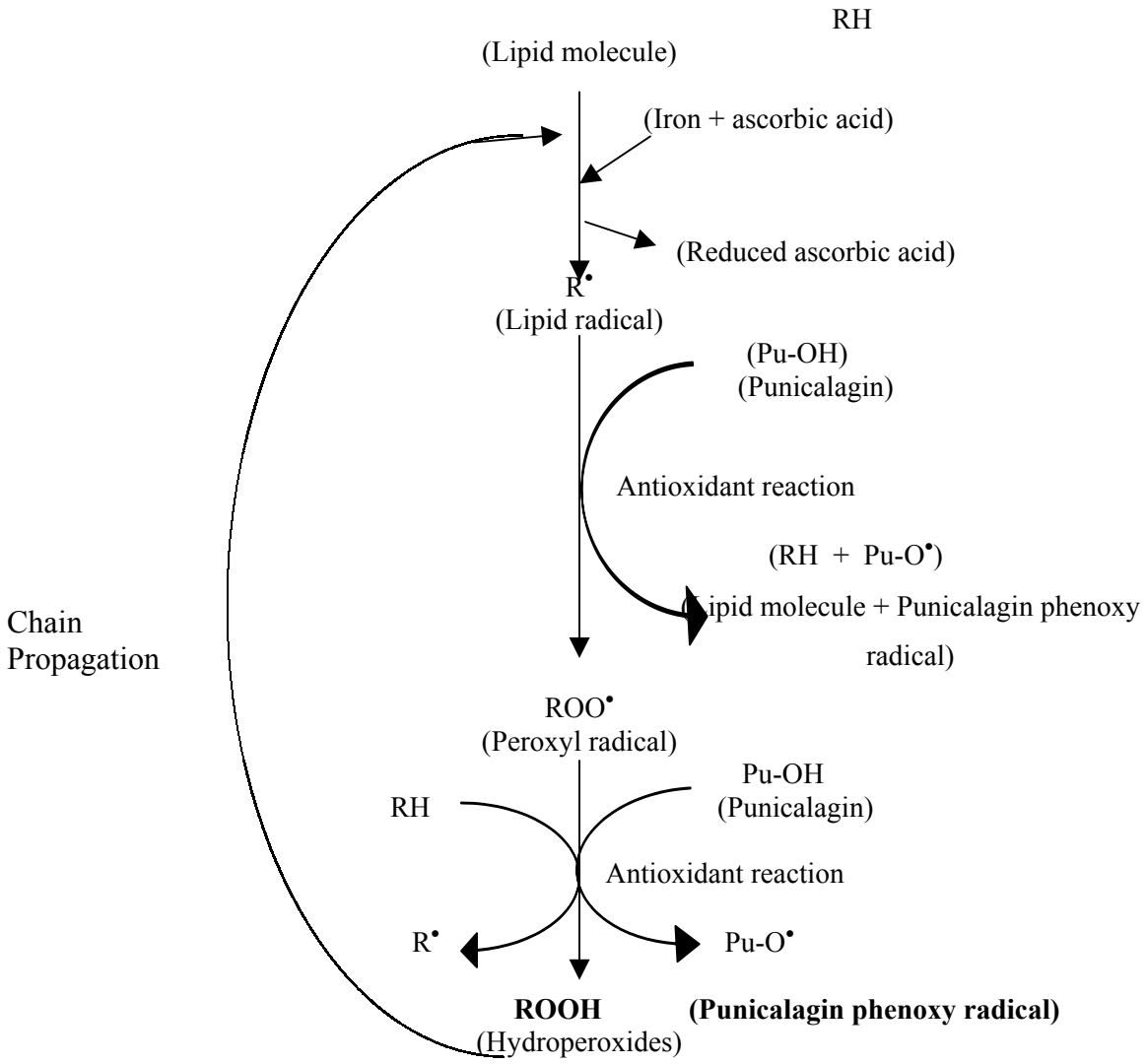
The lipid peroxidation inhibitory activities of punicalagin and methanol extract of pith and CM of pomegranate are shown in Fig 2.5. The $EC_{50}$ value for punicalagin to inhibit lipid peroxidation induced by iron-ascorbic acid system was found to be 54.2 ± 0.9 µg/ml and that of methanol extract was 32.4 ± 1.7 µg/ml. The higher activity of methanol extract as compared to its major component punicalagin may be attributed to

the synergistic effects of its phenolic components. BHA showed very strong antioxidant activity against lipid peroxidation with an $EC_{50}$ of 92.3 ± 1.9 µg/ml.

**Mechanism of action of Punicalagin**

A general reaction of punicalagin in inhibiting the lipid peroxidation involves the quenching of peroxyl free radicals, which is shown in the following reaction

**Hypothetical scheme for mechanism of lipid peroxidation and inhibition by Punicalagin molecule**



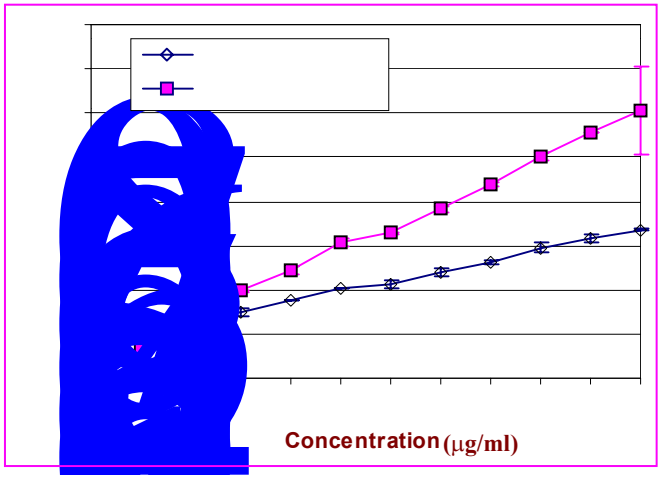

The punicalagin interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals, and also scavenging peroxyl radicals. The resulting punicalagin

phenoxy radical formed is stabilized by the delocalization of unpaired electrons around the aromatic ring, as indicated in the reaction of punicalagin with DPPH radical (Fig. 2.2)

**2.3.1.1.5. Total reducing power**

The reducing capacity of a compound may serve as a significant indicator of its antioxidant capacity (Meir *et al.*, 1995). Total reducing power is the electron donating ability of an antioxidant, which measures its capacity to reduce an oxidant, by donating an electron. In this context; therefore "total reducing power" may also be referred to analogously as "total antioxidant power". In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduction of the $Fe^{3+}$/ferricyanide complex to its ferrous form. Amount of $Fe^{2+}$ complex can be monitored by measuring the formation of Perl's prussian blue at 700 nm. The punicalagin showed concentration dependent reducing power (Fig. 2.6).

**Fig. 2.6. Total reducing power of punicalagin and methanol extract of pith and CM of pomegranate fruit**




The result revealed that the punicalagin is a potential electron donor and also could react with free radicals, converting them to more stable products and terminating

the radical chain reaction. The reducing power of methanol extract was found to be significantly higher than that of punicalagin, which may be due to the synergistic effect of phenolic components.

The protective effects of antioxidants in biological systems are ascribed mainly to their capacity to scavenge free radicals. The radical scavenging ability of punicalagin is because of multiple phenolic hydroxyl groups, which increase the antioxidative activity by additional resonance stability and o-quinone or p-quinone formation (Chen & Ho, 1997; Bouchet *et al.*, 1998). Compared to punicalagin, the methanol extract showed greater ability to scavenge radicals. This may be due to the synergistic effect of other phenolics present in the crude extract, which supplement the radical scavenging ability. Both punicalagin and methanol extract showed potential radical scavenging activities and negligible metal chelating activity. The above experimental results suggest that the mechanism of antioxidant action of punicalagin and methanol extract is by donating electrons to free radicals.

### 2.3.1.2. Pulse radiolysis

Pulse radiolysis experiments were carried out on a 7 MeV linear electron accelerator (Forward Industries Ltd, UK) providing 50 or 500 ns single electron pulses. The pulse irradiates the sample contained in a 1 cm x 1 cm Suprasil quartz cuvette. An optical detection system comprising of a 450 W xenon arc lamp, lenses, mirrors and a monochromator monitors the transient changes in absorbance of the solution following electron pulse. The output from the photo multiplier tube is fed through a DC offset circuit to the Y input of an L & T digital oscilloscope (model 4072). An online computer

was used for data analysis. The absorbed dose was usually ~ 12 Gy / pulse as determined by pulsing an aerated $10^{-2}$ mol $dm^{-3}$ KSCN solution. The formation of $(SCN)_2^{\bullet-}$ radical was monitored at 475 nm. The absorbed dose per pulse was calculated assuming Gε $[(SCN)_2^{\bullet-}] = 2.6 \times 10^{-4}$ $m^2$ $J^{-1}$ at 475 nm.

Water, when subjected to ionizing radiations from linear electron accelerator generates $e^-_{aq,}$ $^{\bullet}OH$ and $^{\bullet}H$ as the three main primary species besides small amounts of molecular products**.** The yield of these primary species being G ($^{\bullet}OH$) ≅ G ($e^-_{aq}$) = 2.9 x $10^{-7}$ mol $J^{-1}$, G ($H^{\bullet}$) = 0.6 x $10^{-7}$ mol $J^{-1}$). The yield of hydroxyl radicals was doubled by irradiating a nitrous oxide ($N_2O$) saturated aqueous solution, where $e^-_{aq}$ was quantitatively converted to the $^{\bullet}OH$ radical. Generation of different oxidizing radicals has been extensively covered in the literature and hence only a brief mention is made here. In aqueous solutions, for example, the energy is used for

Ionization, $H_2O \rightsquigarrow H_2O^+ + e^-$ ---------- (2.7)

Excitation, $H_2O \rightsquigarrow H_2O^*$ ---------- (2.8)

The initial interaction products, $H_2O^+$, $e^-$, and $H_2O^*$, are then converted within 10-12 sec or less into highly reactive primary radical species, like hydrogen atoms $(H)^{\bullet}$ and hydroxyl radicals ($^{\bullet}OH$), hydrated electrons ($e^-_{aq}$), via

$$H_2O \longrightarrow H^+_{aq} + {}^{\bullet}OH \quad \text{---------- (2.9)}$$

$$e^- \longrightarrow e^-_{aq} \quad \text{---------- (2.10)}$$

$$e^- + H_2O^+ \longrightarrow H_2O^* \longrightarrow H^{\bullet} + {}^{\bullet}OH \quad \text{----- (2.11)}$$

The absorption of a pulse of high-energy electrons results in the formation of reactive radical species (reaction 2.9 to 2.11), which are practically homogeneously

distributed throughout the irradiated volume. Their generation is completed within the duration of even the shortest i.e., sub nanosecond pulses are available from an accelerator. These primary radical species can be employed to produce other radical species such as superoxide radical ($^{\bullet}O^{-}$), azide radicals ($^{\bullet}N_3$), nitrous oxide radicals ($NO^{\bullet}_2$), haloperoxyl radicals ($CCl_3OO^{\bullet}$), tryptophanyl radicals ($Trp^{\bullet}$), guanosine radicals ($Gua^{\bullet}$), etc. by changing the chemical system (reactions 2.3 to 2.6). Remarkable point is that, detectors are available to trace the reaction steps of these radicals with the substrates.

$$^{\bullet}OH + N_3^{-} \longrightarrow N^{\bullet}_3 + OH^{-} \quad \text{----------(2.3)}$$

$$^{\bullet}OH + NO_2^{-} \longrightarrow NO^{\bullet}_2 + OH^{-} \quad \text{---------- (2.4)}$$

$$N^{\bullet}_3 + TrpH \longrightarrow Trp^{\bullet} + N_3 + H^{+} \quad \text{---------- (2.5)}$$

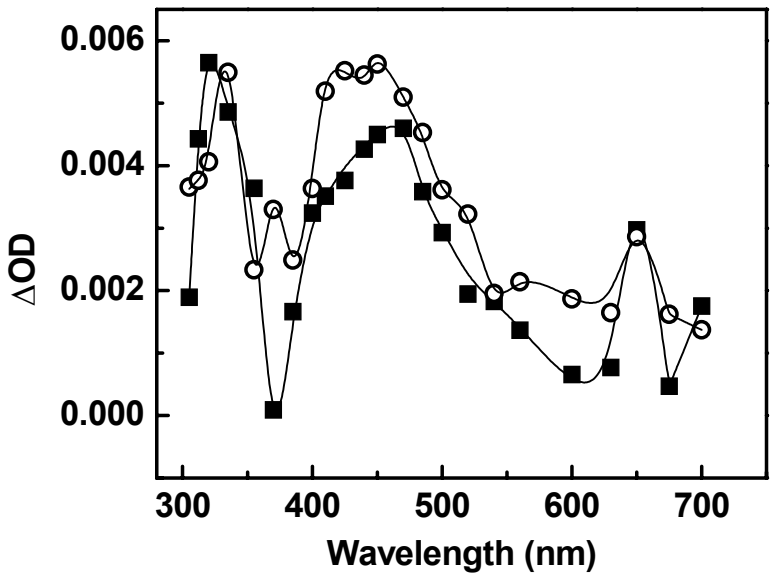
$$N^{\bullet}_3 + ABTS^{2-} \longrightarrow ABTS^{\bullet-} + N_3 + H^{+} \text{---------- (2.6)}$$

In our earlier work on punicalagin showed its potency as an antioxidant molecule, by its radical scavenging potential. In the present work, a pulse radiolysis studies on the reactions of punicalagin with various oxidizing and reducing radicals at near physiological pH in aqueous media was carried out to establish the kinetics and reaction mechanisms. In addition antioxidant ability of the punicalagin has also been assessed in terms of reaction ratio for the repair of radiolytically produced tryptophan radicals.

**Reaction with $^{\bullet}$OH radicals:**

The hydroxyl radicals formed by the radiolysis of water (reactions 2.10, 2.11) are electrophils and react with aromatic substrates by addition, by electron transfer or by abstraction. Its addition to phenols and other substituted aromatic compounds is known to take place preferentially at *ortho*- and *para*-positions (Raghavan & Steenken, 1980). The transient spectrum formed on pulsing a $N_2O$-saturated aqueous solution containing ($10^{-3}$ mol $dm^{-3}$) phosphate buffer and (5 x $10^{-4}$ mol $dm^{-3}$) of punicalagin at pH 6.0 in Fig 2.7 is that of hydroxy cyclohexadienyl radical, formed by the addition of $^{\bullet}$OH radical to punicalagin as shown by the broad $\lambda_{max}$ between 400 – 450 nm.

**Fig. 2.7. Time-resolved absorption spectra of the transient species formed by the $^{\bullet}$OH radical reaction with punicalagin, pH 6.0, measured at 5 and 70 μs after electron pulse.**




The overall reaction between the $^{\bullet}$OH radicals and punicalagin can be given as

$$Pu\text{-}(OH)_{16} + {}^{\bullet}OH \longrightarrow OH^{-} + [Pu\text{-}(OH)_{17}]^{\bullet} \longrightarrow Pu\text{-}(OH_{15})O^{\bullet} + H_2O \text{ ----- (2.12)}$$

The adduct formed at neutral pH loses a water molecule (reaction 2.12) to form phenoxyl radical. In the case of phenols this reaction is facilitated by the presence of phosphate buffer as well as at high and low pH (Land, 1967). Formation of the above transient was exponential with time, which gave the first order rate constants (k′). On plotting the first order rate constants versus the solute concentration (punicalagin), the bi-molecular rate constant for the reaction was 2.5 x $10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$ (Buxton, *et al*, 1988). In general, the rate of reaction of $^{\bullet}OH$ radicals with phenolic derivatives is ~ $10^{10}$ $dm^3$ $mol^{-1}$ $s^{-1}$. Considering the presence of large number of hydroxyl groups in punicalagin that can be oxidized with ease, the above rate constant value is lower by a factor of ~5.

**Reaction with $N_3^{\bullet}$ radicals**

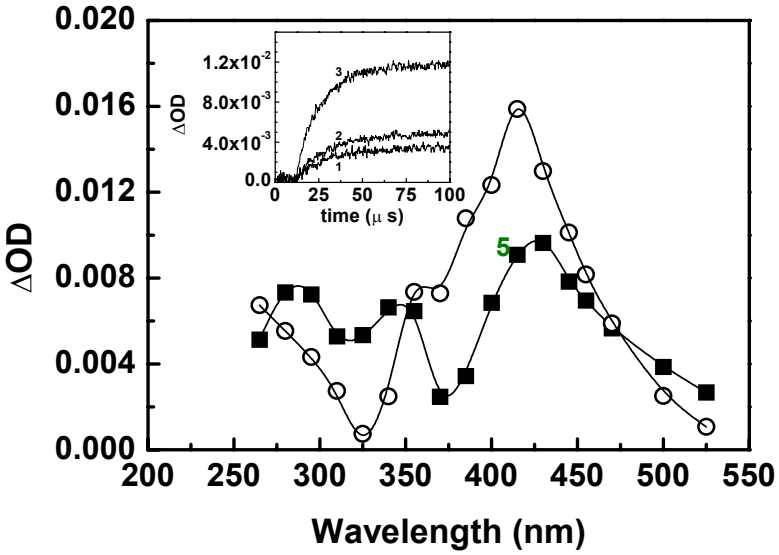
The azide radicals are known to generate phenoxyl radicals from phenols and phenolate ions (Alfassi & Schuler, 1985). Hence, pulse radiolysis of dilute aqueous solution containing of sodium azide (5 x $10^{-2}$ mol $dm^{-3}$), phosphate buffer ($10^{-3}$ mol $dm^{-3}$) and (5 x $10^{-4}$ mol $dm^{-3}$) of punicalagin at pH 6.0 resulted initially in the formation of azide radicals (reaction 2.3) which subsequently result in the generation of punicalagin phenoxyl radicals (reaction 2.13) having an absorption maximum at 425 nm (Fig.2.8).

$$N_3^{\bullet} + \text{Pun-(OH)}_{16} \longrightarrow N_3H + \text{Pun-(OH)}_{15}O^{\bullet} \quad \text{------ (2.13)}$$

The rate constant (*k*) for the formation of transient species, was obtained by plotting the pseudo-first order rates (*k'*) obtained from the build-up traces at 425 nm for different substrate concentrations versus the substrate concentration and was found to be 4.7 x $10^8$ $dm^3$ $mol^{-1}$ $s^{-1}$. This value is quite comparable with some of the reported values for the azide radical reaction with some phenolic derivatives at pH 5.8 (Alfassi & Schuler,

1987). Since these radicals were stable over a long time scale, it was not feasible to determine its decay rate.

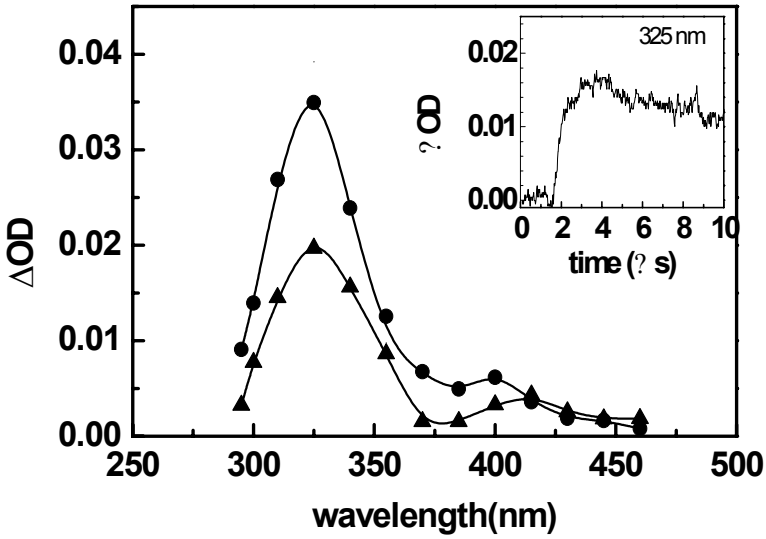
**Fig. 2.8. Transient time-resolved spectra of the semi-oxidized species formed by the reaction of azide radicals with punicalagin at 5 and 15 μs after the pulse. Matrix: $N_2O$-saturated aqueous solution containing (5 x $10^{-2}$ mol $dm^{-3}$) of sodium azide, ($10^{-3}$ mol $dm^{-3}$) phosphate buffer and (5 x $10^{-4}$ mol $dm^{-3}$) of punicalagin at pH = 6.0. (Dose 12 Gy / pulse) Insert: formation of absorbances at 425 nm at (1) 10, (2) 20, (3) 50 x $10^{-6}$ mol $dm^{-3}$ punicalagin concentration.**




**Reaction with $^{\bullet}NO_2$ radicals**

The $^{\bullet}NO_2$ radicals are moderate one-electron oxidants ($E^1$ = 1 V *vs* NHE) that can damage lipids (Byun *et al.*, 1999) and can undergo addition reaction, resulting in nitration of tyrosine (Van der Vliet *et al.,* 1995) present in proteins. It is found in cigarette smoke and exhausts from vehicles. The transient spectrum was recorded by pulsing $N_2O$-saturated aqueous solution containing (5 x $10^{-2}$ mol $dm^{-3}$) of sodium nitrite, ($10^{-3}$ mol $dm^{-3}$) phosphate buffer and (5 x $10^{-4}$ mol $dm^{-3}$) of punicalagin at pH = 7.0 obtained by the reaction of $^{\bullet}NO_2$ radicals with punicalagin is shown in fig 2.9.

**Fig. 2.9. Transient time-resolved spectra of the semi-oxidized species formed by the reaction of $NO2^{\bullet}$ radicals with punicalagin at 5 and 15 μ s after the pulse. Matrix: $N_2O$-saturated aqueous solution containing (5 x $10^{-2}$ mol $dm^{-3}$) of sodium nitrite, ($10^{-3}$ mol $dm^{-3}$) phosphate buffer and (5 x $10^{-4}$ mol $dm^{-3}$) of punicalagin at pH = 7.0. (Dose 12 Gy / pulse). Insert: kinetic trace at 325 nm.**




The absorption maximum at ~ 325 nm is due to the formation of adduct with the substrate and the small peak around 425 nm is that of punicalagin phenoxyl radical. The bimolecular rate constant for the above reaction was 1 x $10^9$ $dm^3$ $mol^{-1}$ $s^{-1}$ at both the wavelengths and was recorded by plotting the pseudo first order rate constants (*k'*) for at least four different concentrations of punicalagin in the range 1 – 6 x $10^{-5}$ mol $dm^{-3}$. This data is indicative of the formation of adduct with punicalagin. The insert in fig. 2.9 shows the time profile of the transient species formed at 325 nm due to the reaction of azide radicals with punicalagin. It could be seen that the formation of the 325 nm species was complete in ~ 4 μs and then decays slightly.

**Repair of ABTS radicals:**

When $N_2O$-saturated aqueous solution containing of sodium azide (5 x $10^{-2}$ mol $dm^{-3}$), phosphate buffer ($10^{-3}$ mol $dm^{-3}$) and of $ABTS^{2-}$ (2 x $10^{-3}$ mol $dm^{-3}$) at pH 7.0 was pulse irradiated, a characteristic absorption observed with $\lambda_{max}$ = 417, 645 and 728 nm is well characterized and attributed to the formation of one-electron oxidized radical of $ABTS^{\bullet-}$ (Scott *et al.*, 1993). In the additional presence of punicalagin (0.5 – 5 x $10^{-5}$ mol $dm^{-3}$), the absorbance at 728 nm showed decay whose rate was found to increase with increasing punicalagin concentration. It might be noted that at this wavelength there is no interference from the punicalagin radicals. Hence, by plotting the pseudo-first order rate constants versus punicalagin concentration the bi-molecular rate constant for the repair of $ABTS^{\bullet-}$ radical by punicalagin was 8.8 x $10^6$ $dm^3$ $mol^{-1}$ $s^{-1}$. This value compares well with the repair of $ABTS^{\bullet-}$ radical by ascorbate (Wolfenden, and Willson, 1982) ($k$ = 8 x $10^6$ $dm^3$ $mol^{-1}$ $s^{-1}$).

$$N_3^{\bullet} + ABTS^{2-} \longrightarrow ABTS^{\bullet-} + N_3^{-} \quad \text{--------} \quad (2.6)$$

$$ABTS^{\bullet-} + \text{Pun-}(OH)_{16} \longrightarrow ABTS^{2-} + \text{Pun-}(OH)_{15}O^{\bullet} \quad \text{--------} \quad (2.15)$$

**Repair of tryptophanyl radicals by Punicalagin**

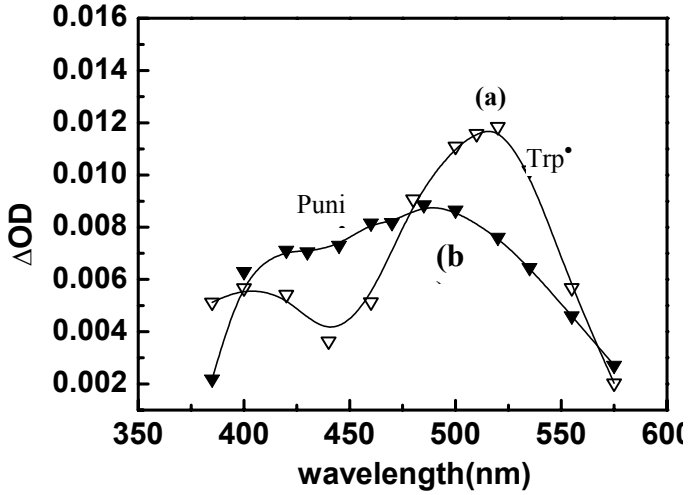
The human body contains ~ 65 % water and may generate $^{\bullet}OH$ radicals as a consequence of radiation. An other possibility of generating $^{\bullet}OH$ radicals *in-vivo* is by Fenton reaction. These radicals are highly toxic and can damage proteins, DNA and lipids. In case of proteins, the amino acids constituting the proteins are prime targets. The azide radicals are initially formed vide reactions (2.3) on irradiating a $N_2O$-saturated aqueous solution containing (5 X $10^{-2}$ mol $dm^{-3}$) sodium azide, ($10^{-3}$ mol $dm^{-3}$)

phosphate buffer, and (3 x $10^{-3}$ mol $dm^{-3}$) tryptophan pH 7. These radicals subsequently react with tryptophan to form the tryptophanyl radicals ($Trp^{\bullet}$) that have well characterized absorbance at 510 nm (Richards *et al.*, 1987). In the additional presence of punicalagin (0.5 – 4 x $10^{-5}$ mol $dm^{-3}$) the tryptophan radicals are scavenged as evidenced by the decrease in the absorption at 510 nm and a consequential increase in the absorbance at 440 nm and adducted to the formation of punicalagin phenoxyl radicals (Fig. 2.10). By a proper choice of experimental conditions such that the reactivity (reactivity = rate constant x concentration) of azide radicals with tryptophan was ≥ 30.

$$N_3^{\bullet} + TrpH \longrightarrow Trp^{\bullet} + N_3^{-} \quad \text{--------} (2.5)$$

$$Trp^{\bullet} + Pun\text{-}(OH)_{16} \longrightarrow TrpH + Pun\text{-}(OH)_{15}O^{\bullet} \quad \text{--------} (2.14)$$

**Fig. 2.10. Transient absorption spectrum attributed to the formation of ($Trp^{\bullet}$) radical on pulsing $N_2O$-saturated aqueous solution containing (5 X $10^{-2}$ mol $dm^{-3}$) sodium azide, ($10^{-3}$ mol $dm^{-3}$ ) phosphate buffer, and of tryptophan, in the absence of punicalagin. pH 7, and at 80 μs after pulse. (B) In the presence of (8 x $10^{-5}$ mol $dm^{-3}$) punicalagin. pH 7, and at 80 μs after pulse. (Dose 12 Gy / pulse).**




The rate constants for the repair of tryptophanyl radicals was determined from the decay rates of tryptophan radicals (λ = 520 nm) with increasing punicalagin concentration

and also from the formation of punicalagin radical as a consequence of repair of tryptophanyl radical at 440 nm were 1.5 and 1.7 x $10^8$ $dm^3$ $mol^{-1}$ $s^{-1}$ respectively and showed good agreement (Richards *et al.*, 1987).

