COMPARATIVE STUDIES ON THE INHIBITORY ACTIVITIES OF PLANT EXTRACTS TOWARDS BANANA PEEL POLYPHENOL OXIDASE (PPO)

A PROJECT REPORT

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BONAFIDE CERTIFICATE

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ABSTRACT

Polyphenol Oxidase (PPO) is a copper containing enzyme present in various fruits and vegetables. It is responsible for the browning reactions when the cells are damaged during handling. It reacts with substrates such as L-DOPA forming a brown colored product, quinone. PPO was extracted from banana peel using an extraction buffer containing sodium phosphate buffer, Triton X-100 and polyvinyl pyrolidone (PVP) and partially purified by ammonium sulphate precipitation, and the enzyme activity was measured spectrophotometrically at 475 nm. PPO inhibitors from plant extracts were identified, extracted using solvents and their inhibitory activities were measured. Polar extracts were prepared from the methanol and aqueous extracts and were used to find the inhibitory effectiveness of various combinations of inhibitors towards PPO. The combination of pomegranate and wheat was found to be more effective than the other combinations in the ratio of 1:1. This inhibitor was tested for its application in fresh-cut fruits, vegetables and their juices. PPO was inhibited by almost 91% in banana juice and in mushroom by about 80%. In fresh-cut fruits and vegetables, enzymatic browning monitored up to 2 hours was inhibited by the inhibitors.

Keywords: Polyphenol Oxidase, L-DOPA, Plants extracts, Pomegranate-Wheat combination

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LIST OF ABBREVIATIONS

PPO	Polyphenol Oxidase
EC	Enzyme Commission Number for Enzymes
L-DOPA	L-3,4-dihydroxyphenylalanine
DHI	5,6-dihydroxyindole
IC	Inhibitory Concentration
SO_2	Sulphur dioxide
Cu	Copper
N-terminal	Amino Terminal
E.coli	*Escherichia coli*
PVP	Polyvinyl Pyrolidone
PBS	Phosphate Buffered Saline
min	Minutes
°C	Degree Celsius
h	Hours
Da	Daltons
cm	Centimetre
NaCl	Sodium chloride
mM	Millimolar
mL	Millilitre
nm	Nanometre
K_m	Michaelis Constant
UV	Ultraviolet
ppm	Parts per million
µmol	Micromole
kg	Kilogram
mmol	Millimole
µM	Micromolar
µg	Microgram

w/w	Weight by weight
GSH	Glutathione
s	Seconds
UF	Ultrafiltration
MPa	Mega Pascal
CO_2	Carbondioxide
mg	Milligram
g	Gram
M	Molar
SHAM	Salicylhydroxamic Acid

INTRODUCTION

CHAPTER 1
INTRODUCTION

1.1 POLYPHENOL OXIDASE (PPO):

Polyphenol oxidases are enzymes with both catecholase (EC 1.10.3.1) and cresolase (EC 1.14.18.1) activity. Based on the substrates they act upon, these enzymes are also referred to as tyrosinase, phenolase, catechol oxidase, catecholase, monophenol oxidase, o-diphenol oxidase and orthophenolase (Mayer and Harel, 1979; Vaughn and Duke, 1984; Mayer, 1987). These enzymes have a dinuclear copper centre, which are able to insert oxygen in a position ortho- to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. Molecular oxygen is used in the reaction. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved.

The enzyme seems to be of almost universal distribution in bacteria, fungi, plants and animals. In addition, they are also found in seafood products like shrimp and lobster. In higher plants the enzyme is localized to the thylakoid membranes of chloroplasts and other plastid organelles.

PPO has been isolated from various sources such as the banana, apple, kiwi, lettuce, pear, cocoa bean, and oil bean. PPO is frequently reported as a latent enzyme, which can be activated in vitro by a number of different factors and treatments such as detergents (Kenten, 1958; Moore and Flurkey, 1990), proteases (King and Flurkey, 1987), low and high pH levels (Kenten, 1957) and exposure to fatty acids in the incubation mixtures (Golbeck and Cammarata, 1981).

1.1.1 Mechanism of Enzymatic Browning:

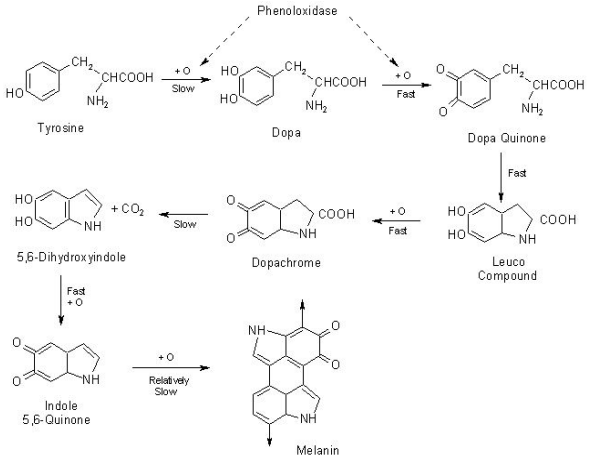

Fig.1.1 Formation of Melanin from Polyphenol Oxidase (PPO)

The pathway of melanogenesis is briefly summarized in Fig.1. When tyrosine is oxidized by PPO, dopaquinone is produced as the immediate product. In the absence of cysteine, dopaquinone undergoes the intramolecular addition of the amino group giving cyclodopa (leuco compound). The redox exchange between leuco compound and dopaquinone gives rise to dopachrome. Dopachrome gradually decomposes to give mostly 5, 6- Dihydroxyindole (DHI). DHI undergoes oxidation to form Indole 5, 6- Quinone which then polymerizes to form the brown coloured pigment, melanin (Shosuke, 2003).

1.1.2 Functions of PPO:

PPO has been implicated in the formation of pigments, oxygen scavenging and defence mechanism against plant pathogens and herbivore insects. Mutations in tyrosinase are thought to contribute to albinism (lack of melanin) in animals.

1.2 ENZYMATIC BROWNING AND INHIBITION:

Enzymatic discoloration often results in the significant loss of post-harvest quality of commercially important fruits and vegetables by causing deterioration in food quality by changing nutritional and organoleptic properties (Mayer and Harel, 1991). This diminishes consumer acceptance, storage life and value of the plant products.

The control of PPO activity is of importance in preventing the synthesis of melanin in the browning of vegetables and fruits (Martinez and Whitaker, 1995; Sanchez-Ferrer *et al.*, 1995; Vamos-Vigyazo, 1981). In addition, PPO inhibitors have been used as depigmenting agents for the treatment or prevention of pigmentation disorders. Hence, PPO inhibitors are supposed to have broad applications in the medical and cosmetic industries. In the food industry, PPO inhibitors could be used as preservatives for foods and beverages of plant origin. Furthermore, PPO is one of the most important key enzymes in the insect molting process, and thus the discovery of inhibitors of this enzyme may be important in the development of new alternatives for insect control.

PPO inhibitors fall into three groups:

i. small molecules or ions that bind to the copper centre in the active site,
ii. aromatic inhibitors that compete with phenolic substrates for binding to the active site, and
iii. compounds that reduce or oxidize copper ions (Burton,1994).

It is estimated that over 50% of losses in fruits occur as a result of enzymatic browning. In addition to the inhibitory mechanism, inhibitory strength is the primary criterion of an inhibitor. Inhibitor strength is usually expressed as the inhibitory IC_{50} value, which is the concentration of an inhibitor needed to inhibit half of the enzyme activity in the tested condition.

1.2.1 Inhibitors from Natural Sources:

As plants are a rich source of bioactive chemicals, which are mostly free from harmful side effects, there is an ongoing effort to search for PPO inhibitors from them. A broad spectrum of compounds have been obtained from the natural plants and investigated for PPO inhibitory activity. These compounds differ from one another in the potency and type of inhibition imposed on the enzyme. Inhibitors from plants are categorized into two main subgroups, namely, polyphenols and aldehydes and other derivatives.

Polyphenols are a group of chemical compounds that are widely distributed in nature and also known as vegetable tannins because they are responsible for the colours of many flowers. Some of them are complex compounds present in the bark, root, and leaves of plants, whereas others are simple compounds present in most fresh fruits, vegetables, and tea.

