ISOLATION AND CHARACTERIZATION OF VALUABLE COMPONENTS FROM THE MANGO PEEL AND BLACK GRAM HUSK

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Dedicated to achan & amma

Without the perseverance, support and guidance of whom, I would not have the goals and the strength to fulfill my dreams ….

DECLARATION

I hereby declare that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF VALUABLE COMPONENTS FROM THE MANGO PEEL AND BLACK GRAM HUSK"** submitted to the **UNIVERSITY OF MYSORE** for the award of the **DEGREE OF DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of research work carried out by me under the guidance of **Dr. U. J. S. PRASADA RAO**, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore-570 020, during the period 2002-2007. I further declare that the results presented in this thesis have not been submitted for the award of any other Degree or Fellowship.

Date: 27th April, 2007 **(Ajila C. M.)**

Place: Mysore

Dr. U. J. S. PRASADA RAO
Scientist
Department of Biochemistry & Nutrition

Date: 27th April, 2007

CERTIFICATE

This is to certify that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF VALUABLE COMPONENTS FROM THE MANGO PEEL AND BLACK GRAM HUSK"** submitted by **Ms. AJILA C.M.,** for the award of the **DEGREE OF DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, to the **UNIVERSITY OF MYSORE** is the result of research work carried out by her in the Department of Biochemistry and Nutrition under my guidance during the period 2002-2007.

(Dr. U. J. S. PRASADA RAO)

Guide

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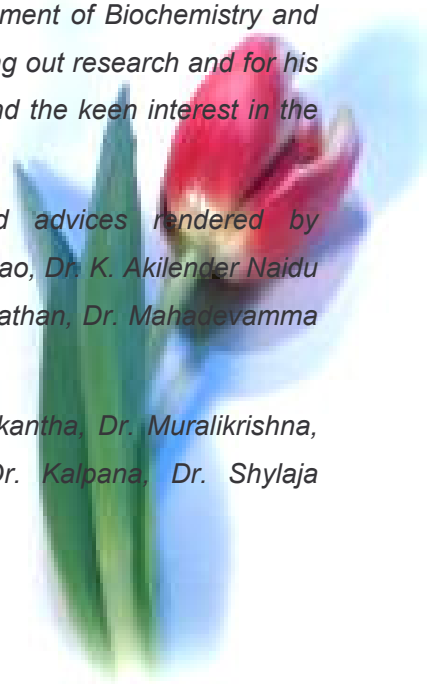

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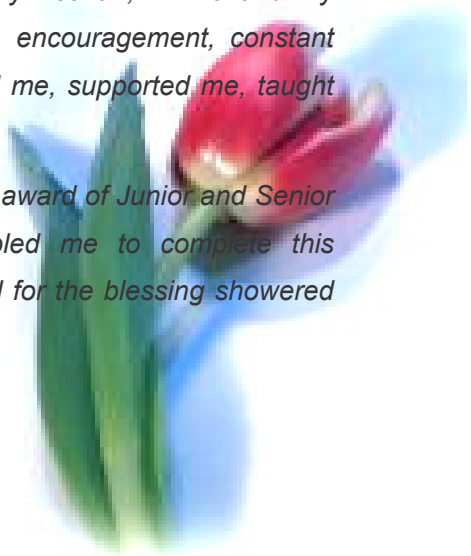


CONTENTS

LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
μg	Micrograms
μl	Microlitre(s)
μM	Micromolar
[S]	Substrate concentration
[V]	Velocity reaction
~	Approximately
APS	Ammonium per sulfate
BHA	Butylated hydroxy anisole
cm	Centimeters(s)
CTAB	Cetyltrimethylammonium bromide
DEAE	Diethylaminoethyl
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTT	Diethyldithiothrietol
EDTA	Ethylenediaminetetra acetic acid
g	Gram(s)
GAE	Gallic acid equivalent
gf	Gram force
GLC	Gas Liquid Chromatography
h	Hour(s)
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration at 50%
kDa	Kilodaltons
kg	Kilogram(s)
kgf	Kilogram force
Km	Michaelis- Menton constant
l	Liter(s)
LCMS	Liquid chromatography Mass Spectrometry
M	Molar concentration
mg	Milligrams(s)

Min	Minute
ml	Millilitre(s)
mM	Millimolar
mm	Millimeters(s)
MPP	Mango peel powder
MW	Molecular weight
nm	Nanometer(s)
p	Page number
PAGE	Polyacrylamide gel electrophoresis
POD	Peroxidase
PPO	Polyphenol oxidase
SDS	Sodium dodecyl sulphate
Sec	Second(s)
-SH	Sulfyhydryl
-SS-	Disulfide
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N, N', N'-Tetraethylmethylenediamine
TIC	Total Ion Current
Tris	Tris (hydroxymethyl) aminomethane
U	Units
w/v	Weight by volume
x g	Times acceleration due to gravity

LIST OF TABLES

LIST OF FIGURES

Synopsis

INTRODUCTION

As a result of rapid advancement made in the field of agricultural and food processing industry, significant quantities of agricultural products are subjected to processing to make them suitable for consumption, increased storage stability and improved nutrition and sensory quality. This has resulted in the generation of huge amounts of waste products causing environmental pollution. Pollution has not only scientific aspects but also sociological, economic, affecting adversely on human beings and its environment. Therefore, currently increased attention has been given for utilization of by-products of different food processing industry. Since many agricultural wastes are good source of nutrients and phytochemicals, and are rich in fermentable substrates, isolation of value added products from agro-waste directly or using fermentation methods are widely used. In the present research work, it is proposed to isolate valuable compounds from mango peel and black gram mill waste, two agricultural waste products generated in large quantity in India.

Mango (*Mangifera indica L.)* is one of the important tropical fruits and India is the world's largest producer of mango fruits. Mango products such as canned slices, puree, nectar, pickles and chutney experienced worldwide popularity and also gained the increasing importance in U.S. and European market. Major by-products of mango processing industry are peel and seeds amounting 15-20% and 10-30% of total fruit weight, respectively. As peel is not currently being utilized for any commercial purpose, it is discarded and becoming a source of pollution. While a number of investigations have been conducted on the composition and possible utilization of mango seed kernel, studies on peels are limited. Earlier, methods have been developed for the

isolation of pectin from mango peel. Recently, it has been reported that mango peel contains polyphenols and dietary fibers. Therefore, one of the objectives in the present study is to isolate and characterize valuable compounds from mango peel.

Black gram (*Phaseolus mungo L.*) is a protein rich legume widely consumed in India. Black gram is milled into dhal and then used in various Indian traditional food preparations. During milling of black gram, about 25% is by-product. This by-product was separated into four fractions namely, husk, germ, plumule and aleurone layer rich husk fraction using air classification. Our preliminary studies indicated that aleurone layer rich husk fraction contained significant amount of peroxidase. Peroxidase catalyzes the oxidation of a variety of compounds like polyphenols, aromatic amines and their derivatives. Because of these, it has a variety of applications in food processing, pharmaceutical and chemical industry, analytical biochemistry, and water purification. Mill waste products of black gram could be used as a source of peroxidase. Therefore, it is proposed to isolate and characterize peroxidase from black gram mill waste.

The major objectives of this study are as follows:

1) To isolate and characterize value added compounds from mango peel and evaluate antioxidant properties and to develop mango peel enriched wheat based food products.
2) To isolate and characterize peroxidase from black gram mill waste and to study the effect of exogenously added peroxidase on wheat proteins.

An outline of these studies forms the subject matter of this thesis and is presented systematically, chapter wise.

CHAPTER I: GENERAL INTRODUCTION AND SCOPE OF THE PRESENT INVESTIGATION

This chapter covers a brief overview of the importance food processing, various food processing by-products generated and their potential application for isolation of the valuable compounds. A review on food processing by-products, problems caused by by-products and different methods of utilization of by-products are presented. Different valuable compounds such as phenolics, carotenoids, dietary fibers and vitamins are briefly described. Isolation of these valuable compounds from by-products of fruit, vegetable and cereal and legume processing industry has been briefly presented. Finally, relevance and objective of the present study has been presented.

CHAPTER II: ISOLATION AND CHARACTERIZATION OF VALUABLE COMPOUNDS FROM MANGO PEEL

This chapter begins with a brief introduction on the current knowledge on mango. The topics covered include mango fruit structure, nutritional and nutraceutical properties of mango and the by-products from mango processing industry. Literature survey on the bioactive compounds from mango processing by-products such as mango peel, mango kernel and mango sap has been given. The results in this chapter are presented and discussed under three sections.

SECTION A: Isolation and characterization of valuable compounds from mango peel

Raspuri and Badami mango varieties in raw and ripe stages were used for the present study. The proximate composition of the peels of these mango fruits were found to be in the following range: moisture content, 66-75%; total protein, 1.45–2.05%; carbohydrate, 11.63-24.60%; ash, 1.16–3.00%; fat, 2.16-2.66% and crude fiber, 3.28-7.40%. The content of total phenolics, anthocyanins, carotenoids, vitamin E and vitamin C in the peels of ripe and raw mangoes were determined, and they were found to be in the following range: total phenolic content, 55-110 mg/g peel; carotenoids, 365-3,945 μg/g peel; vitamin E, 205-509 μg/g peel and vitamin C, 188-392 μg/g peel. The phenolic acids were identified after acid hydrolysis by HPLC using authentic standards. They were gallic acid, protocatechuic acid, gentisic acid, and syringic acid. ESI-LCMS analysis of total polyphenols indicated that some of these phenolic acids were present in glycoside or ester forms and they were tentatively identified. Some of the phenolic compounds identified in Badami mango peel extracts were iriflophenone hexoside, gallic acid, maclurin hexoside, maclurin-tri-O-galloyl hexoside, syringic acid hexoside, mangiferin pentoside, ellagic acid, gentisyl protocatechuic acid, quercetin, and hepta-O-galloyl hexose. The phenolic compounds identified in Raspuri mango peel extracts were gallic acid, maclurin hexoside, protocatechuic acid hexoside, galloyl hexose and gallic acid hexoside.

The carotenoids identified in the mango peel by HPLC were violoxanthin, lutein and β-carotene. The total dietary fiber (TDF) content in dry peel varied from 45 to 78%. In both Raspuri and Badami, soluble and

insoluble fiber contents were higher in ripe peels compared to raw peels. Among these, Badami ripe showed highest TDF content and Raspuri raw showed the lowest. The mango peel was found to contain oxidative and hydrolytic enzymes such as polyphenol oxidase (36-108 U/g), peroxidase (213-275 U/g), xylanase (4.3-9.3 U/g), amylase (0.9-2.8 U/g) and protease (4,573-11,173 U/g).

A two-step method was developed to isolate bioactive compounds viz., polyphenols, carotenoids, vitamin E, enzymes and dietary fiber from mango peel simultaneously using same raw material. The process involves homogenization of the mango peel in a buffer, extraction of homogenate with acetone, filtration, drying of the resulting powder (acetone powder), extraction of powder with buffer, separation of extract into supernatant and residue by centrifugation. Acetone extract contained maximum amount of polyphenols and it also had significant amount of anthocyanins and carotenoids. The acetone peel powder, extracted with buffer, was analyzed for certain hydrolytic and oxidative enzymes and the residue was analyzed for dietary fiber. The enzymes determined in the buffer extract were polyphenol oxidase (37-62 U/g), peroxidase (56-116 U/g), xylanase (1.20-1.86 U/g), amylase (0.91-1.91 U/g) and protease (1,540-6,030 U/g). The total dietary fiber content in the residue was found to be in the range of 40.6-72.5%. The soluble dietary fiber content of the residue was in the range of 12.8 –23.0% and insoluble dietary fiber content varied from 27.8 to 49.5%. The sugar composition in the soluble and insoluble dietary fiber was determined by GLC. Both in soluble and insoluble dietary fibers, galactose, glucose and arabinose were the major neutral sugars The bound phenolic content in mango peel

dietary fiber was found to be in the range of 8.12 to 29.52 mg/g. Gallic acid, protocatechuic acid, syringic acid and ferulic acid were the phenolic acids identified in the bound phenolic fractions.

SECTION B: Evaluation of antioxidant activity of mango peel acetone extract

The antioxidant activity of the acetone extracts of mango peel was determined by different *in vitro* methods such as reducing power method, free radical scavenging activity using stable free radical DPPH, inhibition of lipid peroxidation in microsomes and inhibition of soybean lipoxygenase enzyme and also rat erythrocytes as a cellular model. The reducing power of Badami peel extracts was more compared to Raspuri peel extracts as well as standard antioxidant BHA. In free radical scavenging activity, it was found that acetone extracts of Raspuri raw and ripe mango peels showed more scavenging activity than that of BHA and Badami mango peel extracts. In inhibition of lipid peroxidation assay on liver microsomes, ripe peel extracts showed higher inhibition on lipid peroxidation than raw peel extracts and these peel extracts showed higher IC_{50} values compared to BHA. Raw mango peel extracts inhibited lipoxygenase activity more compared to that of ripe peel extracts. The broad range of antioxidant activity of peel extracts suggests that the synergistic actions of bioactive compounds are responsible for antioxidant activity.

In antioxidant activity studies on erythrocytes as cellular model, it was found that mango peel extracts effectively protected erythrocytes from oxidative damage induced by H_2O_2 by preventing the membrane lipid peroxidation, membrane protein damage thus preventing hemolysis and

structural damage of the erythrocytes. The morphological changes induced by H_2O_2 were greatly prevented when the cells were treated with mango peel extracts and standard antioxidant BHA by scanning electron microscope studies. The peel extracts showed 50% hemolysis inhibition (IC_{50}) at concentrations ranging from 11.5 to 20.9 μg GAE of mango peel extract. The IC_{50} value for lipid peroxidation inhibition on erythrocyte ghost membrane was found to be in the range of 4.5 to 19.3 μg GAE. The alterations in the protein pattern of membrane proteins produced by oxidative stress were observed by SDS-PAGE. In the presence of H_2O_2, the high molecular weight protein bands were disappeared while low molecular weight protein content increased. The mango peel extract effectively protected the erythrocytes membrane from H_2O_2 induced membrane protein degradation.

SECTION C: Development of mango peel enriched biscuits and macaroni products

The studies clearly showed that mango peel is rich in polyphenols, carotenoids and dietary fibers. In order to obtain the maximum nutritional and nutraceutical attributes of mango peel, attempts were made to incorporate mango peel into wheat based products such as biscuits and macaroni. Mango peel was collected from mango pulp processing industry, made into powder and incorporated into biscuits and macaroni. The total dietary fiber content (TDF) in mango peel powder (MPP) was 51.15%, in which insoluble dietary fiber (IDF) constituted 32.11% and soluble dietary fiber (SDF) 19.04%. The total polyphenol content in the MPP was found to be 96.17 mg GAE/g peel powder. The carotenoid content in the MPP was 3,092 μg/g dry peel powder. The antioxidant property of mango peel powder was determined

using free radical scavenging method. The incorporation of MPP in wheat flour at 2.5%, 5%, 7.5% and 10% levels showed significant effect on the dough rheological properties.

Biscuits enriched with MPP showed higher TDF, polyphenol and carotenoid content than the control biscuits. MPP incorporated biscuits showed improved antioxidant activity. The physical, sensory and biochemical studies showed that flour incorporated up to 10% level of MPP yielded biscuits without effecting their over all quality. Incorporation of MPP increased the polyphenol, carotenoid and dietary fiber contents in macaroni and it also exhibited improved antioxidant activity. The cooking quality, sensory and biochemical studies showed that macaroni incorporated with MPP up to 5% level resulted in products with good acceptability.

CHAPTER III: ISOLTAION AND CHARECTERIZATION OF PEROXIDASE FROM BLACK GRAM HUSK

This chapter begins with a brief introduction on the nutritional importance of legumes especially, black gram and its milled products and by-products and their current utilization. A brief over view on the enzyme peroxidase which includes reaction mechanism, substrates and inhibitors, effect of pH and temperature, molecular mass, physiological role, role in food processing and many other applications of the enzyme are reviewed. The results in this chapter are presented and discussed under two sections.

SECTION A: Purification and characterization of peroxidase from black gram husk

By-product of black gram milling, was separated into husk, germ, plumule and aleurone layer rich husk fraction by air-classification. These fractions were analysed for different oxidative and hydrolytic enzymes such as polyphenol oxidase, peroxidase, protease, amylase and xylanase. Aleurone layer rich husk fraction was found to be a rich source of peroxidase (35,800 U/g). On peroxidase staining, whole black gram showed two isoforms, one major and one minor band, where as, black gram husk showed only the major band. Different extraction methods were used to extract the enzyme with maximum activity. The best extraction method for enzyme was grinding with acid washed sand in sodium phosphate buffer (0.05M, pH 7.5) containing 0.01% Tween-20. The enzyme was purified by two-step conventional chromatographic method using DEAE-Sephacel followed by Sephadex G-100. The purity of the enzyme was determined by native PAGE and SDS-PAGE, RP-HPLC and capillary electrophoresis. RZ value of purified enzyme was found to be 1.9. The apparent molecular mass of the purified enzyme was about 38 kDa, as determined by the gel filtration chromatography. On SDS-PAGE, the purified enzyme gave a single band with a molecular weight of 36 kDa and it was found to be a glycoproitein. The MALDI-TOF MS analysis of the purified enzyme showed the molecular weight as 35 kDa. The enzyme activity was found to be maximum at pH 5.5 and stable up to 70°C. The thermal stability of the peroxidase at 50°C was determined and the half-life of the enzyme at this temperature was about 5 h. *o*-Dianisidine was found to be a good substrate for enzyme. Km value for H_2O_2 and *o*-dianisidine were

21.7 mM and 1.67 mM, respectively. The activities of peroxidase from black gram husk were affected by the presence of different metal ions. Zn^{2+}, Li^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Fe^{3+} at 5 mM stimulated the activity of peroxidase. On the other hand, Mn^{2+}, Cd^{2+}, Al^{3+}, Na^{+}, K^{+} moderately inhibited the enzyme activity, while Cu^{2+} inhibited to the maximum. Sodium azide, hydrazine, DTT, thiourea, oxalic acid and citric acid were found to be strong inhibitors. Sodium azide and DTT were found to be non-competitive inhibitors with Ki of 2.0 mM and 2.1 mM, respectively.

SECTION B: Effect of peroxidase enzyme on wheat proteins and its rheological properties

In wheat based product preparation, formation of dough plays an important role in product quality. Cross-linking of proteins/polysaccharides has an important role in dough formation. Peroxidase is an enzyme known to catalyze the cross-linking of proteins. The effect of peroxidase enzyme on the rheological property of wheat flour was studied by Farinograph. The addition of purified black gram peroxidase enzyme on wheat flour showed a significant difference on the rheological properties such as water absorption, dough development time, and dough stability. After treatment with enzymes, the wheat proteins were fractionated into albumins/globulins, gliadins, glutenins and residue protein according to Osborn fractionation method. Significant decrease in extractability of glutenin and increase in residue protein content was observed. The –SH content in the glutenin protein fraction was decreased, while –SS- content in the gluteinin obtained from dough treated with peroxidase was increased. SDS-PAGE of glutenin extracted from peroxidase treated dough showed increase in the intensity of high molecular

weight band compared to control indicating that exogenously added peroxidase induced the formation of the protein-protein interactions in glutenin.

CHAPTER IV: SUMMARY AND CONCLUSION

The results of the thesis are summarized in this chapter with a general conclusion. The present study made an effort for better utilization of two abundantly available agro-wastes in India, namely, mango peel and black gram mill waste. Mango peels were shown to be rich in polyphenols, carotenoids, vitamin E and vitamin C and dietary fiber, which have nutraceutical properties. A method has been developed to isolate these compounds by acetone extraction, the residue was extracted for enzymes and the remaining portion contained dietary fiber. In order to obtain the maximum benefits of these nutraceuticals, mango peel powder was incorporated into food products such as biscuits and macaroni. From black gram aleurone rich husk fraction, peroxidase was isolated, purified and characterized. Peroxidase is a physiologically important enzyme having a number of applications. This purified enzyme was incorporated into wheat flour and studied rheological and protein-protein interactions. This is the first report on peroxidase from black gram.

After chapter IV a collective bibliography for the all chapters was presented.

CHAPTER I

GENERAL INTRODUCTION

1.1. FOOD AND FOOD PROCESSING

1.1.1. Brief history

Food is a more basic need of human than shelter and clothing. It provides components for the body growth, maintenance, repair and reproduction. Man's basic drive is for food to satisfy his hunger. Food is ultimately woven into the physical, economical, intellectual and social life of man. It is part of his culture and is filled with many different meanings and symbolizes for all individuals at various ages and stages of their maturity. There is a proverb that "we are what we eat" which shows the importance of food in our life. Our nutritional status, health, physical and mental status depend on the food we eat and how we eat it. Access to good quality food has been man's main endeavor from the earliest days of human existence.

Primitive men lived as hunters and gatherers. They collected their food from plants and animals. They depended upon fruits, nuts, roots and other plant foods, meat from animals and fishes. They roamed place to place depending on food availability. Gradually, they learned to cultivate and domesticate animals. They settled down, built shelters and raised plants and animals to provide food. Agriculture began in China followed by India, The Eastern Mediterranean area and Africa. The first crops to be grown were wheat and barely (Peterson & Johnson, 1978).

1.1.2. Importance of food processing

Agricultural produces such as cereals, pulses, fruits and vegetables and reared animals for meat, milk, egg etc are foods or food raw materials. After production and before consumption, foods are subjected to numerous

adverse physical, chemical, microbial or parasitic factors, which may cause their spoilage or cause diseases when consumed due to the presence of anti-nutritional or toxic compounds. A number of causes are responsible for food deterioration. These include microorganisms such as bacteria, yeast and mould, activities of enzymes present in the food, insects, parasites and rodents, temperature, moisture, oxygen, light. At any time, many forms of deterioration may take place depending upon the food and environmental conditions. To prevent these and prepare food for immediate or future use raw materials require processing, preservation and storage.

Benefits of food processing include toxin removal, increasing shelf life, improving flavor, raising marketing and distribution task and increasing food consistency. In addition, it increases continuous availability of seasonal foods, enables transportation of delicate perishable foods across long distances and makes many kinds of foods safe to eat by removing microorganisms. Modern food processing also improves the quality of life for allergies, diabetics and also adds extra nutrients.

Food processing is the set of methods and techniques used to transform raw materials into food for consumption by humans. The food processing industries often take harvested or slaughtered food components and all this to produce attractive and marketable food products. The common food processing techniques involve removal of unwanted outer layer such as peeling or skinning, milling, chopping or slicing, mincing and macerating, liquefaction, emulsification, cooking, mixing, addition of gas, proofing, spray drying etc (Potter, 1978).

Food processing mainly falls into two broad categories, primary and secondary processing. The primary processing of rice, wheat and pulses include mainly milling, polishing, cleaning etc. Secondary processing of cereals includes the production of ready to consume products such as bread, biscuits, cakes etc. Secondary processing of pulses includes the preparation of puffed pulses, papad and many break fast and bakery products. Fruit being largely eaten fresh, secondary processing of fruits needs to be considered. The fruits are processed into various products such as juices and concentrates, canned fruit, dehydrated fruit, jam, jellies etc.

1.1.3. Food processing by-products

Rapid advancement in the field of agriculture tremendously increased the availability of agriculture produce. The net impact by the revolution in agriculture is the fast development of food processing industries all over the world. Food industrialization has generated a large quantity of food products, provided employment to large number of people and uplifted the economic status, at the same time; it generated waste in huge quantities causing environmental pollution.

The food wastes can be classified into different categories such as crop waste and residues; fruits and vegetables by-products; sugar, starch and confectionary industry by-products; grain, legumes by-products; distilleries and breweries by-products; milk and dairy by-products; meat, poultry, fish products and egg industry by-products.

The major source of the waste in the fruit and vegetable processing industry are illustrated in the **Table 1.1** and **Figure 1.1**. During apple processing, pomace is the major by-product, which consists of crushed flesh,

stalks, peels, seeds etc (Rahmat et al, 1995). The type of waste from mango processing industry is mainly peel (15-20%), coarse fibrous pulpy waste (5-10%) and kernel (15-20%) (Beerh et al, 1976; Larrauri et al, 1996). The waste from starch industry like tapioca, produce waste in the form of tapioca rind or peeling, spent pulp. Rice husk is a by-product during rice milling. The major wastes from sugar cane industry are bagasse, molasses and sugar cane press mud. Wine making industry produces grape pomace as a by-product consists of skin, seed and stem in an estimated amount of 13% by weight of grapes (Torres et al, 2002).

Table 1.1. Fruit and vegetable processing by-products available in India

Fruit / vegetables	Nature of waste	Approx. waste (%).
Mango	Peel, stones,	45
Banana	Peel	35
Citrus	Peel, rag, seed	50
Pineapple	Skin, core	33
Grape	Stem, skin, seed	20
Guava	Peel, core, seed	10
Pea	Shell	40
Tomato	Skin, core, seed	20
Potato	Peel	15
Onion	Outer leaves	10
Apple	Peel, pomace, seed	25

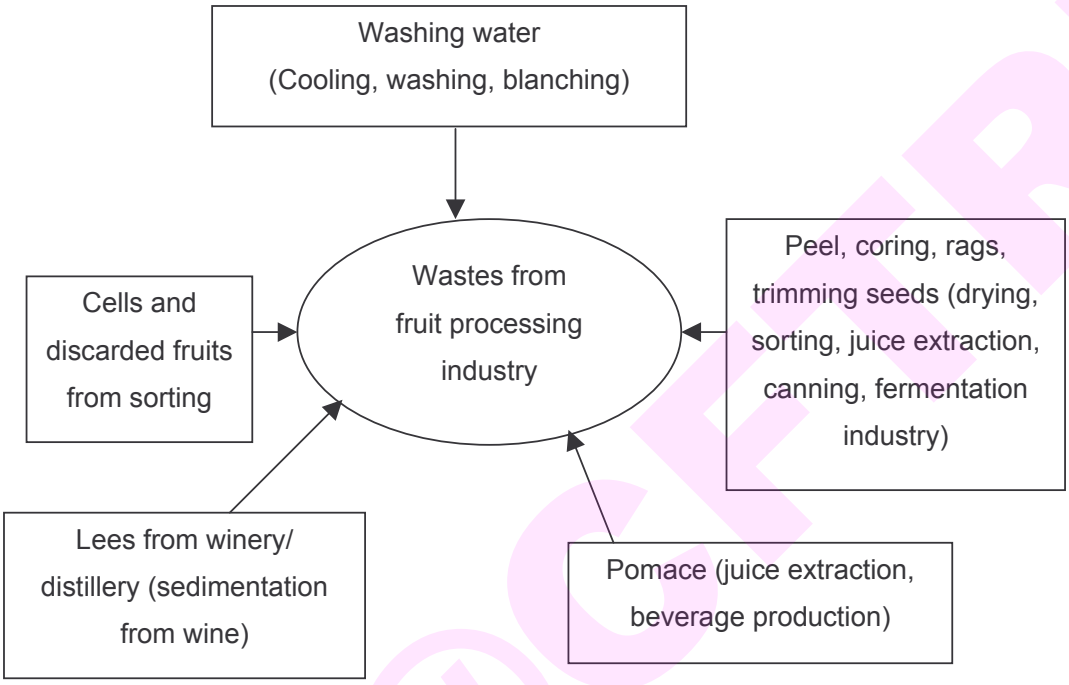


Figure 1.1. Major sources of pollution in fruit and vegetable processing industries (Source: Joshi et al, 1995).

1.1.4. Problems caused by food processing industry by-products

By-products from agriculture and food processing can become one of the most serious sources of pollution (Di Blasi et al, 1997). Because of cheap energy and raw materials following World War II, the role of utilizing by-products was not well studied till recently. An increase in the number of industrial plants in turn increased the volume of by-products, which led to consider about the treatment and environmental friendly disposal of the processing by-products.

Agricultural technology earlier only started to use the by-products as fodder and for oil production from food processing industry (Bulla, 1994). The disposing of waste can be difficult because of the following reasons:

1. **Biological stability and potential growth of pathogens**: Many types of food processing waste contain a large number of microorganisms and will be altered quickly through microbiological activity. If the waste by-products are not processed properly, it will lead to hygienically unacceptable conditions through maggots, microorganisms and moulds. The breakdown of protein is characterized by evolution of strong odors.
2. **Water content:** The water content of the fruit and vegetable processing by-products lies between 70-95% by mass. High water content increases the transport costs of the waste. The mechanical removal of water can lead further problems with water disposal, due to high level of organic material in the water.
3. **Rapid auto-oxidation**: The waste rich in high fat content is susceptible to oxidation, which leads to the releases of foul smelling fatty acids.
4. **Changes due to enzymatic activity**: In many types of by-products from fruit and vegetable processing industry, enzymes are still active which accelerates or intensify the reaction involved in spoilage (Westerndorf & Wohlt, 2002).

1. 2. UTILIZATION OF BY-PRODUCTS

Food industry waste and by-products are substances that originated during processing and can be further utilized in other ways. Food industry wastes and by-products are geographically scattered of large volume and low nutritional value. Consequently, their collection, transportation and processing cost of the by-products can exceed the selling price. If we could produce valuable products from food industry by-products through new scientific and technological methods, environmentally polluting by-products could be converted into products with a higher economic value than the main products.

The different ways of utilization of by-products from food processing industry can be classified mainly into five categories.

A] Directly used as food/feed ingredients

B] Use as carbon source for growing useful microorganisms

C] Use as fertilizer by composting

D] Use for energy generation directly/biogas production

E] Isolation of value added products

1.2.1. Food/feed ingredients

Many by-products of food processing industry can be fed to animals directly. Generally, by-products to be used as feedstuff should be economical, rich in nutrients and free of toxins or other substances that may be unhealthy to animals.

An animal feed from apple pomace has been produced and evaluated (Joshi & Sandhu, 1996). It was reported that *Kloceckera apiculata* and *Candida utilis* could transform apple pomace into an improved stock feed by solid-state fermentation (Rahmat et al, 1995). The carrot pomace has been

incorporated into bread, cakes and dressing (Ohsawa et al, 1994; Ohsawa et al, 1995). A protein rich fibrous food called germinated barley has been made from brewers yeast grain. The effluent from biogas production from mango processing waste has been utilized for the production of fresh water fishes like carp, rohu etc (Mahadevaswamy & Venkataraman, 1990). Milling by-products such as cereal brans and oil cakes obtained after oil extraction are commonly used as animal feed. The utilization of food processing by-products are comparatively very less when compared to the quantity of by-products generated.

1.2.2. Carbon source for growing microorganisms for production of valuable chemicals and enzymes

By-products from the food processing industry as a whole can be used in a number of ways especially for biomass production. Microorganisms are grown on food processing by-products. By-products for this biomass production in view of their use in foods and feeds, in the production of enzymes, single cell protein, amino acids, lipids, carbohydrates and organic acids. This aspect has been done by fermentative utilization of food processing waste as shown in **Table 1.2.**

Wastes from orange, acne, grape, pineapple and apple processing Industries have been utilized for single cell protein production (Krishnamurthy, 1980; Nicoloni et al, 1987; Nigam, 2000). Various fruit and vegetable wastes from the tomato, grape, apple, cabbage, carrot, beetroot and watermelon used as the substrate for the lysine production by *Brevibacetrium spp* (Trifonova et al, 1993). Protein enrichment has been obtained by growing

Aspergillus niger on mango peel, orange peel, green immature banana and carrot wastes in SSF (Davy et al, 1981; Garg et al, 2000).

Table 1.2. Microbial utilization of food processing by-products for production of various products

Products	Wastes
Ethanol	Citrus industry waste, apple pomace, peach waste, cashew apple pomace, pineapple waste, pear cuttings
Biogas	Waste from fruit and vegetable industry, fermentation industry
Single cell protein	Apple pomace, peach waste, cashew apple pomace, citrus waste
Cider, beer and vinegar	Apple pomace, pine apple waste
Pectin, fibers	Citrus waste, apple pomace
Citric acid	Apple pomace, brewery waste
Bakers yeast, Industrial yeast	Waste from wine, beer and distillery
Color	Apple pomace and grape pomace
Flavors/ xanthan gum	Fruits and vegetable waste, citrus waste
Animal feed	Apple pomace, peach waste, potato waste, olive processing waste

(Source: Joshi et al, 1995)

Agriculture and food processing waste can also be used for the production of various acids having a potential use in the food and chemical industries. Vinegar can be prepared from citrus peel or molasses, by-products

of mango processing (Beerh et al, 1976), wastes from potato starch industry (Kumar et al, 1991) and wastes from pineapple juice (Richardson, 1967). The pine apple peel, apple pomace, kiwi fruit peel, wheat barn and rice bran are utilized as substrates for the production of citric acid by solid state fermentation using *Aspergillus spp* (Hang & Woodams, 1987). The manufacture of tartaric acid from grape pomace (Pecker, 1994) and oxalic acid from apple pomace (Kennedy, 1994) has been reported. Gluconic acid has been produced by fermentation of agricultural by-products like grape pomace using *Aspergillus spp* and G*luconobacter oxidans* (Buzzini et al, 1993). Production of lactic acid and butyric acid has been reported from sugar cane molasses (Kanwar, 1995). Citrus peel as a sole source of carbon is used for the production of pyruvic acid by yeast (Moriguchi, 1982).

Many industrially important enzymes are produced from food processing waste by using microorganisms. Wheat bran has been studied extensively for pectinase production by solid-state fermentation (Ghildyal et al, 1981). Lipase was produced from copra waste (coconut oil cake) and rice bran using *Candida rugosa* (Rao, 1993; Benzamin & Pandey, 1996). Production of xylanase has been reported on dried apple pomace (Bhalla & Joshi, 1993). Amylase has been produced from bean waste, banana waste, wheat and maize bran using microorganisms (Hang & Woodams, 1987). Cellulase has been produced from cabbage waste, apple pomace, wheat bran, sugar beet, rice straw, bagassae etc using microorganisms such as *Trichoderma harzianum, Aspergillus ustus, Tricho spp, Bortrytes spp* etc. Recently, kiwi fruit waste has been used as substrate for production of laccase using white rot fungi (Rosales et al, 2005). Fruit processing by-

products such as apple pomace, cranberry pomace, and strawberry pomace were used as substrates for production of polygalactouronase by *Lentinus edodes* through solid-state fermentation (Zheng & Shettty, 2000)

Ethanol can be produced from food processing by-products, which are rich in sugars/starches by the microbial technology, may evolve as an alternative to our limited and renewable source of energy. Ethanol has been produced from food wastes such as citrus, molasses, apple pomace, orange waste, rice husk and banana waste (Govinda Rao 1980; Badger & Broder, 1989; Shankaranand & Lonsane, 1994). Recently, there has been a great interest in use of microbial technology for the production of color, gums and polysaccharides from food processing by-products such as *Rhodotorula, Cryptococcus, Phatlia, Rhodozyma, Monosaus purpureus, Bacillus spp, Xanthomonas campesteris* (Bilanovic et al, 1984).

1.2.3. Food by-products as fertilizer

Food processing by-products can be beneficially used as soil conditioner or fertilizer. The by-product characteristics of interest include the moisture content, BOD, calcium carbonate, O: N ratio, fat and oils, odors, pathogens, pH, soluble salt and toxicity. Composting is a natural aerobic biochemical process in which thermophillic microorganisms transform organic material into a stable soil like product (Rynk, 1992). Composting can reduce the volume of organic by-products up to 40% (Steueville, 1992). It is a managed biological conversion of waste material, under controlled conditions into hygienic humus rich, relatively biostable product that condition soils and nourishes plants. Organic wastes from fruit and vegetable processing industry used for composting include peelings, pulping, outer skins, pomace, cores,

leaves, fruit twigs and sludge's from the processing and packaging of various products.

1.2.4. Use for energy production

In developing countries, production of biogas from waste is an appropriate solution. Biogas generation through anaerobic fermentation is considered to be a simple and economical system of waste treatment and a number of processes have been developed and commercially exploited. Cuzin and Labat (1992) studied methanogenic fermentation of cassava peel with mean methane content of 50%. Moisture of fruit and vegetables wastes subjected to anaerobic degeneration produced 0.12m^3 biogas/kg (Premaviswanath, 1992). Anaerobic digestion of mango peel resulted in biogas production of 0.33m^3/ kg with 53% methane content (Somayaji, 1992). The biomethanation of banana peel and pine apple processing waste suggested their potential and suitability for economically viable waste treatment by technologically anaerobic digester (Bardiya et al, 1996).

1.2.5. Isolation of value added products

In recent years, attempts have been made to convert food by-products into a variety of valuable products. The recycling of by-products and minimizing wastes are crucial aspects of this strategy. The recovery of highly value added compounds is of special interest.

There is an increasing consumer's appreciation of natural products as alternative to synthetic compounds in a variety of goods from food to personal care formulations. Consumers have increasing aware of the diet related health problem, therefore, demanding natural ingredients, which are expected to be safe and health promoting. By-products of plant food processing found

to be promising sources of biological active compounds, which may be used because of their favorable nutraceuticals properties. There are great variety of value added compounds in the by-products and wastes from biological origin. These products may be used as such or may be a starting material for the preparation of novel compounds. The characteristics of commercial interest include antioxidants, carbohydrates, dietary fibers, fat and oils, pigments, proteins and starches. The **Figure 1.2** summarizes the main kind of bioactive compounds, their sources and their applications (Torres et al, 2003).

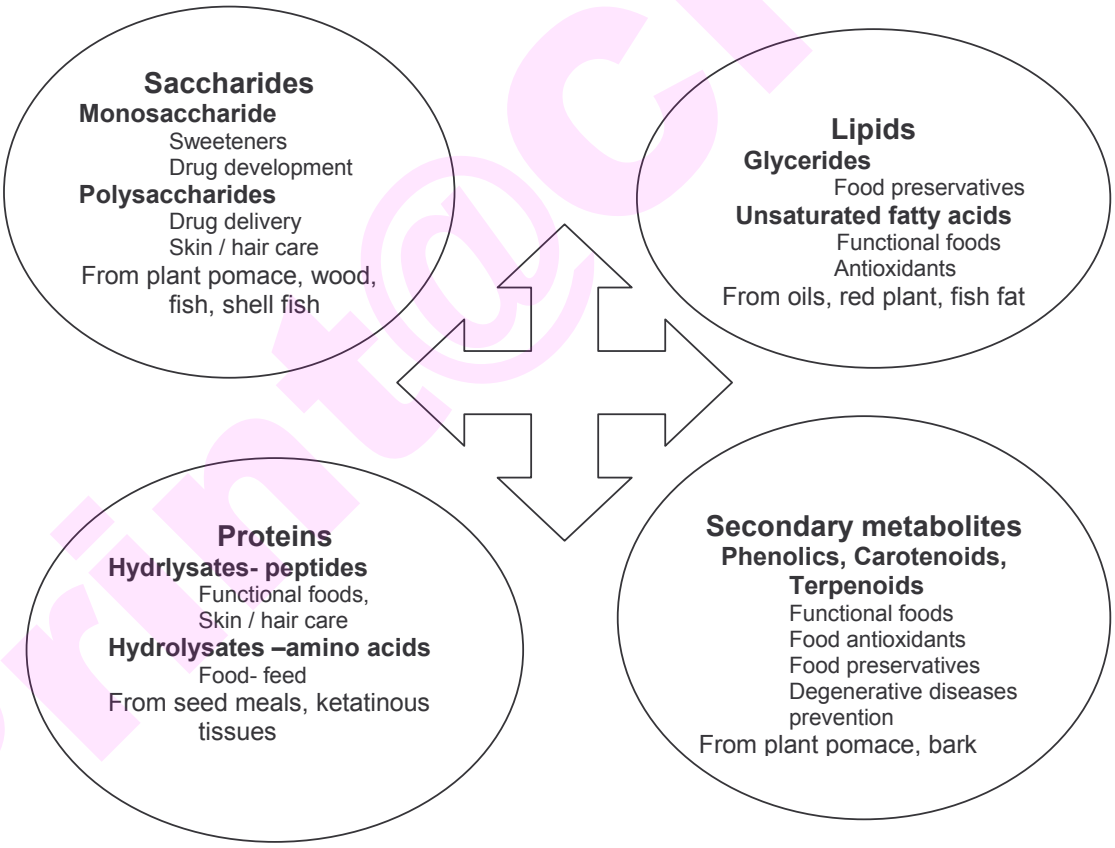


Figure 1.2. Major Bioactive compounds, their sources and applications

(Source: Torres et al, 2003)

Polysaccharides such as pectins from fruit by-products have different applications mostly related to their film properties and pharmaceutical properties (Macleod et al, 1999). Oligosaccharides from fruit processing wastes are used as prebiotic components of functional foods (Sriamornsak, 1998; Roberfriod, 1998) and monosaccharide as starting material for drug development (Rastall & Maitin, 2002). Seeds obtained from fruit beverage and fruit canning industries are rich in oils of high content in unsaturated fatty acids and glycerides of possible direct application in cosmetics and can also be used as starting material for the preparation of novel chemicals such as biocompatible food preservatives (Biermann et al, 2000). By-products from tomato processing industry contain polyunsaturated antioxidant carotenoid such as lycopene, which can use as nutraceuticals. Protein rich meals are by-products from the oil seed processing industry. These proteins and their hydrolysates may be used in animals and poultry feed and in human functional nutrition (Senkoylu, 1999; Baysal et al, 2000). A great variety of secondary metabolites such as phenolic compounds, carotenoids and other bioactive compounds such as dietary fiber, enzymes and vitamins with biological activities can be isolated from food processing by-products (**Table 1.3**). They have been given increasing significance as functionally active components of food and drink and have many nutraceutical properties (Diplock et al, 1998).

Table 1.3. Bioactive compounds from food processing industry by-products

By-product	Bioactive compounds	References
Fruits		
Apple peel	Flavonoids &anthocyanins	Wolfe & Liu (2003)
	Pectin & phenolics	Carle et al (2001)
Citrus peel	Phenolics, dietary fiber	Gorinstein et al (2001)
		Rivera et al (2004)
Banana peel	Phenolic compounds	Someya et al (2002)
Banana bract	Anthocyanins	Pazmino et al (2001)
Apple pomace	Flavonoids & anthocyanins	Sheiber et al (2003)
	Phenolic compounds	
Grape skin & seed	Phenolics	Torres & Bobet, 2001
	Anthocyanins	Mazza (1995)
	Dietary fiber	Larrauri & Saura-Calixto (1998)
Peach pomace	Pectin	Pagan & Ibarz (1999)
Mango seed	Phenolics & phospholipids	Puravankara et al (2000)
	Tannins & gallotannins	Arogba (2000)
Mango peel	Phenolics & dietary fiber	Larrauri et al (1997)
Pineapple waste	Antioxidant principle	Wrolstad & Ling (2001)
Vegetable		
Carrot pomace	Carotenoids	Stoll et al (2001)
Onion waste	Quercetin	Waldron (2001)
Red beet waste	Betalins	Kujala et al (2000)
Potato peel	Phenolic acids	Rodriguez et al (1994)
Tomato seed & peel	Lycopene	Baysal et al (2000)
Others		
Soybean hulls	Peroxidase	Sessa (2003)
Almond hulls	Phenolic acids	Takeoka &Dao (2002)
Peanut hulls	Phenolic compounds	Yen & Duh (1995)
Wheat germ	Vitamin E	Ge et al (2002)
Wheat bran/rice bran	Dietary fiber	Goncalvez et al (1998)

1.3. VALUABLE COMPOUNDS FROM FRUIT, VEGETABLE, CEREAL AND LEGUME PROCESSING BY-PRODUCTS

As the focus of the present investigation was isolation of valuable compounds from fruit, vegetable, cereal and legume processing by-products, few of these compounds and the major by-products are briefly reviewed and presented below.

1.3.1. Phenolic compounds

It is known that polyphenols are a large family of natural compounds. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways in plants.

Phenolic compounds have a major role in growth and reproduction, providing protection against pathogens and predators besides contributing towards the color and sensory characteristics of fruits and vegetables (Alasalvar et al, 2001). Phenolic compounds exhibit a wide range of physiological properties such as antiallergenic, antiinflammatory, antimicrobial, antioxidant, antithrombotic, cardio protective and vasodilatory effects (Middleton et al, 2000; Manach et al, 2004). The several beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim et al, 2002).

The antioxidant properties of phenolics are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans et al, 1996). They also act as chelator of metal ions, preventing metal catalyzed formation of free radical species (Salah et al, 1995). Phenolic antioxidants interfere with the oxidation of lipid

and other molecules by rapid donation of hydrogen atom to radicals as shown in the following equation.

$$ROO^{*} + PPH \longrightarrow ROOH + PP^{*}$$

$$RO^{*} + PPH \longrightarrow ROH + PP^{*}$$

The phenoxy radical intermediates are relatively stable, therefore, a new chain reaction not possible easily. The phenoxy radical intermediate also act as terminator of the propagation route by reacting with other free radical (Shahidi & Wanasundara, 1992).

$$ROO^{*} + PP^{*} \longrightarrow ROOPP$$

$$RO^{*} + PP^{*} \longrightarrow ROPP$$

Structurally phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substitutents, and range from simple phenolic molecules to highly polymerized compounds (Bravo, 1998). Most naturally occurring phenolic compounds are present as conjugates with mono and polysaccharides, linked to one or more of the phenolic groups, and also may occur as functional derivatives such as esters and methyl esters (Shahidi & Naczk, 1995; Harborne et al, 1999;). Phenolic compounds can be classified into several classes as shown in **Table 1.4** (Harborne et al, 1999).

Table 1.4. Classes of phenolic compounds in plants

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	$C_6 – C_1$
Acetophenones, henylacetic acids	$C_6 – C_2$
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	$C_6 – C_3$
Napthoquinones	$C_6 – C_4$
Xanthones	$C_6 – C_1 - C_6$
Stilbenes, anthroquinones	$C_6 – C_2 - C_6$
Flavonoids, isoflavanoids	$C_6 – C_3 - C_6$
Lignans, neolignanas	$(C_6 – C_3)_2$
Bioflavoniods	$(C_6 – C_3 - C_6)_2$
Lignine	$(C_6 – C_3)_n$
Condensed tannins	$(C_6 – C_3 - C_6)_n$

1.3.1.1. Phenolic acids

The name phenolic acids describes phenols that posses one carboxylic acid functionality. Phenolic acids contain two distinguishing constitutive carbon frame works: the hydroxycinnamic and hydroxybenzoic structures. The numbers and position of the hydroxyl groups on the aromatic ring creates the variety. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the $C_6–C_1$ structure. Hydroxycinnamic acids, on the other hand are aromatic compounds with a three carbon side chain ($C_6–C_3$) with caffeic, ferulic, p-caumaric and sinapic acids being the most common (Bravo, 1998). Phenolic acids in plants

have been connected with diverse functions such as nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components and allelopathy (Wu et al, 2000). Caffeic acid known to selectively block the biosynthesis of leukotrienes, components involved in immuno-regulation diseases, asthma and allergic reactions (Koshihara et al, 1984). Caffeic acid and some of its esters might posses antitumour activity against colon carcinogenesis (Koshihara et al, 1984) and act as a selective inhibitors of human immunodeficiency virus type I integrase (King et al, 1999). Chlorogenic acid has been found to inhibit lipid peroxidation in rat liver induced by carbon tetrachloride, a potent liver carcinogen (Huang et al, 1997). Caffeic and ferulic acid found to detoxify carcinogen metabolites of polycyclic aromatic hydrocarbons (Huang et al, 1996).

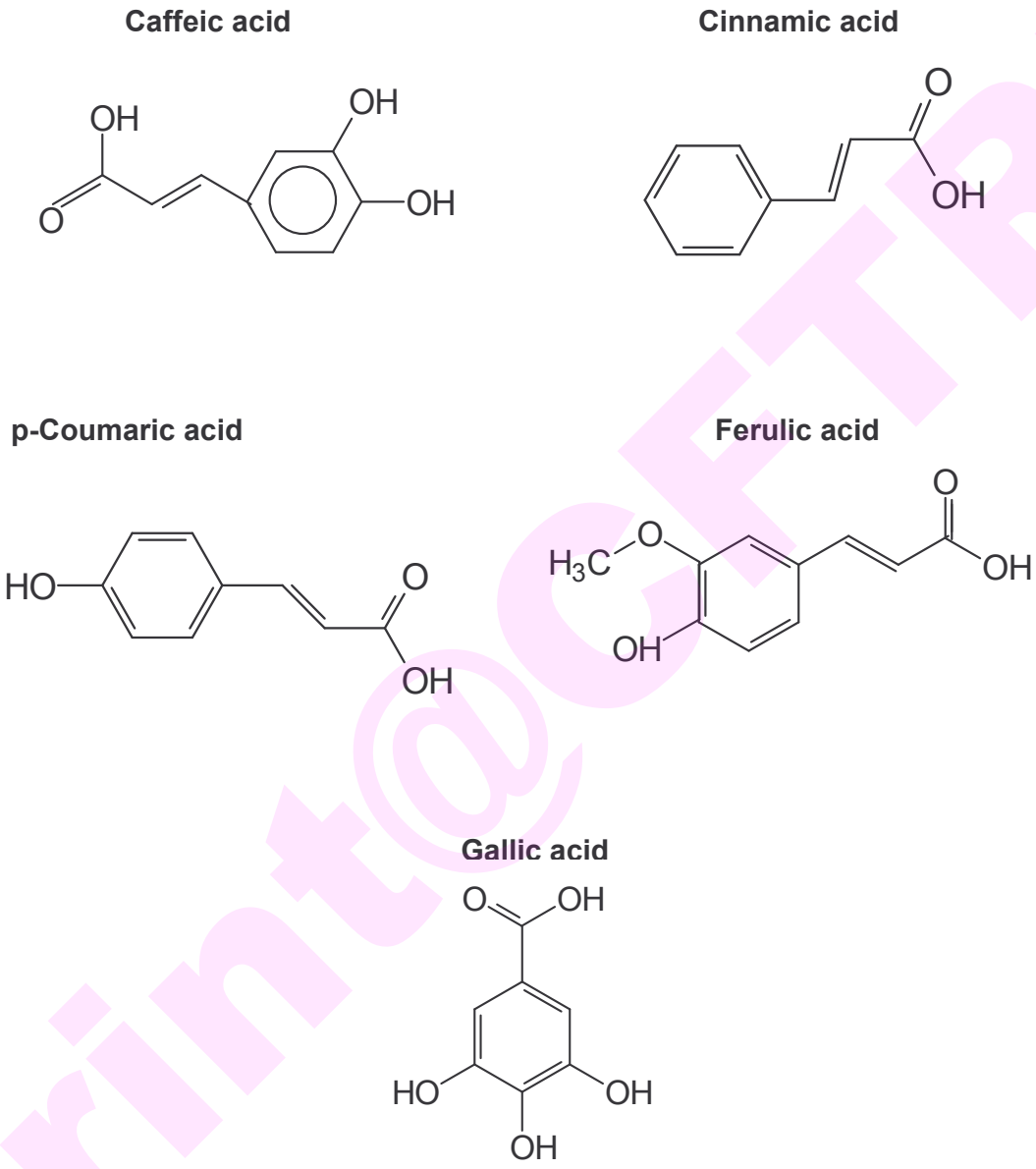


Figure 1.3. Structures of phenolic acids

1.3.1.2. Flavonoids

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne et al, 1999). Flavonoids are low molecular weight compounds

consisting of fifteen carbon atoms, arranged in a C6–C3-C6 configuration. Essentially the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring. Variations in substitution patterns to ring C result in the major flavonoids classes, i.e., flavonols, flavones, flavanones, flavanols (catechins), isoflavones, flavanonols and anthocyanidins (Hallman & Katan, 1997). Flavones and flavonols are the most widely occurring and structurally diverse compounds (Harborne et al, 1999). Substitutions to rings A and B give rise to different compounds of flavonoids and theses substitution may include oxygentaion, alkylation, glycosylation, acylation and sulfation (Hallman & Katan, 1997; Pietta, 2000). The epidemiological studies point out the possible role of flavonoids in preventing cardiovascular diseases and cancer (Chu et al, 2002). Flavonoids are the wonderful molecules with vivid functional properties like anticancer, antiatherosclerotic, antimutagenic, antiviral, antineoplastic, antiallergic, antithrombotic and vasodilatory activity (Hallman & Katan, 1997).

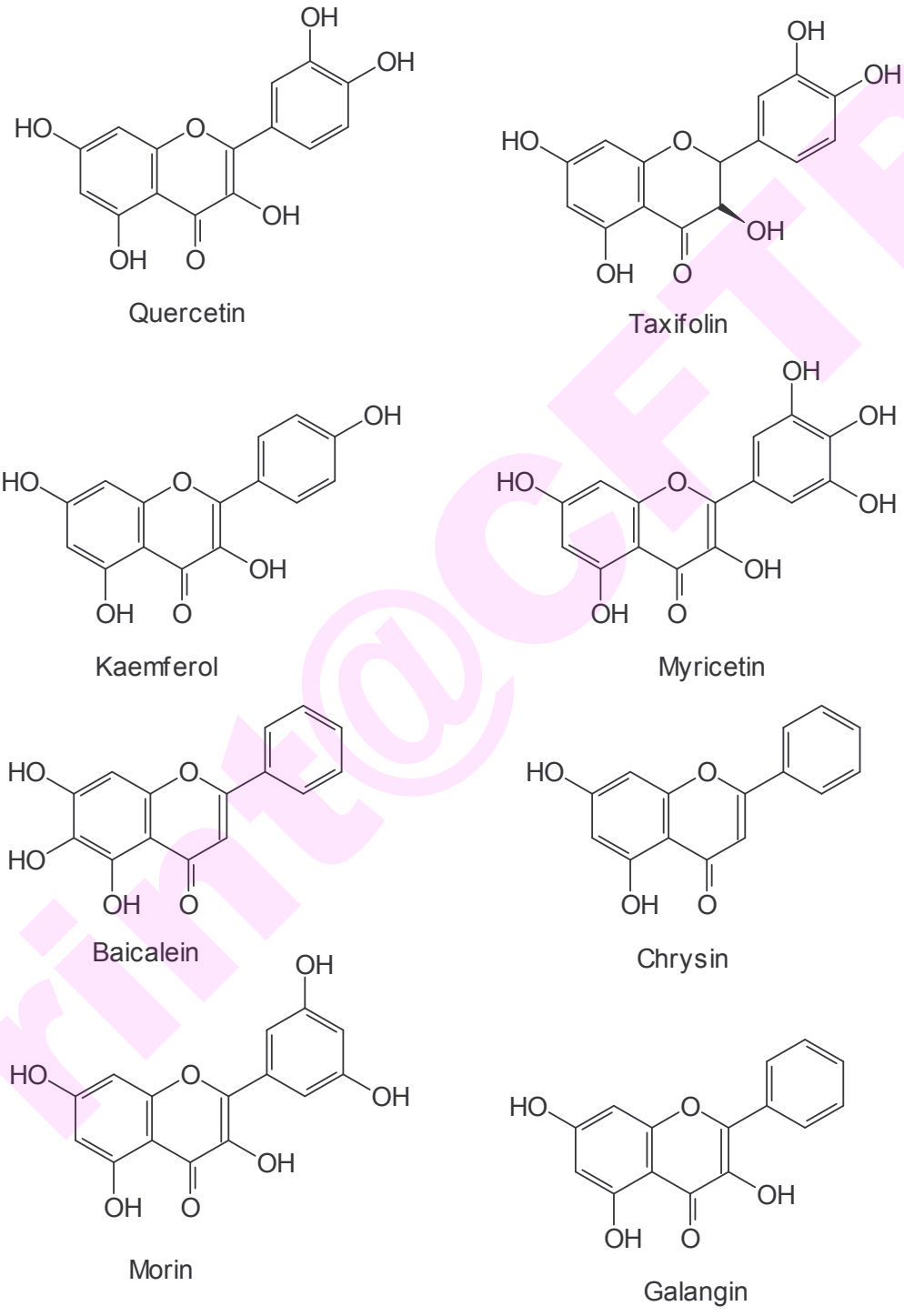
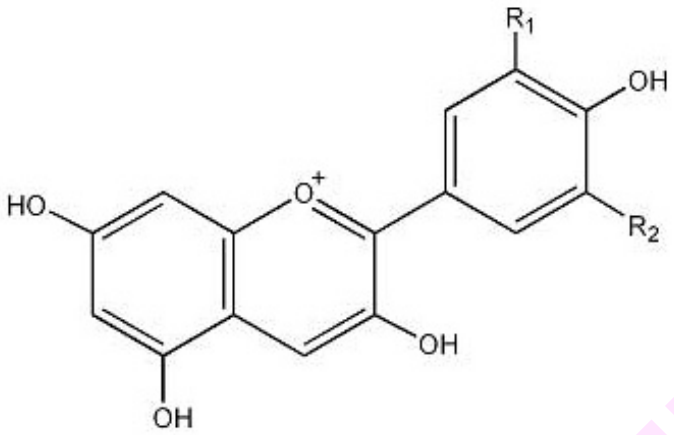


Figure 1.4. Structures of flavonoids

1.3.1.3. Anthocyanins

Anthocyanins are the most important group of water-soluble pigments in the plant kingdom and are responsible for the most red, blue and purple colors of fruits, vegetables, flowers and other plant tissues (Harborne, 1999). Anthocyanins are coming under phenolic compounds in flavonoids groups (**Figure 1.5**). They are generally found in the form of glycosides. The aglycones are rarely found in fresh plants. About 250 different anthocyanins have been isolated from plants (Strack & Way, 1994).

