EVALUATION OF ANTIOXIDANT POTENTIAL IN MIXED FRUIT PEELS

A PROJECT REPORT

Submitted by

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ABSTRACT

Free radicals are produced in the body either naturally or on the exposure of radiation, cigarette smoke, etc., and can be implicated in many diseases such as cancer, atherosclerosis, arthritis, Parkinson's disease, Alzheimer's disease, aging and other age-related diseases. Antioxidants are well known to be playing a role in reducing the risk of succumbing to early diseases, such as heart disease, diabetes, arthritis and some types of cancers. Although, the body produces antioxidants of its own in its defense against free radical damage, it seems to benefit from the extra antioxidants provided in the diet, especially from whole grains, fruits and vegetables. Peels which are usually to be disposed causes a lot of environmental pollution which can be avoided by the usage of peels for a purpose. Peels also have some amount of antioxidants which can be utilized. The project aims to evaluate antioxidant potential from Sweet Lime, Papaya and Avocado. The peels were taken as both individual and combination for synergistic effect. These fruit peel extracts were subjected to *in vitro* free radical scavenging assays like DPPH scavenging assay, ABTS cation radical scavenging assay and total antioxidant capacity assay. Lipid peroxidation assay using goat liver was also carried out. Reducing power assay including FRAP was performed. The results were analyzed statistically, and found that avocado and those combinations with avocado showed greater antioxidant activity compared to other fruit peel extracts.

Keywords: Free radicals, Antioxidant, Synergistic effect, In vitro, Scavenging, Peroxidation.

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre

µm	Micrometer
TAC	Total Antioxidant Capacity
FRAP	Ferric Reducing Antioxidant Potential
DPPH	1, 1 – Diphenyl – 2 – Picryl – Hydrazyl
ABTS	2,2-Azobis (3-ethylbenzothiazoline-6-sulfonic acid)
LPO	Lipid Peroxidation
$FeCl_3$	Ferric Chloride
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric acid
O_2	Oxygen
NO	Nitric Oxide
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Millilitre
NaOH	Sodium Hydroxide

ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
ANOVA	Analysis Of Variance
$^{\cdot}OH$	Hydroxyl Radical

INTRODUCTION

CHAPTER 1

INTRODUCTION

It is ironic that oxygen, an element indispensable for life can, under certain situations, have severely damaging effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and activity of a number of chemical compounds, known as Reactive Oxygen Species (ROS), which have a tendency to donate oxygen to other substances. Many such reactive species are free radicals and have a surplus of one or more free-floating electrons rather than having matched pairs and are, therefore, unstable and highly reactive.

Free radicals and other reactive oxygen species are derived either from normal primary metabolic processes in the human body or from external sources such as exposure to X-rays, cigarette smoke, air pollutants and industrial chemicals. Some of the free radicals include the hydroxyl radical (OH), the superoxide radical (O_2), the nitric oxide radical (NO) and the lipid peroxyl radical (LPO).

Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions which serve as sources of free radicals include those involved in the respiratory chain, in phagocytosis and in the cytochrome P450 system. Free radicals also arise in non-enzymatic reactions of oxygen with organic compounds and also with those initiated by ionizing radiations.

Internally generated sources of free radicals are:

- mitochondria
- phagocytes
- xanthine oxidase
- reactions involving iron and other transition metals
- peroxisomes

- exercise
- inflammation

Externally generated sources of free radicals are:

- cigarette smoke
- environmental pollutants
- radiation
- ultraviolet light
- pesticides
- anaesthetics
- Industrial solvents and
- Certain drugs.

With electrons unhinged, free radicals will be free inside the body, causing infliction. The free radical, in an effort to attain stability, attacks the nearby molecules to obtain another electron. During this process, it damages those molecules. If free radicals are not inactivated, their chemical reactivity can damage all cellular macromolecules including proteins, carbohydrates, lipids and nucleic acids.

The destructive effects on proteins may play a role in the causation of cataracts. Free radical damage to DNA is also implicated in the causation of cancer and its effect on LDL cholesterol is responsible for heart diseases. Cancer and atherosclerosis, two major causes of death, are salient "free radical" mediated diseases. Cancer initiation and promotion is associated with chromosomal defects and oncogene activation. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, most commonly result in tumor formation (Subhasree *et al.*, 2009). The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries and rectum among persons over 55 years may be a reflection of greater lipid peroxidation.