Some potent PPO inhibitory flavonoids such as quercetin, nobiletin and arbutin (Chen and Kubo, 2002) have been isolated from various plants. A lot of work has been done to identify and characterize inhibitors from natural sources and to establish the relationship between their inhibitory activity and structure. All flavonoids inhibit the enzyme due to their ability to chelate copper in the active site.

Quercetin is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. It is used as an ingredient in supplements, beverages and foods. It is a competitive inhibitor of PPO.

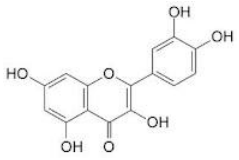

Fig.1.2 Structure of Quercetin

Nobiletin is a o-methylated flavonoid isolated from peels of Citrus fruits. It shows a concentration-dependent reduction in tyrosinase activity using L-DOPA as the substrate (Kenroh and Fumihiko, 2002).

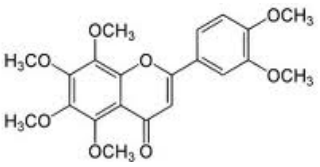

Fig.1.3 Structure of Nobiletin

Arbutin is a glycosylated hydroquinone extracted from bearberry plants. It exhibits competitive inhibition of the substrate binding site of tyrosinase (Parvez *et al.*, 2006). Significant amounts of arbutin levels were found in wheat products, pears, coffee and tea.

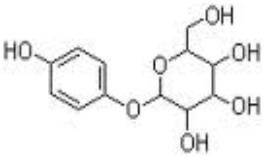

Fig.1.4 Structure of Arbutin

A large number of aldehydes and other derivatives have been isolated and characterized as PPO inhibitors such as cuminaldehyde. It is a natural organic compound with a benzaldehyde structure. It is a constituent of essential oils of eucalyptus, myrrh, cumin and others.

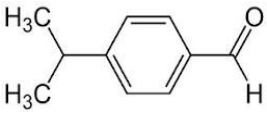

Fig.1.5 Structure of Cuminaldehyde

Ellagic acid is a naturally occurring phenolic compound found in many natural sources such as blackberries, strawberries, raspberries, pomegranates. It has been reported that ellagic acid has a high affinity for copper at its active site of PPO and inhibits its activity (Shimogaki *et al.*, 2000).

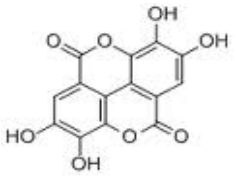

Fig.1.6 Structure of Ellagic Acid

PPO inhibitors find applications in both food and cosmetic industries. In the latter case, they are gaining importance because of their skin-whitening effects. However, as safety is of prime concern for an inhibitor to be used in food industry, there is a constant search for better inhibitors from natural sources as they are largely free of any harmful side effects.

1.3 EXTRACTION OF INHIBITORS:

Extraction of phytochemicals is essential prior to inhibition studies. Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. The solubility of phenolics in solvents is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used.

The toxicity of the solvents used for extraction can render it harmful for application purposes. An aqueous extract containing those solvents with high miscibility in water can be used for application after the removal of solvent by concentration or evaporation.

1.4 INHIBITOR COMBINATIONS:

Inhibitors act on PPO by different mechanisms. To improve the storage life of fresh-cut fruits and vegetables and their products, various methods have been adopted. One method is by using combination of inhibitors with different inhibition mechanisms. These inhibitors act synergistically to produce a cumulative effect with higher inhibition. An inhibitor combination of pomegranate extract rich in ellagic acid (a copper ion chelator) and wheat extract rich in arbutin (a competitive inhibitor of PPO) can prevent substrate binding and thus inhibit enzymatic browning.

1.5 APPLICATION OF INHIBITORS IN FRUITS AND VEGETABLES:

The wide use of chemical preservatives like sulphur dioxide (SO_2) and sodium benzoate in fruit juice preparations can trigger allergic reactions, asthma and skin rashes in sensitive individuals and may sometimes cause brain damage at high concentrations. This has called for the use of natural preservatives that are safe, inexpensive, easily available and free from regulatory constraints.

In the present study, fruits like apple and banana, vegetables like brinjal and potato and mushroom with high PPO activity were used for testing the inhibitory effectiveness of the pomegranate-wheat combination. The inhibitor can be applied as preservatives for fruit juices. A visual examination of the browning inhibition of fresh-cut slices of the selected fruits, vegetables and mushroom was also carried out.

1.6 OBJECTIVES:

The objectives of our work are:

1. To extract Polyphenol Oxidase from Banana peel and partially purify it.
2. To extract inhibitors from plant sources.
3. To study the inhibitory activities of Plant Extracts.

4. To compare the inhibitory effectiveness of the Plant Extracts in fresh-cut fruits, vegetables and their juices.

LITERATURE REVIEW

CHAPTER 2
REVIEW OF LITERATURE

2.1 POLYPHENOL OXIDASE (PPO):

2.1.1 General:

Polyphenol oxidase, also known as tyrosinase, phenolase, catechol oxidase, catecholase, o-diphenol oxidase, monophenol oxidase, and cresolase, was first discovered in mushrooms (Schoenbein, 1856). The enzyme can act on two general types of substrates, a monohydroxyphenol (such as p-cresol) to hydroxylate it in the o-position with respect to the original hydroxyl group [EC 1.14.18.1] (cresolase activity) and on o-dihydroxyphenols (such as catechol) oxidizing them by removal of the hydrogen of the hydroxyl groups, forming benzoquinones [EC 1.10.3.1] (catecholase activity) (John *et al.*, 2002). It is a bi-functional, copper containing enzyme referred to as an oxygen and 4 electron transferring phenol oxidase (Jolley *et al.*, 1974).

PPOs are widely distributed phylogenetically from bacteria to plants to mammals that catalyze the oxidation of phenolics to quinones which produce brown pigments in wounded tissues (Mayer, 1987; Mayer and Harel, 1979; Vaughn and Duke, 1984; Vaughn *et al.*, 1988; Sherman *et al.*, 1991).

2.1.2 Structure of PPO:

The crystal structure of one PPO in its active form, from *Ipomoea batatas* has been solved (Klabunde *et al.*, 1998). The crystal structure of a tyrosinase from *Streptomyces*, bound to a ‘‘caddie protein’’ has been resolved. This tyrosinase shows several features which differ from the plant catechol oxidase (Matoba *et al.*, 2006). The study of another bacterial tyrosinase, from *Ralstonia* has shown that possibly the unusually high ratio of hydroxylase/dopa oxidase activity

of this particular PPO was linked to the presence of a seventh histidine unit, binding Cu (Hernandez-Romero *et al.*, 2006). The importance of the histidine residues of a fungal PPO, the tyrosinase from *Aspergillus oryzyae*, expressed in *Escherichia coli*, has revealed the importance of a previously unrecorded histidine residue (Nakamura *et al.*, 2000). Site directed mutagenesis was used in the study of the enzyme. An active site structure in which the conserved domains CuA is linked to three histidine units and one cysteine, and CuB is liganded by four histidine residues was proposed. No fungal PPO has yet been crystallized, either in its active or its latent form. From the structural studies it is also apparent that PPOs do have distinct features and that not only the amino acid sequences of PPOs differ, but that there are also some differences even at the highly conserved active site (Mayer, 2006).

The amino acid sequence of a considerable number of PPOs, on plants, fungi and other organisms derived from cloning of the enzyme, has now been published (van Gelder *et al.*, 1997; Wichers *et al.*, 2003; Cho *et al.*, 2003; Marusek *et al.*, 2006; Halaouili *et al.*, 2006; Hernandez-Romero *et al.*, 2006; Nakamura *et al.*, 2000; Matoba *et al.*, 2006). Except for the active site, amino acid sequences show considerable variability.

2.1.3 Distribution and Expression of PPO:

The gene coding for PPO in the moss *Physcomitrella patens*, the properties of the enzyme, and changes in the expression of the gene during growth of the protonema of the moss in liquid culture has been reported (Richter *et al.*, 2005). In this moss, the single gene coding for PPO has an intron, absent in most plant PPO genes reported so far. However, in banana an intron is thought to be present (Gooding *et al.*, 2001), and banana tissues contain at least four distinct genes coding for PPO.