Anthocyanins help to attract animals, leading to seed dispersal and pollination (Strack & Wray, 1994). It has a role in protecting plants against ultraviolet induced damage (Bohm et al, 1998). Anthocyanins are regarded as important component in human nutrition (Ross & Kasum, 2002), which is supported by numerous studies that reported a high positive correlation of fruit or vegetable pigment content and antioxidant capacities (Halvorsen et al, 2002; Meyer et al, 2002). Red fruit extracts that are rich in anthocyanins are used in folk medicine and have some positive therapeutic effects as anti-inflammatory agents and in the treatment of various ailments, including microcirculation diseases resulting from capillary fragility and the prevention of cholesterol induced atherosclerosis (Wang et al, 1997). The anthocyanin molecule also protects the gastrointestinal mucus against oxidative damage thus by delaying the onset of stomach, colon and rectal cancer (Halliwell et al, 2001). As a colorant anthocyanins are found to be potential replacement for banned synthetic food dyes.



R_1 = H;	R_2 = H:	Pelagonidin
R_1 = OH;	R_2 = H:	Cyanidin
R_1 = OH;	R_2 = OH:	Delphinidin
R_1 = OCH_3;	R_2 = OH:	Petunidin
R_1 = OCH_3;	R_2 = OCH_3:	Malvidin

Figure 1.5. Structure of anthocyanin

1.3.1.4. Tannins

Tannins are the relatively high molecular weight compounds, which constitute the third important group of phenolics, may be subdivided into hydrolysable and condensed tannins (Porter, 1989). The hydrolysable tannins are esters of gallic acid (gallo- and ellagi-tannins), while the condensed tannins are polymers of polyhydroxyflavan-3-ol monomers (Porter, 1989). A third subdivision, the phlorotannins consisting entirely of phloroglucinol, has been isolated from several genera of brown algae (Porter, 1989). Tannins are polyphenols, which bind with protein, basic compounds such as alkaloids or heavy metallic ions in a solution and making them insoluble and inducing precipitation.

1.3.1.5. Quercetin

Quercetin (3,3,4',5,7-pentahydroxylflavone) is one of the most abundant flavonoids present in fruits and vegetable, tea, coffee, legumes, roots and cereal grains. It occurs mainly as aglycones and glycosides, in which one or more sugar group is bound to phenolic groups by glycosidic bond. Onion ranked first in quercetin content in a survey of twenty-eight vegetables and nine fruits (Hertog et al, 1992). It is mainly found in the less active glycosidic forms and converted to active form by bacterial hydrolysis of these glycosides in the mouth and gut. Quercetin is found to be anticarcinogenic and protective against cardiovascular diseases (Huang et al, 1997).

1.3.1.6. Catechins

Catechins found in many fruits and vegetables. However they are abundant in green and fermented teas. Catechin constitutes 35-52% of the gree tea polyphenols. Catechin is considered as the principle antimutagenic and anti-carcinogenic compound in green tea leaves (Stoner & Mukhtar, 2005). Catechins, epicatechin, epicatechin gallate and epigallocatechin were the major constituents of grape peel tannins (Souquet et al, 1996).

1.3.1.7. Resveratrol

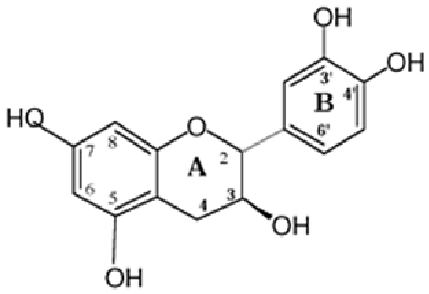
Resveratrol is a polyphenol (3,5,4'-trihydroxystilbene), thought to be a phytoalexin, one group of compound produced during environmental stress or pathogenic attack (Jang et al, 1997). It is mainly found in the skin of grapes and also in peanut. It was found to be an antioxidant and antimutagen (Jang, 1997). There is evidence to suggest that resveratrol inhibits LDL oxidative susceptibility in vitro, platelet aggregation as well as eicosanoid synthesis

(Pace-Asciak et al, 1995). It also has been shown to inhibit the expression of the tissue factor gene (Pendurthi et al, 1999). Resveratrol has been shown to inhibit ribonuleotide reductase and certain other cellular events associated with initiation, promotion and progression of carcinogenesis. It also found to be as phytoestrogen (Lu & Serrero, 1999). Resveratrol act as an antioxidant, promoter of nitric oxide production, increases high-density lipoprotein cholesterol and thereby serve as a cardio protective agent (Olas & Wachowicz, 2006). It is suggested that resveratrol can function as a cancer chemopreventive agent (Signorelli & Ghidoni, 2005). It can act as a signaling molecule within tissues and cells to modulate the expression of genes and proteins. The modulation of genes by resveratrol could act as an explanation for cytoprotective actions as well as its influence on blood flow, cell death and inflammatory cascades (Pervaiz, 2003). It also exhibits anti-inflammatory, neuroprotective and antiviral properties (Pervaiz, 2003). It also showed protective effect on neuron cell death induced by oxidative agents (Wang et al, 2003; Kiziltepe, 2004).

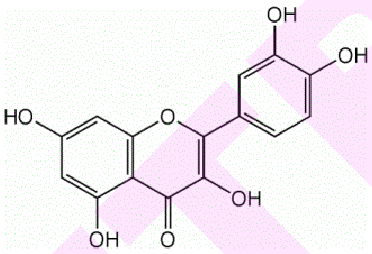
1.3.1.8. Ellagic acid

Ellagic acid is a phenolic lactone, found in many fruits mainly in black and blue berries, raspberries and walnuts. Ellagic acid prevents the metabolic activation of procarciongen, especially nitroso compounds, aflatoxins via non-selective destruction of cytochrome P-450 enzyme which are responsible for activating carcinogens (Stoner, 2005). Carcinogen detoxification is also promoted by ellagic acid via the stimulation of various isoforms of the carcinogen-detoxifying phase II glutathione molecules. It also prevents carcinogen from binding to DNA by binding with DNA itself at the O-6 and N-7

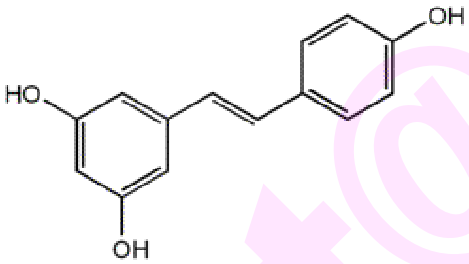
position of guanine and making this site unavailable for carcinogens and their metabolism for methylation (Stoner, 2005).



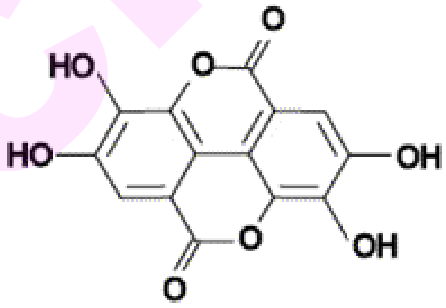
Catechins



Quercetin



Resveratrol



Ellagic acid

Figure 1.6. Structures of phenolic compounds

1.3.1.9. Lignan

Lignans are some of the minor compounds associated with dietary fiber and found to produce important physiological effects. Lignans are a group of relatively simple diphenols. Lignans occur in a wide variety of plant foods, primarily oil seeds, cereal grains, vegetables, fruits and legumes. Flax seed found to be the rich source of lignans. Tea is also found to be a good source of lignan. Plant lignans generally occur in the form of glycosides. They may

occur as monomers as in tea, as a mixture of monomers and oligomers as in case of broccoli or predominantly as oligomers as in case of flax seed. Plant lignan when ingested are converted by bacteria in the large intestine into two simple phenols: enterolactone and enterodiol. These compounds are called mammalian lignans. These compounds are found to exhibit a number of significant physiological effects. Enterolactone is a moderate inhibitor of estrogen synthesis and lower estrogen levels while enterodiol is a weak inhibitor. Both of them also have a role in the level of sex hormone building globulin, which is important in controlling the availability of androgen and estrogen in the body. Hence plant lignans are considered to be one of the several classes of phytoestrogen. Lignans are effective as antioxidants and have a role in reducing the risk of cancer and cardiovascular diseases (Vanharanta et al, 1999; Arts & Hollman, 2005). Effects of lignans on cancer have been studied and it was found that these compounds could provide protection against ovarian and thyroid cancer in women. Also both enterolactone and enterodiol caused dose and time dependent decrease in the number of colon cancer cells (Qu et al, 2005).

1.3.2. Phytosterols

Phytosterols are naturally occurring plant sterols that are present in the unsaponifiable fraction of plant oils. Structurally plants sterols are similar to cholesterol except that they have some substitutions on the sterol side chain at the C24 position. The primary plant sterols in the diet are sitosterol, stigmasterol and campesterol. It was reported that plant sterols could reduce the cholesterol levels in humans by 10% (Krinsky, 1988). Special margarines are the primary food source of plant sterols/stanols. The plant sterol mixtures

are derived from different oil sources, including pine tree wood pulp, soybean oil, rice bran oil and shea nut oil. Sterols present in the unsaponifiable fraction of rice bran oil decrease plasma cholesterol levels (Lichtenstein, 1994) and showed antioxidant activity (Tomeo, 1995).

1.3.3. Carotenoids

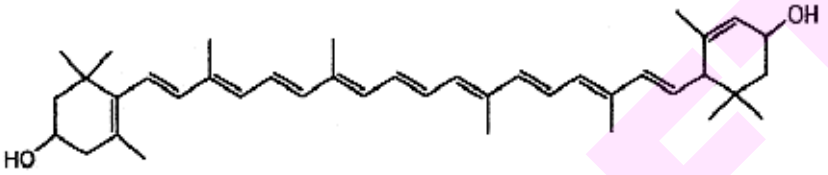
1.3.3.1. Definition and structure

Carotenoids are class of natural fat-soluble pigments. Among the pigments present in living organisms, carotenoids are the most widely distributed in nature. They are found throughout the plant kingdom in both photosynthetic and non-photosynthetic tissues, in bacteria, fungi and animals. They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that two central methyl groups are in 1, 6-position relationship and the remaining non-terminal methyl groups are in 1,5-position relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure having a long central chain of conjugated double bonds by hydrogenation, dehydrogenation, cyclization or oxidation. Some 600 different carotenoids are known to occur naturally (Ong & Tee, 1992). In general carotenoids can be classified into two different groups: carotene, which are strictly hydrocarbons and xanthophylls, which are derived from the former and contain oxygenated functional groups. Structurally the carotenoids may be acyclic or contain a ring of five or six number at one or both ends of the molecules. Carotenoids have been also classified as primary and secondary compounds. Primary carotenoids are those compounds required by plants for photosynthesis (β-carotene, violoxanthin, neoxanthin etc), where as secondary carotenoids are

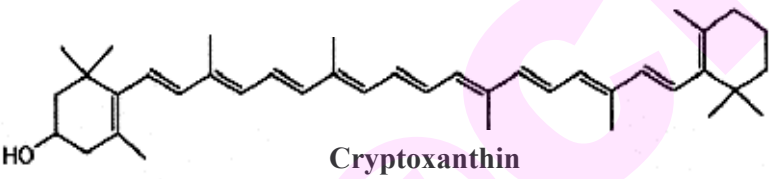
localized in fruits and flowers (α-carotene, β-cryptoxanthin, zeaxanthin, anthrxanthin, capsanthin, capsorubin) (Litchenthaler, 1987).

Carotenoids are lipophyllic substances except in certain cases where highly polar functional groups are present, as in norbixin, a carotenoid with dicarboxyl acid structures (Minguez-Mosquera et al, 2002). The presence of long extensive system of conjugated double bond is responsible for light absorption capacity. The polyene chain makes the carotenoid molecule extremely susceptible to isomerizing and oxidizing conditions such as light, heat or acids.

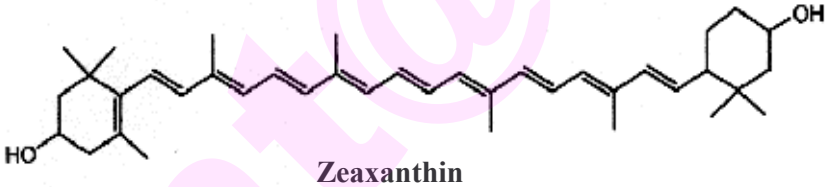
The distribution of carotenoid among the different groups of higher plants does not follow a single pattern. In green plants, beta-carotene found to be the major one followed by xanthophylls, lutein, iolaxanthin and neoxanthin. Zeaxanthin, γ-carotene, cryptoxanthin and anthrxanhin are found in small amounts (Mangles et al, 1993). In case of fruits xanthophylls are the major carotenoids. In maize, the predominant pigments are lutein and zeaxanthin, while in mango and perissmon, the major pigments are β-cryptoxanthin and zeaxanthin. In tomato, the major carotenoid is lycopene. Capsanthin and capsorubin are found almost exclusively in ripe capsicum fruits and are responsible for their attractive red color (Davies, 1976; Goodwin, 1976). The presence and distribution of the most common carotenoid pigments found in nature are shown in the **Table 1.5.**



Lutein



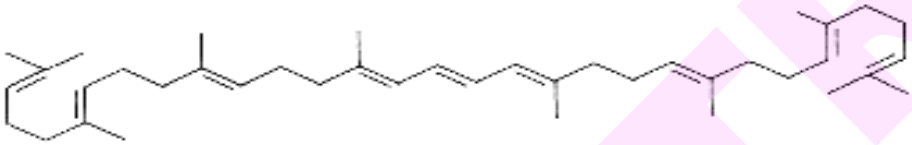
Cryptoxanthin



Zeaxanthin

Figure 1.7. Structures of xanthophylls

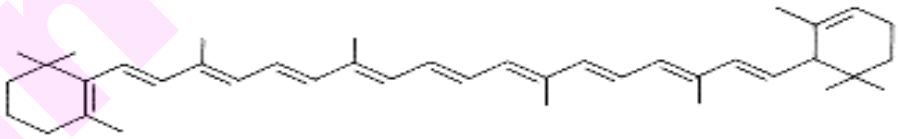
Phytoene ($C_{40}H_{64}$; colorless, λ max, 285 nm)



Lycopene ($C_{40}H_{56}$; red, λ max, 476 nm)

γ-Carotene ($C_{40}H_{56}$; orange, λ max, 460 nm)

α-Carotene ($C_{40}H_{56}$; orange, λ max, 456 nm)



β- Carotene ($C_{40}H_{56}$; orange, λ max, 463 nm)

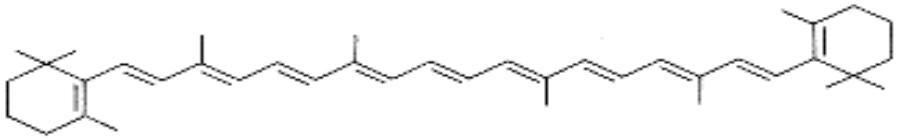


Figure 1.8. Structures of carotenes

Table 1.5. Natural occurrence of some common carotenes and xanthophylls

Carotenoid	Natural occurrence
Carotenes	Carrot, sweet potato, palm tree fruit, rose hips
α-Carotene, β-carotene, δ-carotene, γ-carotene, ε-carotene	Tomato, water melon, rose hips
Lycopene, neurosporene	Carotene rich fruits, flowers and roots
Phytofluene, phytene	
Xanthophylls	Anthers and petals of yellow flowers
Antheraxanthin	Bird feathers, salmon, crustaceans
Astaxanthin	Annatto seeds
Bixin, norbixin	Cyanobacteria and green bacteria
Canthaxanthin	Capsicum fruits
Capsanthin, capsathin-5,6-epoxide	Saffron
Crocetin	Pumkin
Cucurbitaxanthin A	Lettuce,
Lactucaxanthin	Grren fruits, vegetables and flowers
Lutein, violaxanthin, neoxanthin, Luteoxanthin, neochrome, auroxanthin	Vegetables and fruits processed in acid conditions and fermentation
Rubixanthin	Rose hips
Zeaxanthin,β-cryptoxanthin, α-cryptoxanthin, cryptoxanthin-5,6-epoxide	Seeds (corn), flowers, fruits; mango, papaya and persimmon

(Source: Minguez-Mosquera et al, 2002)

1.3.3.2. Functions of carotenoids in plants

In plants, carotenoids are located in specialized subcellular organelles called plastids namely chloroplasts and chromoplats (Goodwin & Britton, 1998). In chloroplast carotenoids are present in the form of chlorophyll-carotenoid –protein complex in thylakoid membranes. Chromoplasts present in flowers, ripe fruits and certain root and tubercles and they are accumulated in lipid rich structures, the plastoglobules (Sitte et al, 1980). In case of fruits and flowers, the main function of carotene is to attract animals and plants in seed dispersion and pollen transport (Bartley & Scolnik, 1995). The carotenoids also taken an active part in plants photo-protective and antioxidant actions.

1.3.3.3. Functions of carotenoids in animals

In animal kingdom, carotenoids are incorporated via the diet and stored in different tissues. The egg yolk owes its yellow color to xanthophylls such as lutein and zeaxanthin and traces of β-carotene. Invertebrates and vertebrates such as birds, fish, reptiles and amphibians show a great diversity of carotenoid pigments and even have the capacity to modify structurally some of the carotenoid ingested in the diet (Schiedt, 1995). In invertebrates, the carotenoids can be ultimately associated to protein giving rise to carotenoid proteins. Such association results in changes of chromatic characteristics of the carotenoids which present coloration including green, blue, purple and gray (Zagalsky, 1985). Carotenoid proteins normally found in the exoskeletons and eggs and ovaries suggesting their role in development, nutrition reserve and protective coloring as a means of camouflage. Within

animals, carotenoids provide bright coloration, serve as antioxidants, and can be a source for vitamin A activity (Ong & Tee, 1992; Britton, 1995).

1.3.3.4. Importance of carotenoids

1.3.3.4.1. Precursor of vitamin A

The main physiological function of the carotenoid is their capacity as precursor of vitamin A and defined as provitamin A value. The condition for a carotenoid to have such activity is that it posses at least one unsubstituted end group with a β ring. β-Carotene presents the greatest activity since the central enzymatic cleavage of its molecule originates two molecules of vitamin A (Olson, 1993). Other carotenoids such as α-carotene, γ-carotene, β-apo-carotenoid and β-cryptoxanthin gives rise to only one β ring in their structure. The conversion of carotenoid to retinal takes place in the intestinal mucosa by the action of the enzyme dioxygenase, β-Carotene giving rise to a two molecules of retinal, subsequently reduced to retinol (vitamin A). The term retinol equivalent (RE) was introduced to express the content in vitamin A (1RE= 6μg carotene) (FAO/WHO, 1967).

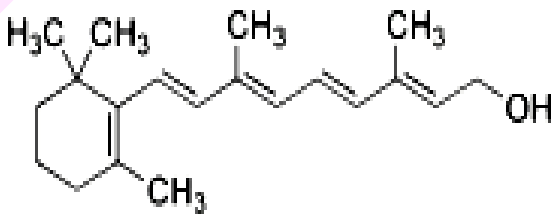


Figure 1.9. Structure of vitamin A

1.3.3.4.2. Antioxidant

Carotenoids are found to be liposoluble antioxidants (Britton, 1995). The major factors responsible for the antioxidants action include the presence of oxygenated functional groups in the pigment (Terao, 1989), the condition of the medium where pigment acts (Lieber, 1993) and the nature of the prooxidant substances (Everett et al, 1996). Lycopene a fat-soluble carotenoid is a precursor of β-carotene (Sandmann, 2001) and has twice the antioxidant capacity of β-carotene (Di Mascio et al, 1991). It was reported that consumption of carotenoid rich fruit and vegetables associated with lower incidence of cancer (Krinsky & Johnson, 2005) cardiovascular disease (Krichersky, 1999), age related macular degeneration and cataract formation (Landrum & Bone, 2001). Epidemiological studies have shown the positive relationship between the level of lycopene ingested in the diet and lower probability of the appearance of prostate cancer and other health benefits (Gann et al, 1999) and inhibit oxidative damage to lymphocyte DNA and cell membranes (Woodall et al, 1997). Carotenoids have been used in therapy to reduce the effect of eryhtropoetic protoporphyria, skin diseases related with metabolism of porphyrin (Van Laar et al, 1996). Lutein and zeaxanthin, xanthophylls found in corn and leafy vegetables are believed to function as protective antioxidants in macular region of the human retina mediated by their ability to quench single oxygen and blue light in the retina (Snodderly, 1995, Landrum & Bone, 2001). Astaxanthin, a xanthophyll found in salmon, shrimp and other seafoods, is another naturally occurring xanthophylls with potent antioxidant activity (Di Mascio et al, 1991).

1.3.3.4.3. Food color

Carotenoid pigment extract obtained from natural sources are used to enhance, correct or contribute the color of foods. Currently these carotenoid rich colorants are permitted by the US Food and Drug Administration (FDA). The advantage of using carotenoid, as a colorant is that it is of natural origin preferred by consumer and it has provitamin A activity and liposoluble antioxidant. Carotenoid pigments are used in cosmetic products in the form of suspension, emulsion or lotion in lipsticks and in make up foundations.

1.3.4. Dietary fiber

1.3.4.1. Definition and composition

Dietary fiber includes the compounds found in plant foods, which are non-digestible in the mammalian small intestine. These compounds include cellulose, non-cellulose polysaccharides (Southgate, 1982). It plays an important role in the prevention and treatment of diseases. Cereals, legumes, vegetables, fruits, lentils, nuts and seeds are found to be rich source of dietary fiber. Dietary fibers do not constitute a defined chemical group, but are combination of chemically heterogeneous substances such as cellulose, hemicellulose, pectins, lignins, gums and polysaccharides (Asp et al, 1992). The most widely accepted definition of dietary fiber is physiological one, in which 'dietary fibers' correspond to the vegetable cell wall residues that are resistant to enzymatic hydrolysis in the small intestine. A chemical definition describes dietary fibers as non-starch polysaccharides. The most commonly used definition is dietary fiber is that dietary fibers are oligosaccharides, polysaccharides and the derivatives which can not be digested by the human digestive enzymes to absorbable components in the upper alimentary tract

(Trowell et al, 1976). Dietary fibers can be generally classified into two main categories insoluble and soluble dietary fiber depending on their solubility in water (Periago et al, 1993). Insoluble dietary fiber (IDF) is a coarse material that does not dissolve in water. The chemical component of insoluble fiber includes cellulose, lignin and hemicellulose found in primary and secondary cell walls of plants. The cellulose in cell wall is chemically bound to hemicelluose and lignin (Salisbury & Ross, 1992). The hemicellulose and lignin act to give significant rigidity and resistance to the action of enzymes and acids. It is roughage. Soluble dietary fiber (SDF) is made up of sticky substances like gums and dissolves in water. Each fraction has different physiological effects (Schneeman, 1987). The insoluble part is related to both water absorption and intestinal regulation, whereas the soluble fraction is associated with the reduction of cholesterol in blood and the decrease in the intestinal absorption of glucose. In terms of health benefits, both fibers complement each other and a 70-50% insoluble and 30-50% soluble dietary fiber is considered a well-balanced proportion (Schneeman, 1987). In cereals the SDF content is quiet low where as in fruits the ratio between soluble and insoluble DF fractions is more balanced (Saura-Calixto, 1993). SDF consist of mainly hemicelluloses, pectic substances, gums and mucilages. Hemicellulose includes xylan, mannans and xyloglucans (Aspinall, 1980). The pectic substances comprise the second major category of SDF and include the arabinans and galactans (Schneeman, 1987). The third major category in SDF are gums and are considered to be hydrophilic polymeric material, generally polysaccharide in nature, that can be dissolved or dispersed in water to give a gelling effect (Whistler, 1973). The monomeric units found in

gums include the neutral sugars, uronic acids and other acid groups (Schneeman, 1986).

The physical and chemical properties of the components of dietary fiber appear to be important in determining the physiological response to sources of fiber in the diet. Mainly four properties have been associated with physiological responses to various sources include fermentability by bacteria, binding to organic compounds, ion-exchange capacity and water holding capacity which has also been associated with viscosity and solubility of various fiber sources (Schneeman, 1986).

1.3.4.2. Functions of dietary fiber

The physiological effects of total dietary fiber, in the form of insoluble and soluble fractions, have a significant role in human nutrition (Schweitzer & Edwards, 1992). Numerous health organizations suggest daily intake of total dietary fiber, with specific recommendations of 30-45 g per day with approximately one third of that as soluble dietary fiber (Schweitzer & Edwards, 1992). The nutritional value of fruit fiber dietary fiber is considerable due to the presence of bioactive compounds such as phenolics, water and oil holding capacities and colonic fermentabilty, low phytic acid and caloric value contents (Saura-Calixto, 1998). Recent studies indicated that dietary fiber might be protective against constipation, cardiovascular diseases, diabetes, colon cancer, obesity and diverculosis (Marlett, 2001; Spiller, 2001). Dietary fibers have been reported to decrease the digestion and absorption of carbohydrate and postprandial serum glucose levels (Ou et al, 2001). Fibers as fermentative substrates can also modify the activity of digestive microflora and lead to a modification or reduction in the production of mutagens and

some fibers can adsorb mutagenic agents, leading to their excretion in the faeces (Roberton et al, 1991). Short chain fatty acids are produced in the colon from the bacterial breakdown of dietary fibers and these fatty acids have been proposed by many researchers to inhibit hepatic cholesterol synthesis.

Dietary fibers also have technological properties that can be used in the formulation of foods, resulting in texture modification and enhancement of the stability of the food during production and storage. Dietary fibers are also used to increase the total dietary fiber content of cereal bars, breakfast cereals, fruit products and yogurts. They can be used in sauces and soups for their water retention and textural properties. They can also be used in dietetic drinks, meal substitutes and break fast drinks.

1.3.5. Vitamins

Vitamins are organic substances with very high biological activity that are required in small amounts for the growth and maintenance of human health. As they cannot be synthesized in the human body, they need to be supplied in the diet. The vitamins can be generally classified into two major groups, water-soluble and fat-soluble depending on their solubility. Water-soluble vitamins include vitamin B complexes and vitamin C and in general perform catalytic functions as cofactors for enzymes. Vitamin A, D, E and K are called fat-soluble vitamins being non-polar, hydrophobic molecules associated with lipid fraction of the food.

1.3.5.1. Thiamin: Thiamin also called aneurin and is an amine consisting of a substituted pyrimidine and thiazole linked by methylene bridge. It is rich in dietary sources like unrefined cereals, nuts, legumes, green leafy vegetables,

organ meats, pork, liver and egg. Thiamins are involved in the energy metabolism of proteins, lipids and carbohydrates and also have a role in the nerve conduction and in some functions of the brain. In liver and brain, thiamin is converted into its pyrophosphate form (TPP) to become biologically active as a coenzyme.

1.3.5.2. Riboflavin: Riboflavin contains a substituted isoalloxazine ring with a D-ribityl side chain attached at position 10. This vitamin functions metabolically as the essential component of two flavin coenzymes, flavin adenine dinucleotide (FAD) and favin mononucleotide (FMN) and complexed with proteins, which acts as intermediaries in transfers of electrons in biological oxidation-reduction reactions function as coenzymes for flavoproteins and flavoenzymes. Flavoproteins are essential for the metabolism of carbohydrates, amino acids and lipids and for pyridoxine and folate conversion to their respective coenzyme forms. Dairy products, animal proteins and green leafy vegetables are found to be good sources of riboflavin (Merrill et al, 1981).

1.3.5.3. Niacin: Niacin is the generic name of compounds having the nutritional activity of nicotinic acid and includes two vitamers, nicotinic acid and nicotinamide. Nicotinamide in the form of coenzymes NAD^+ and $NADP^+$ is concerned with the functioning of a large number of dehydrogenases. They are concerned with the oxidation of glucose, synthesis of high-energy phosphate, fatty acid breakdown and synthesis, amino acid deaminations and reactions in photosynthesis in plants. Coffee is found to be the richest source. Liver, kidney, heart, lean meat, fish, peanuts, wheat germ and dried legumes

are excellent sources. It has role in redox and non-redox functions in the body (Henderson, 1983).

1.3.5.4. Pyridoxine (Vitamin B_6): It occurs in three vitamers; pyridoxine, pyridoxal and pyridoxamine in which alcohol, aldehyde, and amine groups are located at the 4-position of the pyridine ring. Pyridoxol and pyridoxine amine are labile compounds readily destroyed by exposure to air, light and heat in dilute solutions. Pyridoxal phosphate is produced by phosphorylation of pyridoxal by an ATP-dependent kinase. Human brain is a richest source of the enzyme, which is also activated by Zn^{2+} ions. It is a co-enzyme in transamination reactions of a large number of amino acids, in decarboxylation reactions of amino acids. It is involved in the transport of amino acids and certain metal ions across cell membranes. This vitamin also has relation with the endocrinal metabolism and hormone synthesis. Meat and cereals are the richest sources of pyridoxine.

1.3.5.5. Folic acid: Folic acid contains three parts, a pteridine nucleus, para-aminobenzoic acid and glutamic acid. The active coenzyme form of vitamin is tetrahydrofolic acid formylated in the 5- and 10- positions. The primary role of the folic acid co-enzyme is in the synthesis of purines and pyrimidines used in the formation of nucleic acids and certain amino acids. In association with vitamin B_{12}, it also appears to be involved in methylation reactions concerned with the synthesis of methionine from homocysteine, thymine from uracil and choline from ethanolamine. It is abundant in green leafy vegetables, pulses, liver and egg yolk.

1.3.5.6. Vitamin B_{12}: It is a group of active vitamers called cobalamines, which contain cobalt. Microbial synthesis is the singular source of this vitamin.

It acts as a co-enzyme in the mitochondria functions. The vitamin with its combination of a porphyrin-like moiety and nucleotide type portion suggests an association with other active substances like cytochromes and the co-enzymes. In association with pteroylglutamic acid, the vitamin is concerned with the metabolism of one carbon fragments and labile methyl groups. It has been suggested that it has a role in the synthesis of nucleic acids, particularly that of the deoxyribose moiety.

1.3.5.7. Biotin: Biotin is a bicyclic compound and is a sulphur-containing vitamin. Biotin can bind to avidin a toxic factor in raw egg white and convert into non-toxic. Biotin is an extremely potent growth factor for microorganisms. Biotin acts as prosthetic group in combination with the ε-amino group of lysine in the protein. Biotin also has a role in fatty acid synthesis. Liver, kidney, peanuts, eggs and poultry meats are found to be richest source of this vitamin.

1.3.5.8. Vitamin C: Vitamin C is widely distributed in plant cells where it plays many crucial roles in growth and metabolism. L-ascorbic acid and L-dehydroxyascorbic acid are the two biologically vitamin C active compounds. It is related to the C_6 sugars, being the aldono-1,4-lactone of hexonic acid. Biological function of L-ascorbic acid can be defined as an enzyme co-factor, a radical scavenger and as a donor/acceptor in electron transport chain at the plasma membrane. Ascorbic acid is able to scavenge super oxide and hydroxy radical as well as regenerate α-tocoferol (Davey et al, 2001). Vitamin C is a potent antioxidant and has the capacity to eliminate several reactive oxygen species, keeps the membrane-bound antioxidant α-tocoferol in the reduced state, act as a co-factor maintaining the activity of a number of

enzymes and has a role in stress resistance (Arrigoni & De Tullio, 2002; Davey et al, 2001). Vitamin C has been reported to inhibit carcinogens and protect against cardiovascular diseases. The vitamin C has specific function in collagen synthesis, formation and maintenance of cartilage, bones, gums, skin, and teeth. Also recognized that the activity of leucocytes and other aspects of immune system are enhanced by ascorbic acid (Davey et al, 2001). Block et al (2004) found that vitamin C can reduce the levels C-reactive protein, a marker of inflammation and possibly a predictor of heart diseases. More than 85% vitamin C in human diet is supplied by vegetables and fruits (Davey et al, 2001). Guava and amla are rich source of vitamin C.

1.3.5.9. Vitamin A: Vitamin A or retinol was the first essential lipid soluble substances identified. Vitamin A and its derived retinoids are required for vision, growth, reproduction, epithelial cell differentiation and mucus secretion. Retinol occurs as long chain fatty acid esters in mammalian liver and in fish oils. In fruits and vegetables, vitamin A exists as provitamin in the form of carotenoids. Retinol has an important role in retinal vision. The other functions of vitamin A are in reproduction, growth and differentiation. It is involved in the synthesis of glycoprotein. It is also found to alter gene expression resulting in the prevention of certain neoplastic transformations. The dietary deficiency of vitamin A can lead to blindness and child mortality (Mayne, 1996).

1.3.5.10. Vitamin D: Vitamin D is chemically sterol derivatives. The two most important forms are: ergocalciferol (plant origin) and cholecalciferol (animal origin). The ergocalciferol is derived from the plant sterol, ergosterol. The cholecalciferol from 7-dehydrocholesterol, present in mammalian skin and the conversion occurs on exposure to sunlight. Certain marine fishes are found to

be good sources of vitamin D. It is considered to be a prohormone, giving rise to the hormone 1,25 $(OH)_2$ D_3 whose primary function is to regulate serum calcium.

1.3.5.11. Vitamin E: Vitamin E belongs to a group of lipid soluble antioxidants. Vitamin E is comprised of two homologous series of tocochromanols, termed “tocoferols” and “tocotreinols”. They are structurally related, having a common chromanol ring, but distinguished by their side chains. Tocoferols have a saturated phytyl tail, whereas the tocotrienols posses an unsaturated isoprenoid side chain. Four homologous of each type characterized by their Greek prefixes (α,β,γ,δ) are known to exist in nature (Kamal-Eldin & Appelqvist, 1996). Vegetable oils, nuts and whole grains are the richest natural sources of vitamin E. It is well recognized as a major antioxidant and the primary defense against lipid peroxidation (Tomeo et al, 1995). The predominant antioxidant reaction responsible for tocoferol antioxidant activity is mainly because of hydrogen atom donation, where a tocoferoxyl radical is formed. It provides protection to mitochondria and microsomes against oxidative damage. Vitamin E has a protective role in the prostaglandin-mediated disorders such as inflammation, premenstrual syndrome and circulatory irregularities. Vitamin E shows protective effect against the coronary heart diseases mainly due to inhibition of LDL oxidation (Stampfer & Rimm, 1993). Tocotrieinols have been reported to have possible antitumour activities in different cancer systems. The therapeutical properties of tocotrienols have recently been reviewed by Theriault et al (1999).

1.4. ISOLATION OF VALUABLE COMPOUNDS FROM FOOD PROCESSING INDUSTRY BY-PRODUCTS

Valuable compounds that are isolated from food industry wastes or by-products mainly from fruit and vegetable processing industry, and cereal and legume processing industry are briefly presented.

1.4.1. Fruit processing by-products

Several valuable substances – fibers, coloring agents, phenolics, gelling agents and many other nutraceutical bioactive compounds are extracted from the by-products of fruit processing industry. The way and goal of utilization is determined by the economic efficiency of extraction and the market potential of the compound.

1.4.1.1. Apple

Highly variable compositions of apple pomace and possible strategies of utilization have been reviewed (Kennedy et al, 1999). Apple pomace was found to be rich sources of pectin with superior gelling properties. Apple pomace has been found to be good source of polyphenols, which are predominantly localized in the peel. Major compounds isolated and identified include catechins, hydroxycinnamates, phloretin glycosides, qurcetin glycosides and procyanindins (Foo & Lu, 1999; Lu & Foo, 1998, 1997; Schieber et al, 2001). Phenolics and other bioactive compounds from apple peel showed antioxidant, anticariogenic and anticancer activity (Lu & Foo, 2000; Shoji et al, 1999; Eberhardt et al, 2000). Recently a method has been reported for combined recovery of pectin and polyphenols from apple pomace (Carle et al, 2001).

1.4.1.2. Grape

Grape pomace, a major by-product from grape processing industry, which contributes about 20% of the weight of grape processed (Mazza & Miniati, 1993). A great range of compounds such as phenolics, anthocyanins and dietary fibers are recovered from grape pomace. Anthocyanins have been considered as most valuable components and methods for extraction have been reported (Mazza, 1995) and have been commercially extracted for food industrial uses. In chardonnay grape pomace, 17 polyphenolc constituents were identified by NMR spectroscopy (Lu & Foo, 1999) and also a source of two unusual dimeric flavanols (Foo et al, 1998). Catechins, epicatechin, epicatechin gallate and epigallocatechin were the major constituents of grape peel tannins (Souquet et al, 1996). Amico et al (2004) identified 16 flavonoids and flavonol glycosides from grape pomace scilian cultivar Nerello mascalese. The antioxidant activity of grape pomace (Larrauri et al, 1998) has led to the development of a new concept of antioxidant dietary fibers (Saura Calixto, 1998). Enzymatic treatment of grape pomace enhanced release of phenolic compounds (Meyer et al, 1998). Crude extract and fractions from the grape by-product showed strong free radical scavenging and antioxidant activity and inhibitory effect on proliferation of tumor cell lines (Torres et al, 2002). It has been reported that pomace extracts from Kalecik Karasi and Emirs grape cultivars have antibacterial activity on pathogenic bacteria and can be used as food preservatives (Gulcan et al, 2004). Grape seed contain about 15% of oil that can be removed by mechanical processing or solvent extraction. The predominant fatty acids in grape seed oil are linoleic acid, which represents 71.5% of the fatty acid (Mattick & Rice, 1976).

1.4.1.3. Citrus

Residues of citrus processing industry are sources of dietary fiber, pectin, cold pressed oils, essence, limonene, limonoids and flavanoids (Bradock, 1995). Fiber pectins are easily recovered from lime peels and as characterized by high fiber content (Siliha et al, 1995). The main flavanoids found in citrus species are hesperidin, narirutin, naringen and eriocitrin (Mouly et al; 1994; Coll et al, 1998). Recently, flavonid content and antioxidant activity from the residue of sweet orange peel (*Citrus sinensis*) have been reported (Anagonostopoulou et al, 2005). The nonvolatile component in lemon peel was identified by HPLC-MS and evaluated for its antioxidant activity (Baldi et al, 1995). High dietary fiber powder prepared from lemon juice by products with high functional and microbial quality, as well as favorable physico-chemical characteristics to be used in food formulation (Lario et al, 2004). High dietary fiber prepared and their dietary fiber composition and antioxidant capacities determined from Persian and Mexican lime peels (Rivera et al, 2004). Citrus seed and peel found to posses high antioxidant activity (Bocco et al, 1998).

1.4.1.4. Other fruits

Pineapple stem and pulp waste material from pineapple processing industry can be used for the production of bromelain enzyme. The antioxidant principles have been structurally elucidated (Ling et al, 1999) and method for recovery from the pineapple processing industry has been described. Banana represents one of the most important fruit crops. Peel constitutes about 35% of the ripe fruit. Anthocyanin pigments in banana were evaluated for their potential application as natural food colorants and identified anthocyanins are

delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin (Pazmeno-Duran et al, 2001). Most of the carotenoids found in banana peels were demonstrated to be xanthophylls esterified with myristate and to a lesser extent with laurate, palmitate and caprate (Subaigo et al, 1996). The guava processing by-products found to be rich in methoxylated pectins. The guava seed contain about 5-13% oil rich in essential fatty acid (Adsule & Kadam, 1995). Recently it was reported that guava fruit could be used as source of antioxidants and dietary fiber (Jimenez-Eserig et al, 2001). Papain, a proteolytic enzyme has been recovered from the latex of papaya fruit. The defatted papaya seed meal contains high amounts of crude protein (40%) and crude fiber (50%) (Jagtiani et al, 1988). Passion fruit processing industry by-products contribute about 75% of the raw material and the rind constitutes about 90% of the waste and is good sources of pectin. Passion fruit seed oil is rich in linoleic acid (Askar & Treptow, 1998). Passion fruit seed was found to be rich in insoluble dietary fiber, which is mainly composed of cellulose, pectic substances and hemicelluloses (Chau & Huang, 2004). During kiwi fruit processing 30% of the raw material wasted as by-product. Kiwi fruit pomace contains about 25% of dietary fiber (Martin-Cabrejans et al, 1995). Blue berry press cake has high amount of anthocyanins and polyphenolics (Lee & Wrolstad, 2004) and is a potential source of natural colorants and nutraceuticals. Recently, a method was developed for the extraction of polyphenolics and anthocyanins using enzymes from blue berry (Lee & Wrolstad, 2004). An antioxidant compound punicalagin has been isolated and identified from pomegranate processing by-products and also studied the

antioxidant activity (Kulkarni et al, 2004). Antioxidant rich fractions from seed and peel extracts of pomegranate evaluated for their antioxidant activity using various *in vitro* models (Singh et al, 2002).

1.4.2. Vegetable processing by-products

By-products of vegetable processing industry also found to be good sources of bioactive compounds. Tomato juice is the most important vegetable juice followed by carrot juice with respect to per capita consumptions. Tomato seeds account for approximately 10% of the fruit, they accounts for 60% of the total waste and are source of protein (35%) and fat (25%). Tomato seed oil found to be rich in unsaturated fatty acids such as linoleic acid (Askar & Treptow, 1998, Roy et al, 1996). Baysal et al (2000) reported the higher content of carotenoids and lycopene in tomato processing waste. Enzymatic treatment of tomato enhanced lycopene extractability (Bohm et al, 2000). Carrot pomace represents valuable natural sources of carotene and can be applied as functional food ingredients in their genuine proportion (Stoll et al, 2001). The major by-products resulting from onion processing are brown skin, outer fleshy leaves and bottom bulb. They are sources of flavones and fiber components, and are rich in quercetin glycosides (Hertog et al, 1992; Waldron, 2001). The pomace from beetroot processing industry constitutes about 15-30% of the raw material (Otto & Sulc, 2001). The beetroot peel was rich in betalins, betacyanins, betaxanthins and phenolic acids such as p-coumaric acid and ferulic acid as well as cyclodopa glucoside derivatives (Kujala et al, 2000). Peel is the major by-product of potato processing industry, which constitutes about 15-40% depending on the

processing method (Putz, 1991). Aqueous peel extract was shown to be a good source of phenolic acids especially chlorogenic, gallic, protocatechuic and caffeic acids (Onyeneho & Hettiararchchy, 1993; Rodriguez de Sotillo et al, 1994a). The antioxidant activity of freeze-dried water extract of potato peel was comparable to that of butylayted hydroxyl toluene (Rodriguez de Sotillo et al; 1994a, Singh & Rajini, 2004).

1.4.3. Cereal and Legume processing by-products

Wheat and rice are the important cereal consumed worldwide. Basic staples like rice, wheat and pulses reach the market after going through many primary mechanical processing operations that convert the grain into an edible material.

During processing of rice, bran (3-7%), husk (21-24%), broken rice and germ are the major by-products. Bran, which comprises the testa and pericarp, is known to be rich in number of component such as oil, vitamins, sugars and proteins. The rice bran is a rich source of oil around 20% (Zachraiassen, 1964) and has balanced fatty acid composition and contains oryzanol. Oryzanol and ferulate esters of triterpenoid alcohols and plant sterols and are well known for their strong hypocholestermic properties (Rukumni & Raghuram, 1991) and antioxidant activity (Seetharamaiah & Prabhakar, 1986). The rice bran was found to be rich in anthocyanins. Cyanindins 3-O-β-D-glucopyranoside and pronidin 3-O-β-D-glycopyranoside were identified to be the anthocyanin pigment responsible for the activity of ethanol extracted bran fractions in pigmented rice, evaluated by linoleic acid auto-oxidation and rabbit erythrocytes membrane systems (Choi et al, 1996).

The proteins in rice bran are of a complex nature. Rice bran proteins contain 37% albumin, 36% globulin, 22% glutenin and 5% prolamin (Betschart, 1977). The rice bran contain high fiber content (Juliano, 1985).

Wheat is an important staple food and germ and bran are by-products obtained during milling and they constitute 3 and 15 % of the grain respectively. Germ is a good source of vitamin E, beta-carotene and valuable proteins (Ge et al, 2002). Wheat germ was found to be a good source of enzymes such as lipase and esterase enzymes. Wheat and rice bran is found to be rich source of dietary fiber (Goncalvez et al, 1998). Barely rootlets mass produced as a by-product in brewing industry is a rich source of dietary fiber, protein, vitamin and 5'nucleotidase (Iwamatsu et al, 1991).

1.5. NUTRACEUTICAL PRODUCTS

1.5.1. Definition and importance

Nutracueticals are the most interesting topic of research in the world today. The concept of nutracuetical gained impetus and importance in the last twenty years. M.D. Stephan de Feluice, Director of New York Foundation for Innovation in medicine coined the term 'Nutraceutical' in 1989. Word nutracueticals is a combination of two words nutrition and pharmaceutical. The nutracuetical can be defined as any substance that may be considered as a food or part of a food and provides medical or health benefits including the prevention and treatment of disease (De Felice, 1992). Nutracueticals may range from isolated nutrients, dietary supplements and diets to genetically engineered "designer foods", herbal products and processed products such as cereals, soups and beverages. These can be either ingested as such by

using its sources as food or can be extracted from its sources and then used to fortify different types of foods such as fortified foods, health foods, designer foods or functional foods.

Major nutracueticals from plants include antioxidants (carotenoids, polyphenols, vitamins, tocoferols) phytosterols, fatty acids, dietary fiber etc are gaining importance in scientific research due to their beneficial role in preventing and even curing certain chronic diseases like cancer, cardiovascular diseases, neurodegenerative diseases etc. High consumption of fruit and vegetables has been associated with a lower incidence of degenerative diseases including cancer, heart diseases, inflammation arthritis, brain dysfunction, cataract etc (Feskanich et al, 2000; Michels et al, 2000). These protective effects are considered to be related to the various bioactive or nutracuetical compounds present in them. Antioxidant can inhibit or delay the oxidation of oxidisable substrates in a chain reaction and therefore, seem to be very important in the prevention of these diseases (Pratico & Delanty, 2000; Wang et al, 2003). Few nutraceutical products relevant to the subject of thesis available in market are described below.

1.5.2. Commercial nutraceutical products

Many commercial nutraceutical products are available in the market. Green Power Syner TeaTM and Luo Han Guo are nutraceutical products marketed by Amax Nutrasource, which are rich source of antioxidants made from herbal extracts. Berry Force TM and Berry Bites are antioxidant fruit chew for kids marketed by Artemis International Inc and used for their antioxidant, antiviral, anti-inflammatory and antimutagenic properties. Nutra VeggieTM is a polyphenolic ingredients obtained from a phytinutrient extract.

XtraSaladTM is a standardized extract of salad greens providing 30% of caffeic acid derivatives and 50% polyphenols. PotentOnionTM is a standardized extract of onions providing 30% quercetin in the highly bioavailabe glycosylated form (Ohr, 2005). Mega Natural Gold grape seed extract and MegaNatural Red Color marketed by Polyphenolics showed antioxidant capacity similar to fresh fruit servings. MegaNatural Gold grape pomace extract and MegaNatural Rubired grape juice extract are self-affirmed as GRAS for functional beverages and most food and confection applications (Ohr, 2005). Some of the innovative natural products include Oxyphyte@ natural antioxidants, ChocamineTM and Talin@ which are marketed by RIF ingredients. Dark Red Concentrates and Grape Skin Extract are widely used in the food industry as stable, natural colorants with positive health benefits. Activin Grape Seed Extract is not only the most widely researched grape seed extract in the market but also the only grape seed extract approved by both FEMA and FDA for use in U.S foods and beverages (Ohr, 2005). High-grade tea and green tea antioxidants are offered under the Sunphenon@ brand. Matcha powder is a natural green tea leaf powder high in polyphenols, vitamins and fibers. Sunfiber@ is a water-soluble dietary fiber marketed by Taiya (www.taiyointernational.com).

Phytosterols are marketed as CardioAidTM with many nutraceutical properties. Novasoy@ brand soy isoflavone concentrates are ingredients for healthy functional food and beverage applications. Natural Source d-Alpha Vitamin E is ideal for food and beverage fortification and these products are marketed by ADM Natural Health & Nutrition. Diminicol is another plant sterol product, which can be used in a wide variety of dairy, meat, food and

beverages products. Because of the microcrystalline structure it has no gritty mouth feel and can be used in a broad range of products. FloraGLO lutein is sourced from marigold flowers and marigold oeoresins was found to be a nutraceutical product rich in lutein. Lycomato@ and LycoPen@ are the tomato complex rich in lycopene, phytoene, phyofluene, tocoferols, phytosterols and beta-carotene. These antioxidant phytonutrients, acting in synergy with lycopene, transform an ordinary food into a functional food with numerous health benefits, including the support of cardiovascular, prostate health and prevention of DNA damage. Lycomato@ is extracted from non-GMO, specially selected, lycopene rich tomato containing four times the lycopene content of ordinary varieties.

The beverages are available in six fruit flavor developed with AuraTM and CommonsenseTM flavour technologies. They are fortified with Litesse@ polydextrose, which is a good source of fiber. It is prebiotic, nonglycemic, and widely recognized as a source of dietary fiber suitable for enriching numerous applications like beverages (Ohr, 2005). FI-Soy fiber is an insoluble dietary fiber rich product produced from soybean hulls which reduces calories, adds dietary fiber improves yield marketed by The Fibred group. Fibrex@ is a natural, non-GMO, organic dietary fiber with an excellent composition of soluble and insoluble fiber and unique water holding capacity and is gluten free. Solka-Floc@ and Justfiber@ are also two functional fiber food products marketed by International Fiber Crop. FiberAid@ is a multifunctional prebiotic fiber that has a beneficial effects on the gastrointestinal system. A slowly fermenting prebiotic fiber present in the product preferentially increases beneficial microflora like lactobacilli and bifidobacteria and decreases

endogenous pathogenic bacteria. Fibersol-2 is used for beverages, including sport drinks and fortified waters, processed foods, baked goods, dairy products, dietetic foods, fiber supplements and functional foods. It also has acid and heat stability, low viscosity, prebiotic and high stability. Oatwell@ natural oat bran ingredients are in compliance with FDA standards for oat and fiber based health products which are soluble fiber from oat bran and can be a part of a diet low in saturated fat and cholesterol and reduce the risk of heart diseases. Frutalose L-85 is 50% sweet as sucrose and is a replacement for sugar and sugar alcohols. It has 85% fiber and all the health benefits associated with inulin/ fructo-oligosacchride marketed by Sensus America.

1.6. OBJECTIVES AND SCOPE OF THE INVESTIGATION

It is clear from the review of literature presented under 'introduction' that the processing of agricultural commodity yields many by-products (wastes) having significant potential for value addition. The exploitation of these by-products as a source of functional compounds and their application in food is a promising field, which requires interdisciplinary research by food technologists, food scientists and nutritionists. As many agricultural by-products are good source of nutrients, phytochemicals and fermentable substrates, isolation of value added products directly from agro-wastes or using fermentation methods are widely used approach for better utilization of these by-products. In the present study, it is proposed to isolate and characterize valuable compounds from mango peel and black gram mill waste, two agricultural waste products generated in large quantities in India.

Mango (*Mangifera indica* L.) is one of the most important tropical fruits and India is the world's largest producer of mango fruits. Mango products such as canned slices, puree, nectar, pickles and chutney experienced worldwide popularity and also gained the increasing importance in U.S. and European market. Major by-products of mango processing industry are peel and seeds amounting to about 60% of total fruit weight and peel constitutes about 15-20%. As peel is not currently being utilized for any commercial purpose, it is discarded and becoming a source of pollution. While a number of investigations have been conducted on the composition and possible utilization of mango seed kernel, studies on peels are limited. Recently, it has

been reported that mango peel contains polyphenols and dietary fibers. Therefore, one of the objectives in the present study was to isolate and characterize valuable compounds from mango peels.

Black gram (*Phaseolus mungo* L.) is a protein rich legume widely consumed in India. Black gram is milled into dhal and then used in various Indian traditional food preparations. During milling of black gram, about 25% is by-product and it is being wasted. These by-prduct is separated into four fractions, viz.,husk , germ, plumule and aleurone rich husk fraction using air classification. Our preliminary studies indicated that aleurone layer rich husk fraction contained significant amount of peroxidase. Peroxidase is a physiologically important enzyme having a number of applications. It catalyzes the oxidation of a variety of compounds like polyphenols, acylamines, halides and thiols. Because of these, it has a variety of applications in food processing, pharmaceutical and chemical industry, analytical biochemistry, and water purification. Therefore, it is proposed to isolate and characterize peroxidase from black gram mill waste.

Therefore, major objectives of this study are as follows:

3) To isolate and characterize value added compounds from mango peel and evaluate antioxidant properties and to develop mango peel enriched food products such as biscuits and macaroni.
4) To isolate and characterize peroxidase from black gram mill waste and to study the effect of exogenously added peroxidase on wheat proteins.

Mango peel will be analyzed for few important phytonutrients such as polyphenols, carotenoids, vitamins and dietary fibers. These phytonutrients in recent years are receiving increased attention for their nutraceutical properties. The feasibility of using mango peel, as a source of these valuable compounds will be attempted either by incorporating peel as such or isolated compounds into food products to increase nutraceutical properties of the food products. In addition certain oxidative enzymes like polyphenol oxidase and peroxidase and hydrolytic enzymes such as protease, amylase and xylanase will be studied in both mango peel and black gram husk. As these enzymes are involved in protection as well as use of stored energy during maturation/germination, these by-products could be a good source of these enzymes. Thus, use of these by-products for isolation of above mentioned value added product not only provides an alternate source of these valuable compounds but also reduces the pollution.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF VALUABLE COMPOUNDS FROM MANGO PEEL

2.1. INTRODUCTION

The aim of fruit processing is to transform fresh fruits into different types of processed products. The selection and elimination of components unsuitable for human consumption leads to products and by-products. Significant quantities of agricultural products are subjected to primary and secondary processing. This has resulted in the generation of huge amounts of waste products causing environmental pollution. Therefore, currently increased attention has been given for utilization of waste products.

2.1.1. Mango

The mango commonly referred to as the king of fruits, in Asia, is very important tropical fruit (Purseglove, 1974). The world's annual mango production is 25 MMT and India contributes about 46% of the world mango production with an annual production of 12.7 million metric tones (FAO, 2005). Although most of the major mango producing countries like India, China, Thailand, Indonesia, Pakistan, Philippines, Bangladesh are located in Asia, the mango fruits are also cultivated in countries like Mexico, Brazil, Madagascar, Tanzania, Dominican Republican, Haiti, Brazil and Australia (FAO, 2005).

2.1.1.1. Mango as a fruit

The common English term mango and the botanical name *Mangifera indica* L. originate from the ancient Tamil name manga (Singh, 1960). The *Mangifera indica* belongs to the dicotyledons family Anacardiaceae that consists of 64 genera mostly of trees and shrubs, some of which are poisonous. Mango is one of the oldest tropical fruits and has been cultivated

for over 4,000 years, originating apparently in the Indo-Burma region (Hulme, 1971). The mango fruit is a large fleshy drupe containing edible mesocarp of varying thickness. It varies in size, shape, colour and flavor. The shape and specific names of the various physical features of the mango are shown in **Figure 2.1**. The exocarp (peel) is thick and glandular. The mesocarp (pulp) can be fibrous or fiber free with flavor ranging from terpentine to sweet. The mesocarp provides the edible pulp, which is firm containing a sweet, well flavored juice. The endocarp is woody, thick and fibrous. The endocarp develops into a thick, tough, leathery glandular covering of the seed and termed as husk. The seed is exalbuminus. It is solitary, large and flat, ovoid oblong and is surrounded by the endocarp at maturity. The testa is thin and papery (Litz, 1997).