### 2.3.1.3. Cyclic Voltametric experiments

The antioxidant action of punicalagin can be assessed from the ease with which they can donate an electron to an oxidizing radical in aqueous solution (Jovanovic *et al.*, 1996). In order to investigate this, differential pulse voltametric technique was employed to transfer electrons from punicalagin to a glassy carbon electrode in aqueous media at different pH. The differential pulse voltammogram peaks of all punicalagin solutions at different pH were well resolved. This effect has been exemplified in Fig 2.11.,

**Fig. 2.11: Effect of pH on the dependence of peak potential of punicalagin (5 x $10^{-4}$ mol $dm^{-3}$) containing ($10^{-3}$ mol $dm^{-3}$) phosphate buffer and ($10^{-1}$ mol $dm^{-3}$) KCl at pH 7.5 and at 28° C.**

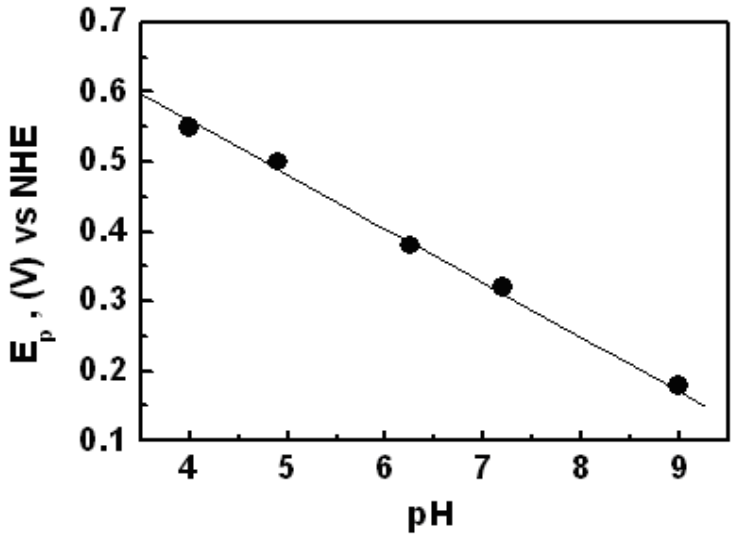


Figure shows a linear plot of peak potential $E_p$ versus solution pH over the range 4 – 9. Since there was no change in the slope of the line over the pH range 4 – 9, one can conclude that the protons are still participating in the oxidation process over this range. From the above figure one concludes that the compound can be easily oxidized at near neutral pH than at lower pH of 4.

**2.3.1.4. Interactions of punicalagin with metal ions**

Redox active metal ions such as iron and copper are believed to play a central role in the formation of reactive oxygen species in biological system (Halliwell, & Gutteridge, 1990). Free radicals and reactive oxygen species are associated with many pathological conditions such as ischemia-reperfusion and aging (Comporti, 1985; Halliwell, & Gutteridge, 1990). Superoxide anion is readily produced through the one-electron reduction of oxygen by ferrous ion (reaction 2.16), and is largely dismuted in to hydrogen peroxide by enzymatic and nonenzymatic mechanisms (Fridovich, 1989). Hydrogen peroxide is further converted to hydroxyl radical by the Fenton reaction (reaction 2.19), which requires reduced iron or copper (Halliwell, & Gutteridge, 1990). Reduced iron also binds to molecular oxygen, and forms perferryl ion ($Fe^{2+}$-$O_2$) (Svingen *et al.*, 1971; Aust, & Svingen, 1982; Miller, & Aust, 1989). Hydroxyl radical and perferryl ion are highly reactive, and act as the actual initiating species for cellular lipid peroxidation (Svingen *et al.*, 1971; Aust, & Svingen, 1982; Fridovich, 1989).

Although iron has received the most attention in this capacity (perhaps because of its greater cellular abundance), the role of copper may be particularly crucial because copper occurs in the mammalian cell nucleus where it may be involved the condensation

of DNA-histone fibres into higher order chromatin structures (Lewis & Laemmli, 1982; Cramp *et al.*, 1989).

$$O_2 + Fe^{+} \longrightarrow {}^{\bullet}O_2^{-} + Fe^{2+} \text{ ---------- (2.16)}$$

$$O_2 + Cu^{+} \longrightarrow {}^{\bullet}O_2^{-} + Cu^{2+} \text{ ---------- (2.17)}$$

$$2O^{\bullet}{}_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \text{ ---------- (2.18)}$$

$$Fe^{+} + H_2O_2 \longrightarrow Fe^{2+} + {}^{\bullet}OH + OH^{-} \text{ ------- (2.19)}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-} \text{ ------- (2.20)}$$

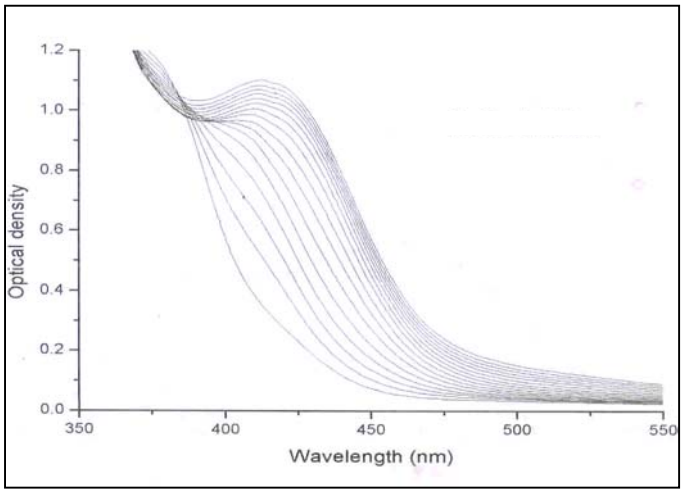
Phenolics are capable of forming metal ion complexes. They mimic animal iron – binding proteins such as transferrin and protect plants by withholding iron from pathogens (Hynes & O'Coinceanainn, 2004). Interactions of several phenolic acids viz. chlorogenic acid, caffeic acid, ferulic acid, naringin, etc. with iron has been widely investigated (Hynes & O'Coinceanainn, 2004). The phenolic molecule, Caffiec acid, which is known to be absorbed into blood stream, can inhibit *in vitro* human low-density lipoprotein oxidation (Laranjinha, Cadenas, 1999; Fito, *et al*, 2000). It is also known to protect against metal ion catalysed oxidation of olive oil samples (De Leonardis, & Macciola, 2002) and shows a potent inhibitory effect on iron-dependent oxidative DNA damge *in vitro* (Lodovici *et al.*, 2001).

A detail study on the interaction of punicalagin with iron and copper was carried out employing UV-Visible spectroscopy technique and presented below. The punicalagin molecule contains four moieties of gallic acid and one of ellagic acid, it is likely that the hydroxyl groups of gallic acid units present in punicalagin are actively involved in binding with the metal ion. The presence of 16-OH groups in punicalagin makes it a good candidate ligand to bind transition metal ions at neutral pH.

### 3.1.7.1. Characteristics of the Punicalagin–$Cu^{2+}$ complexes

Figures 2.12 shows the spectra of punicalagin complex at pH 7.5, in the presence of different concentrations (0.25 – 4 x $10^{-4}$ mol $dm^{-3}$) of copper (II). Addition of copper (II) ions to punicalagin solution results in the oxidation of –OH groups of punicalagin (O'Brien, 1991) to form a semiquinone type of radical and a consequential reduction of copper (II) to Cu (I). The punicalagin *ortho*-semiquinone can be further oxidized to *o*-quinone (Pattison *et al.*, 2002). Chelation of the metal ions occurs with the oxygen atoms of o-quinone and results in stabilization of the complex having an absorption maximum at 411 nm. The metal:ligand ratio was found to be (1:1). A small red shift of ~ 7 nm was observed on increasing the Cu (II) / punicalagin molar ratios. Increasing the solution pH of the complex to ~ 12 did not show any shift in the absorption maximum.

**Fig. 2.12. Change in UV-Vis. absorption spectra of aqueous solution containing 2 x $10^{-3}$ mol $dm^{-3}$ phosphate buffer, (5 x $10^{-4}$ mol $dm^{-3}$) punicalagin and (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 400) x $10^{-6}$ mol $dm^{-3}$ $CuCl_2$, pH = 7.5 at 28° C.**



Keeping the concentration of the ligand constant and by varying the concentration of metal ion it was possible to determine the stability constants. Stability constants of a 1:1 M:L complex was determined from the following relation

M is the amount of metal ion, and L is the amount of the ligand such as phenol.

$$M + L = ML \quad \text{---------- 1}$$

$$K = \frac{[ML]}{[M][L]} \quad \text{---------- 2}$$

$$[M]_0 = [M] + [LM] \quad \text{and} \quad [L]_0 = [L] + [LM]$$

where, $M_0$ is the total concentration of the metal ion and $L_0$ is the total concentration of the ligand.

$$K = \frac{[ML]}{([L]_0 - [ML])([M]_0 - [ML])} \quad \text{---------- 3}$$

The equilibrium constant *K* for a (1:1) complex was determined by employing Benesi-Hildebrand equation 4. (Doyle, 1974)

$$\frac{1}{[\Delta A]} = \frac{1}{K\Delta\varepsilon[M]_0[L]_0} + \frac{1}{\Delta\varepsilon[M]_0} \quad \text{---------- 4}$$

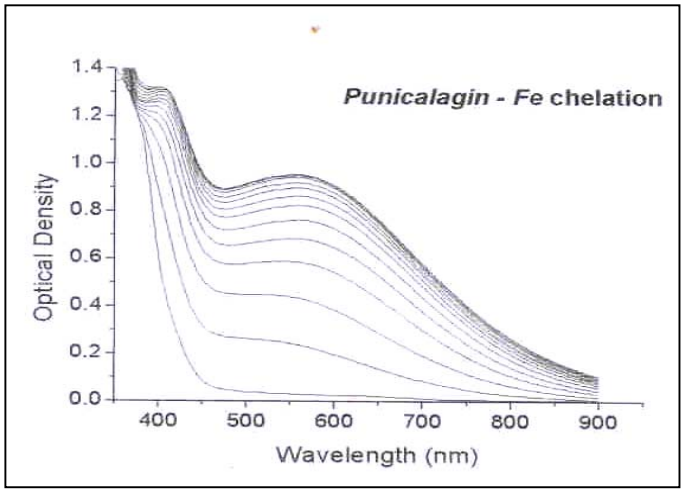
Where, *K* is the stability constant (expressed as logarithm).

From the linear plot of 1 / ΔA *versus* 1 / [M], with slope = 1 / $K\,\Delta\varepsilon[L]_0$ the value of *K* the equilibrium constant was determined to be $K = 1.8 \times 10^7\ M^{-1}$. This binding constant is quite low and fairly comparable to the binding constant of Cu (II) with nucleosomes (Martell, 1971) is ~ $4.5 \times 10^4\ M^{-1}$.

**Characteristics of the Punicalagin–$Fe^{2+}$ complexes**

The blue colored $Fe^{2+}$ punicalagin complex (Fig. 2.13) was formed in a similar manner to the $Cu^{2+}$-punicalagin complex, and showed two maxima, one at 400 nm and another one at 555 nm. The peak at 555 nm is attributed to metal to ligand charge transfer band. A similar blue colored complex ($\lambda$ = 555 nm) was also observed in the case of Fe (II)-n propyl gallate (nPG) at pH ~ 7.5 (Guo, *et al*, 1996). Increasing the solution pH from ~ 7 to 12 resulted in a shift of absorbance maximum from 405 to 420 nm, while the band at ~ 550 nm showed a hipsochromic shift of about 50 nm at higher pH. The metal:ligand ratio at this pH 7 was (1:1) and the binding constant for the complex was $K$ =1 x $10^7$ $M^{-1}$. This value is slightly higher than that reported by Reddan *et al.* (2003) for (1:1) Fe(II):nPG complex but comparable to the (1:1) Cu(II)-punicalagin complex. Metal ion binding of punicalagin may thus help in mitigating oxidative damage due to metal ions

**Fig. 2.13. Change in UV-Vis. absorption spectra of aqueous solution containing 2 x $10^{-3}$ mol $dm^{-3}$ phosphate buffer, (5 x $10^{-4}$ mol $dm^{-3}$) punicalagin and (0, 50, 100, 150, 200, 250, 300 350, 400, 450, 500, 550, 600) x $10^{-6}$ mol $dm^{-3}$ ferrous ammonium sulphate, pH = 7.5 at 28° C. Inset: double reciprocal plot of optical density *versus* iron (II) concentration at 550 nm.**



Binding of iron and copper to the punicalagin can suppress the accessibility of the iron and copper to oxygen molecules. Preferential ligand binding to $Fe^{2+}$ will change the redox potential for converting the ferrous ion to ferric state and thereby inhibit oxidative damage. Polyphenols were also reported to reduce iron and then form $Fe^{2+}$ - polyphenol complexes that are inert. (Laughton *et al.,* 1987). Information from this study and the literature shows that the following functional groups are important for Fe and Cu binding viz. *ortho*-dihydroxyl groups and presence of large number of OH groups.

The literature on the adverse effects of polyphenols on iron bioavailability have emphasized that the polyphenols bind to metal ions resulting in the reduced iron absorption both *in vivo* and *in vitro* (Brune *et al.*, 1989).

### 2.3.2. Interaction of punicalagin with HSA

Human serum albumin (HSA) is one of the most abundant proteins in the blood plasma involved in the transportation and disposition of endogenous and exogenous ligands (Robertson, & Brodersen 1991), such as drugs, which are poorly soluble in water, fatty acids, etc. The structure of HSA has been determined crystallographically (He, & Carter 1992; Carter, & Ho, 1994) and discovered that it consisted of three structurally homologous, predominantly helical domains (domain I, II and III), of which each domain contained two sub domains (A and B). Two principal binding sites were located in sub domains IIA and IIIA, which were named as site I and site II respectively. The drugs bound in site I are generally bulky heterocyclic anions with the charge situated in a central position of the molecules and that bound in site II are aromatic carboxylic acids with an extended conformation and the negative charge located at one end of the

molecule (Peters, 1996; Dockal *et al.,* 2000). Human serum albumin (HSA) has been usually used as a model protein to study the drug to protein interactions (Zsila *et al.,* 2003; Jiang *et al.,* 2004).

Punicalagin is hydrophilic molecule with sixteen phenolic OH groups. Interactions between many phenolics and HSA have been studied extensively. It has been found that 99.4% of quercetin in plasma binds to human serum albumin (Boulton *et al.,* 1998). Zsila *et al*. (2003) probed the binding of the quercetin to HSA using Circular Dichroism and Electronic Absorption Spectroscopy, and the interactions of quercetin and other flavonoids with proteins were also investigated by fluorescence spectroscopic method (Sengupta, & Sengupta, 2002; Sengupta, & Sengupta, 2003; Gutzeit *et al.,* 2004).

A study on bioavailability of punicalagin in the rats reported that punicalagin and its metabolites were observed in plasma, urine, and faeces. (Cerda *et al.,* 2003). However, it is not known whether HSA can function as carrier molecule. To assess this, the present investigation was undertaken to study the interaction of punicalagin with serum protein. The interaction between punicalagin and HSA has been investigated by UV absorption and fluorescence spectroscopy.

Binding of punicalagin with HSA was analyzed in terms of the quenching of relative fluorescence intensity of HSA in presence of punicalagin using an established method (Lehrer & Fasman, 1966), with the assumption that the binding of each molecule of punicalagin causes some degree of fluorescence quenching. The percentage quenching of the fluorescence intensity of proteins by punicalagin was corrected empirically for internal absorption and filtration by subtracting the percentage quenching by the same concentration of punicalagin of the fluorescence of N-acetyl tryptophan amide equivalent

in absorption to protein at 285 nm. The equilibrium constant, *K,* is given by the following equation:

$$K = \frac{\beta}{1-\beta} \times \frac{1}{Cf} \qquad \text{----------(i)}$$

Where,

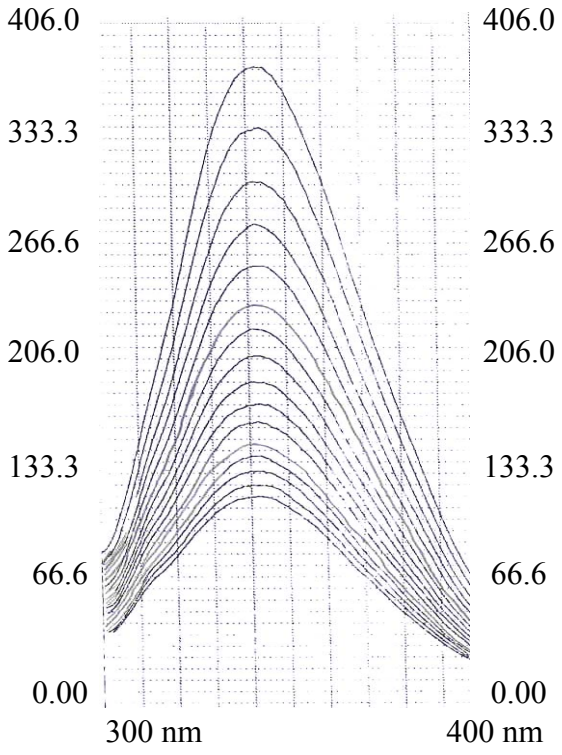
$$\beta = Q/Q_{max}$$

$Q_{max}$ = maximum quench, which was determined by extrapolation of a double reciprocal plot of Q against $C_T$ to intercept. $C_T$ is the total concentration of punicalagin added.

$$Cf = C_T - n\beta T$$

The value of *K* is given by the slope of a plot of β/(1-β) against C*f*, the free ligand concentration.

**Fig. 2.14. Fluorescence quenching spectra of HSA with the addition of a series increasing concentration of punicalagin $\lambda_{ex}$ 295 nm, $\lambda_{em}$ 334 nm in physiological condition (pH 7.4)**



There is one tryptophan residue in HSA, *TRP214*, which is situated in sub domain IIA (He & Carter, 1992). When the excitation wavelength is 295 nm, only the tryptophan residue has a fluorescence emission at about 333 nm. At the excitation wavelength of 280 nm, both tryptophan and tyrosyl amino acid residues have fluorescence emission. Fig. 2.14 shows the fluorescence quenching spectra of HSA before and after addition of a series concentration of punicalagin in physiological condition (pH 7.4) at an excitation of 295 nm. Punicalagin has no fluorescence emission at the range measured and the background was subtracted by using the corresponding concentration of punicalagin solution as reference while recording the spectra. It can be seen from Fig. 2.14 that the intensities of fluorescence emission of HSA decreased gradually with the increase of punicalagin concentration. The relative fluorescence emission intensities at 333 nm were plotted against punicalagin concentration (fig. 2.15).

**Fig. 2.15. Plot of fluorescence quenching of HSA by punicalagin (concentration of HSA (1.3 μM), Concentration of punicalagin (0 – 13.8 μM))**

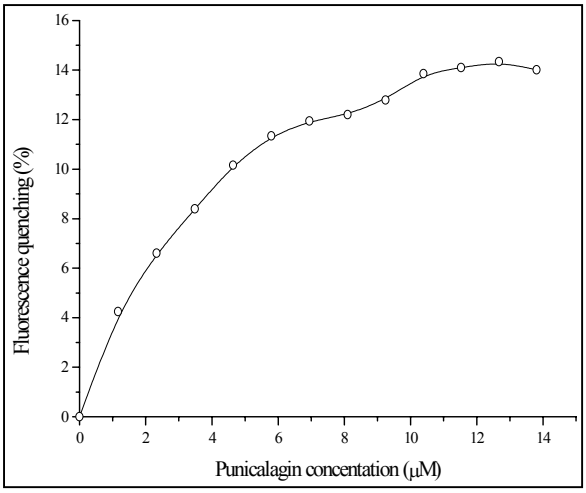
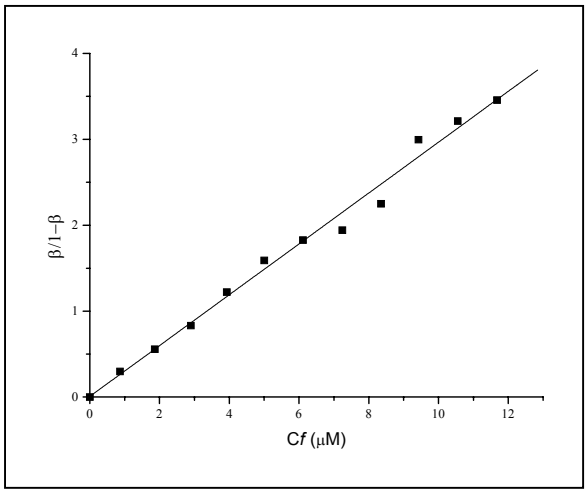


**Fig. 2.16. Mass action plot (β/1-β) against free punicalagin concentration (C*f*). The slope of the straight line was used to obtain the binding constant. (K*b*)**




Fluorescence emission of HSA was decreased by 14 % at a punicalagin concentration of 13.8 μM. Quenching of fluorescence elucidated that the microenvironment of tryptophan residues in HSA have been changed and the conformation of protein has been perturbed due to the punicalagin–protein interaction. From mass action plot (Fig. 2.16), the binding constant of punicalagin with HSA was found to be 2.9 X $10^5$ $M^{-1}$

**Table 2.1 Effect of temperature on the binding constant of punicalagin with HSA protein**

| Temperature °C | Binding constant |
|---|---|
| 17 | 3.5 x$10^5$ $M^{-1}$ |
| 27 | 2.9 x$10^5$ $M^{-1}$ |
| 37 | 1.6 x$10^5$ $M^{-1}$ |
| 47 | 9.5 x$10^5$ $M^{-1}$ |

Punicalagin being a high molecular weight polyphenol with sixteen phenolic OH groups, it can reversibly complex with protein via hydrogen bonding and hydrophobic interactions (Maliwal *et al.*, 1985). The involvement of hydrophobic groups in the formation of phenolics has been established in several independent studies (Oh *et al.,* 1980; Haslam, 1996). Oh *et al.* (1980) drawn the attention to the fact that hydrophobic interactions may dominate the ligand protein interaction. The presence of hydrogen donors in the form of phenolic OH groups in the punicalagin molecule and hydrogen acceptors of the peptide linkage of the protein, may lead to formation of hydrogen bonds. However, our studies on the equilibrium constant as a function of temperature showed that, binding constant decreased with increase in the temperature from 17°C to 47°C (Table 2.1) to favor the concept of hydrogen bonding as a contributing factor for punicalagin binding to HSA. The variance of $K_{eq}$ in the temperature ranges studied support the role of hydrogen bonding (Table 2.1).

HSA is the primary carrier of both exogenous and endogenous ligands. The reversible association of punicalagin with serum transport protein (HSA) in the plasma by non covalent linkages may helps in its transport to appropriate target cells where it can elicits its pharmacological effects.

### 2.3.3. Interaction of Punicalagin with DNA

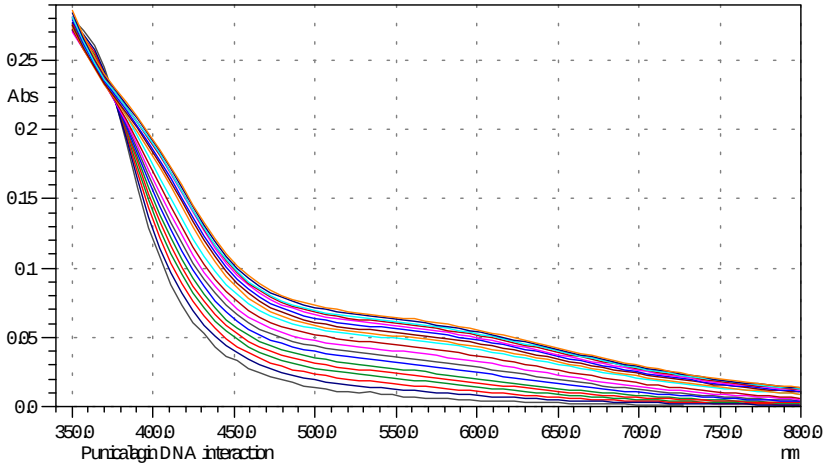
The study of the interaction of many naturally occurring and synthetic organic ligands is an active area of research in chemistry and biology. In several years, the interaction has been widely studied using various spectrometric techniques (Dougherty *et al.*, 1985; Sari *et al.*, 1990; Monnot *et al.*, 1991; Ismail *et al.*, 1998; Medina *et al.*, 1998;

Ghosh *et al.*, 1999; Tjahjono *et al.,* 1999), such as UV-Visible, NMR, ESR, fluorescence, resonance Raman spectroscopy, CD, and FT-IR, and so does electrochemical methods (Feng *et al.*, 1997; Wang *et al.*, 2000).

A great interest in this field arises, on the practical level, from the fact that many molecules, which bind to DNA, are effective pharmaceutical agents, especially in cancer chemotherapy (Mudasir *et al.*, 1999). Understanding the binding of small molecules to DNA is therefore, potentially useful in developing design principles to guide the synthesis of new improved drugs which can recognize a specific site or conformation of DNA and to provide a good tool for biotechnology (Pyle *et al.*, 1989; Naing *et al.*, 1994).

Figure 2.17, shows the spectral changes recorded on stepwise addition of punicalagin to DNA solution. The figure does not show any significant shift in the absorption maximum as a consequence of binding between punicalagin and DNA. The equilibrium constant value ($K$ = 45 $dm^3$ $mol^{-1}$) calculated form this data was quite low and is suggestive of non-specific interactions.