Differential, tissue specific, expression of six genes coding for PPO in potatoes has been reported by Thygesen *et al.* (1995), and for seven genes in different tissues of tomatoes (Thipyapong *et al.*, 1997). Other early contributions to this aspect are the observations that apple PPO is encoded by a multiple gene family, whose expression is up-regulated by wounding of the tissue (Boss *et al.*, 1994; Kim *et al.*, 2001). The sequences of PPO in any one species are highly conserved, but there is a lot of divergence in the sequences among different species (Thygesen *et*

al., 1995). Surprisingly, early work by Robinson and his co-workers indicate the presence of only a single PPO gene in grape vine (Dry and Robinson, 1994).

The response of expression of PPO to wounding has been shown in poplar (Constabel *et al.*, 2000), who also showed that methyl jasmonate induced expression of PPO genes (Constabel and Ryan, 1998). However, not all species respond to methyl jasmonate by induction of PPO. It is now well established that methyl jasmonate induces formation of other proteins involved in the defense response of plants (Constabel *et al.*, 1995; Howe, 2004).

The presence of PPO has been described in a variety of plants, some unusual or exotic. The PPO from the aerial roots of an orchid *Aranda* was found to be present in four iso-forms, which were partially characterized, including the N-terminal sequences of the iso-forms (Ho, 1999). Two distinct PPOs are present in leaves and seeds of coffee (Mazzafera and Robinson, 2000), in the parasitic plant *Cuscuta* (dodder) (Bar Nun and Mayer, 1999), and in Chinese cabbage (Nagai and Suzuki, 2001). The PPO present in red clover, which has an important role during ensiling of leaves, has been cloned and characterized. At least three PPO genes were detected, which had a high degree of identity, and which were differentially expressed in different parts of the plant (Sullivan *et al.*, 2004). One of these genes was successfully expressed in *E. coli*. The proteins encoded by these three genes all had sequences which predict that they would localize in chloroplast thylakoids.

2.1.4 Functions of PPO:

PPO has been implicated to function in the formation of pigment (Vaughn and Duke, 1984), oxygen scavenging and pseudocyclic phosphorylation in chloroplast (Trebst and Depka, 1995) and defence mechanism against insects and plant pathogens (Li and Steffens, 2002). Some PPOs have been implicated in biosynthetic pathways including the biosynthesis of yellow aurone pigments in snapdragon flowers (Nakayama *et al.*, 2001) and a specific lignin in creosote bush (Cho *et al.*, 2003).

2.1.5 Enzymatic Browning:

Phenolic compounds serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. The quinones formed by PPOs can bind plant proteins, reducing protein digestibility and their nutritive value to herbivores (Ryan, 2000). On the other hand, the oxidation of phenolic substrates by PPO is thought to be the major cause of the brown coloration of many fruits and vegetables during ripening, handling, storage and processing. This problem is of considerable importance to the food industry as it affects the nutritional quality and appearance, reduces the consumer's acceptability and therefore causes significant economic impact, both to food producers and to food processing industry (Nunez-Delicado *et al.*, 2005). It is estimated that over 50% of losses in fruits occur as a result of enzymatic browning and tropical and subtropical fruits and vegetables are the most susceptible to these reactions.

2.1.6. Plant PPO:

PPO has been regarded to be a critical enzyme in food technology and it has been intensively studied in several plants (Arogba, 2000; Qudsieh *et al.*, 2002; Spagna *et al.*, 2005; Kavrayan and Aydemir, 2001; Colak *et al.*, 2005). Among many other plants, PPO in banana showed a much higher activity (Matuschek and Svanberg, 2005).

It is known that plant PPOs are synthesized as preproteins and contain putative plastid transit peptides at the N-terminal region, which target the enzyme into chloroplast and thykaloid lumen (Marusek *et al.*, 2006). PPO from some plants has been described as a multiple gene family.

The mechanism of action proposed for PPO is based on its capacity to oxidize phenolic compounds. When the tissue is damaged, the rupture of plastids, the cellular compartment where PPO is located, leads to the enzyme coming into contact with the phenolic compounds released by rupture of the vacuole, the main storage organelle of these compounds (Mayer and Harel, 1979).

Plants PPOs have broad substrates specificities and are able to oxidize a variety of mono, di or polyphenols. Phenolic compounds are natural substances that contribute to the sensorial

properties (colour, taste, aroma and texture) associated with fruit quality (Es-Safi *et al.*, 2003). Structurally they contain an aromatic ring bearing one or more hydroxyl groups together with a number of other substituents. Some of PPO substrates occur naturally in fruits and vegetables, e.g., apples, very suitable to enzymatic browning, are rich in chlorogenic acid, catechin and epicatechin (Podsedek *et al.*, 2000).

2.1.7 Extraction of PPO:

Some scientists have reported the extraction of banana polyphenol oxidase using a buffer system containing no detergent (Padron *et al.*, 1975; Yang *et al.*, 2000; Unal, 2007). In contrast, some others applied an extraction medium containing an insoluble Poly vinyl pyrolidone (PVP) and Triton X-100 (Thomas and Janave, 1986; Jayaraman *et al.*, 1987; Ngalani *et al.*, 1993). Wuyts *et al.* (2006) reported the optimised amount of these substances in the extraction medium for PPO from *Musa acuminata* roots. Detergents increase the extractability of PPO while the removal of phenols in the presence of PVP is sufficient to avoid browning of the enzyme solution. However, it seems necessary to dialyze the extract against PBS since it improves the stability and activity of the enzyme (Fatemh *et al.*, 2008). This effect could be due to the renaturation of PPO in the presence of phosphate ions and the removal of inhibitory substances and detergents during dialysis (Galeazzi *et al.*, 1981).

2.1.8 Purification of PPO:

The banana pulp crude extract was subjected to a two-stage protein precipitation using ammonium sulphate to avoid the possible risk of denaturating effect of acetone (Thomas and Janave, 1986). Ammonium sulphate powder was added to the supernatant from the former step to make a 30% saturated solution. The resulting solution was stirred in an ice bucket for 30 min and then centrifuged for 30 min at 20000xg and 4°C. After removing the precipitate at this stage, the supernatant was saturated to 65% of ammonium sulphate. The solution was left stirring on ice for 2 h, followed by centrifugation at 20000xg and 4°C for 30 min. After discarding the supernatant, the resulting precipitate was dissolved in PBS and dialysed against similar PBS at 4°C overnight.

The dialysed protein solution was loaded onto and ion exchange (DE-52) column, 2.5x45 cm. The proteins were eluted from the column by a gradient of NaCl solution 0-200 mM, with a flow rate of approximately 1 mL min^{-1} controlled by a peristaltic pump. The output of the column was monitored spectrophotometrically at 280 nm. The enzymatic assays revealed the highest PPO activity in the fraction eluted by 150 mM NaCl while the other fractions were inert or showed low activity (Fatemh *et al.*, 2008).

2.1.9 Characterization of PPO:

The molecular weight of the enzyme extracted from banana peel (*Musa acuminata*) was estimated by gel filtration to be about 41000 Da (Chang-Peng *et al.*, 2001). This weight is almost the same as that of banana pulp PPO (Yang *et al.*, 2000), but is different from that of pulp PPO from the other banana species (*Musa cavendishii* L, var. Nanica) being estimated to be about 62000 Da by sucrose gradient ultra centrifugation (Galeazzi *et al.*, 1981). The difference in the molecular weight may be due to the different banana species used.

Comparison of substrate specificity of PPO purified from banana peel (*Musa* acuminata) revealed that the enzyme strongly oxidized dopamine. However, the oxidation rates of catechol, catechin, and chlorogenic acid by the peel PPO were below one-third that of dopamine. The enzyme had no activity toward resorcinol and phloroglucinol (Chang-Peng *et al.*, 2001). Similar substrate specificity was also found in banana pulp PPO (Griffiths, 1959; Palmer, 1963; Yang *et al.*, 2000). These results indicate that the purified banana peel PPO can be considered as a dopamine oxidase like the pulp PPO. Therefore, the substrate specificity for banana PPOs is different from those of apple (Murata *et al.*, 1992), Japanese pear (Tono *et al.*, 1986), eggplant (Fujita and Tono, 1988), and lettuce (Fujita *et al.*, 1991) PPOs; the latter oxidized chlorogenic acid markedly.