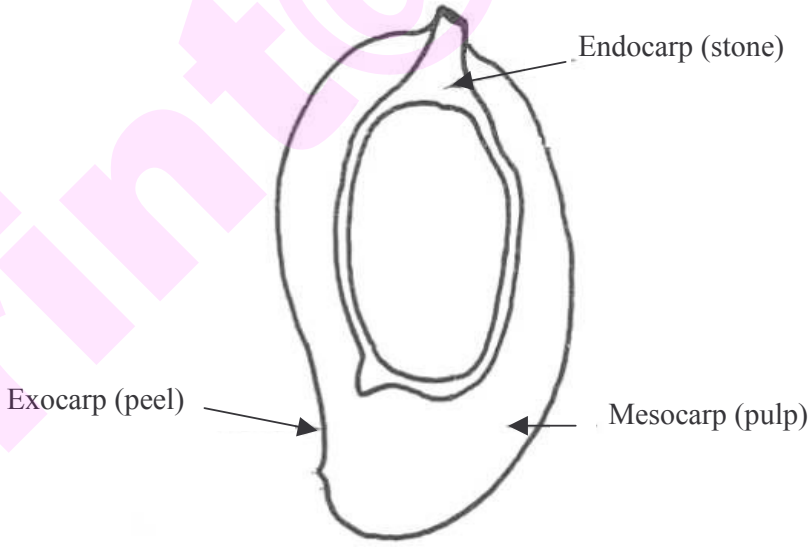


Figure 2.1. Mango fruit cross section

(Source: Hulme, 1971)

2.1.1.2. Mango products

Mango is an important component of the diet in many less developed countries in the subtropics and tropics. Mango fruit is valued because of excellent flavor, attractive fragrance, delicious taste and health giving properties (Morton, 1987). Most of the fruits are consumed as such or processed as relished products. As mango is a seasonal fruit, mango fruit is processed and used at almost every stage of its growth. The range of products includes products derived from both raw as well as ripe fruit. The raw fruits are utilized for products like chutney, pickle, amchur, green mango beverage etc and ripe fruits are used in making pulp, juice, nectar, squash, leather sheets etc. Mango and mango products are experiencing worldwide popularity and also gained increasing importance in European market (Loeillet, 1994). Therefore, processing and product development is an important factor in mango marketing.

2.1.1.3. Nutritional and nutraceutical value of mango

The mango is used as food in all stages of its development. Green mango is a rich source of pectin, which gradually diminishes after the formation of stone. During the ripening process, the fruit is initially acidic, astringent and rich in ascorbic acid. Mango fruit contains amino acids, carbohydrates, fatty acids, minerals, organic acids, proteins and vitamins (**Table 2.1**). The fruit flesh of ripe mango contains about 15% sugar. Sugars present in mango comprise sucrose, glucose, fructose and maltose. Sucrose is the principal sugar of ripe mango. The sucrose content of ripe fruit of three important cultivars viz., Alphonso, Pairie and Totapuri ranges from 11-20% (Lakshminarayana, 1980). Free sugars including glucose, fructose and

sucrose generally increases during ripening. Mango fruit contains 0.5 to 1.0% protein on a fresh weight basis (Lakshminarayana, 1980). The presence of amino acids including the essential ones like lysine, leucine, cysteine, valine, arginine, phenylalanine and methionine in fruits of various cultivars has been recorded. Concentration of amino acids varied from 10-100 mg/100 g edible pulp (Majumdar & Shrama, 1985). Selvaraj and Kumar (1989) reported that total lipid content in seven commercial mango cultivars ranged from 0.26 to 0.67% at harvest.

Table 2.1. Food value of mango (100 g fresh fruit)

Component	Content
Calories	62.10-63.70
Moisture	78.90-82.80 g
Protein	0.36- 0.40 g
Fat	0.30-0.53 g
Carbohydrates	16.20-17.18 g
Fiber	0.85-1.06 g
Ash	0.34-0.52 g
Calcium	6.10-12.80 g
Phosphorus	5.50 – 17.90 g
Iron	0.20-0.63 g

(Source: Morton, 1987)

Mango fruits are particularly rich source of vitamin C. Siddappa and Bhatia (1954) determined the vitamin C content of Raspuri mango fruit in different maturity stages. The raw mango contains more vitamin C than that of ripe or fully ripen mango (Siddappa & Bhatia, 1954; Matto & Modi, 1969). The ascorbic acid content in mango varied from 3.2 to 62.3 mg/100 g of pulp (CSIR, 1962). Fruit acidity is mainly due to the presence of malic and citric

acids. The presence of folic acid (3.6 mg/100 g), vitamin B1 (35-63 mg/100 g) and vitamin B2 (37-73 mg/100 g) in mango has been reported by Majumdar and Sharma (1985). Gopalan et al (1999) reported 0.08 mg of thiamine and riboflavin each and 0.09 mg of niacin per 100 g of ripe mangoes. The vitamin A equivalent in mango ranges between 1000-6000 I U. Beta-carotene was found to account for 60% of total carotenoids in mango. Modi et al (1965) showed that ripe mangoes are ten times richer in carotene than partially ripe ones and they reported that mevalonic acid, a precursor of carotenoids, increased progressively during the ripening of mangoes. Ash content decreased during fruit development with some increase near maturity, while crude fiber remained constant (Kalra et al, 1995).

Mango is known for its medicinal properties at both raw and ripe stages. The unripe fruit is astringent and stimulant tonic. The acid content in the green mango increases the secretion of bile and has a role in intestinal absorption. Ripe mangoes are highly beneficial in the treatment of night blindness. The ripe mango is antiscorbetic, diuretic, laxative and astringent. It increases seven body nutrients called *dhatus* namely, food, juice, blood, flesh, fat, bone marrow and semen in ayurveda. The fruit is beneficial in liver disorders, loss of weight and other physical disturbances (www.indian Gyan.com).

2.1.1.4. Nutracuetical components of mango

Mango is found to be a rich in bioactive compounds such as polyphenolics, carotenoids, vitamins and dietary fibers and many of the pharmacological properties attributed due to the presence of phenolic acids present in them (Singh et al, 2004). El Ansari et al (1969) reported the

presence of gallic acid, *m*-digallic acid, *m*-trigallic acid, gallotannin, quercetin, isoquercetin, mangiferin and ellagic acid in mango fruit. El Ansari et al (1971) made quantification study of the polyphenolic components of Rumani mango at different ripening stages. Digallic acid seemed to undergo a small degree of polymerization, where as quercetin and isoquercetin disappeared during ripening. Another type of polyphenols found in mango fruit is alkyl resorcinols, which are predominantly, found in unripe fruit and decrease in quantity during ripening (Prusky et al, 1988).

2.1.2. Valuable compounds from mango processing by-products

Major by-products of mango processing industry are peels and stones amounting from 15-20% and 10-30% total fruit weight respectively (Beerh et al., 1976; Larrauri et al, 1996). They are currently not being utilized for any commercial purpose; it is becoming a source of pollution.

2.1.2.1. Mango kernel

Mango seed is major waste by-products of mango processing industry and it constitutes about 10-30% of the total fruit weight. The chemical composition of mango seed kernel is given in **Table 2.2**.

Table 2.2. Chemical composition of mango kernel

Component	Content (%)
Moisture	10.55-11.35
Protein	4.76-8.5
Fat	6-15
Starch	40-72
Sugar	1.07
Fiber	1.17-2.6
Ash	1.72-3.66
Silica	0.41
Iron	0.03
Calcium	0.11-0.23
Magnesium	0.34
Phosphorus	0.21-0.66
Sodium	0.28
Potassium	1.31
Sulphur	0.23
Carbonate	0.09

(Source: Morton, 1987)

Tannin content in kernel varied from 0.12 to 0.18% or much higher, depending on the cultivar (Arogba, 2000). Mango seed kernel fat is a good source of edible oils and its fatty acid and tryglyceride profile is similar to that of cocoa butter. It is a promising source of fat, cocoa butter substitutes and other food substitutes (Laksminarayana et al, 1983). Paramar and Sharma (1980) reported that mango seed kernel enhanced the oxidative stability of cheese and ghee. The antioxidant principles were characterized as phenolic compounds and phospholipids (Puravankara et al, 2000). The phenolics reported in mango kernels are mainly gallic acid, ellagic acid, gallate and

gallotannin and condensed tannin related polyphenols (Arogba, 2000). Ethanolic extracts of mango kernel displayed a broad antimicrobial spectrum and were effective against gram-positive bacteria. The active components responsible for this bioactivity was found to be a polyphenolic type structure, however, its exact nature still remains to be elucidated (Kabuki et al, 2000).

2.1.2.2. Mango sap

Sap is another by-product obtained from mango industry. Sap is present in fruit ducts under pressure and when the pedicel is broken at the abscission zone during harvest, the sap spurts out and is deposited on the surface of the same or other fruits causing sap injury (Johnson et al, 1993). De-sapping of mangoes is one of the methods practiced to control sap-injury and the sap thus obtained is currently being wasted. Saby John et al (1999) reported that 100 kg of mango fruits yield 100-250 ml of sap depending on the variety. Sap is a viscous liquid with a pH around 4 and has the characteristic raw mango aroma. Sap can be separated into non-aqueous and aqueous phase. Non-aqueous phase was found to be rich in aroma compounds such as terpenoids (Loveys et al, 1992; Saby John et al, 1999) and it has been shown to posses antimicrobial activity (Negi et al, 2002). The aqueous phase contains various enzymes such as polyphenol oxidase/laccase, peroxidase, polyphenols and carbohydrates (Robinson et al, 1993; Saby John et al, 2003).

2.1.2.3. Mango peel

Mango peel is another major by-product of mango processing industry. It is a rich source of nutrients as shown in **Table 2.3.** Ripe mango peel was reported to be a good source of quality pectin (12.8%) (Tandon et al, 1991).

Srirangarajan and Srikande (1977) investigated the chemical and physical characteristics of mango peel pectin and suggested its commercial exploitation. Sudhakar and Mani (2000) developed a standardized method for the recovery of pectin from Totapuri mango peels. Larrauri et al (1996) reported that mango peels were a good source of dietary fiber containing large amounts of total extractable polyphenolics. Flavonol-O-glycosides and Xanthone-C-glycosides were extracted from Tommy Atkins mango peels and characterized using HPLC-ESI-MS (Scheiber et al, 2003). During mango fruit development the content of total phenolics are higher in the peel than in the flesh at all stages (Lakshminarayana & Subramanyam, 1970). HPLC analyses of peel extracts of Peruvian mangoes revealed that quercetin 3-glucosides were the prominent phenolic compounds (Schieber et al, 2000). The crude fiber content in peel was reported to be 8.4% (Beerh et al, 1976) and total dietary fiber was 28.1% (Larrauri et al, 1998). The total polyphenol content in the peel ranged from 4.4 to 7 % (Larrauri et al, 1998). The mango peel contains antifungal compounds such as 5-(12)-cis heptadecyl-resorcinol and 5-penta decyl resorcinol (Cojocara, 1980).

Table 2. 3. Proximate composition of mango peel

Components	Content (%)*
Total sugars	48.1
Reducing sugars	40.8
Starch	2.9
Pectin	12.9
Protein	3.9
Crude fiber	8.4
Tannin	2.3
Ash	2.9

*Dry weight.

(Source: Beerh et al, 1976)

In the present study, valuable bioactive compounds such as polyphenols, carotenoids, dietary fibers and enzymes in the raw and ripe peels of two Indian mango varieties viz. Badami and Raspuri were determined with the aim of exploiting the potential value of the peel.

2.2. MATERIALS AND METHODS

2.2.1. MATERIAL

2.2.1.1. Plant material

Raspuri and Badami mango varieties grown in CFTRI campus, Mysore, India were used in this study. Mango varieties were harvested at harvest maturity and peel was removed using a sharp knife and the underlying pulp removed by gently scraping with its blunt edge. To obtain the ripe peel, some fruits were kept to ripen at room temperature and the peel was removed as described earlier. The fresh peels thus obtained were used for analysis.

For some experiments, freshly processed mango peel (5 kg) was collected from mango processing industry (Kodagu Foods, Mysore, India) and washed with water, peel was spread in trays and dried at 50±2°C using a cross flow drier (Model PTD-48E, Premium Industries Ltd., Ahemadabad, India). The dried peel was powdered using a hammer mill (M/S Apex instruments, England) and was sieved through 150 micron sieve.

Commercial wheat flour and commercial semolina were used in the study. Commercially available bakery fat, sugar powder and skimmed milk powder were procured from the local market. Food grade dextrose, sodium chloride, sodium bicarbonate and ammonium bicarbonate were used in biscuit making.

2.2.1.2. Chemicals

Vitamin C, pyrogallol, $\propto$-amylase (termamyl), pepsin, pancraetin, celite, α-tocoferol, γ-tocoferol, δ-tocoferol, tocotreinol, bovine serum albumin, Coomassie blue G-250, Coomassie blue R-250, catechol, *o*-dianisidine,

azocaesin, soluble starch, xylan, xylose, bipyridyl pyridine, Tris, 2,5-dinitrosalicylic acid, 2,4-dinitrophenyl hydrazine, gallic acid, caffeic acid, p-coumaric acid, cinnamic acid, ferulic acid, gentisic acid, protocatechuic acid, syringic acid, vannilic acid, β-carotene, butylated hydroxyanisole (BHA), adenosine diphosphosphate (ADP), ascorbic acid, thiobarbituric acid (TBA), soybean lipoxygenase, linoleic acid, rhamnose, glucose, arabinose, galacotse, mannose, xylose were obtained from Sigma Fine Chemicals, St.Louis, USA. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Himedia Laboratories Limited, Mumbai, India. All other chemicals and solvents were of analytical grade.

2.2.2. METHODS

2.2.2.1. Determination of proximate composition

2.2.2.1.1. Determination of moisture content

Moisture content of the mango peel was determined according AOAC method (2005). Sample (2 g) was weighed in an aluminum dish and placed in a hot air oven maintaining at 130±1°C for 4 h. It was cooled to room in a desicator and the loss in weight in percentage was reported as moisture content.

2.2.2.1.2. Determination of ash

Ash content in mango peel was determined according to the procedure described in AOAC method (2005). The mango peel was accurately weighed in a clean silica crucible. The contents of the crucible were dried on a hot plate (100-200ºC) and after the sample stopped emitting smoke, the crucible was kept in a muffle furnace. The temperature of the muffle furnace was

slowly raised to 250-300ºC. Later, the temperature was raised to 550ºC and ashing was continued for 5 h at 550°C. The weight of crucible with its ash content was recorded and the ash content was calculated and expressed as percentage of original sample.

2.2.2.1.3. Determination of fat

Total fat content in the mango peel was determined according to the AOAC method (2005). Sample (10 g) was accurately weighed into a dry thimble and extracted using petroleum ether (60-80°C boiling range) as solvent for 12 h. The extract was collected in a previously weighed dry flat bottom flask and the solvent was evaporated over hot water bath. The flask was dried in an oven at 100°C, cooled and weight was taken. The fat content expressed as g/100 g of sample.

2.2.2.1.4. Determination of crude fiber content

Crude fiber content of the mango peel was determined according AOAC method (2005). Sample (2 g) was taken into a digestion flask, 200 ml of 1.25% sulphuric acid was added, connected to a condenser and boiled for 30 min. Immediately filtered the digested material through a linen cloth and washed with near boiling water until free of acid. To the residue, 200 ml of NaOH solution (1.25%) was added, connected to a condenser and boiled for 30 min. Immediately filtered through a sintered crucible and washed thoroughly with near boiling water to remove the alkali. Finally washed the residue with alcohol followed by ether. Then dried at 100°C and weight was taken. The sintered crucible was transferred to muffle furnace and the

material was made into ash and determined its weight. The crude fiber in percentage of original sample was calculated.

2.2.2.1.5. Determination of total protein content

Total protein content in mango peel was determined by micro-Kjeldhal method according to AOAC (2005). Mango peel (1 g) and 1 g of digestion mixture were taken into a Kjeldhal flask. To this, 20 ml of sulphuric acid was added and digested until the organic matter was oxidized. The digest was cooled and volume was made up to 50 ml with distilled water.

An aliquot of 5 ml was taken for steam distillation with 20 ml of 40% NaOH solution. The liberated ammonia was absorbed in 10 ml of 2% boric acid containing a few drops of mixed indicator. This was titrated against N/70 HCl. Simultaneously, standard (ammonium sulphate) was done to estimate the amount of nitrogen content of the sample. The protein content of sample was calculated in percentage using the factor 6.25.

2.2.2.2. Preparation of acetone extract and acetone powder of mango peel

Both raw and ripe mango peels were removed from the fruits. Peel was homogenized with chilled phosphate buffer (0.05 M, pH 7.5**)** using a homogenizer**.** The homogenate was made upto 80% acetone with respect to acetone by adding chilled acetone and mixed thoroughly and filtered using cheesecloth. The residue was washed with 80% chilled acetone, filtered and air dried (acetone powder). The filtrates (80% acetone extract) were combined and kept in 4°C for further studies. The extractability of 80% ethyl alcohol for

the extraction of bioactive compounds from mango peel also carried out in the same way using ethyl alcohol instead of acetone.

The 80% acetone extract used for the estimation of total phenolic compounds, anthocyanin and carotenoid contents, and evaluated for antioxidant activity. The dried powder obtained after filtering was used for the estimation of dietary fiber and enzymes.

2.2.2.3. Estimation and identification of phenolics

2.2.2.3.1. Determination of total phenolics content

Mango peel extracts of 80% acetone, 80% ethanol and 0.05 M sodium phosphate buffer (pH 7.5) obtained in the previous Section (2.2.2.2) were centrifuged for 15 min at 10,000Xg. The clear supernatants obtained were subjected to total polyphenol estimation using the method of Swain and Hills (1959). To 0.5 ml of extract, 4.5 ml of ethanol was added and to this 0.5 ml phenol reagent (Folin–Ciocalteu reagent, diluted 1: 2 with water) was added and the contents were incubated at room temperature for 3 min. To this, 1 ml of saturated Na_2CO_3 was added and the reaction mixture was incubated at room temperature for 60 min. The absorbance was recorded at 675 nm. Gallic acid was used as a standard (**Figure 2.2**). The total polyphenols content in the extract was expressed as gallic acid equivalents (GAE).

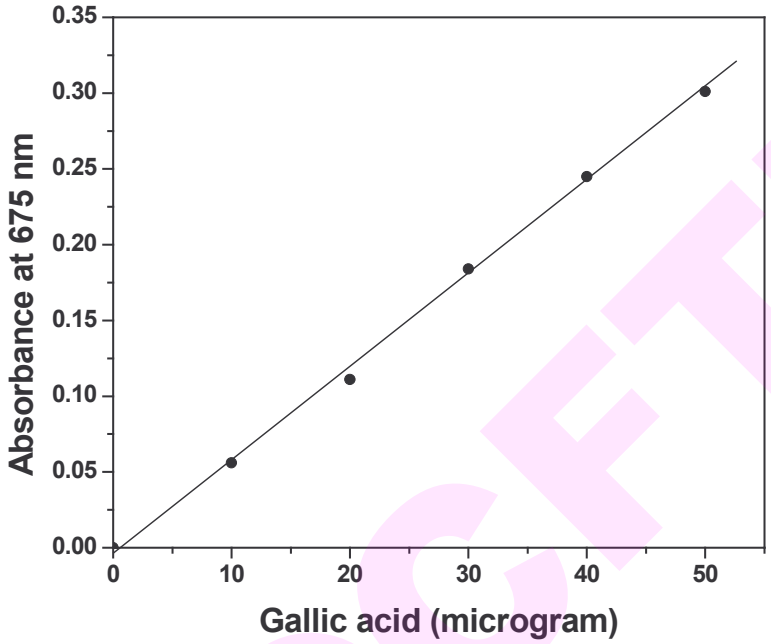


Figure 2.2. Standard graph for the estimation of total phenolics

2.2.2.3.2. Identification of phenolics in acetone extract by HPLC and LCMS

Polyphenols were separated on a reverse phase C18 column (4.6X 250 mm) using HPLC system (Shimadzu, Model LC-10A) using a diode array detector (operating at 280 nm and 320 nm). A solvent system consisting of water: methanol: acetic acid (83:15:2) was used as mobile phase (isocratic) at a flow rate of 1 ml/min (Glowniak et al, 1996). Known quantities of phenolic acid standards such as caffeic acid, p-coumaric acid, cinnamic acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, syringic acid, vanillic acid were used for identification and quantification of phenolic acids present in the acetone extracts.

HPLC-ESI-MS analyses were done on a Waters platform ZMD 4000 system composed of a micro mass ZMD Spectrophotometer, a Water 2690 HPLC and a Waters 996 photo diode array detector (Waters corporation, MA, USA). Data were collected and processed via a personal computer running Mass Lynx software version 3.1 (Micromass, a diversion of Waters corporation, MA, USA). The samples in 10 μl aliquot were separated on a reversed phase C18 column (4.6X 250 mm), using a diode array detector (operating at 280 and 320 nm). A solvent system consisting of water: methanol: acetic acid (83:15:2) was used as mobile phase (isocratic) at a flow rate of 1 ml/min. UV-VIS absorption spectra were recorded on-line during HPLC analysis. Spectral measurements were made over the range of 200-600 nm. The following ion optics was used- capillary voltage 3 KV, cone voltage 100 V and collision voltage 10 V. The source block temperature was 80°C and the desolvation temperature was 150°C. ESI-MS was performed using argon as cone gas (50 L/h) and hydrogen as desolvation gas (50 L/h). The electron spray probe flow was adjusted to 70 ml/min. Continuous mass spectra were recorded over the range M/Z 100-200 with scan time 1 sec and interscan delay 0.1 sec.

2.2.2.3.3. Extraction, estimation and identification of phenolic acids liberated from the soluble bound phenolic compounds

Phenolic acids from acetone soluble bound phenolics were isolated according to the method of Krygier *et al* (1982) with some modifications. The pH of the 80% acetone extract (20 ml) was adjusted to 2 with 4 M HCl and refluxed in boiling water bath for 1 h. Phenolic acids were separated by ethyl

acetate phase separation (5X50 ml) and the pooled ethyl acetate fractions were treated with anhydrous sodium sulphate for overnight to remove moisture, filtered and evaporated to dryness and re-dissolved in methanol. Phenolic content was determined by the method of Swain & Hills (1959) and the phenolic acids were determined separation on reverse phase C18 column by HPLC, according to the procedure described in Section 2.2.2.3.2.

2.2.2.3.4. Extraction, estimation and identification of phenolic acids from dietary fiber bound phenolics (insoluble bound phenolics)

Bound phenolic acids were isolated according to the method of Krygier et al (1982) with some modifications. Mango peel dietary fiber (2 g) was hydrolyzed with 1 M sodium hydroxide containing 0.5% sodium borohydride under nitrogen atmosphere. The hydrolysate was acidified to pH 2 with 4 M HCl and centrifuged and the phenolic acids were obtained using procedure described earlier (Section 2.2.2.3.3). Phenolic content was determined by the method of Swain & Hills (1959) and the phenolic acids were determined by HPLC, according to the procedure described in Section 2.2.2.3.2.

2.2.2.4. Estimation and identification of carotenoids

2.2.2.4.1. Estimation of carotenoids

Mango peel (1g) or acetone extract (2.5 ml) was homogenized with 40 ml of methanol containing 1 g KOH. The mixture was saponified for over night and the saponified mixture was transferred to separating funnel containing 25 ml of hexane and gently shaken for 60 sec, the phases were allowed to separate. The aqueous phase was separated and was re-extracted in the separating funnel with 25 ml hexane. This was repeated until the hexane

extract was colorless. The hexane extracts were pooled, washed with water until free of alkali, dried over sodium sulphate and concentrated in a vacuum evaporator at room temperature. The resulting solution was made up to a suitable volume with hexane.

The total carotenoid content in the hexane extract was estimated using two different colorometric methods reported by Litchenthaler (1987) and by Davis (1976) as described below. The carotenoid content in mango peel acetone extract was calculated by Litchenthaler method using the following formula.

$$\text{Chlorophyll a } (C_a) = 12{,}25A_{663.2} - 2.79A_{646.8}$$

$$\text{Chlorophyll b } (C_b) = 21.50A_{646.8} - 5.10A_{663.2}$$

$$\text{Total Carotenoid} = \frac{1000A_{470} - 1.82C_a - 85.02\,C_b}{198}$$

The carotenoid content in the peel acetone extract was calculated by Davis method using the following formula.

$$\text{Total carotenoid} = \frac{A_{450} \text{ X total volume of sample X dilution factor X } 10}{2500}$$

2.2.2.4.2. Identification of carotenoid by HPLC

Carotenoids in the hexane extract were separated on a reverse phase C18 column (4.6X 250 mm) using HPLC system (Model LC- 10A, Shimadzu) with a diode array detector (operating at 460 and 425 nm) using a gradient of two solvents, solvent A-100% acetone and solvent B-90% methanol and 10% water (Sarada et al, 2006). The gradient for separation consists of 1% B traversing to 80% in 40 min at a flow rate of 1 ml/min. The detection was carried out using diode array detector at 445 nm and 460 nm. Peak

identification was based on the comparison of retention time value with authentic standards of carotenoids such as β-carotene, lutein and violoxanthin. The carotenoids were quantitated based upon peak areas relative to standard calibration plots by external standard method.

2.2.2.5. Estimation of Vitamin C

Vitamin C (ascorbic acid) content was determined according to the method of Omaye et al (1973). Peel (100 mg) was extracted with 10 ml of ice-cold 10% TCA and centrifuged for 20 min at 1,500Xg. Supernatant (0.5 ml) was mixed with 0.1 ml of DTC reagent (2,4 dinitrophenyl hydrazine, thiourea, copper sulfate reagent) and incubated for 3 h at 37°C. To this, ice-cold 750 μl of 0.5% sulfuric acid was added, mixed thoroughly and allowed to stand at room temperature for 30 min. Absorbance was measured at 520 nm. Ascorbic acid was used as a standard **(Figure 2.3).**

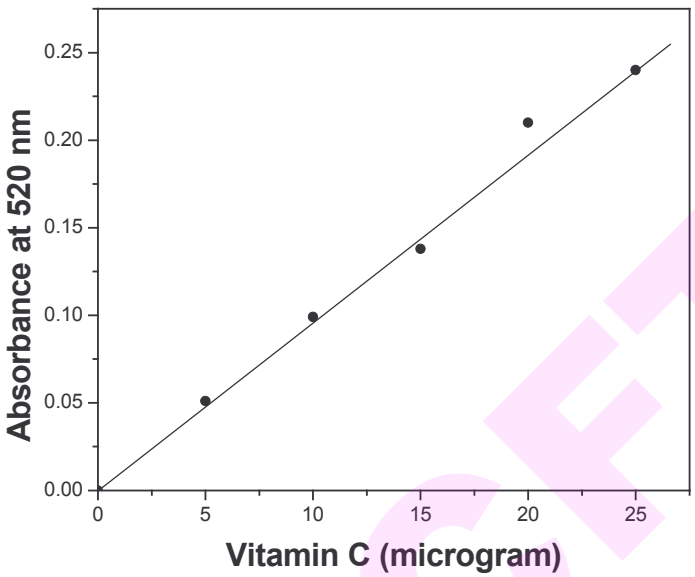


Figure 2.3. Standard graph for the estimation of vitamin C

2.2.2.6. Estimation of Vitamin E

Vitamin E content in mango peel was determined according to the method of Joshi and Desai (1952). Peel (2 g) was homogenized with 4 ml of 0.5% pyrogallol ethanolic solution, refluxed for 1 min and added 1 ml of 40% KOH solution. After cooling to room temperature, 25 ml of water was added. The mixture was transferred to a separating funnel, 25 ml of diethyl ether was added, gently shaken for 1 min and the phases were allowed to separate. The aqueous layer was separated and re-extracted with 25 ml of diethyl ether. This was repeated until the extract was colorless. The diethyl ether extracts were pooled, washed with water until free of alkali, dried over anhydrous sodium sulphate and concentrated using vacuum evaporator at room

temperature. The resulting solution was made up to a suitable volume with hexane. The appropriate amount of sample was made upto 4 ml by ethyl alcohol. To this, 200 μl of 0.2% $FeCl_3$ solution and 0.2% bipyridyl pyridine solution were added and measured the absorbence at 520 nm. α-Tocoferol was used as a standard to estimate total vitamin E content (**Figure 2.4**).

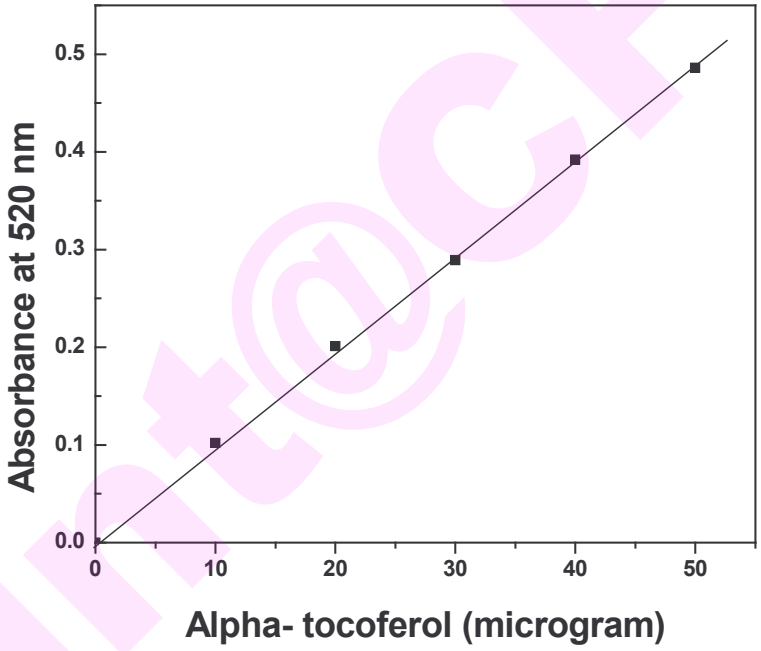


Figure 2.4. Standard graph for the estimation of vitamin E

2.2.2.7. Estimation and characterization of dietary fiber

2.2.2.7.1. Soluble and insoluble dietary fibre determination

The dietary fibre estimation was done by an enzymatic gravimetric method (Asp et al, 1983). Fresh peel/peel acetone powder (0.5 g) was homogenized in 20 ml of sodium phosphate buffer (pH 6.0). α-Amylase enzyme (Termamyl) 50 µl was added and incubated at 50ºC for 30 min with occasional stirring. The contents were cooled to room temperature, 10 ml of water was added and pH was adjusted to 1.5 with 4 N HCl. To this mixture, pepsin (50 mg) was added and incubated in a shaking water bath at 37ºC for 1 h. The contents were again cooled to room temperature, 10 ml of water was added and adjusted the pH to 6.8 with 4 N NaOH solution. To this mixture, pancreatin (50 mg) was added and incubated for 1 h at 37ºC. The contents were cooled and adjusted the pH to 4.5 with 4 N HCl and filtered through a dried and weighed crucible containing 0.5 g of celite (Drying and weighing of crucible was done by adding 0.5 g of celite into the crucible and washing it with 20 ml of 95% ethanol and 20 ml acetone. It was then dried in oven at 105ºC for 30 min and weighed).

The residue (insoluble fibre) retained on the crucible, washed with 20 ml of 95% ethanol and 20 ml of acetone, the crucible was dried at 105 ºC overnight and weight was taken.

To the filtrate (soluble fibre), four volumes of 95% ethanol was added and kept at room temperature for one hour for precipitation. The precipitate was filtered through a dried and weighed crucible containing celite as described earlier and washed with 20 ml of 95% ethanol and 20 ml of acetone. Crucibles were dried at 105ºC for over night and weight was taken.

The crucibles were incinerated at 500ºC in a muffle furnace for 8 h. Blank was processed as above without sample for both soluble and insoluble dietary fibre analysis.

2.2.2.7.2. Determination of sugar composition of dietary fibre by Gas Liquid Chromatography

Dietary fiber was isolated from mango peel according to the method of Asp et al (1983). The isolated dietary fibres (10 mg) were suspended in water (0.5 ml) and solubilized with concentrated sulphuric acid (0.6 ml) at ice-cold temperature, after which the concentration of sulphuric acid was brought down to 8% by the addition of water. The above mixture was refluxed in a boiling water bath for 10 to 12 h, volume was made up to 20 ml. The mixture was neutralized with barium carbonate, concentrated, deionized and reduced with sodium borohydride. Alditol acetates were prepared according to the method of Sawardekar et al (1965). The component sugars were separated and identified on a 3% OV-225 (1/8" X 6') column using a Shimadzu 14-B Gas Liquid Chromatograph equipped with flame ionization detector at 200°C column temperature, and 250°C injector and detector port temperatures. Nitrogen (40 ml/min) was used as the carrier gas. A sugar mixture consisting of rhamnose, arabinose, xylose, mannose, galactose and glucose was used as reference sugars.

2.2.2.8. Extraction of enzymes from mango peel

To 1 g of fresh peel/mango peel acetone powder, 0.5 g of acid washed sand was added and ground into paste using 5 ml of 50 mM sodium phosphate buffer (pH 7.5) using mortar and pestle at 4°C. To the resultant

paste, 20 ml of buffer was added, stirred for 1 h and was centrifuged at 8,000 X g for 15 min at 4°C. The supernatant obtained was used to estimate the protein content and to assay peroxidase, polyphenol oxidase, xylanase, protease and amylase activities.

2.2.2.8.1. Protein estimation

The protein content in the mango peel extract was determined using the dye binding method described by Bradford (1976). Bovine serum albumin (BSA) was used as a standard (**Figure 2.5**).

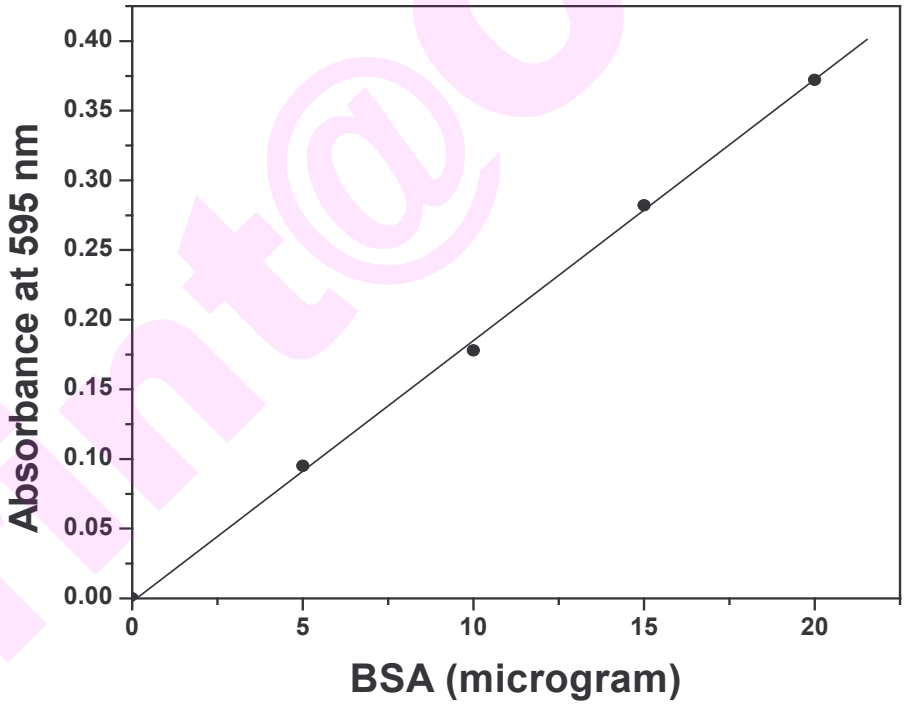


Figure 2.5. Standard graph for the estimation of protein

2.2.2.8.2. Peroxidase assay

Peroxidase activity in the peel extracts was measured using H_2O_2 and *o*-dianisidine (ODA) as substrates as described by Saby John et al (2003). The 1 ml reaction mixture contained varying amount of the appropriately diluted enzyme, 0.1 ml of 0.25% ODA and 0.1 ml of 1% H_2O_2 in 0.05 M sodium acetate buffer (pH 5.5). One unit of peroxidase activity was defined as the amount of enzyme that caused increase in the absorbance of 1 per min at 460 nm.

2.2.2.8.3. Polyphenol oxidase assay

Polyphenol oxidase activity in the peel extracts was assayed using catechol as substrate as described by Saby John et al (2003). The 1 ml reaction mixture contained varying amount of appropriately diluted enzyme, 0.1 ml of 0.5 M catechol and the remaining volume was made up with 0.05 M sodium phosphate buffer pH (7.0). One unit of enzyme activity was defined as the amount of enzyme that caused increase in absorbance of 1 per min at 420 nm.

2.2.2.8.4. Protease assay

Protease activity in the mango peel extracts was estimated using the azocaesin hydrolysis (Sarath et al, 1989). To 0.45 ml of 0.05 M Tris- HCl buffer (pH 8.0), 0.05 ml of azocaesin solution (25 mg/ ml) was added and the solution was pre-incubated at 37°C for 10 min. To this, 0.2 ml of appropriately diluted enzyme extract was added and it was incubated at 37°C for 30 min. To the reaction mixture, 0.5 ml of 10% trichloroacetic acid was added, kept in ice (4-8°C) for 10 min, centrifuged at 8,000Xg for 10 min and the supernatant was

transferred into a fresh tube. To the supernatant, 0.04 ml of 10 M sodium hydroxide was added and was kept at room temperature for 5 min. The absorbance of the solution was measured at 440 nm. An increase in absorbance of 1 was regarded as one unit of activity.

2.2.2.8.5. Amylase assay

Amylase activity in the mango peel extract was determined according to the method described by Bernfeld (1955). Gelatinized soluble starch (1%, 1 ml) in sodium acetate buffer (50 mM, pH 4.6) was incubated with appropriately diluted enzyme extract at 45ºC for 30 min. The reaction was stopped by adding 1 ml of 2,5-dinitrosalicylic acid (DNS) reagent (1 g of 2,5-dinirtosalicylic acid and 30 g of sodium potassium tartarate in 100 ml of 0.4 N NaOH) and boiling for 10 min. The reducing sugar released was estimated by DNS method (Luchsinger & Cornesky, 1962). Glucose was used as standard for the DNS method. One unit of enzyme activity was defined as µmole maltose equivalent released per min under the assay conditions.

2.2.2.8.6. Xylanase assay

Xylanase activity in the mango peel extract was determined according to the method described by Miller (1959). The reaction mixture containing 1 ml of xylan solution (0.5% in sodium acetate buffer (pH 4.8, 0.1 M) was incubated with 100 µl of appropriately diluted enzyme extract at 50ºC for 60 min with constant stirring. The reaction was stopped by the addition of 1 ml of 2, 5-dinirtosalicylic acid reagent and boiling for 10 min. The reducing sugar released was estimated by DNS method (Luchsinger & Cornesky, 1962). Xylose was used as a standard. One unit of xylanase activity was defined as

the amount of enzyme required to release 1 μmole xylose per min under the experimental conditions.

2.2.2.9. Determination of antioxidant activity by different methods

2.2.2.9.1. Measurement of reducing power

The reducing power of the mango peel extract and synthetic standard, BHA was determined according to the method of Yen and Chen (1995). The mango peel extract containing 5 to 20 μg of gallic acid equivalent (GAE) was made up to 500 μl with 0.2 M phosphate buffer (pH 6.6) and mixed with 1 ml of potassium ferricyanide (0.1%) and the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (500 μl, 10%) was added to the reaction mixture and centrifuged at 3,000xg for 10 min. The supernatant obtained was mixed with equal volume of distilled water and 300 μl of 1% ferric chloride was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated the increased reducing power. The antioxidant activity of the extract was compared with BHA.

2.2.2.9.2. Measurement of free radical scavenging Activity

The effect of acetone extracts of mango peel and synthetic standard, BHA on DPPH radical was determined according to the method described by Blois (1958) with modification described by Brand–Williams *et al* (1995). A 100 mM solution of DPPH in methanol was prepared and mango peel extract (200 μl) containing 1 to 5 μg GAE was mixed with 1 ml of DPPH solution. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured at 517 nm. The

control contained all the reagents except peel extract/BHA. The capacity to scavenge DPPH radical was calculated by following equation.

Scavenging activity (%) = 1- (A_s/ A_0) X100

Where A_0 is the absorbance at 517 nm of the control and A_s is the absorbance in the presence of peel extract or BHA. The results were plotted as the % of scavenging activity against concentration of the sample. The half-inhibition concentration (IC_{50}) was defined as the amount of GAE required for 50% of free radical scavenging activity. The IC_{50} value was calculated from the plots as the antioxidant concentration required for providing 50% free radical scavenging activity.

2.2.2.9.3. Measurement of inhibitory effect on microsomal lipid peroxidation

Liver microsomes were prepared according to the method of Kemp and Writz (1974). The protein content in the microsomes was determined according to the method described by Lowry (1951).

The liver microsomal lipid peroxidation was performed according to the method of Miller and Aust (1989) with some modifications. To 100 μl peel extract containing 1- 5 μg of GAE, 1 ml of microsomes suspension (100 μg protein) was added. Lipid peroxidation was induced by adding 25 μl ADP (2 mM), 25 μl of $FeSO_4$ (4 mM) and 25 μl of ascorbic acid (0.1 mM). After incubation for 1 h at 37°C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% TCA and 0.375% thiobarbituric acid. The reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm. The blank contained all the reagents and peel

extract except microsomes while control contained all the reagents and microsomes except peel extract/ BHA. Inhibition of lipid peroxidation (%) by the extract was calculated using following equation. Lipid peroxidation inhibition (%) = 1- (A_s/ A_0) X100

Where A_0 is the absorbance value of the fully oxidized control and A_s is the absorbance in presence of extract.

The percentage of inhibition was plotted against the concentration of the peel extract that was expressed in terms of GAE. The half-inhibition concentration (IC_{50}) value was defined as the amount of GAE required for inhibition of 50% of lipid peroxidation. The IC_{50} value was calculated from the plots as the antioxidant concentration required for providing 50% of the lipid peroxidation inhibition.

2.2.2.9.4. Measurement of inhibitory effect on lipoxygenase activity

Lipoxygenase inhibiting activity was measured according to the method described by Shobana and Naidu (2000) with some modification. The enzymatic lipid peroxidation was measured spectrophotometrically following an increase in absorbance of lipid hydroperoxide formation at 234 nm. The 1 ml reaction mixture contained 250 µM linoleic acid substrate solution, 5 nM soybean lipoxygenase and 50 mM Tris buffer (pH 9.0). Different concentrations of mango peel extracts (2.5-40 µg GAE) were incubated with soybean lipoxygenase for 2 min prior to the initiation of the reaction with linoleic acid. The decrease in hydroperoxide formation in presence of peel extracts or BHA was calculated. Control was done without using any inhibitors (peel extract/BHA). The IC_{50} value was determined by plotting the graph with

extract concentration versus percentage of inhibition of lipid peroxidation. The half-inhibition concentration (IC_{50}) value was defined as the amount of GAE required for inhibition of 50% of lipoxygenase activity.

2.2.2.9.5. Preparation of rat erythrocytes

Blood samples were obtained from male wistar rats with a body weight of 180-220 g by heart puncture. Blood was collected in heparinized tubes and centrifuged (1,500Xg, 10 min) at 4°C using a refrigerated centrifuge. Erythrocytes were separated from the plasma and buffy coat, and were washed three times by centrifugation (1,500Xg, 5 min) in ten volumes of 10 mM sodium phosphate buffer containing 0.9% saline (pH 7.4, PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4°C and used with in 6 h for further studies.

2.2.2.9.6. *In vitro* assay inhibition of rat erythrocyte hemolysis

The inhibition of rat erythrocyte hemolysis by the mango peel extract was evaluated according to the procedure described by Tedesco et al (2000) with some modifications. The rat erythrocyte hemolysis was performed by H_2O_2 as free radical initiator. To 100 μl of 5 % (v/v) suspension of erythrocytes in PBS, 50 μl of mango peel extracts with different concentrations (5-25 μg of GAE in PBS, pH 7.4) were added. To this, 100 μl of 100 μM H_2O_2 (in PBS, pH 7.4) were added. The reaction mixture was shaken gently while being incubated at 37°C for 3 h. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 2,000Xg for 10 min. The absorbance of the resulting

supernatant was measured at 540 nm by spectrophotometer. Like wise, the erythrocytes were treated with 100 μM H_2O_2 and without inhibitors (mango peel extract) to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. The inhibitory effect of the extract was compared with standard antioxidant BHA. The percentage of inhibition was calculated, plotted against the concentration of the samples and IC_{50} values were determined.

2.2.2.9.7. *In vitro* assay for the inhibition of lipid peroxidation on rat erythrocyte ghost membrane

The erythrocyte ghost membranes were prepared following the procedure of Fairbanks (1971) by hypotonic lysis of erythrocytes in 5 mM phosphate buffer (pH 8.0) with the addition of 1 mM EDTA to the lysis buffer. Protein content in the membrane was determined according to the method described by Lowry (1951) using bovine serum albumin as a standard.

The erythrocyte ghost membrane lipid peroxidation was performed according to the method of Stocks and Dormandy (1972) with slight modification. Peel extract containing 5-25 μg of GAE concentration was added to 1 ml of erythrocyte ghost membrane (200 μg protein) and lipid peroxidation was induced in the membrane by the addition of 100 μl of 200 μM H_2O_2. After incubation for 1 h at 37^{o}C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% TCA and 0.375% thiobarbituric acid. The reaction mixture was boiled for 15 min, cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm. The appropriate blanks and controls were run along with the test samples. The percentage of inhibition was

calculated and plotted against the concentration of the samples and IC_{50} values were determined.

2.2.2.9.8. Evaluation of oxidative damage on rat erythrocyte ghost membrane proteins by SDS–PAGE

The erythrocyte ghost membranes were prepared by following the procedure of Fairbanks (1971) by hypotonic lysis of erythrocyte in 5 mM phosphate buffer (pH 8.0) with the addition of 1 mM EDTA to the lysis buffer. The oxidative modifications on membrane proteins were done according to the method described by Carini *et al* (2000) with slight modifications. The membrane protein oxidation was induced by addition of 100 μl of 200 μM H_2O_2 to 1 ml of reaction mixture containing 200 μg of protein and with or without mango peel extract (10 μg of GAE) and incubated for 1 h at room temperature. Appropriate controls were run along with the test samples. SDS–PAGE was performed on 7.5% gel according to the method of Laemmli (1970). The protein bands were visualized by staining with Coomassie brilliant blue.

2.2.2.9.9. Evaluation of protective effect on rat erythrocyte by Scanning Electron Microscopy

Erythrocytes (50 μl) were incubated with or with out mango peel extracts and treated with 100 μl of 200 μM H_2O_2 for 1 h at 37^{o}C. After incubation, the erythrocytes were centrifuged at 2,000Xg for 10 min and the cell pellets were processed for scanning electron microscope studies according to the method described by Agarwal and Sultana (1993). For

scanning electron microscopy, cell pellets were fixed in 3% glutaraldehyde on a cover slip (Hoyer & Bucana., 1982). After fixation, the cover slips were dehydrated in an ascending series of acetone (30-100%). The dried samples were mounted on an aluminum stubb (100-200 A^{o}) using double sided tape and coated with gold film to a thickness of 10-20 nm using a sputter coater (Polaron, E 5000, SEM coating system). The cells were examined under a scanning electron microscope (Model No. LEO 425 VP, Electron microcopy LTD, Cambridge, UK) with an acceleration voltage of 20 KV. The magnification used was 4000X.

2.2.2.10. Preparation of mango peel incorporated food products

2.2.2.10.1. Chemical analysis of mango peel powder

Mango peel powder (MPP) was analyzed for moisture, ash, protein and fat, according to the standard AOAC methods (2005) as described earlier in Section 2.2.2.1. Nitrogen content was estimated by micro-Kjeldhal method and was converted to protein by using the factor 6.25.

MPP was extracted with 80% acetone and the supernatant obtained was analyzed for total phenolics and carotenoids as described earlier (Section 2.2.2.3.1 & 2.2.2.4.1).

2.2.2.10.2. Effect of mango peel on dough characteristics

Blends of 2.5%, 5.0%, 7.5% and 10% were prepared by substituting wheat flour with MPP. The effect of MPP on dough rheology was determined using Brabender Farinograph (Model E-380, Brabender OHG, Duisburg, Germany) according to the standard AACC methods (2000). Brabender Farinograph is the most universally used physical dough testing instrument by

means of which water absorption and mixing profile of the dough are determined.

Fifty gram flour (14% moisture basis) was transformed into Farinograph mix bowl. The flour was dry mixed for one min. The amount of water was added in such way that the consistency of the dough was maintained at 500 BU. Parameters measured were water absorption, dough development time, dough stability and mixing tolerance index.

2.2.2.10.3. MPP incorporated biscuits

2.2.2.10.3.1. Biscuit making studies

Baking of biscuits was carried out for blends containing different levels of MPP according to the method of Leelavathi and Haridas Rao (1983). The formula used was as follows: 200 g flour, 60 g sugar, 50 g shortening, 2 g sodium chloride, 0.8 g sodium bicarbonate, 3 g ammonium bicarbonate, 4 g dextrose, 4 g skimmed milk powder and 40-42 ml water. Sugar and fat were creamed in a Hobart mixer (N-50) with a flat beater for 3 min at 61 rpm (speed 1) to obtain a smooth cream. Sodium bicarbonate, sodium chloride and ammonium bicarbonate were dissolved in water and added to the cream. Skimmed milk powder was made into suspension with water and transferred to the cream. The above contents were mixed for 6 min at 125 rpm (speed 2) to obtain a homogeneous cream. Flour was added to the above cream and mixed for 2 min at 61 rpm (speed 1) to obtain the biscuits dough. The biscuit dough was sheeted to a thickness of 3.5 mm, cut using a circular mould (51 mm dia) and baked at 205°C for 8-9 min. After baking, biscuits were cooled to

room temperature and were packed in polypropylene pouches and sealed till further analysis.

2.2.2.10.3.2. Evaluation of physical characteristics of biscuits

Diameter (W) of biscuits was measured by laying six biscuits edge-to-edge with the help of a measuring scale. The same set of biscuits were rotated 90° and the diameter was remeasured. Average values of these biscuits are reported in mm. Thickness (T) of biscuits was measured by stacking six biscuits on top of one another and taking average of six biscuits in mm. The spread ratio was calculated by dividing width (W) by thickness (T).

The objective evaluation of texture, expressed as breaking strength (kg force), was measured using the triple beam snap (three-point break) technique as described by Gains (1991) using a Texture Analyzer (TA-HDi, Stable Microsystems, UK). A cross-head speed of 10 mm/min with a load cell of 50 kg was used in these studies. Force required to break biscuits individually was noted and the average was calculated.

2.2.2.10.3.4. Color measurement of biscuits

The surface color (brightness L) was measured using Hunter Color Measuring System (Labscan, XE, Hunter Lab Inc, USA). Average of six values was taken for each set of samples.

2.2.2.10.3.5. Sensory evaluation of biscuits

The sensory characteristics of the MPP incorporated biscuits were conducted to determine the acceptability of the product. Biscuit samples were presented in a sealed pouch to six panelists and were asked to rate each

sensory attribute. Biscuits were evaluated for surface color, surface appearance, texture, taste, flavor and overall quality in a 9-point hedonic scale (Hooda & Jood, 2005).

2.2.2.10.3.6. Chemical and biochemical analysis of biscuits

Biscuits were powdered and were used for further analysis. Moisture content of biscuits was determined according to Bureau of Indian Standards (IS 1011-1981). Five grams of biscuit powder was dried in an oven at 105±1°C for 4 h. The difference in weight before and after drying was recorded and expressed as percent moisture content.

One gram of powdered biscuit was extracted with 20 ml of 80% acetone and centrifuged at 8,000Xg at room temperature. The supernatant obtained was used for the analysis of total phenolics and carotenoids and for the determination of antioxidant activity. The total phenolics content and total carotenoids content was estimated according to the method described in Section earlier as in 2.2.2.3.1 and 2.2.2.4.1 respectively. Soluble, insoluble and total dietary fiber content in the biscuits powder was estimated according to the method described earlier as in Section 2.2.2.7.

2.2.2.10.3.7. Measurement of antioxidant activity by free radical scavenging activity

Biscuits powder was extracted with 80% acetone and the extracts corresponding to 5-25mg powder were subjected to antioxidant assays. The antioxidant activity in the acetone extract of biscuit samples and MPP was determined by free radical scavenging activity as described earlier in section

2.2.2.9.2. Free radical scavenging activity of mango peel powder ranging from 25 to 100 μg was also determined in the same way.

2.2.2.10.4. MPP incorporated macaroni

2.2.2.10.4.1. Macaroni preparation

Macaroni samples were prepared by incorporating 2.5%, 5% and 7.5% levels of MPP. Blends of semolina and MPP were pre-mixed with pre-calculated amount of warm distilled water (40°C) in a Hobart mixer (Model N-50, Richmond Hill, Ontario, Canada) at speed 1 (61 rpm) for 5 min to make 500 g dough using 150 ml water. The premixed mixture of semolina, MPP and water was transferred to a laboratory single screw pasta machine (La Monferrina, Model dolly, Asti, Italy) and further mixed and kneaded for 10 min at 60 rpm. The dough was extruded using single screw laboratory pasta machine. The temperature of extruded dough 40±2°C. A die was used to shape the dough and obtain the spiral shaped macaroni strands. The extruded macaroni sample was dried at 85°C for 3 h in a laboratory dryer (Model Sakav, Mumbai, India).

2.2.2.10.4.2. Evaluation of macaroni cooking quality

Macaroni samples (10 g) were cooked in 200 ml of boiling distilled water for 10 min according to the method of Bureau of Indian Standards (BIS, IS 1485, 1993).

a) Cooking loss (total solids in gruel) - Cooking loss of different macaroni samples was determined according to BIS method (IS 1485: 1993) with some modifications. Macaroni samples (10 g) were cooked in 200 ml of boiling water for a period of 10 min with occasional stirring. After cooking, the gruel

was drained using a Buchner funnel into a flask and the residue was rinsed with 50 ml of distilled water for 30 sec and was allowed to drain for 2 min. The collected gruel was pooled and the total volume (V) was measured. The gruel was shaken well for even distribution of the solid content, and 20 ml of the gruel was taken into a petri dish and evaporated to dryness on a water bath. The petri dish was transferred to a hot air oven maintained at 105±2°C and dried to constant weight. The cooking loss was calculated using the following equation

$$\text{Cooking loss (\%)} = \frac{(M2-M1) \text{ X } V \text{X} 100}{20 \text{ X } 10}$$

Where,

M2= Mass, in g of petri dish with total solids

M1 = Mass, in g of empty petri dish

V = Total Volume of gruel (ml)

Weight of the macaroni sample- 10g

b) Cooked weight: The cooked weight was determined by weighing the drained and rinsed macaroni and reported in grams.

c) Macaroni firmness: Macaroni firmness was measured according to the method described by Walsh and Gilles (1971) with some modifications using a Universal texture measuring system (LLOYDS instruments, LR-5K, Hampshire, UK). Cooked macaroni samples were immediately transferred to a 250 ml beaker containing distilled water, at room temperature. Two cooked macaroni strands were placed on a sample holder parallel to each other, sheared using a specifically designed aluminum-shearing blade with a contact surface of 1 mm. The shear was performed at a crosshead speed of 10

mm/min and a load cell of 5 Kg. The force (gf) required to shear the macaroni was measured in triplicate and the average value was reported.

2.2.2.10.4.3. Sensory evaluation of macaroni

Macaroni products were cooked in boiling water without addition of salt and drained as described earlier in Section 2.2.2.10.4.2a. Five-membered panel evaluated the cooked macaroni for different sensory attributes such as color, texture, taste, and flavor.

2.2.2.10.4.4. Biochemical analysis of macaroni

Both control and peel incorporated macaroni samples were made into powder using mortar and pestle and extracted at room temperature with 20 ml of 80% acetone for 1 h with occasional stirring using vertex mixer. The extract was centrifuged at 8,000Xg. The supernatant obtained was subjected for the estimation of bioactive compounds such as total phenolics, carotenoid and antioxidant activity. The total phenolics content and total carotenoid content was estimated according to the method described in Section earlier as in 2.2.2.3.1 and 2.2.2.4.1 respectively. Soluble, insoluble and total dietary fiber content in the macaroni powder was estimated according to the method described earlier as in Section 2.2.2.7.