**Fig. 2.17. Change in UV-Vis. absorption spectra of aqueous solution of DNA on the addition of punicalagin. Matrix: To a 1ml aliquot of 50 μl of 1mg /ml of DNA and 950 μl of Tris HCl buffer at pH 7.4, was added (10 μl) aliquots of (5 x $10^{-4}$ mol $dm^{-3}$) punicalagin.**



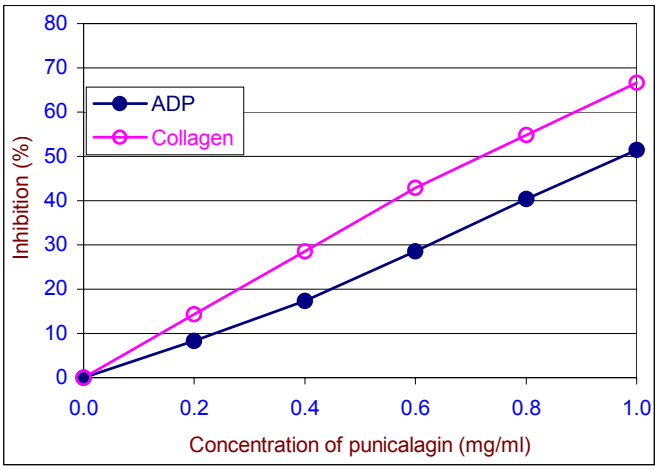
Many DNA binding molecules are known to have chemotherapeutic activity against cancer. Since the binding of DNA can leads to altered replication and transcription, which play a crucial role in cancer therapies and also can be a source for designing the anticancer drugs.

### 2.3.4. Platelet aggregation inhibitory activity

Platelets are tiny corpuscles continuously surveying the inner lining of blood vessels, the vascular endothelium. Any break in the continuity of the vessel wall, leading to hemorrhage, or a break in the atherosclerotic plaque is met with an instant response from the platelets, which contact the zone of injury, spread and aggregate, forming thrombi that seal off the break.

Regulation of platelet activity by using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Antiplatelet agents, such as aspirin, dipyridamole, thienopyridines, and platelet glycoprotein IIb/IIIa antagonists have amply demonstrated their utility in preventing and treating coronary artery thrombosis. (Van De Graa & Steinhubl, 2000; Calverley, 2001) Despite their benefits, administration of thienopyridines can in certain cases be associated with several undesired side effects like neutropenia, agranulocytosis, gastrointestinal toxicity (Berger, 1999), whereas a problem in clinical use of all the currently available antiplatelet agents is the interference of physiological platelet function in haemostasis (Schror, 1995). Therefore, the development of antiplatelet agents, showing limited side effects, is still a relevant goal of the pharmaceutical research. Thus, the purpose of the present study was to investigate the effects of punicalagin isolated from pomegranate fruit pith and CM on platelet function stimulated by different agonists.

**Fig. 2.18 Platelet aggregation inhibitory activity of punicalagin against ADP and collagen induced aggregation (Incubation time for ADP 1 min and for Collagen 10 min)**




The punicalagin showed concentration dependent platelet aggregation inhibitory activity (Fig 2.18) against both the agonists used in the study viz. ADP and collagen with an $IC_{50}$ value of 0.98 ± 5.6 µM and 0.72 ± 3.8 µM respectively. Platelet activation may be inhibited by a number of dietary components such as alcohol (Rorondo *et al.*, 1996), some dietary fats (Cerbone *et al.,* 1999) and polyphenols (Tzeng *et al.,* 1991). Several *in vitro* studies showed that polyphenols such as resveratrol, the flavonoids quercetin (Qc) and catechin inhibit platelet aggregation (Pece-Asciak *et al.*, 1995; Kelly *et al.,* 1996; Pignatelli *et al.,* 2000). Results obtained by the incubation of human or animal platelets with isolated polyphenols suggest that the antiplatelet properties may be attributed to the inhibition of TxA2 formation (You, *et al* 1999), thromboxane receptor antagonism (Hubbart *et al.,* 2003), protein kinase C activation (Ganet-Payrastre *et al.,* 1999) and phosphoinositide synthesis.

## 2.3.5. *In-vitro* assay for cytotoxicity of punicalagin

A critical module in the characterization of a functional molecule is to assess the safety of the candidate in a series of acute and sub acute toxicity. An ideal antioxidant molecule should exert antioxidant properties in a concentration range that has no toxic effect on the health. A simplest method to determine the toxicity of a molecule is *in vitro* cytotoxicity studies against the animal cell lines cultured under laboratory conditions. It is well known that different cell lines exhibit different sensitivities to a chemical compound. Therefore, the use of more than one cell line was considered necessary in assessing the cytotoxicity of punicalagin. Punicalagin as well as its source extracts were tested against three different cell lines viz. Vero, HEp-2, and A-549, which are from different origins and possess different morphology and tumorigenic properties. A linear increase in cytotoxic activity was observed in relation with concentration gradient of punicalagin and methanol extract against all the three cell lines tested.

**Table 2.2 Cytotoxicity of punicalagin against cell lines in in-vitro conditions**

| Cell lines | Cytotoxicity $CTC_{50}$ value (μM) | |
|---|---|---|
| | **Punicalagin** | **Methanol extract** |
| Vero | 587± 21 | 545 ± 13 |
| HEp-2 | 950± 12 | 740 ± 18 |
| A-549 | 975± 18 | 830 ± 21 |

The $CTC_{50}$ value of punicalagin was about 587 ± 21, 950 ± 12 and 975 ± 18 μM against Vero, HEp-2 and A-549 cell lines respectively, where as that of methanol extract was 545 ± 13, 740 ± 18, 830 ± 21 μM respectively. Results reveal that punicalagin is

having very low cytotoxicity. Interesting to note down is that the constituent molecules of punicalagin viz ellagic acid and gallic acid were reported to be toxic at lower concentration i.e. in the range of 1-185 μM against digestive cells of the freshwater mussel *Unio tumidus* (Labieniec *et al.*, 2003). The wide variation in the cytotoxicity may be attributed the structural variation in a molecule as well as cell lines used for the studies. For example, in a study on cytotoxicity of phenolics viz. quercetin and rutin, the two pharmacologically well-studied antioxidant phenolics revealed that quercetin at higher doses (50–100 μM) was cytotoxic, on the contrary, rutin had no cytotoxic effect at any dose on Human hepatoma cell line (HepG2) (Alía *et al.*, 2005). Gupta *et al.* (1999) reported that tea polyphenols produce cytotoxicity at high concentrations. Though many reports are available on cytotoxicity of phenolics, their mode of action is not yet well established, perhaps Damianaki *et al.* (2000) have proposed that protein binding ability, in particular membrane protein of cell lines may affects the cell growth and its viability. True to this, our results also revealed the potential protein (HSA) binding ability of punicalagin. However, variation in cytotoxic activity of punicalagin against different cell lines suggests its non-specific type of toxicity and its mode of action is not conclusive. However, this kind of investigation allows us to apply these functional phytochemicals in the treatments using their beneficial properties at the concentration, which has no toxic effect.

However, punicalagin has showed cytotoxic properties *in vitro*, the *in vivo* behavior of the molecule will be different. Usually phenolics found to be non-toxic or toxic only at very high concentration. Hence it can be used as a pharmaceutical molecule because of its multifunctional activities

### 2.3.6. Antibacterial activity

An estimated 76 million cases of food borne disease occur each year in the world. The great majorities of these cases are mild and cause symptoms for only a day or two. Some cases are more serious, and reports are there that there are 325,000 hospitalizations and 5,000 deaths related to food borne diseases each year. The most severe cases tend to occur in the very old, the very young, those who have an illness already that reduces their immune system function, and in healthy people exposed to a very high dose of an organism. Many different disease causing microbes or pathogens, can contaminate foods, so there are many different food borne infections. The most commonly recognized food borne infections are those caused by the bacteria, *Salmonella*, and *E. coli* O157:H7, *Enterobacter, Pseudomonas*, *Shigella, Proteus, Campylobacter*, etc.

Due to the indiscriminate use of antimicrobial drugs the microorganisms have developed resistance to many antibiotics. This has created immense clinical problem in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects on host, which include hypersensitivity. Therefore there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen local medicinal plants for possible antimicrobial properties. Medicinal herbs represent a rich source from which novel antibacterial molecules may be obtained. The present investigation represents a study on antibacterial activity of punicalagin against clinically important food borne pathogenic bacteria. Along with punicalagin other extracts of pith and CM of pomegranate were tested for their antibacterial activity against important gram-negative pathogenic bacteria (clinical isolates).

**Table 2.3. Clinical isolates used for studying the antibacterial potential of punicalagin**

| Clinical isolates | Diseases |
|---|---|
| *E. aerogenes* | Urinary tract infections (UTI), neonatal meningitis, and gastroenteritis |
| *E. coli* | Urinary tract infections (UTI), neonatal meningitis, and gastroenteritis |
| *P. mirabilis* | Formation of stuvite and carbonate-apatite crystals |
| *P. aeruginosa* | Urinary tract infections, respiratory system infections, dermatitis, |
| *S. typhi* | Salmonellosis |
| *S. dysenteriae* | Shigellosis |

**Agar-well diffusion assay**

Among the six extracts of pith and CM, viz. chloroform, ethyl acetate, acetone and methanol extracts from pith and CM of pomegranate fruit and its major bioactive component punicalagin showed significant antibacterial activity against all the bacteria tested (Table 2.4).

**Table 2.4. Antibacterial activity of extracts of pomegranate fruit waste (pith and CM) and punicalagin on clinically important human pathogenic bacteria**

| Bacteria | Diameter of the zone of inhibition (mm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Solvent extracts | | | | | | Punicalagin |
| | Hexane | Chloroform | Diethyl ether | Ethyl acetate | Acetone | Methanol | |
| **E. aerogenes** | 17±1 | 20±1 | 13±0.5 | 20±0.5 | 25±0.5 | 22±0.5 | 21±0.5 |
| ***E. coli*** | 18±0 | 21±0.5 | 14±0.5 | 21±1 | 24±1 | 25±0.5 | 25±0 |
| ***P. mirabilis*** | 17±0 | 22±0.5 | 16±0.5 | 21±1 | 29±0.5 | 25±0 | 25±0 |
| ***P. aeruginosa*** | 17±0 | 20±0 | 13±0.5 | 20±0.5 | 21±0.5 | 19±0 | 18±0 |
| ***S. typhi*** | 17±1 | 19±0 | 15±0 | 20±0.5 | 20±0.5 | 25±0 | 25±0 |
| ***S. dysenteriae*** | 16±0 | 19±0.5 | 12±0 | 21±0.5 | 28±1 | 21±1 | 20±1 |

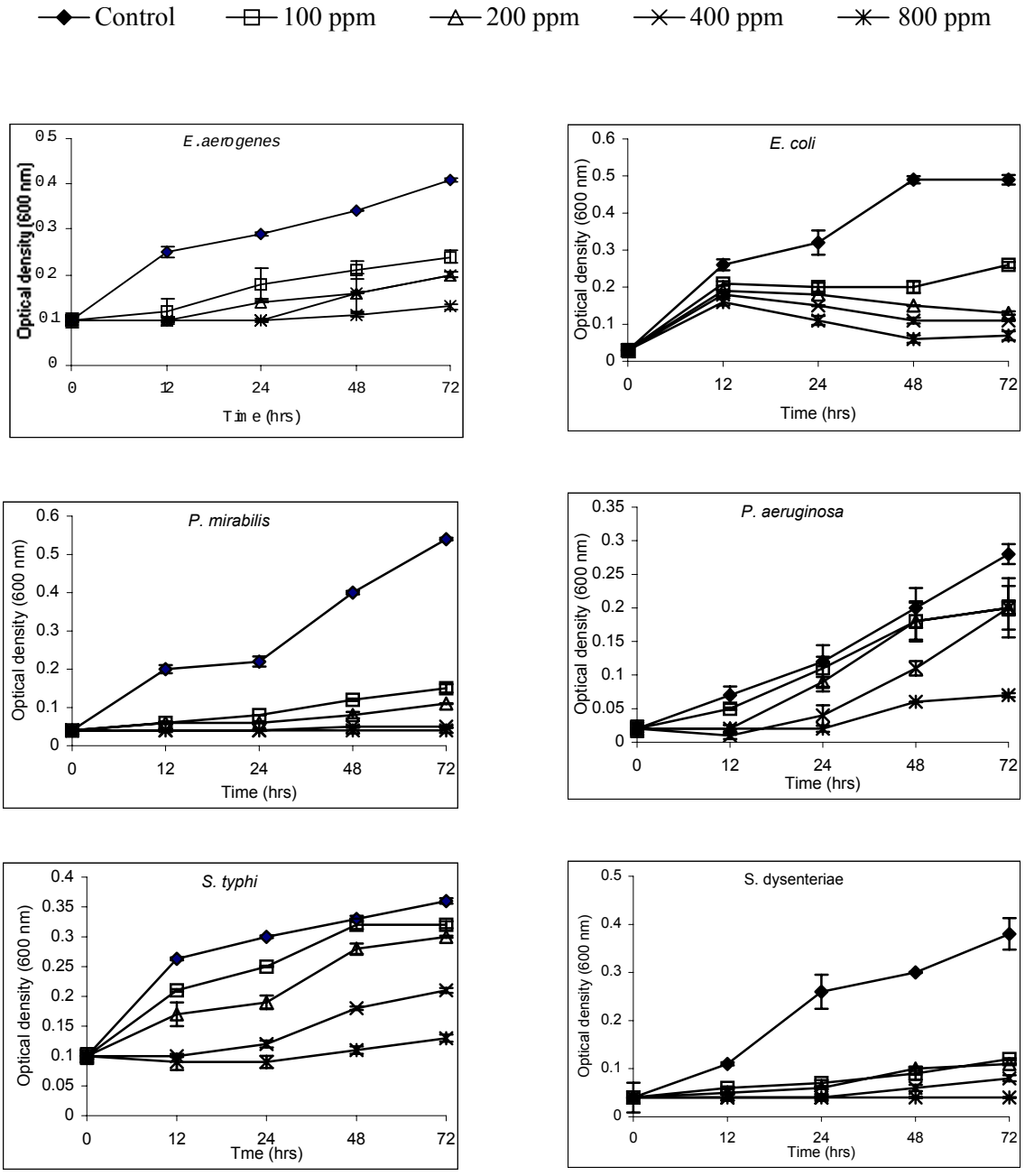
Data shown are the mean value of triplicates ± SD

Among the different solvent extracts acetone, methanol extract and punicalagin showed highest antibacterial effect against all the bacterial species tested while diethyl ether extract showed lowest antibacterial activity. Highest zone of inhibition of acetone extract was observed against *P. mirabilis* (29 mm) followed by *S. dysenteriae* (28 mm). Methanol extract and punicalagin showed highest zone of growth inhibition against *S. typhi* (25 mm) and *P. mirabilis* (24 mm). The broad spectrum antibacterial activity of methanol extract may be attributed to the punicalagin and other phenolics present in it.

**Bacterial Growth inhibitory assay**

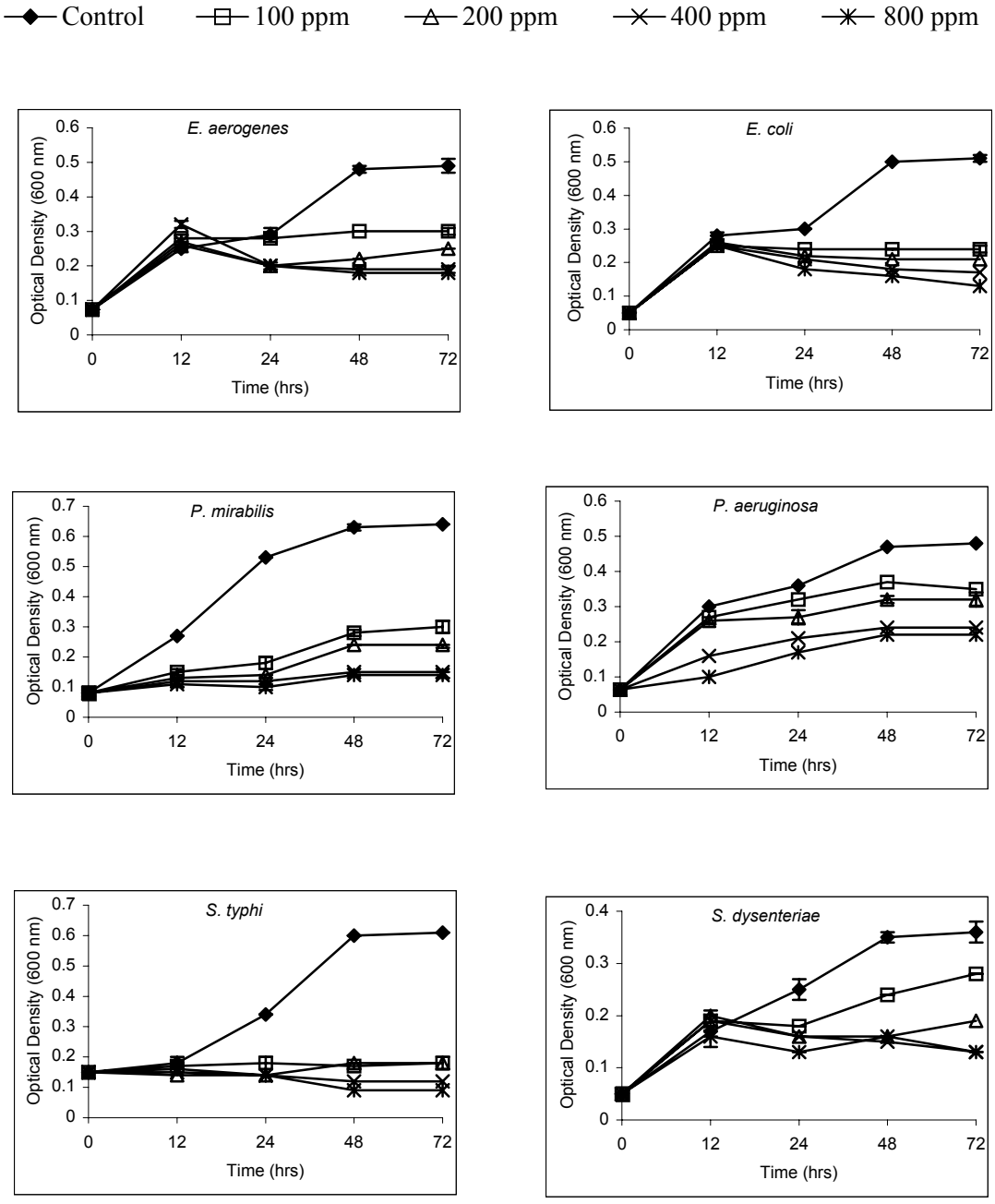
Acetone, methanol extract and punicalagin that showed high antibacterial activity at low concentration (Table 2.4) were further evaluated for their antibacterial effects at different concentration by spectrophotometric method. (Fig. 2.19, 2.20, 2.21). The dose-response curves obtained from plotting the concentration of acetone extract, methanol extract and punicalagin against the bacterial growth at different time intervals are shown in Fig. 2.19, 2.20, and 2.21 respectively. Acetone extract showed an excellent growth inhibitory activity against *P. mirabilis* and *S. dysenteriae* with $MIC_{50}$ value of 60 and 75 ppm respectively (Fig. 2.19). It also inhibited 50% growth of two other enteric opportunistic pathogens viz. *E. aerogenes* and *E. coli* at 190 and 150 ppm respectively. But *P. aeruginosa* and *S. typhi* required high doses of acetone extract viz. 570 and 560 ppm.

**Fig. 2.19. Dose response growth curves of clinically important pathogenic bacteria against acetone extract from pith and carpellary membrane of pomegranate fruit**



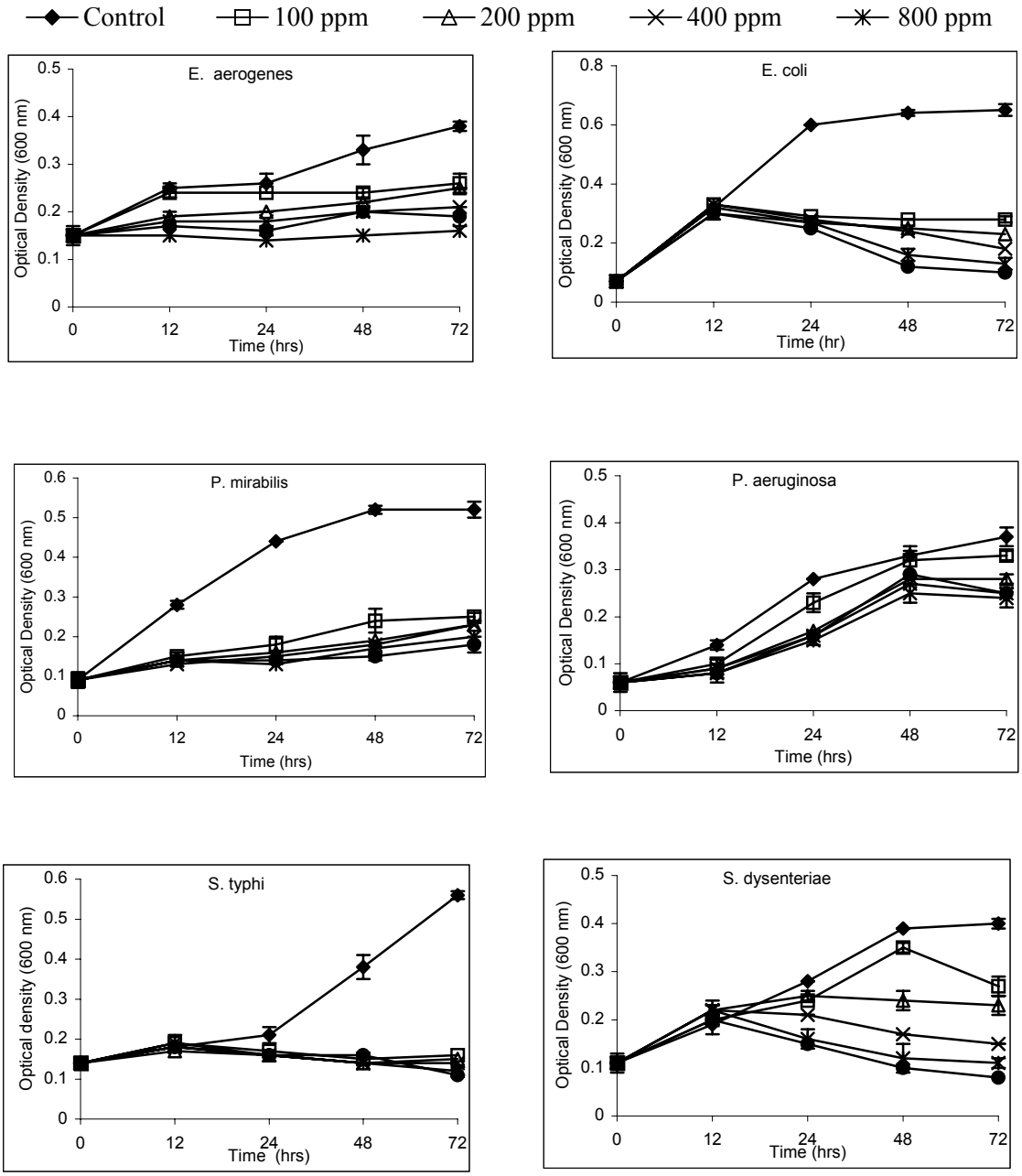

- Values are mean ± standard error of four replicates

**Fig. 2.20. Dose response growth curves of clinically important pathogenic bacteria against methanol extract from pith and carpellary membrane of pomegranate fruit**




- Values are mean ± standard error of four replicates

**Fig. 2.21. Dose response growth curves of clinically important pathogenic bacteria against punicalagin from pith and carpellary membrane of pomegranate fruit**

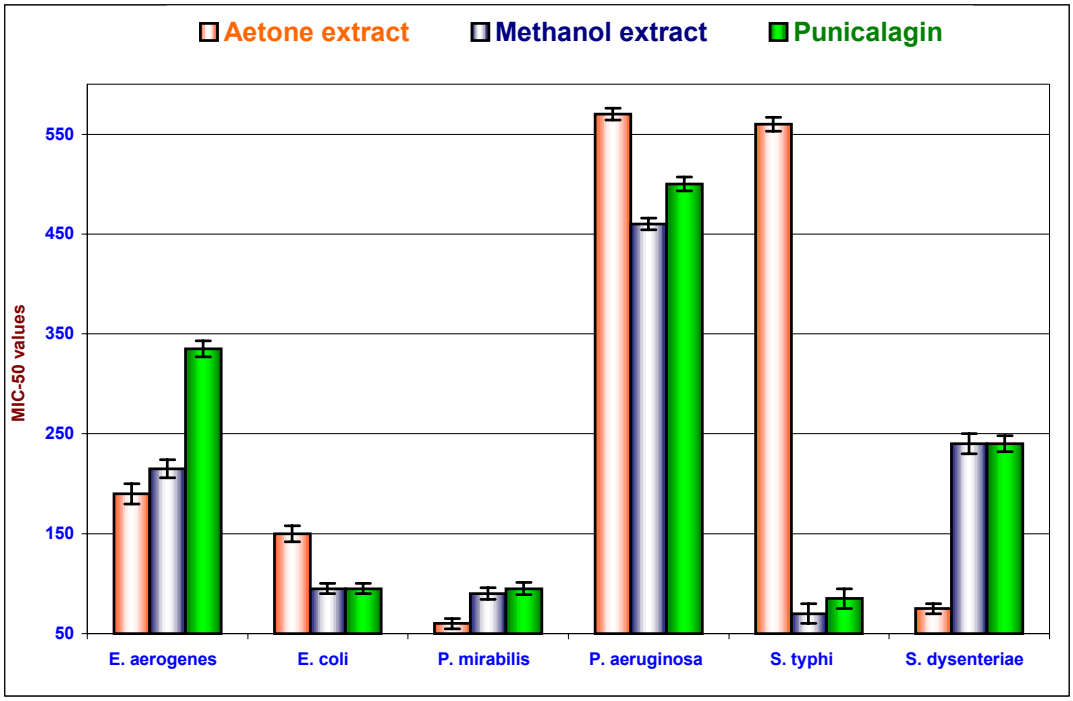



- Values are mean ± standard error of four replicates

Methanol extract showed potential bacterial growth inhibitory activity against *S. typhi, P. mirabilis* and *E. coli* with an $MIC_{50}$ value of 70, 90 and 95 ppm respectively

(Fig. 2.20). It also inhibited 50% growth of *E. aerogenes* and *S. dysenteriae* at 215 and 240 ppm respectively.

**Fig. 2.22. Minimum inhibitory concentration ($MIC_{50}$) of extracts and punicalagin from pith and carpellary membrane of pomegranate fruit against clinically important pathogenic bacteria**




The punicalagin isolated from the methanol extract of pith and CM of pomegranate showed similar trend in antibacterial activity compared to its source methanol extract. The $MIC_{50}$ value of punicalagin for *E. coli, P. mirabilis* and *S. typhi* was 95, 95 and 85 ppm respectively.