The Michaelis constant (K_m value) of peel PPO for dopamine oxidation was 3.9 mM (Chang-Peng *et al.*, 2001), which is similar to that of pulp PPO (2.7 mM, Yang *et al.*, 2000), but was higher than that for PPO from the inner part of banana pulp of the variety Nanica (0.17 mM, Galeazzi and Sgarbieri, 1981). Banana peel contains a large amount of dopamine (Riggin *et al.*, 1976; Tono *et al.*, 1999), and dopamine is strongly oxidized by peel PPO. These results

suggested that the browning of banana peel is mainly due to the enzymatic oxidation of dopamine.

The optimum pH of the banana peel PPO for dopamine oxidation was 6.5 (Chang-Peng *et al.*, 2001). The value is similar to that of PPOs in banana pulp (Yang *et al.*, 2000), kiwi fruit (Park and Luh, 1985), royal ann cherry (Benjamin and Montgomery, 1973), guava (Augustin *et al.*, 1985), and Satsuma mandarin (Fujita and Tono, 1979). However, it was different from the PPOs of Japanese pear (Tono *et al.*, 1986) and apple (Murata *et al.*, 1992).

The enzyme activity of banana peel PPO is quite stable in the range of pH 5-11 (Chang-Peng *et al.*, 2001). The pH stability is similar to those of banana pulp PPO (Yang *et al.*, 2000) and cabbage PPO (Fujita *et al.*, 1995; Fujita *et al.*, 1997), but is different from the PPOs of apple (Murata *et al.*, 1992) and eggplant (Fujita and Tono, 1988).

The optimum temperature of the PPO from banana peel was 30°C. The enzyme was relatively stable at high temperatures: about 90% of the activity remained after heat treatment at 60 °C for 30 min (Chang-Peng *et al.*, 2001). Similar high thermal stability has been found for PPOs in banana pulp (Yang *et al.*, 2000), kiwi fruit (Park and Luh, 1985), mango (Park *et al.*, 1980), lettuce (Fujita *et al.*, 1991), and cabbage (Fujita *et al.*, 1995; Fujita *et al.*, 1997).

2.2 INHIBITORS OF PPO:

For the past few decades, tyrosinase inhibitors have been a great concern solely due to the key role of tyrosinase in both mammalian melanogenesis and fruit or fungi enzymatic browning. Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, i.e., melanin. Melanin is formed by a combination of enzymatically catalyzed and chemical reactions. The biosynthetic pathway for melanin formation in various life forms has firstly been elucidated by Raper, (1928), Mason, (1948) and recently been modified by Cooksey *et al.*, (1997) and Schallreuter *et al.*, (2008).

Melanin plays an important role in protecting human skin from the harmful effects of UV radiation from the sun. Melanin also determines our phenotypic appearance. Although melanin

has mainly a photoprotective function in human skin, the accumulation of an abnormal amount of melanin in different specific parts of the skin resulting in more pigmented patches might become an aesthetic problem. In addition, enzymatic browning in fruit and fungi is undesirable in, for example, fresh fruits, beverages, vegetables, and mushrooms (Artes *et al.*, 1998). Browning after harvest is a common phenomenon in crops such as mushrooms, which decreases the commercial value of the products. Hyperpigmentation in human skin and enzymatic browning in fruits are not desirable. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in antibrowning of foods and skin whitening.

Te-Sheng, (2009) gives an updated review on tyrosinase inhibitors. 'True inhibitors' of tyrosinase are those specific inhibitors which can either inhibit tyrosinase activity by inducing the enzyme catalyzing "suicide reaction" or by reversibly binding to tyrosinase to reduce its catalytic capacity. These inhibitors are classified into four types, including competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/ uncompetitive) inhibitors and non-competitive inhibitors.

Inhibitor strength is usually expressed as the inhibitory IC_{50} value which is the concentration of an inhibitor needed to inhibit half of the enzyme activity in the tested condition. Kojic acid, the most intensively studied inhibitor of tyrosinase, is a fungal metabolite used as a cosmetic skin-whitening agent and as a food additive for preventing enzymatic browning (Chen *et al.*, 1991). The inhibitors are classified into five major classes including polyphenols, benzaldehyde and benzoate derivatives, long-chain lipids and steroids, other natural and synthetic inhibitors and irreversible inactivators based on either the chemical structures or the inhibitory mechanism.

2.2.1 Natural Inhibitors:

Polyphenols, the largest group in tyrosinase inhibitors, apart from providing protection against UV radiation, pathogens and herbivores (Harborne and Williams, 2000), are also responsible for the characteristic colour of fruits and vegetables. Many flavonols, a sub-group of polyphenols, have been isolated from plants and some were identified as tyrosinase inhibitors. In terms of

inhibitor strength, the flavonol inhibitors are ranked as quercetin > myricetin > kaempferol > galangin > morin (Xie *et al.*, 2003; Matsuda *et al.*, 1995).

Quercetin is a natural compound widely distributed in the plant kingdom (Aherne and O'Brien, 2002; Herrmann, 1976), occurring as various O-β-glycosides with D-glucose as the most common sugar residue (Herrmann, 1988). Fruit, vegetables and beverages are the major sources of quercetin in diet (Crozier *et al.*, 1997; Hertog *et al.*, 1993). Quercetin is a strong *in vitro* antioxidant with ability to chelate metals and scavenge free radicals, which in turn inhibits lipid peroxidation (Terao *et al.*, 1998; Afanas'ev *et al.*, 1989). It can also inhibit platelet aggregation (Pace-Asciak *et al.*, 1995) and/or broad spectrum of enzymes (Cos *et al.*, 1998) and has been demonstrated to have anti-inflammatory properties (Guardia *et al.*, 2001). Hollman *et al.*, (1997) showed that quercetin bioavailability from onions was 30% higher than that from apples or from pure rutinoside.

Citrus peel as a by-product of the citrus juice industry contains a large amount of flavonoids. Some of the flavonoids were identified as tyrosinase inhibitors, including nobiletin (Kenroh and Fumihiko, 2002), naringin, and neohesperidin (Zhang *et al.*, 2007). However, the inhibitory strength of the three inhibitors was found to be poorly active toward mushroom tyrosinase compared with kojic acid (Zhang *et al.*, 2007; Itoh *et al.*, 2009). Nobiletin has been reported to significantly lower blood pressure and reduce blood glucose levels in mice, as well as have inhibitory effects on production of oxygen radicals that are considered carcinogenic. Several studies have shown that nobiletin can reduce progression of colorectal cancer and cutaneous carcinoma in animal models (Kohno *et al.*, 2001; Murakami *et al.*, 2000a and b; Sato *et al.*, 2002).

Arbutin is a naturally occurring chemical found in the dried leaves of bearberry, blueberry, cranberry and pear trees. It is used as a stabilizer for colour photographic images. Therapeutically, it is used as an anti-infective for the urinary system as well as a diuretic (Budavari, 1996). The mechanism of action for arbutin is competitive inhibition of the substrate binding site of tyrosinase (Parvez *et al.*, 2006). Significant amounts of arbutin levels were found in wheat products (1-10 ppm [4-37 µmol/kg]), pears (4-15 ppm [0.01-0.055 mmol/kg]), and

coffee and tea (0.1 ppm [0.4 µM]) (Deisinger *et al.*, 1996). A PPO-inhibiting compound was found in the acid-methanolic extract of the durum variety of wheat bran which lowered PPO activity by three-fourfold (Moses *et al.*, 2001).

Ellagic acid is a naturally occurring phenolic compound found in many natural sources like strawberries, raspberries, blackberries and pomegranates. It has been found to have anticarcinogenic (Mukhtar *et al.*, 1986), antifibrosis (Thresiamma and Kuttan, 1996) and antioxidative properties (Osawa *et al.*, 1987). It has a high affinity for copper at the active site of tyrosinase and inhibits its activity (Shimogaki *et al.*, 2000). It has been reported that ellagic acid inhibited mushroom tyrosinase with an IC_{50} value of 182.2 µg/mL when compared to arbutin (162.2 µg/mL) and L-ascorbic acid (18.4 µg/mL), which are known tyrosinase inhibitors (Mineka *et al.*, 2005).