2.2.2.10.4.5. Measurement of antioxidant activity by free radical sc avenging method

Macaroni powder was extracted with 80% acetone and the extracts corresponding to 10-50 mg powder were subjected to antioxidant assays. The antioxidant activity in the acetone extract of macaroni samples was

determined by free radical scavenging activity as described earlier in Section 2.2.2.9.2.

2.2.2.11. Statistical analysis

All analyses were performed in triplicate and data were reported as means±SD unless otherwise described. Duncan's new multiple range tests was used to determine the difference of means, and $P \leq 0.05$ was considered to be statistically significant (Steel & Torrie, 1980).

2.3. RESULTS AND DISCUSSION

2.3.1. SECTION A: Isolation and characterization of valuable compounds from mango peel

Peels were removed from raw and ripe mango fruits of different varieties and the proximate composition of the peels were determined. Further, compounds such as polyphenols, carotenoids, vitamin C and E, different enzymes and dietary fiber were isolated from these peels. Some of the polyphenols, carotenoids and sugar constituents of dietary fiber were also determined and the results are presented below.

2.3.1.1. Proximate composition

The proximate composition of raw and ripe mango peels (fresh) of Badami and Raspuri is shown in **Table 2.4**. The moisture content ranged from 66 to 75% and it was found to be more in ripe mango peels. The total protein content in peel ranged from 1.45 to 2.05%. The fat content ranged from 2.16 to 2.66%. The carbohydrate content in the mango peel ranged from 11.63 to 24.60%. The crude fiber content ranged from 3.28 to 7.40% and was found to be higher in ripe mango peels. The ash content ranged from 1.16 to 3.00%. Selvaraj and Kumar (1989) reported that total lipid content in mango fruits of seven cultivars ranged between 0.26 to 0.67% at harvest. The proximate composition of mango fruit pulp was 81% moisture, 0.6% protein, 0.4% fat, 0.4% minerals, 0.7% fiber and 16.9% carbohydrate as reported by Gopalan *et al* (1999). Pulp being the juicy portion of the fruit, moisture content of the pulp is more than the peel, while most of the other components in pulp are lower compared to peel.

Table 2.4. Proximate composition (%) of fresh mango peel

Mango variety	Moisture	Protein	Carbohydrate	Fat	Crude fiber	Ash
Raspuri raw	66.00 ±0.50^{a}	1.76 ±0.50^{b}	24.60 ±0.46^{d}	2.49 ±0.03^{b}	3.80 ±0.30^{b}	1.40 ±0.20^{a}
Raspuri ripe	72.50 ±0.50^{c}	2.05 ±0.02^{c}	16.27 ±0.35^{b}	2.22 ±0.02^{a}	5.80 ±0.10^{c}	1.16 ±0.14^{a}
Badami raw	70.25 ±0.25^{b}	1.45 ±0.11^{a}	19.86 ±0.22^{ac}	2.16 ±0.06^{a}	3.28 ±0.14^{a}	3.00 ±0.20^{b}
Badami ripe	75.25 ±0.25^{d}	1.76 ±0.08^{b}	11.63 ±0.17^{a}	2.66 ±0.03^{c}	7.40 ±0.20^{d}	1.30 ±0.10^{a}

All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$),

2.3.1.2. Total phenolic content in mango peel

Mango peels were extracted with 80% (v/v) acetone or 80% (v/v) ethyl alcohol or sodium phosphate buffer (50 mM, pH 7.5) separately and the total phenolic contents in the extracts were determined. Of the three extracts, acetone extracted maximum amount of polyphenols followed by ethanol from both raw and ripe peels (**Table 2.5**). Polyphenol contents in acetone extracts of raw and ripe fresh peels varied from 55 to 110 mg GAE/g peel (dry weight basis). The polyphenol content was found to be significantly higher in raw peels compared to ripe peels. Earlier, Larrauri et al (1996) reported the total polyphenol content in aqueous methanol extract of ripe peel of Hayden variety of mango to be 70 mg/g. These values are in the range reported in the

present study. Ueda *et al* (2000) determined the total polyphenol content in Irwin variety of mango peels and they reported that the polyphenol content in ripe peel was higher compared to that of raw peel. The content of total polyphenol was higher in the peel than the pulp at any stage of mango fruit development (Lakshminarayana et al, 1970; Ueda et al, 2000). The total polyphenol content in grape pomace extracts was reported to range from 68.8 to 98.3 mg GAE/g (Gulcan et al, 2004) which is comparable to polyphenol content in mango peels, where as in apple pomace it was reported to be 33.42 mg GAE/g (Wolfe et al, 2003) much lower than mango peels.

As 80% acetone extracted maximum amount polyphenols from the raw and ripe peels of different mango varieties, acetone extract was used for further studies such as identification of polyphenols and antioxidant properties.

Table 2.5. Total polyphenol content* in fresh mango peel extract

Mango variety	Acetone extract (mg/g)	Alcohol extract (mg/g)	Buffer extract (mg/g)
Raspuri Raw	109.7±0.82^{d}	73.88±0.35^{c}	29.40±0.60^{d}
Raspuri Ripe	100.00±1.90^{c}	46.31±3.50^{b}	13.90±1.24^{b}
Badami Raw	90.18±0.57^{b}	37.92±0.86^{a}	20.10±0.72^{c}
Badami ripe	54.67±1.50^{a}	33.31±1.20^{a}	9.84±0.88^{a}

*Values are expressed on dry weight basis as gallic acid equivalents.

All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differ significantly ($P \leq 0.05$)

2.3.1.3. Anthocyanin content in mango peel

Anthocyanins are a group of phenolic compounds in the plant kingdom and they exhibit good antioxidant properties. As can be seen from **Table 2.6** the anthocyanin content in the peel extracts ranged from 2.0 to 5.7 mg/g and was more in ripe mango peel compared to raw peel extracts. Wolfe et al (2003) determined the anthocyanin content in apple peel and reported that it ranged from 0.021 to 0.268 mg of cyanidin 3-glucoside equivalent/g of apple peel depending on the variety.

Table 2.6. Anthocyanin content in acetone extracts of mango peel †

Variety	Anthocyanin content (mg/g)
Raspuri raw	2.0± 0.05[a]
Raspuri ripe	3.6± 0.06[b]
Badami raw	3.3± 0.03[c]
Badami ripe	5.7± 0.04[d]

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

† Dry weight basis

2.3.1.4. Estimation and identification of phenolic acids from the soluble bound phenolic compounds

Initially, efforts were made to identify phenolic compounds by separating on C18 column using HPLC. The extracts obtained from each variety were separated into 5-8 peaks. Efforts to identify these peaks by comparing the retention times of authentic standards of phenolic acids, or spiking with known phenolic acids did not help in identifying the peaks, as

there were little variations with retention times. This is mainly due to the complex nature of polyphenols.

Phenolic acids can be classified as free, soluble and insoluble bound phenolic acids (Renger & Steinheart, 2000). The free forms of phenolic compounds are very rarely present in plants. Majority of phenolic acids are linked through ester, ether or acetal bonds either to structural compounds of plants such as protein, dietary fiber or to longer polyphenols like flavonoids or smaller organic molecules such as glucose, maleic acid or to other natural products (Harborne, 1989, Robbins, 2003; Nardini et al, 2004). Acid hydrolysis and saponification are the commonly used methods by different workers. Esterases are also used to cleave the ester bond, but not as popular as the other two methods (Robbins, 2003).

Therefore, in the present study acid hydrolysis was employed to determine the phenolic content in the acetone extract (soluble phenolics) of mango peel. As shown in **Table 2.7**, the phenolic content in the hydrolyzed acetone extracts of different peels ranged from 8.08 to 22.12 mg/g peel. The phenolic content was found to be more in raw peel compared to ripe peels. The phenolic content determined after acid hydrolysis was found to be much lower than total phenols estimated. Low recovery of phenolic acids after acid hydrolysis was observed by others also and it was reported to be due to the destruction of some phenolic acids during acid hydrolysis (Robbins, 2003). Therefore, the low value obtained in the present study may be attributed to the destruction and loss of certain phenolic acids in the acidic medium.

The phenolic acids in phenolic fractions after acid hydrolysis of raw and ripe Badami and Raspuri peels were separated on reverse phase C18 column

on HPLC using water: methanol: acetic acid (83:15:2). The compounds eluted were monitored at 280 and 320 nm to detect various compounds. The retention times of these peaks were compared with the authentic standards of phenolic acids. However, wherever there is a little variation in retention times, their identifications were confirmed by spiking with authentic standards. HPLC chromatogram and content of phenolic acids present in phenolic fractions of mango peel are shown in **Figure 2.6a, 6b, 6c** and **Table 2.8**. Gallic acid, protocatechuic acid and gentisic acid were the phenolic acids identified in phenolic fractions of Raspuri raw and ripe mango peels, where as gallic acid, protocatechuic acid, gentisic acid and syringic acid were the phenolic acids identified in phenolic fractions of Badami raw and ripe mango peel extracts.

Table 2.7. Content of soluble bound phenolics in the mango peel acetone extract

Variety	Free phenolics* (mg/g peel)
Badami raw	12.20± 0.42^{b}
Badami ripe	8.08± 0.19^{a}
Raspuri raw	22.12±0.30^{c}
Raspuri ripe	17.17±0.40^{d}

Values are expressed on dry weight basis. All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differ significantly ($P \leq 0.05$),

* GAE

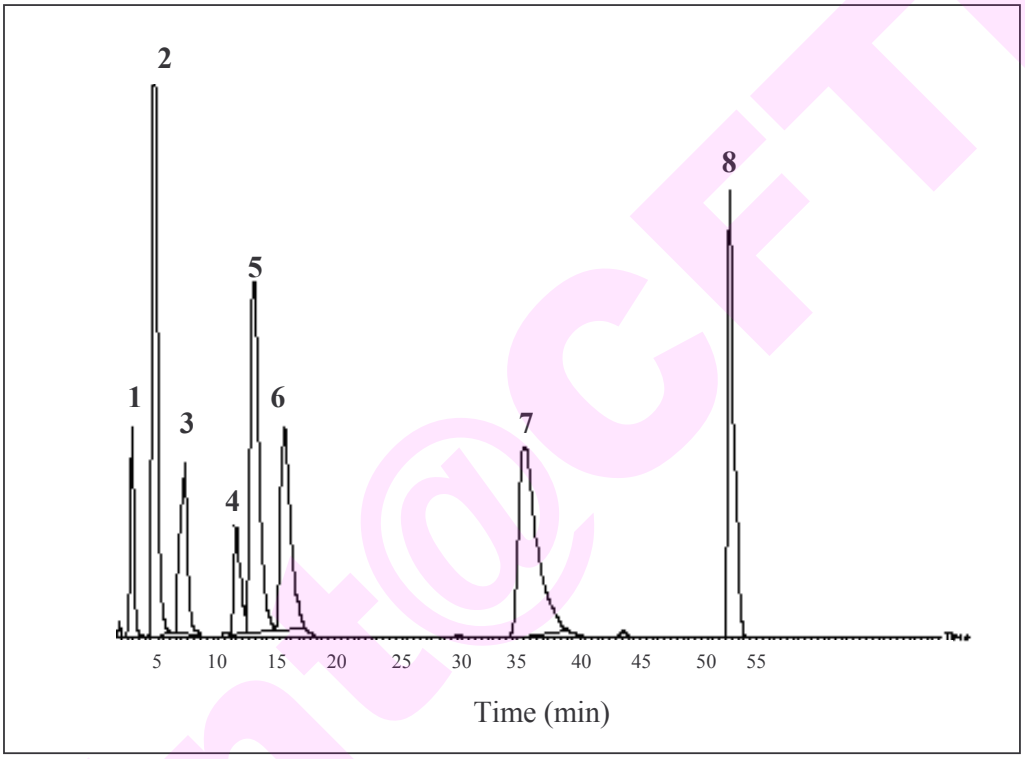


Figure 2.6a. HPLC profile of standard phenolic acid mixture

Standard mixture, Peak identification: 1. gallic acid, 2. protocatechuic acid, 3. gentisic acid, 4. vannilic acid, 5. syringic acid, 6.caffeic acid, 7. p-coumaric acid, 8. cinnamic acid

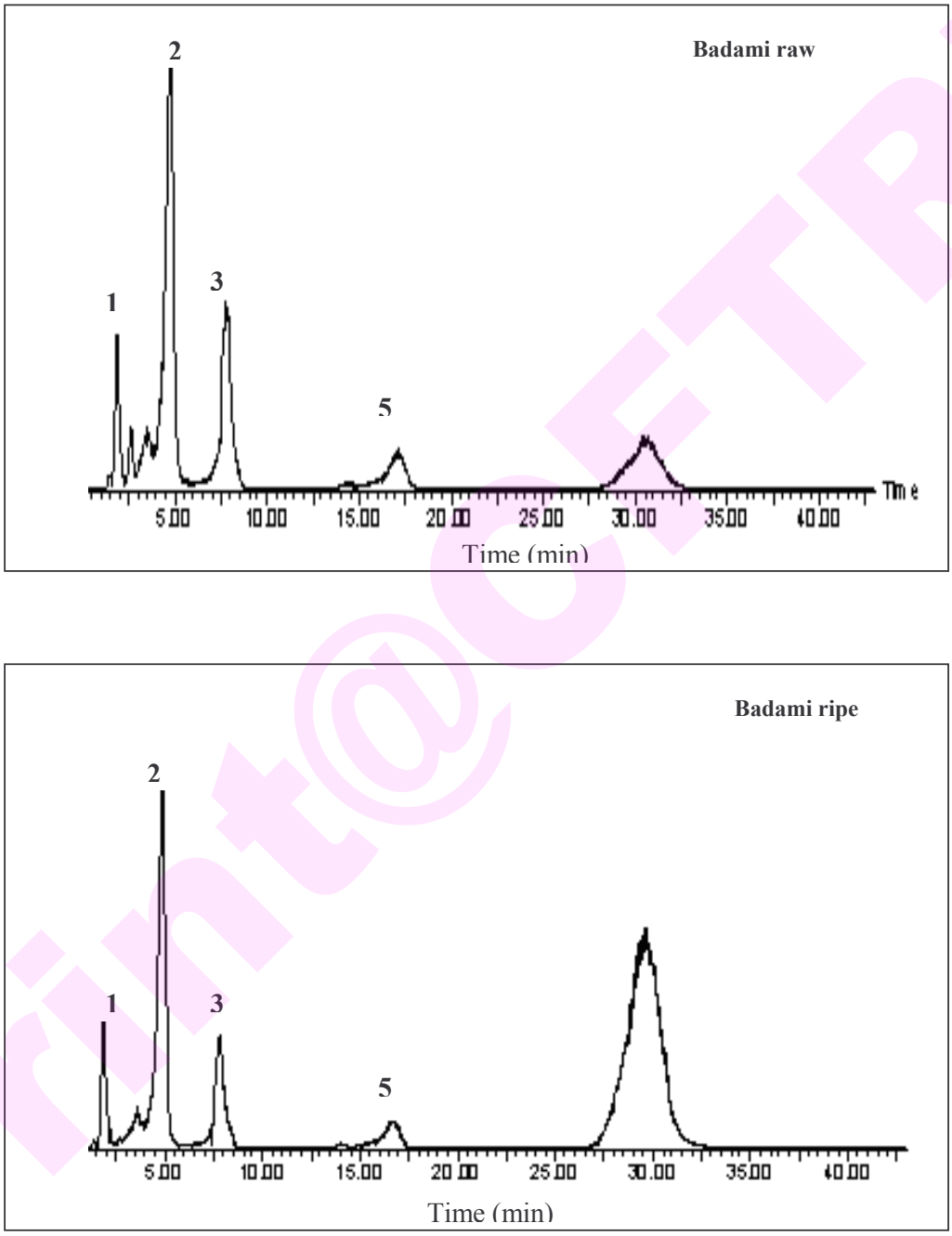


Figure 2.6b. HPLC profiles of phenolic acids in Badami mango peel extracts

Peak identification: 1. gallic acid, 2. protocatechuic acid, 3. gentisic acid, 5. syringic acid

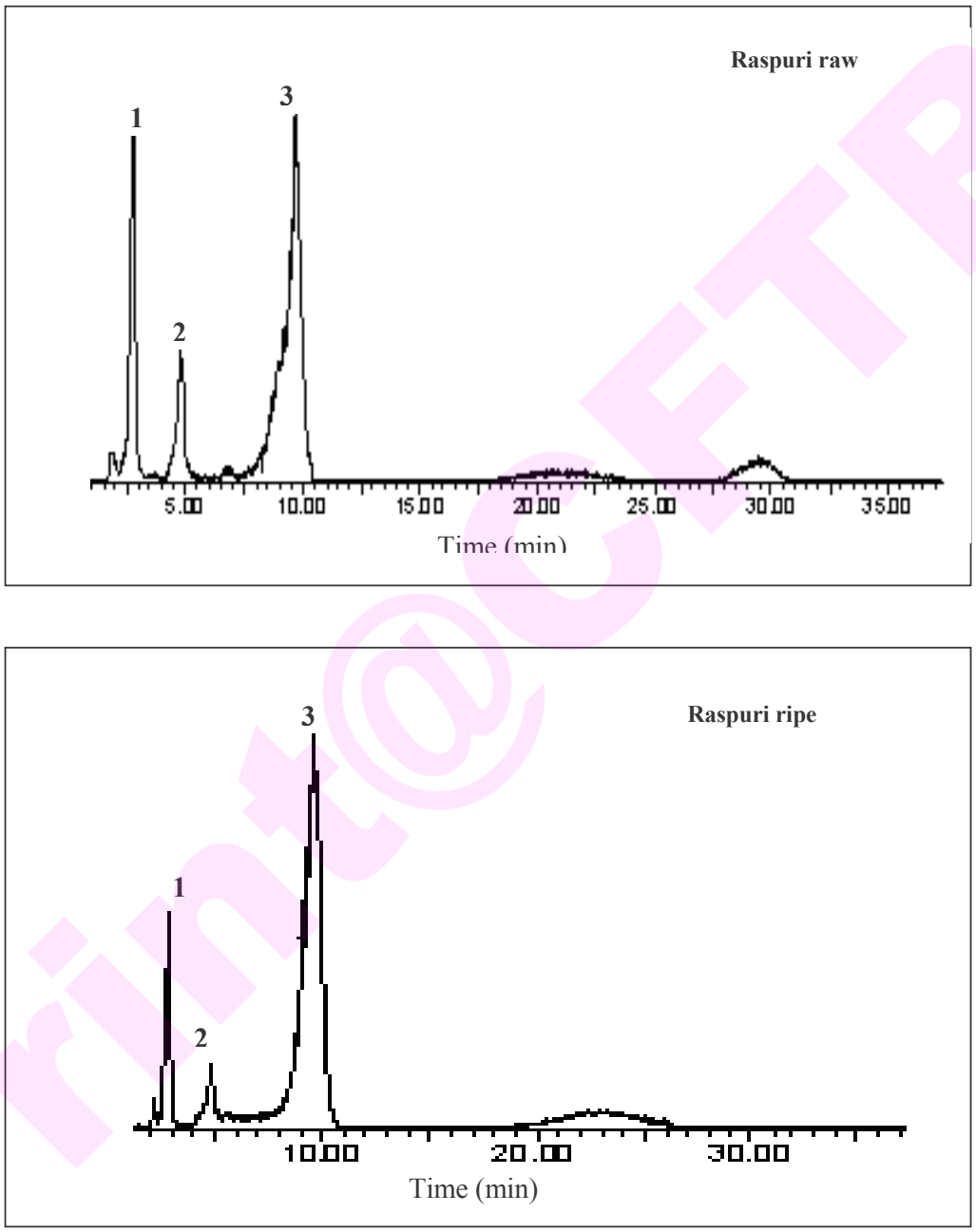


Figure 2.6c. HPLC profiles of phenolic acids in Raspuri mango peel extract

Peak identification: 1. gallic acid, 2. protocatechuic acid, 3. gentisic acid.

Table 2.8. Identification of phenolic acids in the soluble bound Phenolics in mango peel acetone extracts

Phenolic acids	Badami raw (mg/g extract)	Badami ripe (mg/g extract)	Raspuri raw (mg/g extract)	Raspuri ripe (mg/g extract)
Gallic acid	0.92	0.86	5.4	2.75
Protocatechuic acid	2.70	2.49	1.6	0.28
Gentisic acid	1.94	1.04	5.6	4.65
Syringic acid	0.08	0.05	ND*	ND*

ND*- Not Detected

Insoluble bound phenolics are mainly present in the residue, and are associated with dietary fiber. Therefore, isolation and identification of dietary fiber bound phenolics are described in later Sections (2.3.1.18).

2.3.1.5. Identification of phenolic compounds in the acetone extract of mango peel

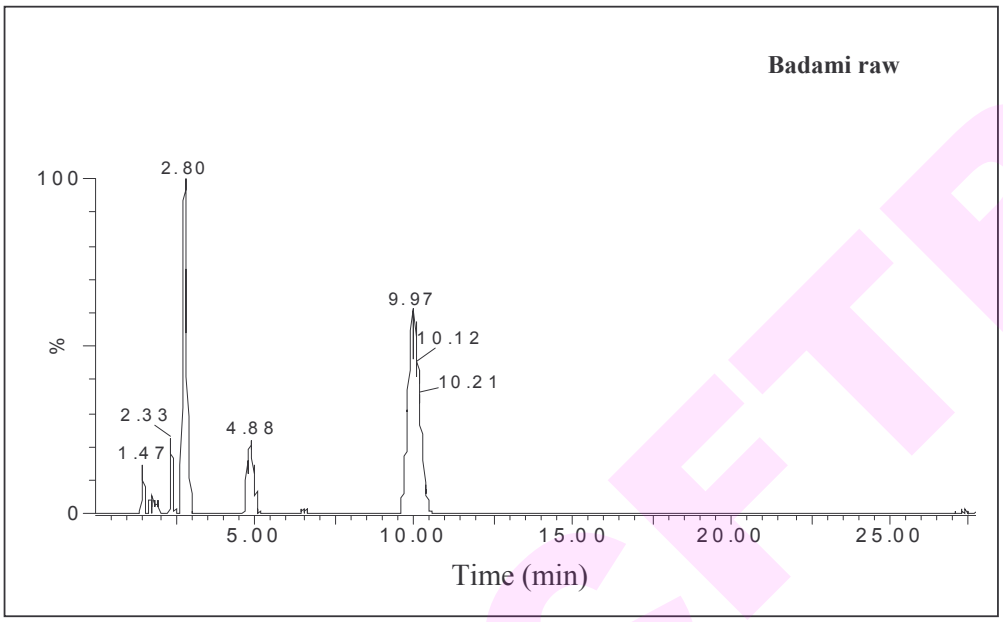
As mentioned earlier, polyphenol exists as esters or glycosides. In order to identify these polyphenols in the acetone extract of mango peel, they were analyzed by ESI-LCMS. The acetone extracts of raw and ripe peels were separated on reverse phase C18 column using conditions described earlier. The LCMS-TIC profiles of polyphenolics in mango peel extracts of raw and ripe Raspuri and Badami were shown in **Figures 2.7a and 2.7b**.

Based on mass spectrometric analysis, phenolic compounds were tentatively identified. The phenolic compounds identified in Badami raw peel extract are listed in **Table 2.9.** The major peak with a retention time of 2.8 min showed mixture of syringic glycoside and mangiferin pentoside. Another major peak was identified as quercetin. Iriflophenone hexoside, maclurin-tri-O-

galloyl hexoside, ellagic acid were also identified in the peel extract. Two minor peaks, eluted very closely (1.68 min, 1.90 min), were identified as gallic acid (sodium adduct) and maclurin hexoside, respectively. A small peak at 6.67 min was identified as gentisyl protocatechuic acid. In case of ripe peel extract most of the polyphenols present were similar to that of raw peel extract (**Table 2.10**). Iriflophenone hexoside was not detected in ripe peel extract, while hepta-O-galloyl hexose was identified in ripe peel extract of Badami and it was not present in raw peel of Badami.

The polyphenols identified in raw peel of Raspuri mango variety are given in **Table 2.11**. The major peak having a retention time of 1.78 min was identified as gallic acid. Maclurin hexoside, galloyl hexose were the other two compounds identified. A minor peak was identified as protocatechuic acid hexoside. Raspuri ripe peel extract also showed the presence of similar compounds in addition to gallic acid hexoside (**Table 2.12**).

The results indicated that there are few differences in the composition of raw and ripe peels of the same variety, but the difference were found to be more between the varieties. Recently, Schieber et al (2003) and Berardini et al (2004) reported the identification of polyphenols from Tommy Atkins mango peel. Of the several compounds identified by them, some of them were mangiferin and its derivatives, iriflophenone di-O-galloyl glucoside, maclurin derivatives including maclurin-tri-O-galloyl glucoside, hepta-O-galloyl glucose. They did not report the presence of ellagic acid, syringic or gentsyl or protocatechuic acid derivatives. Earlier, Prabha and Patwardhan (1986) reported the presence of ellagic acid in mango peel by paper chromatographic method.



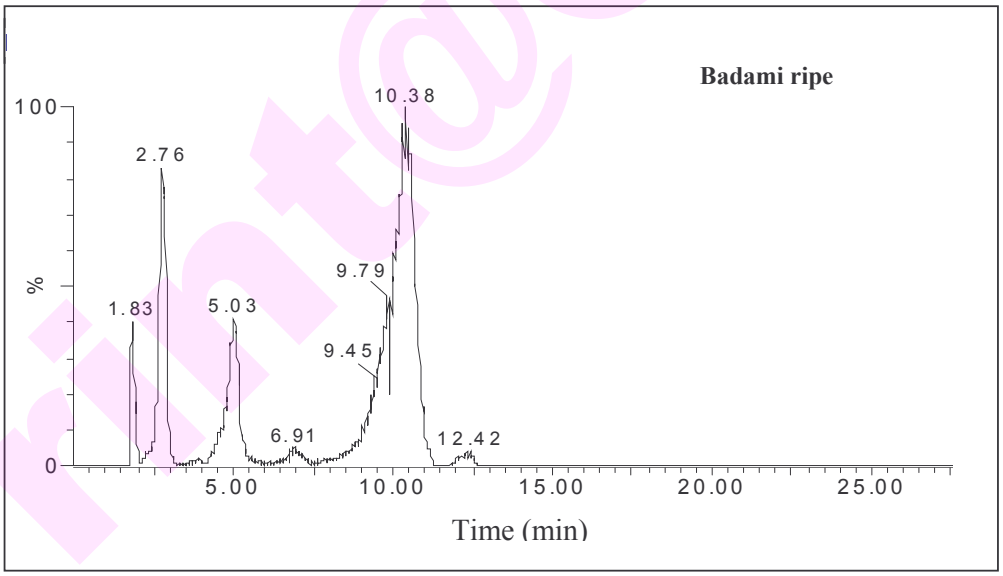


Figure 2.7a. LCMS- TIC profiles of phenolic compounds in the mango peel extract

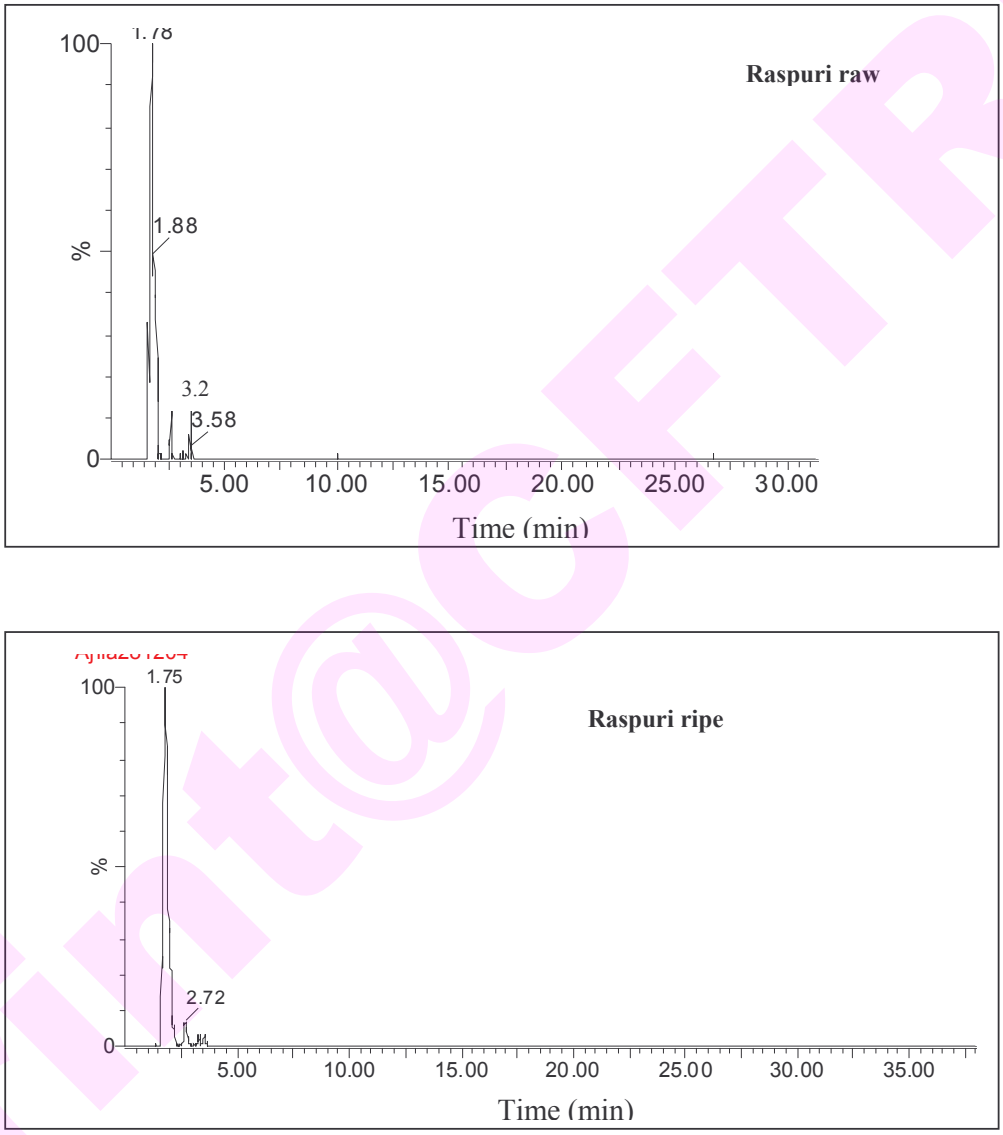


Figure 2.7b. LCMS-TIC profiles of phenolic compounds mango peel extract.

Table 2.9. UV spectra and characteristic ions of polyphenols extracted from the raw mango peel of Badami

Retention time (min)	Identity*	HPLC-DAD λ- max (nm)	MW	HPLC-ESI (-) MS
1.47	Iriflophenone hexoside	280 ; 320	408	391[M+H-H_2O]
1.68	Gallic acid (sodium adduct)	278 ; 320	170	191[M+Na-2H]
1.90	Maclurin hexoside	230 ; 280	424	405 [M-H-H_2O], 191
2.33	Maclurin-tri-O-galloyl hexoside (glucoside)	280 ; 321	880	879 [M-H]
2.80	Syringic acid hexoside	280 ; 320	360	361 [M+H]
	+			
	Mangiferin pentoside	280 ; 320	554	553 [M-H]
4.88	Ellagic acid (monohydrate)	280 ; 230	302	321[M+H+H_2O], 169
6.67	Gentisyl protocatechuic acid	281	290	289 [M-H], 153
9.97	Quercetin (monohydrate)	321 ; 280	302	321[M+H+H_2O]

* Tentatively identified

Table 2.10. UV spectra and characteristic ions of polyphenols extracted from the ripe mango peel of Badami

Retention time (min)	Identity*	HPLC-DAD λ- max (nm)	MW	HPLC-ESI (-) MS
1.83	Hepta-O-galloyl hexose	230 ; 280	1244	1244[M+]
2.42	Gallic acid	281 ; 320	170	169 [M-H]
2.76	Syringic acid hexoside	281 ; 324	360	361[M+H]
	+			
	Mangiferin pentoside	280 ; 320	554	553 [M-H]
5.03	Ellagic acid (monohydrate)	281	302	321[M+H+H_2O], 169
6.91	Gentisyl-protocatechuic acid	280 ; 321	290	289 [M-H], 153
10.38	Quercetin (monohydrate)		302	321[M+H+H_2O, 301

* Tentatively identified

Table 2.11. UV spectra and characteristic ions of polyphenols extracted from the raw mango peel of Raspuri

Retention time (min)	Identity *	HPLC-DAD λ- max (nm)	MW	HPLC-ESI (-) MS
1.78	Gallic acid (sodium adduct)	278 ; 230	170	191[M+Na-2H]
2.65	Maclurin hexoside	278 ; 323	424	405 [M-H-H_2O], 191
3.20	Protocatechuic acid hexoside	278 ; 230	316	315 [M-H], 153
3.51	Galloyl hexose	230 ; 280	332	331[M-H], 191

* Tentatively identified

Table 2.12. UV spectra and characteristic ions of polyphenols extracted from the ripe mango peel of Raspuri

Retention time (min)	Identity *	HPLC-DAD λ- max (nm)	MW	HPLC-ESI (-) MS
1.751	Gallic acid (sodium adduct)	278 ; 230	170	191[M+Na-2H]
2.118	Gallic acid hexoside	278 ; 230	332	331 [M-H]
2.721	Maclurin hexoside	280 ; 323	424	405 [M-H-H_2O], 191
3.274	Protocatechuic acid hexoside	278 ; 323	316	315 [M-H]
3.540	Galloyl hexose	230 ; 280	332	331[M-H]

* Tentatively identified

2.3.1.6. Carotenoid content in mango peel

Carotenoids are widely distributed in nature and they are liposoluble antioxidants. Carotenoids show good absorption at 470 nm. However, a small amount of absorption is contributed by chlorophyll b and negligible absorption comes from chlorophyll a. The concentration of total carotenoids content can, therefore, be determined by deducting the absorption of chlorophyll a and b from the absorbance read at 470 nm followed by division by the absorption coefficient of total carotenoids at 470 nm (Litchenthaler, 1987). The carotenoid content in mango peel was estimated using two different spectrophotometric methods described by Davis (1976) and Litchenthaler (1987). As can be seen from **Table 2.13** carotenoid content determined using both the methods are comparable. The carotenoid content was found to be more in ripe mango peels compared to raw peels. Modi and Reddy (1967) showed that ripe mangoes are ten times richer in carotenoids than partially ripe ones. The present study showed that carotenoid content in mango peels was 4 to 8 times higher in ripe mango peels than in raw mango peels.

Table 2.13. Total carotenoid content of fresh mango peel acetone extract

Mango variety	Davis method† (μg/g)	Litchenthaler method†† (μg/g)
Raspuri Raw	493±31[a]	547±18[b]
Raspuri Ripe	3,945±85[c]	3,337±65[d]
Badami Raw	365±10[a]	387±35[a]
Badami ripe	1,400±40[b]	1,520±84[c]

Values are expressed on dry weight basis. All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differ significantly ($P \leq 0.05$),
† Davis (1976); †† Litchenthaler (1987)

2.3.1.7. Identification of carotenoids by RP- HPLC

The carotenoids present in the mango peels of Badami and Raspuri were separated by reverse phase HPLC on C18 column. The carotenoids were separated into 4-5 major peaks with few minor peaks. However, only three peaks were identified using the authentic standard carotenoids (**Figures 2.8a, 2.8b and 2.8c**). The carotenoids identified in the mango peel extracts were violoxanthin, lutein and β-carotene. As can be seen from **Table 2.14**, the β-carotene content was more in ripe mango peels than the raw mango peels in both Badami and Raspuri.

It was reported that the major carotenoid present in mango fruits were trans-violaxanthin, trans-β-carotene, 9-cis-violaxanthin and luteoxanthin (Mereadante et al, 1997, Godoy et al, 1989). Recently, Chen et al (2004) identified carotenoids such as β-carotene, violaxanthin, neochrome, luteoxanthin, neoxanthin and zeaxanthin in Taiwanese mango pulp. Earlier, Lizada et al (1993) identified β-carotene, xanthophyll esters and xanthophyll as principal carotenoids in the peel of Alphonso mango.

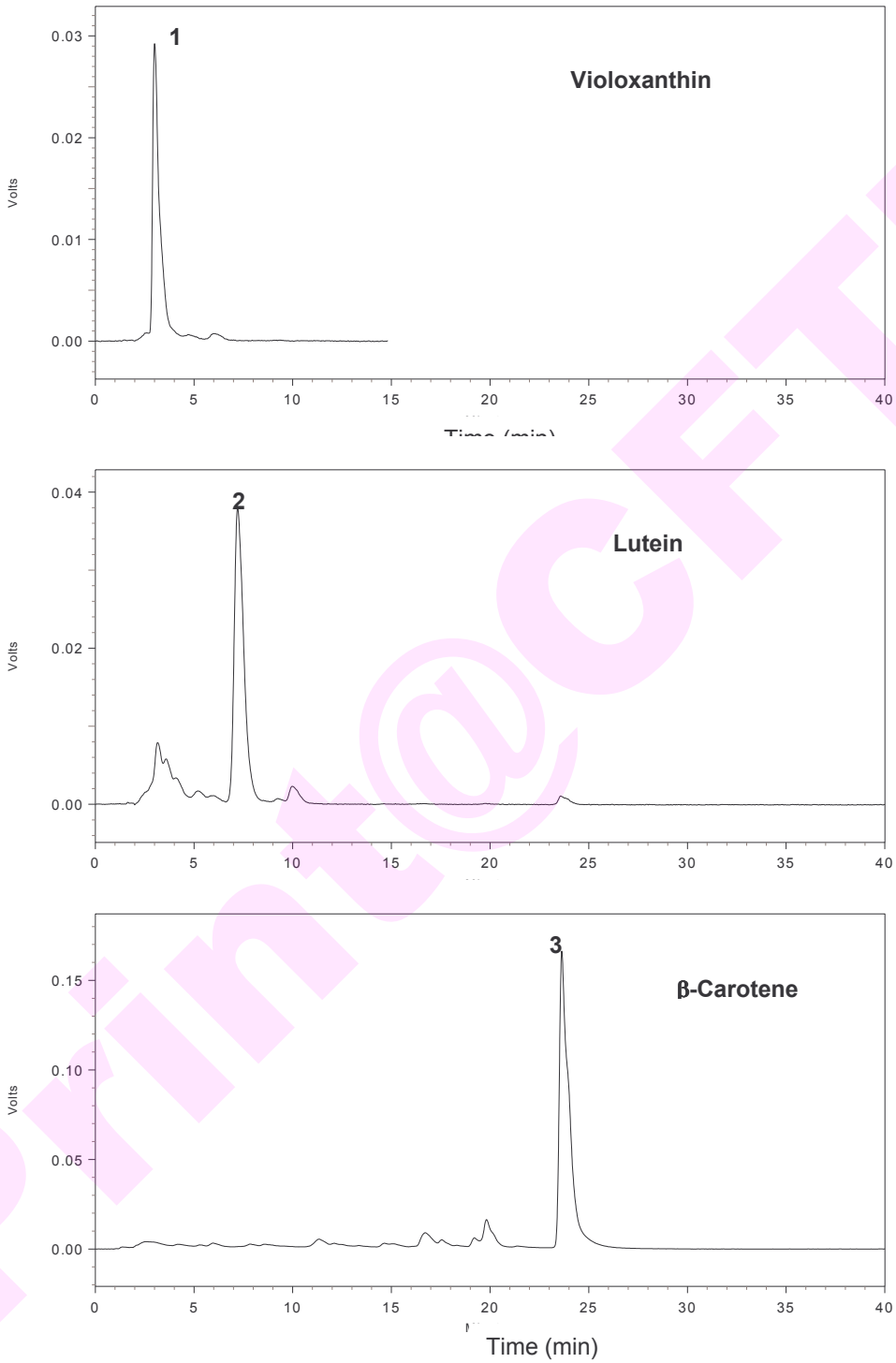


Figure 2.8a. HPLC profiles of standard carotenoids

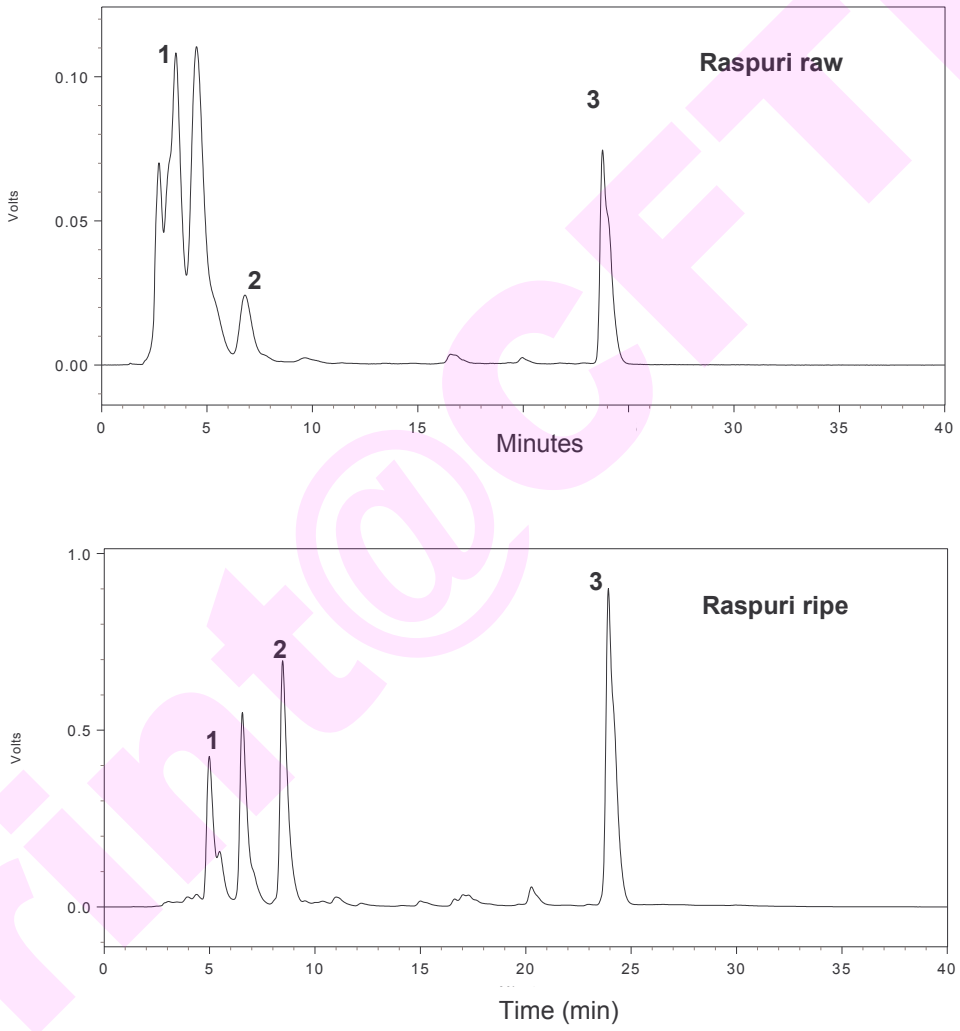


Figure 2.8b. HPLC Profiles of carotenoids in Raspuri mango peel

Peak identification: 1. Violoxanthin, 2. Lutein, 3. β-Carotene

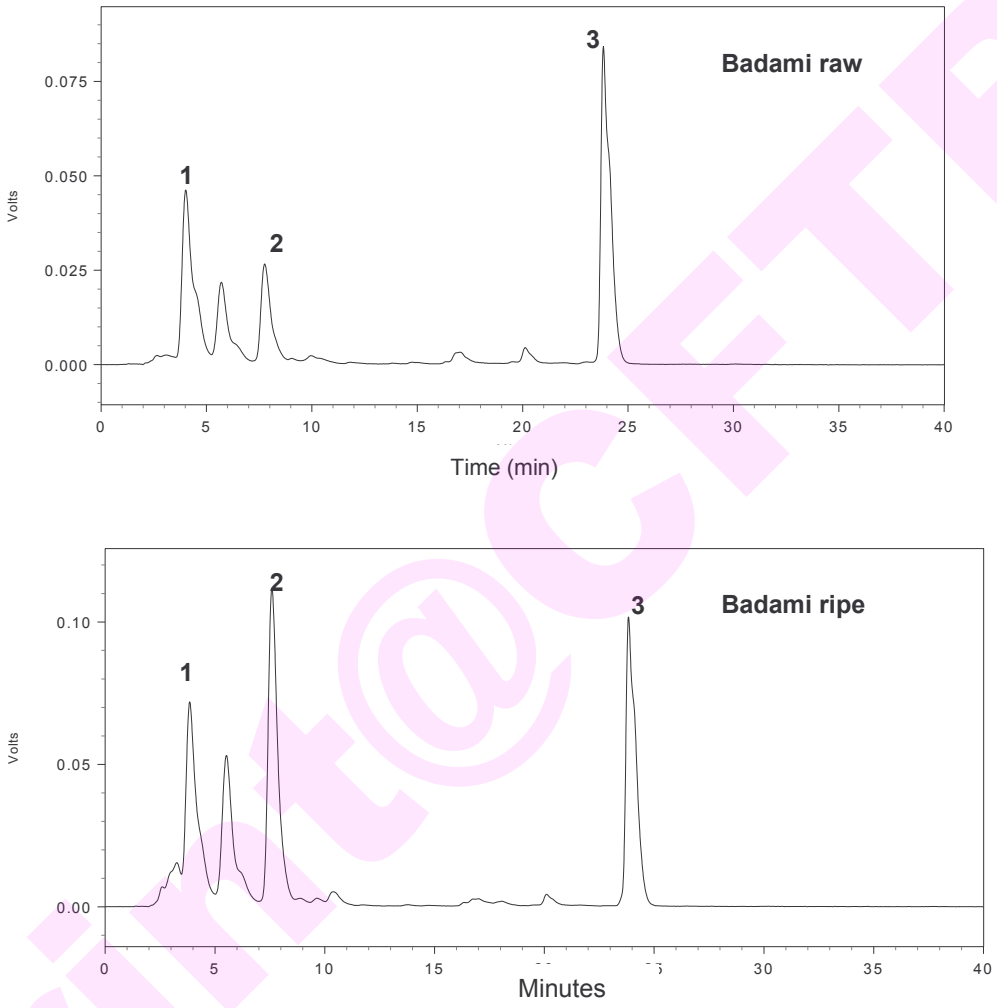


Figure 2.8c. HPLC profiles of carotenoids in Badami mango peel

Peak identification: 1. Violoxanthin, 2. Lutein, 3. β-Carotene

Table 2.14. Identification of carotenoids in mango peel

Variety	Raspuri raw (μg/g)	Raspuri ripe (μg/g)	Badami raw (μg/g)	Badami ripe (μg/g)
Violaxanthin	121	420	95	235
Lutein	26	740	27	260
β-Carotene	169	1836	183	520

2.3.1.8. Vitamin C content in mango peel

The vitamin C content of mango peel ranged from 188 to 392 μg/g of the peel and in both varieties it was more in ripe peels compared to raw peels (**Table 2.15**). Earlier, vitamin C content in mango peel of five different varieties was reported which ranged from 190 to 2,570 μg/g (Teotia et al, 1987).

Table 2.15. Vitamin C content of mango peel

Variety	Vitamin C (μg/g)
Raspuri Raw	188±18[a]
Raspuri Ripe	349±11[c]
Badami Raw	315±10[b]
Badami ripe	392±21[d]

Values are expressed on dry weight basis. All data are the mean±SD of three replicates.

Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.9. Vitamin E in mango peel

The vitamin E content in mango peel was determined by spectrophotometric method. Its content in peel ranged from 205 to 509 μg/g and was higher in ripe mango peel than raw mango peel (**Table 2.16**). Recently, Burns et al (2003) reported the presence of α-tocopherol in mango pulp. However, no report is available with respect to vitamin E content in mango peel.

Table 2.16. Vitamin E content of mango peel

Variety	Vitamin E (μg/g)
Raspuri Raw	205±4[a]
Raspuri Ripe	308±11[b]
Badami Raw	337±3[c]
Badami ripe	509±14[d]

Values are expressed on dry weight basis. All data are the mean±SD of three replicates.
Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.10. Enzymes in mango peel

Peroxidase, polyphenol oxidase (PPO), protease and carbohydrases are reported in mango pulp and their role in fruit ripening was studied by different workers (Gomez-Lim, 1997; Tharanathan et al, 2006). However, very few reports are available with regard to enzymes in mango peel. Therefore, both raw and ripe mango peels were assayed for various enzymes. PPO activity varied between 36 and 108 U/g peel and was higher in ripe peel. Badami ripe peel had more PPO activity, while Raspuri raw had less activity (**Table 2.17**). Earlier, presence of PPO was reported in mango peel by

different researchers (Prabha & Patwardhan, 1986; Saby John et al, 2002; Robinson et al, 1993). Prabha and Patwardhan (1986) reported that PPO content in ripe peel was more than that of raw peel. PPO has been widely studied in various fruits such as pineapple (Das et al, 1997) and apple (Jonovitz–Klap & Sagribien, 1989), among others. PPO activity was detected in the skin and pulp of Irwin mango variety and a gradual increase in the enzyme activity was reported from raw to ripe stages of mango during maturation (Ueda *et al*, 2000). The PPO activity (214 U/mg protein) in mango sap was reported by Saby John et al (2003) and it was found to be higher than in most fruits and vegetables. Hobson (1967) reported that PPO activity was increased during the growth phase of fruit and subsequently declined as it ripened.

Peroxidase activity was highest in Raspuri raw peel and lowest in Badami raw mango peel (**Table 2.17**). Increase in peroxidase activity in fruit ripening was reported in mango (Mattoo et al, 1968) and apples (Gorin & Heidema, 1976), while decrease in peroxidase activity with ripening was reported in tomato (Thomas et al, 1981). However, no such trend was seen in case of peroxidase in mango peel in the present study. A correlation between peroxidase activity in pulp and fruit ripening was reported earlier (Prabha & Patwardhan, 1986). Mattoo and Modi (1970) found that ethylene promoted a three-fold increase in peroxidase activity in preclimacteric mango fruit following 24 h ethylene treatment.

Peel extracts showed good protease activity. Protease activity was found to be significantly more in ripe mango peel than in the raw mango peels. Protease activity ranged from 4,573 to 11,173 U/g of peel and it was

found to be higher in Badami ripe peel (**Table 2.17**). Protease activity may be involved in protein turn over and particularly during the final stages of senescence by protein catabolism, which is responsible for increase in free amino acids and amides. Presence of protein hydrolyzing enzymes in fruits have been well studied especially papain from papaya fruit (Howard & Glazer, 1969).

Xylanases are a group of cell wall degrading enzymes. The xylanase activity ranged from 4.0 to 9.9 U/g peel. Xylanase activity was found to be maximum in raw mango peel than the ripe mango peel (**Table 2.17**). Ali et al (1995) reported the presence of xylanase activity in fruit pulp and their activities were unchanged throughout the ripening stages of mango.

Enzymatic hydrolysis of starch can be performed by amylases. Amylase has a role in fruit ripening. Amylase activity in mango peel ranged from 1.1 to 2.8 U/g peel. It was found to be more in ripe peel compared to raw peel (Table 2.17). It has been reported that mango fruit at the mature green stage contains some accumulated starches (Subramanyan et al, 1976), which is mobilized during ripening (Morga et al, 1979). Different enzymes like amylases mediate sucrose and starch metabolism. α-Amylase activity has been found to increase at least four times during ripening of mango fruit (Mattoo et al, 1975, Majmudar et al, 1981). Ueda et al (2000) reported that β-amylase activity increased with fruit maturity reaching a maximum in fruit harvested at 19 weeks after flowering.

Table 2.17. Enzyme activities of mango peels

Mango variety	Polyphenol oxidase (U/g)	Peroxidase (U/g)	Protease (U/g)	Xylanase (U/g)	Amylase (U/g)	Protein (mg/g)
Raspuri raw	36.4±1.2^{a}	275±10^{d}	4,573±14^{a}	5.9±0.45^{b}	1.1±0.08^{a}	9.9±1.1^{b}
Raspuri ripe	74.7±0.1^{b}	242±2^{b}	10,363±363^{b}	4.3±0.33^{a}	2.2±0.13^{b}	4.0±0.8^{a}
Badami raw	72.0±4.0^{b}	213±8^{a}	11,058±381^{c}	9.3±1.12^{c}	0.9±0.07^{a}	12.7±0.4^{c}
Badami ripe	108.0±1.0^{c}	260±4^{c}	11,173±74^{c}	6.4±0.80^{b}	2.8±0.05^{c}	5.2±0.7^{a}

Values are expressed on dry weight basis. All data are the mean±SD of three replicates.

Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.11. Dietary fiber content in mango peel

Dietary fiber (DF) is well established to have many health beneficial effects. It is shown to have a very important role in prevention of colon cancer, lowering of blood cholesterol level, blood glucose attenuation, laxation etc (Lupton, 1995, Anderson 1995, Greenwald & Clifford 1995). In food processing, dietary fiber is used as fat replacer to increase viscosity of the product and reduce oil uptake during frying (Dreher, 1995). The nutritional value of fruit DF additionally attributed due to the presence of significant amount of bioactive compounds such as flavonoids and carotenoids, which are linked covalently to different dietary fiber components.

The crude fiber content of mango peel is presented earlier in **Table 2.4.** This represents mainly cellulose fractions, which is a major part of insoluble dietary fiber. The dietary fiber content in mango peels of different varieties

was estimated (**Table 2.18**). The total dietary fiber (TDF) content in peel varied from 45 to 78%. In both Raspuri and Badami, soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) contents were higher in ripe peels compared to raw peels. Among these, Badami ripe showed highest TDF content and Raspuri raw showed the lowest. Earlier, Larrauri et al (1996) reported that the dried mango peel contained 28.1% of SDF and 43.4% IDF. In the present study, IDF/SDF ratio varied from 1.7 to 2.0. The soluble dietary fiber content in both raw and ripe mango peels are more than 35% of TDF. For health benefits, it is reported that 30-50% SDF and 50-70% insoluble dietary fiber are considered to be well-balanced proportions (Schnneman, 1987). Though in terms of health benefits, both IDF and SDF complement each other, each fraction has different physiological effect. Insoluble dietary fiber helps in better water absorption and intestinal absorption of nutrients whereas SDF is associated with cholesterol in blood and diminishes its intestinal absorption. The total dietary fiber content in Mexican and Persian lime peel was 70.4 % and 66.7%, respectively (Rivera et al, 2004). The characteristic feature of mango peel is that it has high content of soluble dietary fiber, which is reported to have better health beneficial effects. SDF content in apple waste was reported to be 23% of the TDF, while it was 36% in orange byproducts. However, SDF contents in wheat bran and oat bran were 6.6 and 15.0%, respectively, which are low compared to mango peel (Grigelmo et al, 1999).

Table 2.18. Dietary fiber content of fresh mango peel

Mango variety	SDF (%)	IDF (%)	TDF (%)
Raspuri Raw	15.70±0.30[a]	28.99±0.51[a]	44.70±0.71[a]
Raspuri Ripe	23.81±0.91[c]	39.99±2.90[b]	63.80±3.80[b]
Badami Raw	21.42±0.73[b]	42.61±0.20[b]	64.13±0.25[b]
Badami ripe	28.06±0.41[d]	50.33±0.75[c]	78.40±0.20[c]

SDF-Soluble dietary fiber; IDF-Insoluble dietary fiber; TDF-Total dietary fiber.
Values are expressed on dry weight basis. All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.12. Development of a process for the isolation of valuable compounds from mango peel

The results presented in the earlier sections indicated that mango peel contained a number of valuable compounds such as polyphenols, carotenoids, vitamins E and C, enzymes and dietary fibers and also showed that extraction with acetone was superior to alcohol extraction giving higher yield of phenolic compounds and other bioactive compounds. Acetone is widely used to extract lipophyllic compounds, to release membrane bound enzymes and to precipitate proteins, hence acetone was used to isolate various compounds simultaneously starting with the same material. In the present study, we have developed a process for simultaneous isolation of valuable bioactive compounds such as polyphenols, carotenoids, dietary fibers and enzymes from mango peel (**Figure 2.9**). The mango peel was homogenized in sodium phosphate buffer and the homogenate was made up to 80% acetone extract with respect to acetone and filtered. The supernatant was designated as acetone extract and the residue was air dried and

designated as acetone peel powder. The acetone extract was analyzed for phenolics, carotenoids and vitamin E. The acetone powder was extracted with buffer, separated into supernatant and residue by centrifugation, the supernatant obtained was used for the analysis of different hydrolytic and oxidative enzymes, and the residue was characterized for dietary fibers.