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments.

One important line of defense is a system of enzymes, including glutathione peroxidases, superoxide dismutases and catalase, which decrease concentrations of the most harmful oxidants in the tissues. Several important minerals including selenium, copper, manganese and zinc are necessary for the formation or activity of these enzymes. Hence, if the nutritional supply of these minerals is inadequate, enzymatic defenses against those free radicals may be impaired.

The second line of defense against free radical damage is the presence of antioxidants. An antioxidant is a molecule, stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Such antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body. Other lighter antioxidants are found in the diet. Although about 4000 antioxidants have been identified, the best known are vitamin E, vitamin C and the carotenoids. Many other non-nutrient food substances, generally phenolic or polyphenolic compounds, display antioxidant properties and, thus, may be important for health.

A wide variety of antioxidants in foods contribute to disease prevention. The antioxidant nutrients in these peels have specific activities and they often work synergistically to enhance the overall antioxidant capability of the body. The balance between the production of free radicals and the antioxidant defenses in the body has important health implications. If there are too many free radicals produced and too few antioxidants, a condition of "oxidative stress" develops which may cause chronic damage.

Antioxidants are present in most fruits and vegetables. The major antioxidants from a fruit or a vegetable may be from compounds such as Vitamin C, Vitamin E or β carotene. Foods rich in antioxidant phytochemicals are important for the prevention of diseases related to oxidative stress such as heart diseases and cancer (Riyaz *et al.*, 2010).

Papaya is a popular fruit, loaded with nutrition and effective in preventing and treating a number of health issues. It has a number of functions and health benefits, and owes its efficacy to its nutrient rich content. Most varieties turn yellow to orange when ripe, while some remain green. Papaya is a rich source of antioxidants, especially β carotene, which is a form of Vitamin A. It has the second highest content of β carotene among fruits, and derives its orange color from it. A raw papaya is very rich in Vitamin C. Papaya has a calorific value

of 37 to 42 calories per 100 g; carbohydrate-7.5% ; protein-1% ; dietary fibre-4.5% ; vitamin C-103% ; 0% cholesterol ; β carotene-276 mcg ; calcium-2.5% ; iron-1% ; niacin-2% and folates-9.5%.

Figure 1.1: Papaya fruit (*Carica papaya*)

Figure 1.2: Papaya peel

Sweet lime is a citrus fruit variety which is most common in Asian countries and has a high content of Vitamin C. It also has a high amount of potassium among most fruits. For every 100g, this fruit contains 11g of carbohydrates; 3g of dietary fibre; 0.7g of protein; 88g of water; 35% of ascorbic acid. This citrus fruit has a variety of health benefits. The peel of the sweet lime may provide a natural treatment for jaundice.

Figure 1.3: Sweet lime fruit **Figure 1.4: Sweet lime peel**

With their flavor, texture, nutritional value and culinary versatility, avocados might be one of nature's highest achievements (Nicoletta *et al.*, 2003). Avocados provide nearly 20 essential nutrients, including fiber, potassium, Vitamin E, B-vitamins and folic acid. They also act as a "nutrient booster" by enabling the body to absorb more fat-soluble nutrients, such as α and β-carotene and lutein, in foods that are eaten with the fruit. This fruit contains 0.75% of polyunsaturated fat; 0% cholesterol; 4% of potassium; 8% of dietary fibre; ascorbic acid-4%; iron-2%; riboflavin-4%; niacin-4%; folate-6%; phosphorus-2%; magnesium-2% (Yuuko *et al.*, 2010). The high potassium content helps in regulating blood pressure and prevents circulatory diseases**.** The mono saturated fats in avocados not only help in reducing LDL cholesterol, but are also good for the heart. Avocado also lowers the triglycerides in the blood and thus is useful for diabetic patients. It is a powerhouse of vitamin E which protects our skin from free radicals.

Figure 1.5: Avocado fruit (*Persia americana*)

Figure 1.6: Avocado fruit peel

Fruit peel is the outer skin or the covering of fruits. In general, the peel in some thick coated fruits like pomegranate, passion fruits, etc,., is known as rind, where as in citrus category fruits like in oranges, sweet lime, it is better termed as zest. While the outer cover protects the fruit from environment, micro and macro organisms, it indeed has several phyto-nutrients which help keep up good health. Peel of some of the fruits like berries, grapes, guava, and avocado contain more antioxidants such as anthocyanin pigments, tannins, catechins, than in the pulp or flesh. Blue or purple color fruit peels are rich in anthocyanidin glycosides while yellow color fruits have xanthin, carotenes and lutein pigments. Major component of these pigments are present just underneath the skin.