Both methods of antibacterial assay revealed that acetone, methanol extract of pith and CM and major bioactive molecule punicalagin were effective antibacterials and their effect was found to be concentration dependent (Fig. 2.19, 2.20, 2.21). Acetone, methanol extract and punicalagin showed highest antibacterial effect on *P. mirabilis* with $MIC_{50}$ value lesser than 100 ppm. In addition, they were also found to be very effective

against *E. coli* and *S. dysenteriae* at low concentration. Methanol extract and punicalagin were strong antibacterials against *E. coli, P. mirabilis* and *S. typhi* at low concentration. The antibacterial effects of pomegranate fruit pith and CM extracts may be attributed to the phenolics present in it with the major constituent punicalagin

The antibacterial properties of polyphenols is usually attributed to their protein binding properties, membrane binding properties, metal ion chelating property, which results in the altered extra cellular enzyme activity, thus making the bacteria devoid of essential metal ions for normal metabolic activities. Punicalagin being a high molecular weight polyphenol may not enter the bacterial cell. Its antibacterial effect may be due to complexing with cell wall protein and extra cellular enzymes of the bacteria. Thus might have affected the transport of nutrients in to the bacterial cell.

It was also found that tested bacteria showed varied tolerance to the acetone, methanol extract and punicalagin. Secretion of extra cellular polysaccharides by the bacteria that separates the microbial cell wall from reactive molecule may be the factor responsible for the tolerance of bacteria to the above tested phenol rich extracts and punicalagin (Brooker *et al*., 2000). The antibacterial effect of methanol extract was found to be superior to punicalagin, which may be due to the synergistic effect of other phenolics present in it. Punicalagin and methanol extract from pith and CM of pomegranate fruit may be utilized to control *E. coli, P. mirabilis* and *S. typhi,* while acetone extract to control *P. mirabilis and S. dysenteriae* as they were effective antibacterials at lesser concentration i.e. below 100 ppm. The antibacterial effect of punicalagin against clinically important pathogenic bacteria is in supplement to earlier reported health benefit as an antioxidant.

## 2.4. Summary and conclusion

Studies on functional properties of punicalagin revealed a variety of its functional attributes like antioxidant, binding with HSA, binding with DNA, platelet aggregation inhibitory activity, low cytotoxicity, and antibacterial activity.

The punicalagin showed potential radical scavenging properties against DPPH, superoxide, and ABTS radicals and also showed potential lipid peroxidation inhibitory activity against iron-ascorbate induced peroxidation of liver microsomes. In addition, punicalagin showed very high reducing power. The pulse radiolysis studies revealed the ability of punicalagin to scavenge one-electron oxidants and repair of tryptophan radicals and ABTS radical species produced under *in-vitro* conditions using pulse radiolysis technique. Further metal chelating property of punicalagin proves that the punicalagin is one of the most potent antioxidant molecules, which acts in several ways against pro oxidants.

The binding ability of punicalagin with human serum albumin as revealed in our study proved its availability across the body through blood system. The DNA binding property of punicalagin rendered it an anticancer property. The punicalagin molecule also showed moderate platelet aggregation inhibitory activity. The platelet aggregation has been reported to result in the diseases like atherosclerosis. The low cytotoxic potential of punicalagin renders it as a promising nutraceutical molecule towards human health benefits. Further the antibacterial activity against clinical isolates added up to the list of functional properties of punicalagin towards human health benefits.

The vivid bioactivity, bioavailability and low toxicity, rendered the punicalagin in to a promising multifunctional bioactive molecule towards human health benefits

# *Chapter III*

## *Elucidation of mechanism of internal browning in pomegranate fruit and role of bioactive molecules in it*

## 3.1. Introduction and review of literature

### 3.1.1. Introduction

Pomegranate is a widely grown horticulture crop in many tropical and subtropical countries. It is one of the hardiest fruit crops and thrives well under arid and semi-arid climatic conditions. The fruits are generally harvested when fully ripe and possess a waxy shining surface of reddish yellow (Biale, 1981). In India, the area under this crop has increased substantially mainly because of the versatility, adaptability, drought resistance, low maintenance cost, steady and high yields of the crop. Pomegranate fruit has better keeping quality than other tropical fruits such as mango, grape, and banana. It can be stored up to 1 month in a cool dry place and up to 3 months under cold storage (Wasker & Roy, 2000). In spite of the above keeping qualities, export of fresh fruit is below 1% while its utilization for processing is less than 2% of its production. The major bottleneck is its internal discoloration and browning.

Internal browning also referred as internal breakdown, is a serious problem for pomegranate industry in India, accounting for about 10-12% loss of fruits. During internal browning the pomegranate arils does not develop the typical pink/red color and are somewhat flattened than plump. Flavor of the arils is atypical and many have a streaked appearance due to fine white lines radiating from the seeds. There are no visible external symptoms of this disorder on the fruit. The cause of the disorder was so far not known; it originates during growth in some seasons, usually only in limited areas (Ryall & Pentzer, 1974). Khodade (1987) reported the incidence of internal browning of crops after 120 days of fruit set in G137 variety of pomegranate and its intensity increases if the fruits are left on the tree up to 135 days. He also noted that the incidence of browning increased with an increase in the weight of fruit from 150 g to more than 350 g. Desai (1989) reported that total soluble solids, acidity, ascorbic acid, total sugars, reducing

sugars, potassium, and phosphorous were low, whereas non-reducing sugars, starch, phenolics, calcium polyphenol oxidase and peroxidase enzymes were high in affected arils of Ganesh variety of pomegranate.

So far the etiology of this disorder was not elucidated; hence an attempt was made in the present study to have mechanistic insight regarding the mechanism of internal browning in pomegranate arils and factors influencing it. An attempt was also made to elucidate the role of bioactive molecule isolated from pith and CM towards the internal browning of arils.

### 3.1.1.Browning in fruits and vegetables and their products

The primary belief regarding the browning reactions in fruits and vegetables is that, disruption of cellular compartmentalization initiates browning, and browning reactions involves physical, physiological and functional interaction among different chemical constituents of disrupted cell resulting in the brown colored end products. Literature review reveals the involvement of several classes of molecules in the browning of fruit and vegetables and their products. Mechanism of browning in fruits and vegetables and their products can be broadly classified in to two major types as enzymatic and non-enzymatic browning. The later can be divided based on the interacting chemical constituent.

## Browning reactions in fruits and vegetables

1. **Non-enzymatic browning:**
    - ❖ Maillard reaction
    - ❖ Active aldehyde theory
    - ❖ Ascorbic acid mechanism
    - ❖ Breakdown of pigments
        - ➢ Carotenoids
        - ➢ Chlorophylls
        - ➢ Anthocyanins
    - ❖ Polyphenolic browning
        - ➢ Metallic interactions

2. **Enzymatic browning:**
    - ❖ Polyphenol oxidase activity
    - ❖ Peroxidase activity
    - ❖ Phenyl alanine lyase activity

### 3.1.1.1. Mechanisms of non-enzymatic browning reactions

❖ **Maillard reaction**

The term 'Maillard reaction' is used to indicate browning reactions involving the interactions of reducing sugars and amino compounds. Maillard reaction is a multi stage reaction between sugars and amino acids. The general equation of Maillard reaction can be given as follows

Aldose sugar + Amino compound ⟶ Melanoidins

(Brown nitrogenous polymers and copolymers)

As such, this kind of reaction has not been reported so far in intact fruit and vegetables, but it is the major cause for browning in many processed products. For example in apple juice, Maillard reaction, taking place between α-amino groups and reducing sugars is the most important cause of browning (Toribio & Lozano, 1984). Maillard reaction results in the loss of quality in fruit and vegetables and their products; also it causes losses in nutritional value of foods (Daniel & Whistler, 1985). The decreased water activities in processed products up to 0.6 –0.7 found to favor the Maillard reactions (Eskin, 1990). In many fruits and vegetables Maillard reaction is unpleasant, but Maillard browning is desirable in the manufacture of coffee, tea, bear, and in the toasting and baking of bread (Arnoldi *et al.*, 1988; Eskin, 1990).

❖ **Active aldehyde theory**

Another mechanism of browning reaction i.e. active aldehyde theory addresses the caramelization of sugars during processing at higher temperature resulting in the browning reaction. Garza *et al.,* (1999) reported that reducing sugars present in the peach puree, mainly glucose and fructose, participate directly in the non-enzymatic browning reactions, in which furfural and hydroxymethylfurfural (HMF) were found. Furfural and

HMF are the most important chemical substances produced in non-enzymatic browning processes, and their content indicates the degree of heating during processing (Resnik & Chirife, 1979). Some disaccharides, such as sucrose, can also hydrolyze during thermal treatment, leading to glucose and fructose formation. In consequence, the evolution of sugar content can be used as an indicator of non-enzymatic browning variation (Babsky *et al.,* 1986)

❖ **Ascorbic acid mechanism**

Ascorbic acid (AA), the most important antioxidant molecule in biological system and is also found involved in browning reactions in fruits and vegetables. Ascorbic acid on oxidation yields reactive products that may polymerize or react with the nitrogenous constituents to form brown pigments like furaldehyde and 5-hydroxymethyl-furaldehyde (HMF) resulting in the browning (Huelin, 1953; Huelin *et al.*, 1971; Kanner *et al.*, 1981; Robertson & Samaniego, 1986). In freshly produced commercial citrus juice, browning was mainly due to carbonyl compounds formed from L-ascorbic acid degradation (Roiga *et al.,* 1999). Presence of amino acids and other amino compounds found to enhance nonenzymatic browning involving ascorbic acid (Roiga, *et al*, 1999).

In contrast to the above, Veltman *et al*, (1999) found that ascorbic acid acts as a browning inhibitor molecule in pears, wherein the inhibition was due to its antioxidant potential. Thus AA plays a dual role in fruits and vegetables as browning inducer as well as browning inhibitor.

❖ **Breakdown of pigments**

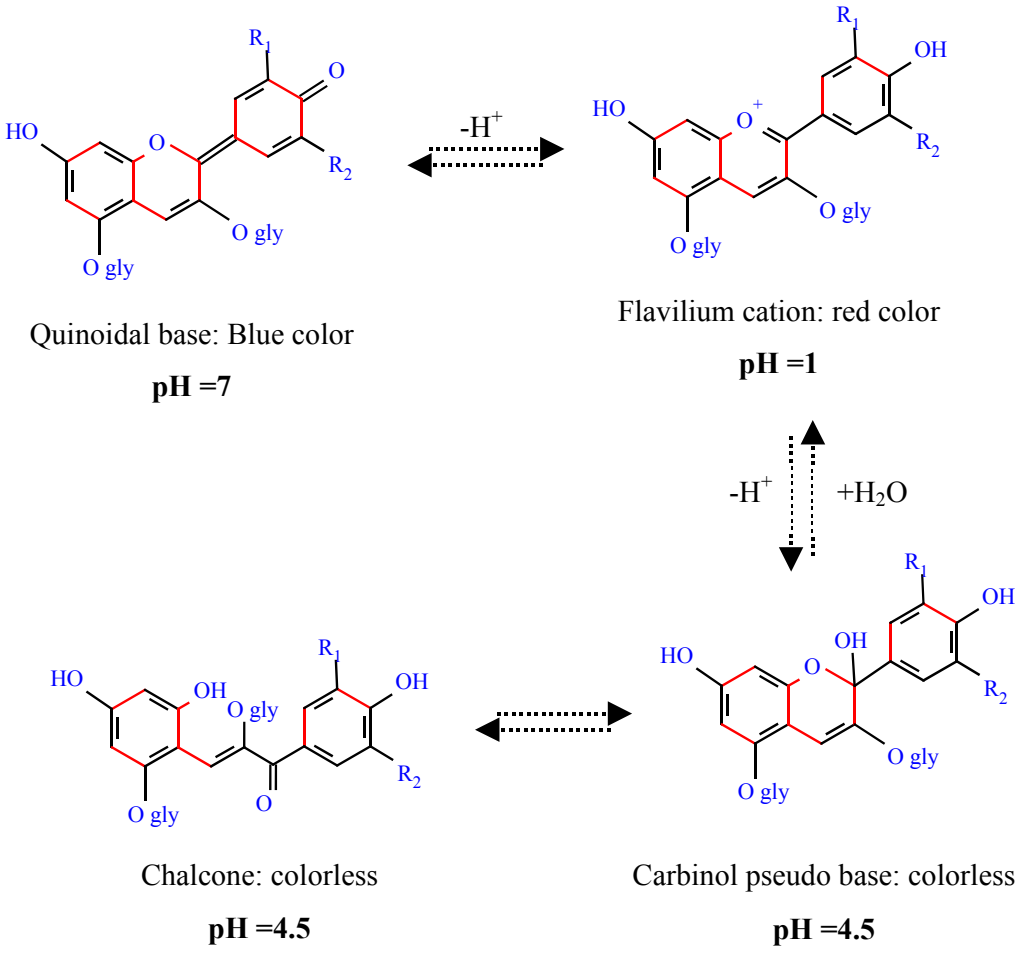
Pigments play an important role in plant system by providing a distinguishing color to the different parts of the plant. The three major classes of pigments in plants

include chlorophyll, carotenoids, and anthocyanins. In most of the cases, pigments were found to be very unstable outside their source system even sometimes within the tissues resulting in the discoloration and some times browning also due to the degraded products.

**Chlorophyll:** Almost any type of food processing or storage causes some deterioration of the chlorophyll pigments. Phenophytinisation (with consequent formation of a dull olive brown phenophytin) is the major change; this reaction is accelerated by heat and is acid catalyzed. Other reactions are also possible. For example, dehydration and photo-oxidation results in the loss of desirable colour (Cuny *et al.*, 1999).

**Anthocyanin:** Anthocyanins are a group of more than 150 water-soluble pigments that are very widespread in the plant kingdom and responsible for the pink, red, purple and blue hues seen in many flowers, fruits, and vegetables. The pomegranate fruit arils color is due to pelargonidin, delphinidin and cyanidin pigments belonging to the group of anthocyanins. The anthocyanins are very unstable molecules outside the source system (Gradinaru *et al.*, 2003). The color and stability of an anthocyanin in solution is highly dependent on the pH and temperature. They are most stable at low pH and gradually lose color as the pH is increased. At around pH 4 to 5, the anthocyanin is almost colorless. This color loss is reversible and red hue will appear upon acidification. The change in the anthocyanin coloration with a change in pH is attributed to the reversible structural transformation of anthocyanin molecules with a change in pH (Fig 3.1).

**Fig. 3.1. Predominant structural forms of anthocyanins present at different pH level**




Quinoidal base: Blue color

**pH =7**

Flavilium cation: red color

**pH =1**

Chalcone: colorless

**pH =4.5**

Carbinol pseudo base: colorless

**pH =4.5**

Some metal ions, such as $Fe^{3+}$ and $Al^{3+}$ form deeply colored co-ordinaton complexes with anthocyanins, which also lead to the loss of anthocyanin coloration in fruits and vegetables and their products. Their interaction with metal ions may bring discoloration and browning (Toyama-Kato, *2003*). Anthocyanin pigments are also sensitive to thermal processes, yielding a loss in the desirable hue and an increase in a brown hue as the pigment degrades and polymerizes.

**Carotenoids:** The carotenoids are a group of mainly lipid soluble compounds responsible for many of the yellow and red colors of plant and animal products. The main cause of carotenoid degradation in foods is oxidation. The mechanism of oxidation

in processed foods is complex and depends on many factors. The pigments may auto-oxidize by reaction with atmospheric oxygen at rates dependent on light, heat and the presence of pro- and antioxidants (Krinsky, & Yeum, *2003*)

❖ **Polyphenol reactions**

Polyphenols are highly reactive group of phytochemicals. Oxidation of polyphenols leads to the formation of brown colored quinones, and also polyphenols readily interact with other chemical components, such as amino acids, sugars, and metal ions yielding brown colored complexes. Rate of all the above reactions are determined by time, temperature, pH, moisture content, dehydration, and oxidation.

Compounds formed between polyphenols and metal ions are of particular interest in wine where several different metal ions such as copper, iron, tin, magnesium, calcium and potassium have been implicated in polyphenol interactions (Mathew & Parpia, 1971; Beveridge, 1997). Studies by Oszmianski *et al.*, (1996) indicated that the degradation of catechin in wine like solutions increased in the presence of iron. Thus, the presence of these two metal ions is associated with increased rates of oxidation of catechin and other phenolics to their brown products.

Multiple non-enzymatic phenomena may also bring browning in fruits, vegetables and products. In apple juice concentrate, browning during storage is attributed to caramelization, ascorbic acid degradation and Maillard reaction (Babsky *et al.*, 1986; Ibarz *et al.*, 1990). While caramelization occurs on heat treatment of sugars at high temperatures and ascorbic acid degradation occurs by an oxidative path (Clegg, 1964). However, the Maillard reaction, taking place between α-amino groups and reducing sugars is the most important cause of browning in apple juice (Toribio & Lozano, 1984).

### 3.1.1.2. Mechanism of enzymatic browning reactions

Enzymatic oxidation of phenols is the most common cause of naturally occurring tissue browning in fruits and vegetables. The enzyme predominantly responsible is polyphenol oxidase (Joslyn & Pomting, 1951; Mathew & Parpia, 1971). However, the contributions of other enzymes like peroxidase, phenylalanine lyase are also not less in bringing browning (Vamos-Vigyazo 1981).

**Polyphenol oxidase** (PPO)

Polyphenol oxidase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is an oxidative enzyme widely distributed in the plant kingdom and has been detected in most fruits and vegetables. PPO has been partially purified from many fruits, including grape (Sanchez-Ferrer *et al.*, 1989), apple (Oktay *et al.*, 1995), guava (Augustin *et al.*, 1985), peach (Laveda *et al.*, 2000), banana (Sojo *et al.*, 1998), pear (Siddiq *et al.*, 1994), kiwi (Park & Luch, 1985), strawberry (Ebeling & Montgomery, 1990), plum (Siddiq *et al.*, 1992), cherry (Pifferi & Cultera, 1974), and pineapple (Das *et al.*, 1996). The localization of the enzyme in the plant cell depends upon the species, age, and in fruits and vegetables – on maturity. In potato tubers nearly all-sub cellular fractions were found to contain PPO. In freshly harvested apples, the enzyme is localized almost exclusively in chloroplasts and mitochondria (Vamos-Vigyazo, 1981). Where as its substrates i.e. phenols are localized in the vacuoles.

PPO is a copper-containing enzyme, which catalyses two entirely different reactions (a) the hydroxylation of monophenols to the corresponding o-dihydroxy compounds; (b) The oxidation of o-dihydroxy phenols to o-quinones. The reactions require molecular oxygen. The quinones formed from above reactions are very unstable and rapidly react with amino acids or proteins, generating brown pigments by

polymerization (Garciýa-Carmona *et al.*, 1988). These reactions are more important in fruits with high phenol contents such as eggplant, apple, potato (Sakamura & Obata, 1963; Bajaj *et al.*, 1979). In the healthy cellular system PPO is separated from its substrates due to membrane compartmentation. Upon the loss of membrane integrity due to ripening/senescence/physical injury in the cells of fruit and vegetables, the contact of the enzyme and its substrates initiates browning reactions (Moskowiz and Hradzina, 1981; Mayer, 1987).

## Peroxidase (POD)

Peroxidase (donor: hydrogen peroxide oxidoreductase, E.C.1.11.1.7) are, similar to PPO, member of the group of oxidoreductases and both enzymes catalyse more than one reaction and acts on a great number of substrates; Both are involved in enzymatic browning of fruit and vegetables (Williams *et al.*, 1985; Nicolas *et al.*, 1994). In plant cells POD is located in cytoplasm as a soluble form, and partly cell wall bound, which is in insoluble form, (Vamos-Vigyazo, 1981). Plant peroxidase is an iron-containing enzyme, which catalyses four types of reactions: (1) peroxidatic, (2) oxidatic, (3) catalatic, and (4) hydroxylation. It degrades hydrogen peroxide in the presence of a hydrogen donor. POD is highly specific to peroxide substrate, where as it has very low specificity for the hydrogen donor substrate. It uses wide variety of hydrogen donor substrates to decompose hydrogen peroxide. It can oxidize phenols to quinones, and then condense tannins to brown polymers in the presence of $H_2O_2$, which may then contribute to enzymatic browning (Robinson, 1991). Increased POD activity has been observed in pineapple upon exposure to ozone, pollution, nutritional disorders, wounding, and chilling injury (Campa, 1991). The browning of pineapple and litchi fruit has been attributed to the POD activity (Zhang *et al.*, 2005).

**Phenyl alanine lyase**

Being a key enzyme in phenolic biosynthesis, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has also been considered to be associated with browning and the accumulation of chlorogenic acid and lignin-like materials (Hahlbrock & Scheel, 1989). PAL has been reported to play an important role in the browning process of many fruits and vegetables (Ke & Saltveit, 1989). During infection process of pathogens, the PAL activity was increased in order to render resistance to invading pathogen by excessive synthesis of polyphenols. Further oxidation reaction of these polyphenols leads to browning and cell death (Saltveit, *2000*).

Different fruits have showed different mechanism of browning. For example phenolics, PPO, PAL, and iron play important role in blackening reactions of stored artichoke heads (Lattanzio, *et al* 1994), whereas PPO, peroxidase and anthocyanase enzymes together play important role in browning of litchi fruit (Zhang *et al.*, 2005). Blackheart development in pineapple fruit (*Ananas comosus*, Smooth Cayenne) has been attributed to activity of PPO, peroxidase and phenylalanine ammonia-lyase (Zhou *et al.*, 2003). Thus browning mechanisms in fruit tissues may involve any one of the above phenomenons or many interlinked phenomena.

A summary of overall biochemical factors associated with browning reactions in fruits and vegetables can be listed as follows

- pH
- Temperature
- Sugars
- Proteins & amino acids
- Polyphenols
- Ascorbic acid
- Metal ions (Fe, Cu, Zn, Mg).
- Polyphenol oxidase enzyme
- Peroxidase enzyme
- Phenyl alanine lyase

### 3.1.1.3. Control of browning in fruits and vegetables

Browning results from both enzymatic and non-enzymatic phenomenon. Non-enzymatic browning is observed commonly in processed products of fruits and vegetables, whereas enzymatic browning is in fresh produce, mostly in ripening/senescing fruits and vegetables. On altering the processing conditions like temperature, pH, water activity, etc non-enzymatic browning can be controlled, whereas control of enzymatic browning needs special techniques.

Enzymatic browning does not occur in the intact plant cells since phenolic compounds in cell vacuoles are separated from the polyphenol oxidase, which is present in the plastids and peroxidase enzyme, which is partly located in cytoplasm. Once the tissue is damaged by physiological injury or physical injury, leads to the browning reactions. The most important factors that determine the rate of enzymatic browning of

fruits and vegetables are the concentrations of active PPO, POD enzymes, phenolic compounds, pH, temperature and the oxygen availability in the tissue (Martinez & Whitaker, 1995). Based on these factors, different methods have been used to prevent deterioration and browning of fresh-cut produce.

Heat inactivation of PPO is feasible by applying temperatures of >50°C, but it may produce undesirable colors and/or flavors as well as undesirable changes in texture. Polyphenols can be removed by β-cyclodextrins and by insoluble polyvinyl polypyrrolidinone or polyethylene glycol. The adjustment of the pH with citric acid, malic acid or fumaric acids to pH 4 or below can be used to control browning in juices, fruit slices (Martinez & Whitaker, 1995). The use of reducing compounds/antioxidants is the most effective controlling strategy for browning.

Traditionally, sulphites have been used to prevent browning, however, the Food and Drug Administration banned their use in processed fruits and vegetables in 1986 (FDA, 1986) as a result of adverse reactions developed in certain consumers. An alternative method is to inhibit browning and deterioration by using natural antioxidants/reducing agents derived from plants.

### 3.1.1.4. Phytochemicals as browning inhibitors

Various endogenous organic acids and reductants such as ascorbic acid have been proposed or used as potential antibrowning agents (Wiley, 1994; Avhenainen, 1996). Recently, the use of natural compounds and their derivatives such as 4-hexylresorcinol, N-acetylcysteine, ascorbic acid, iso-ascorbic acid, citric acid, potassium sorbate, calcium chloride and propionate alone or in combination at different concentrations, have been found effective in retarding browning and decay of different fruits and vegetables

(Monsalve-Gonzalez *et al*., 1995; Gunes & Lee, 1997; Kim & Klieber, 1997; Saper & Miller, 1998; Buta *et al*., 1999; Buta & Abbott, 2000).

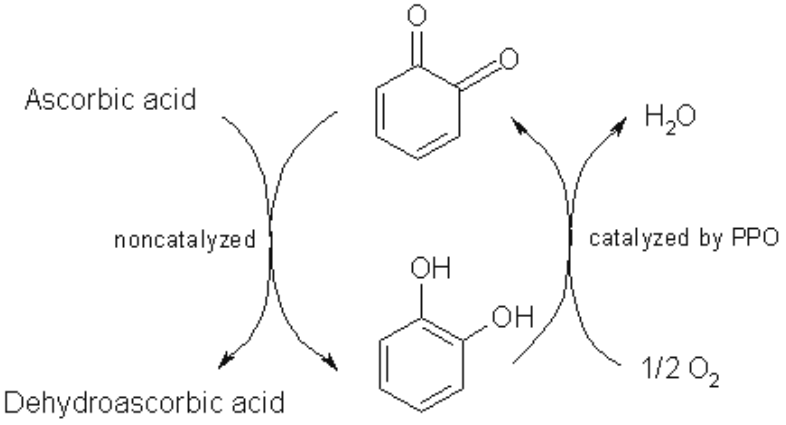
The phytochemicals used to inhibit the enzymatic browning can be divided in to two groups based on their mechanism of action. First group consists of antioxidants and second one includes metal chelators.

## Antioxidants

Antioxidants play a role in the prevention of enzymatic browning either by reducing *o*-quinones to colorless diphenols, or by reacting irreversibly with *o*-quinones to form stable colorless products. Antioxidant compounds are very effective in the control of browning (Guerrero-Beltrán *et al*., *2005*).

**Ascorbic acid:** Polyphenol oxidase inhibition by ascorbic acid has been attributed to its antioxidant potential, by reduction of enzymatically formed *o*-quinones to their precursor diphenols (Walker, 1977). Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in polyphenol oxidase reactions. Ascorbic acid is however irreversibly oxidized to dehydroascorbic acid during the reduction process, thus allowing browning to occur upon its depletion by both enzymatic and non-enzymatic means.

**Fig. 3.2. Mechanism of prevention of colour formation by ascorbic acid**



**Phenolic antioxidants:** Plant phenolic compounds such as tocopherols, flavonoid compounds, chalcones, cinnamic acid derivatives, and coumarins are naturally occurring compounds, which have an antioxidant effect that renders them inhibitory to polyphenoloxidase, and thus browning. Elimination of reactive oxygen species results in the inhibition of browning due to phenolics (Vamos-Vigyazo, 1981).

## Metal chelators

Enzymes generally possess metal ions at their active sites. Removal of these metal ions by chelating agents can therefore render enzymes inactive. Polyphenol oxidase is a copper containing enzyme, where as peroxidase contains iron. Chelating agents complex copper / iron ions, through an unshared pair of electrons in their molecular structures, rendering the active site of enzyme to inactive. Ascorbic acid also has a chelating effect on the prosthetic group of polyphenol oxidase. Metal chelating ability of many plant phenolics including flavonoids, renders them as browning inhibitors (Roux *et al.*, *2003*).

The detailed review evidence that browning is a universal phenomenon in both fresh and processed fruits and vegetables. It is influenced by the structural, compositional and functional attributes of the system, which in turn controlled by time, temperature, and storage system. Phytochemicals also play important role in controlling the browning due to enzymes.