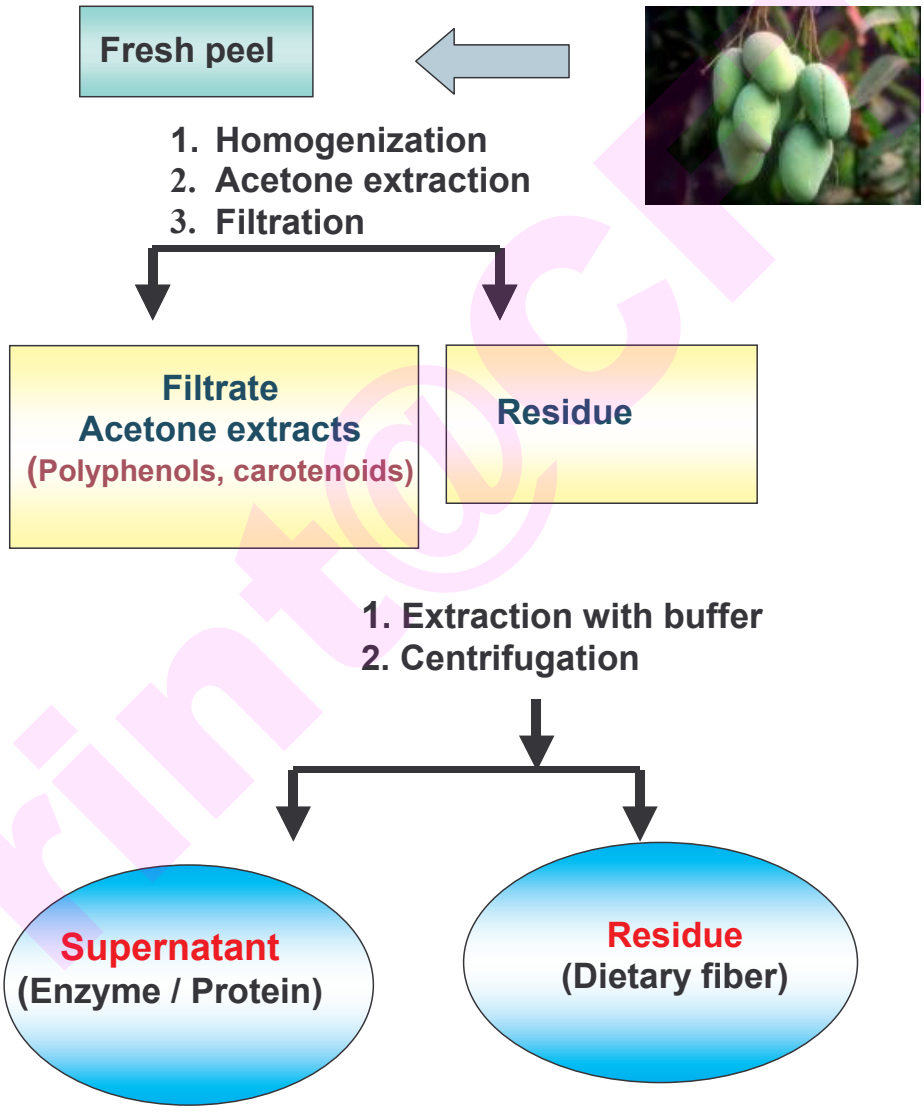


Figure 2.9. Two step method for the isolation of valuable compounds from mango peel

2.3.1.13. Polyphenols, carotenoids and vitamin E content in acetone extract

Acetone extract is analyzed for various valuable compounds namely polyphenols, carotenoids, anthocyanins and vitamin E. The polyphenol and anthocyanin content in the acetone extract were reported earlier in Section 2.3.1.2 and Section 2.3.1.3, respectively. This fraction when analyzed for carotenoid contents, it showed 74 to 436 μ/g peel. However, these values are less than that were obtained directly from peels, which were 547-3,337 μ/g peel carotenoid present in fresh peel (**Table 2.14**). This clearly showed that only a portion of carotenoid content of mango peel was extracted in the acetone fraction. Saponification using KOH- methanol is carried out to avoid the effect of fat in the estimation of carotenoids. It has also been reported that organic acids present in tissues affect the estimation of carotenoids. In order to avoid such artifacts, during extraction, addition of alkaline compounds are suggested to neutralize the acids (Minguez-Mosquera et al, 2002). Mango fruit is rich in organic acids such as citric, malic and oxalic acids (Hulme, 1971). Mango peel is rich in polyphenols and it contains about 2.5% fat, which may interfere while estimation of carotenoids in the absence of alkali. Thus, the results indicated that extraction using KOH-methanol may be a more suitable method to extract and estimate carotenoids in mango peel. Similarly, vitamin E content in the acetone extract of mango peel varied from 82-230 μg/g of peel, which is about 31-45% of vitamin E present in the fresh peel extracted by saponification. These results also indicated that extraction using KOH-methanol may be a more suitable method to extract and estimate carotenoids and vitamin E in mango peel.

Table 2.19. Total phenolic, carotenoid and anthocyanin contents in acetone extracts of mango peel †

Variety	Total phenolic content * (mg/g)	Anthocyanin (mg/g)**	Carotenoid content (μg/g)	Vitamin E (μg/g)
Raspuri raw	109.70± 0.82^{d}	2.0±0.05 ^{a}	73.5± 0.53^{a}	82± 5.03^{a}
Raspuri ripe	100.00± 1.90^{c}	3.6±0.06 ^{b}	436.0± 0.22^{d}	121± 5.40^{c}
Badami raw	90.18± 0.57^{b}	3.3±0.03^{c}	81.0± 0.42^{b}	104± 3.20^{b}
Badami ripe	54.67± 1.50^{a}	5.7±0.04^{c}	194.0± 0.26^{c}	230± 12^{d}

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).
† Dry weight basis
* Reported earlier in Section 2.3.1.2; ** Reported earlier in Section 2.3.1.3

2.3.1.14. Enzyme activities in mango peel acetone powder

Buffer extract of acetone powder was analyzed for oxidative and hydrolytic enzyme activities as done for mango peel. The protein content in the extract ranged from 4.9 to 14.5 mg/g. The total extractable protein was comparable with fresh peel. However, the enzyme activities in the acetone extract were lower. Among the different enzymes, peroxidase, xylanase and protease activities were much lower than the polyphenol oxidase and amylase. The low enzyme activities in the acetone powder may be either due

to the inactivation of enzyme by acetone or proteolytic degradation of enzyme protein by the protease present in the extract.

As shown in **Table 2.20**, PPO activity varied between 37 to 62 U/g. Badami ripe peel had more polyphenol oxidase activity than other mango peel extracts and Raspuri raw had less activity. The highest peroxidase activity was found in the Badami ripe peel extract (116 U/g) and lowest in Raspuri raw peel extract (56 U/g). Protease activity was found to be the highest in Badami ripe peel extract and the lowest in Raspuri raw extract. Activities of all these enzymes were higher in ripe peel extracts compared to the raw peel extracts. Xylanase activities were found to be in the range of 1.20 to 1.86 U/g while amylase activities ranged from 0.91 to 1.91 U/g (**Table 2.20**).

Table 2.20. Hydrolytic and oxidative enzymes in mango peel acetone powder

Variety	Polyphenol oxidase (U/g)	Peroxidase (U/g)	Protease (U/g)	Xylanase (U/g)	Amylase (U/g)	Protein (mg/g)
Raspuri raw	37±3.0^{a}	56±2.0^{a}	1,540±70^{a}	1.86±0.02^{d}	0.91±0.05^{a}	10.0±0.17^{c}
Raspuri ripe	58±1.4^{c}	83±2.0^{c}	1,800±50^{b}	1.20±0.01^{a}	1.91±0.02^{c}	4.9±0.14^{a}
Badami raw	52±1.4^{b}	68±1.8^{b}	1,960±40^{c}	1.73±0.04^{c}	0.94±0.04^{a}	14.5±0.71^{d}
Badami ripe	62±4.2^{c}	116±4^{d}	6,030±34^{d}	1.28±0.10^{b}	1.22±0.11^{b}	6.2±0.80^{b}

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.15. Soluble and insoluble dietary fiber content

As indicated in earlier Section 2.3.1.11., the total dietary fiber content in fresh peel of raw and ripe Raspuri and Badami varieties varied from 45 to 78% (dry weight, **Table 2.18**). The total dietary fiber content in acetone powder of mango peel after enzyme extraction was found to be in the range of 40.6 to 72.5% (**Table 2.21**). The soluble dietary fiber content in acetone peel powder was in the range of 12.8 to 23.0% and insoluble dietary fiber content varied from 27.8 to 49.5%. In the present study, IDF/SDF ratio varied from 2.0 to 2.2. The soluble dietary fiber content was found to be 31-33% of TDF. It may be noted that the content of soluble dietary fiber increased during ripening. Content of dietary fiber is more in Badami variety in both raw and ripe compared to Raspuri. Compared to the dietary fiber content in fresh mango peel, acetone powder had low dietary fiber. This decrease in TDF is mainly due to the decrease in SDF content. It is to be noted that in the present study acetone powder was extracted with buffer for the isolation of enzymes whereas earlier, whole peel was used for fiber extraction (**Table 2.18**).

Table 2.21. Soluble and insoluble dietary fiber content in mango peel acetone powder

Dietary fiber	Soluble dietary fiber (%)	Insoluble dietary fiber (%)	Total dietary fiber (%)
Raspuri raw	12.8±.1.8^{a}	27.8±2.8^{a}	40.6±1.1^{a}
Raspuri ripe	17.2±1.8^{b}	37.7±0.9^{b}	54.9±2.6^{b}
Badami raw	19.6±1.0^{b}	39.9±1.9^{b}	59.5±2.5^{b}
Badami ripe	23.0±1.2^{c}	49.5±.2.1^{c}	72.5±2.4^{c}

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.1.16. Carbohydrate composition of soluble and insoluble dietary fiber

The contents of total sugar uronic acid and sugar composition of the soluble and insoluble dietary fibers isolated from Badami and Raspuri raw and ripe mango peel is shown in **Table 2.22**. The total sugar content in the IDF and SDF fractions was in the range of 66 to 82% and uronic acid content varied from 9.8% to 26.0%. Uronic acid in mango peel is likely to be galacturonic acid. The uronic acid content in the soluble and insoluble dietary fiber of peel of Hayden variety was reported to be 6.5 and 6.8%, respectively (Larrauri et al, 1996). Variation in the present and reported data may be due to varietal differences as well as maturity stages.

The sugar composition of soluble and insoluble dietary fiber isolated from Badami and Raspuri raw and ripe mango peels was determined by GLC as aldiol acetates (**Figure 2.10a-c**). Arabinose, galactose and glucose were the major neutral sugars in the insoluble dietary fiber fractions (**Table 2.22**). Soluble dietary fiber also had arabinose, galactose and glucose as the major sugars. Higher amounts of arabinose and galactose could be due to neutral arabinogalactan type polysaccharides or could also be pectic type, which are linked to galacturonic acid residues. Higher amount of glucose could be due to cellulosic cell wall polysaccharides and could also be due to associated β-glucan type polysaccharides. Small amounts of galactomannan type or glucomannan type or mannan type of polysaccharides may also form cell wall polysaccharides in mango. Arabinoxylan and xylan type of polysaccharides may be in minor amounts in mango peel.

The content of glucose was increased in IDF during ripening in Raspuri variety and in contrast it decreased in Badami variety. In Badami, content of galactose in IDF was increased during ripening suggesting them to be galactan or arabinogalactan type polysaccharides. IDF in Raspuri peel is likely to be more of cellulosic in nature. This may indicate possible change in the nature of IDF in cell wall polysaccharide between the two mango varieties. No significant difference in SDF of Raspuri between raw and ripe was observed. Galactose was absent in SDF of both in raw and ripe Raspuri variety. Content of mannose was high in SDF compared to IDF in Raspuri mango peel. The presence of arabinose and galactose may be due to arabinogalactan type of polysaccharides and was higher in SDF of Badami raw and that during ripening increase in arabinose content indicating arabinose rich polysaccharides or increased substitution of arabinose to arabinogalactan or pectic type polysaccharides (Aspinall, 1980; Neill et al, 1990). Rhamnose content was found to be low mango peels. This indicated their close association with arabinogalactan type polysaccharides or complex pectic polysaccharides. Content of glucose decreased in IDF during ripening in Badami variety. Decrease in galactose content during ripening of SDF of Badami can also be noted. Arabinose, galactose and glucose were found to be the major neutral sugars in both soluble and insoluble fractions of Hayden variety (Laurrari et al, 1996). Hemicellulosic xylan is reported to be the major polysaccharide in grape seed dietary fiber (Igatuburu et al., 1998). These results indicate complex nature of cell wall polysaccharides in mango peel and changes during ripening are complex and need to be investigated in detail.

Table 2.22. Carbohydrate composition (%) of insoluble and soluble dietary fiber of mango peel.

Dietary fiber	Total sugar	Uronic acid	Rha	Ara	Xyl	Man	Gal	Glu
Raspuri raw IDF	72.6	9.8	2.62	19.35	13.03	7.48	25.21	32.28
Raspuri raw SDF	65.9	20.3	1.42	27.37	5.51	36.66	-	29.02
Raspuri ripe IDF	79.4	17.4	3.90	35.70	6.20	1.43	11.75	41.02
Raspuri ripe SDF	69.7	15.2	1.36	27.87	6.36	38.93	-	26.36
Badami raw IDF	76.5	21.0	0.73	25.65	7.02	5.73	1.26	59.59
Badami raw SDF	74.1	26.0	1.59	29.72	15.70	7.15	21.23	24.57
Badami ripe IDF	82.0	17.0	1.63	19.77	2.43	10.62	39.89	26.66
Badami ripe SDF	71.6	21.7	1.30	38.41	23.54	4.80	5.21	31.26

Rha-rhamnose; Ara- arabinose, Xyl-xylose, Man-mannose, Gal-galactose, Glu-glucose

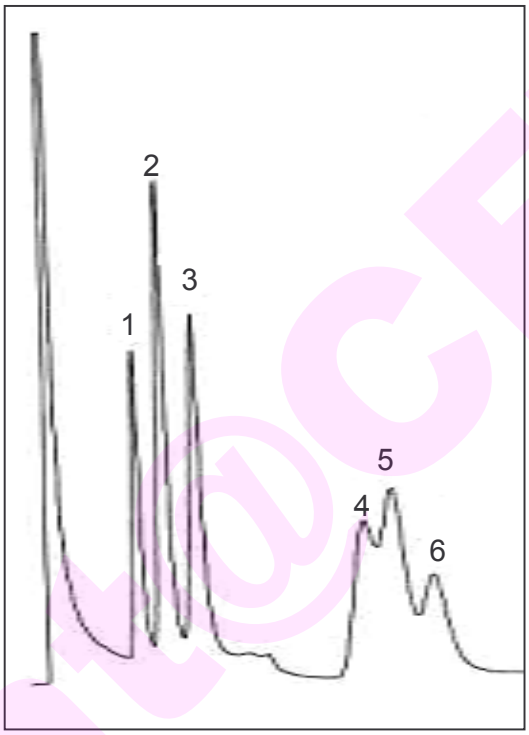


Fig 2.10a. GLC chromatogram of standard sugar mixture

Identified peaks 1. Rhamnose 2. Arabinose, 3. Xylose, 4. Mannose, 5. galactose, 6. glucose.

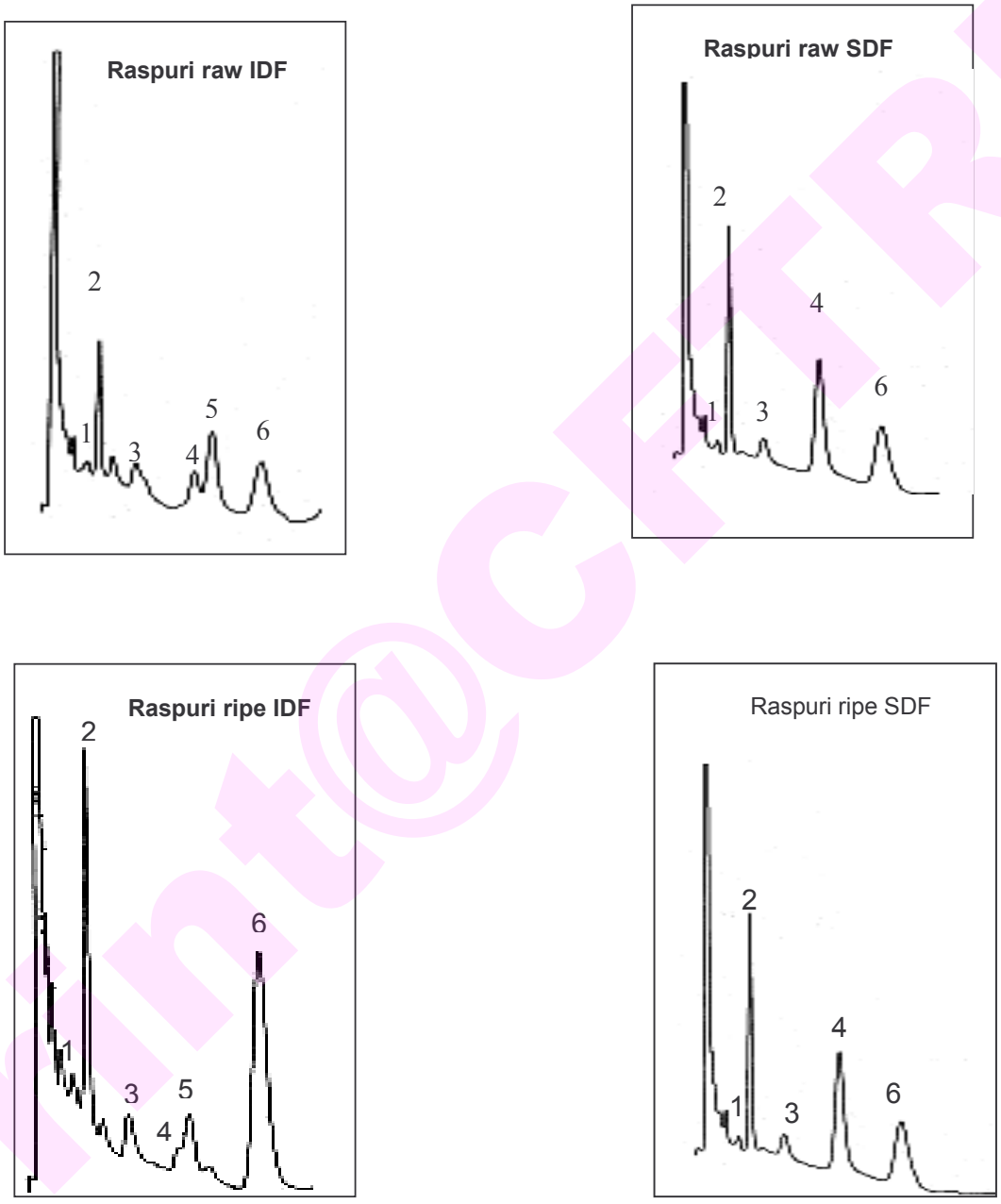


Fig 2.10b. GLC chromatogram of soluble and insoluble dietary fiber isolated from mango peel acetone powder.

Identified peaks 1. Rhamnose 2.Arabinose, 3. Xylose, 4. mannose, 5. galactose, 6. glucose.

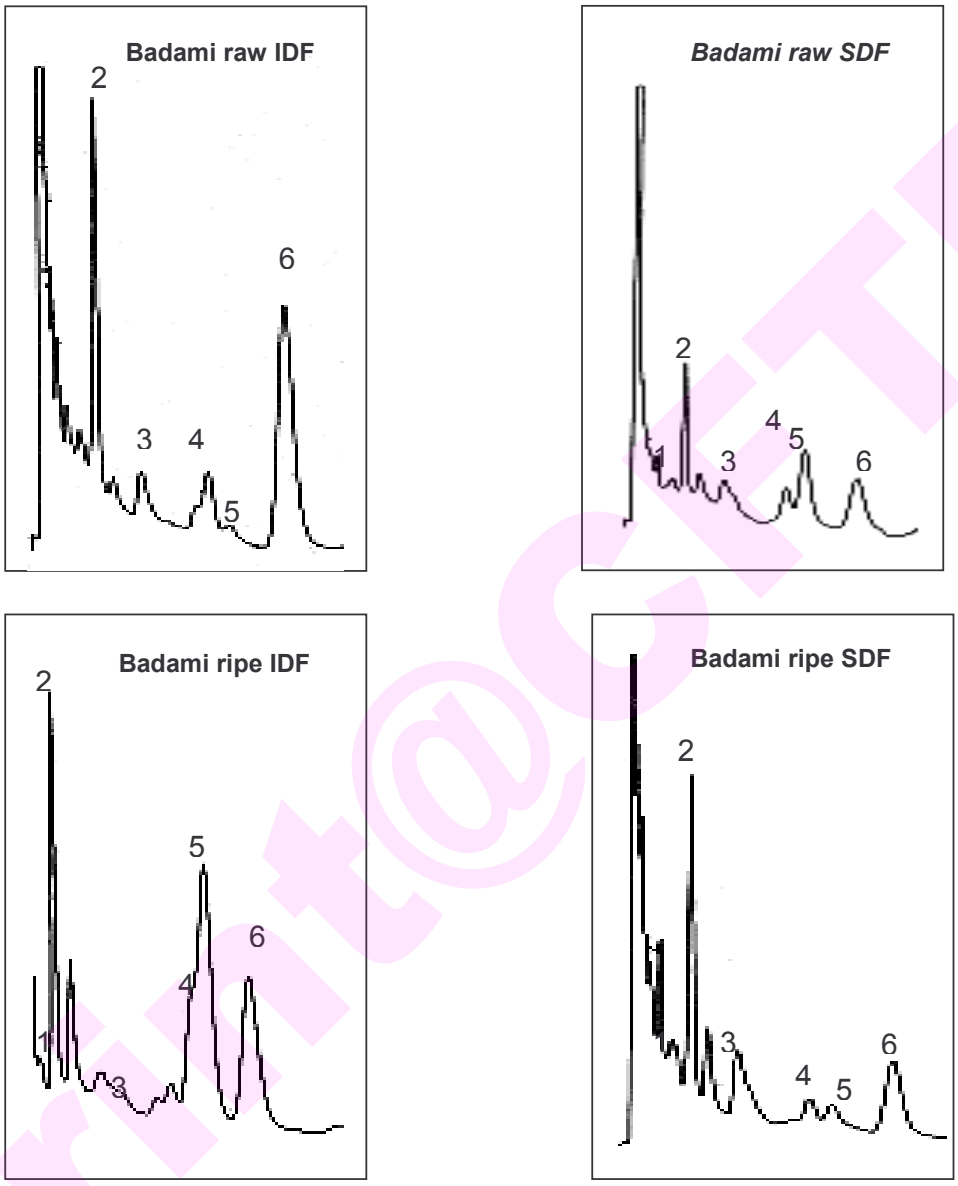


Fig 2.10c. GLC chromatogram of soluble and insoluble dietary fiber isolated from mango peel acetone powder.

Identified peaks 1. Rhamnose 2.Arabinose, 3. Xylose, 4. mannose,5. galactose, 6. glucose.

2.3.1.17. Insoluble bound phenolics content in mango peel dietary fiber

Phenolic acids may form both ester and ether linkages owing to their bifunctional nature through reactions involving their carboxylic and hydroxyl groups, respectively. This allows phenolic acids to form cross-links with cell wall macromolecules such as polysaccharides, proteins or fatty acids (Yu et al, 2001). Cross-linking of lignin components via phenolic acids appears to have a profound effect on the growth of cell wall and its mechanical properties and biodegradability. Such cross-linked phenolic fractions are unextractable in aqueous organic solvents, and therefore, can be referred as insoluble bound phenolics. Insoluble bound phenolics may be released by alkali, acid or enzymatic treatment of sample prior to extraction, for isolation and identification. In the present study, alkaline hydrolysis was adopted to extract the phenolic acids.

The bound phenolic content of mango peel dietary fiber was determined and is shown in **Table 2.23**. The bound phenolic content in mango peel dietary fiber was found to be in the range of 8.12 to 29.52 mg/g. The bound phenolic content was more in ripe mango peel than the raw mango peel. Earlier (Section 2.3.1.2), it was reported that extractable phenolics were more in raw peel compared to ripe peel. However, in case of bound phenolics it was found to be different. It indicates that during ripening the phenolics may be involved in cross-linking of polysaccharides and as a result of this insoluble dietary fiber is increased during ripening (**Table 2.18**).

Table 2.23. Insoluble bound phenolic content in the mango peel dietary fiber

Variety	Bound phenolics (mg/g)
Raspuri raw	8.12± 0.37[a]
Raspuri ripe	29.52± 1.73[c]
Badami raw	10.45±0.51[b]
Badami ripe	28.10± 0.10[c]

Values are expressed on dry weight basis. All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differ significantly ($P \leq 0.05$),

HPLC profiles of phenolic acids present in bound phenolic fractions are shown in **Figures 2.11a & 2.11b**. The phenolic acids separated by C18 column were identified by spiking with authentic standards. Gallic acid, protocatechuic acid, syringic acid and ferulic acid were found to be the phenolic acids identified in the bound phenolic fractions (**Table 2.24**). Gallic acid was found to be the major phenolic acid in both raw and ripe Badami and Raspuri mango peel. Ferulic acid was identified only in Raspuri peel, but it was absent in both Badami raw and ripe peel dietary fiber. It was reported that derivatives of hydroxycinnamic acids such as sinapic acid, p-coumaric acid and ferulic acid occur mainly in bound phenolic fractions of lemon, orange, and grapes (Gorinstein et al, 2001). There is a similarity between the compositions of extractable phenolic acids present in the acetone extract of mango peel (**Table 2.5**) and the phenolic acids associated (bound) with peel dietary fiber except that ferulic acid is present only in the dietry fiber bound phenolics.

Table 2.24. Identification of bound phenolic acids from mango peel dietary fiber

Phenolic acids (mg/g extract)	Raspuri raw	Raspuri ripe	Badami raw	Badami ripe
Gallic acid	4.54	14.79	6.29	16.60
Protocatechuic acid	0.39	1.23	0.39	0.63
Syringic acid	1.42	4.88	1.45	2.67
Ferulic acid	0.94	3.57	-	-

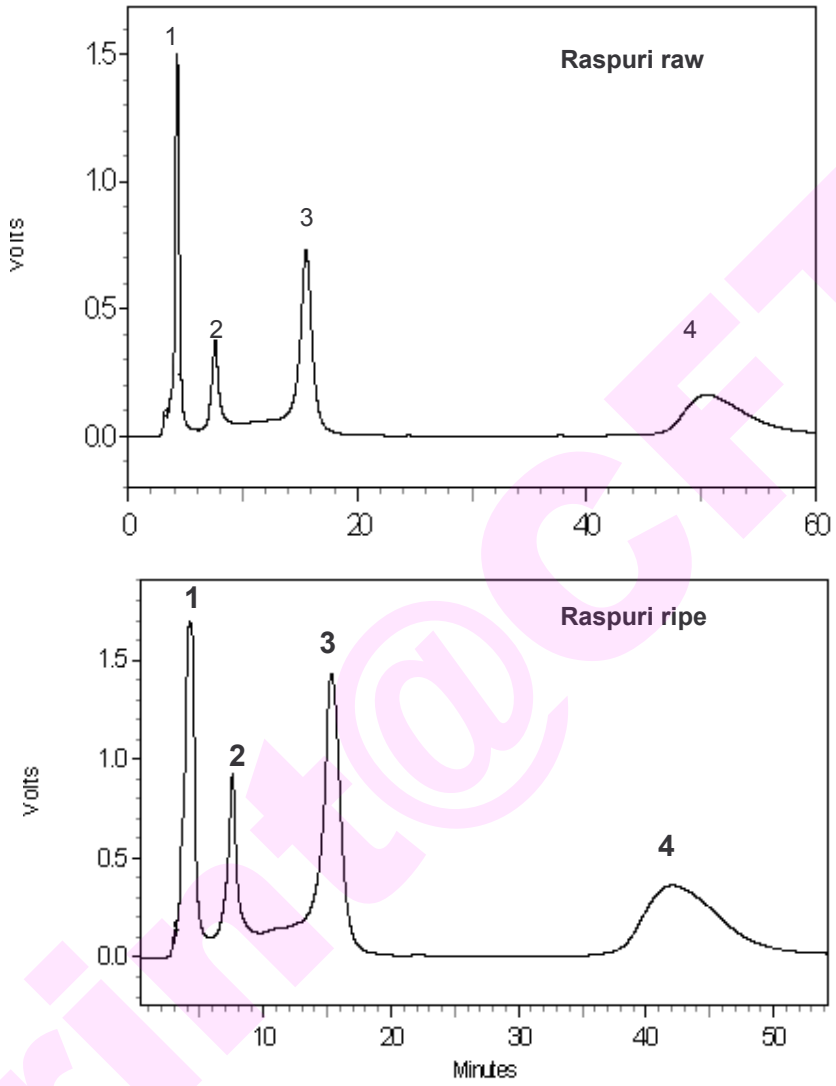


Figure 2.11a. HPLC chromatogram of insoluble bound phenolics isolated from Raspuri mango peel dietary fiber

1. Gallic acid, 2. Protocatechuic acid, 3. Syringic acid, 4. Ferulic acid

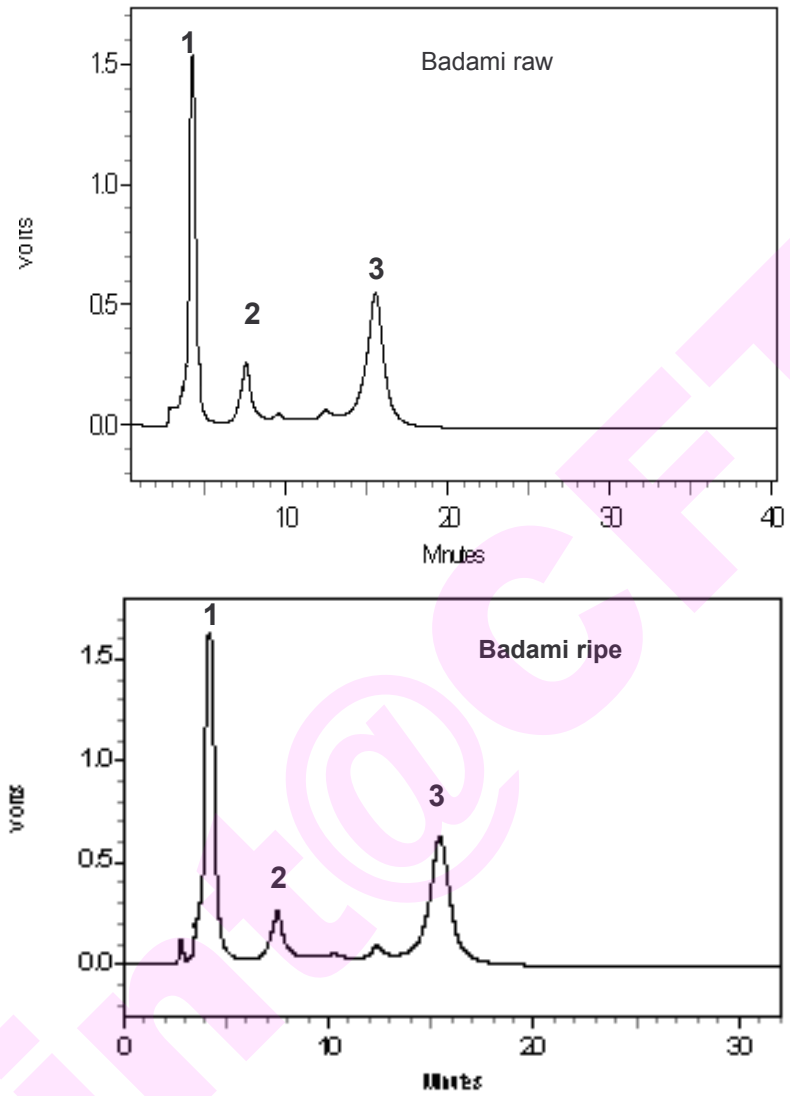


Figure 2.11b. HPLC chromatogram of insoluble bound phenolics isolated from Badami mango peel dietary fiber

1. Gallic acid, 2. Protocatechuic acid, 3. Syringic acid

Thus, the results show that mango peel is a rich source of valuable compounds such as polyphenols, carotenoids, anthocyanins, vitamins C and E, enzymes, and dietary fibers. A simple two-step process to isolate these bioactive compounds simultaneously from mango peel, which is a byproduct from processing industry, has been developed. This new source will be potential as a functional food or value added ingredient in future for our diet system.

2.3.2. SECTION B: Antioxidant activity of mango peel acetone extract

Fruits and vegetables contain phenolic compounds, carotenoids, anthocyanins and tocopherols and these compounds exhibit antioxidant properties (Bartosz, 1997; Naczk & Shahidi, 2006). Many studies have shown that free radicals in the living organisms cause oxidative damage to different molecules such as lipids, proteins, nucleic acids and these are involved in the interaction phases of many degenerative diseases. Antioxidants are substances that delay or prevent the oxidation of cellular oxidisable substrates. They exert their effect by scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS) or preventing the generation of ROS/RNS (Halliwell, 1996).

Earlier, in Section 2.3.1., it was reported that mango peel contained a number of valuable compounds such as polyphenols, carotenoids, vitamin E and vitamin C. Therefore, the objective of the present study was to evaluate the antioxidant activity of acetone extract of mango peel. The antioxidant potential of the acetone extract of mango peel was determined by different *in vitro* methods such as reducing power methods, free radical scavenging activity using stable free radical DPPH, inhibition of lipid peroxidation in microsomes and inhibition of soybean lipoxygenase enzyme and also erythrocytes as a cellular model.

2.3.2.1. Reducing power of mango peel acetone extract

The reducing power of a compound is related to its electron transfer ability and may, therefore, serve as a significant indicator of its antioxidant activity (Meir et al, 1995). **Figure 2.12** shows the reducing power of the

Raspuri and Badami mango peel extracts. The reducing power increased with the concentration of peel extract. The reducing power of Badami peel extracts was more compared to Raspuri peel extracts and BHA. For example, the absorbance at 700 nm was found to be 0.358 for Badami raw peel extract at a dose level of 5 μg GAE, while it was 0.125 for Raspuri raw extract and 0.136 for BHA. Phenolics, carotenoids and anthocyanins present in the peel are good electron donors and could reduce Fe^{3+}/ferricyanide complex to ferrous form, which indicates the antioxidant activity (Yen & Chen, 1995; Chung et al, 2002).

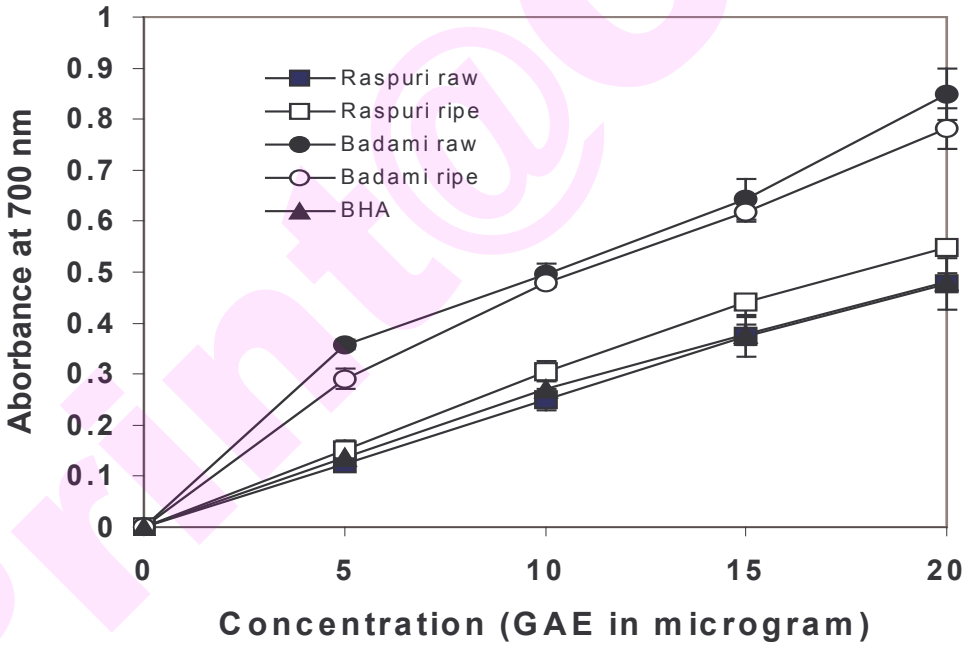


Figure 2.12. Reducing power of the raw and ripe Raspuri and Badami mango peel extracts

2.3.2.2. Scavenging effect of mango peel acetone extract on DPPH radical

Scavenging the stable DPPH radical model is another widely used method to evaluate antioxidant activity. DPPH is a stable free radical with characteristic absorption at 517 nm and antioxidants react with DPPH and convert it to 2, 2-diphenyl-1-picrylhydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extract, which is due to the hydrogen donating ability (Van Gadow et al, 1997).

Figure 2.13 shows the dose dependence curve for the radical scavenging activity of the mango peel extracts. The IC_{50} values ranged from 1.83 to 4.54 μg of GAE (**Table 2.25**). The mango peel extracts showed a concentration dependent scavenging of DPPH radical, which may be attributed to its hydrogen donating ability. Raspuri extracts showed low IC_{50} values (1.83 μg to 1.98 μg of GAE) compared to that of Badami peel extracts (3.67 to 4.54 μg of GAE). The free radical scavenging activity of mango peel extracts were compared with BHA. It was found that acetone extracts of Raspuri raw and ripe mango peels showed more scavenging activity than that of BHA (**Table 2.25**).

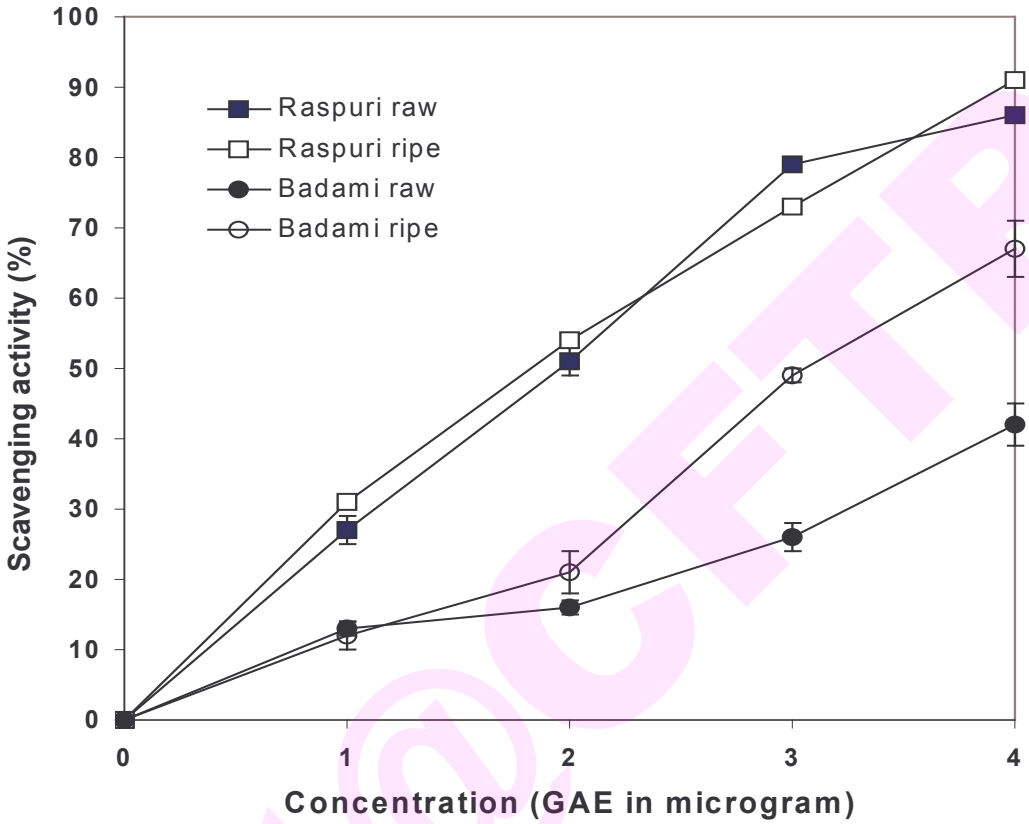


Figure 2.13. DPPH radical scavenging effects of raw and ripe mango peel extracts of Raspuri and Badami

2.3.2.3. Inhibition of microsomal lipid peroxidation

In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acids in the cell membrane) generates a large number of degradation products such as malonaldehyde (MDA), which are found to be an important cause of cell membrane destruction and cell damage (Kubow, 1992). MDA, one of the major products of lipid peroxidation, has been extensively used as an index for lipid peroxidation and as a marker for oxidative stress. The reaction of MDA with TBA has been widely adopted as a sensitive assay method for lipid peroxidation (Ohkawa et al, 1978).

Figure 2.14 shows the dose dependence curve for the inhibition of lipid peroxidation by acetone extracts of peels from different mango varieties. Badami extracts showed IC_{50} values from 1.39 μg to 2.68 μg of GAE compared to that of Raspuri peel extracts (3.13 to 4.59 μg GAE). The lipid peroxidation inhibitory activity of mango peel extracts was compared with BHA (**Table 2.25**). In this method, ripe peel extracts showed higher inhibition on lipid peroxidation than raw peel extracts and these peel extracts showed higher IC_{50} values compared to BHA.

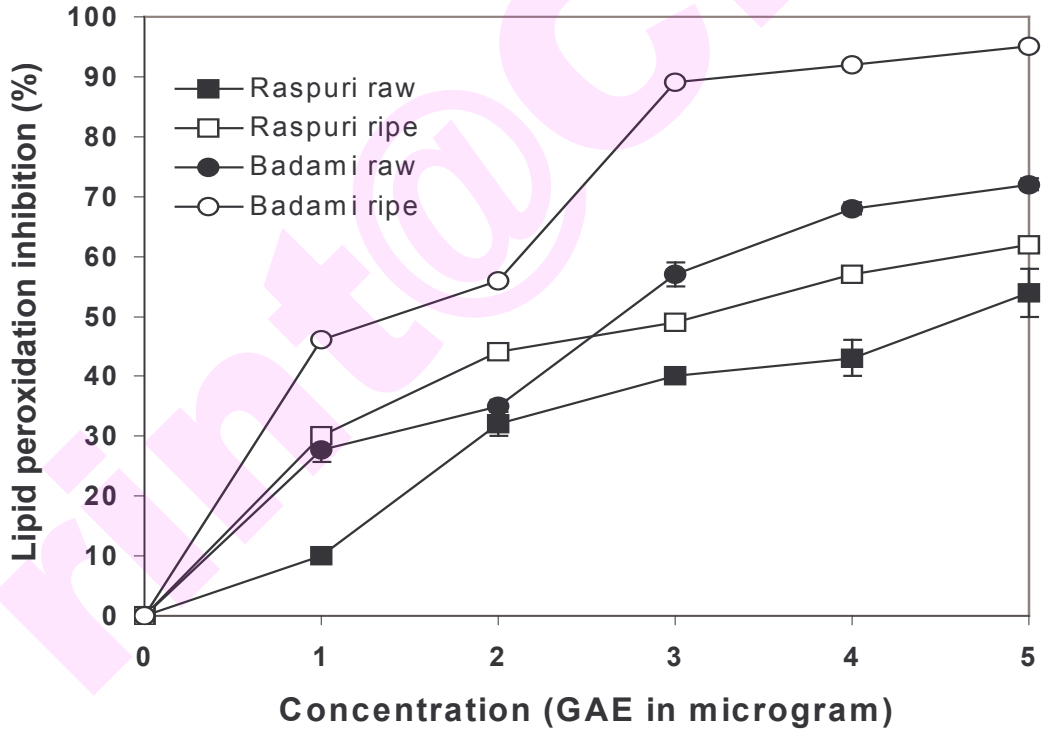


Figure 2.14. Inhibition of lipid peroxidation by raw and ripe mango peel acetone extracts of Raspuri and Badami

2.3.2.4. Inhibition of soybean lipoxygeanse activity by mango peel acetone extract

Lipoxygenase is a biological target for many diseases such as asthma, atherosclerosis, cancer (Mogul & Holman, 2001) and tumor angiogenesis (Nie & Honn, 2002). Lipoxygenase (E.C. 1.13.11.12) constitutes a family of non-heme containing dioxygenase group of enzymes that are widely distributed in plants and animals. In mammalian cells these enzymes play key role in the biosynthesis of a variety of bio-regulatory compounds such as hydroxyeicosatetraenoic acid, leukotrienes, lipoxins and hepoxylines (Lands, 1985). Lipoxygenases are potential targets for the rational drug design and discovery of mechanism based inhibitors for the treatment of a variety of disorders and autoimmune diseases, therefore, inhibition of this enzyme is widely used to evaluate antioxidant activity. Antioxidants interact non-specifically with lipoxygenase by scavenging radical intermediates and/or reducing the active heme site (Cao et al, 1996).

Acetone extracts of mango peel showed concentration dependent inhibition of soybean lipoxygenase activity (**Figure 2.15**). Raw mango peel extracts showed higher inhibition of lipoxygenase activity compared to that of ripe peel extracts (**Table 2.25**). Of the different extracts, Badami raw peel extract showed maximum inhibition with an IC_{50} value of 2.02 μg of GAE, which is better than BHA (2.82 μg of GAE, **Table 2.25**).

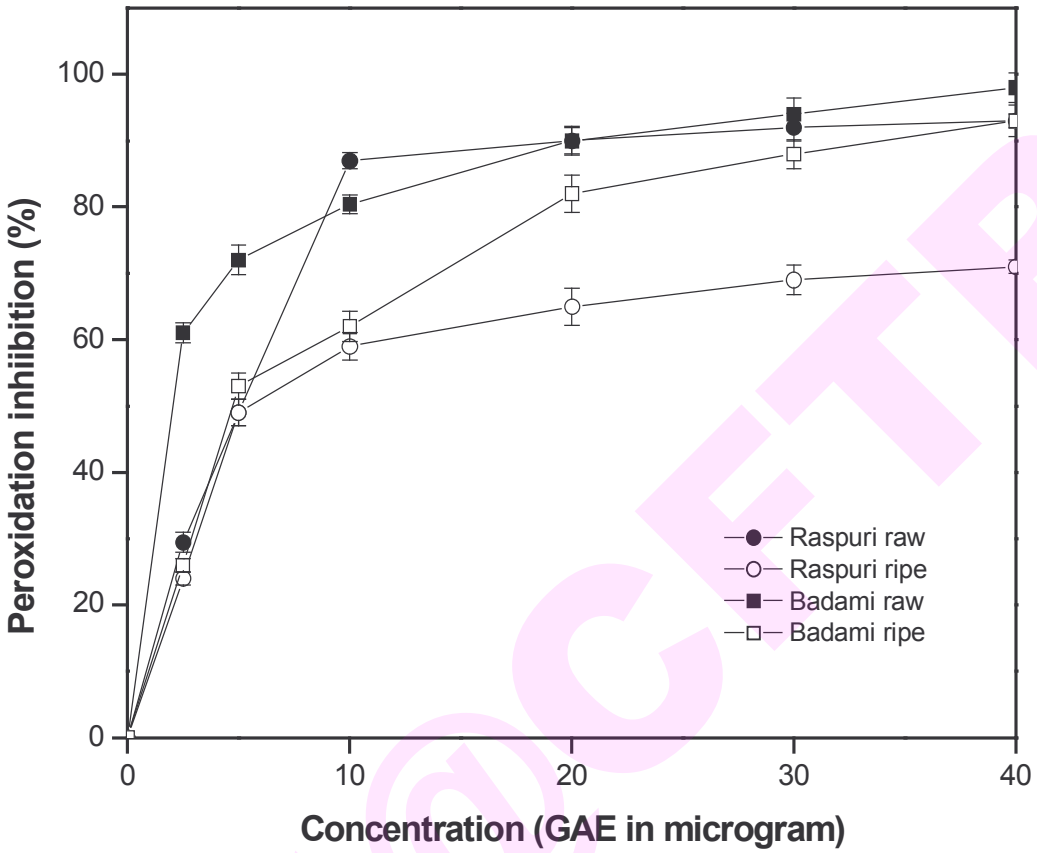


Figure 2.15. Inhibition of soybean lipoxygeanse activity by raw and ripe mango peel acetone extract of Raspuri and Badami

Table 2.25. IC_{50} values of acetone extract of Raspuri and Badami raw and ripe mango peel on different antioxidant model systems.

Mango variety	DPPH (μg of GAE)	LPO (μg of GAE)	Lipoxygenase (μg of GAE)
Raspuri Raw	1.98±0.05^{b}	4.59±0.04^{e}	5.14±0.08^{d}
Raspuri Ripe	1.83±0.02^{a}	3.13±0.02^{c}	5.24±0.09^{d}
Badami Raw	4.54±0.02^{e}	2.68±0.01^{d}	2.02±0.10^{a}
Badami ripe	3.67±0.06^{d}	1.39±0.02^{b}	4.73±0.12^{c}
BHA	3.40±0.08^{c}	0.80±0.04^{a}	2.82±0.14^{b}

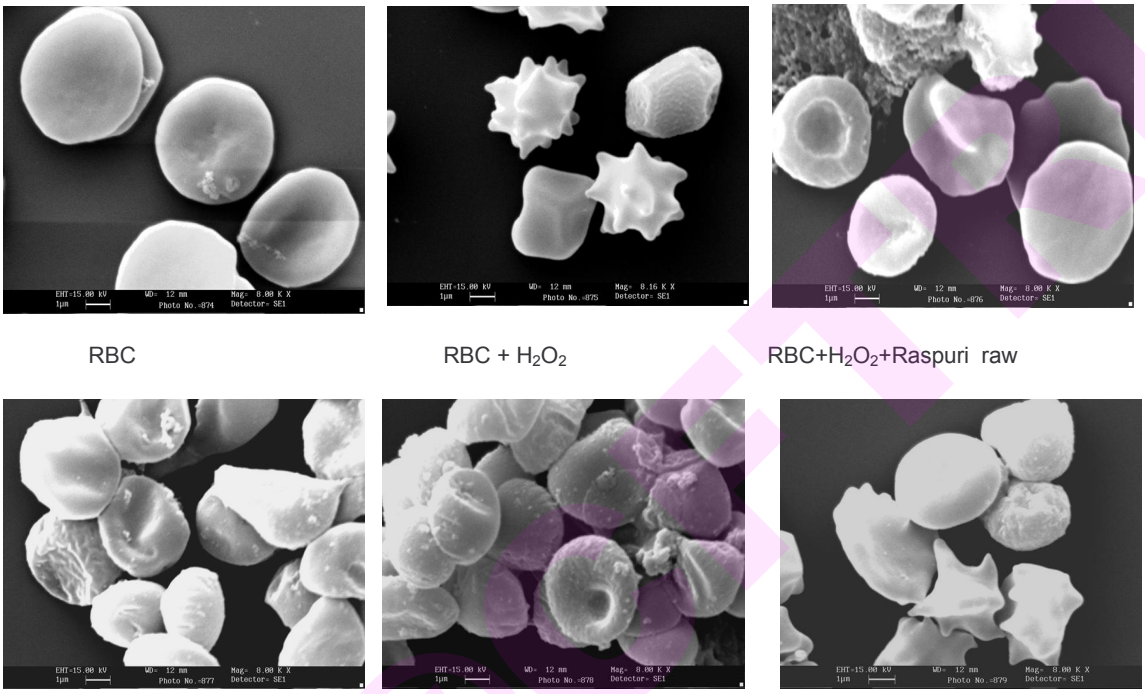
IC_{50} values were calculated from the dose responses curves. All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.2.5. Inhibition of hydrogen peroxide induced oxidative damage in rat erythrocytes by mango peel extract

Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O_2 transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O_2 species (Sadrazadeh et al, 1984). Moreover, the free hemoglobin exposed to H_2O_2 causes heme degradation with the release of iron ions which catalytically active in initiating free radicals and lipid peroxidation. Because of their susceptibility to oxidation, erythrocytes have been used as cellular model to investigate oxidative damage in biomembranes and have been used as *in vitro* model for the evaluation of antioxidant potential of antioxidant compounds. It has been reported that aqueous extracts of stem bark (Vimang) from selected mango varieties protected the human erythrocytes damage by H_2O_2 (Rudriguez et al, 2006) and also prevented age associated oxidative stress in elderly humans (Pardo-Andreu et al, 2006). Polyphenolics or anthocyaninins extracted from different fruits such as blue berry, grapes are found to protect or increase resistance of erythrocytes to oxidative stress (Youdim et al, 2000; Carini et al, 2000; Tedesco et al, 2001). The aim of the present study is to evaluate the protective role of mango peel extract against H_2O_2 induced oxidative damage in normal rat erythrocytes.

2.3.2.5.1. Effect of mango peel extract on rat erythrocyte structure/ morphology

Scanning electron micrographs of rat erythrocytes treated *in vitro* with H_2O_2 and treated in the presence of mango peel extracts are shown in **Figure 2.16**. Untreated erythrocytes appeared as typical discocytes while exposure to H_2O_2 resulted in a significant change in the cell shape and distinct echinocyte formation. The morphological changes induced by H_2O_2 were greatly prevented when the cells were treated with mango peel extracts and standard antioxidant BHA (**Figure 2.16**). Oxidative damage to cell membrane leads to alterations in cell rigidity and shape. This oxidative stress leads to echinocyte formation because of membrane damage, which in turn leads to change in whole red blood cell structural conformation and its functioning (Linderkamp et al, 1997). Earlier inhibitory effect of gallic acid (25 μM) on structural damage of mice erythrocytes by 0.3% H_2O_2 was reported (Ohinishi et al, 1994).



RBC | RBC + H_2O_2 | RBC+H_2O_2+Raspuri raw

RBC+H_2O_2+Raspuri ripe | RBC+H_2O_2+Badami raw | BC+H_2O_2+Badami ripe

Figure 2.16. Scanning electron micrograph of normal rat erythrocyte and protective effects of different variety of mango peel extracts at different stages against H_2O_2 induced oxidative damage on RBC.

2.3.2.5.2. Inhibition of rat erythrocyte hemolysis

Figure 2.17 shows inhibitory effect of different concentrations of mango peel extract (5-25 μg GAE) on H_2O_2 induced hemolysis of rat erythrocytes for 3 h. The mango peel extract inhibited the hemolysis of rat erythrocytes in a dose dependent manner with 81.7% as maximum inhibition of erythrocyte hemolysis at 25 μg GAE. The peel extract showed 50% hemolysis inhibition (IC_{50}) at concentrations ranging from 11.5 to 20.9 μg GAE of mango peel extract (**Table 2.26**). The BHA exhibited with an IC_{50} value of

20.5 μg GAE that is comparable to that of peel extracts. Tedesco et al (2000) reported that red wine had showed protecting properties against erythrocyte hemolysis that was induced by H_2O_2.