Peel is rich source of dietary fiber also known as NSP (non soluble polysaccharides) like hemi-cellulose, pectin, tannins, gum. These compounds increase bulk of the food and helps prevent constipation by reducing gastro-intestinal transit time. They also bind to toxins in the food which helps to protect the mucus membrane of gut and thus cuts colon cancer risk. Furthermore, dietary fibers bind to bile salts (produced from cholesterol) and decrease their re-absorption, thus help lower serum LDL cholesterol levels. Peel is low in calories, sugar, and fats; and is from cholesterol. It adds to the bulk of the food and helps cut down overall food intake. Nevertheless, the peel of some fruits contains considerable amounts of mineral and vitamins, especially in guava and citrus fruits. Certain fruits peel like in orange contains more vitamin C (ascorbic acid) than its juice. The peel provides 136 mg per 100 g of

vitamin C whereas the same in its pulp is just about 71 mg. Likewise the peel is rich source of vitamin A, B-complex vitamins, minerals such as calcium, selenium, manganese, zinc, etc,.

This project work has focused on the evaluation of antioxidants that are present in three different fruit peels namely Sweet lime, Papaya and Avocado both individually as well as in combinations (dual and triple) to assess the synergistic effect on the antioxidants of these fruit peels.

1.1 OBJECTIVES

- Evaluation and comparison of antioxidant potential in individual fruit peels.
- Evaluation of comparison of antioxidant potential in mixed fruit peels (dual and triplet combinations).

LITERATURE REVIEW

CHAPTER 2
LITERATURE REVIEW

2.1 Free Radicals

A free radical is an oxygen molecule with more or less than its normal number of electrons. This imbalance makes the atom or molecule unstable, reactive, and destructive.

Some free radical activity is normal. White blood cells use peroxide (H_2O_2) to kill ingested bacteria, viruses, and other foreign invaders. Too much free radical activity, however, is harmful. Cell damage and cell death occur when energy production (ATP) is reduced and excess peroxide and hydroxyl (OH) radicals are formed.

2.2 Antioxidants as Free Radical Scavengers

Certain vitamins, minerals, enzymes, and amino acids help keep excessive free radical reactions in check. These are called free radical scavengers or Antioxidants. Some of the best free radical scavengers are:

- Vitamins: A, B_3, B_5, B_6, C Complex, and E Complex
- Minerals: Germanium, Manganese, Selenium, and Zinc
- Amino Acids: L-Cysteine, DMG (N,N-Dimethylglycine), and GSH (as Glutathione Peroxidase and Reduced Glutathione), L-Methionine, and L-Taurine
- Enzymes: Catalase, Coenzyme-Q-10 (Co-Q-10), Sorbic Acid, and Superoxide Dismutase (SOD)

One of the most bioactive natural antioxidants is a product called Pycnogenol. It is about 50 times stronger than Vitamin E and about 20 times more potent than Vitamin C as an antioxidant. One of the richest sources for this proanthocyanidin (procyanidin) is the French maritime pine tree (needles, primarily, and bark). Pycnogenol contains about 85% proanthocyanidins by weight. Red wines, grapes, other foods and herbs also contain lesser levels of proanthocyanidins.

Elemental oxygen exists in two allotropic forms: as an invisible gas composed of two oxygen atoms; and as a perceptibly blue form composed of three oxygen atoms that we call ozone. Unlike nitrogen or carbon dioxide, oxygen is paramagnetic. This paramagnetic property is explained as two electrons in the molecule that are not paired to each other.

These “free unpaired” electrons can interact with a variety of packaging materials and food products to create “unpaired electron fragments,” which we call “free radicals.” The usual path is through hydrogen abstraction and formation of peroxides, which can decompose to free radicals. The presence of peroxides further degrades packaging materials and food products.

Most of us are aware our aging process can be accelerated by oxygen, and doctors recommend we consume food rich in antioxidants, such as fruit, fresh produce, wines, and vitamins. Nature does a good job in controlling the degradation process by maintaining high levels of antioxidants.