In the present investigation the mechanism of internal browning of arils was traced out by holistic approach, for which two strategies were employed to understand the phenomenon of browning in pomegranate fruit,

(a) Factors associated with development, maturation, ripening and senescence of pomegranate fruit;

(b) Factors associated with the different stages of browning in pomegranate arils

## 3.2. Materials and Methods

### 3.2.1. Chemical composition analysis of arils

#### 3.2.1.1. Experimental material

**Arils under different developmental stages**

Pomegranate fruits, var. Ganesh, at different developmental stages, namely, 20-, 40-, 60-, 80-, 100-, 120- and 140-day old from the day of fruit set, were harvested from a pomegranate orchard located in the Bagalkot district, India, and brought immediately to the Central Food Technological Research Institute, Mysore. The arils were collected manually and analyzed for major chemical composition and antioxidant activity. Time delay, from harvesting to analysis, was about 24 h. Four replicates were maintained for each analysis, each replicate indicating a single pomegranate fruit.

**Arils under different stages of browning**

Internally browned pomegranate fruits var. Ganesha were harvested freshly with the help of local experts and experienced farmers from orchards near Kaladagi Village, Bagalkot District and brought immediately to Central Food Technological Research Institute, Mysore. Arils were collected from internally browned fruits and divided into five groups viz. healthy, 25% brown, 50% brown, 75% brown and 100% brown, based on the intensity of browning disorder by visual assessment. Though visual assessment of degree of browning was used as a initial guide in terms of grouping of arils. Later, optical densities at 420 nm, and L, a, b, color values were designated to provide the numerical background required to complement the visual data. The arils were immediately analyzed for biochemical attributes. Four replicates were maintained for each analysis.

The pomegranate arils were squeezed in two-layered muslin cloth to extract the complete juice from arils. For each chemical analysis, juice was freshly extracted. All the

above steps were carried out at low temperature (4°C) to avoid enzymatic reactions. The juice was centrifuged at 5000 rpm for 10 min at 4°C prior to estimation of total phenolics, total protein content and antioxidant activity.

**Chemicals**

Fehling's A, Fehling's B, Sodium hydroxide, L-Ascorbic acid**,** Metaphosphoric acid, Citric acid, Sodium phosphate (dibasic), Sodium phosphate (monobasic), Tris HCl, Folin–Ciocalteau's phenol reagent, Sodium carbonate, Orthophosphoric acid, Potassium ferricyanide, Trichloroacetic acid, Ferric chloride, L-Dopa, Dopamine, Tyrosine, Catechol, Hydrogen peroxide (AR), Guiacaol and O-Dianisidine were purchased from Sisco Research Laboratories, India. Acetonitrile (HPLC grade) was purchased from Merck, Mumbai. Gallic acid, Coomassie Brilliant Blue G-250, Bovine serum albumin (BSA), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma chemicals, USA.

### 3.2.1.2. Color measurement

The pomegranate juice color was measured using a Hunter lab color measuring system (Model *Lab Scan XE*, Hunter Associates Laboratory Inc, Virginia, USA) and recorded as *L a b.* The *L a b* color system consists of a luminance or lightness component '*L*' and two chromatic components: the component 'a' for green (-*a*) to red (+*a*) and the '*b*' for blue (-*b*) to yellow (+*b*) colors. The colorimeter was calibrated using a standard white plate. Values of the white standard were $L = 99.899$, $a = +0.236$, $b = +0.042$. Color was measured in a quartz cuvette of 50 ml capacity. Each measurement was replicated.

### 3.2.1.3. Relative water content

Relative water content in the arils was determined using a modified standard procedure (AOAC 1990a) as follows. About 5 g of arils were placed in a pre-dried and weighed glass petridish (50 mm diameter) and dried in a vacuum oven at 90°C for 48 hours, at a pressure of 200 mm Hg. Dried samples were placed in a desiccator, and cooled for 30 min to room temperature. The weight was recorded and percentage water content based on initial wet weight was calculated. Each measurement was replicated.

### 3.2.1.4. pH

The pH of pomegranate juice was measured using a *Control Dynamics* pH meter, calibrated using standard buffers of pH 4 and 7 (HiMedia). The pH of the juice was measured by placing about 15 ml of juice in a 25 ml beaker and immersing the pH electrode in to the sample. Refrigerated samples were allowed to room temperature prior to pH measurement. Each measurement was replicated.

### 3.2.1.5.Total soluble solids

The °Brix, a measure of soluble a solid was determined by placing 3-4 drops of pomegranate sample juice over a prism of the digital refractometer (ATAGO RX-5000), calibrated with triple distilled water. The °Brix value was recorded directly from the digital readout of the refractometer as *% sucrose* content. Each measurement was replicated.

### 3.2.1.6. Titrable acidity

Titrable acidity was determined by AOAC method [1990]. About 2-3 g of freshly extracted pomegranate juice was titrated against 0.1N NaOH with phenolphthalein as

indicator until pink color persisted. Titrable acidity was calculated using the following formula and expressed as *% citric acid* content.

$$\text{Titrable acidity (\% citric acid)} = \left( \frac{64 \text{ x } N_{(NaOH)} \text{ x Titre value}}{\text{Wt. of sample x } 1000} \right) \text{x } 100$$

**3.2.1.7. Total sugars and reducing sugars**

Total sugars and reducing sugars were estimated according to the method of Lane and Eynon (1923). In principle, reducing sugars reduces the copper in Fehling's solution to red, insoluble cuprous oxide. The sugar content can be estimated by determining the volume of unknown sugar solution required to completely reduce a measured volume of Fehling's solution.

**Determination of factor for Fehling's solution**: About 40 ml Fehling's solution (Fehling's A: Fehling's B: Water, 1:1:2) was pipetted in to a 250 ml conical flask, heated to boiling. Under boiling condition standard invert sugar solution prepared by inverting aliquot quantity of sucrose with 5 ml HCl was titrated. Methylene blue was used as indicator. The formation of brick red color was considered as endpoint. Burette reading was recorded, and factor for Fehling's solution was calculated using following formula

$$\text{Factor for Fehling's solution (g of invert sugar)} = \frac{\text{Titre X } 2.5}{1000}$$

**Estimation of reducing sugars:**

**Preparation of sample:** About 10 g of filtered pomegranate juice sample was transferred to 250 ml volumetric flask, diluted to ≈100 ml and neutralized with 1N NaOH in presence of phenolphthalein indicator. Volume was raised to 250 ml.

**Titration:** About 40 ml of Fehling's solution (Fehling's A : Fehling's B: Water, 1:1:2) was pipetted in to a 250 ml conical flask, heated to boiling. Under boiling condition sample juice was titrated with methylene blue as indicator. Formation of brick

red color was considered as endpoint and burette reading was recorded. Reducing sugar content was calculated using the following formula and expressed as % content.

$$\text{Reducing sugars (\%)} = \frac{\text{Factor X dilution}}{\text{titre X weight of sample}} \text{X } 100$$

**Estimation of total sugars:**

**Preparation of sample:** About 10 g of filtered pomegranate juice sample was transferred to 250 ml volumetric flask, diluted to ≈25 ml. Sample was allowed for inversion by adding about 5 ml of concentrated HCl and keeping it overnight. Inverted sample juice was neutralized with 1N NaOH in the presence of phenolphthalein indicator and volume was raised to 250 ml.

**Titration:** Titration was carried out as described in estimation of reducing sugars. Total sugar content was calculated as per the following formula and expressed as % content.

$$\text{Total sugars (\%)} = \frac{\text{Factor X dilution}}{\text{titre X weight of sample}} \text{X } 100$$

**3.2.1.8. Ascorbic acid content**

Ascorbic acid content was determined by HPLC method, modified from Wimalasiri & Wills (1983) as follows: About 5g of pomegranate arils were blended in 3% (w/v) metaphosphoric acid, centrifuged at 10,000 rpm for 10 min at 4°C and the volume was made up to 10 ml. HPLC analysis was carried out on an analytical liquid chromatograph LC-10A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 20µl sample loop and a 250 x 4.6 mm, i.d. 5 µm, SS Excil Amino column (SGE, Australia). The sample (10 µl) was eluted with an isocratic solvent mixture comprising 0.1M citrate–phosphate buffer (pH 2.6): acetonitrile (1:3 v/v) with a

flow rate at 1.5 ml/min. The UV detection was carried out at 254 nm with a Shimadzu diode array detector, series SPD-M10 Avp, Shimadzu (Singapore).

**3.2.1.9. Total phenolics**

Total phenolic content was determined with the modified method of Taga *et al.*, (1984). About 100 µl of pomegranate juice was mixed with 2 ml of 2% aqueous sodium carbonate solution. After 3 min, 100 µl of 50% Folin–Ciocalteau's phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents.

**3.2.1.10. Total anthocyanin content**

Anthocyanin content of pomegranate arils was determined according to the method described by Ranganna (2001a). About 5 g of arils were blended with 10 ml of ethanolic HCl (ethanol with 5% HCl) in a pestle and mortar and kept in dark for overnight. Centrifuged at 5000 rpm for 15 min. Supernatant was collected and diluted with ethanolic HCl to yield optical density measurements at 535 nm within the range of 0.1 to 0.6. The molar extinction coefficient 98.2 used here for calculating the total anthocyanin content was the average molar extinction coefficient of anthocyanin pigments viz. cyanidin, pelargonidin and delphinidin pigments reported from pomegranate arils. Total anthocyanin content was determined using the following formula

$$\text{Total anthocyanin (mg/100g)} = \frac{Total\ OD_{533}}{98.2} \text{x} 100$$

$$\text{Total OD}_{533} = \frac{\text{OD}_{533} \text{ X Volume made upto}}{\text{Sample weight}} \text{X} 100$$

### 3.2.1.11. Total protein content

The protein dye binding procedure of Bradford (1976) was used for estimation of total protein content.

**Preparation of coomassie blue reagent:** About 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. About 100 ml of orthophosphoric acid (85% w/v) was added to above solution and diluted to 1000 ml with cold water.

**Estimation of protein:** To the aliquot of sample solution, about 2 ml of Coomassie blue reagent was added and change in optical density was read at 595 nm. Protein content was calculated on the basis of the calibration curve of bovine serum albumin (BSA) and expressed as BSA equivalents.

### 3.2.1.2. Mineral composition

Mineral composition of pomegranate juice was estimated by direct aspiration of the sample juice into the AA-6710F Atomic Absorption Flame Emission Spectrophotometer, Shimadzu, equipped with ASC-6000 Auto sampler. A linear gradient of standard solutions of calcium, potassium, copper and iron were prepared using potassium chloride (KCl), calcium carbonate ($CaCO_3$) dry, copper sulphate ($CuSO_4.5H_2O$) and iron alum [$Fe_2(SO_4)3(NH_4)_2SO_4.24H_2O$] respectively according to the standard procedures described by Ranganna (2001b). Test samples of pomegranate juice were prepared as follows. About 10 g of pomegranate arils were homogenized in a pestle and mortar with 5 ml of 1 N HCl, filtered in a muslin cloth followed by centrifugation at 10,000 rpm for 30 min, at 4°C, in a 'Sorvall RC 5B Plus' refrigerated centrifuge. The supernatant was directly used to estimate the mineral content by direct aspiration in to the atomic absorption flame emission spectrophotometer. For calcium estimation, 0.5% of lanthanum chloride was added to both standard and sample solution. For each mineral, separate sample solutions in triplicates were prepared and each

analysis was replicated three times. Mineral content was expressed as ppm, calculated based on the linear regression curve of the standard mineral solutions.

### 3.2.1.13. Antioxidant activity

#### 3.2.1.13.1. DPPH free radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of sample juice was measured according to the method described earlier by Moon and Terao (1998). The test sample (10- 100 µl) was mixed with 0.8ml of tris-HCl buffer (pH 7.4) to which 1ml DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517nm in a UV-Visible Spectrophotometer (UV-160A, Shimadzu co. Japan). Radical scavenging potential was calculated with the following formula.

$$\text{DPPH radical scavenging activity} = \left(1 - \frac{A_{\text{sample}\,(517nm)}}{A_{\text{control}\,(517nm)}}\right) x100$$

Radical scavenging potential was expressed as $IC_{50}$ value, which represents the concentration, which scavenged 50% of the DPPH radicals.

#### 3.2.1.13.2. Total reducing power

The reducing power of the pomegranate juice was quantified by the method described earlier by Yen & Chen (1995) with minor modifications. Reaction mixture, containing test samples at different concentrations (10-100 µl) in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50ºC for 20 min. The reaction was terminated by the addition of TCA solution (10% w/v) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

### 3.2.2. Enzyme studies

#### 3.2.2.1. Preparation of pomegranate enzyme source

About 10 grams of arils were crushed in a pre-chilled pestle and mortar and then blended in 5 ml of phosphate buffer (pH 6.4). The suspension was shaken for 30 min and centrifuged in a 'Sorvall RC 5B Plus' refrigerated centrifuge at 10,000 g for 15 min at 4°C, and the supernatant was collected. The supernatant was used as source of pomegranate enzymes through out this experiment. All steps were carried out at 4°C. For each assay, enzyme source was prepared freshly.

#### 3.2.2.2. Protein estimation

Protein content of the enzyme extracts was determined by the Bradford method (1976), using bovine serum albumin (BSA) as standard protein.

#### 3.2.2.3. Polyphenol oxidase enzyme activity

PPO activity was determined spectrophotometrically in UV-visible spectrophotometer, Cintra 10A instrument. Unless otherwise stated, the reaction mixture (1 ml) was composed of 0.1 ml of substrate, 0.8 ml of buffer and 0.1 ml of enzyme extract. Reactions were carried out at room temperature unless specified. Substrate blank and enzyme blanks were used as reference blanks, which were prepared by mixing all components except substrate or enzyme source respectively. The enzymatic activity was calculated from the slope of the linear portion of the curve. Four replicates were maintained for each assay.

##### 3.2.2.3.1. Evaluation of substrate specificity

PPO activity was tested using crude enzyme source against four substrates: Dopa, Dopamine, Tyrosine, and Catechol. Both, the reaction medium and the determination of enzymatic, activity accorded with the procedures described in the previous section, the

concentration used for all the substrates was 5 mM. All assays were done with four replicates.

**3.2.2.3.2. Evaluation of optimum pH**

Optimum pH studies were carried out using Citrate-phosphate buffer between pH 2.8 and 7. Enzymatic activity of the crude enzyme extract was determined as described above. All assays were performed with four replicates.

**3.2.2.3.3. Evaluation of optimum temperature**

PPO activity of the crude enzyme extract was measured between 0 and 60°C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached.

**3.2.2.2. Peroxidase activity**

Peroxidase activity was determined spectrophotometrically in UV-visible spectrophotometer, Cintra 10A instrument. Unless otherwise stated, the reaction mixture (1 ml) was composed of 0.05 ml of substrate, 0.05 ml of $H_2O_2$, 0.85 ml of buffer and 0.05 ml of enzyme extract. Reactions were carried out at room temperature unless specified. Substrate blank and enzyme blanks used as reference blanks were prepared by mixing all components except substrate or enzyme extract respectively. The enzymatic activity was calculated from the slope of the linear portion of the curve. Four replicates were maintained for each assay.

**3.2.2.2.1. Evaluation of substrate specificity**

Peroxidase activity of the crude enzyme extract was tested against two substrates: Guiacaol and O-Dianisidine. Both, the reaction medium and the determination of

enzymatic activity accorded with the procedures described above. The concentration used for all the substrates was 2 mM. All assays were done with four replicates.

**3.2.2.2.2. Evaluation of optimum pH**

Optimum pH for peroxidase activity of the crude enzyme extract of pomegranate arils was studied using citrate-phosphate buffer between pH 2.8 and 7. Enzymatic activity of the crude enzyme extract was determined as described above. All assays were performed with four replicates.

**3.2.2.2.3. Evaluation of optimum temperature**

Effect of temperature on peroxidase activity of the crude enzyme extract of pomegranate arils was measured between 30 and 55°C. All assays were performed in triplicate, and the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached.

### 3.2.3. Estimation of bioactive molecules in pith and carpellary membrane

Sample preparation

**Arils:** The pomegranate juice was obtained from the arils (5 g) by squeezing in a double-layered muslin cloth, diluted to 10 ml with triple distilled water followed by centrifugation at 10,000 rpm for 30 min at 4°C. Supernatant was subjected for analytical HPLC for estimating the bioactive molecules.

**Pith and carpellary membrane:** The pith and CM (2 g) was homogenized in pestle and mortar with 10 ml of methanol (HPLC grade). The extract was squeezed in muslin cloth, followed by centrifugation at 1500 rpm for 10 min at 4°C. The supernatant was used for estimating the bioactive molecule content using analytical HPLC.

## HPLC analysis

Sample was analyzed in analytical HPLC, using a Shimadzu Analytical Liquid Chromatograph LC-10A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 20 µl sample loop and a 250 x 4.6 mm, i.d. 5 µm, Shimpack C-18 column (Kyoto, Japan). The fraction was eluted with a binary mobile phase comprising: (A) 2% formic acid in HPLC grade water; (B) Acetonitrile. The elution profile was 0-5 min, 95-90 % A (linear gradient); 5-10 min, 90-85 % A (linear gradient); 10-15 min, 85-80% A (linear gradient); 15-20 min, 80-40% A (linear gradient); 20-25 min, 40-10% A (linear gradient); 25-28 min, 10 % (isocratic); 29 min stop. The injection volume was 10 µl. Flow rate, 1 ml/min. The UV detection was carried out at 280 nm with a Shimadzu diode array detector, series SPDM10 Avp, Shimadzu (Singapore). Gallic acid, ellagic acid, gallagic acid, punicalin, and punicalagin were used as internal standards and data used to identify the respective molecules and quantified.

## 3.3. Results and Discussion

The arils constitute 52% of pomegranate fruit (w/w), comprising 78% juice and 22% seeds. The fresh juice contains 85.4% moisture and considerable amounts of total soluble solids (TSS), total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid and proteins (El-Nemr, et al, 1990) and has also been reported to be a rich source of antioxidants (Gil, et al, 2000).

Incidence of internal browning of arils is one of the major problems in pomegranates, which usually occurs in over-ripe fruits (Waskar & Roy, 2000). Prabhu Desai (1989) reported that TSS, acidity, ascorbic acid and reducing sugar contents were low, whereas non-reducing sugars and tannins were high in affected arils. Browning of tissues is generally attributed to oxidation of phenolics. Harvest maturity is also known to influence quality and the nature of disorders during storage life of fresh fruits (Desai, 1989).

Early harvest may impede the development of the characteristic colour, taste and aroma of pomegranates, while late-harvested fruits exhibit a reduced shelf life; early deterioration was observed in apple, mango and other tropical fruits (Medlicott *et al.*, 1988; Fellman *et al.*, 2003). There is an increased concern about the quality of fruit during development and prior to harvest aimed at minimizing post-harvest deterioration. The present study was undertaken to investigate changes in the major biochemical composition, along with bioactive molecule content in pomegranate arils during different stages of fruit development and different stages of browning.

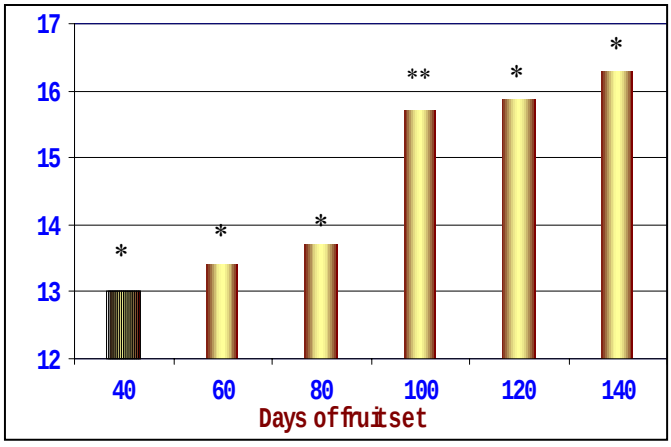
### 3.3.1. Biochemical studies of pomegranate arils from fruits under different developmental and maturation stages

#### 3.3.1.1. Chemical composition

##### 3.3.1.1.1 Total soluble solids, total sugars and reducing sugars

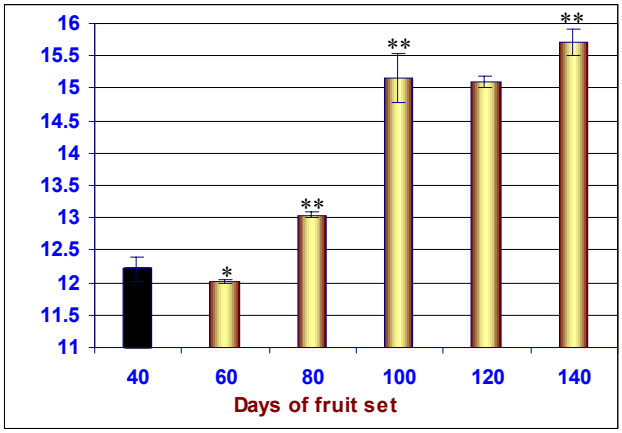
Pomegranate arils showed an increase in the concentration of TSS, total sugars and reducing sugars during fruit development (Fig 3.3, 3.4, and 3.5). The lowest TSS (13%), total sugar (12.6%) and reducing sugar (12.2%) content were recorded in 40 days old fruit. A sudden and significant increase in all of the above three constituents were recorded in 100 days old fruit followed by a steady state. Hence the 100 days from fruit set can be marked as an ultimate stage of development (optimum maturity) of pomegranate fruit. However, the highest TSS (15.3%), total sugar (16.6%) and reducing sugar (15.7%) contents were recorded in 140 day-old fruit. The increases in TSS, total sugar and reducing sugar after 100 days of fruit set may be attributed to hydrolysis of starch into simple sugars (Biale, 1960), involving ripening changes.

**Fig. 3.3 Total soluble solids content of pomegranate arils during fruit development and maturation**



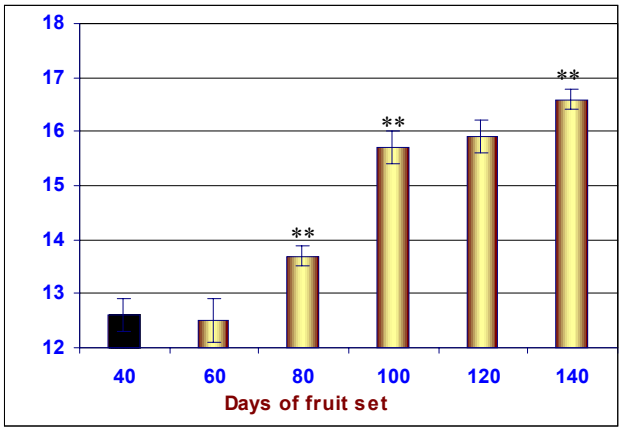

- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Fig. 3.4 Reducing sugar content of pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Fig. 3.5 Total sugar content of pomegranate arils during fruit development and maturation**



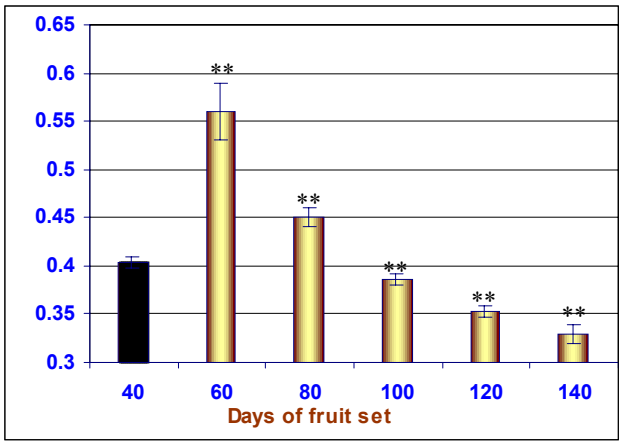

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

### 3.3.1.1.2. Titrable acidity

Organic acids found in the pomegranate include citric, malic, acetic, fumaric, tartaric and lactic acids; however, the major acid accounting for titrable acidity in pomegranate arils is citric acid (Melgarejo *et al.*, 2000). The highest titrable acidity (0.56 as % citric acid) was recorded in 60 day-old pomegranate fruit arils. This was followed

by a continuous, but significant decrease in titrable acidity to the lowest concentration of 0.33 (as % citric acid), which was recorded in 140-day-old fruit (Fig. 3.6).

**Fig. 3.6. Titrable acidity of pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

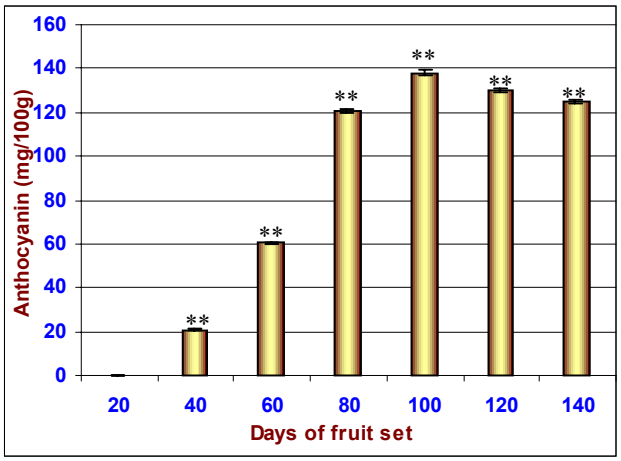
The decrease in acidity coincided with the increase in sugar concentration. A gradual decrease in acidity, concomitant with increased TSS and total sugar content, is an inherent process during ripening of pomegranate to impart the characteristic flavor. However, it also induces discoloration of anthocyanin pigments because of reversible structural transformation of the anthocyanins (Cabrita *et al.*, 2000; Cordenunsi *et al.*, 2003).

**3.3.1.1.3. Anthocyanin pigments**

A rapid increase (100%) in the anthocyanin pigment concentration was observed in pomegranate arils between 20 and 80 days of fruit development (Fig. 3.7). The highest concentration of anthocyanins (138 mg/100 g) was recorded in 100 day-old fruit, which was followed by a slight, but significant decrease of 9.3% up to 140 days of fruit development (Fig.3.7). The increase in anthocyanin content up to 100 days of fruit set may mark the developmental period of the fruit with 100 days as a optimum maturity of the fruit. The decrease in anthocyanin content afterwards may be attributed to ripening

changes. The anthocyanins are located in the vacuoles and during ripening changes due to membrane disintigrity they may be exposed to different oxidative enzymes like peroxidases. Hence decrease in their content may be observed. This decrease in the anthocyanin content after 100 days of fruit development may also influenced by decrease in acidity (Fig. 3.6), since the anthocyanin pigments undergo reversible structural transformation with a change in the acidity (Cabrita *et al*., 2000).