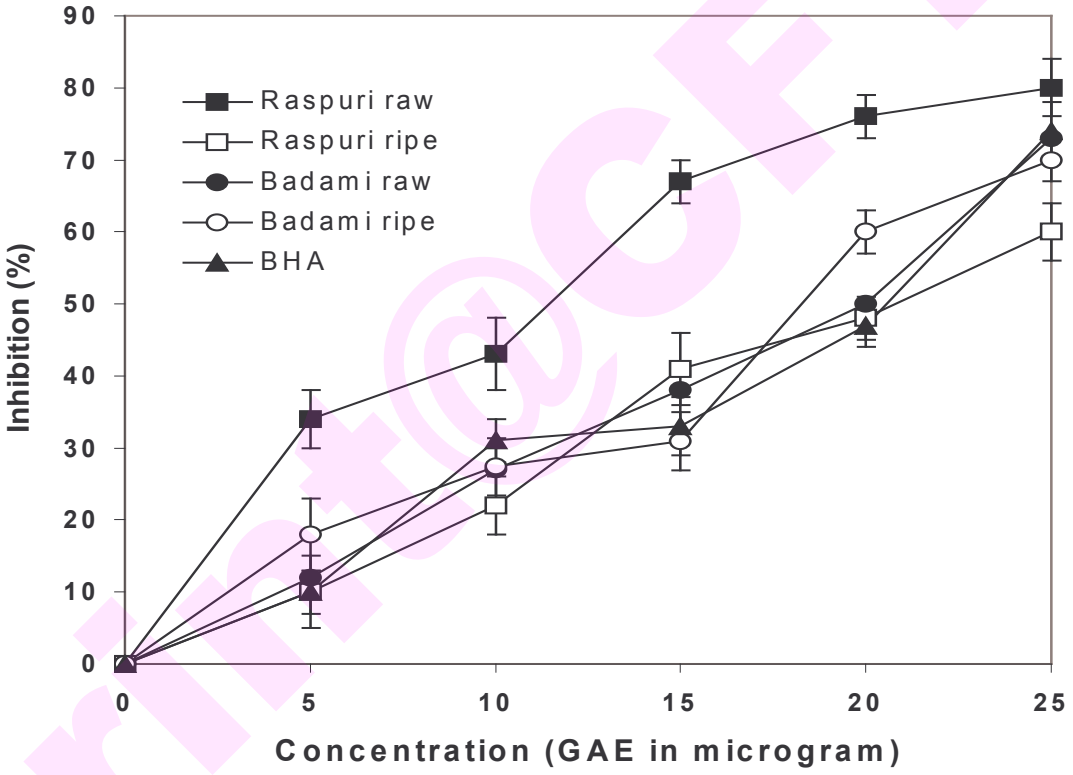


Figure 2.17. *In vitro* protective effects of mango peel extracts against H_2O_2 induced hemolysis of rat erythrocytes

2.3.2.5.3. Inhibition of lipid peroxidation on rat erythrocyte ghost membrane

The results presented in **Figure 2.18** indicated that lipid peroxidation could be effectively inhibited by mango peel extract. The IC_{50} value for lipid peroxidation inhibition on erythrocyte ghost membrane was found to be in the range of 4.5 to 19.29 μg GAE. As can be seen from **Table 2.26**, Raw peel extracts showed higher inhibition on lipid peroxidation than ripe peel extracts as well as BHA. Of the different extracts, Raspuri raw extract showed the maximum inhibition of lipid peroxidation, while Raspuri ripe extract showed less inhibition.

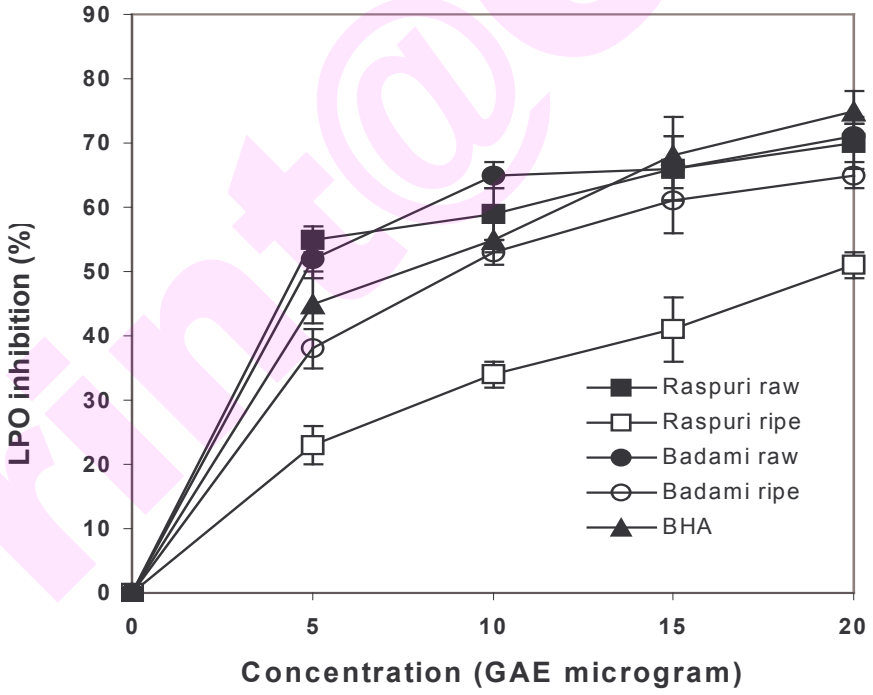


Figure 2.18. In vitro protective effects of mango peel extract against H_2O_2 induced lipid peroxidation on rat erythrocytes ghost membrane

Table 2.26. IC_{50} values of acetone extract of Raspuri and Badami raw and ripe mango peel for inhibition of hemolysis and lipid peroxidation of rat erythrocytes

Mango variety	Inhibition of Hemolysis (μg GAE)	Inhibition of LPO (μg GAE)
Raspuri Raw	11.5±0.04[a]	4.5±0.05[a]
Raspuri Ripe	20.9±0.08[d]	19.3±0.04[e]
Badami Raw	19.9±0.05[c]	4.7±0.02[b]
Badami ripe	18.3±0.06[b]	9.2±0.03[d]
BHA	20.5±0.08[c]	7.5±0.04[c]

IC_{50} values were calculated from the dose response curves. All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.2.5.4. Protection of oxidative damage on rat erythrocyte ghost membrane proteins

The alterations in the protein pattern of membrane proteins produced by oxidative stress were observed by SDS-PAGE. Ghost membrane was prepared by hypotonic lysis of normal erythrocytes. Membranes were treated with H_2O_2 and with or without mango peel extract and were analyzed on SDS-PAGE (**Figure 2.19**). The membrane proteins, spectrin (band 1 and band 2) and actin bands diminished after 1h of incubation with H_2O_2. Upon treatment with H_2O_2, most of the bands present in the high molecular weight region, diminished and new bands in the low molecular weight region appeared (**Figure 2.19**, lane 6). However, bands 1, 2, 3 and 4, and actin band were still clearly evident in the membranes treated with antioxidant rich mango peel extracts even after 1 h incubation with H_2O_2 (**Figure 2.19**, lanes 2,3,4,5). The mango peel extract thus effectively protected the erythrocytes membrane from H_2O_2 induced membrane protein degradation. Changes in erythrocyte

membrane ion permeability, lipid peroxidation, and formation of disulfide bonds and activation of proteolysis have been reported following the challenge of erythrocytes by different oxygen radical generating system (Chakrabarti et al, 1990; Tavazzi et al, 2000). It was reported that a commercially available antioxidant mixture effectively protects cells from 2,2'-azobis-2-amidinopropane dihydrochloride (AAPH) induced membrane protein degradation under the experimental condition (Zou et al, 2001). In the present study, the high molecular weight bands disappeared while low molecular weight content increased indicating under stress induced by H_2O_2, proteolysis might have taken place. Earlier, Davis and Goldberg (1987) reported that the membrane protein degradation due to increased proteolysis in erythrocytes exposed to H_2O_2.

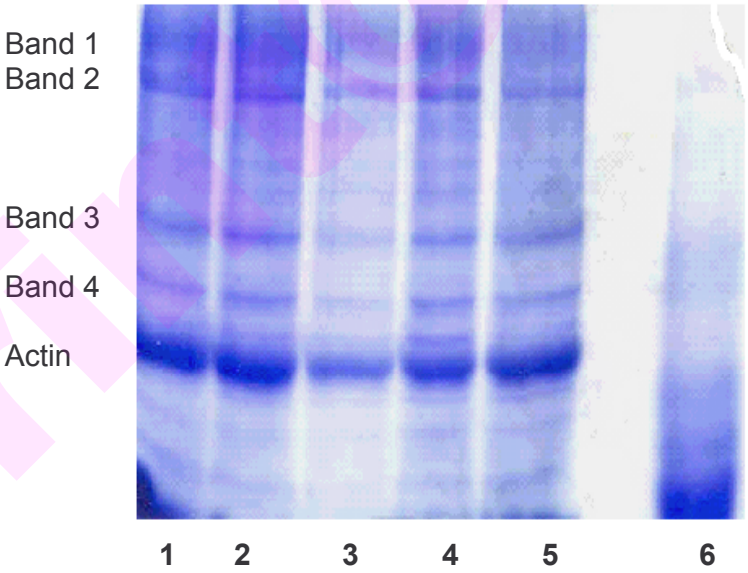


Figure 2.19. SDS–PAGE of rat erythrocyte membrane proteins showing protective effects of mango peel extract against H_2O_2 induced oxidative damage on erythrocyte ghost membrane.

(1) Membrane proteins (untreated), (2) H_2O_2 and Raspuri raw peel extract, (3) H_2O_2 and Raspuri ripe peel extract, (4) H_2O_2 and Badami raw peel extract, (5) H_2O_2 and Badami ripe peel extract, (6) membrane protein treated with H_2O_2.

The present study revealed that acetone extracts exhibited good antioxidant activity by effectively scavenging various reactive oxygen species and other free radicals such as DPPH radicals, ferric ion, hydroxyl radicals and peroxyl radicals in different antioxidant systems. The difference in antioxidant activity of the peel of different varieties at different stages of maturity may be due to variation in composition and content of antioxidants such as polyphenols, carotenoids and anthocyanins. The present studies also showed that mango peel acetone extract demonstrated the inhibition of the oxidative hemolysis of erythrocytes induced by H_2O_2 under experimental conditions. The mango peel extract showed protection against lipid peroxidation, membrane protein degradation and morphological changes caused by H_2O_2. In the present study, the results indicated the antioxidant potential of mango peels could be due to synergistic actions of bioactive compounds present in them. Thus, acetone extract of mango peel due to its excellent antioxidant activity may find application as an antioxidant in food preservatives and as nutraceutical.

2.2.3. SECTION C: Incorporation of mango peel powder into biscuits and macaroni and their sensory and nutraceutical properties

Earlier in Section 2.3.1., it clearly demonstrated that mango peel is a good source of bioactive compounds such as polyphenols, carotenoids, vitamins, enzymes and dietary fibers. It was also shown that mango peel extract exhibited antioxidant activities such as scavenging of hydroxyl radical, lipid peroxyl radical, DPPH radical and thus may be used in nutraceutical and functional foods. Another approach for better utilization of mango peel attempted in this was by incorporating the mango peel into food products. For these studies fresh mango peel was collected from a mango pulp industry immediately after their processing, dried and powdered. The powdered mango peel was incorporated into soft dough biscuit and macaroni formulation. Both the products were evaluated for their rheological, sensory and nutraceutical properties of products.

2.3.3.1 Chemical analysis of mango peel powder

The proximate composition of MPP is shown in **Table 2.28**. The moisture, fat and total protein content was 10.5%, 2.2% 3.61%, respectively. The carbohydrate content in the MPP was 80.65%. The crude fiber content was 24%.

The total dietary fiber content (TDF) in MPP was 51.15%, of which insoluble dietary fiber (IDF) constituted 32.11% and soluble dietary fiber (SDF) 19.04%. The total polyphenol content in the MPP was found to be 96.17 mg GAE/ g. The carotenoid content in the MPP was 3,092 μg/g dry peel

powder. The antioxidant property of mango peel powder was determined using free radical scavenging method found to be 79.6 μg of MPP.

Earlier in Section 2.3.1., it was reported that the polyphenols, carotenoids and dietary fiber content in raw and ripe peels of Raspuri and Badami mango varieties, ranged from 55-110 mg/g, 387-3,337 μg/ g, 44-78%, respectively. The values obtained in the present study are within above range. The polyphenol and carotenoid contents determined in the MPP were comparable to the peel of ripe Raspuri mango peel. However, total dietary fiber was lower than that reported for peel from ripe Badami and Raspuri varieties.

Table 2.28. Chemical composition and IC_{50} value for free radical scavenging activity of MPP.

Component	Content
Moisture (%)	10.50±0.50
Fat (%)	2.20±0.06
Ash (%)	3.04±0.18
Total protein (%)	3.61±0.60
Total carbohydrate (%)	80.65±1.20
Crude fiber (%)	24.00±1.10
Total dietary fiber (%)	51.15±1.08
Soluble dietary fiber (%)	19.04±0.26
Insoluble dietary fiber (%)	32.11±1.34
Total polyphenols (mg GAE/g MPP)	96.17±1.40
Total carotenoids (μg/ g MPP)	3,092±98.0
Free radical scavenging activity ($IC_{50,}$ μg peel)	79.60±2.20

Values are expressed on dry basis

All data are the mean±SD of three replicates.

2.3.3.2. Influence of MPP on dough characteristics

The effect of MPP on dough mixing properties was measured using Brabender Farinograph. Incorporation of MPP at 2.5%, 5%, 7.5% and 10% showed significant difference on the dough mixing properties as shown in **Table 2.29.** Addition of MPP increased the water absorption from 60 (control) to 68% (10% MPP). Similar increase in water absorption was reported when bran from different cereals was incorporated into flour (Pomeranz et al, 1977; Sudha et al, 2007). Rosell et al (2001) reported that difference in water absorption is mainly caused by the greater number of hydroxyl groups, which exists in the fibre structure and allowed more water interaction through hydrogen bonding. The higher content of dietary fiber in MPP may be responsible for this increase in water absorption during mixing of dough. The dough development time increased from 4.2 to 5.8 min with 10% incorporation of MPP. Dough stability, which indicates the dough strength decreased from 6.7 to 4 min with incorporation of 10% MPP. There was an increase in mixing tolerance index.

Table 2.29. Effect of MPP on Farinographic characteristics[a]

MPP (%)	WA (%)	DDT (min)	DS (min)	MTI (BU)
0*	60.0	4.2	6.7	27
2.5	61.0	4.5	6.5.	65
5.0	64.2	5.3	5.7	56
7.5	66.5	5.6	4.5	52
10	68.1	5.8	4.0	49

[a] average of two values; *Control

WA-water absorption, DDT dough development time, DS, dough stability and MTI mixing tolerance index.

2.3.3.3. Physical characteristics of biscuits incorporated with MPP

Biscuits are convenient food products and the most popular bakery items consumed by both rural and urban population of the society. Some of the reasons for such wide popularity are its ready-to-eat nature, affordable cost, good nutritional quality, availability in different taste and longer shelf life (Gandhi et al., 2001). Reports are available on the use of oat bran, wheat bran and rice bran as source of dietary fiber content in bread and other bakery products (Pomeranz et al, 1977; Leelavathi & Rao, 1993; Saunders et al, 1990; Laurikainen et al, 1998; Sidhu et al, 1999). Recently, Sudha et al (2007) reported the influence of different cereal brans on biscuit quality. On the other hand, fruit fiber concentrates have better nutraceutical quality than those found in cereals due to higher proportion of SDF and significant content of dietary fiber associated bioactive compounds (Laurauri et al, 1996; Grigelmo-Miguel & Martin Belloso, 1999; Chau & Huang, 2003). Recently, Vergara-Valencia (2007) reported the improvement in nutraceutical properties of cookies and bread with the incorporation of mango dietary fiber obtained from unripe mango fruit (whole fruit).

In the present study, commercial wheat flour having 10.7% moisture, 10.1% protein and 0.48% ash was used for the preparation of biscuits. Biscuits were prepared by incorporating 5%, 7.5%, 10%, 15% and 20% levels of MPP in the formulation and were determined for physical and sensory attributes. Physical characteristics of biscuits such as thickness, diameter and spread ratio were affected slightly with the increase in the level of MPP (**Table 2.30**). Incorporation of MPP decreased the diameter of the biscuits from 55.6 mm (control) to 52.2 mm (20%). But there was no significant difference

observed upto 10% level of incorporation. The thickness biscuits incorporated with MPP was comparable to control up to 10% level. No significant difference in spread ratio was observed in control and MPP incorporated biscuits. However, the breaking strength of biscuits increased with incorporation of MPP. Biscuits prepared from flour containing 20% MPP had a breaking strength 1.97 kg compared to 0.88 kg of the control biscuits.

Figure 2.21. Mango peel powder enriched biscuits: C- control 1) 5% MPP, 2) 7.5% MPP, 3) 10% MPP, 4) 15% MPP, 5) 20% MPP.

Table 2.30. Influence of MPP on the physical characteristics of biscuits

MPP (%)	Diameter (mm) W	Thickness (mm) T	Spread ratio (W/T)	Breaking strength (kg f)
0% (control)	55.6±0.2^{cd}	1.11±0.005^{cd}	50.1±0.36^{a}	0.88±0.08^{a}
5%	56.0±0.3^{d}	1.12±0.005^{d}	49.8±0.40^{a}	1.27±0.02^{b}
7.5%	55.6±0.5^{cd}	1.10±0.008^{c}	50.1±1.50^{a}	1.29±0.17^{b}
10%	55.1±0.3^{c}	1.00±0.005^{c}	49.9±1.50^{a}	1.42±0.09^{bc}
15%	53.2±0.6^{b}	1.07±0.005^{b}	50.6±1.70^{a}	1.69±0.17^{cd}
20%	52.2±0.3^{a}	1.04±0.005^{a}	50.1±1.10^{a}	1.97±0.18^{d}

All data are the mean±SD of six replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.4. Color measurement of biscuits

The color of the biscuit was measured by Hunter system using L, a and b values (Brien et al, 2003). L value is a measure of the light-dark fraction of biscuit surface color. The 'L' value decreased with the increase in levels of MPP (**Table 2.31**). Control biscuits had the highest brightness compared to the MPP enriched biscuits. No specific trend is seen in change in 'a' values upon addition of MPP. The change in 'b value, which indicates the yellowness, gradually decreased with increase in MPP level.

Table 2.31. Influence of MPP on the color of the biscuits

MPP (%)	L value	'a' value	'b' value
0(Control)	64.87±0.80 ^{e}	9.52±0.35^{e}	26.26±0.27^{e}
5	61.78±0.78^{d}	6. 62±0.41^{a}	25.33±0.57^{d}
7.5	59.30±0.29^{c}	7.65±0.23^{bcd}	23.99±0.24^{c}
10	56.38±1.05^{b}	8.04±0.52^{cd}	22.85±0.24^{b}
15	52.90±0.82^{a}	8.11±0.40^{d}	22.26±0.28^{a}
20	52.90±0.61^{a}	7.71±0.25^{bcd}	22.42±0.29^{a}

All data are the mean±SD of six replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.5. Sensory evaluation of biscuits

Sensory studies showed that (Table 2.32) the crust color and appearance of biscuits containing MPP were equally acceptable as those of control biscuits up to 10% level of incorporation. Color and appearance of biscuits containing 15% MPP were also quite acceptable. Above this level the color of the biscuits were relatively dark, therefore, less acceptable. Even though, the objective measurement showed that the biscuits containing MPP were relatively hard compared to control biscuits, sensory studies showed that texture of biscuits was acceptable up to 10% level of incorporation. Taste and flavor of biscuit was also highly acceptable up to 15% level of MPP incorporation. MPP incorporation imparted a pleasant mango flavor and aroma to the biscuits, which was found to be highly desirable. However, at 20% level of incorporation a slight bitter taste was noticed, may partly be due to higher content of polyphenols. From this study it can be concluded that

biscuits with highly acceptable sensory parameters can be prepared with the incorporation of 10% MPP.

Table 2.32. Influence of MPP on the sensory properties of biscuits

MPP (%)	Crust color (9)	Crust appearance (9)	Crumb color (9)	Texture (9)	Taste/ flavor (9)	Overall quality (9)
Control	**8.50±0.27^{d}**	**8.70±0.25^{d}**	**8.79±0.25^{d}**	**8.71±0.25^{d}**	**8.60±0.24^{b}**	**8.66±0.10^{d}**
5	**8.25±0.27^{cd}**	**8.66±0.20^{cd}**	**8.39±0.44^{cd}**	**8.58±0.41^{cd}**	**8.58±0.19^{b}**	**8.49±0.16^{cd}**
7.5	**8.00±0.42^{c}**	**8.41±0.38^{cd}**	**8.12±0.44^{c}**	**8.33±0.41^{cd}**	**8.30±0.38^{b}**	**8.23±0.16^{c}**
10	**7.97±0.43^{c}**	**8.20±0.46^{c}**	**7.88±0.44^{bc}**	**8.25±0.27^{c}**	**8.30±0.36^{b}**	**8.10±0.21^{c}**
15	**7.38±0.37^{b}**	**7.50±0.47^{b}**	**7.41±0.49^{b}**	**7.75±0.26^{b}**	**7.80±0.41^{b}**	**7.56±0.18^{b}**
20	**6.13±0.41^{a}**	**6.75±.0.40^{a}**	**6.75±0.52^{a}**	**6.80±0.26^{a}**	**6.92±.0.20^{a}**	**6.67±.0.29^{a}**

All data are the mean±SD of six replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.6. Cooking characteristics of macaroni incorporated with MPP

Pasta is a traditional cereal-based food product which origins from the first century BC (Agnesi, 1996). Macaroni products are well-accepted world wide because of their low cost, ease of production, versatility, sensory attributes and long shelf life (Bergman et al, 1994). Recently, macaroni products have been fortified with β-carotene, inulin and various protein concentrates to improve its nutritional and nutraceutical value (Nielson et al, 1980; Pereira et al, 1999; Brennan et al, 2004). In the present study, commercial semolina having 11.87% moisture content, 12.3% protein and 0.72% ash was used for the preparation of macaroni. MPP was incorporated at 2.5%, 5% and 7.5% levels in the preparation of macaroni.

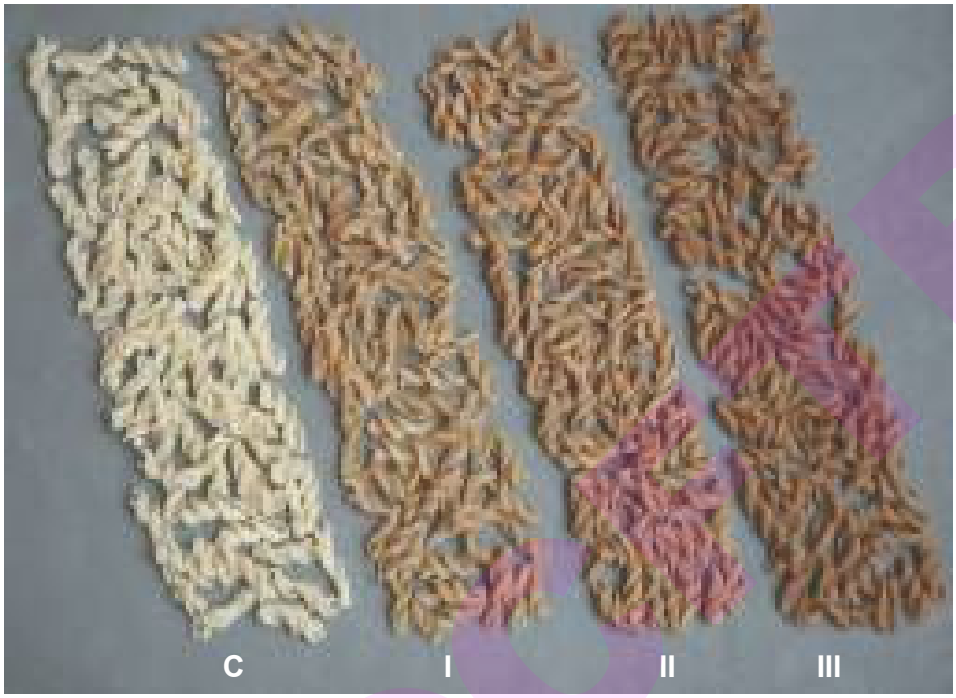


Figure 2.22. MPP enriched macaroni. C- Control, I) 2.5% MPP, II) 5.0 % MPP, III) 7.5 % MPP

Cooking quality is an important parameter for evaluation of macaroni. **Table 2.33** shows the cooking quality parameters of macaroni prepared using MPP. For consumers, cooking quality, which includes cooking loss, cooked weight, and texture of the cooked macaroni, are the most important quality attributes (Feillet, 1988). Measurement of cooking loss of macaroni is one of the important parameters in assessing its over all quality. Cooking loss has been associated with both starch pasting properties and protein quality (Bately & Curtin, 2000). The results indicated that cooking loss of macaroni significantly increased with the increase in level of MPP (**Table 2.33**). Cooking loss increased from 5.84 to 8.71% with increase in the incorporation of MPP from 0 to 7.5%. Taha (1992) reported that there were higher cooking losses in noodles prepared from whole durum wheat flour supplemented with defatted

soy flour. Cooking losses could be attributed to the changes in the gluten protein network because of the interference of mango peel powder, which is rich in dietary fiber content. Earlier, Tudorica et al (2002) reported that the increase in cooking loss could be due to the disruption of protein-starch matrix and uneven distribution of water within the macaroni matrix due to the competitive hydration tendency of the fiber. Hoseney (1992) reported that cooking loss above 9% in macaroni making is undesirable and in the present study cooking loss by the incorporation of MPP was found to be less than 9% (**Table 2.33**).

The cooked weight of macaroni containing MPP at 2.5%, 5% and 7.5% decreased with increased levels of incorporation (**Table 2.33**). The results indicated that cooked weight decreased from 37 g (control) to 35.9 g (7.5%MPP). The decrease in cooked weight was indirectly proportional to the cooking loss.

Table 2.33. Cooking* and textural characteristics of control and MPP enriched macaroni

MPP (%)	Cooking loss (%)	Cooked weight (g)	Firmness (gf)
0 (Control)	5.84±0.07^{a}	37.0±0.20^{c}	44.0±1.70^{a}
2.5%	5.90±0.02^{a}	36.3±0.30^{b}	52.4±0.63^{b}
5%	8.24±0.08^{b}	36.0±0.48^{a}	62.9±3.16^{c}
7.5%	8.71±0.36^{c}	35.9±0.14^{a}	73.5±1.22^{d}

*10 g of raw material was used for cooking

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.7. Firmness of cooked macaroni

The firmness of cooked macaroni samples is listed in **Table 2.33**. Results clearly indicated that addition of MPP increased the firmness of the macaroni. The firmness of macaroni product increased from 44 to 73.46 gf with incorporation of 7.5% MPP. Dexter and Matsuo (1979) pointed out that starch is the major component of semolina, and firmness in cooked macaroni would be influenced by gelatinized starch properties. Present study indicated that higher content of dietary fiber in the flour by the incorporation of MPP may also have a contributory role in the firmness of macaroni products.

2.3.3.8. Sensory evaluation of macaroni

The hedonic test conducted on parameters such as colour, texture and tastes of cooked macaroni, supplemented with MPP, are presented in **Table 2.34**. As can be seen from **Figure 2.22**, there was a decrease in macaroni lightness as MPP level increased to 7.5%. The color of macaroni was quite acceptable up to 5% level. It is to be noted that after cooking, at 2.5% level the color was more appealing/ acceptable than the control. However, at 7.5% level, the color was the darkest and was not acceptable. Macaroni containing 7.5% scored less for taste and flavor. From the sensory analyses, it was concluded that mango peel powder could be incorporated up to 5% level in the formulation of macaroni with out significantly affecting its sensory qualities.

Table 2.34. Influence of MPP on the sensory acceptability of macaroni

MPP (%)	Color	Texture	Taste	Overall quality
0 (Control)	7.4±.0.52^{b}	7.7±.0.42^{b}	7.9±.0.13^{b}	7.7±.0.22^{b}
2.5%	7.8±.0.26^{c}	7.9±0.21^{b}	7.8±.0.26^{b}	7.83±.0.50^{b}
5.0%	7.5±0.29^{bc}	7.9±0.20^{b}	7.6±.0.34^{b}	7.66.± 0.19^{b}
7.5%	6.3±.0.26^{a}	6.9±.0.21^{a}	6.8±.0.54^{a}	6.80±.0.05^{a}

All data are the mean±SD of five replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.9. Content of dietary fiber, polyphenols and carotenoids in MPP incorporated biscuits

The moisture content increased from 3.75% (control) to 5.2% at 10% level of incorporation and increased to 6% in case of biscuits containing 20% MPP. The increase in moisture content may be due to the increase in water absorption by dietary fiber present MPP. It is also to be noted that during mixing water required for the biscuits dough incorporation increased in the presence of MPP.

Biscuits formulated with MPP exhibited increased total dietary fiber content (**Table 2.35**) with a good balance of SDF and IDF. The TDF content increased from 6.47 to 20.70%, the SDF content from 2.8 to 8.2% and IDF content from 3.67 to 12.50% with the incorporation of 20% MPP. The ratio of IDF/SDF, which is nutritionally significant, increased from 1.31 to 2.04. The results indicated that there is a significant increase in the total dietary fiber content in biscuits incorporated with MPP. The increase in TDF seems to be more than the dietary fiber contributed by the MPP. It is possible that during baking some of the components such as phenolics, carbohydrates and

proteins present in mango peel would have contributed to the formation of dietary fiber. Earlier, the formation of resistant starch in wheat flour and soybean during baking, extrusion or cooking was reported (Bjorck et al, 1986; Kutos et al, 2003; Kim et al, 2006). Mango peel contains a significant amount of polyphenols, which may form cross-linking of polysaccharides and proteins. Thus, the increase in dietary fiber content in MPP incorporated biscuits could be not only due to MPP, but may be also due to the contribution by other factors described. The TDF content in commercial fiber rich cookies has been reported to be between 3.73 and 5.95% with marked predominance of IDF (Sangronis & Rebolledo, 1993).

The total polyphenol content in the control and enriched biscuits showed that the incorporation of 20% MPP increased the content of phenolics in the biscuits from 540 to 4,500 μg GAE/ g (**Table 2.35**). Even though, there was some loss in the polyphenol content during processing, nevertheless, there was an increase in polyphenol content in biscuits with the incorporation of MPP.

The carotenoid content in the biscuits increased from 17 to 247 μg/g in biscuits containing 20% MPP (**Table 2.35**). There was a 14 fold increase in the carotenoid content in biscuits by the enrichment of 20% MPP. Therefore, it was clear from the chemical analysis of the product that enrichment of mango peel increased the content of bioactive compounds, thus increasing the nutraceutical properties of the product.

Table 2.35. Total polyphenol, carotenoids, dietary fiber and free radical scavenging activity (IC_{50} value) of MPP enriched biscuits

MPP (%)	TDF (%)	IDF (%)	SDF (%)	Polyphenolics (μg GAE/g)	Carotenoids (μg/g)
0 (Control)	6.47±0.2[a]	3.67±0.3[a]	2.80±0.1[a]	540±2[a]	17±3[a]
5	11.00±0.2[b]	7.40±.0.1[b]	3.60±.0.2[b]	1,800±32[b]	53±8[b]
7.5	12.42±0.8[c]	8.20±0.4[c]	4.22±0.4[c]	2,250±10[c]	89±14[c]
10	14.54±0.6[d]	9.76±.0.6[d]	4.78±0.3[c]	2,630±40[d]	145±23[d]
15	18.37±0.6[e]	11.80±0.2[e]	6.57±0.3[d]	4,350±80[e]	214±22[e]
20	20.70±0.8[f]	12.50±0.2[f]	8.20±0.4[e]	4,500±50[e]	247±14[f]

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

SDF- soluble dietary fiber, IDF- soluble dietary fiber, TDF Total dietary fiber

2.3.3.10. Content of dietary fiber, polyphenols and carotenoids in MPP incorporated macaroni

Incorporation of MPP as an ingredient in macaroni formulation, significantly improved its total phenolics, carotenoids and dietary fiber content in comparison with macaroni made using semolina (control) (**Table 2.36**).

Results showed that incorporation of 7.5% MPP increased the content of total phenolics in macaroni from 458 to 1,800 μg GAE/g of macaroni. There was a 3.9 fold increase in phenolic content in macaroni with the incorporation of MPP.

The carotenoid content in the macaroni increased from 4.65 to 84 μg/g macaroni by the incorporation of MPP from 0 to 7.5% level. There was 18 fold increase in the carotenoid level in macaroni by the enrichment of MPP. It has been reported that cooking did not decrease total carotenoid content in macaroni (Pereira et al, 1999).

Estimation of dietary fiber content in macaroni containing different levels of MPP showed a significant increase in its content (**Table 2.36**) with a better balance of soluble and insoluble dietary fiber. Total dietary fiber content increased from 8.58 to 17.83%, and the SDF content from 3.59 to 5.63% and IDF content from 4.99 to 12.20% in macaroni containing 7.5% MPP. The ratio of SDF/IDF, which has nutritional significance, increased from 1.3 (control) to 2.16 (7.5% MPP).

Table. 2.36. Total polyphenol content, carotenoids, dietary fiber and free radical scavenging activity (IC_{50} value) of MPP enriched macaroni

MPP (%)	Polyphenolics (μg/g GAE)	Carotenoid (μg/g)	SDF (%)	IDF (%)	TDF (%)
0 (Control)	460±8[a]	4.65±0.15[a]	3.59±0.17[a]	4.99±0.2[a]	8.58±0.2[a]
2.5%	1470±30[b]	26.50±1.0[b]	4.46±0.2[b]	9.34±0.2[b]	13.80±0.3[b]
5%	1605±15[c]	41.00±0.8[c]	5.18±0.05[c]	10.60±0.15[c]	15.80±0.09[c]
7.5%	1803±86[d]	84.00±2.0[d]	5.63±0.20[d]	12.20±0.6[d]	17.83±0.4[d]

All data are the mean±SD of three replicates. Mean followed by different letters in the same column differs significantly ($P \leq 0.05$).

2.3.3.11. Evaluation of antioxidant properties of mango peel enriched biscuits and macaroni

As described in earlier section 2.3.2.2. scavenging the stable DPPH radical model is a widely used method to evaluate antioxidant activity. The antioxidant potential of biscuits and macaroni incorporated with MPP was determined by this method. **Figure 2.23** shows the dose dependence radical scavenging activity of the acetone extract of the biscuits. With increase in level of MPP incorporation, the DPPH radical scavenging activity increased. In

case of 10% MPP incorporated biscuits, acetone extracts corresponding to 5, 15, 25 mg of biscuit powder showed 47, 51, 60% scavenging activities, respectively. The increase in the free radical scavenging may be attributed to the increase in the contents of polyphenols and carotenoids through the incorporation of MPP. The results suggested that baking does not show significant impact on the antioxidant compounds. Thus, the mango peel powder enriched biscuits not only increased the nutritional quality of the product but also increased the nutraceutical property by increasing their antioxidant activity.

Figure 2.24 shows the dose dependence radical scavenging activity of the acetone extract of the macaroni samples. In case of 5% MPP incorporation, macaroni acetone extracts corresponding to 10, 20, 30, 40, 50 mg of macaroni showed 38, 60, 76, 82, 85% scavenging activity of the DPPH radical. The increase in the free radical scavenging activity may be attributed to the increase in the content of polyphenols and carotenoids as a result of incorporation of MPP. The results suggested that macaroni processing did not have significant impact on the antioxidant compounds.

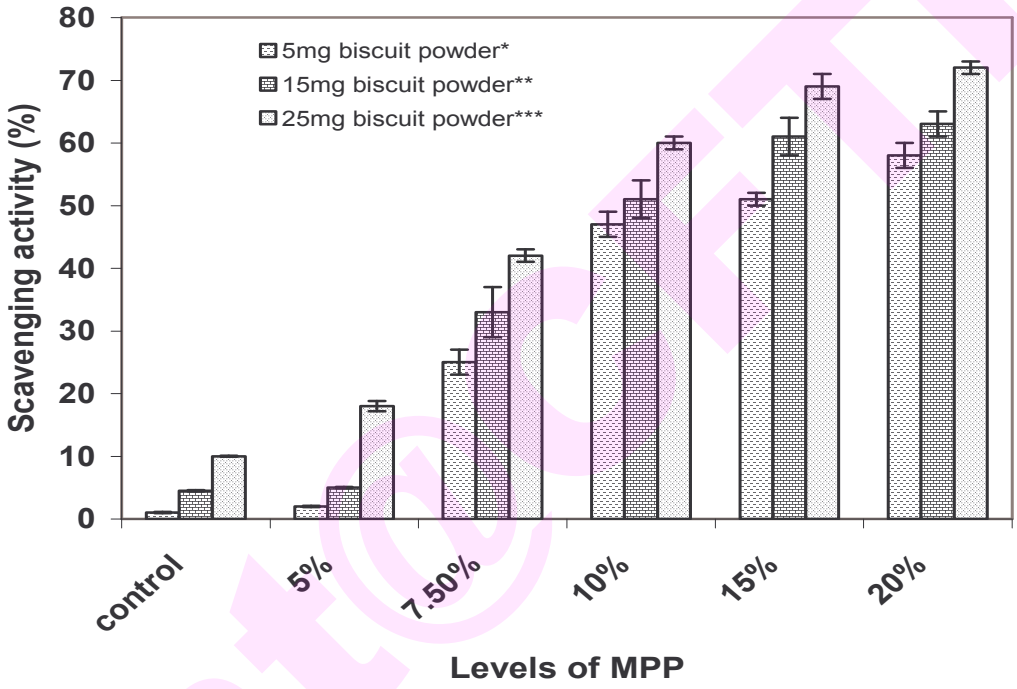


Figure 2.23. DPPH radical scavenging activity of control and MPP incorporated biscuits

*- Extract equivalent to 5 mg biscuit powder; **- Extract equivalent to 15 mg biscuit powder; ***- Extract equivalent to 25mg biscuit powder;

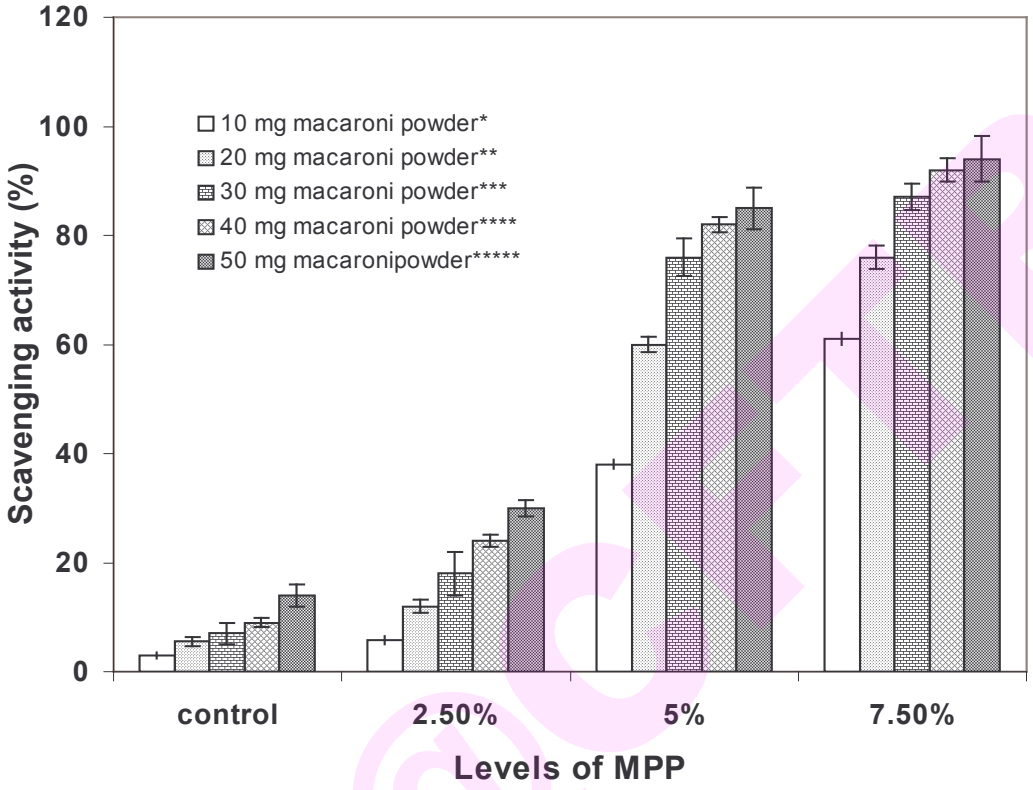


Figure 2.24. DPPH radical scavenging activity of control and mango peel powder incorporated macaroni at different levels. * Extract equivalent to 10 mg macaroni powder; ** Extract equivalent to 20 mg macaroni powder; *** Extract equivalent to 30 mg macaroni powder; **** Extract equivalent to 40 mg macaroni powder. ***** Extract equivalent to 50 mg macaroni powder.

Biscuits enriched with MPP showed higher polyphenol and carotenoid contents than the control biscuits and as a result of this, MPP incorporated biscuits showed significant improved antioxidant activity. In the MPP incorporated biscuits, the total dietary fiber content also increased significantly. It may be concluded from the present study that MPP could be incorporated up to 10% level in the formulation of biscuits without affecting their overall quality. At 10% MPP level incorporation, the biscuits had TDF of 14.54%, polyphenols of 2,630 μg of GAE, carotenoids of 145 μg/g biscuit

powder which are 2 fold, 5 fold, 8 fold more than that of control biscuits. Similarly, incorporation of MPP increased the polyphenol, carotenoid and dietary fiber contents in macaroni and it also exhibited improved antioxidant activity. The cooking quality, sensory and biochemical studies showed that macaroni incorporated with MPP up to 5% level resulted in products with good acceptability. Thus, mango peel, a by-product from mango processing industry could be utilized for the preparation of biscuits and other food products with improved functional and nutraceutical properties.

As reported earlier, mango peel contains various valuable components such as polyphenols, carotenoids, vitamin C and E and dietary fibers. In the present study, whole mango peel powder was incorporated into biscuits and macaroni to obtain maximum nutraceutical benefit of mango peel powder. However, acetone extract, which has good antioxidant activity and the residue rich in dietary fiber, could also be incorporated into these products. Earlier, under General Introduction (Section 1.5), few commercially available nutraceutical products containing antioxidants and dietary fiber were mentioned. Acetone extract of mango peel dietary fiber isolated from mango peel and whole mango peel powder as such may find their application in the preparation of wide variety of nutraceutical food products.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF PEROXIDASE FROM BLACK GRAM HUSK

3.1. INTRODUCTION

Cereals and legumes form staple food for majority of the population. Cereals accounts for about 50% of the total dietary proteins of the world. The share of food legumes and animal production in the total world protein resources is about 25% each. The grain legumes containing low oil (1-5%), high protein (20-30%) and carbohydrate (50% or more) are called pulses. In India pulses are the second major sources of dietary proteins (~27%) after cereals (~55%). Legumes are the relatively inexpensive sources of dietary proteins and posses other desirable attributes such as abundance of complex carbohydrates, ability to lower cholesterol in humans, high fiber content, low fat content, high concentration of polyunsaturated fatty acids, long shelf life and diversity of foods that can be made from them. Legumes also contain bioactive compounds whose beneficial effects in human health need to be fully exploited (Beninger & Hosfield, 2003).

Pulses are the major dietary protein sources in India. India is one of the major pulse growing countries of the world sharing about 26% of the world pulse production. Black gram or urad is one of the important pulse crops in India. Black gram (*Phaseolus mungo L.)* reported to be originated in India belonging to the family Leguminosae. Its reference has also been found in Vedic texts such as Kautilya's "Arthasasthram" and "Charaka samhita". India is largest producer and consumer of black gram in the world. It is commonly referred as urd bean, urad, urid, black gram, black lentil or white lentil. The production of pulses during 2005-06 estimated at 10.6 million tones where urad constitutes around 1.1 million tones (India Infoline, 2005).

3.1.1. Nutritional importance and food products of black gram

Black gram is a rich protein food. It has a protein content ranging from 26-28% on a dry-weight basis, which is almost three times that of cereals. Black gram proteins are characterized by the presence of a major storage protein, globulin, accounting for 81% of the total protein followed by albumin, prolamine and glutelins and they deficient in sulphur-containing amino acid and rich in lysine (Reddy et al, 1981). It is consumed in the form of split pulses as well as whole pulse, which is an essential supplement of cereal-based diet. The combination of dhal-chawal (pulse-rice) or dhal-roti (pulse-wheat) is an important ingredient in the average Indian diet. The biological value improves greatly, when wheat or rice is combined with black gram because of the complementary relationship of the essential amino acids such as arginine, leucine, lysine, isoleucine, valine and phenylalanine etc. The nutritional composition of black gram is given in **Table 3.1**.

Black gram contains trypsin and chymotrypsin inhibitors, phytic acid and flatulence factors. Phytic acid in black gram accounts for about 79% of the total phosphorus. Raffinose, family of oligosaccharides accounts for about 61% of total sugar and is shown to cause flatulence in both rats and humans. Black gram is a good source of water-soluble vitamins, certain minerals and polyunsaturated fatty acids (Reddy et al, 1981). Black gram lipids shown to have cholesterol-reducing effect in both humans and experimental animals (Devi & Kurup, 1972).

Table 3.1. Proximate composition (%) of black gram

Component	Content
Calorific value (cal/100 g)	350.0
Crude protein (%)	26.20
Fat (%)	1.20
Carbohydrate (%)	56.60
Ca (mg/100 g)	185.0
Fe (mg/100 g)	8.70
P (mg/100 g)	345.0
Vitamin B1 (mg/100 g)	0.42
Vitamin B2 (mg/100g)	0.37
Niacin (mg/100 g)	2.00

(Sources: Gopalan et al, 1999)

Black gram is used in the preparation of fermented foods such as idli, dosa, hopper, papad and waries (spicy hollow balls) (Batra & Millener, 1974). In India, idli, dosa and hopper are used as breakfast and snack foods while papad and waries are generally consumed with other cooked preparations such as rice, vegetables and others.

3.1.2. Milling and milled by-products of pulses

Basic staples like rice, wheat and pulses reach the market after going through primary mechanical processing operations that convert the grain into edible raw material. After primary processing, the products subjected for secondary processing and yield popular products such as bread, biscuits and many other products. During primary processing the major by-products obtained are husk, hull and germ.

The milling of legumes is done to transform grains into split dhal without any breakage of cotyledons. About 75% of total pulse produced in

India is milled into splits. It is done at domestic, cottage and small-medium scale industries. The major operations involved in pulses processing are cleaning, drying, milling, packaging, handling and storage. Dhal milling is the third largest food grain processing industries in India after rice and wheat milling. Dhal milling basically consists of removal of the pericarp seed coat, which is attached to the cotyledon through a layer of biochemically complex gum. Premilling treatment involves loosening the pericarp from cotyledons through chemical, mechanical or heat treatment.

Milling of pulses essentially consists of the removal of the tightly bound external 'husk' from the grain and recovery of the cotyledon in the form of dhal. The average yield of dhal in the commercial mill is about 75% and the rest of grain material (about 25%) is obtained in the form of various type of by-products. These comprise of husk, powder, large and small brokens, shriveled and under processed grains (Ramakrishnaiah et al, 2004).

The cereal and pulse by-products were found to be a rich and economically inexpensive source of bioactive compounds such as antioxidants, dietary fibers and enzymes (Aspinalle, 1969; Moure et al, 2001; Sessa, 2003). The extraction of antioxidant compounds from cereal and legume by-products such as hulls, seed coat etc have been reported (Moure et al, 2001). The seed coat of cereals and legumes posses large quantities of endogenous antioxidants such as phenolic compounds (Moure et al, 2001; Tsuda et al, 1994). Ramarathnan et al (1989) identified isovitexin as natural component in white rice hull, which showed strong antioxidant activity. Another study showed that rice hulls contained several kinds of strong antioxidants such as anisole, vanillin and syringaldehyde (Asamarai, 1994).

The effect of navy bean crude hull extract on the oxidative stability of edible oil was reported by Onyeneho and Hettiarachchy (1991). Anthocyanins were isolated from *Phaseolus vulagris* seeds and their antioxidant activities were reported (Tsuda et al, 1994). Soybean hulls with an US production in excess of $9.1X10^8$ kg are an underutilized co-product of the soybean processing industry (Sessa, 2003). Aspinall et al (1969) estimated that soy hull contains about 11% protein, 9-11% galactomannan, 10-12% acidic polysaccharides, 9-10% xylan hemicelluolse and about 40% cellulose with the remainder probably lignin. At present soy hulls are primarily used as fiber sources for dairy and beef cattle. Soy hull proteins have been investigated for almost two decades, but only four proteins, namely peroxidase, a hydroxy proline rich glycoprotein termed extensin, glycin rich proteins and a bowman-birk type protein inhibitor have been identified (Gilliki, 1991).

Husk and germ rich fractions are the major waste by-products obtained during black gram milling. Around 20-25% of the total grain was wasted as germ, plumule and husk rich fractions. The proximate composition of black gram husk on dry-weight basis has been reported as shown in **Table 3.2**. The husk of black gram was investigated as a new biosorbent of cadmium from low concentration aqueous solutions. During milling of the black gram, about 20-25% of the pulse obtained as waste by-product, in which husk and husk rich fraction constitutes about 17% and it does not have currently any use except as cattle feed.

Table 3.2. Proximate composition (%) of black gram husk

Component	Content
Moisture	15.5
Crude protein	3.32
Fat	0.7
Carbohydrates	39.15
Crude fiber	51.9
Ash	4.93

(Sources: Saeed & Iqbal, 2003)

Pulses are found to be rich in oxidative enzymes like peroxidase, which has many physiological roles, and industrial and analytical applications. Roots of horseradish represent the traditional source of commercial production of peroxidase (Krell, 1991). An alternative/additional indigenous source of cost effective peroxidase with increased availability, higher stability, different substrate specificity and lower isolation cost is of much value. Many studies have been done in this direction. Earlier, a peroxidase was extracted from soybean hulls, an inexpensive food industry by-product (Sessa, 2003). Therefore, it is attempted in this study to isolate and characterize peroxidase from black gram husk fraction.

A brief review on peroxidase is presented below.

3.1.3. Peroxidase

Peroxidases (EC, 1.11.1.7; donor: hydrogen-peroxide oxidoreductase) are enzymes that catalyze the oxidation of a wide variety of substrates using H_2O_2. They are ubiquitous in nature and have diverse physiological functions (Dunford, 1999). They can be divided into two main classes (1) iron containing peroxidases and 2) flavoprotein peroxidases. The iron containing peroxidases

can be subdivided into two groups: ferriprotoporphyrin peroxidases and verdoperoxidases. The former contain ferriprotoporphyrin III (Hematin) as prosthetic group and are brown in pure form and occur mainly in higher plants, animals and microorganisms. The verdoperoxidases contain an iron ferriprotoporphyrin but different from ferriprotoporphyrin III and green in pure form and present in animal organs and in milk. The flavoprotein peroxidases contain flavine-adenine-dinucleotide as prosthetic group and occur in microorganisms and animal tissues (Whitaker, 1995).

The heme peroxidase superfamily has been classified into three groups on the basis of amino acid sequence comparisons. Class I comprises intracellular peroxidases, including cytochrome peroxidase, ascorbate peroxidase and the gene duplicated bacterial catalase peroxidases. Class II contains the secretary fungal enzymes such as manganese peroxidases and lignin peroxidases. Finally, class III peroxidases consist of the secretory plant peroxidases such as horseradish peroxidasse (HRP). Evidences are available on the role of peroxidase in stress related process such as wounding (Breda et al, 1993), salt stress (Botella et al, 1994) and disease resistance (Moerschbacher, 1992). Hydrogen peroxide is produced in the plant under oxygen stress during photosynthesis and peroxidases degrade toxic hydrogen peroxide to water (Droillard et al, 1987).

3.1.3.1. Biochemistry of peroxidase

3.1.3.1.1. Reaction mechanism: Peroxidases reduce hydrogen peroxide to water while oxidizing a variety of substrates. Peroxidases catalyses mainly four types of reactions (1) peroxidatic, (2) oxidative (3) catalytic and

(4) hydroxylation (Whitaker, 1995). Thus peroxidases are oxidoreductase, which use hydrogen peroxide as electron acceptor for catalyzing different oxidative reactions. The overall reaction is as follows:

$$H_2O_2 + 2AH_2 \xrightarrow{POD} 2AH^* + 2\,H_2O$$

In course of the reaction, intermediate compounds are formed.

$$POD + H_2O_2 \longrightarrow \text{compound I}$$

$$\text{Compound I} + AH_2 \longrightarrow \text{compound II} + AH$$

$$\text{Compound II} + AH \longrightarrow POD + A$$

Figure 3.1. Overall reaction of the peroxidatic reaction

The catalytic reaction of peroxidase involves the decomposition of H_2O_2 in the absence of hydrogen donor. The reaction occurs as follows:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Figure 3.2. Catalytic reaction of the peroxidase

The hydroxylating reaction involves the production of *o*-dihydroxy phenols from monophenols and O_2, in presence of hydrogen donor (Whitaker, 1995).

Many plant peroxidases have specific requirements for peroxide and may be considered as peroxide scavengers (Robinson, 1989). In the presence of peroxide, the peroxidases were able to oxidize a wide range of phenolic compounds such as guaiacol, pyrogallol, chlorogenic acid, catechins, catechol, aniline, benzidine, *o*-phenylene diamine, *o*-dianisidine, reduced nicotinamide-adenine dinulceotide and reduced nicotinamide adenine

dinucleotide phosphate (Richard-Forget & Gauillard, 1997). Oxidation of a wide range of phenolic compounds has led to speculation that they may be associated with losses in color and flavour of raw and processed foods (Nicolas et al, 1994). The oxidative reaction of POD may take place in the absence of hydrogen peroxide. It requires oxygen and cofactors such as Mn^{2+} and phenol.

3.1.3.1.2. Substrates of peroxidase: Peroxidase is highly specific to the peroxide substrates and hydrogen peroxide is the main substrate. The enzyme is inactivated by higher concentration of hydrogen peroxide. The rate of inactivation of enzyme activity depends on the concentration of enzyme and hydrogen peroxide. It was reported that at higher concentration, hydrogen peroxide attacked non-heme, as well as heme site of the enzyme (Weinryb, 2003, Arnao et al, 1990). It was reported that peroxidase requires a free HOO^- group in order to react with peroxides (Marklund, 1971).

o-Dianisidine, *o*-phenylene diamine, *o*-tolidine, 3-amino-9-ethyl-carbazole, 3,3'-diaminobenzidine tetrachloride, 2,2'azinobis(3-ethylbenzothiazoline -6-sulphonate), guaiacol, syringaldazine, p-phenylene diamine, N',N' dimethyl-p-phenylene diamine and *o*-toludine are some of the widely used substrates for peroxidase (McDougal et al, 1992). The wide substrate specificity for the peroxidase in plants may be mainly due to the presence of isoenzymes. The affinity of peroxidase for a given substrate was found to depend on the source of the enzyme and its purity.

3.1.3.1.3. Inhibitors of peroxidase: The inactivation of peroxidase can be achieved by chemicals acting on enzyme itself, by chemicals acting on substrates or the reaction products. Compounds that are known to inhibit

peroxidases are cyanide, sulphide, azide, nitric oxide, hydroxylamine, sodium metabisulphite, sodium thionate etc. Bisulphite inactivated the peroxidase enzyme in the presence of weak acids through destabilization of the linkage between the iron–containing prosthetic group and the protein. Cyanide, azide and fluoride forms irreversible complexes with the heme iron (Embs& Markakis, 1965). Natural competitive inhibitors of peroxidase were extracted from unripe mango and banana and they are found to be heat labile and protein in nature (Matoo & Modi, 1970).

3.1.3.1.4. Effect of pH and temperature: The pH optimum of the peroxidase activity varies with the enzyme source, the isoenzyme composition, the donor substrate and the buffer used (Nagle, 1975). The broad pH optima are mainly due to the presence of isoenzymes of different pH optima. Activity decreases at low and high pH values. The loss of activity observed on acidification is attributed to the change in the protein from the native state to the reversible denatured state due to the detachment of heme. The disturbance of the heme-protein interaction causes loss of protein stability (Lopez-Molina et al, 2003).

Peroxidases have high thermal stability, attributed to the presence of sugar in their structure (Mellon, 1991). However, this thermostability cannot be extended to all peroxidase isoenzymes due to the existence of isoenzymes with different temperature resistance (Moulding et al, 1987). The main process found to be involved in the thermal denaturation of peroxidase was mainly due to the dissociation of prosthetic groups from the holoenzyme, a conformation change in the apoenzyme and modification or degradation of the prosthetic group (Tamura & Morita, 1975). The inactivation curve showed the

apoenzyme to be more heat stable than the holoenzyme, the difference being more pronounced at 70°C than at 90°C. Heat inactivation of peroxidase from many sources is in certain conditions, a biphasic and partly reversible process.

3.1.3.1.5. Molecular mass and homogeneity: The heterogeneity of peroxidase has been known for years. The isoenzymes are known to differ in isoelectric points, substrate specificity, resistance to heat and inhibitors. Isoenzmyes were isolated from radish with molecular masses of 44 and 45 kDa (Lee et al, 1994) and from strawberry with 58 and 65 kDa (Civello et al, 1995). However membrane bound peroxidase from wheat germ showed no isoenzymes (Converso & Fernandez, 1995)

3.1.3.1.6. Structure of peroxidase: The primary structure of enzymes is based on amino acids present in them. The number of amino acids differs from one peroxidase chain to another. The horseradish peroxidase consists of 306 amino acids and one heme group. The secondary structure of peroxidase monomer consists mostly of α-helices. A typical chain consists of 10 α-helical segments. In tertiary structure peroxidase monomer consists of two domains with two antiparralel helices from the two domains form a crevice in which the heme group is inserted. In quaternary structure, the number of subunits or chains depends on the enzyme (Veitch, 2004). The three dimensional structure of HRP is shown in **Figure 3.3**.