A large number of aldehydes and other derivatives have been isolated and characterized as mushroom tyrosinase inhibitors such as transcinnamaldehyde (Lee *et al.*, 2000), (2E)-alkenals (Kubo and Kinst-Hori, 1999a), 2-hydroxy-4-methoxybenzaldehyde (Kubo and Kinst-Hori, 1999b), anisaldehyde (Kubo and Kinst-Hori, 1998a), cuminaldehyde and cumic acid (Kubo and Kinst-Hori, 1998b), and 3, 4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid (Lee, 2002). The inhibitory effect is due to the formation of a Schiff base by the aldehyde group with the primary amino group of the enzyme. Comparison of the inhibitory activities of various aldehydes and closely related compounds such as cinnamic acid, anisic acid, cumic acid, and benzoic acid proved cuminaldehyde to be the strongest inhibitor (Kubo and Kinst-Hori, 1998b). The electron donating groups (isopropyl and methoxy) at the para position in cuminaldehyde provide stability to the Schiff base at the active site of the enzyme through inductive effect. Cuminaldehyde is the biologically active component of cumin seed oil.

2.2.2 Synthetic Inhibitors:

Chemical anti-browning agents have been commonly used to prevent browning of fruits and fruit products. The most widespread methodology used in the fruit industry for control of enzymatic browning is the addition of sulfiting agents. As a reducing agent, sulfites reduce the o-quinone produced by PPO catalysis to the less reactive diphenol, preventing the development of melanin.

Other non-sulfite anti-browning agents include ascorbic acid (Golan-Goldhirsh *et al.*, 1984), erythorbic acid (Borestein, 1965) as reducing agents and ethylenediamine tetraacetic acid (Mc Evily *et al.*, 1992) as a chelating agent.

2.3 EXTRACTION OF NATURAL PPO INHIBITORS:

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals prior to inhibition studies. Phenolics can be extracted from fresh, frozen or dried plant samples. Before extraction, plant samples are treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying. Generally, freeze-drying retains higher levels of phenolics content in plant samples than air-drying (Abascal *et al.*, 2005).

Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability. The yield of chemical extraction depends on the type of solvents with varying polarities, extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples. The solubility of phenolics is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used. Depending on the solvent system used during extraction, a mixture of phenolics soluble in the solvent will be extracted from plant materials.

Solvents, such as methanol, ethanol, acetone, ethyl acetate, hexane, butanol and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted (Xu and Chang, 2007). In particular, methanol has been found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone (Jin and Russell, 2010).

Tyrosinase inhibitors were screened from hexane, methanol, ethyl acetate, butanol and aqueous extracts of seashore plants. Ethyl acetate extract showed potent inhibitory activity. Two inhibitors were further purified and structurally determined which showed varying inhibitory activities depending on the substrate used (Toshiya *et al.*, 2005).

2.3.1 Toxicity of Solvents:

Methanol is a commonly employed organic solvent and reactant in organic synthetic procedures. It has long been recognised as a human intoxicant due to its role in causing blindness (ocular toxicity) after injestion of 10 mL of pure methanol and death at higher doses of 30 mL. Toxicity and lethality vary widely among individuals due to nutritional differences such as folate deficiency (Thomas, 1991).

Hexane is widely used as a cheap, relatively safe, largely unreactive and easily evaporated non-polar solvent. The acute toxicity of hexane is low. The long term toxicity can lead to extensive peripheral nervous system failure when exposed to levels of n-hexane ranging from 400 to 600 ppm, with occasional exposures up to 2,500 ppm. In severe cases, atrophy of the skeletal muscles is observed, along with loss of coordination and problems of vision (Hathaway *et al.*, 1991).

Ethyl acetate is a clear, colourless, flammable liquid with a pleasant, fruity odour. It is an irritant of eyes and upper respiratory tract at concentrations of above 400 ppm. It occasionally causes sensitization, with inflammation of the mucous membranes and eczema of the skin (Hathaway *et al.*, 1991).

Butanol is primarily used as a solvent. Like many alcohols, it is considered toxic. Brief, repeated overexposure with the skin can result in depression of the central nervous system. It may also cause severe eye irritation and moderate skin irritation (McCreery and Hunt, 1978).

2.3.2 Solubility of Solvents in Water:

When comparing the miscibility of methanol, hexane, ethyl acetate and butanol in water, methanol has 100% (w/w) solubility being highly polar, ethyl acetate and butanol with 8.7% (w/w) and 0.43% (w/w) respectively being moderately polar and hexane with 0.001% (w/w) being highly non-polar.

2.4 COMBINATIONS OF INHIBITORS:

Various approaches are being investigated to increase the shelf-life of fresh-cut fruits and vegetables. One method is by using combinations of different PPO inhibitors with different types of biochemical functions. The cumulative effect produced by the combination depends on the individual effect of the inhibitors. In most cases, the inhibition is higher when combinations are used. Pomegranate extract along with ascorbic acid was studied as agents to control enzymatic browning (Federico *et al.*, 2011). Arbutin is extensively used in cosmetic industries. A study on the effect of co treatment of arbutin and aloesin, a natural tyrosinase inhibitor from *Aloe vera*, revealed that both inhibit the enzyme in a synergistic manner by acting through different mechanisms; aloesin inhibits non-competitively whereas arbutin inhibits competitively (Jin *et al.*, 1999).

L-Ascorbic acid is often used in combination with other PPO inhibitors like kojic acid (Chen *et al.*, 1991b), anisaldehyde (Kubo and Kinst-Hori, 1998a), lemon juice combined with citric acid (Michel *et al.*, 2005), 4-hexylresorcinol (Arias *et al.*, 2007), oxyresveratol (Haitao *et al.*, 2007), sucrose-GSH derived Maillard reaction products (Jia-Jun *et al.*, 2007) and L(+) Lactic acid as a skin whitening agent(Walter, 1999). The selection of L-ascorbic acid is not only a well-known tyrosinase inhibitor (Ros *et al.*, 1993) but its effect on tyrosinase has been well studied because of its extensive use in food processing (Golan-Goldhirsh and Whitaker, 1984).

Another approach is by using browning inhibitors in combinations with modified (reduced oxygen) atmospheres and low-temperature storage (Friedman, 1996). Reducing agents such as ascorbic acid or sodium erythorbate (isoascorbate), often with the addition of calcium chloride, have been widely employed to prevent the browning of apple slices as when used in conjunction with vacuum infiltration, by which inhibition of browning was achieved for 7 days in 4°C storage (Sapers *et al.*, 1990). Combinations of 4-hexylresorcinol, ascorbic acid, and calcium chloride have been effective in the prevention of browning of apple slices stored for up to 8 weeks at 0.2°C with vacuum packaging (Luo and Barbosa-Canovas, 1996).

2.5 APPLICATION OF INHIBITORS IN FRUIT INDUSTRIES:

Browning in fruit and fruit products is a major problem in the fruit industry and is believed to be one of the main causes of quality loss during post-harvest handling and processing. Many studies have focussed on either inhibiting or preventing PPO activity in foods.

2.5.1 Physical Treatments:

Refrigeration (0-4°C) retards browning but during fruit juice processing, the cellular tissue is practically destroyed and low temperatures are not enough to control oxidation.

Steam treatment of cloudy apple juice at 65-70°C for 15-20 s was very effective not only in inactivating oxidative enzymes but also in stabilizing cloudiness (Genovese *et al.*, 1997). Thermal treatment at 65°C for 20 min of plum juice was effective in inhibition of enzymatic browning (Siddiq *et al.*, 1994).

Ultrafiltration (UF) has been shown to be effective in stabilizing the colour of fruit juices (Flores *et al.*, 1988).

High-pressure treatments may be related to reversible or irreversible changes in protein structure. The effects of high-pressure treatments up to 400 MPa combined with mild heat treatments up to 607°C on PPO in strawberry puree and orange juice was determined (Cano *et al.*, 1990).