**Fig 3.7. Anthocyanin content of pomegranate arils during fruit development and maturation**

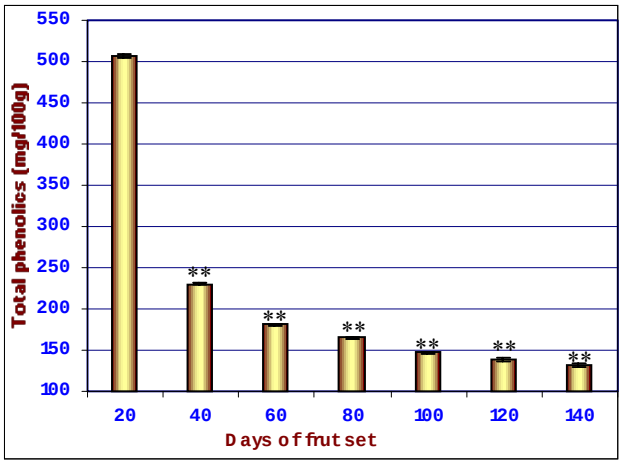



- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at P ≤ 0.005) compared to previous

### 3.3.1.1.4. Total phenolics

Pomegranate arils showed a rapid and significant (P≤0.005) depletion (by 54.5%) in total phenolics during the initial stage of fruit development from 20 to 40 days; later, the decrease was gradual but significant up to 140 days (Fig. 3.8). The highest phenolic content (506 mg/100 g arils) was recorded in 20 day-old fruit. There was a nearly 73.9% reduction in total phenolics from 20 to 140 days of fruit development.

**Fig 3.8. Total phenolic content of pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

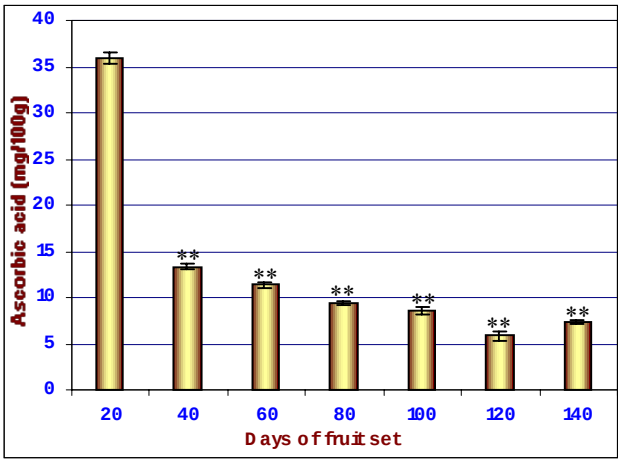
A decrease in the total phenolics reduces the astringency of fruit (Goldstein & Swain, 1963; Ozawa *et al.*, 1987), which is a desirable sensory attribute in pomegranate. A decrease in phenolic compounds with maturation and ripening has also been reported in banana (Goldstein & Swain, 1963) and guava fruits (Bashir & Abu- Goukh, 2003). A decrease in total phenolics (Fig. 3.8) during developmental period of the fruit i.e. up to 100 days is coincided with an increase in anthocyanin pigment content (Fig. 3.7); former may contribute to the biosynthesis of the flavylium ring of anthocyanin. The phenolics may also be used as antioxidant molecules to overcome the oxidative stress due to high rate of metabolic activity during developmental stages. Further decrease in phenolic content after 100 days of fruit set may be attributed to the ripening changes in the fruit, which involves the interaction of various substrates with oxidative enzymes due to altered membrane permeability.

**3.3.1.1.5. Ascorbic acid content**

Ascorbic acid present abundantly in all plant cells and perform many biological functions. As an antioxidant, it is known to inhibit oxidative browning directly or indirectly (Smirnoff, 1996). However, ascorbic acid losses during the development of

fruits and vegetables have often been reported (Mercado-Silva *et al.*, 1998; Yahia *et al.*, 2001;). Pomegranate arils also showed a similar trend with rapid and significant ($p \leq 0.005$) depletion (63.1%) in the ascorbic acid content during the initial stages, from 20 to 40 days of fruit development. This was followed by a gradual but significant decrease up to 140 days (Fig. 3.9). The highest ascorbic acid content (360 mg/100 g) was recorded in 20 day-old fruit. A similar trend of decrease in ascorbic acid content during development and maturation was recorded in mango and guava fruits (Bashir & Abu-Goukh, 2003).

**Fig 3.9. Ascorbic acid content of pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

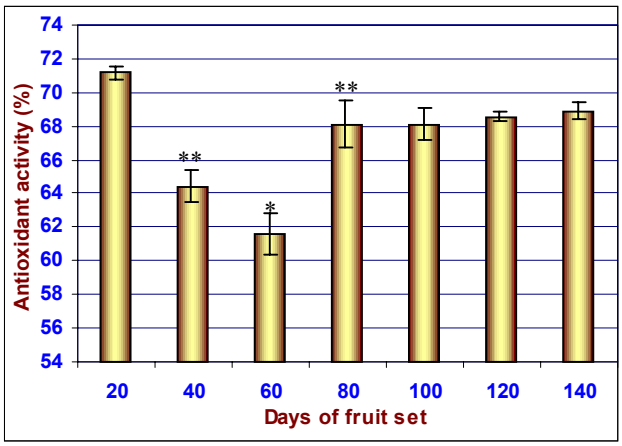
Ascorbic acid as such involved in variety of metabolic reactions as a reducing agent, its decrease in pomegranate arils may be attributed to decreased rate of metabolic activity as the fruit attains the maturity. A decrease in ascorbic acid (Fig. 3.9) and phenolics (Fig. 3.8) contents and an increase in total sugar (Fig. 3.5) and anthocyanin (Fig. 3.7) levels during 80 days of fruit development marked the shift in the metabolic activity toward biosynthesis of anthocyanins, wherein polymerization of phenolics and further glycosylation lead to the formation of anthocyanin pigments. The equilibrium concentration of the above chemical constituents in 100 days old fruit marks the

optimum maturity, and further changes in them may be attributed to the ripening changes.

### 3.3.1.1.6. Antioxidant activity

Antioxidant activity of pomegranate juice was measured in terms of its radical scavenging potential. The pomegranate arils showed a rapid decrease in antioxidant activity (by 13%) from 20 to 60 days of fruit development, which immediately replenished to its peak activity with 10.6% increase on the 80$^{th}$ day (Fig. 3.10).

**Fig 3.10. Antioxidant activity of pomegranate arils during fruit development and maturation**



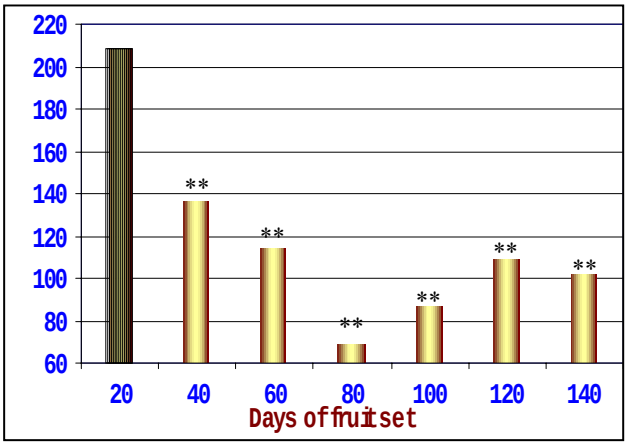

- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

The lowest antioxidant activity (61.6%), recorded in 60 day-old fruit, might be due to a reduced concentration of total phenolics and ascorbic acid in arils by 73.9% and 80.1%, respectively (Fig. 3.8 & Fig 3.9.). The surge in antioxidant activity from the 80$^{th}$ day onwards might be attributed to an increased concentration of anthocyanin pigments (Fig. 3.7). Anthocyanin, ascorbic acid and phenolic acids, either alone or in combination, may be responsible for the antioxidant activity of pomegranate arils (Miller & Rice-Evans, 1997; Scalzo *et al.*, 2004). The major phenolic acids reported from pomegranate fruit include punicalagin, punicalin, gallagic acid, ellagic acid and gallic acids (Chen & Ho, 1997; Bouchet *et al.*, 1998).

### 3.3.1.1.7. Total protein content

Pomegranate arils showed the highest total protein (209 mg/100 g) in 20 day-old fruit followed by a rapid decrease (66.9 %) in total protein up to 80 days of fruit development (Fig. 3.11).

**Fig 3.11. Total protein content of pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

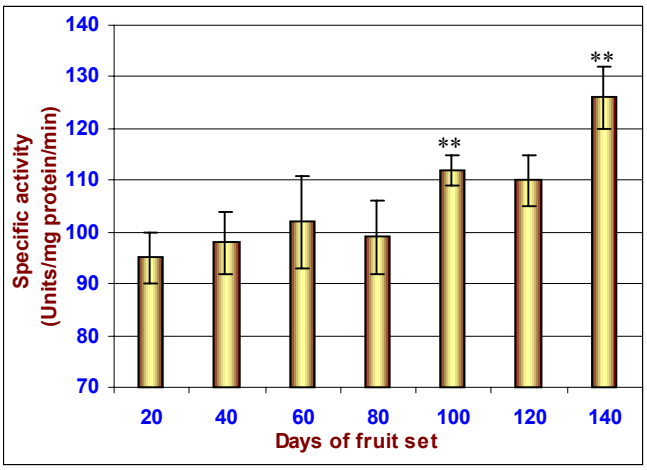
The decrease in total protein content may be a consequence of a reduction in demand of endogenous enzymes associated with anabolic activities, which decrease with fruit development and maturity (Frenkel *et al.*, 1968). An increase in the total protein content (by 58.7%) between 80 and 120 days might be due to an acceleration of ripening changes that initiate the array of enzyme activities. A slight but significant decrease (6.3%) in protein content after 120 days might be attributed to breakdown of proteins, which is normally observed during senescence of fruits (Abu-Goukh & Abu-Sarra, 1993; Bashir & Abu-Goukh, 2003).

### 3.3.1.2. Enzyme activities during developmental stages of arils

#### 3.3.1.2.1. Polyphenol oxidase activity

Polyphenol oxidase is an oxidative enzyme, and its activity is reported to be the major contributing factor in browning of many fruits and vegetables. Enzyme extract of pomegranate arils revealed an increase in PPO activity during fruit development and maturation. Lowest PPO activity (95 Units/mg protein/min) was observed in the arils of 20 days old pomegranate fruit, whereas its highest activity (126 Units/mg protein/min) was observed in the arils of 140 days old fruit (Fig 3.12).

**Fig 3.12. Polyphenol oxidase activity in pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

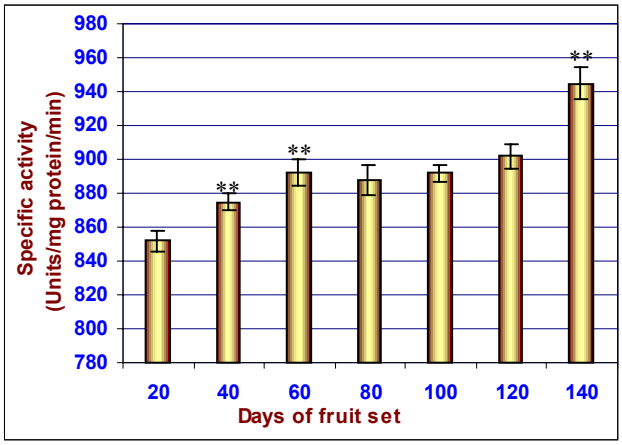
The increase (13%) in its activity observed from 20 to 120 days of fruit development and maturation was slight but significant. In contrast, a sudden significant ($P \leq 0.005$) increase by about 12.7% was observed from 120 to 140 days of fruit development. The increase in the activity after 120 days of fruit set supports the fact that it is active during ripening period and also incidence of internal browning of arils in over mature pomegranate fruit. However PPO activity was observed in enzyme extract of arils under all stages of fruit development, its activity in *in-vivo* condition would be influenced by many endogenous enzyme inhibiting factors. Most important being, which

makes it inactive, is a well-defined cellular compartmentation in developmental stages, which prevents the enzyme from the contact with substrates, whereas in ripening or senescing fruit cellular compartmentation is known to be disrupted (Wade & Bishop, 1978) bringing it in contact with endogenous substrate. Other than cellular compartmentation, ascorbic acid, anthocyanins, and low pH are also known to inhibit PPO activity (Vamos-Vigyazo, 1981).

### 3.3.1.2.2. Peroxidase activity

Similar to PPO, peroxidase is an oxidative enzyme, and also reported to involved in browning reactions of fruits and vegetables. Its activity is usually observed in ripening or senescing tissues of fruits and vegetables. Lowest POD activity (852 Units/mg protein/min) was observed in the arils of 20 days old pomegranate fruit, whereas its highest activity (945 Units/mg protein/min) was observed in the arils of 140 days old fruit (Fig 3.13).

**Fig 3.13. Peroxidase activity in pomegranate arils during fruit development and maturation**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

The increase (5.5%) in its activity observed from 20 to 120 days of fruit development and maturation was slight. In contrast, a sudden increase by about 4.6%

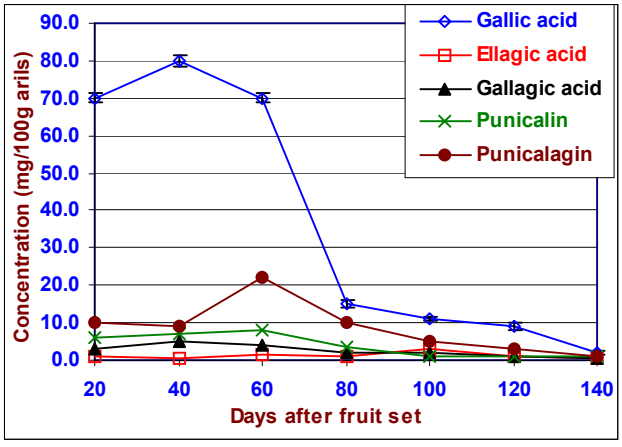
was observed from 120 to 140 days of fruit development. The increase in the activity after 120 days of fruit set supports the view of incidence of internal browning of arils in over mature fruits. Like PPO, POD activity was observed in enzyme extract of arils under different stages of development, but its activity in *in-vivo* condition would be influenced by enzyme inhibiting factors present in the arils in particular low pH of the arils and well defined cellular compartmentation. The increase in its activity after 120 days of fruit development may be attributed to the altered membrane permeability and exposure of the various hydrogen donor substrates like phenolics in to cytoplasm.

### 3.3.1.3. Bioactive molecule content

#### 3.3.1.3.1. Concentration of bioactive molecules in arils

The bioactive molecules isolated from pith and CM viz. gallic acid, ellagic acid, gallagic acid, punicalin and punicalagin were estimated in the pith and CM and arils from pomegranate fruits under different developmental stages. In the arils gallic acid was found to be the major molecule (Fig. 3.14) with a concentration of 80 ± 6 mg/100g in the arils of 40 days old fruit followed by punicalagin, (9.0 ± 1.2 mg/100g), punicalin (7.2 ± 0.9 mg/100g), gallagic acid (5.0 ± 0.6 mg/100g) and ellagic acid (0.7 ± 0.03 mg/100g).

**Fig 3.14. Bioactive molecule content of pomegranate arils during fruit development and maturation**



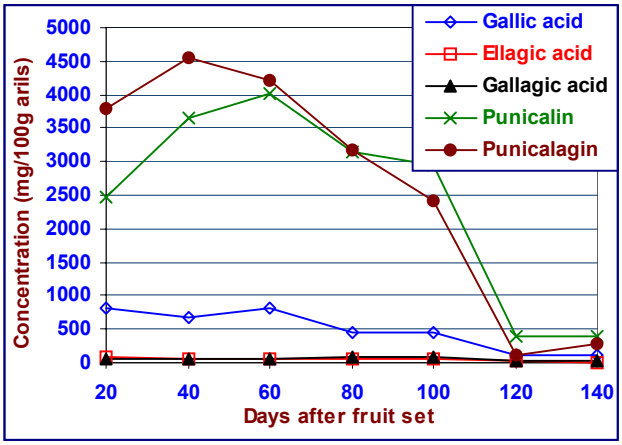

- Values are mean ± standard error of four replicates

The drastic decrease (78.57 %) in the concentration of gallic acid from 60 - 80 days old fruit may be attributed to its use as precursor for the biosynthesis of anthocyanin molecules. Gallic acid is the basic structural unit of flavylium ring of the anthocyanin molecules. The later contains two gallic acid moieties along with two sugar moieties in its structure. A drastic increase (50.8%) in the anthocyanin content (Fig. 3.7) of the arils from 60 – 80 days old fruit coinciding to decreased gallic acid content in the same period supports the above view.

**3.3.1.3.2. Concentration of bioactive molecules in pith and carpellary membrane**

Punicalagin was found major bioactive molecule content of pith and CM as recorded in 40 days old pomegranate fruit (Fig 3.15) with a concentration of (4,540 ± 225 mg/100g arils), followed by punicalin (3,660 ± 156 mg/100g), gallic acid (670 ± 24 mg/100g), ellagic acid (70 ± 6 mg/100g), and gallagic acid (60 ± 5 mg/100g).

**Fig 3.15. Concentration of bioactive molecules in pomegranate pith and CM during fruit development and maturation**




- Values are mean ± standard error of four replicates

The highest concentration of punicalagin and punicalin (4,540 ± 225 and 3660 ± 125 mg/100g arils respectively) was recorded in the pith and CM of 40 days old pomegranate fruit, followed by a drastic decrease in their concentration by 97.35% and 90.27 % respectively up to 120 days of fruit development. The higher concentration of

these high molecular weight polyphenols (punicalagin/punicalin) in the pith and CM and low molecular weight phenolics (gallic acid) in the arils and their period of decrease and increase in parallel with increase in anthocyanin content during the same period suggests a close association among them. The pith and CM may acts as a synthesis/storage tissue of high molecular weight polyphenols, and high molecular weight polyphenols as a storage form of low molecular weight phenolics. The decrease in these polyphenols may be attributed to their usage in the biosynthesis of anthocyanin pigments, which begins after 60 days of fruit development.

### 3.3.2. Biochemical studies in pomegranate arils during internal browning of the fruit

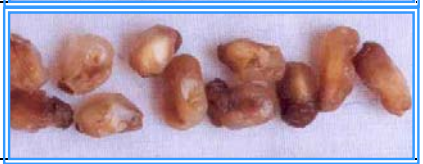
**Visual characteristics of internal browning of pomegranate fruit and arils**

The internal browning of pomegranate fruit is a typical disorder of pomegranate fruit, in which no external symptoms can be observed though the fruit is completely browned inside (Ryall & Pentzer, 1974). This disorder can only be observed after opening the fruit. The locus of browning initiation is non-specific with respect to different parts of the fruit (Fig 3.16). But once initiated, it spreads from that locus in all directions in that compartment of the pomegranate fruit and gradually covers the whole fruit

In internally browned pomegranate fruit, the arils does not develop the typical red color and become somewhat flattened than plump (Table 3.1), finally mummified with blackish color (Ryall & Pentzer, 1974; Desai 1989). Flavor of the arils is atypical and many have a streaked appearance due to fine white lines radiating from the seeds. But mechanism of browning is not known

**Fig 3.16 Visual characteristics of pomegranate fruit at different stages of internal browning**

**Table 3.1. Visual characteristics of arils at different stages of browning**

| Browning (%) | Visual characteristics | Visual appearance of arils |
|---|---|---|
| Normal | : Pink colored, turgid, juicy arils, with firm juice sac membrane, edged |  |
| 25 | : Discolored, less turgid, Juicy, intact juice sac, browning initiated at lower part of aril |  |
| 50 | : Semi browned, partly flaccid, partly browned arils with wrinkled juice sac membrane |  |
| 75 | : Browned, flaccid, partially dehydrated, saggy, wrinkled juice sac, |  |
| 100 | : Dark brown / black, hard to touch, mummified juice sac. |  |

During development of the internal browning in the arils, there is a gradual decrease in the turgidity, juice content followed by browning and blackening of the arils. This disorder found to initiate with discoloration of the arils followed by slight browning, browning and blackening of the arils (Table 3.1). In the final stage arils appears as mummified.

**Color measurement**

Visual characterization of intensity of browning in arils has been subjected to instrumental color measurement to provide numerical scale for measurement of browning. In the present study Hunter L a b color measuring system was used, in which L indicates lightness, (white =100 to black = 0), whereas 'a' indicates red (+a) to green (-a) and 'b' indicates yellow (+b) to blue (-b).

**Table 3.2. Color measurement of pomegranate juice from the arils grouped in to five groups based on visual observation**

| Parameter | Healthy | 25% brown | 50% brown | 75% brown | 100% brown |
|---|---|---|---|---|---|
| **L** | 83.39 | 85.93 | 83.14 | 80.97 | 69.81 |
| **a** | 9.07 | -0.28 | -1.39 | -2.27 | 6.43 |
| **b** | 5.86 | 20.22 | 25.61 | 32.44 | 38.82 |
| **ΔE** | 19.82 | 24.86 | 29.73 | 37.56 | 49.46 |

The above data revealed that, arils have passed through pinkish red (Healthy) color to light yellowish red (25% brown) followed by light brown (50% brown), brown (75% brown) and dark brown (100% brown) color stages indicating initial discoloration followed by browning.

### 3.3.2.1. Chemical composition associated with different stages of browning

#### 3.3.2.1.1. pH and titrable acidity

The measurement of pH of pomegranate juice extracted from different stages of browning revealed that, there is a gradual and significant (P≤0.005) increase (33.3%) in pH from healthy arils to 100% brown arils (Fig. 3.17). Coinciding to this a drastic decrease (97.7%) in titrable acidity was also recorded from healthy to 100% browned arils (Fig. 3.18).

**Fig 3.17. pH of the pomegranate arils at different stages of internal browning**

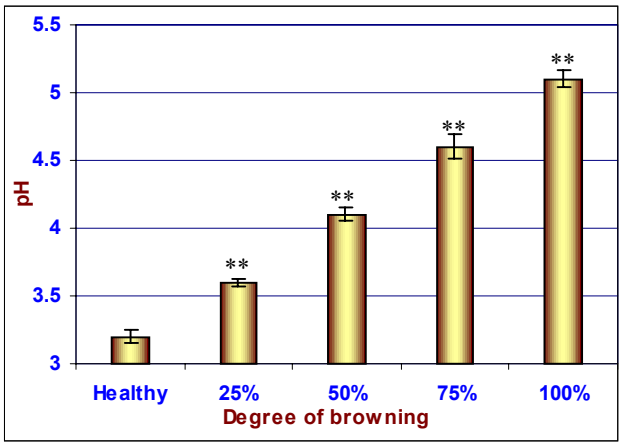
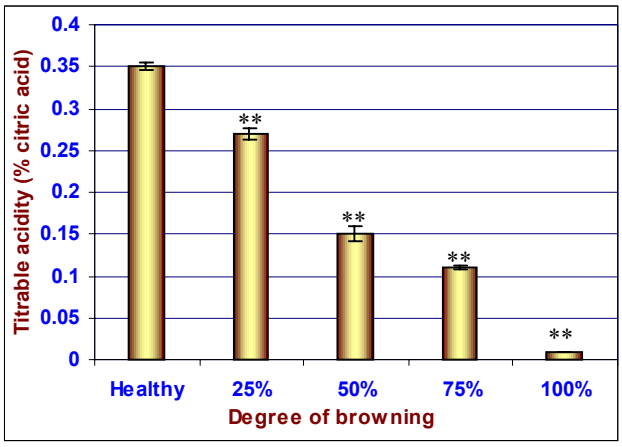



**Fig 3.18. Titrable acidity of pomegranate arils at different stages of internal browning**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at P ≤ 0.005) compared to previous

In terms of quality of a fruit, acids and sugars are important components, which provide characteristic taste and flavor to fruits and their products, and may also influence the color of the anthocyanin rich fruits and vegetables. There are two basic ways to quantify the acid content of samples viz. direct measure of pH and measuring the titrable acidity in comparison with standard acids viz. citric acid. The magnitude of pH gives immediate or actual acidity (actual hydrogen ion concentration) of sample. On the other hand, titrable acidity gives the total or potential acidity, i.e. total number of acid molecules, both protonated and unprotonated.

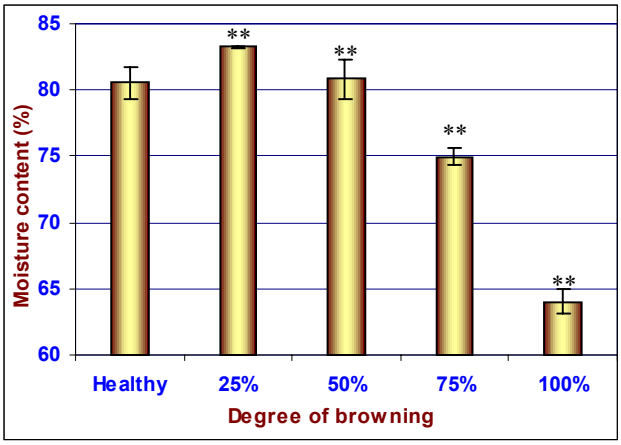
Organic acids reported from pomegranate include citric, malic, acetic, fumaric, tartaric and lactic acids; the major acid accounting for titrable acidity in pomegranate arils is citric acid (Melgarejo *et al.*, 2000). Decrease in acid content is usually associated with the respiration process, since acids forms the necessary respiratory material. This kind of decrease in titrable acidity was observed during ripening/senescence of fruits including pomegranate (Bashir *et al.*, 1989). The increase in the pH in pomegranate arils is due to the decreased acid content. Change in acid content plays important role in the fruits, which contain anthocyanins as coloring pigments. Because anthocyanin pigments undergo reversible structural transformation (Fig. 3.1) with a change in acidity (Cabrita *et al.*, 2000; Cordenunsi *et al.*, 2003). Thus decrease in acidity might have initiated early discoloration of arils during initiation of browning of arils. Though the decrease in acidity may not brings up the browning of arils, but it influences the other factors responsible for browning of arils in pomegranate.

**3.3.2.1.2. Relative water content**

Pomegranate aril is pink or red colored juicy sac with a soft white seed in the center and contains about 85% water. Pomegranate arils under different stages of browning showed an initial increase by 3.25% in water content from healthy arils to 25%

brown arils, but in later stages there is a drastic decrease by about 23.13% in water content up to 100% browning stage (Fig. 3.19).

**Fig 3.19. Water content of the pomegranate arils at different stages of internal browning**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

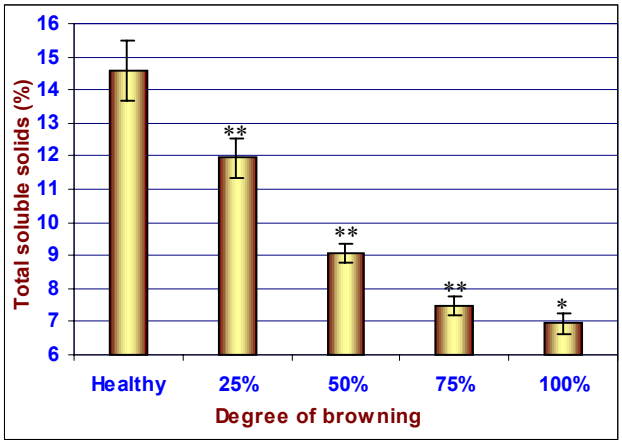
The influx or efflux of organic solutes is mainly due to the altered cell membrane permeability (Landrigan *et al.*, 1996). The loss of membrane integrity was recorded due to the desiccation of litchi fruit after harvest (Lin *et al*., 1988; Chen and Hong, 1992; Jiang and Fu, 1999). In litchi, the severity of membrane leakage and the rate of desiccation were found influenced by humidity (Jiang and Fu, 1999). Water stress affects many of the fruit species in many ways since water is the medium for most of biochemical reactions (Landrigan *et al.*, 1996). Enzymatic browning is the phenomenon occurs only in high water activity foods, where as non-enzymatic browning is in intermediate moisture (45 to 50%) foods. The decreased water stress also leads to decreased turgidity of the cell, which further leads to the loss of membrane integrity (Landrigan *et al.*, 1996*;* Jiang, & Fu, 1999).