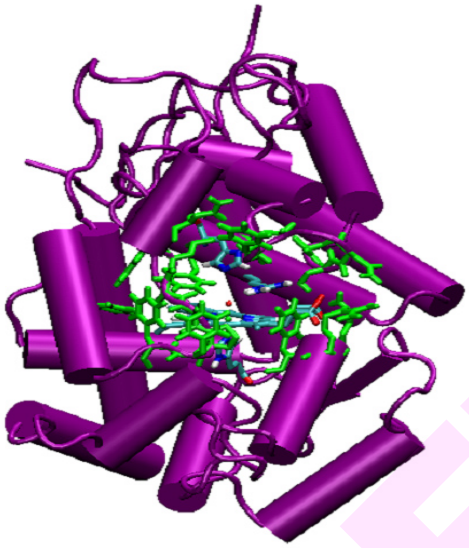


Figure 3.3. Three dimensional structure of HRP

The heme group for a peroxidase monomer is embedded deeply in the crevice between two domains. A helix contains the fifth iron for the heme group. For HRP the heme iron is covalently bound to nitrogen in histidine 170's imidazole ring. The heme porphyrin plane is perpendicular to the imidazole ring. This histidine is conserved residue that is present in almost all types of peroxidases. Aside from histidine, there are three other residues that are conserved in most peroxidase at the heme's distal side. These three function as a ligand pocket for H_2O_2. They are responsible for catalyzing heterocyclic cleavage of H_2O_2 . For HRP, these amino acids are Arg38, Phe41 and His42 (Veitch, 2004).

The three dimensional structures of peroxidase isolated from fungi and bacteria, which are grouped in the plant peroxidase family, are remarkably similar despite a limited sequence identity (Poulos & Fenna, 1996; Banci, 1997). The molecular architecture includes 10 α-helices, a small amount of β-sheets, and a single heme group located in a cavity between two antiparallel α-helices (Poulos & Fenna, 1996). Moreover, a number of invariant residues

namely distal histidine and the adjacent arginine, along with the proximal histidine and aspartic acid hydrogen bonded to it, play a key catalytic role.

3.1.3.2. Physiological role of peroxidase

The higher number of isoenzymes and their remarkable catalytic versatility allow them to be involved in a broad range of physiological and developmental process all along the plant life cycle (Passardi et al, 2006). These enzymes have various physiological roles in plant cells, some of them are excreted and participate in many reactions including lignification, cross linking of cell wall polysaccharides, oxidation of indole acetic acid, regulation of cell elongation, wound healing, phenol oxidation and also have role in abiotic stress and against pathogenic organisms ((Gasper & Penel, 1982; Castillo, 1992; Kaspera et al, 2001). Peroxidases are involved in the modulation of plant cell growth both by promoting cell wall rigidity through lignin synthesis and oxidative cross-linking of polysaccharide components (Ros Barcelo, 2002) as well as by generating H_2O_2 needed for these reactions through the oxidation of NADH with molecular oxygen (Halliwell, 1978). Plants contain several peroxidase isoenzymes whose pattern of expression is tissue specific and developmentally regulated or responsive to environmental stimuli.

Cellular metabolic pathways may generate cytotoxic oxygenated by-products, such as peroxides and the superoxide anion (Cowan, 1997). Peroxidase along with superoxide dismutase and catalase, are redox metallo-enzymes involved in cell defense against oxidative stress (Cowan, 1997) in particular, heme peroxidases catalyses the oxidation of a wide variety of substrates (eg: fatty acids, amines and phenols) by H_2O_2 or other organic

peroxides, thereby combining cell defense with biodegradative and biosynthetic pathway (Banci, 1997). It was reported that horseradish peroxidase decreased the levels of total serum cholesterol, triglyceride, blood glucose and lipid peroxidation in hyperlipidemic mice (Wang et al, 2002).

3.1.3.3. Role of peroxidase in food processing

Peroxidase enzyme can participate in a great number of oxidative reactions such as color change, degradation of chlorophyll, oxidation of phenols and indoleacetic acid and many of theses factors are associated with the flavor, color, texture and nutritional qualities of foods which leads to flavor loss and odor of foods (Clemente & Pastore, 1998). Oxidative enzymes like peroxidase have role in protein polymerization and consequently on the rheological properties of dough (Dunnewind, 2002; Takasaki et al, 2005). A positive correlation exists between brownness of whaet based products such as pasta, chapatti and the peroxidase activity (Fraigner, 2000; Hemalatha et al, 2007).

3.1.3.4. Applications of peroxidase

Peroxidases have attracted industrial attention because of its usefulness as a catalyst in clinical examinations and other application. The great diversity of applications is due to the wide substrate specificity of peroxidase catalysis (McEldoon & Dordick, 1996). Perhaps the best-known peroxidase is HRP due to its broad specificity for hydrogen donors and its high catalytic efficiency. Due to its wide substrate specificity peroxidase has a number of industrial, analytical and biomedical applications (McEldoon & Dordick, 1996). Peroxidases are used commercially as a catalyst for phenolic

resin synthesis (Dordick, 1987), as an indicator for reactive species formed during food processing and as components of kit for research medical diagnosis (Thomson, 1977; Wung, 1997). Their novel application include in the treatment of waste-water containing phenolic compounds and aromatic amines (Wu et al, 1998). It is also used as a reagent for organic synthesis and biotransformation as well as in coupled enzyme assays, chemiluminiscent assays and immuno assays (Veitch & Smith, 2001; Krieg & Halbhuber, 2003). Combination of horseradish peroxidase with indole 3-acetic acid may offer new potential for targeted cancer therapy (Folkes et al, 2002; Wardman, 2002). Also the enzyme can be used in bio-bleaching process and lignin degradation in fuel and chemical production from wood pulp, or in the production of dimeric alkaloids, oxidations, biotransformations of organic compounds (Macek et al, 1993). It can also be used in the enzymatic determination of serum metabolites such as uric acid (Agostini et al, 1999). Soybean peroxidase has been found to be a very effective biocatalyst and biosensor, which has been used in waste-water treatment and phenolic resin synthesis (Wright, 1999). Manganese peroxidase can oxidize a wide range of substrates, including various phenolic compounds, high MW chlorolignins (Wariishi et al, 1989) and lignin (Deguchi et al, 1997) rendering it an interesting enzyme for potential applications in various industries such as the pulp industry and paper industry and also as a detergent (Bermek, 2004).

3.2. MATERIAL AND METHODS

3.2.1. MATERIAL

3.2.1.1. Plant material

Black gram (Thiram variety) was procured from National Seed Corporation, Beej Bhavan, Pusa, New Delhi, India. Commercial wheat flour was obtained from the local market.

3.2.1.2. Chemicals

DEAE-Sephacel and Sephadex G-100 procured from Pharmacia Fine Chemicals, Uppasala, Sweden. Bovine serum albumin, ovalbumin, pepsin, trypsionogen, β-lactoglobulin, lysozyme, alcohol dehydrogenase, β-galactosidase, carbonic anhydrase, cytochrome C, blue dextran, catechol, *o*-dianisidine, guaiacol, benzidine diaminobenzidine, p-phenylene diamine, tetramethyl benzidine, pyrogallol, SDS, APS, TEMED, sodium azide, hydrazine, citric acid, oxalic acid, thiourea, DTT, EDTA, trifluroacetic acid and Ellman's reagent (5, 5'- dithiobis 2-nitrobenzoic acid) were obtained from Sigma Chemical Company, St.Louis, USA. CTAB was procured from Fluka, Switzerland. All other chemicals and solvents were of analytical grade.

3.2.2. METHODS

3.2.2.1. Milling of black gram and separation of milled by-products

Black gram (10 kg) was pitted in Versatile Dhal Mill (CFTRI design) mixed with 30 ml of oil, kept for overnight for tempering and dried at 60°C for 8h. The black gram thus obtained after treatment was milled using Versatile

Dhal Mill according to the procedure described by Narasimha et al (2002). The by-products obtained were separated into different fractions by air classification as shown in **Figure 3.4.**

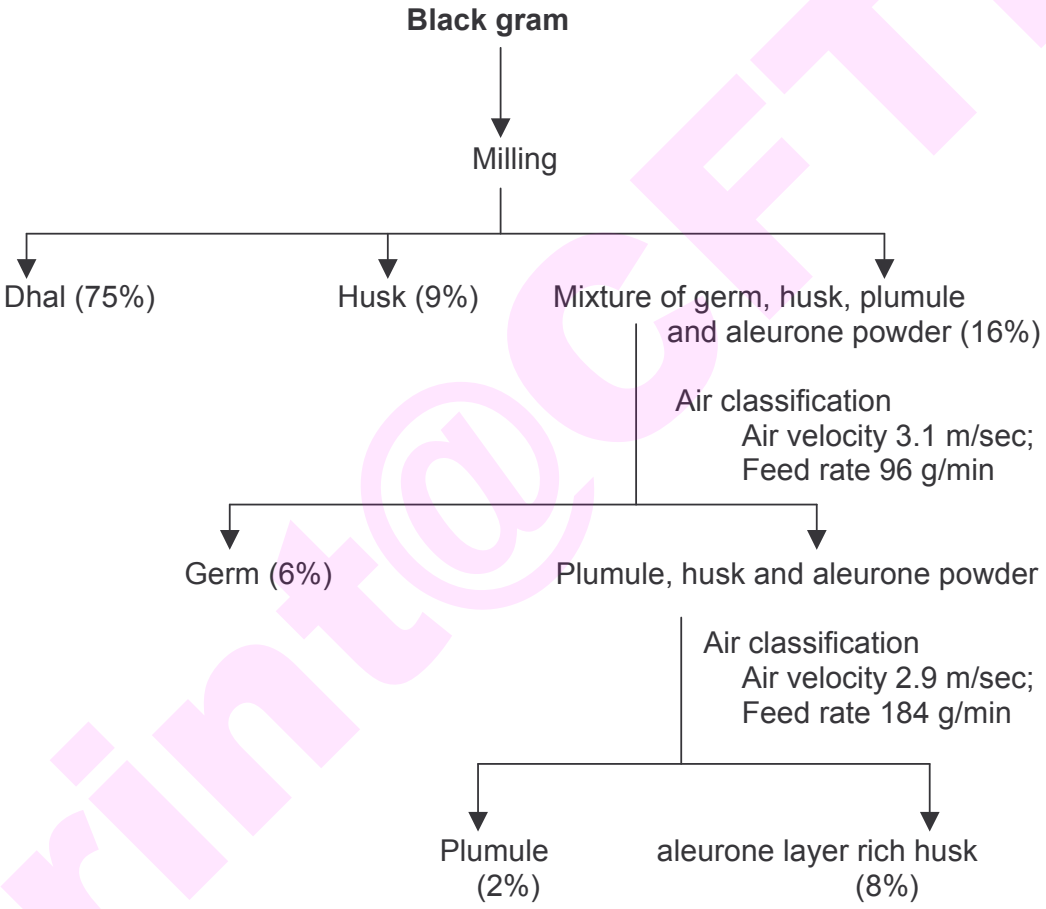


Figure 3.4. Scheme for separation of black gram milled by-products

3.2.2.2. Determination of total protein content

The total protein content in the black gram flour and its milled fractions was determined using micro-Kjeldhal method as described in Chapter II Section 2.2.2.1.5.

3.2.2.3. Enzyme extraction from black gram flour and its milled fractions

To 1 g of sample, 0.5 g of acid washed sand was added and was ground into paste using 5 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 0.01% Tween-20 using mortar and pestle at 4°C. To the resultant paste, 20 ml of buffer was added, stirred for 1 h and was centrifuged at 8,000Xg for 15 min at 4°C. The supernatant obtained was used to estimate the protein content and to assay peroxidase, polyphenol oxidase, xylanase, protease and amylase activities as described earlier (Chapter II, Section 2.2.2.8).

3.2.2.4. Determination of optimum peroxidase extraction

Extraction was done with different solutions by soaking, grinding or by homogenizing to obtain maximum activity of peroxidase from black gram aleurone layer rich husk fraction. Water, 0.05 M sodium phosphate buffer (pH 7.5) and also buffer containing 0.01% Tween-20 was used for extracting peroxidase.

3.2.2.4.1. Soaking method: Aleurone layer rich husk fraction (1 g) was soaked in 25 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20, and kept for different time intervals of 2 h and 24 h. The solution obtained was centrifuged at 8,000Xg for 15 min at 4°C. The

supernatant obtained was used to estimate the protein content and to assay peroxidase activity.

3.2.2.4.2. Homogenization method: Aleurone layer rich husk fraction (1 g) was homogenized using a homogenizer with 25 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20. The resultant homogenate was centrifuged at 8,000Xg for 15 min at 4°C. The supernatant obtained was used to estimate the protein content and to assay peroxidase activity.

3.2.2.4.3. Grinding method: To aleurone layer rich husk fraction (1 g), 0.5 g of acid washed sand was added and was ground into paste using 5 ml of water or 0.05 M sodium phosphate buffer (pH 7.5) or buffer containing 0.01% Tween-20. To the resultant paste, 20 ml of corresponding aqueous medium was added, stirred for 1 h and centrifuged at 8,000Xg for 15 min at 4°C. The supernatant obtained was used to estimate the protein content and to assay peroxidase activity.

3.2.2.5. Purification of peroxidase

3.2.2.5.1. Ion–exchange chromatography on DEAE-Sephacel

Extract of aleurone layer rich husk fraction obtained using grinding method and buffer containing 0.01% Tween-20 was loaded onto DEAE-Sephacel column (2.5 X 18 cm) and the unbound proteins were eluted with 0.05 M Tris-HCl buffer (pH 8.0). The bound enzyme was eluted with a gradient of 0-0.5 M sodium chloride in 0.05 M Tris-HCl buffer (pH 8.0). Fractions (2 ml) were collected with a flow rate of 12 ml/h and monitored for protein by determining the absorbance at 280 nm. The protein fractions were

assayed for peroxidase as described earlier in Section 2.2.2.8.2. The active enzyme fractions were pooled and used for further purification by gel filtration.

3.2.2.5.2. Gel filtration chromatography on Sephadex G-100

The active enzyme fraction obtained from ion-exchange chromatography was concentrated by lyophilization and chromatographed on Sephadex G-100 column (1.2 X 140 cm) using 0.05 M Tris-HCl buffer (pH 8.0) as eluant. Fractions (2 ml) were collected with a flow rate of 12 ml/h and monitored for protein by determining the absorbance at 280 nm. The protein fractions were assayed for peroxidase. The active enzyme fractions were pooled and concentrated by lyophilization and used as enzyme for further studies.

3.2.2.6. Characterization of peroxidase

3.2.2.6.1. Determination of purity of peroxidase

3.2.2.6.1. 1. Polyacrylamide gel electrophoresis (Native PAGE)

The purified protein was dissolved in sample buffer, centrifuged at 8000Xg and 40 μl of supernatant was applied on 10% native gel. The electrophoresis was carried out at 50 V and continued till the tracking dye was about 0.5 cm above the lower end of the gel. The gel composition, sample buffer compositions, staining and distaining were followed according to the method described by Lammeli (1970) without SDS and β-mercaptoethanol.

After the electrophoresis the gel was cut into different parts and each part was subjected to protein staining, glycoprotein staining and peroxidase staining.

3.2.2.61.2. Peroxidase staining: Peroxidase staining was done according to the method described by Hoffman (1970). After electrophoresis, the gel was washed twice (for 5 min each) in 0.05 mM sodium acetate buffer, pH 5.5. The gel was transferred into the freshly prepared solution containing 1 mg/ml diaminobenzidene in the above buffer. The color reaction was started by the addition of 0.05 ml of 6% H_2O_2. The gel was allowed to remain in the solution till the bands were visible. The reaction was stopped by the addition of 5% acetic acid and washed several times with water to remove traces of substrate. The gel was stored in 5% ethanol.

3.2.2.6.1.3. Glycoprotein staining: Initially the gel was soaked for in 12% trichloroacetic acid for overnight and later it was immersed in 3% acetic acid solution containing 1% periodic acid for 1h. After repeated washings of the gel with water to remove traces of periodic acid and it was immersed in Schiff's reagent at 4°C in dark until pink color developed. The gel was destained with 7% acetic acid (Zacharius et al, 1969).

3.2.2.6.1.4. HPLC of purified peroxidase

Reverse phase HPLC of the purified protein was carried out using a C18 column (25 cm X 5 mm, 100 A°) on LC-10 A system (Shimadzu LC) using a gradient of two solvents: Solvent A- 0.01% trifluroacetic acid (TFA) in water; and solvent B- 0.01% TFA in 70% acetonitrile. The gradient for separation consists of 1% B traversing to 100% in 60 min at a flow rate of 1ml/min. The sample detection was carried out using diode array detector.

3.2.2.6.1.5. Capillary electrophoresis

Capillary electrophoresis of purified enzyme was carried out on a Prince Capillary Electrophoresis (Prince 550, Prince Technologies, The Netherlands) using fused silica capillary (id-100 micron, length-100 cm) connected to an UV detector (220 nm) at 10 KV, 100 mbar, 26±10ºC. Before the run, the capillary was rinsed with Tris-glycine buffer (pH 8.0). The sample was introduced in the capillary using a low pressure (0.5 psi) hydrodynamic injection of 2 sec. The data acquisition and control were performed on DAx software.

3.2.2.6.2. Determination of molecular weight

3.2.2.6.2.1. Determination of molecular weight of the purified enzyme by gel filtration chromatography

The molecular weight of the purified enzyme was determined by gel filtration chromatography on Sephadex G-100 using 0.05 M Tris- HCl buffer (pH 8.0) as eluant. It was calibrated with alcohol dehydrogenase (150 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). The void volume (V_o) was determined using blue dextran. The log molecular weight of each standard protein was plotted against its V_e/V_o (V_e elution volume of protein) and molecular weight of peroxidase enzyme calculated from the calibration graph (**Figure 3.5**).

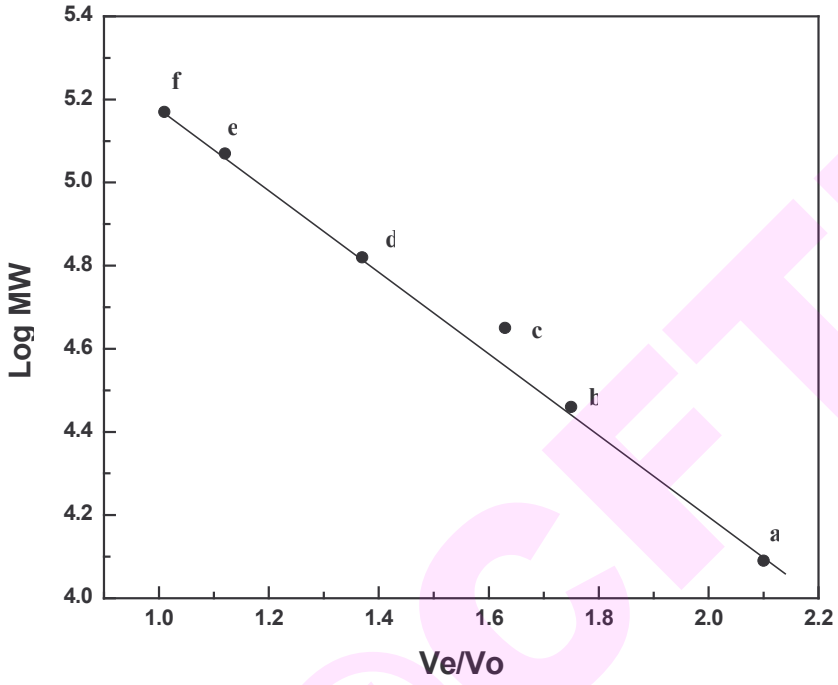


Figure 3.5. Calibration graph for molecular weight determination using Sephadex G-100 gel filtration chromatography

a. Cytochrome C (12.4 kDa); b. carbonic anhydrase (29 kDa); c. ovalbumin(45 kDa); d. bovine serum albumin (66 kDa); e. β-galactosidase (116 kDa); f. alcohol dehydrogenase (150 kDa).

3.2.2.6.2.2. Determination of molecular weight SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of purified fractions was performed using 10% gel at 50 V according to the method of Lammeli (1970). Samples containing a mixture of molecular weight markers containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (36 kDa), trypsin (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa) as well as the enzyme sample prepared in buffer containing 1% SDS and 5% mercaptoethanol were boiled for 5 min. After the electrophoresis, proteins were stained with

Coomassie brilliant blue for 5 h and destained with solution containing 10% methanol and 7.5% acetic acid. The mobility of the each protein was determined by calculating the distance moved by each protein from the origin. A calibration graph was plotted with the mobility versus log molecular weight of the standard protein markers (**Figure 3.6**). From the mobility of the enzyme, its molecular weight was obtained by interpolation.

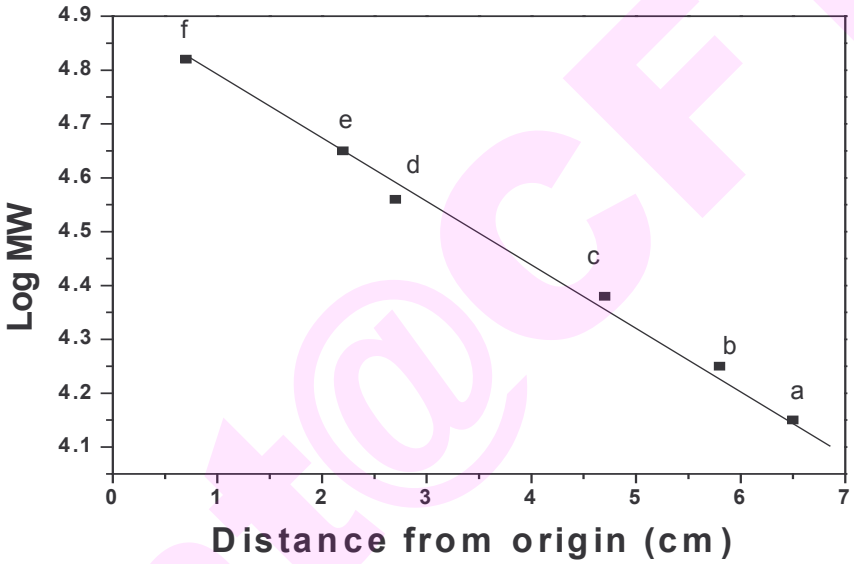


Figure 3.6. Calibration graph for molecular weight determination using SDS-PAGE. a. lysozyme (14.3 kDa); b. β- lactoglobulin (18.4 kDa); c. Trypsin (24 kDa); d. pepsin (36 kDa), e. Ovalbumin (45 kDa); f. Bovine serum albumin (66 kDa).

3.2.2.6.2.3. MS analysis of purified peroxidase by MALDI-TOF

Molecular mass of black gram husk peroxidase was confirmed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). The enzyme was subjected to MS on a MALDI-TOF Ultima (Waters Corporation, MA, USA) with nitrogen laser at 280 nm wavelength and

5 ns pulse width. The laser beam was focused onto the sample at an angle of 45° to the surface normal. Typical spot size ranged from 10-30 μM. Ions were accelerated to energy of 3 KV before entering the spectrophotometer. At the detector, ions were post accelerated to a maximum kinetic energy up to 30 KV for more efficient detector. The matrix was prepared by dissolving α-cyano-4-hydroxy cinnamic acid in 10% ethanol. Protein (100 μg/ml) was dissolved in triple distilled water and then diluted with matrix. Aliquots of resulting mixture (1 μl) were placed on a piece of silver plate; solvent removed by air-drying and the sample matrix mixture was transferred into a vacuum chamber of the mass spectrophotometer. Bovine serum albumin was used for external calibration.

3.2.2.6.3. Properties of peroxidase

3.2.2.6.3.1. Substrate specificity

Substrates such as *o*-dianisidine, tetramethylbenzidine, benzidine, 3,3-diaminobenzidine, guaiacol, *p*-phenylenediamine, pyrogallol were tested for substrate specificity for peroxidase. The compounds tested as substrates, the solvent used to dissolve them and the wavelengths used in the assays indicated in the **Table 3.3**. The assay protocol remained otherwise unchanged.

Table 3.3. Substrates used for substrate specificity of peroxidase

Compound	Solvent	Wavelength (nm)
o-Dianisidine	Water	460
Guiacol	Ethanol	470
Benzidine	Methanol	655
Diaminobenzidine	Water	460
p-phenylenediamine	Water	460
Tetramethyl benzidine	Water	655
Pyrogallol	Ethanol	440

3.2.2.6.3.2. Determination of Km value (Michaelis constant)

Peroxidase activities at varying concentration of *o*-dianisidine and hydrogen peroxide were determined. Double reciprocal plots were plotted against 1/[V] versus 1/[S] and Km values were calculated according to Lineweaver and Burk (1934).

3.2.2.6.3.3. Effect of inhibitors on peroxidase activity

The effect of varying concentrations of inhibitors on the peroxidase activity was determined. The compounds tested were sodium azide, hydrazine, citric acid, oxalic acid, thiourea, DTT, EDTA, and CTAB. With the exception of hydrazine, which was alcohol soluble, all the compounds were water-soluble. Appropriately diluted enzyme (0.1 ml) was incubated with 0.1 ml of varying concentrations of inhibitors in 0.6 ml of 0.05 M sodium acetate buffer (pH 6.0) at room temperature for 5 min. To the reaction mixture, 0.1 ml of 1% H_2O_2 and 0.1 ml 0.25% *o*-dianisidine were then added, and the absorbance was recorded at 460 nm for 3 min.

3.2.2.6.3.4. Determination of inhibition kinetic analysis

Peroxidase activity was carried out with 0.1 ml of 1% H_2O_2 in the presence of inhibitors (DTT or sodium azide) at different concentrations of inhibitors and *o*-dianisidine. The type of inhibition was determined from the Lineweaver Burk plot of 1/[V] versus 1/[S]. The Ki value was determined from the slope of Lineweaver Burk plot against the concentration of corresponding inhibitor as described by Segel (1994).

3.2.2.6.3.5. Determination of optimum pH

The effect of pH on the enzyme activity was studied at pH values ranging from 4 to 6 (0.05 M sodium acetate buffer) 6 to 8 (0.05 M sodium phosphate buffer) and 8 to 9 (0.05 M Tris-HCl buffer). The enzyme assays were carried out as described earlier.

3.2.2.6.3.6. Determination of temperature stability

The purified enzyme was incubated at room temperature**,** 40ºC, 50ºC, 60ºC, 70ºC, 80ºC and 90ºC for 10 min and assayed for enzyme activity under standard conditions as described earlier. The temperature stability of peroxidase enzyme at 50ºC was determined by incubating the enzyme at different time interval such as 30 min, 1 h, 2 h, 6 h, 10 h, 12 h, 20 h, 24 h, 36 h and the activity of the enzyme was determined under standard assay conditions. The half-life of the enzyme was calculated.

3.2.2.6.3.7. Effect of metal ions

The effects of various metal ions such as Fe^{2+}, Ca^{2+}, Cu^{2+}, Al^{3+}, Mg^{2+}, Zn^{2+}, Li^{+}, Ba^{2+}, Na^{+}, K^{+}, Cd^{2+}, Mn^{2+} were determined by pre-incubating peroxidase with the individual metal ions of different concentrations (5 mM

and 10 mM) in 0.05 M sodium acetate buffer at pH 5.5 for 10 min. The activity of the enzyme was determined under standard assay conditions in the presence and absence of metal. Residual activity was calculated taking activity of control as 100%.

3.2.2.7. Effect of peroxidase on wheat proteins

3.2.2.7.1. Effect of peroxidase on dough rheology of wheat flour

To study the effect of peroxidase on dough rheology, Farinograph experiments were carried out using 50 g wheat flour containing exogenously added 10,000 U of purified black gram peroxidase and 250 μl of 0.1% H_2O_2 using the procedure described in Section 2.2.2.10.2.

3.2.2.7.2. Isolation of various wheat protein fractions from dough

Wheat proteins viz., albumin, globulin, giladin and glutenin were extracted from the control dough and dough prepared in presence of enzymes according to the procedure described by Prasada Rao and Nigam (1987) and Saxena et al (1997). Doughs prepared from 25g of flour samples were extracted with 300 ml of 0.15 M NaCl, with 500ml ethanol (70% v/v) and with 500 ml of 0.1M acetic acid solution to give four protein fractions: salt-soluble (albumin and globulin), ethanol soluble (gliadin), acetic acid soluble (glutenin) and residue protein, respectively. All extraction steps and centrifugation were performed at 4°C. Supernatant from each fraction was combined. All the fractions were reduced to minimum volume by evaporation using a rotary evaporator (Buch Laboratories, Technik, Flawil/ Schweiz, Switzerland). Concentrated fractions were dialyzed against water and freeze dried. The percentage of yield of the fractions was calculated. The total protein content in

the fractions was determined by micro-Kjeldahl method using 5.25 as the protein converting factor (AOAC, 2005).

To study the effect of peroxidase enzyme on extractability of protein fractions, doughs were prepared using 25 g wheat flour with water containing 125 μl of 0.1% H_2O_2 and 1ml (5000 U of activity) of purified black gram husk peroxidase. Wheat protein fractions were isolated as described above.

3.2.2.7.3. Determination of sulfhydryl and disulfide contents

Sulfhydryl (-SH) and disulfide (-SS-) contents in glutenin obtained from the peroxidase treated or untreated wheat flour dough was determined by Ellman's reagent according to the procedure of Beveridge et al (1974). About 20 mg of protein fractions were dissolved in 2 ml of 5 M guanidine HCl in Tris-glycine buffer (10.4 g Tris, 6.9 g of glycine, 1.2 g of EDTA, in 1 L of water, pH 8.0; TG buffer) and centrifuged at 8,000Xg at room temperature and the clear supernatant obtained was used to determine the protein content and –SH and -SS- content. The protein content in the extract was determined using the method described by Bradford (1976).

For determination of –SH group, 0.5 ml of protein solution was diluted to 1 ml with 5 M guanidine HCl –TG buffer. To this, 4 ml of 8 M urea- 5M guanidine HCl in TG buffer was added. Color was developed with 50 μl of Ellman's reagent [4 mg of 5, 5'- dithiobis (2-nitro benzoic acid) in 1 ml of TG buffer].

For the determination of –SS- groups, 50 μl of β-mecaptoethanol and 4 ml of 8 M urea in 5 M guanidine HCl in TG buffer were added to 1 ml of the protein solution, and the mixture was incubated for 2 h at room temperature.

After additional 1.5 h incubation with 10 ml of 12% TCA, the samples were centrifuged at 8000Xg at room temperature. The precipitate obtained was suspended in 10 ml of 12% TCA and centrifuged at 8000Xg. The supernatant was decanted, and the residue washed three more times as above. The protein precipitate thus obtained was dissolved in 10 ml of 8 M urea in TG buffer, and color was developed with 50 μl Ellman's reagent.

Both for the -SH and –SS-, the OD of the color was read at 412 nm using spectrophotometer. Free sulfhydryl and disulfide content were calculated using the following formula.

$$\mu M\ SH/g = 73.53\ A_{412}.D/\ C$$

Where A_{412} = the absorbance at 412 nm, C = the sample concentration in mg solids per ml. D = dilution factor [D is 5.02 for -SH; 10.00 for total SH (ie., -SH + reduced -SS-).

3.2.2.7.4. Reducing and non-reducing SDS-PAGE analysis of glutenin from peroxidase treated dough

Polyacrylamide gel electrophoresis of wheat protein fractions was performed using 10% gel at 50 V according to the method of Lammeli (1970). Samples containing a mixture of molecular weight markers containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (36 kDa), trypsin (24 kDa), β- lactoglobulin (18.4 kDa), lysozyme (14.3 kDa) as well as the protein fractions prepared in buffer containing 1% SDS and 5% mercaptoethanol were boiled for 5 min. In case of non-reducing SDS-PAGE, the sample was dissolved in sample buffer having no mercaptoethanol. After the electrophoresis proteins were stained with Coomassie brilliant blue for 5 h and destained with solution containing 10% methanol and 7.5% acetic acid.

3.3. RESULTS AND DISCUSSION

3.3.1. SECTION A: Purification and characterization of peroxidase from black gram husk

3.3.1.1. Enzyme activities in different milled fractions

During milling of black gram into dhal about 25% was obtained by-product. This by-product was separated into four fractions namely, husk, germ, plumule and aleurone layer rich husk fraction using air classification (Section 3.2.2.1). These fractions and whole black gram flour were analyzed for few oxidative and hydrolytic enzymes such as polyphenol oxidase, peroxidase, protease, amylase and xylanase and the results are shown in **Table 3.4.** All the fractions exhibited more peroxidase activity compared to other oxidative and hydrolytic enzymes and the aleurone layer rich husk fraction showed maximum peroxidase activity (35,800 U/g).

Table 3.4. Enzyme activities in different black gram milled fractions

Fraction	Content (%)	Protein (mg/g)	Peroxidase (U/g)	Polyphenol oxidase (U/g)	Protease (U/g)	Amylase (U/g)	Xylanase (U/g)
Whole gram	-	96±8^{d}	3,200±102^{d}	30±8^{a}	2,124±82^{d}	16±1.2^{c}	1.3±0.1^{c}
Dhal	75	145±12^{e}	500±54^{a}	54±6^{b}	1,823±48^{c}	14±1.8^{c}	1.7±0.1^{d}
Germ	6	95±10^{d}	2,910±16^{c}	172±12^{c}	2,014±82^{d}	8±0.8^{b}	1.0±0.1^{b}
Plumule rich	2	64±5^{c}	572±24^{a}	24±4^{a}	102±10^{a}	4±0.5^{a}	0.4±.0.1^{a}
Aleurone rich husk	8	42±3^{b}	35,800±140^{e}	160±15^{c}	2,020±56^{d}	12±2^{c}	2.0±0.1^{e}
Husk	9	10±1^{a}	1800±23^{b}	32±4^{a}	828±14^{b}	6±1.2^{b}	0.5±0.1^{a}

All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$),

The native PAGE of the enzyme extracts from whole black gram and different milled fractions were carried out and subjected for peroxidase staining and the results are shown in **Figure 3.7**. Peroxidase enzyme staining clearly showed that black gram contains two isoenzymes, one major and one minor form. Aleurone layer rich husk fraction showed only one form of the peroxidase enzyme (major form) and all other fractions showed both major and minor form of the enzymes. Since the aleurone layer contained significant portion of the peroxidase activity of black gram and has only one isoform of the enzyme, this fraction has been taken up for further studies.

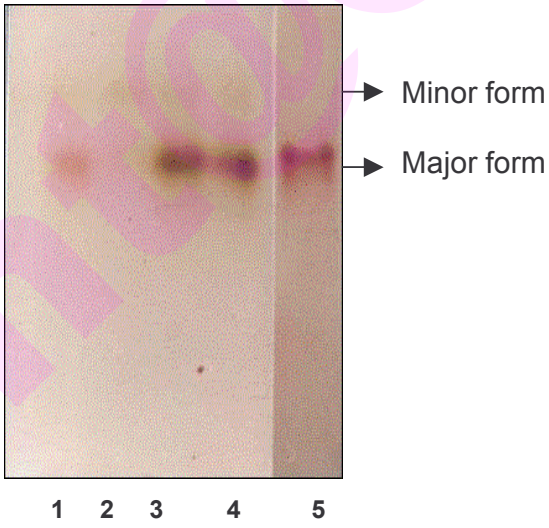


Figure 3.7. Native PAGE of enzyme extract of black gram and its different milled fractions of black gram on peroxidase staining; Lanes-1.whole flour; 2.dhal; 3. germ; 4. plumule; 5. aleurone layer rich husk

3.3.1.2. Determination of optimum condition for the extraction of peroxidase

Different extraction methods were employed to extract maximum peroxidase enzyme activity and the results are shown in **Table 3.5.** Soaking the aleurone layer rich husk fraction in water and sodium phosphate buffer (0.05 M, pH 7.5) and the same buffer containing 0.01% Tween-20 extracted the enzyme with an activity of 3,687, 4231 and 7,430 U/g, respectively. When the aleurone rich husk fraction was homogenized with water and phosphate buffer (0.05 mM, pH 7.5) and buffer containing Tween-20 the enzyme activity obtained was more than the soaking method (8,736-25,512 U/g). Maximum enzyme was extracted by grinding with acid washed sand using mortar and pestle with an activity of 35,800 U/g. It has been reported that peroxidases have been ionically bound to the cell wall and to the tonoplast membrane in many plant materials (Lutz, 1987). The presence of peroxidase in the soluble fraction suggested that the enzyme localization would be in vacuoles so that they were slightly bound to the tonoplast in such a way that during the grinding process in presence of nonionic detergent such as Tween-20 the vacuoles would have been broken and all their content could be solubilized and extracted in the soluble fractions. It was reported that 0.5% Triton X-100 was found to enhance the extractability of peroxidase from black berry fruits (Gonzalez et al, 2000).

Table 3.5. Different methods of extraction of peroxidase from aleurone layer rich black gram husk fraction

Extraction medium	Protein (mg/g)	Activity (U/g)	Specific activity (U/mg protein)
Soaking			
Water			
2h	1.19±0.20^{a}	3,687±41^{a}	3,098±12^{j}
24h	1.22±0.10^{a}	3,560±35^{b}	2,918±18^{i}
Buffer*			
2h	2.12±0.20^{b}	4,231±24^{d}	1995±21^{h}
24h	2.48±0.30^{b}	4,180±20^{c}	1685±12^{g}
Buffer* +Tween-20**			
2 h	4.43±0.15^{c}	7,430±28^{g}	1,677±14^{g}
24 h	5.93±0.15^{d}	6,528±66^{f}	1,100±17^{f}
Homogenization			
Water	12.00±1.20^{e}	8,736±14^{h}	728±20^{c}
Buffer*	21.00.±1.40^{f}	12,810±24^{i}	610±41^{b}
Buffer* +Tween-20**	39.7±1.37^{h}	25,504±357^{j}	643±14^{b}
Grinding			
Water	12.45±1.4^{e}	5870±32^{e}	471±23^{a}
Buffer*	32.18±1.5^{g}	26,140±21^{k}	812±12^{d}
Buffer*+ Tween-20**	42.00±3.0^{h}	35,800±140^{l}	852±12^{e}

* Sodium phosphate buffer (50 mM, pH 7.5); **0.01% Tween-20

All data are the mean±SD of three replicates. Mean value followed by different letters in the same column differs significantly ($P \leq 0.05$),

3.3.1.3. Purification of peroxidase

The peroxidase was extracted from the aleurone layer husk rich fraction by grinding method using sodium phosphate buffer (0.05 M, pH7.5) containing 0.01% Tween-20. This extract was subjected to DEAE-Sephacel chromatography and the unbound fraction without enzyme activity was washed off and bound proteins were eluted by sodium chloride gradient from 0 to 0.5 M. **Figure 3.8** shows elution profile of POD on DEAE-Sephacel. POD eluted just before the major protein peak. The fractions having POD activity were pooled and concentrated by lyophilization. The elution profile suggested that peroxidase enzyme is a cationic protein, which possesses positive net charge, which allow them to adsorbed onto negatively charged surface such as DEAE-Sephacel. Peroxidase purification by ion-exchange chromatography yielded 63% activity with 26 fold purification.

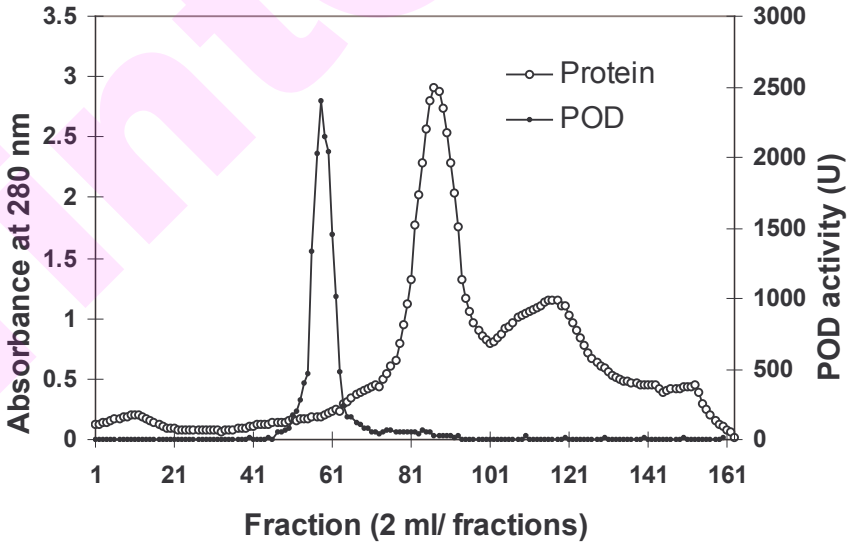


Figure 3.8. Elution profile of black gram peroxidase from DEAE– Sephacel

The concentrated enzyme fraction obtained after ion-exchange chromatography was subjected to gel filtration chromatography. **Figure 3.9** shows the elution profile of peroxidase on Sephadex G-100. At this step of purification enzyme was purified to 44 fold with 44% recovery as shown in **Table 3.6.** Active fractions containing peroxidase activity were pooled and concentrated by lyophilization. This concentrated enzyme was used for further studies.

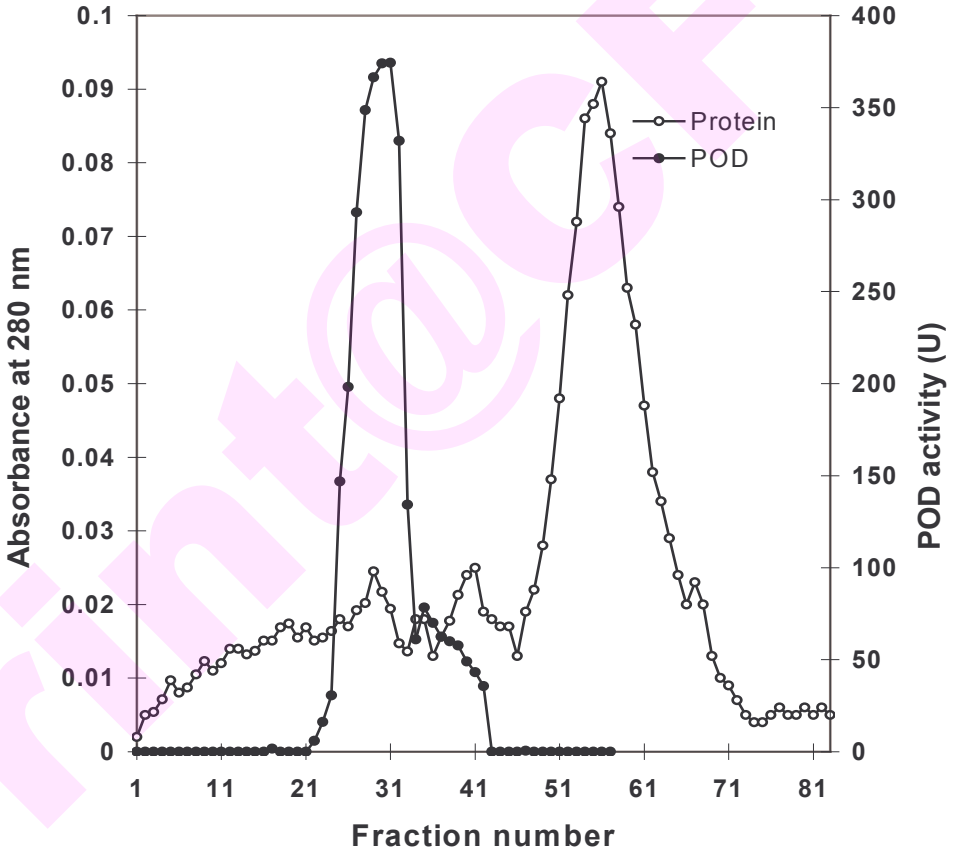


Figure 3.9. Elution profile of black gram peroxidase from Sephadex G-100

Table 3.6. Purification of peroxidase

Fractions	Total POD activity (U)	Protein (mg)	Specific activity (U/mg protein)	Fold purification	Yield (%)
Crude	18,432	22.2	830	-	100
DEAE-Sephacel fraction	11,682	0.54	21,633	26	63
Sephadex G-100 fraction	8,102	0.22	36,827	44	44

3.3.1.4. Determination of purity of peroxidase

The purity of the enzyme was determined by native PAGE, HPLC and capillary electrophoresis. Single protein band was obtained on native PAGE, which showed peroxidase activity on zymogram studies and was stained for carbohydrate staining indicating that this peroxidase from black gram is a glycoprotein (**Figure 3.10.A, B & C**).

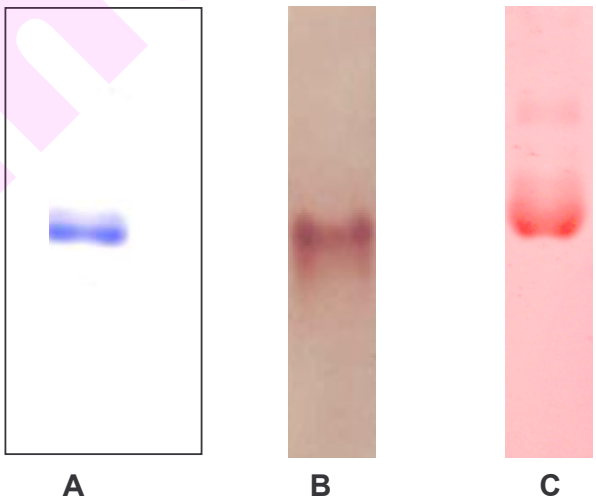


Figure 3.10. Native PAGE of purified peroxidase A) Coomassie staining; B) peroxidase staining and C) glycoprotein staining

When the purified enzyme was subjected for HPLC on C18 column and the capillary electrophoresis as shown in **Figure 3.11** and **3.12,** respectively, in both the cases single peak was obtained confirming the purity of the enzyme.

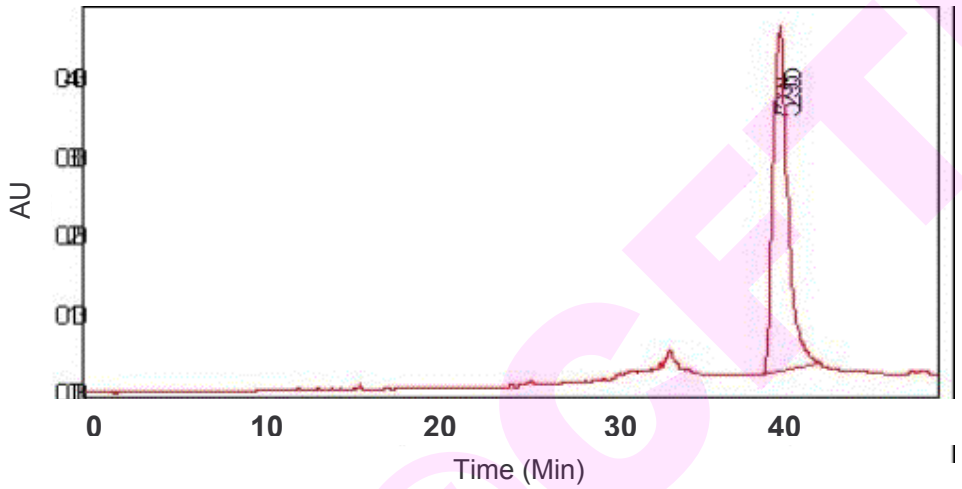


Figure 3.11. HPLC of purified peroxidase

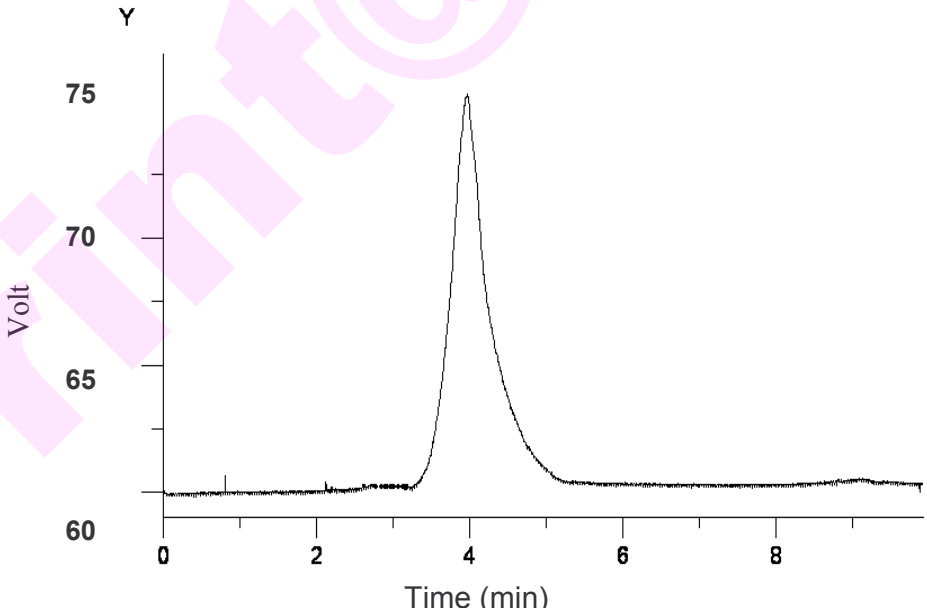


Figure 3.12. Capillary zone electrophoresis of purified peroxidase

3.3.1.5. Properties of peroxidase

3.3.1.5.1. Spectral characteristics

The UV-Visible spectrum of the purified enzyme shown in **Figure 3.13.** The purified enzyme showed absorbance at 280 nm and 408 nm (Soret band), which are characteristics of protein and in particular heme protein. RZ value (Reinheitszahl), which is a measure of hemin content of the peroxidase, was found to be 1.9.

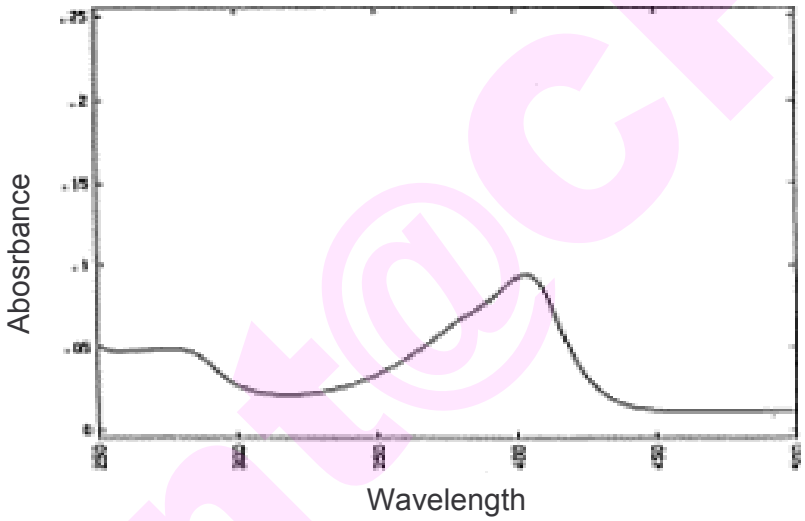


Figure 3.13. UV-Visible spectrum of purified peroxidase

3.3.1.5.2. Molecular weight determination

The molecular weight of the peroxidase enzyme was determined by gel filtration, SDS-PAGE and MALDI-TOF-MS.

The purified enzyme was chromatographed on Sephadex G-100 column, which was calibrated with standard proteins. The molecular weight of the enzyme was calculated from the plot of Ve/Vo versus log of molecular weight as shown in **Figure 3.5** (Section 3.2.2.6.2.1) and was found to be 38

kDa. The purified enzyme showed a single protein band on SDS-PAGE by Coomassie staining (**Figure 3.14**). The molecular weight of the protein was also calculated using the plot shown in **Figure 3.6** (Section 3.2.2.6.2.2.) and was found to be 36 kDa. The molecular weight of the enzyme determined by MALDI-TOF-MS was found to be 35 kDa (**Figure3.15**). MALDI-TOF, gel filtration and SDS-PAGE studies showed that enzyme was a monomeric protein with a molecular weight of approximately 36 kDa.

The molecular mass of black gram husk peroxidase was found to be in the same range those reported for peroxidase from wheat germ with molecular mass of 35 kDa (Converso & Fernandez, 1995), from tobacco with 36 kDa (Gazaryan & Lagrimini, 1996).

Figure 3.14. SDS PAGE of purified peroxidase enzyme

Lanes, 1. Purified enzyme; 2. Molecular weight markers; 66 kDa-bovine serum albumin; 45 kDa- ovalbumin; 36 kDa - pepsin; 24 kDa- trypsionogen; 18.4 kDa- β lactoglobulin and 14.3 kDa- lysozyme

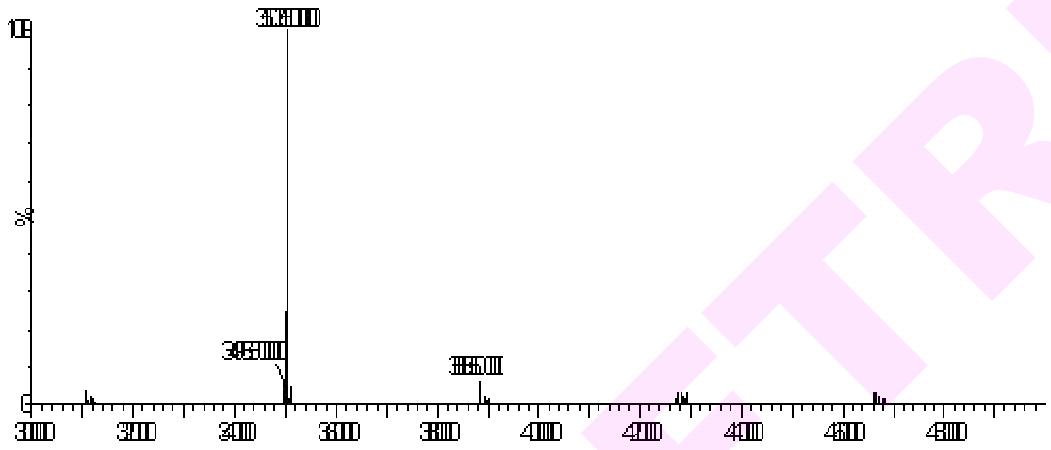


Figure 3.15. MALDI TOF mass spectrum of the native purified peroxidase. The POD peak was 35,035 Da

3.3.1.5.3. Substrate specificity of peroxidase

Different compounds were tested as hydrogen donor substrates for the enzyme. The enzyme showed activity with different substrates such as *o*-dianisidine, *p*-phenylenediamine, diaminobenzidine, benzidine, guaiacol, *p*-coumaric acid, ferulic acid, tetramethyl benzidine and pyrogallol. *o*-Dianisidine was found to be the best substrate for the enzyme followed by guaiacol. All other substrates showed 2-4% of activity obtained with *o*-dianisidine, indicating that these are poor substrates (**Table 3.7**)

Table 3.7. Effect of different substrates on the activity of peroxidase

Substrate	Concentration	Activity	% Activity
o-dianisidine	1.0mM	504	100
Guaiacol	1.0mM	92	18
3,3, diaminobenzidine	1.0mM	21	4
Tetramethyl benzidine	1.0mM	14	3
p-Phenylenediamine	1.0mM	18	4
Benzidine	1.0mM	18	4
Pyrogallol	1.0mM	8	2
Ferulic acid	1.0mM	20	4
p-Coumaric acid	1.0mM	21	4

3.3.1.5.4. Effect of substrate concentration and Km value for the peroxidase

The effect of substrate concentration on purified peroxidase was studied by assaying the activity at different concentration of substrates. For peroxidase hydrogen peroxide and *o*-dianisidine were used as substrates. The specific constant for an enzyme-catalyzed reaction is given by Km value that provides an index to describe the catalytic efficiency of the enzyme towards a substrate. The peroxidase activity increased with increasing concentration of substrate and reached maximum at 1.25 mM for *o*-dianisidine and 29.4 mM for hydrogen peroxide. Beyond these, substrates concentration, the enzyme activity decreased drastically. The Km value was calculated from the Lineweaver-Burk plot and was found to be 3.7 mM for *o*-dianisidine and 43.5 mM for hydrogen peroxide (**Figure 3.16 & 3.17**). The very low Km value

of the enzyme towards *o*-dianisidine shows its increased affinity towards the substrate. The peroxidase enzyme displayed Michael's Menton kinetics within a concentration of 0.2 to 1.25 mM for *o*-dianisidine and 7-29 mM for hydrogen peroxide. Above this concentration, enzyme inhibition occurred probably because of the substrate inhibition combined with oxidation of the iron at the heme group most likely forming oxyperoxidase (Childs & Barsley, 1975).