A major role of food packaging is to retard the natural processes that lead to food spoilage by reducing oxygen and moisture. Antioxidants and free radical scavengers are used for this purpose.

Among the oxygen reduction advances in packaging have been the introduction of PVDC-coated films, incorporation of PVOH as an oxygen barrier layer, and the use of vacuum-deposited aluminium to reduce oxygen penetration to packaging products. Also, vacuum packaging and use of inert atmosphere significantly extend the shelf life of many food products.

Antioxidants exist in many natural products. For instance, beta-carotene, vitamins C and E, and polyphenols are all potent antioxidants. Red beans, raisins, and blueberries have some of the highest levels of antioxidant activity. However, most antioxidants added to packaging materials are synthetic materials.

Antioxidants used in packaging materials are a diverse group of chemicals that combine with free radicals that would otherwise attack and oxidize packaging materials. Common antioxidant synthetic substances used in packaging are butylated hydroxytoluene [BHT] and

butylated hydroxyanisole [BHA]. As a chemical class, these materials are phenolic chemicals that can react with peroxide radicals by hydrogen donation to form hydroperoxides and prevent the process from forming more reactive radicals.

Oxygen scavengers include inorganic materials that can absorb oxygen, as well as organic reactive materials that can consume oxygen through chemical reaction.

Free radical scavengers can include hydroquinones, thiols, hydroxylamines, plus many other materials that will trap free radicals by chemical reaction and prevent the normal degradation process.

Fruits are packed with powerful antioxidants that can lower the risk of heart disease, diabetes-related damage and even slow down the body’s natural aging process. Epidemiological evidence links high intake of ascorbic acid (AA) and other antioxidant micronutrients to health promotion. It would be useful to know the over all, or 'total' antioxidant capacity of foods, to establish the contribution of AA to this, and to assess how this information may translate into dietary intakes to meet the new US daily reference intake for AA. In this study, the total antioxidant capacity, as the ferric reducing-antioxidant power (FRAP) value, and DPPH free radical scavenging assay was analyzed.

The total antioxidant activities of 64 fruits including Avocado, Banana, Berries, etc., 34 beverages and 6 oils were measured by different assays – Total Antioxidant Capacity Assay, FRAP Assay and TEAC Assay (Nicoletta *et al.,* 2003). Among fruits, the highest antioxidant activities were found in berries (i.e., blackberry, redcurrant and raspberry) regardless of the assay used. Among beverages, coffee had the greatest TAC, regardless of the method of preparation or analysis, followed by citrus juices, which exhibited the highest value among soft beverages. Finally, of the oils, soybean oil had the highest antioxidant capacity, followed by extra virgin olive oil, whereas peanut oil was less effective.

The study of four plant species *Trigonella foenum-graecum, Centella asiatica, Pisonia alba, Sauropus androgynus* imply their enormous nutritive value and their significance in the prevention of free radical-induced diseases. The observations may be used to substantiate the scientific reasoning that free radical-scavenging is indeed the mode of operation of these

plants in the treatment or prevention of the onset of deadly disorders like arthritis, breast cancer, artherosclerosis, etc. (Subhasree *et al*., 2009)

Foods rich in antioxidant phytochemicals are important for the prevention of diseases related to oxidant stress such as heart disease and cancer. Supportive evidences from phytochemical analysis indicating the presence of flavones (flavonoids) and phenolic contents, contribute as natural antimicrobials as well as antioxidants. This in turn reveals the anticancerous activity of fruit peel extract against liver and breast cancer which can insight, in future, in drug development strategies including apoptosis. (Riyaz *et al*., 2010)

The present study aims at evaluating the antioxidant potential of three fruit peels, Papaya, Avacado and Sweet lime both individually and in combination (dual and triple). The main objective is to assess the effect of synergism on the antioxidant activities of these fruit peels.

2.3 Papaya (*Carica papaya)*

Papaya contains 'papain' which helps to digest food. The papaya is the fruit of the plant Carica papaya, in the genus Carica. Papaya in plants in the myrtle family (Caricaceae). It has a nutritional value where this fruits contain about 9% sugars and only a low organic acid content about 0.2%. It also has the vitamin C level is about 60mg/100g.Their flesh is a rich orange color due to carotenoids contain. These nutrients help prevent the oxidation of cholesterol. The fruits of papaya have the shape like spherical which can be as long as 20 inches. The seeds of the fruit are black and round shape.