2.5.2 Chemical Inhibition:

Anti-browning agents are compounds that either act primarily on the enzyme or react with the substrates and/or products of enzymatic catalysis in a manner that inhibits coloured product formation. The enzyme PPO can be inhibited by acids, halides, phenolic acids, chelating agents, sulphites, reducing agents such as ascorbic acid, quinone complexes such as cysteine and some other substrate-binding complexes.

The most widespread methodology used in fruit industry for control of enzymatic browning is the addition of sulfiting agents. Inactivation of PPO by application of sulphur

dioxide (SO_2) has been successful in preventing enzymatic browning, but its use was restricted by regulations due to its link to allergic reactions. Dosage is reasonably self-limiting, since too little is ineffective and too much gives an unpleasant, pungent aroma. Levels of 30 to 100 ppm, at a pH below 4.0 are effective. Caution is advised in use and labelling in foods if over 10 ppm is mandatory.

Other preservatives such as benzoic acid, sorbic acid and to a lesser extent, CO_2 can be used individually or synergistically. U.S. Federal regulations limit benzoates to 0.1%. Benzoates and sorbates are often used together in combination with low temperatures to extend the shelf life of minimally processed juice drinks (Somogyi *et al.*, 1996).

Sodium benzoate or benzoic acid appears to be safe for most people, though they cause hives, asthma or other allergic reactions in sensitive individuals. When used with ascorbic acid, the two substances can react together in acidic solutions to form small amounts of benzene, a chemical that causes leukaemia and other cancers. Sorbic acid is considered to be safe.

A preservative must be nontoxic, stable, compatible and inexpensive and have an acceptable taste, odour and colour. Chemical preservatives have to be used only at a dosage level which is needed for a normal preservation and not more. The use of preservatives must be strictly limited to those substances which are recognised as being without harmful effects on human beings' health and are accepted by national and international standards and legislation. This calls for use of natural preservatives with minimal side effects.

2.5.3 Natural Preservatives:

Salt, sugar, alcohol and vinegar are the traditional preservatives used in food that are used at home. A number of natural compounds such as ascorbic acid, thiol-containing amino acid, kojic acid, oxalic acid, 4-hexylresorcinol, oxyresveratrol, lincorice extract, and mulberry twig extract were reported to demonstrate sufficient inhibitory activities when added to fruit products (Gacche *et al.*, 2004; Jeon and Zhao, 2005; Nerya *et al.*, 2006; Oms-Oliu *et al.*, 2006; Rojas-Grau *et al.*, 2006; Haitao *et al.*, 2007).

Pineapple, papaya and lemon juices have been reported to prevent discolouration of cut surfaces of fruits and vegetables (Bennion, 1990). Pineapple juice contains a neutral compound

of low molecular weight that is an effective browning inhibitor in both fresh and dried apple (Patricia *et al.*, 1993).

Studies have been conducted using various other sources of anti-browning agents. The butanol and ethyl acetate extracts of *Rumex crispus* L. seed, a perennial wild plant which exists in Korea and other Asian countries, effectively inhibited browning in apple juice at a concentration below 0.3 mg mL^{-1}. This indicates that it can be used as anti-browning agents to reduce oxidative damage (Hwa-Jin *et al.*, 2011).

Extracts from vegetables too have been studies for its effectiveness as anti-browning agents. Rhubarb juice at concentrations above 20% inhibited browning of apple effectively. The inhibition effect is due to oxalic acid and analysis of oxalic acid in rhubarb juice showed that 20% solution contained about 67 mg/100 g. It is a very effective natural anti-browning agent for fresh-cut fruits and vegetables and has a strong potential for commercial use (Son *et al.*, 2000).

Anti-browning agents those are easily obtainable, inexpensive, safe, natural and free of regulatory constraints can be used to increase the storage life of fruits, vegetables and their products.

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS:

3.1.1 Plant Materials:

Fresh banana fruits, Orange, Pomegranate, Cumin seeds, Wheat Flour, Onion, Apple, Brinjal, Potato and Mushroom were purchased from the local market.

3.1.2 Chemicals:

All chemicals used-Sodium dihydrogen phosphate, Disodium hydrogen phosphate, Poly vinyl pyrolidone (PVP), Triton X-100, Ammonium Sulphate, L-Dihydroxy phenyl alanine (L-DOPA)-were of analytical grade. They were purchased from HIMEDIA Laboratories Pvt. Ltd, Mumbai and RFCL Limited, New Delhi. The solvents used for extraction-Methanol, n-butyl alcohol, n-hexane, Ethyl acetate-were purchased from RFCL Limited, New Delhi.

3.1.3 Instruments:

pH Meter from (Make: ELICO, India, Model: L1120) was used to measure the pH of the buffer. Absorbance was measured using Spectrophotometer (Make: Systronics, India, Model: 106). The extracted sample was concentrated using a Flash evaporator (Make: Heidolph, Germany, Model: 591-00100-00-0).

3.2 METHODOLOGY:

3.2.1 Enzyme Isolation:

1. Banana peel was homogenised by using an extraction buffer containing 0.2M Sodium Phosphate buffer (pH 6.5), 1 part(s) of Poly vinyl pyrolidone (PVP) and 6% Triton X-100 in the ratio of 1:10.
2. The obtained crude extract was filtered through a cheese cloth.

3. The homogenate was incubated at 4°C for 10 min followed by centrifugation at 10,000 rpm for 30 min.
4. To the supernatant obtained, 4% Triton X-100 was added and was incubated at 4°C for 15 min and then at room temperature for 10 min.
5. The extract was centrifuged at 10,000 rpm for 10 min.
6. The pellet was discarded and the supernatant was subjected to fractionation.

3.2.2 Partial Purification of Enzyme:

1. The enzyme solution was fractionated by using solid Ammonium sulphate.
2. After addition of ammonium sulphate salt, the extract is centrifuged at 10,000 rpm for 10 min.
3. The pellet obtained was suspended in a minimum volume of 0.2M Sodium Phosphate buffer, pH 6.5.
4. The enzyme is kept for dialysis overnight. The enzyme solution thus obtained was used for enzyme assay and inhibition assay.

3.2.3 Enzyme Assay:

The assay solution was prepared by mixing 1 mL of 20 mM substrate (L-DOPA), 1 mL 0.2M sodium phosphate buffer, 0.9 mL double distilled H_2O and 0.1 mL of enzyme solution. Absorbance was measured spectrophotometrically at 475 nm against a blank containing no enzyme.

3.2.4 Inhibition Assay:

The assay solution was prepared by mixing 1 mL of 20 mM substrate (L-DOPA), 1 mL 0.2M sodium phosphate buffer, 0.8 mL double distilled H_2O, 0.1 mL inhibitor and 0.1 mL of enzyme solution. Enzyme and Inhibitor are subjected to 2 min incubation at room temperature before the preparation of the assay solution. Absorbance was measured spectrophotometrically at 475 nm against the blank containing no inhibitor for both test and the duplicate.

3.2.5 Extraction of PPO Inhibitors from Natural Sources:

1. 500 g of dry, powdered sample was soaked in 1000 mL of methanol for two days.

2. The sample was filtered to obtain the methanol extract.
3. The extract was concentrated to 100 mL using a Flash Evaporator.
4. The concentrated sample was partitioned with 100 mL n-hexane to obtain a methanol layer and hexane layer.
5. The hexane layer was further extracted with methanol-water (9:1, 50 mL).
6. The obtained methanol layer (**Methanol-1 Extract**) and hexane layer (**Hexane Extract**) was concentrated to 10 mL each.
7. The first partitioned methanol solution was concentrated to 50 mL and suspended in 50 mL double distilled water.
8. This suspension was extracted twice with ethyl acetate (50 mL each) to give ethyl acetate layer (**Ethyl acetate Extract**) and methanol layer (**Methanol-2 Extract**). Both the extracts were concentrated to 10 mL each.
9. The ethyl acetate layer was next extracted with 50 mL water-saturated butanol and 100 mL of double distilled water.
10. The solution separates into an aqueous layer (**Aqueous Extract**) and a butanol layer (**Butanol Extract**) both of which were further concentrated to 10 mL each.
11. The six extracts obtained for each natural source was used to carry out inhibition assays against a blank containing no enzyme.