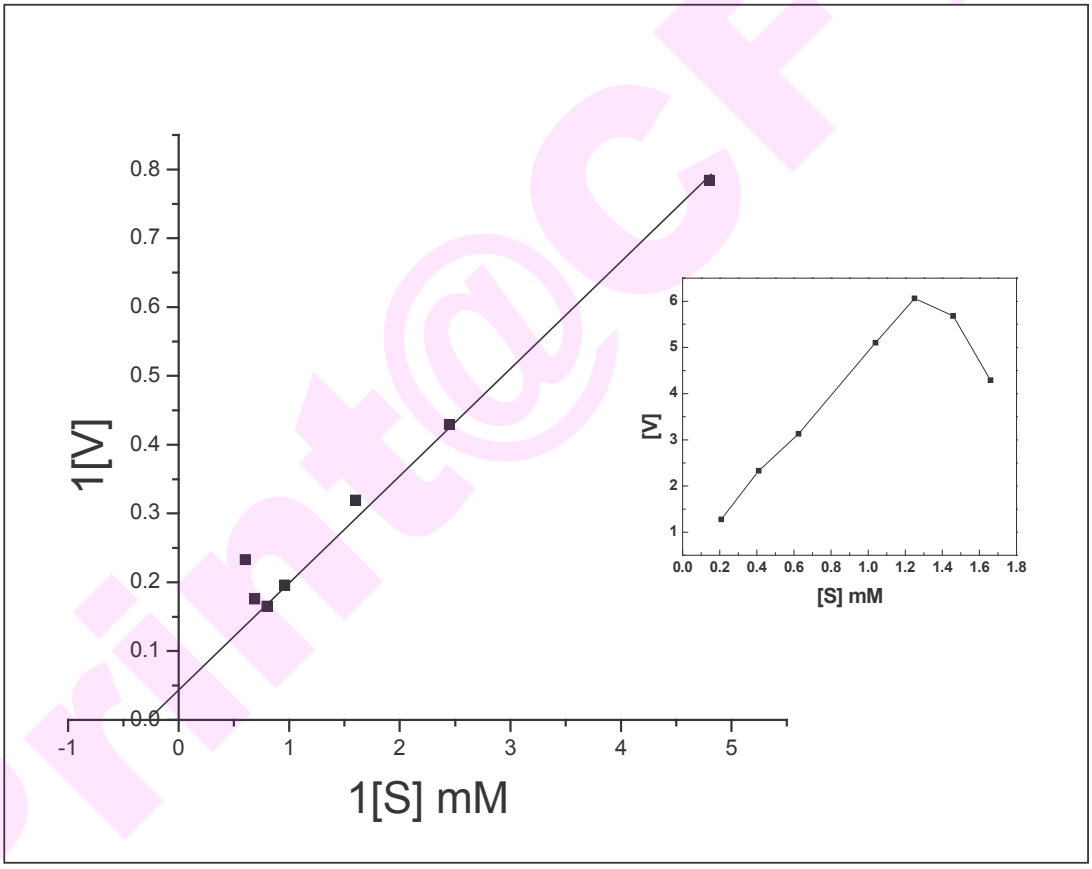


Figure 3.16. Lineweaver Burk double reciprocal plot of the effect of concentration of *o*-dianisidine on the initial velocity of peroxidase from black gram husk; Inset: Michaelis-Menten Curve

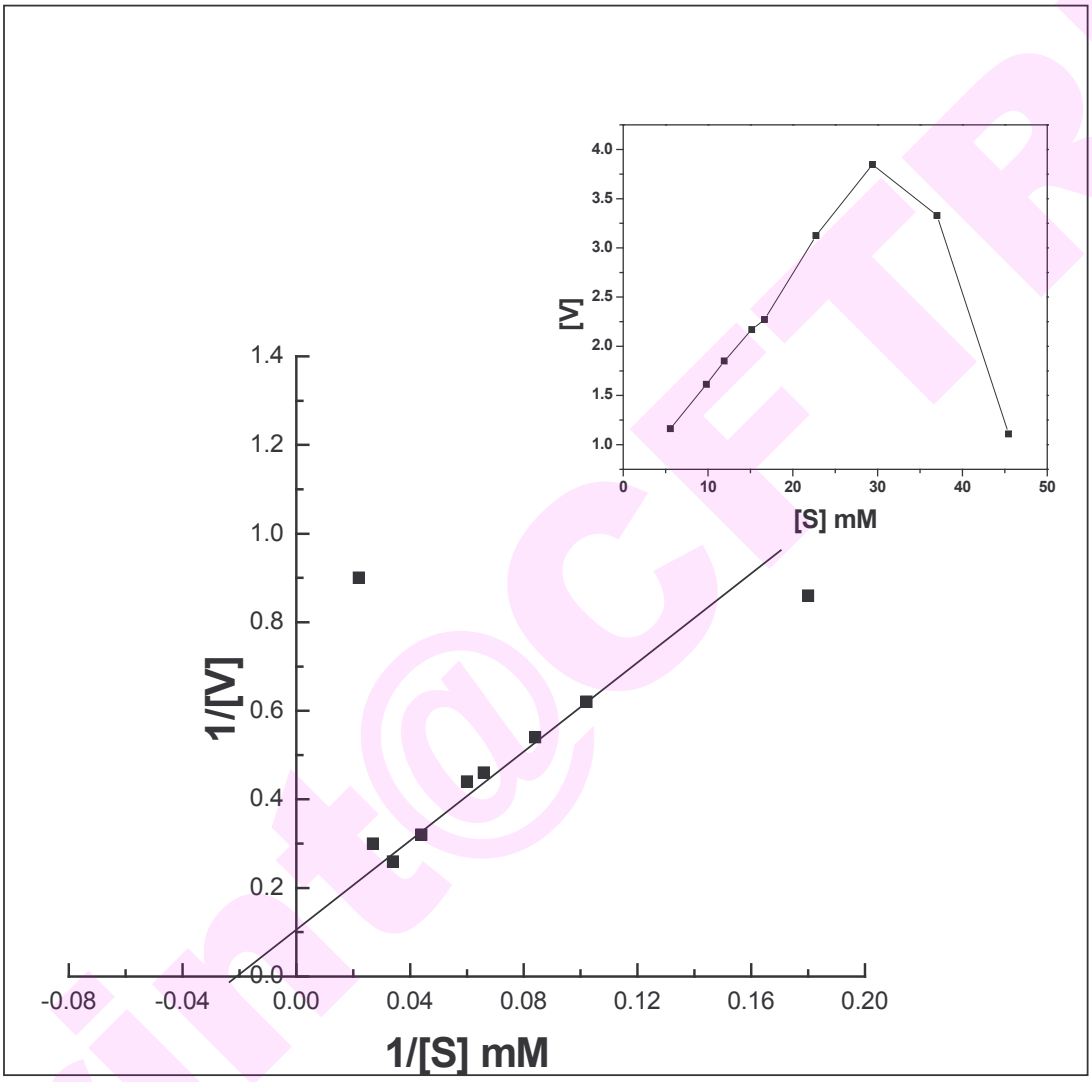


Figure 3.17. Lineweaver Burk double reciprocal plot of the effect of concentration of H_2O_2 on the initial velocity of peroxidase from black gram husk; Inset: Michaelis- Menten Curve

3.3.1.5.5. Effect of inhibitors and inhibition kinetics

The inhibition of peroxidase activity by different inhibitors was determined. Inhibitors like thiourea, sodium azide, dithiotheretiol and hydrazine exhibited more than 90% inhibition at 5 mM, where as carboxylic acids such as oxalic acid and citric acid which are known to be a peroxidase inhibitors showed 93% and 73% inhibition respectively (**Table 3.8**). EDTA and CTAB were found to be weak inhibitors with an inhibition of 33% and 66%, respectively at 20 mM. EDTA, metal chelator, was unable to fully combine with Fe^{2+} ions; consequently, the active site maintained its integrity (Onsa et al, 2004).

Table 3.8. Effect of different inhibitors on peroxidase activity

Inhibitors	Concentration	% Inhibition
Sodium azide	5 mM	95
Hydrazine	5 mM	97
DTT	5 mM	95
Thiourea	5 mM	92
CTAB	20 mM	66
EDTA	20 mM	33
Oxalic acid	10 mM	93
Citric acid	10 mM	73

The inhibition kinetics were determined following the procedure of Lineweaver Burk (1934). Non-competitive inhibition was observed for DTT and sodium azide (**Figure 3.18 & 3.19**). Non-competitive inhibition was

observed when the inhibitor combine with the enzyme substrate complex and prevents transformation of the substrate to products; the substrate and the inhibitors do not compete with each other in the binding to the enzyme active site (Segel, 1994; Wong, 1995). The affinity of inhibitor (Ki) of black gram husk peroxidase by DTT and sodium azide were determined and they were found to be 2.00 mM and 2.08 mM, respectively.

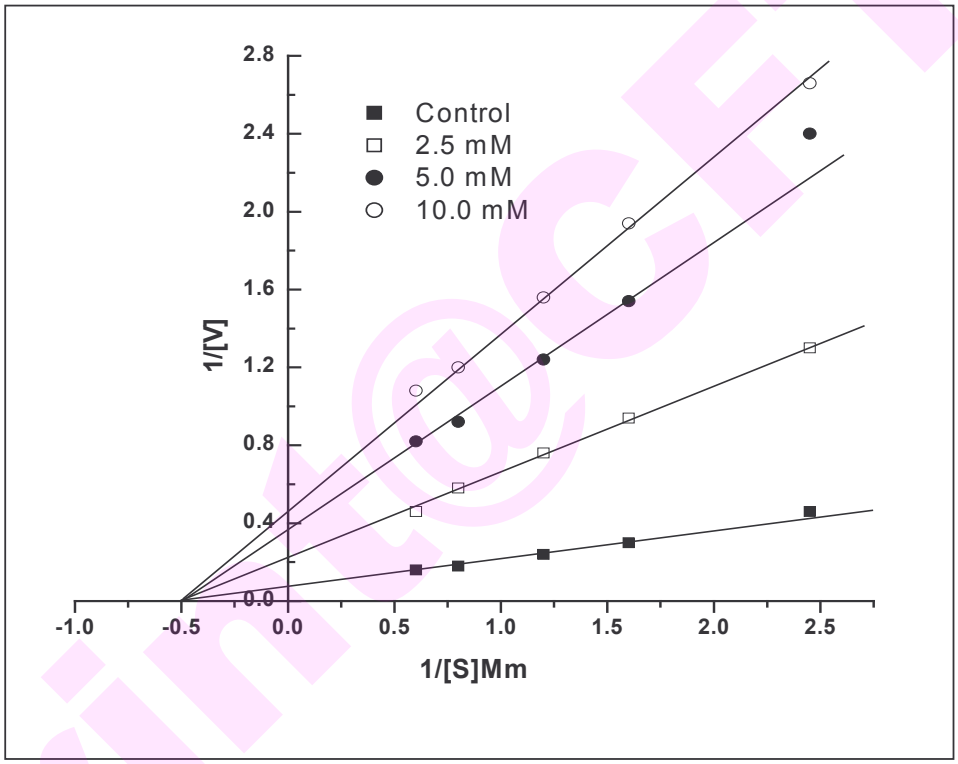


Figure 3.18. Lineweaver Burk double reciprocal plot of 1/[V] versus 1[S] in the presence of different concentrations of sodium azide as an inhibitor for peroxidase at different concentrations of *o*-dianisidine

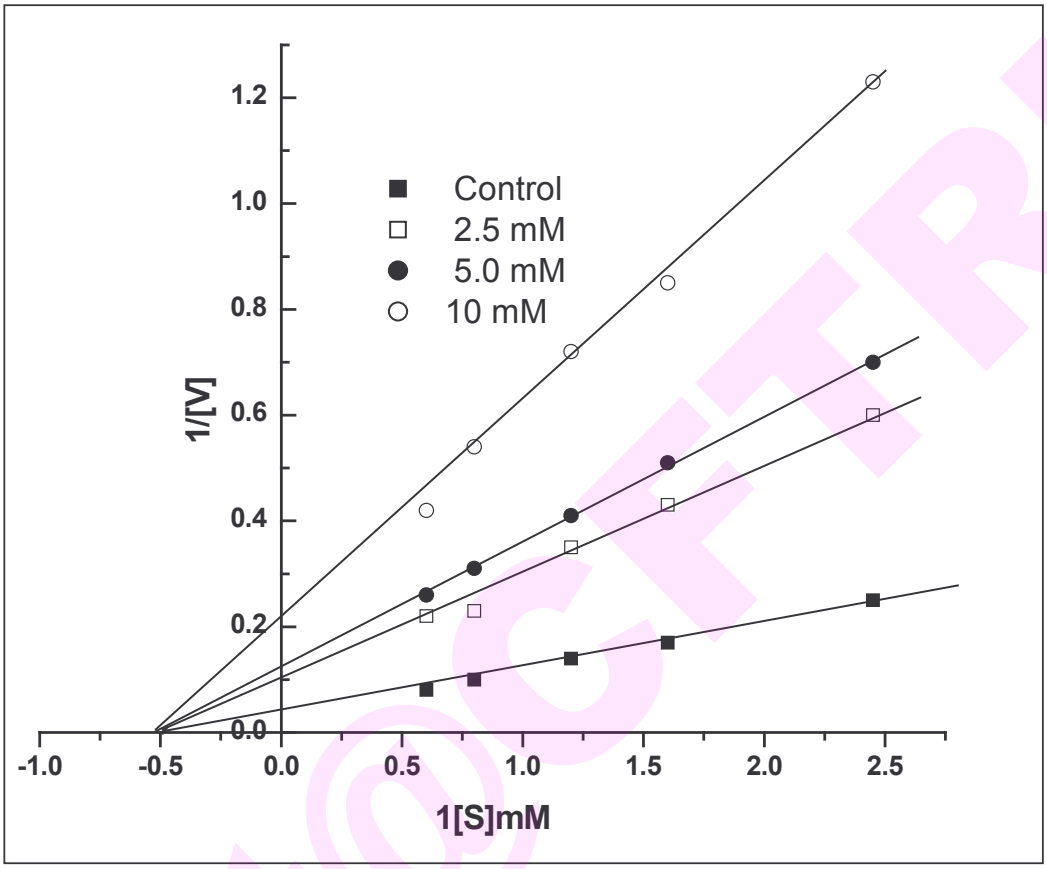


Figure 3.19. Lineweaver Burk double reciprocal plot of 1/[V] versus 1[S] in the presence of different concentrations of DTT as an inhibitor for peroxidase at different concentrations of *o*-dianisidine

3.3.1.5.6. Effect of pH on peroxidase

pH is a determining factor in the expression of an enzyme activity as it alters the ionization state of amino acid chains or the ionization of the substrate (Voet & Voet, 1990). The effect of pH on the activity of enzyme was shown in **Figure 3.20,** the enzyme showed maximum enzyme activity at pH 5.5 and it decreased sharply with increase in pH. The pH optima of peroxidase from grape were 5.4, banana 4.5-5.0, pineapple 4.2, HRP 4.6-5.8,

potato 5.0-5.4 (Kay et al 1967; Vamos & Vigyazo, 1981), wheat germ 5.5-6.3 (Billiaud et al, 1999). Lopez and Burgos (1995) reported that the release of heme group from the enzyme active site was pH dependent and occurred most rapidly at lower and higher pH and lead to the loss in activity. The active site of enzyme is mainly composed of ionic groups (prosthetic group) that must be in the proper ionic form in order to maintain the conformation of the active site of enzyme for substrate binding or reaction catalysis (whitaker, 1995).

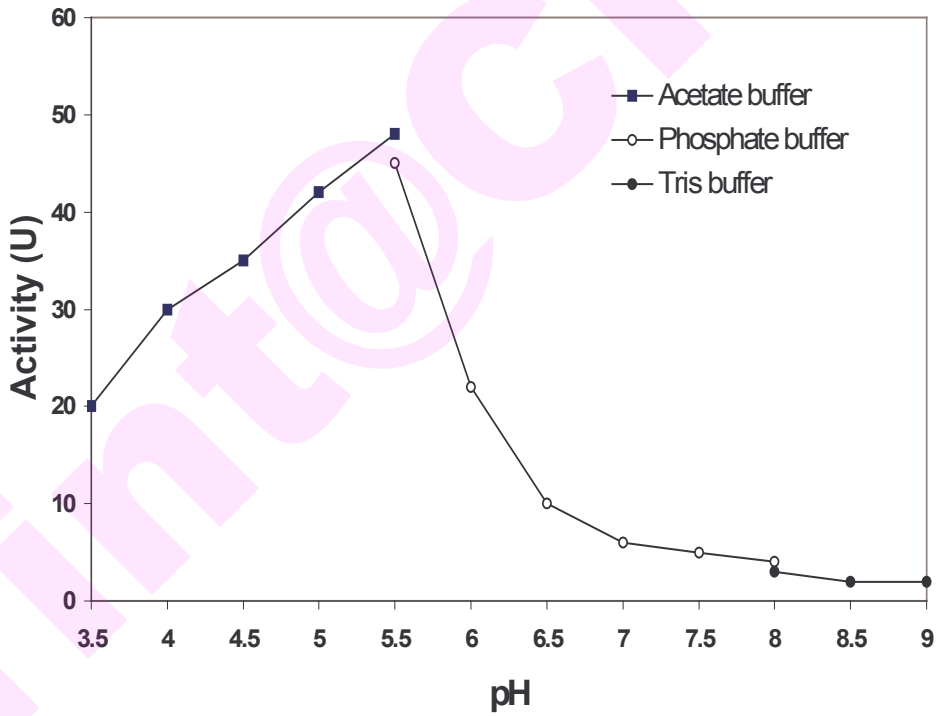


Figure 3.20. Effect of pH on the activity of peroxidase enzyme

3.3.1.5.7. Temperature stability of peroxidase

In order to determine the temperature stability of the enzyme, aliquots of enzyme were incubated at different temperatures varying from 27°C (room temperature) to 90°C, for 10 min as described in methods. Temperature stability studies indicated that POD slightly increased with increase in temperature incubated up to 60°C and the enzyme was stable up to 70°C. Above 70°C a drastic loss of activity was observed (**Figure 3.21**).

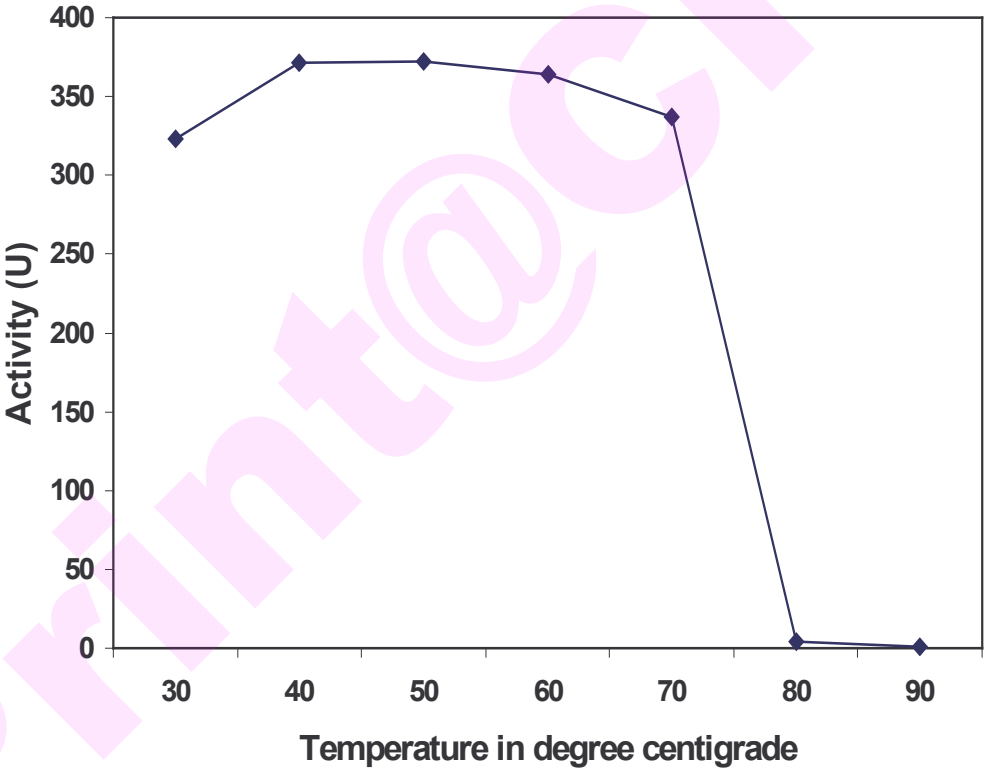


Figure 3. 21. Temperature stability of peroxidase

The thermal stability of the peroxidase at 50°C was determined by incubating the enzyme at 50°C for different time intervals from 30 min to 24h. The half-life of the enzyme at 50°C was found be 5h and 15 min (**Figure 3.22**). The enzyme showed 25% activity even after 24 h of incubation at 50°C. The peroxidase has high thermal stability, attributed to the presence of sugar in their structure (Mellon, 1991). However, this thermostability cannot be extended to all peroxidases due to the existence of isoenzymes with different resistance to temperature (Moulding et al, 1987). The main process found to be involved in the thermal denaturation of peroxidase was due to the dissociation of prosthetic groups from the holoenzyme, a conformation change in the apoenzyme and modification or degradation of the prosthetic group (Tamura & Morita, 1975).

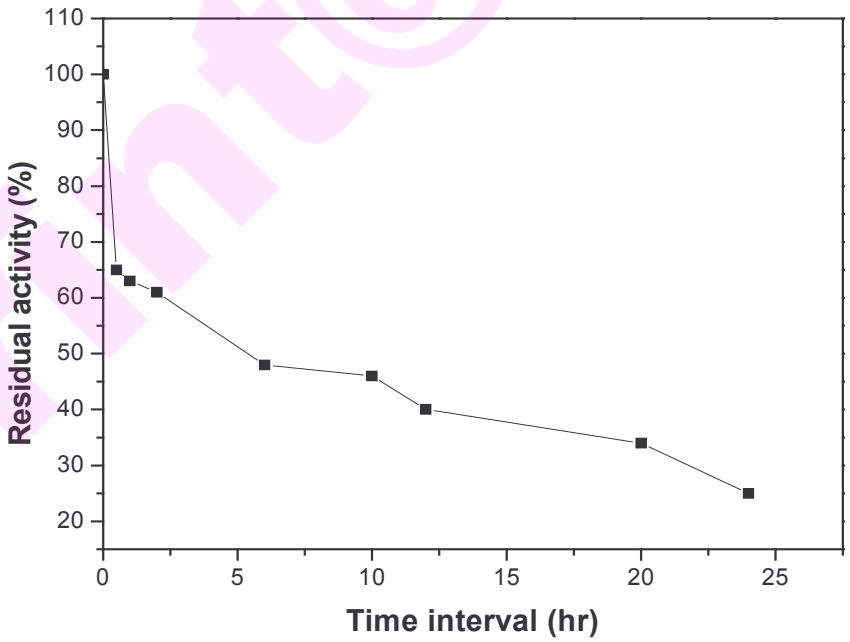


Figure 3.22. Thermal stability of peroxidase at 50°C at different time intervals

3.3.1.5.9. Effect of metal ions on peroxidase

The activities of peroxidase from black gram husk were affected by the presence of different metal ions. Zn^{2+}, Li^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Fe^{3+} stimulated the activity of peroxidase at 5 mM. On the other hand, Mn^{2+}, Cd^{2+}, Al^{3+}, Na^{+}, K^{+} moderately inhibited the enzyme activity at 5 mM, where as Cu^{2+} decreased the enzyme activity to 9% even at 5 mM (**Table 3.9**). At 5 mM concentration Fe^{3+} was able to enhance the activity of peroxidase to 145%. Iron is considered essential for the activity of most of the plant peroxidase as it is involved in binding of H_2O_2 and formation of compound I (Whitaker, 1995, Wong 1995). Ca^{2+} also stimulated the enzyme activity to 110%. Ca^{2+} is a cofactor that serves to maintain the conformational integrity of the enzyme active site (Adams et al, 1990). Activation by Ca^{2+} was reported for peroxidases of avocado (Sanchez-Romera et al, 1994), barely grain and wheat germ (Billaud et al, 1999).

Table 3.9. Effect of different metals on peroxidase enzyme activity

Compound	Concentration	Activity	% Activity
Control	0	91	100
$ZnCl_2$	5mM	110	120
LiCl	5mM	112	120
$MgCl_2$	5mM	95	104
$BaCl_2$	5mM	98	107
$MnCl_2$	5mM	86	94
$CuCl_2$	5mM	8	9
$CdCl_2$	5mM	76	83
$CaCl_2$	5mM	100	110
$FeCl_3$	5mM	132	145
$AlCl_3$	5mM	85	93
NaCl	5mM	74	81
KCl	5mM	89	98

3.3.2. SECTION B: Effect of peroxidase on wheat flour

Wheat is unique among cereals and it forms viscoelastic dough. Proteins especially glutenin, play a major role in contributing the viscolelastic property of dough. In addition, pentosans present in wheat also play a significant role in dough quality. Wheat flour is made into dough before preparation of various bakery products such as bread, chapatti, biscuits etc. During dough formation new covalent and non-covalent interaction and disulfide (-SS-) and sulphydryl (-SH) interchange reactions are reported to take place. These reactions may be controlled by the presence of chemical constituents as well as enzymes present in the flour endogenously or added exogenously. When the compounds are added exogenously to improve the dough quality, they are referred as dough improver.

Wheat flours are broadly classified into strong (hard), medium strong and soft flour depending on the dough quality. Strong wheat flours are reported to be more suitable for bread, medium strong wheat flours are suitable for chapati and soft wheat flours are suitable for biscuits. Several workers have reported the differences in the quality and quantity of protein present in the flour (MacRitchie et al, 1990; Kolster & Vereijken, 1993; Saxena et al, 1997; Srivastava et al, 2003). It is a common practice to add chemical oxidizing agents such as potassium bromate, potassium iodate, ascorbic acid and calcium peroxide to improve the dough quality (Allen, 1998). Due to increase in demand by consumers for more natural products and especially concerns about the possible risks of bromate in food have created a need for bromate replacers. Therefore, studies were carried out to incorporate enzymes such as glucose oxidase, transglutaminase, peroxidase, xylanase

and amylase into flour to improve the dough characteristics (Whitehurst & Law, 2002).

Peroxidase can use a wide range of compounds such as phenols, acylamines, halides and thiols as electron donors. *In vitro,* the combination of peroxidase and peroxide, catalyses the gelation of arabionxylans via formation of diferulic acid linkages (Newkom & Markwalder, 1978; Schooneveld-Bergmans et al, 1999). Further, peroxidase has been suggested to cross-link arabinoxylan to side chains of amino acids in wheat proteins (Neukom & Markwalder, 1978). In dough such cross-linking was suggested to be responsible for the improvement of dough properties (Van Oort, 1996).

Peroxidase has been reported to have a role in the dough improvement properties (Matheis & Whitaker, 1987; Takasaki et al, 2005). Proteins and carbohydrates undergo a variety of reactions in the presence of peroxidase. It is well documented that peroxidase catalyses the formation of inter-dityrosyl, and intra-isodityrosyl bonds in protein molecules. However, there are few reports to indicate that it may also form intermolecular disulfide bonds by oxidizing –SH group in protein ((Matheis & Whitaker, 1987).

In the present study, purified black gram husk peroxidase was incorporated into flour and studied the dough characteristics, changes in protein extractability and effect of peroxidase on glutenin.

3.3.2.1. Effect of peroxidase on wheat flour dough rheology

The effect of peroxidase on the rheological property of wheat flour was determined by Farinograph. As shown in **Table 3.10**, addition of peroxidase decreased the water absorption from 61.2 to 58.5%. The dough development

time increased from 5.0 to 5.5 with incorporation of black gram husk peroxidase. The increase in dough development time shows that the dough was strengthened with the addition of peroxidase. Dough stability, which indicates the dough strength increased from 6.7 min to 7.9 min with the addition of peroxidase. The strength of the dough increased with the dough stability, which in turn imparts gas-holding capacity in the preparation of bread, which is an essential criteria for the product quality.

Table 3.10. Effect of peroxidase on dough rhoelogy property of wheat flour

Sample	WA (%)	DDT (min)	DS (min)
Control	61.2	5.0	6.7
BGHP	58.5	5.5	7.9

Average of two values
WA-water absorption, DDT- dough development time,
DS-dough stability; BGHP- black gram husk peroxidase

3.3.2.2. Effect of peroxidase on wheat protein extractability from dough

Based on Osborne's classification, wheat proteins are classified into albumins, globulins, gliadins and glutenins. Wheat proteins especially gluten proteins play an important role in the formation of cohesive dough and its functionality. Different protein fractions were extracted from dough prepared from flour containing exogenously added peroxidase, and flour with out peroxidase (control). As shown in **Table 3.11**, the extractability of different protein fractions decreased significantly compared to dough that did not have exogenously added peroxidase, i.e., control. Of these, decrease in extractability of glutenin was more (17%) compared to other fractions. The

decrease in solubility may be due to the formation of protein-protein, protein-carbohydrate cross-links. As a result of decrease in solubility of theses fractions, residual protein content increased (**Table 3.11**). Allen (1999) reported that giladin were not susceptible to protein-protein cross-linking. Earlier, Vemulapalli and Hoseney (1998) reported that glucose oxidase had no effect on gluten protein solubility, while it affected the water soluble protein fractions.

As extractability of glutenin from dough having exogenously added peroxidase was decreased, -SH and -SS- content in glutenin fractions was determined (**Table 3.12**). The -SH content in glutenin obtained from control dough was 3.3 ± 0.1 μmole/g protein, while it was 0.16 ± 0.01 μmole/g glutenin obtained from black gram husk peroxidase treated doughs. On the other hand, disulfide bond content was increased in glutenins extracted from exogenous peroxidase treated doughs. The -SS- content in glutenin obtained from control dough was 76.5±3.2 μmole/g protein, while it was 83±2.6 μmole/g protein in glutenin treated black gram husk peroxidase treated dough.

Table 3.11. Effect of peroxidase on wheat protein extractability

Protein fraction	Control (g/100g)	Peroxidase treated (g/100g)
Albumin+ gloubulin	2.485	2.202
Gliadin	2.863	2.573
Glutenin	2.125	1.755
Residual protein	2.426	3.205

Average of two values; Value expressed as protein extracted from dough made from 100g flour

3.3.2.3. SDS-PAGE analysis of glutenin from peroxidase treated dough

SDS-PAGE of glutenin obtained from dough having exogenously added peroxidase was performed in presence and absence of β-mercaptoethanol (**Figure 3.23**). Results indicated that in case of glutenin that was extracted from dough treated with peroxidase showed high molecular weight bands compared to control indicating that exogenously added peroxidase induced the formation of the protein-protein interactions in glutenin. When electrophoresis was carried out in the presence of β-mercaptoethanol the difference in electrophoretic pattern of glutenin between the control and peroxidase treated dough still exist, but to a lesser extent compared to the unreduced conditions. The results indicated that peroxidase is involved in the covalent linking of proteins during dough formation, which results in decreased extraction of protein from dough. Earlier, several workers reported the formation of dityrosine linkages between glutenin proteins in addition to disulfide bonds by peroxidase (Matheis & Whitaker, 1984a, b; Tilley et al, 2001).

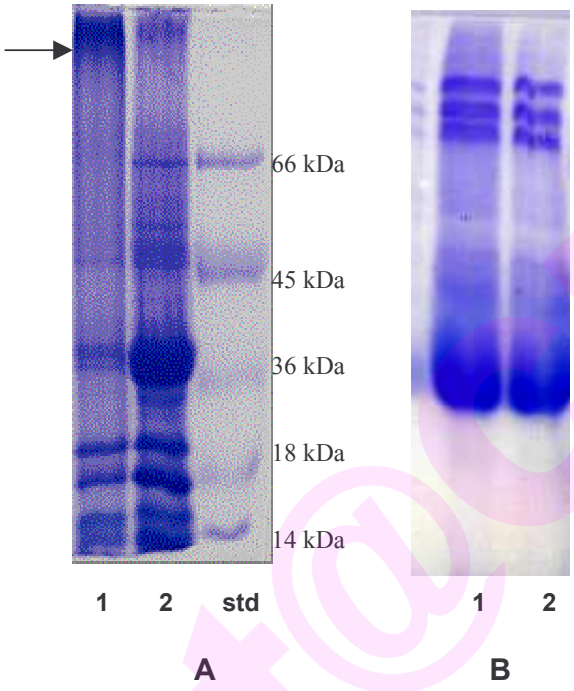


Figure 3.22. SDS-PAGE patterns of reduced and non-reduced glutenin proteins of control and peroxidase treated doughs: A: non-reduced condition, B: reduced condition.

Lanes 1- BGH treated glutenin; 2- control; std- standard molecular weight protein markers. Arrow mark indicates increase in HMW proteins.

CHAPTER IV

SUMMARY & CONCLUSION

4. 1. SUMMARY

Food processing industry generates large quantity of waste by-products causing environmental pollution. Being the agricultural waste, it is rich source of nutrients and some other valuable compounds. Currently, increased efforts are made for better utilization of these by-products that will result in reduction in pollution. In the present study, two abundantly available waste products in India namely mango peel and black gram mill by-products were processed to isolate valuable compounds.

4.1.1. Valuable compounds from mango peel

Mango is one of the most important tropical fruits and India ranks first in the world production. As mango is a seasonal fruit, they are processed for products such as puree, nectar, leather, pickles, canned slices etc, which have worldwide popularity. During processing of mango, peel is one of the major by-products. Peel constitutes about 15-20% of mango fruit. As peel is not currently utilized for any commercial purpose, it is discarded as a waste and becoming a source of pollution. Therefore, the objectives of the present study was to isolate and characterize valuable products from peels, and evaluate the antioxidant activity of mango peel and develop mango peel incorporated products such as biscuits and macaroni and evaluate for their nutraceutical property. The results of this investigation are summarized below.

1) Raw and ripe mango peels from two popular local mango varieties namely; Badami and Raspuri were used for the studies.

2) The proximate composition of the peels were as follows: moisture content, 66-75%; total protein content, 1.45-2.05%; fat content, 2.16-2.66%; carbohydrate content, 11.63-24.60% and crude fiber content, 3.28-7.40%.
3) Of the different solvents used, acetone extracted maximum amount of total phenolic compounds (55-110 mg/g peel) and total phenol content was significantly higher in raw mango peels.
4) The phenolic acids present in the acetone extract of mango peel after acid hydrolysis separated by C18 column using HPLC. Gallic acid, protocatechuic acid, gentisic acid and syringic acid were the phenolic acids identified.
5) The polyphenols in the acetone extracts of peel were also characterized using ESI-LCMS. Based on the mass spectrophotometric analysis, phenolic compounds were tentatively identified. The phenolic compounds identified in Badami mango peel extracts were iriflophenone hexoside, gallic acid, maclurin hexoside, maclurin-tri-O-galloyl hexoside, syringic hexoside, quercetin monohydrate, mangiferin pentoside, ellagic acid, gentisyl protocatechuic acid and hepta-O-galloyl hexose. The phenolic compounds identified in Raspuri mango peel extracts were gallic acid, maclurin hexoside, protocatechuic acid hexoside, galloyl hexose and gallic acid hexoside.
6) The carotenoid content was in the range of 365 to 3,945 μg/g peel and it was found to be more in ripe peel than the raw mango peel.
7) The carotenoids identified in mango peel were violaxanthin, lutein and β-carotene.

8) Mango peel was found to contain vitamin C in the range of 188 to 392 μg/ g peel and ripe peel had more vitamin C than the raw peel.

9) The vitamin E content in the mango peel ranged from 205 to 509 μg/g peel.

10) Mango peel was analyzed for few oxidative and hydrolytic enzymes. The mango peel was found contain polyphenol oxidase (36 to 108 U/g), peroxidase (213 to 275 U/g) and protease (4,573 to 11,173 U/g), amylase (0.9 to 2.8 U/g) and xylanase (4.3 to 9.3 U/g) activities.

11) The dietary fiber content in mango peel varied from 45 to 78% (dry weight basis) and it was found to be more in ripe mango peel in both the varieties. The soluble dietary fiber content varied from 15.7 to 28.1 and insoluble dietary fiber content varied from 28.9 to 50.3%.

12) A method was developed to isolate valuable bioactive compounds such as polyphenols, carotenoids, enzymes and dietary fibers from mango peel simultaneously using the same raw material. This process involves homogenization of the mango peel in a buffer, extraction of homogenate with acetone and air drying of the resulting powder (acetone powder) and extraction of the this powder with buffer. The acetone extract contained phenolics (55-110 mg/g peel), carotenoids (74-436 μg/g peel), anthocyanins (2.0-5.7 mg/g peel) and vitamin E (82-230 μg/g peel). The acetone powder of the peel was found to be rich in oxidative and hydrolytic enzymes and dietary fibers. Carotenoid and vitamin E of acetone extract was lower as saponification was not performed before extracting with acetone.

13) The buffer extract of acetone powder showed significant oxidative and hydrolytic enzyme activity. The peroxidase, 56 to 116 U/g; polyphenol oxidase, 37 to 62 U/g; protease, 1,540 to 6,030 U/ g ; amylase, 0.91 to 1.91 U/g; xylanase, 1.20 to 1.86 U/g.

14) The total dietary fiber content in acetone powder of mango peel after enzyme extraction was found to be in the range of 40.6 to 72.5%, of these soluble dietary fiber content in acetone peel powder was in the range of 13 to 23% and insoluble dietary fiber content ranged from 27.7 to 49.5% with an IDF/SDF ratio varied from 2.1 to 2.8.

15) The sugar composition in the soluble and insoluble dietary fiber was determined by GLC. Galactose, glucose and arabinose were the major neutral sugars in both insoluble and soluble dietary fiber fractions.

16) The insoluble bound phenolic content in the mango peel dietary fiber was found to be in the range of 8.1 to 29.5 mg/g.

17) The bound phenolic content in dietary fiber was more in ripe peel than raw peel. Gallic acid, protocatechuic acid and syringic acid were found to be the phenolic acids identified in the bound phenolic acids of dietary fiber of raw and ripe mango peels of Badami and Raspuri. Ferulic acid was identified only in dietary fiber of Raspuri raw and ripe peels.

18) Since the mango peel was found to be rich in polyphenols, carotenoids vitamin E, the antioxidant activity of the acetone extract of mango peel was determined by different *in vitro* methods such as reducing power method, free radical scavenging activity using stable free radical DPPH, inhibition of lipid peroxidation in microsomes, inhibition of lipoxygenase enzyme and also erythrocytes as a cellular model to

asses the antioxidant potential of the mango peel extract. Mango peel extract showed concentration dependent antioxidant activity as measured by above methods.

19) The reducing power of Badami peel extracts was more compared to Raspuri peel extracts and BHA.

20) Raspuri extracts showed low IC_{50} values (1.83 μg to 1.98 μg of GAE) compared to that of Badami peel extracts (3.67 to 4.54 μg of GAE). It was found that acetone extracts of Raspuri raw and ripe mango peels showed more free radical scavenging activity than that of BHA.

21) Badami extracts showed IC_{50} values from 1.39 μg to 2.68 μg of GAE compared to that of Raspuri peel extracts (3.13 to 4.59 μg GAE) in inhibiting microsomal lipid peroxidation.

22) Of the different extracts, Badami raw peel extract showed maximum lipoxygenase inhibition activity with an IC_{50} value of 2.02 μg of GAE.

23) Antioxidant potential of mango peel extract was studied using rat erythrocytes. Erythrocytes are widely used as cellular model to assess the antioxidant potential by measuring the morphological change, inhibition of lipid peroxidation, hemolysis and effect on membrane proteins on H_2O_2 exposure to rat erythrocytes.

24) The morphological changes induced by H_2O_2 were greatly prevented when the cells were treated with H_2O_2 in presence of mango peel extracts.

25) The mango peel extract inhibited the hemolysis of rat erythrocytes in a dose dependent manner. The peel extract showed 50% hemolysis

inhibition (IC_{50}) at concentrations ranging from 11.5 to 20.9 μg of GAE of different mango peel extract.

26) The lipid peroxidation on erythrocyte membrane was effectively inhibited by mango peel extract. The IC_{50} value for lipid peroxidation inhibition on erythrocyte ghost membrane was found to be in the range of 4.5 to 19.3 μg of GAE.

27) The mango peel extract showed protection against membrane protein degradation induced by hydrogen peroxide.

28) Mango peel was collected from mango processing industry and made into powder with a particle size of 150 micron and incorporated into biscuits and macaroni.

29) The incorporation of MPP at 2.5%, 5%, 7.5% and 10% showed significant effect on the dough rheological properties. Addition of MPP at different levels increased the water absorption from 60 to 68%. The dough development time increased from 4.2 to 5.8 min with 10% incorporation of MPP. Dough stability decreased from 6.7 to 4.0 min with incorporation of 10% mango peel powder.

30) The Influence of MPP on the physical characteristics of biscuits prepared using 5%, 7.5%, 10%, 15% and 20% of mango peel powder were evaluated. Physical characteristics of biscuits such as thickness, diameter and spread ratio were affected slightly with the increase in the level of MPP. Biscuits prepared from flour containing 20% MPP had a breaking strength 1.97 kg compared to 0.88 kg of the control biscuits.

31) The incorporation of 20% MPP increased the content of phenolics in the enriched biscuits from 540 (control) to 4,500 μg GAE/g biscuit

powder. The total carotenoid content increased from 17 to 247 μg/g biscuit with the incorporation of 20% MPP. TDF content increased from 6.47 to 20.70% and the SDF content from 2.8 to 8.2% and IDF content from 3.67 to 12.50%. MPP incorporated biscuits exhibited improved antioxidant activity.

32) Mango peel powder incorporated biscuits were analysed for sensory quality with respect to surface color, surface appearance, texture, taste, flavor and overall quality. The results indicated that biscuits incorporated up to 10% level of MPP are acceptable with out any change in the sensory quality. At 10% MPP level incorporation, the biscuits had TDF of 14.54%, polyphenols of 2,630 μg GAE/g, carotenoids of 145 μg/g biscuit powder which are 2 fold, 5 fold and 8 fold more than that of control biscuits, respectively. Taste and flavor of biscuits was also highly acceptable up to 15% level of MPP and imparted a very pleasant mango flavor and aroma, which was found to be highly desirable.

33) Macaroni were prepared by incorporating 2.5%, 5% and 7.5% levels of mango peel powder. Cooking loss increased from 5.84 to 8.71% with the increase in the incorporation of MPP from 0 to 7.5%. The firmness of macaroni product increased from 44.0 to 73.5 gf.

34) The incorporation of 7.5% MPP increased the content of total phenolics in the macaroni from 460 to 1,803 μg GAE/g of macaroni sample. There was a 3.9 fold increase in phenolic content in macaroni by the incorporation of MPP. The carotenoid content in the macaroni

increased from 4.65 to 84 μg/g macaroni by the incorporation of MPP from 0 to 7.5% level. Total dietary fiber content increased from 8.58 to 17.83%, and the soluble dietary fiber (SDF) content from 3.59 to 5.63% and insoluble dietary fiber (IDF) content from 4.99 to 12.20%. The antioxidant potential of macaroni incorporated with mango peel was significantly improved.

35) Mango peel powder incorporated macaroni were analysed for sensory attributes like color, texture, taste, flavor and over all quality. The results indicated that macaroni incorporated up to 5% level of MPP are acceptable with out any change in the sensory quality. MPP incorporation also imparted mild mango flavor, thus increasing the sensory quality of the macaroni. In macaroni incorporated with 5% level of MPP the phenolic content, total carotenoid content and total dietary fiber content was increased from 460 to 1,605 μg/g, 4.65 to 41.00 μg/g and 8.58 to 15.8%, respectively.

4.1.2. Peroxidase from black gram husk

Black gram (*Phaseolus mungo L.)* is one of the important pulse crops in India and it supplies a major share of protein requirement of vegetarian population. It is mainly consumed in the form of dhal. During milling of the black gram about 25% of the pulse obtained as waste by-product and it does not have any use except as cattle feed. Aleurone layer rich husk fraction constitutes about 8% of by-products. Aleurone layer is rich in protein and metabolically active part of the seed. Attempts were made to screen for certain enzymes in these fractions. The results showed that aluerone layer

rich husk fraction was found to be rich in peroxidase activity. Therefore, the second objective of the present study was to isolate, purify and characterize peroxidase from black gram aleurone layer rich husk fraction.

1) During milling, 25% of black gram is end up as mill waste. By air classification these mill wastes were separated into husk, germ, plumule and aleurone layer husk rich fraction.
2) Above fractions were analyzed for few oxidative and hydrolytic enzymes such as polyphenol oxidase, peroxidase, protease, amylase and xylanase. Of these enzymes, peroxidase activity was found to be more than the other enzymes and aleurone layer rich husk fraction showed maximum peroxidase activity (35,800 U/g). On peroxidase staining, whole black gram showed two isoforms, one major and one minor band, whereas, aleurone layer rich husk fraction showed only the major band.
3) Different extraction methods were employed to extract the peroxidase activity from aleurone layer rich husk fraction. The best extraction method for enzyme was grinding with acid washed sand in phosphate buffer (0.05 M, pH 7.5) containing 0.01% Tween-20.
4) The extracted enzyme was purified to homogeneity by two step chromatographic method using DEAE-Sephacel followed by Sephadex G-100.
5) The purity of the enzyme was determined by native PAGE, SDS-PAGE, HPLC and capillary electrophoresis. Purified enzyme showed single band on native PAGE, which showed peroxidase activity in zymogram and stained for carbohydrate.

6) RZ (Reinheitszahl) value, which is a measure of hemin content of the peroxidase, was found to be 1.9. The UV-Visible spectrum of the native enzyme showed Soret maximum at 408 nm.

7) The peroxidase from black gram was found to be a monomeric glycoprotein as indicated by the presence of a single band in SDS-PAGE by Coomassie staining and glycoprotein staining with a molecular weight of approximately 36 kDa. The apparent molecular mass of the purified enzyme was ~38 kDa, as determined by the gel filtration chromatography. The MALDI-TOF MS analysis of the purified enzyme showed the molecular weight as 35 kDa.

8) The peroxidase enzyme showed maximum enzyme activity at pH 5.5.

9) Temperature stability studies indicated that POD was stable up to 70°C. Above 70°C a drastic loss of activity was observed. Half-life of the enzyme at 50°C was found be 5 h and 15 min.

10) *o*-Dianisidine was found to be the best substrate for the enzyme followed by guaiacol. All other substrates showed 2 to 4% of activity compared to *o*-dianisidine. The Km value was found to be 43.5 mM for hydrogen peroxide and 3.7 mM for o-dianisidine.

11) Sodium azide, thiourea, dithiotheretiol and hydrazine and carboxylic acids such as oxalic acid and citric acid were found to be strong inhibitors of peroxidase enzyme, where as EDTA was found to be a weak inhibitor.

12) Non-competitive type of inhibition was observed for both DTT and sodium azide and the Ki for these inhibitors were determined and were found to be 2.0 mM and 2.1 mM, respectively.

13) The activities of peroxidase from black gram husk were affected by the presence of different metal ions. At 5 mM concentration Zn^{2+}, Li^{+}, Mg^{2+}, Ba^{2+}, Ca^{2+}, Fe^{3+} stimulated the activity of peroxidase. On the other hand at 5 mM concentration Mn^{2+}, Cd^{2+}, Al^{3+}, Na^{+}, K^{+} moderately inhibited the enzyme activity, where as inhibition by Cu^{2+} was maximum.

14) The effect of peroxidase enzyme on the rheological property of wheat flour was measured by Farinograph. The addition of purified black gram peroxidase enzyme to wheat flour showed significant difference on the rheological properties of the flour. Addition of peroxidase enzyme decreased the water absorption from 61.2 to 58.5%, the dough development time increased from 5.0 to 5.5 min and dough stability increased from 6.7 min to 7.9 min.

15) The free sulfhydryl (-SH) and disulfide (-SS-) content in the glutenin protein isolated from control and peroxidase treated wheat flour were determined. The –SH content in the glutenin protein fractions decreased from 3.3 to 0.16 μmole/g protein, where as the –SS- content in the gluteinin proteins treated with peroxidase increased from 76.5 to 83 μ mole/g protein. SDS-PAGE of glutenin, extracted from peroxidase treated dough showed increased intensity of high molecular weight band compared to control indicating that exogenously added peroxidase induced the formation of cross-linking between proteins.

4.2. CONCLUSION

The present study made an effort for better utilization of two abundantly available agro-wastes in India namely mango peel and black gram mill waste.

Mango peel is edible in nature. Unripe mango, in the form of chutney and pickle, is consumed with the peel. On the other hand, mango peel of the ripe fruit due to its leathery nature, not so acceptable in taste, therefore, peel is generally removed and wasted. However, a number of people consume mango with the peel especially some of the mango varieties like Totapuri, where the taste of the mango is not greatly affected by the presence of mango peel. However, in food industry mango peel is removed for technological and sensory advantages. Thus, in food processing industry mango peel generally ends up as a waste by-product.

The present study clearly showed that mango peel is rich in number of valuable components such as polyphenols, carotenoids and dietary fiber. All these components have nutraceutical properties. It is clearly demonstrated using two different varieties of mango at both raw and ripe stages that mango peel is a valuable source of these natural compounds. Therefore, attempts were made to exploit benefits of the mango peel in two ways. In one approach, valuable components were extracted from mango peel. The acetone extract of mango peel showed excellent antioxidant properties. The total antioxidant property of mango peel extract could be due to the synergistic action of polyphenols, carotenoids and vitamin E, the presence of which were clearly established by the detailed analytical studies. In addition, hydrolytic and oxidative enzymes were isolated from the acetone powder,

though the activity was lower than that of fresh peel. The remaining residue was found to be rich in dietary fiber with a well balanced proportion of IDF/SDF for better health benefits.

Another approach for better utilization of mango peel attempted in this study was by incorporating the mango peel into food products namely biscuits and macaroni. Mango peel from mango processing industry, was collected, washed and subjected for mild drying condition and powdered into fine powder. This mango peel powder (MPP) was incorporated into biscuits and macaroni. Biscuits incorporated up to 10% level of MPP were acceptable in terms of its sensory characters. At this level of MPP, the phenolic content, total carotenoid content and total dietary fiber content increased which improved nutraceutical property to the product. Macaroni prepared at 5% level of MPP were acceptable and at this level of MPP, the phenolic content, total carotenoid content and total dietary fiber content was increased which in turn increased the nutraceutical property. MPP incorporation also imparted mild mango flavour, thus increasing the sensory quality of the products. In this study, feasibility of incorporating MPP into two wheat based products such as biscuits and macaroni were documented. However, the MPP and different products isolated from mango peel namely mango peel extracts and mango peel dietary fiber may be incorporated into a number of other convenient ready to eat confectionary products such as other bakery products, fruit bars, health drinks and dairy products. Such utilization of the mango peel not only promotes the better utilization of natural food resources, but also helps to reduce the environmental pollution.

Black gram milled by-products are not utilized for human consumption. It is mainly used as fodder. Therefore, isolation of value added components were attempted from the black gram husk in the study. The aleurone layer reported to be rich in protein and it is associated with the husk fraction of black gram mill waste. Therefore, attempts were made to isolate functionally active proteins such as enzymes. Among the several enzymes studied, peroxidase activity was maximum. Peroxidase is a physiologically important enzyme having a number of applications. It was shown that black gram contained two isoenzymes of peroxidase (one major and one minor form). Aleurone layer rich husk fraction contained major form of isoenzyme of peroxidase. A simple purification method was developed to isolate peroxidase in homogeneous form in good yield (specific activity-36,827 U/mg protein, recovery-44%) from black gram husk. Thus, black gram husk fraction could be a good and easily available source of peroxidase. As far as we know, this is the first study on black gram husk peroxidase.

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Appendix

RESEARCH PAPERS

a) Papers published/ communicated so far based on the work reported in the thesis

1) **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. Valuable components of raw and ripe peels from two Indian mango varieties- *Food Chemistry,* 2007**,** 102: 1006-1011.
2) **C. M. Ajila**, K. A. Naidu, S. G. Bhat and U. J. S. Prasada Rao. Bioactive compound and antioxidant potential of mango peels extract-. *Food chemistry* (Accepted).
3) **C. M. Ajila** and U. J. S. Prasada Rao. Inhibition of hydrogen peroxide induced oxidative damage in erythrocytes by mango peel extracts- *Food Chemical and Toxicology* (Under revision).
4) **C. M. Ajila**, K. Leelavathi and U. J. S. Prasada Rao. Effect of mango peel powder on rheological, baking and antioxidant properties of biscuits. *Journal of Cereal Science* – Communicated.
5) **C. M. Ajila**, M. Aalami, K. Leelavathi and U. J. S. Prasada Rao. Mango peel powder: A potential source of antioxidant and dietary fiber in macaroni preparations. *European Food Research and Technology*- Communicated.

b) Papers under preparation based on the work reported in the thesis

1) **C. M. Ajila** and U. J. S. Prasada Rao. A process for the isolation of bioactive compounds from mango peel and characterization of dietary fiber for bound phenolics.
2) **C. M. Ajila** and U. J. S. Prasada Rao. Purification and characterization of peroxidase enzyme from black gram husk.

PATENTS

1) A two-step process for extraction of bioactive compounds from mango peel. U. J. S. Prasada Rao, S. G. Bhat, C. M. Ajila, Mahadevamma [443/DEL/ 03].

POSTER PRESENTATIONS AT ACADEMIC CONFERENCES

1. **C. M. Ajila,** S. G. Bhat and U. J. S. Prasada Rao. Isolation and characterization of peroxidase from black gram (*Phaselous mungo*). Presented at the 71st Annual meeting of Society of Biological chemists (I), Ludhiana, P.124, Nov 14-16, **2002**.
2. **C. M. Ajila,** S. G. Bhat and U. J. S. Prasada Rao. Mango peel- A potential source of value added products. Presented at the National Symposium

(IFCOST-2002) jointly organized by AFSTI and CFTRI, Mysore, p.29, Dec 12-13, **2002.**

3. **C.M. Ajila**, K. A. Naidu, S. G. Bhat and U. J. S. Prasada Rao. Antioxidant properties of mango peel – A byproduct of mango processing industry. Presented at the 10th Congress of Federation of Asian & Oceanic Biochemists and molecular Biologists and 72nd Annual meeting of Society of Biological chemists (I), (FAOMB- 2004), Bangalore, India, P.124, Dec7-11, **2003**.
4. **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. Black gram husk- A new source of peroxidase. Presented at the International Food Convention (IFCON-2003) jointly organized by AFSTI and CFTRI, Mysore, p.16, Dec 5-8, **2003**.
5. **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. Bioactive compounds from mango peel. Presented at 73rd Annual meeting of Society of Biological chemists (I), G.B Pant University, Pantnagar, India, P.74, Nov 21-24, **2004**.
6. **C.M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. Partial purification and characterization of peroxidase from mango peel. Presented at the 16th Indian Convention of Food Scientist and Technologists on focal theme Food technology: rural out reach – vision 2020 (IFCOST-2004) jointly organized by AFSTI and CFTRI, Mysore, p.23, Dec 9-10, **2004**.
7. **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. "Hydrolytic and oxidative enzymes of mango peel of different varieties. Presented at the Colloquium on Novel proteins in nutrition and health". Jointly organized by CFTRI, Mysore and The Solae Company on March 22nd, **2005**.
8. **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao. Inhibition of hydrogen peroxide induced oxidative damage in erythrocytes by mango peel extracts. Presented at the National Symposium on value addition of Food. IFCOST-2005, organized by AFSTI and CFTRI, Mysore, Dec 9-10, **2005**.
9. **C. M. Ajila**, S. G. Bhat and U. J. S. Prasada Rao "Peroxidase from food processing waste". Jointly organized by CFTRI, Mysore and Biosensor society of India.Jan-20-22, **2006**.
10. **C. M. Ajila**, K.Leelavathi and U. J. S. Prasada Rao. Effect of mango peel powder on rheological, baking and antioxidant properties of biscuits. Presented at the IFCOST-2006, organized by AFST(I), Hyderabad, November 16-17, **2006**.