**3.3.2.1.3. Total soluble solids**

The pomegranate arils showed a drastic decrease (52.23%) in total soluble solids (TSS) content in 100% browned arils (Fig. 3.20), indicating the decrease in sugar,

minerals, organic acids content. The decrease in TSS content in pomegranate arils under different stages of browning is whether due to enzymatic break down or due to leakage through the disintegrated cell wall material is not known. But this kind of decrease in TSS is observed during the ripening/senescence in the fruits and vegetables. However, loss in soluble solids content in particular sugars, proteins rules out the possibility of nonenzymatic browning due to Maillard reaction.

**Fig 3.20. Total soluble solid content of pomegranate arils at different stages of internal browning**



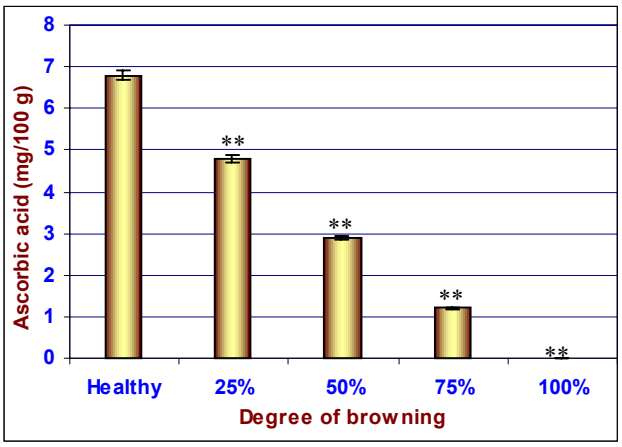

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

### 3.3.2.1.4. Ascorbic acid content

A drastic decrease (100%) in ascorbic acid content was observed in 100% brown pomegranate arils (Fig. 3.21). Ascorbic acid as such is a very unstable molecule and mechanism(s) for ascorbic acid losses are still not fully understood. Multiple factors such as oxidation, pH, relative humidity and temperature were ascribed to its degradation in the cells. Oxidation reaction of ascorbate to dehydroascorbic acid (DHA) with the concomitant reduction of molecular oxygen to water by ascorbic acid oxidase (AAO, EC 1.10.3.3) is also reported (Yahia *et al.*, 2001; Nakamura *et al*., 1968). Decrease in ascorbic acid adds up to the decrease in acidity of arils, which has negative impact on

anthocyanin coloration. The loss in ascorbic acid content in pear fruit has been reported to increased incidence of browning in pears (Veltman *et al*., 1999).

**Fig 3.21. Ascorbic acid content of the pomegranate arils at different stages of internal browning**



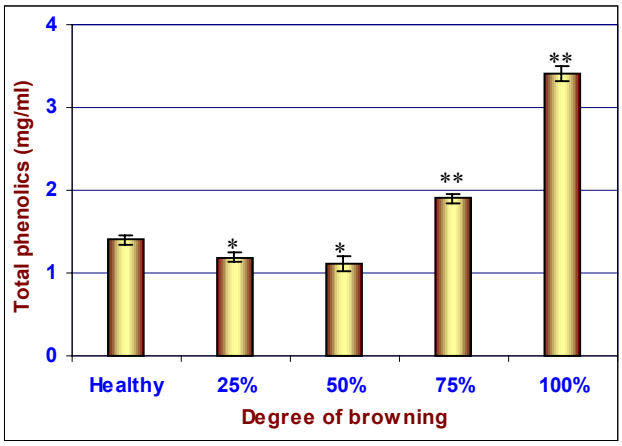

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

### 3.3.2.1.5. Total phenolics

Total phenolic content recorded slight decrease (20%) in initial stages of browning of arils (healthy stage to 50% browning), whereas in later stages of browning, a drastic increase (58.8 %) in phenolic composition was observed. Highest phenolic content (3.4 ± 0.05 mg/ml) was recorded in 100% brown arils (Fig. 3.22). Initial decrease in phenolic composition may be attributed to the breakdown of phenolics, but later increase may be due to the concentration of phenolics owing to loss of water in the pomegranate arils after 50% browning. The phenolic content in fruits and vegetables plays important role in enzymatic as well as nonenzymatic browning reactions. In the former case, phenolic compounds act as substrates for a number of oxidoreductases, namely, polyphenoloxidases (PPO) and peroxidases (POD) resulting in the brown colored end products, while in later case, their interaction with metal ions brings up the formation of brown metal complexes. The exact mechanism, in which browning via

metal ion interaction occurs, is not well understood (Oszmianski *et al.*, 1996) but it may be due to complex formation of phenolic compounds with metal ions (Cheng & Crisosto, 1997). Thus increase in phenolic content might have adverse affects on the pomegranate arils.

**Fig 3.22. Total phenolic content of the pomegranate arils at different stages of internal browning**




- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

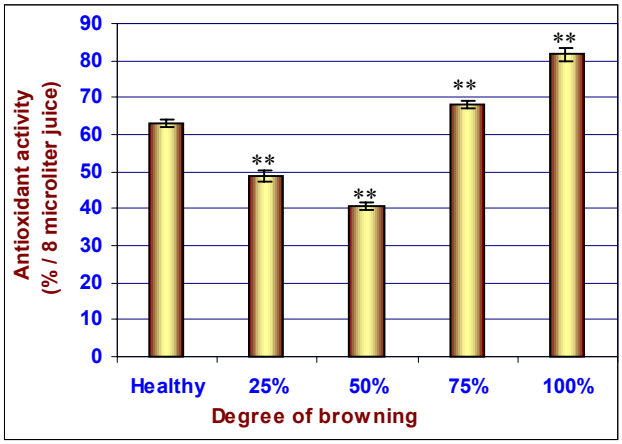
### 3.3.2.1.6. Antioxidant activity

#### DPPH radical scavenging activity

Browning in fruits and vegetables is an oxidation phenomenon (Veltman *et al.*, 2000). Despite the fact that pomegranate arils are a rich source of antioxidants (Ascorbic acid, anthocyanins and other phenolics), pomegranate fruit is at the risk for internal browning of arils. During initial stages of browning (healthy to 50% brown), decrease (35.4%) in the radical scavenging activity (Fig 3.23) was observed, which may be attributed to the decrease in ascorbic acid content and total phenolics (Fig. 3.21, 3.22). Interestingly, the arils have showed an increase (50.3%) in the radical scavenging activity in 100% brown arils, which may be attributed to the increased concentration of

phenolics due to loss of water content in the arils. The increased browning with increased phenolic content may be the cause of browning rather than the consequence.

**Fig 3.23. DPPH radical scavenging activity of pomegranate arils under different stages of internal browning**

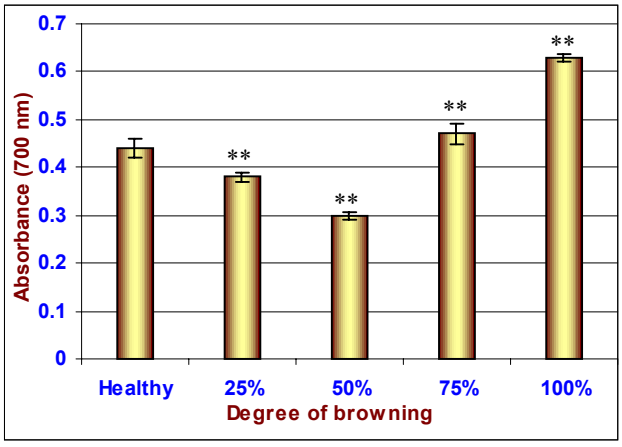



- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Total reducing power**

Similar to DPPH radical scavenging activity, arils showed initial decrease (31.8%) in total reducing power up to 50% browning of arils (Fig. 3.24) followed by significant increase (52.4%).

**Fig 3.24. Total reducing power of pomegranate arils under different stages of browning**



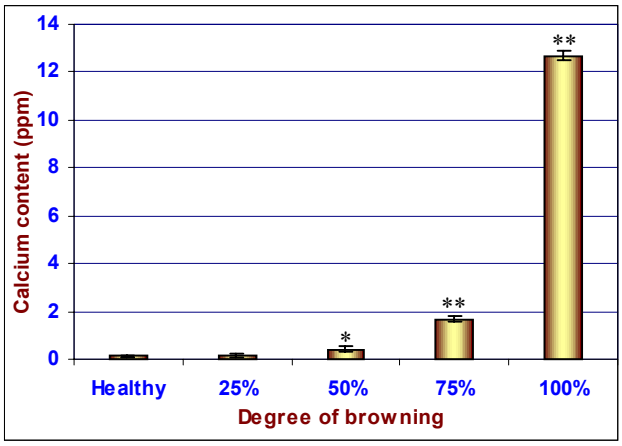

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

The reducing capacity of a compound may serve as a significant indicator of its antioxidant capacity (Meir *et al.*, 1995). The reducing power of pomegranate arils may be mainly attributed to its ascorbic acid content, and phenolics. Despite of 100% decrease in ascorbic acid content in 100% brown arils (Fig. 3.21), the higher reducing power of the arils may be due to the high phenolic content (Fig. 3.22).

**3.3.2.1.7. Mineral content**

Minerals in particular transition metals like iron and copper and electrolytes like calcium and potassium play an important role in cell physiology. Iron and copper have been reported to involved in non-enzymatic browning by interacting with various biomolecules in the cellular system (Marsilio *et al.*, 2001; Lombardi, & Sebastiani, 2005). A study on the mineral content of arils viz. calcium, potassium, copper, iron, and zinc during different stages of browning revealed that there is a decrease in the content of potassium, iron, and copper by 4.9%, 83.4%, and 24.4% respectively, where as a drastic increase in the calcium content by about 98.97% in 100% brown arils (Figs 3.25; 3.26; 3.27; and 3.28).

**Fig 3.25. Calcium content of the pomegranate arils under different stages of internal browning of arils**




- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Fig 3.26. Potassium content of the pomegranate arils at different stages of internal browning of arils**

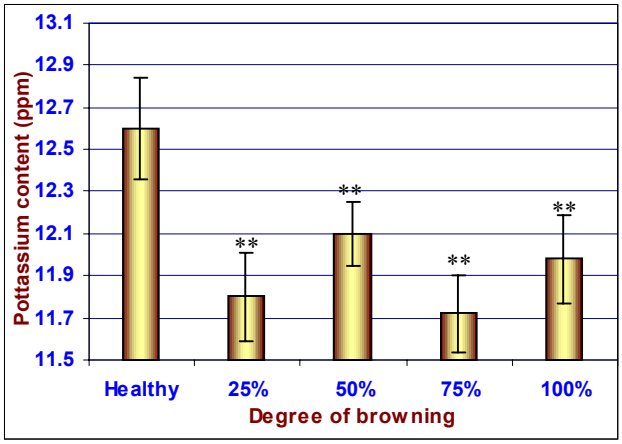



**Fig 3.27. Iron content of pomegranate arils at different stages of internal browning**

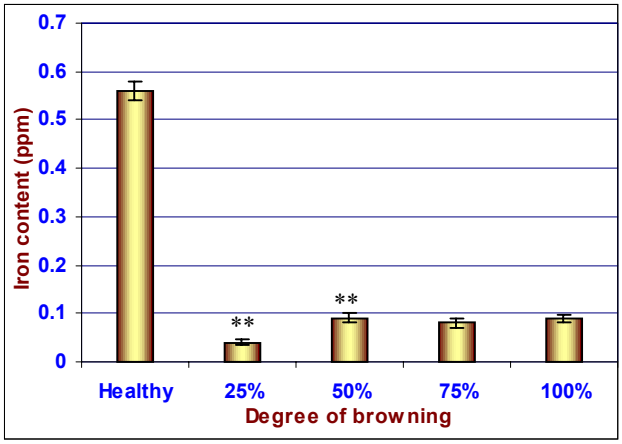
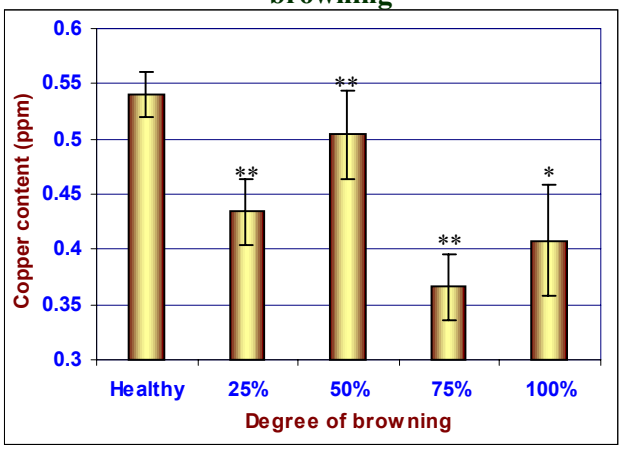



**Fig 3.28. Copper content of pomegranate arils at different stages of internal browning**



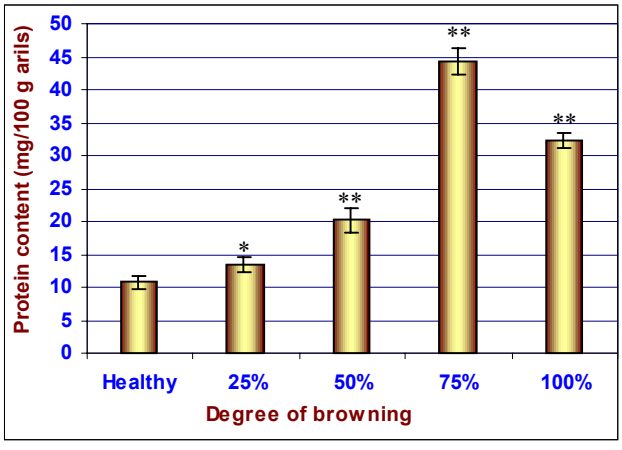

- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

Decrease in iron and copper content from arils rules out the possibility of non-enzymatic browning of arils due to their interaction with phenolics. An increase in phenolics, protein content and decrease in electrolytes suggests a disruption to membrane integrity in fruits and vegetables (Landrigan *et al.,* 1996) and marks the over maturation/ripening/senescence changes in pomegranate fruit. A loss of membrane integrity may in turn contribute to the development of browning symptoms by releasing the oxidative enzymes and substrates in to the cytoplasm.

### 3.3.2.1.8. Total protein content

During browning, an increase (75.73 %) in the protein content was observed till 75% browning of arils, followed by a drastic decrease (27.09%) in 100 % browned arils (Fig. 3.29). Initial increase in early stages followed by a decrease in protein content is observed in ripening and senescing fruits and vegetables (Bashir, & Abu-Goukh, *2003*). The initial increase in protein content up to 75% browning in pomegranate arils may be attributed to synthesis of oxidative enzymes involved in ripening processes, while decrease in protein content during later stages may indicates the process of senescence, autolysis and apoptosis of the cell (Frenkel *et al.*, 1968)**.**

**Fig 3.29. Protein content of pomegranate arils at different stages of internal browning of arils**



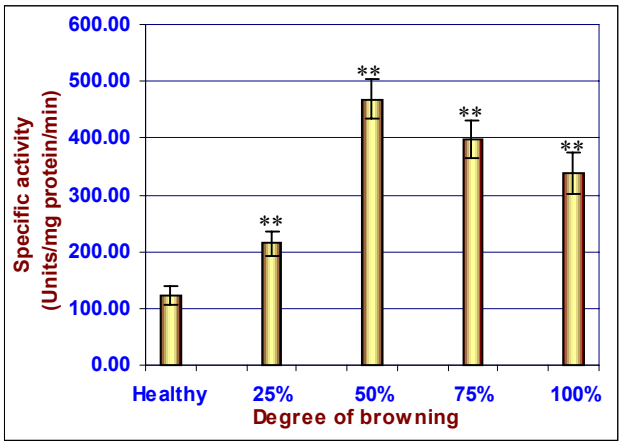

- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

### 3.3.1.2. Enzyme activities in arils

#### 3.3.1.2.1. Polyphenol oxidase activity

Polyphenol oxidase is a bifunctional copper-containing enzyme that uses molecular oxygen to catalyze the co-oxidation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and their subsequent oxidation to *o*-quinones (diphenolase or catecholase activity). The *o*-quinones thus generated polymerize to form brown colored compounds, which are responsible for browning. This metallo-enzyme is considered to be the main contributor to the browning, discoloration and darkening in fruits and vegetables (Vamos-Vigyazo, 1981; Zawistowski *et al.*, 1991). During browning of pomegranate arils, PPO activity was found to increase drastically (73.72%) in 50% browned arils, followed by decrease in its activity significantly by 28% in 100% browned arils (Fig. 3.30).

**Fig 3.30. Specific activity of polyphenol oxidase from pomegranate arils under different stages of internal browning of arils**

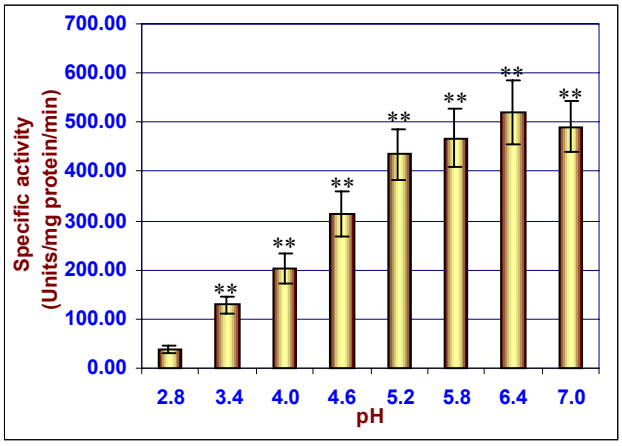



- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

The increase in PPO activity coincides with increased protein content till 50% browning of arils. Further decreased activity of PPO enzyme, though the protein content increased till 100% browning, may be due to the inactivated enzymes because of feed back inhibition phenomenon, since, quinones, the end products of PPO catalyzed

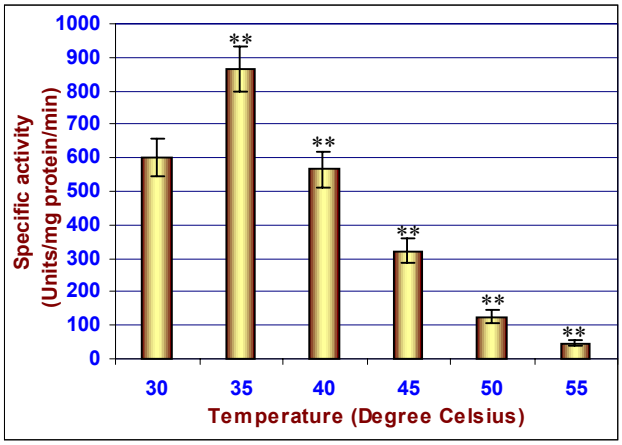
reaction, are known to bind enzymes irreversibly making them inactive. Hence decrease in PPO activity in later browning stages may be attributed to their interaction with quinones. The increase in PPO activity in coincidence with increased intensity of browning in pomegranate arils suggests the close association of PPO enzyme activity with the phenomenon of browning in pomegranate arils.

**Fig 3.31. Effect of pH on the enzymatic activity of PPO from pomegranate arils**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Fig 3.32. Effect of temperature on the enzymatic activity of PPO from pomegranate arils**



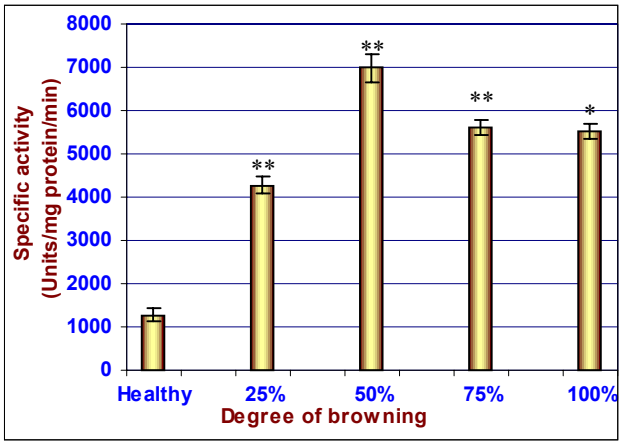

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

The optimum pH for pomegranate PPO activity was found 6.4 (Fig. 3.31) and optimum temperature was 35°C (Fig. 3.32). Though the optimum pH is 6.4, its significant activity was observed at pH 3.4 to 5.2. The PPO activity at low pH may be a key factor in the browning of arils, since, the pH of the healthy pomegranate arils was increased up to 5.2 during browning stages from healthy to 100% browning due to corresponding decrease in acid content, which may provided optimum conditions for PPO activity. Resulting in the browning reactions of pomegranate arils.

**3.3.1.2.2. Peroxidase (POD) activity**

Peroxidase is also a member of the group of oxidoreductases (Anon, 1973). They decompose hydrogen peroxide in the presence of a hydrogen donor and are considered to be indices of ripening and senescence in many fruits and vegetables (Haard, 1973). The ability of POD to act on a variety of substrates including, phenols, anthocyanins, make it more destructive enzyme compared to PPO. POD can catalyze oxidation of many kinds of phenols in the presence of peroxides. During browning POD activity was found to increase drastically by 81.17% in 50% browned arils followed by slight but significant decrease in the peroxidase activity by 20.6% in 100% browned arils (Fig. 3.33).

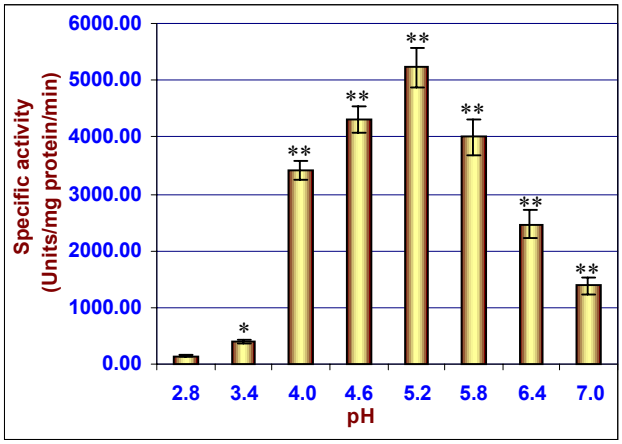
**Fig 3.33. Specific activity of peroxidase from pomegranate arils under different stages of internal browning of arils**




- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

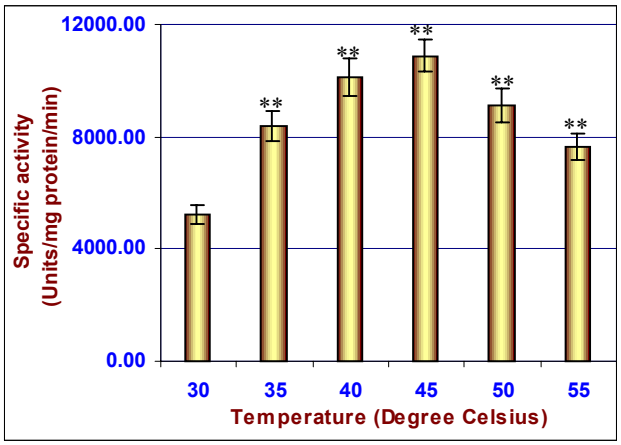
The decrease in the POD activity during later stages of browning, may be attributed to corresponding decrease in protein content, which has been attributed to the senescence changes and also lack of substrates, feed back inhibition.

**Fig 3.34. Effect of pH on the enzymatic activity of peroxidase from pomegranate arils**




- Values are mean ± standard error of four replicates
- Single asterisk denote significant difference (at $P \leq 0.05$) compared to previous
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

**Fig 3.35. Effect of temperature on the enzymatic activity of peroxidase from pomegranate arils**



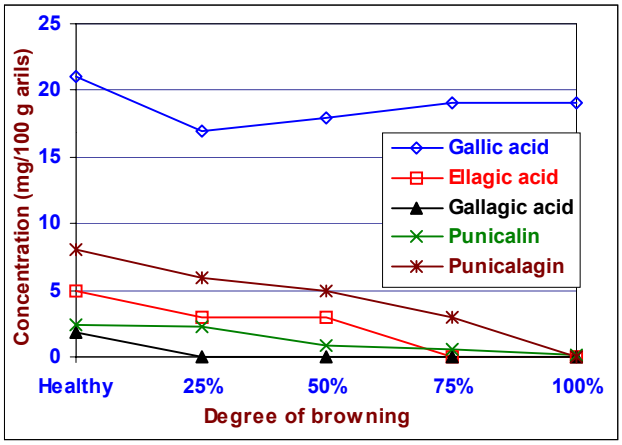

- Values are mean ± standard error of four replicates
- Double asterisk denote significant difference (at $P \leq 0.005$) compared to previous

Optimum pH for POD activity was found 5.2, and optimum temperature was 45°C (Fig. 3.34, 3.35). The low pH optima of peroxidase may be the factor responsible initiation of senescence process in the arils and browning. The significant activity of POD at pH 4, may be the factor behind the browning in 50% brown arils, which have recorded a pH of about 4.2 (Fig. 3.17). Enzyme activity has been ascribed as a major cause of browning in many fruits like pear (Richard & Gauillard, 1997), pineapple (Selvarajah *et al.*, 1998) and Litchi (Underhill & Critchley, 1995). The higher activity of POD at low pH, in the range of that of pomegranate arils suggested its close association with browning phenomenon in pomegranate arils.

### 3.3.2.3. Concentration of bioactive molecules in arils

The concentration of bioactive molecules viz. gallic acid, ellagic acid, gallagic acid, punicalin, and punicalagin in browning arils was significantly decreased with increase in browning (Fig. 3.36). Decrease in above phenolics may be attributed to the enzymatic breakdown by peroxidase and polyphenol oxidase enzymes.

**Fig. 3.36. Concentration of bioactive molecules in pomegranate arils under different stages of browning**




1. Values are mean ± standard error of four replicates

### 3.3.2.4. Enzyme inhibitory activity of bioactive molecules

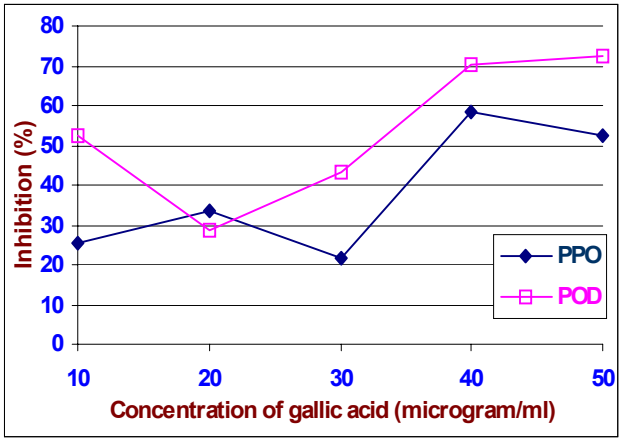
Presence of high concentration of gallic acid at different stages of browning prompted us to study its inhibitory effect on pomegranate PPO and POD enzyme activity, further, the ellagic acid and punicalagin were also included in our study of enzyme inhibition Among the three molecules tested, gallic acid showed potential enzyme inhibitory activity against both PPO enzyme and peroxidase enzyme followed by ellagic acid and punicalagin (Table 3.3).