3.2.6 Preparation of Polar Extracts:

10 mL each of Methanol-1, Methanol-2 and aqueous extracts was concentrated to 10 mL at 60°C in a water bath. The obtained aqueous extracts were measured for its inhibition effectiveness. These extracts were also used to carry out studies using combination of inhibitors.

3.2.7 Inhibition Assay using Combination of Inhibitors:

Inhibition assay was carried out as per 3.2.4 using varying volumes of inhibitors.

3.2.8 Application of Inhibitor Combination in Juices:

1. 0.5 g of the fruit or vegetable sample was homogenised both in the presence and absence of inhibitor using 10 mL of 0.2M Sodium Phosphate buffer (pH 6.5).
2. The homogenised samples were filtered through a cheese cloth.

3. The sample without inhibitor was taken as the control and that with inhibitor was taken as the test sample.
4. The absorbance of the control was measured spectrophotometrically at 475 nm against blank containing distilled water and that of the test sample was measured against the control.

RESULTS AND DISCUSSION

CHAPTER 4
RESULTS AND DISCUSSION

Table 4.1 Percentage Inhibition of Sample Extracts

S. No	Solvent Extracts	Percentage Inhibition of Sample Extracts (%)				
		Cumin	Onion	Orange	Pomegranate	Wheat
1	Methanol-1	20	15	**38**	30	18
2	Hexane	-	-	-	-	-
3	Ethyl Acetate	**39**	47	19	2	9
4	Methanol-2	15	**38**	37	**59**	6
5	Aqueous	8	24	7	26	**48**
6	Butanol	5	9	-	-	11

The inhibitory activity of Cumin was found to be higher in Ethyl acetate extract, Onion and Pomegranate in Methanol-2 extract, Orange in Methanol-1 extract and Wheat in Aqueous extract. Almost negligible or no inhibitory activity was found in the Hexane and Butanol extracts of all the samples.

Table 4.2 Percentage Inhibition of Polar Extracts

S. No	Sample	Percentage Inhibition (%)
1	Cumin	33
2	Onion	**76**
3	Orange	69
4	Pomegranate	**76**
5	Wheat	75

Polar extracts of Pomegranate and Onion showed maximum inhibition by 76%. Those of Wheat and Orange inhibited PPO by about an average of 73%. Among all the extracts, cumin showed the least inhibitory activity of 33%.

Table 4.3 Percentage Inhibition of Combinations of Polar Extracts

S. No	Combinations of Polar Extracts	Percentage Inhibition (%)
1	Cumin + Onion	56
2	Cumin + Orange	60
3	Cumin + Pomegranate	**94**
4	Cumin + Wheat	49
5	Onion + Orange	70
6	Onion + Pomegranate	76
7	Onion + Wheat	72
8	Orange + Pomegranate	**86**
9	Orange + Wheat	71
10	Pomegranate + Wheat	**81**

The combinations of pomegranate with other samples exhibited highest inhibitory activity. The combination of pomegranate with both cumin and orange were not used for application purposes because of it odour. The combination of pomegranate and wheat was selected for further studies.

Table 4.4 Percentage Inhibition of Varying Volumes of Pomegranate and Wheat

S. No	Pomegranate + Wheat (mL)	Percentage Inhibition (%)
1	0.0 + 0.1	75
2	0.01 + 0.09	70
3	0.02 + 0.08	80
4	0.03 + 0.07	83
5	0.04 + 0.06	80
6	0.05 + 0.05	**92**
7	0.06 + 0.04	**92**
8	0.07 + 0.03	80
9	0.08 +0.02	82
10	0.09 + 0.01	88
11	0.1 + 0.0	76

Inhibitory activities of all the probable combinations with varying volumes (a total of 0.1 mL) were measured. The combinations in the ratio of 1:1 and 3:2 inhibited PPO by about 92%.

Table 4.5 Percentage Inhibition of Inhibitor Combination in Juices

S. No	Juice Sample	Percentage Inhibition (%)		
		Pomegranate-Wheat	Citric Acid	SHAM
1	Apple	54	45	64
2	Banana Pulp	91	45	47
3	Brinjal	54	70	68
4	Mushroom	82	60	56
5	Potato	50	48	67

The inhibitory value of Pomegranate-Wheat combination taken in a ratio of 1:1 was measured using the homogenised solutions of apple, banana pulp, brinjal, mushroom and potato. PPO was effectively inhibited by 85% in banana pulp and mushroom while for apple, brinjal and potato, the inhibitory value was only 50%.

Inhibition studies were carried out for the same sample using two specific inhibitors of PPO at their IC_{50} values-Citric Acid (IC_{50} at 21 mM) and Salicylhydroxamic Acid (SHAM) (IC_{50} at 16 µM). The former showed an inhibition of 40-60% for all the samples while the latter had a higher inhibitory activity of 65%.

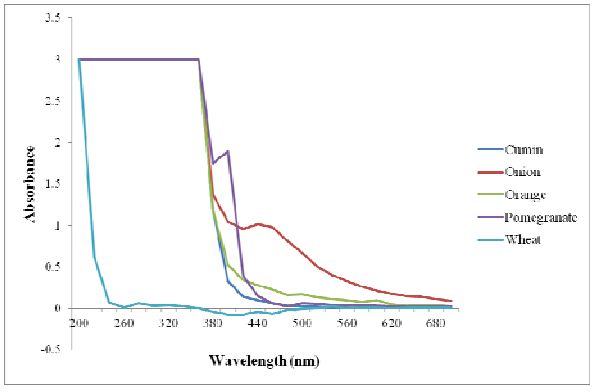

Fig.4.1 Absorption Spectrum of Aqueous Extracts of Inhibitor Samples

The absorption spectrum of polar extracts were measured from 200-700 nm. Maximum absorption lies in the range between 200-380 nm which indicates the presence of aromatic compounds in the extracts and the absence of aliphatic compounds. These aromatic compounds might be responsible for the inhibitory actiivty of PPO.

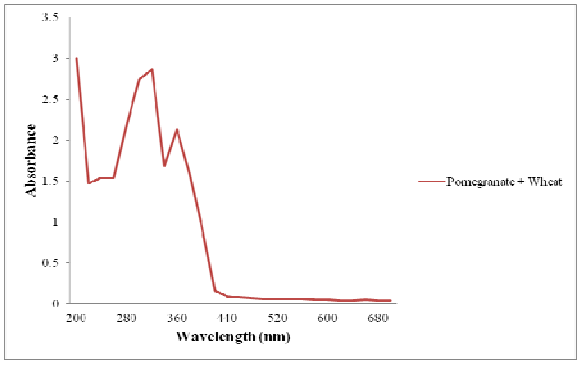

Fig.4.2 Absorption Spectrum of Pomegranate-Wheat Combination

The absorption spectrum for Pomegranate-Wheat combination is maximum at 320 nm. At the absorption maxima of L-DOPA, 475 nm, the spectrum shows a low absorbance value. The absorbance value measured during the reaction between inhibitor and enzyme in the presence of L-DOPA, can be thought to be due to the brown colored pigment, melanin.

A visual examination of application of Pomegranate-Wheat combination on fresh-cut fruits and vegetables were carried out over a period of 2 h. No inhibitor was added to the control while to the test sample a 30 times diluted solution of the inhibitor was added.