Table 3.3. The inhibitory effect of bioactive molecules from pomegranate fruit pith and CM on pomegranate polyphenol oxidase and peroxidase enzyme activity

| Bioactive molecule | PPO inhibition (%) | POD inhibition (%) |
|---|---|---|
| Gallic acid (50µg/ml) | 52.5 ± 2.3 | 72.3 ± 3.5 |
| Ellagic acid (500 µg/ml) | 22.3 ± 1.5 | 42.6 ± 1.3 |
| Punicalagin (500 µg/ml) | 14.2 ± 1.2 | 38.5 ± 2.1 |
| KMS (50 µg/ml) | 100 ± 0.0 | Not tested |

1. Values are mean ± standard error of four replicates

**Fig. 3.37. Dose response curve of inhibition of PPO activity and POD activity by gallic acid**




Further the dose response curve of inhibitory activity of gallic acid against both the enzymes revealed that, the enzyme inhibitory activity is of non-specific type. Since, competitive, non-competitive inhibitions show a concentration dependent linear gradient

of inhibitory activity. The inhibitory activity shown by gallic acid against both PPO and POD is neither concentration dependent nor linear (Fig. 3.37). The enzyme inhibitory activity of gallic acid may be due to its protein binding ability or may be due to metal chelating activity, or both. The gallic acid was reported as a strong metal chelator and can also binds to proteins (Khokhar, & Apenten, 2003). The non-linear enzyme inhibitory activity of gallic acid (Fig. 3.37) may be attributed to possibility of different mechanism of enzyme inhibition i.e. by protein binding and metal chelating or both.

## 3.4. Summary and conclusion

### Stages of fruit development

The study of biochemical profile of pomegranate fruit during different stages of fruit development clearly demarked in to four phases viz. growth phase up to 80 days of fruit set, maturation phase 80-100 days, ripening 100-120 days and senescence 120 days onwards. The developmental period of arils was extended up to 80 days from fruit set, which was associated with a continuous increase in concentration of TSS, total sugar, reducing sugar and anthocyanin pigments. This was accompanied by a significant ($P \leq 0.05$) reduction in phenolics, ascorbic acid and titrable acidity up to 80 days. The increase in anthocyanin content and decrease in phenolics may be related to each other; since phenolics may be used up in the biosynthesis of the flavylium ring during anthocyanin pigment formation, leading to a reduction in their content. Decreased gallic acid content coinciding with anthocyanin content, suggests the role of gallic acid in the arils as precursor for anthocyanin biosynthesis.

Maturation period (80 and 100 days) of pomegranate fruit was associated with a sharp and significant increase of TSS, total sugar and reducing sugar, along with a gradual decrease of total phenolics and ascorbic acid. The equilibrium concentration of these chemicals on the 100th day marked the attainment of optimum maturity and onset of ripening.

Ripening phase was highly distinct, with array of physico-chemical changes like change in surface color, texture, and characteristic sensory attributes. During this phase, there was an increase in TSS, total sugars and reducing sugars, PPO, POD enzyme activities. The ripening phase in pomegranate fruit was extended up to 120 days of fruit set. A slight but significant decrease (9.3%) in anthocyanin pigment content was observed after 100 days. However, enzyme extract from pomegranate arils of different

stages of fruit development showed a slight increase in peroxidase activity and PPO activity as tested under in vitro conditions but their activity might be inhibited by low pH of arils, ascorbic acid, and anthocyanin content hence no browning reactions were observed during this period. After 100 days of fruit set, pomegranate arils showed a sudden increase in the activity of these two deteriorative enzymes. The increase in the activity of these two oxidative enzymes associated with initiation of anthocyanin discoloration with a decrease in acidity was also associated with ripening changes.

Decreased chemical components and increased activity of oxidative enzymes heralds the onset of senescence in 140 days old fruit. This phase heralds the cellular disintegration due to autolysis of the cells, which can be visualized by distinct browning.

**Mechanism of internal browning in pomegranate arils**

The studies on biochemical profile during different stages of fruit development revealed that, 100 days after fruit set marks the physiological maturity of the pomegranate fruit, then onwards, ripening changes initiate in the fruit followed by senescence. The initial observations on the internally browned fruits by different researchers and field experts of pomegranate orchards revealed its incidence in over mature fruits.

Further, the studies on biochemical profile of arils under different stages of browning clearly explained associated biochemical factors in pomegranate fruit browning. Browning phenomenon in pomegranate arils was initiated with discoloration of arils followed by browning and hardening of the arils. The initial discoloration of arils be attributed to increased pH of the arils. The increase in pH was due to the decreased content of organic acids. Decrease in the acidity has been attributed to the consequence of onset of ripening/senescence in the fruits. The increase in pH associated with

decreased acid content resulted in the non-enzymatic initial discoloration of anthocyanins due to structural transformation of later.

Further, loss of total soluble solids, minerals like potassium, iron and copper content was recorded from the arils under different stages of browning, supports the view of senescence in the arils under different stages of browning. The loss in the mineral content in early stages of browning suggests the loss of membrane integrity in arils prior to the drastic browning reactions. Loss of membrane integrity and cellular decompartmentation are the common phenomenon associated with ripening of fruits. However, increased calcium content due to its crystallization property adds up to the increase in the pH of arils. The loss of metal ions viz copper and iron rules out the phenomenon of non-enzymatic browning due to the interaction of these metal ions with polyphenols.

Excessive loss of water content during browning of arils adds up to the process of loss in membrane integrity. The loss of membrane integrity also leads to the cellular decompartmentalization resulting in exposure of different oxidative enzymes and substrates including PPO and POD. The phenolics, which are located predominantly in the vacuoles, may be released in to the cytoplasm on cellular decompartmentation. The exposure of oxidative enzymes as well as substrates in to the cytoplasm provided an optimum condition for oxidation reaction and hence browning reaction in pomegranate arils of over mature fruit.

Optimum pH for the activity of pomegranate PPO enzyme found to be 6.2, whereas for peroxidase enzyme it was 5.2. Study on changes in pH during browning of arils exhibited that there is an increase in the pH of the arils from pH 3.2 in healthy arils to pH 5.2 in 100 % browned arils. The increase in the pH has been attributed to the decreased acid content and increased calcium content**.** The increased pH has provided the

optimum pH conditions for PPO and peroxidase enzymes. The high activity of peroxidase enzyme as compared to the PPO enzyme may be attributed to its optimum activity at low pH compared to that of PPO.

Though the optimum pH for the activity of peroxidase enzyme from pomegranate arils is 5.2, but it has showed a significant activity at pH 4.0 also. Hence peroxidase enzyme may be considered as the major enzyme responsible for enzymatic browning of pomegranate arils. The initial discoloration (25% brown) of the arils may be due to pH change, but later browning (25 – 100% brown) of arils involves the activity of peroxidase enzyme. The peroxidase enzyme has been reported to involve in the enzymatic browning of many fruits and vegetable. Though the activity of PPO enzyme from the pomegranate arils is less at low pH as compared to that of peroxidase activity in terms of specific activity, its significant activity was observed at the pH of 4.6 and onwards and it is also a potential contributor for browning of pomegranate arils.

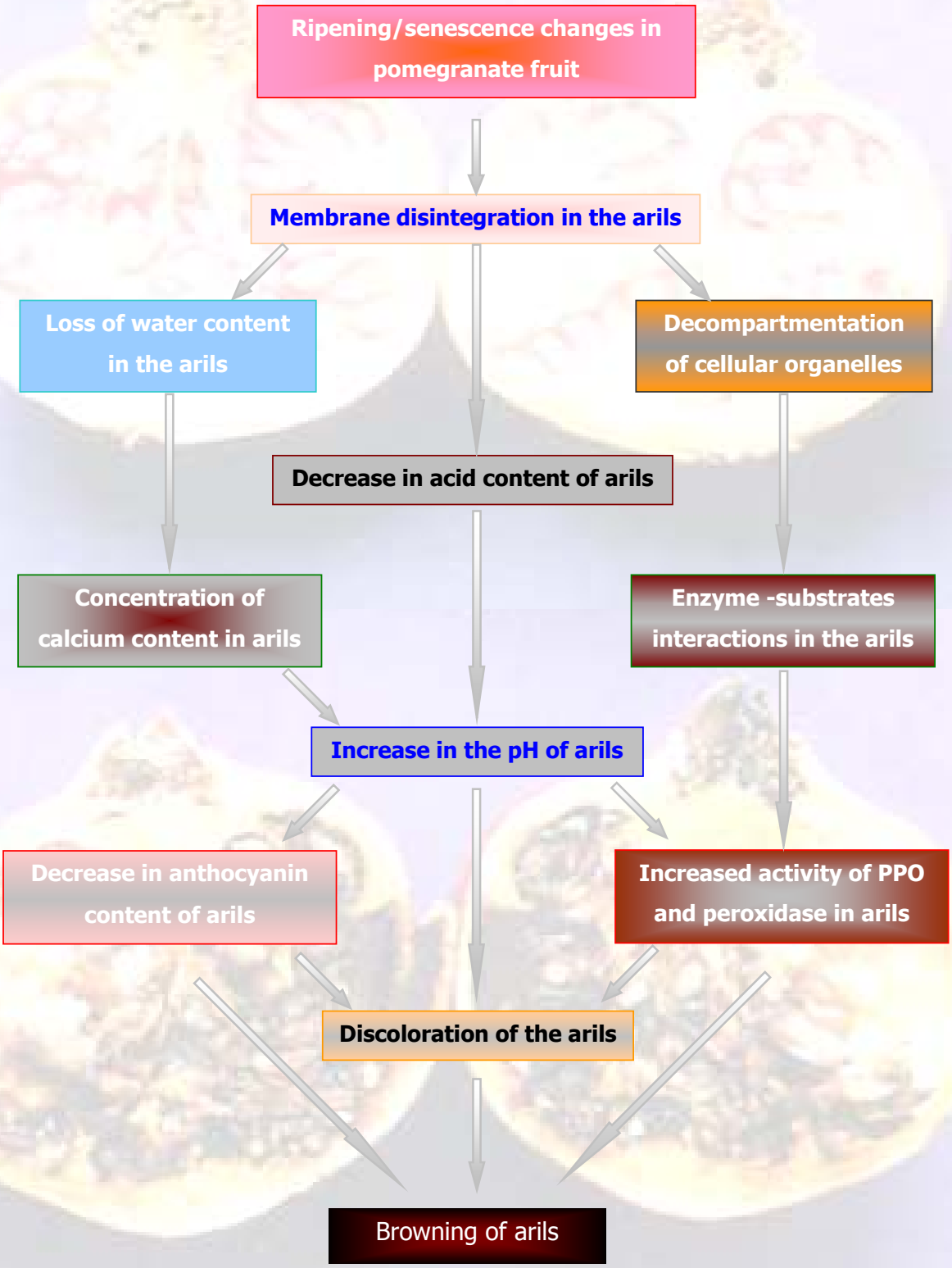
Though, peroxidase and PPO activity was observed in the enzyme extract of healthy pomegranates arils also. But in the healthy arils, presence of the anthocyanins ascorbic acid and punicalagin and its derivatives may play an enzyme inhibitory role along with gallic acid to arrest browning reaction. Numerous examples are given in the literature regarding the inhibitory activity of anthocyanins, ascorbic acid. In addition low pH and well-defined cellular compartmentation due to rigid cell membrane may not allow for the activities of these deteriorative enzymes in healthy arils.

In conclusion, advance of maturity leads to ripening. Ripening is characterized by an array of catabolic reactions. These catabolic reactions found to credit favorable biochemical conditions necessary for the initiation of browning reactions. However, clear manifestation of browning was observed at the advanced stage of the ripening,

which heralds the senescence process. Senescence is a natural process of cell death. In plant cells, browning may be the visible manifestation of senescence.

The overall steps involved in browning of pomegranate arils are as follows. The ripening changes in pomegranate arils associated with disintegration of membrane permeability, which may leads to the loss of water & acid content, and increase in the calcium content resulting in increase in the pH of arils. The increase in the pH results in the discoloration of arils due to the structural transformation of anthocyanin pigments. Discoloration of arils is the first step towards the browning of arils. The exposure of oxidative enzymes viz. PPO and POD enzymes and associated substrates due to degenerated membrane integrity, leads to the enzymatic browning reactions in the arils. The decreased anthocyanin content and ascorbic acid content and increased pH favors the oxidative enzyme activity. Further accumulation of calcium crystals in the arils leads to the hardening of the arils. The research findings towards browning of arils in pomegranate arils were consolidated and interlinked to elucidate the possible mechanism of internal browning.

*Flow chart: 3.1. Proposed mechanism of internal browning of arils in pomegranate fruit*



**Role of bioactive molecules in the internal browning of pomegranate arils**

Pith and CM in addition to their anchoring and protective teleological role, also performs the function as storage tissue for bioactive molecules viz. punicalagin and its derivatives (punicalin, gallagic acid, ellagic acid, gallic acid). The decrease in the bioactive content of pith and CM during development, maturation, ripening and senescence indicates its role as supportive tissue for supply of the same to perform multiple functions of anthocyanin source material. Punicalagin was successfully demonstrated as the most active molecule present in pith and CM and also in arils. A rapid decrease in punicalagin and its derivatives in arils during browning may be due to its utilization as an antioxidant to prevent oxidative damages like browning of arils. Retention of gallic acid may be as a last line defense against oxidative stress including browning.

The PPO and POD enzyme inhibitory activity of punicalagin, gallic acid, and ellagic acid was though non-specific type, but it proves their role as inhibitor of browning of pomegranate arils. The results suggest that there may be minimum threshold concentration of bioactive molecules with PPO and POD enzyme activities indicating a delicate balance between them in healthy arils. The intact cell membrane of cell organelle, low pH, high concentration of bioactive molecules; anthocyanin pigments during development and maturation of pomegranate arils favors the balance in favor of bioactive molecules. The decreased organic acids including ascorbic acid, anthocyanin content, concentration of bioactive molecules favors the activity of PPO and POD enzymes leading to the browning reactions.

The complexity of living fruit matrices adds a complexity to the understanding of browning phenomenon in pomegranate arils. To accurately elucidate internal browning of fruit arils models must account for chemical, structural and environmental conditions

of cytosol and cell organelles. The extensive biochemical evidences accumulated in the present study are convincing to indicate that ripening induced changes are the major pre requisite conditions that alters the intracellular, chemical, biochemical, structural and functional profiles that favors the activity of PPO and POD, the enzymes responsible for browning.

Ripening and senescence induced changes precedes browning in pomegranate arils, altered membrane permeability, decrease in acid content, anthocyanin, ascorbic acid, increase in pH, calcium, PPO, POD activity appears to be a major contributor to the up regulation browning. Peroxidase appears to be a major enzyme for initiation and persistence of browning in pomegranate arils due to its low pH optima of activity. However PPO was also supplementary for the peroxidase activity.

Phenolic compounds spearheaded by punicalagin and its derivatives are the major endogenous phytoprotectants in pomegranate arils. However metabolic demand of highly active phase of growth viz. ripening may be contributing factor for early depletion of these bioactive molecules due to utilization in flavylium rings for anthocyanin formation and also for antioxidant action.

# *General summary and conclusion*

General summary and conclusion encompasses the salient features inferred from this investigation are as follows:

- Pomegranate fruit is a botanical enigma classified as 'Balausta' type of fruit, and occupies a unique position in the fruit world. The edible portion of pomegranate fruit called arils originates from the pith and is well protected by carpellary membrane (CM). Incidence of internal browning of arils is one of the major problems in pomegranates, wherein degeneration of pith and CM found to be always associated. They together constitute 13% (w/w) of the fruit. There was an expressed doubt regarding association pith and carpellary membrane's structural and functional protective role to the arils. Literature survey revealed that, so far, no attempt has been made to study the chemical composition and bioactive properties of pomegranate fruit pith and CM. Hence, a maiden attempt was undertaken with the objective of isolating and identifying the potential bioactive component from pith & CM of pomegranate fruit var. Ganesh.

- The pith and carpellary membrane was screened initially for its bioactivity by extraction and subsequent bioactivity assay like antioxidant activity. The methanol extract exhibited highest activity, hence selected as source of bioactive molecule, which was then subjected for repeated column chromatography using silica gel, and eluted with a series of solvents of increasing polarity, composed of various combinations of hexane, chloroform, ethyl acetate, and methanol. Finally the active molecule was purified by preparative HPLC. The spectroscopic data accumulated by UV-visible, IR, NMR, and Mass spectroscopy of the purified molecule, indicated the structure of the molecule as punicalagin, which is a high molecular weight

polyphenol. The molecular weight was 1081 and it possesses 16 functional phenolic OH groups. This is the first report on isolation and identification of punicalagin from pith & CM of pomegranate fruit waste and its bioactive property. Further punicalagin was acid hydrolyzed and its products were identified as gallic acid, ellagic acid, gallagic acid, and punicalin by analytical HPLC and mass spectroscopy.

- In order to measure functional ability of punicalagin and its source extract, a range of antioxidant assays in many in-vitro systems like DPPH, Superoxide, $ABTS^{\bullet}$ radical, lipid peroxidation inhibitory assay, total reducing power assay, metal chelating activity assay were carried out. The punicalagin showed potential radical scavenging activity against the DPPH, superoxide radical, and $ABTS^{\bullet}$ radicals. Further the punicalagin showed potential lipid peroxidation inhibitory activity against iron-ascorbic acid induced lipid peroxidation. The protective effects of antioxidants in biological systems are ascribed mainly to their capacity scavenge free radicals. In support to this, the punicalagin showed a very high electron donating activity as recorded from the total reducing power assay. The high electron donating ability was observed at physiological pH range, using differential pulse voltametric technique. Further the antioxidant potential of punicalagin was measured in terms of its metal chelating activity against iron and copper ions - the well-known potential pro oxidants, which can initiate oxidative damage of cell membrane. The punicalagin showed potential metal ion-chelating activity against both the metal ions used in the present study. The metal chelating activity of punicalagin is also due to is large number of phenolic OH groups. The results indicate that the punicalagin is a potential antioxidant and acts in multiple ways like radical scavenging, metal chelating.

- Pulse radiolysis studies experiments were carried out in order to know the kinetics of radical reactions of punicalagin with various radical species produced radiolytically and rate constants were calculated based on the rate of decay or formation of the radical species in presence and absence of the punicalagin. The results of above experiments demonstrated the ability of punicalagin to scavenge a variety of ROS and RNS known to produce disease status through cellular damage and points out its protective function. At pH 7 it exhibited good ability to reduce not only $^{\bullet}OH$ and $NO_2^{\bullet}$ radicals, but it also showed good ability to repair $ABTS^{\bullet-}$ and tryptophan radicals, which added up to its antioxidant potential

- Punicalagin exhibited low binding affinity towards DNA indicated, which may be attributed to the hydrogen bonding. This property may provide an opportunity to use punicalagin to inhibit DNA replication hence incomplete cell cycle- a strategy to target against cancer cells. The potential binding ability of punicalagin with HSA suggests its biological availability. Since HSA is known to act as carrier protein to many drug molecules. The platelet aggregation inhibitory activity of punicalagin renders the molecule as a potential pharmacological agent for the prevention of thrombosis. Platelet aggregation inhibitory activity added up to the functional properties of punicalagin.

- Further the antibacterial activity assay of the punicalagin against clinically important pathogenic bacteria indicated its potential bacterial growth inhibitory activity, The methanol extract and acetone extract showed higher antibacterial activity than punicalagin which can be attributed to the synergistic effect of the phenolic content of the above extracts. The antimicrobial activity of pith and

carpellary membrane extracts and punicalagin suggests the possible protective role of them against microbial damage to arils

- Since the animal experiments are expensive, and governed by complicated ethical issues, the in-vitro toxicity of punicalagin was carried out in the present work against three different cell lines, results indicated that, the punicalagin is not toxic at low concentration, the range in which the punicalagin is potential antioxidant molecule. The low cytotoxicity and high functional attribute at its low concentration, bioavailability across the body, rendered the punicalagin as multifunctional bioactive molecule, which can be employed for human health benefits.

- The changes in the biochemical profile of pomegranate arils, from 20 to 140 days, clearly explained their growth, development, maturation and ripening stages. The developmental period of arils was extended up to 80 days from fruit set, which was associated with a continuous increase in concentration of TSS, total sugar, reducing sugar and anthocyanin pigments. This was accompanied by a significant reduction in phenolics, ascorbic acid and titrable acidity up to 80 days, followed by a steady state. This clearly indicates that the increase in anthocyanin content and decrease in phenolics were related to each other; phenolics were being used up in the biosynthesis of the flavylium ring during anthocyanin pigment formation, leading to a reduction in their content. A sharp and significant increase of TSS, total sugar and reducing sugar between 80 and 100 days, along with a very slow decrease of total phenolics and ascorbic acid during the same period, was also observed. The equilibrium concentration of these chemicals on the 100th day may mark the attainment of optimum maturity and onset of ripening. A further increase in TSS,

total sugars and reducing sugars was due to progress in ripening in pomegranate fruit. A slight but significant decrease (9.3%) in anthocyanin pigment content was observed after 100 days. This early initiation of anthocyanin discoloration, associated with a decrease in acidity, may be a cause for the internal breakdown of arils in over-ripe pomegranate fruits.

- Based on the extensive research data generated through biochemical analysis of arils during fruit development and during browning of arils, and estimation of the bioactive molecules of pomegranate (punicalagin and its derivatives) for the first time teleological role of them was elucidated in the health and disease status of the pomegranate arils. A gradual decrease in the concentration of punicalagin and its derivatives in the arils during fruit development, maturation, coinciding with increase in anthocyanin content suggests their possible role in the synthesis of anthocyanin pigments. Similarly the arils under different stages of browning recorded a drastic decrease in the concentration of punicalagin and its derivatives except gallic acid. It appears that there is a delicate balance between internal browning of pomegranate arils with bioactive molecules along with biochemical attributes of arils. Further punicalagin and its derivatives appears to have multiple role in pomegranate fruit like phyloprotectants, either substrate for oxidative enzymes (POD & PPO) or for synthesis of flavylium ring towards development of various characteristics of sensory attributes. Their role may be regulated by cytosolic biochemical configuration, which in turns governed by developmental phases of pomegranate fruit.

## Futuristic note

The work envisaged in this thesis provides a new, cheap source (i.e. pith & CM) for isolation of punicalagin and its derivates, potential bioactive molecules. The knowledge base generated on various functional activities and bioavailability of punicalagin and its source extract can be effectively used to produce new age functional beverages or specialty foods targeting specific consumer groups. The causative factors and functional role of bioactive molecules addressed can be effectively used to draw new strategies to control internal browning of arils.

The work may be a testimony for scientific validation of functional properties of pomegranate fruit and its therapeutic use in Ayurveda. Thus, in terms of their health benefits and applications, the fruit and its bioactive molecules can be placed within the 'nutraceutical landscape'.

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# *List of patents/publications/presentations*

## Patent filed

✉ **Anand P. Kulkarni,** S. Divakar*, S. M. Aradhya. "A Process For The Extraction of an Antioxidant Principle From Pomegranate Fruit Waste Viz. Pith And Carpellary Membrane." *Indian patent,* Submitted to IPMD, New Delhi, WO 2004, /056444A1

## Research publications

🗁 **Anand P. Kulkarni,** Somaradhya, Mallikarjuna Aradhya and Soundar Divakar, Isolation and identification of a radical scavenging antioxidant - punicalagin from pith and carpellary membrane of pomegranate fruit. *Food Chemistry,* 2004 (87), 551-557.

🗁 **Anand P. Kulkarni**, S. Divakar and S. M. Aradhya. Lipid peroxidation inhibitory activity of pomegranate fruit waste extract. *Food and Nutritional Security: Agrotechnology and Socio-economic aspects* (Vol. 2). 2003, PP 75-77.

🗁 **Anand P. Kulkarni**, and Somaradhya Mallikarjuna Aradhya Chemical Changes and Antioxidant Activity in Pomegranate Arils During Fruit Development, *Food Chemistry*. 93: 319-324 (2004).

🗁 **Anand P. Kulkarni,** and Somaradhya Mallikarjuna Aradhya. Inhibition of selected pathogenic gram-ve bacteria by pomegranate fruit waste (pith and carpellary membrane) extracts and its major bioactive molecule - punicalagin. *Food Microbiology*, 2004, Communicated.

🗁 **Anand P. Kulkarni,** H. S. Mahal, S. Kapoor, Somaradhya Mallikarjuna Aradhya. Binding, Antioxidant and Cytotoxic actions of Punicalagin- A High Molecular Weight Polyphenol present in pomegranate. (2005) communicated to *Free Radicals Biology and Medicine.*

- **Anand P. Kulkarni**, and S. M. Aradhya. Pomegranate: A potential nutritional and nutraceutical fruit (2005) to be communicated to *CRC critical Reviews in Food Science and Nutrition.*

**Poster Presentations:**

- **Anand P. Kulkarni,** S. Divakar, and S. M. Aradhya. Potential of pomegranate fruit waste (pith and carpellary membrane) as a source of an antioxidant - an in vitro approach, International Conference on Role of Free Radicals and Anti-oxidants in Health & Disease Lucknow, February 10-12, 2003.

- **Anand P. Kulkarni,** S. Divakar* and S. M. Aradhya. Lipid Peroxidation Inhibitory Activity of Pomegranate Fruit Waste Extract. National Symposium on "Food and Nutritional Security: Technological interventions and Genetic Options" CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, HP. September 18 –19, 2003.

- **Anand P. Kulkarni;** H. M. Swaroop Kumar; and S. M. Aradhya. Antibacterial effect of Punicalagin- a high molecular weight polyphenol from pomegranate fruit waste on food borne human pathogens. 44th annual conference of the association of microbiologists of India. University of Agricultural Sciences, Dharwad. November 12 - 14, 2003.

- **Anand P. Kulkarni,** Sivaprasad M, Policegoudra R.S. and Aradhya S.M. Studies on changes in chemical composition and antioxidant properties in pomegranate arils during fruit development. International Food Convention [IFCON) Mysore. Dec. 5-8, 2003

  AWARDED WITH SECOND BEST POSTER PAPER

- **Anand P. Kulkarni** and S. M. Aradhya. Punicalagin - A Natural Product Isolated from Pomegranate Fruit with Free Radical Scavenging and Anticancer Activity An Invitro approach. International Conference on "Natural Products, Free Radicals and Radioprotectors in Health" (NFRH - 2004), Jan. 17-19, 2003.

  AWARDED WITH SECOND BEST POSTER PAPER