Fig.4.3 Inhibitory Effectiveness on Apple

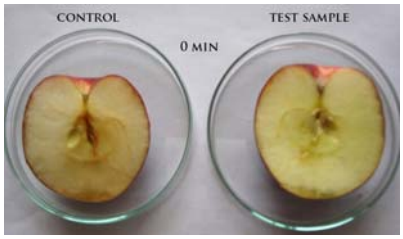

Fig.4.3(a) Inhibitory Activity on Apple at 0 min

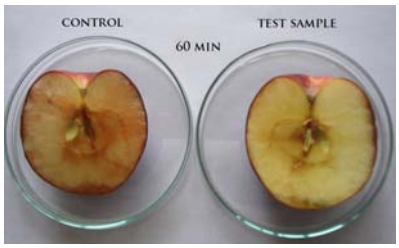

Fig.4.3(b) Inhibitory Activity on Apple at 60 min

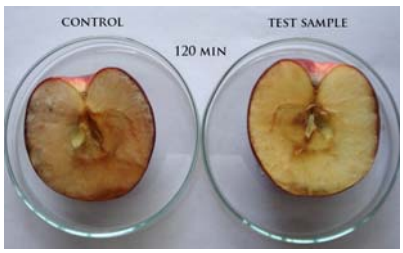

Fig.4.3(c) Inhibitory Activity on Apple at 120 min

Fig.4.4 Inhibitory Effectiveness on Banana Pulp

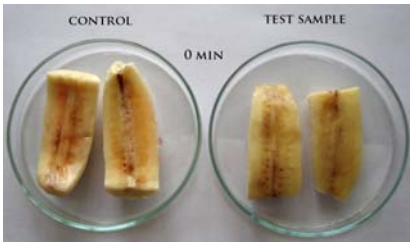

Fig.4.4(a) Inhibitory Activity on Banana Pulp at 0 min

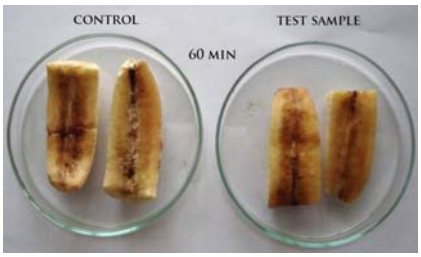

Fig.4.4(b) Inhibitory Activity on Banana Pulp at 60 min

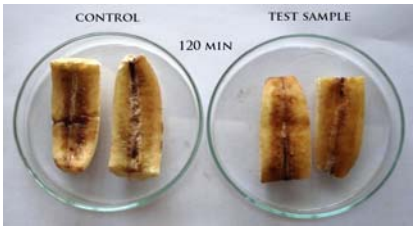

Fig.4.4(c) Inhibitory Activity on Banana Pulp at 120 min

Fig.4.5 Inhibitory Effectiveness on Brinjal

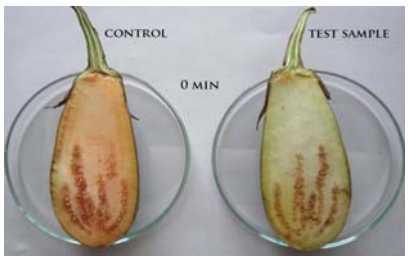

Fig.4.5(a) Inhibitory Activity on Brinjal at 0 min

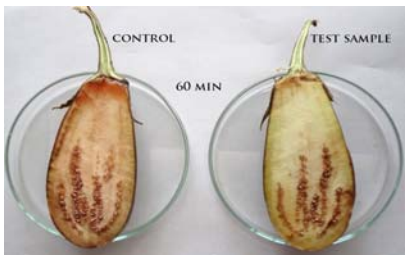

Fig.4.5(b) Inhibitory Activity on Brinjal at 60 min

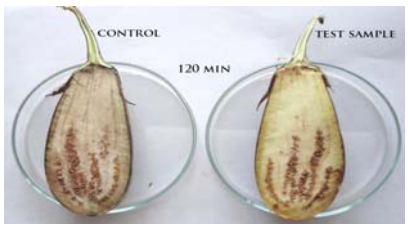

Fig.4.5(c) Inhibitory Activity on Brinjal at 120 min

Fig.4.6 Inhibitory Effectiveness on Mushroom

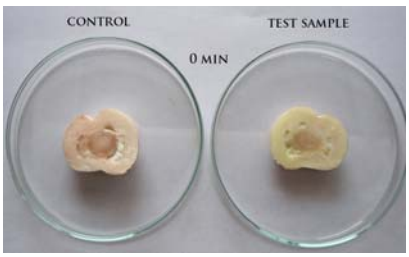

Fig.4.6(a) Inhibitory Activity on Mushroom at 0 min

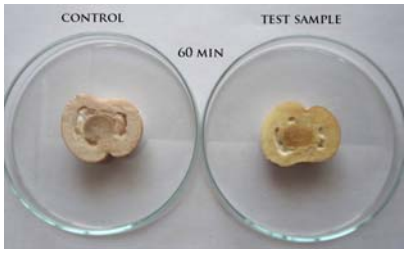

Fig.4.6(b) Inhibitory Activity on Mushroom at 60 min

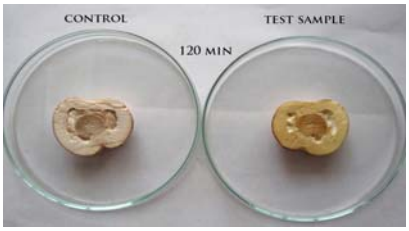

Fig.4.6(c) Inhibitory Activity on Mushroom at 120 min

Fig.4.7 Inhibitory Effectiveness on Potato

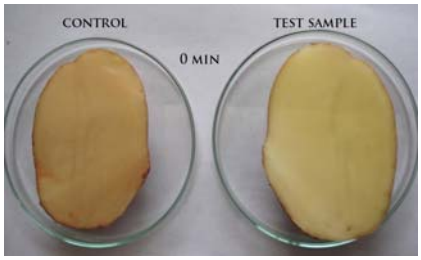

Fig.4.7(a) Inhibitory Activity on Potato at 0 min

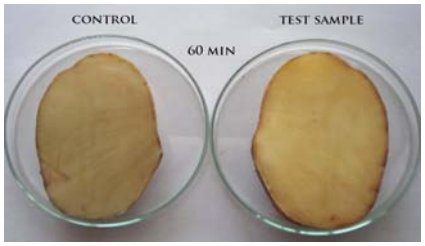

Fig.4.7(b) Inhibitory Activity on Potato at 60 min

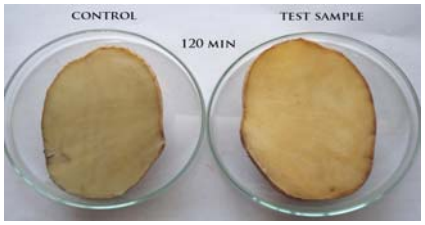

Fig.4.7(c) Inhibitory Activity on Potato at 120 min

The inhibitor was found to be more effective against PPO of brinjal and potato as browning did not occur over the observation period of 2 hours at room temperature. In apple, browning was prevented for 1 h when observed at room temperature exposed to air. The inhibitor was found to less effective against PPO of banana pulp and mushroom since enzymatic browning was inhibited for 30 min at room temperature. This can be because of the high enzymatic activity of PPO in banana pulp and mushroom.

CONCLUSION

CHAPTER 5
CONCLUSION

Polyphenol oxidase (PPO) extracted and partially purified from Banana peel was measured for its enzyme activity spectrophotometrically at 475nm. PPO inhibitors from various plant sources were extracted using solvents and the percentage inhibition of each sample extracts were determined. Polar extracts prepared from methanol and aqueous extracts were measured for their inhibitory activity. Maximum inhibition was found in pomegranate and onion extracts. A combination of polar extracts of PPO inhibitors revealed highest percentage inhibition in combinations of pomegranate-cumin, pomegranate-orange and pomegranate-wheat. The former two combinations were not selected for further studies because of their odour. Further, pomegranate – wheat combination was found to be more effective in the ratio 1:1. Absorption spectrum analysis of all the polar extracts revealed that the inhibitory activity was due to the presence of aromatic compounds. The inhibitor combination was tested for its effectiveness in fresh-cut fruits, vegetables and their juices. The percentage inhibition was almost 91% for banana juice and 80% for mushroom. Comparing the obtained results with those obtained using specific PPO inhibitors-Citric acid and Salicylhydroxamic Acid (SHAM)-the inhibitor combination was found to be more effective. A visual examination of inhibitor effectiveness revealed that the pomegranate-wheat combination could inhibit PPO in brinjal and potato for up to 2 h at room temperature. However, for banana and mushroom, browning reaction was observed at 30 min. This can be due to the high enzymatic activity of PPO in these sources. Thus, it can be concluded that the pomegranate-wheat inhibitor combination against PPO can be effectively used for commercial applications in the preparation of fruit juices to prevent the browning reaction. This plant extract is inexpensive and easily available. Therefore it can be further studied for application in food processing industry.

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