2.4 Sweet lime (*Citrus limetta*)

It is a small tree which may reach 8 m in height. The sweet lemon has irregular branches, and relatively smooth, brownish-grey bark. It possesses numerous thorns which may grow to anywhere from 1.5 to 7.5 cm long. The petioles of the sweet lemon are narrowly but distinctly winged, and are 8 to 29 mm long. It has leaflets rather than leaves, which are obviate and 5.5 to 17 cm long, 2.8 to 8 cm wide. The apex of the leaflet is acuminate, and the base of the leaflet is rounded. Flowers are white in bud and in bloom, ranging from 2 to 3 cm wide. The petals soon fall away, leaving the fruit to grow. The skin of the fruit is light yellow at maturity; the rind is white and about 5 mm thick. The pulp is greenish and the juice is sweet rather than acidic. The fruit has high levels of ascorbic acid (Vitamin C).

2.5 Avocado (*Persea americana*)

The avocado (*Persea americana*) is a tree enative to Central Mexico, classified in the flowering plant, family Lauraceae along with cinnamon, camphor and bay laurel. Avocado or alligator pear also refers to the fruit (botanically a large berry that contains a single seed) of the tree, which may be pear-shaped, egg-shaped or spherical. Avocados are commercially valuable and are cultivated in tropical and Mediterranean climates throughout the world. They have a green-skinned, pear-shaped fleshy body that ripens after harvesting. Trees are partially self-pollinating and often are propagated through grafting to maintain a predictable quality and quantity of the fruit

Peels of fruits such as Avocado, mango and star fruit has a much higher phenolic content than the edible parts. Mango, Avocado and Star fruit peels contain the largest phenolic content of 123, 75 and 80 mg/g DW, respectively (Yuuko *et al.*, 2010). Non-edible parts (seed and peel) of mango, avocado and star fruit were found to have high radical scavenging capacity.

The seeds of Avocado which is commonly called as Butter fruit is rich in antioxidants and has a highly potent antimicrobial activity. Avocado is an edible fruit and the seeds which are considered as waste can be used for curing many human diseases. According to Nagaraj *et al.*, (2010), as an edible and a natural resource, these seeds can be readily used for many diseases and also to identify antioxidant and antibacterial drugs which will be helpful for human shelf life.

The radical scavenging activity of avocado epicarp extract was investigated and found to be about 2 times higher than those of tocopherol were measured by the thiocyanate method with a linoleic acid system (Naoko *et al.*, 2006).

2.6 Antioxidant studies in other fruit varieties

Consumers are currently demanding less use of chemicals or minimally processed fruits and vegetables, so more attention had been paid to search for naturally occurring substances. This is true for plant materials that act as alternative antioxidant sources. Studies have shown that, by performing several *in vitro* assays to evaluate the antioxidant potential of different fruits and vegetable peels, it is identified as *M. indica* peel may become important as a cheap and

noticeable natural source of compounds with health protective potential which can be used in pharmaceutical, nutraceutical and food preparation (Rakholiya *et al.,* 2010).

The phenolics extracted from a variety of pineapple fruits exhibited excellent antioxidant activity. The results of all the assays (Antioxidant Capacity assay, DPPH assay, Superoxide radical scavenging assay) are in agreement that methanol extracts of pineapple phenolics displaying high antioxidant activity. They act as hydrogen donating agent in the DPPH assay, were effective in scavenging superoxide anion produced by hypoxanthine-xanthine oxidase and hydroxyl radicals. These effects may be correlated with its phenolic structure, which can react with a free radical to form the phenoxyl radicals. These results show that pineapple and its active constituents may be used in future antioxidative therapy and provides a valuable source of nutraceutical supplements (Adhikarimayum *et al.,* 2010).

Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological and free radical scavenging activities. To identify their potential sources, extracts of some underutilized fruits (*Muntingia calabura, Averrhoea bilimbi and Artocarpus altilis*) were assessed with regard to their total phenolics and flavonoid content, as well as antioxidant capacity in different solvent systems and distilled water. (Firdose *et al.,* 2011)

Studies to evaluate the antioxidant and antibacterial powder of banana fruit peel and to identify the responsible compounds for those activities were done (Matook and Fumio 2005). Ethyl acetate and water soluble fractions of green banana peel displayed high antimicrobial and antioxidant activity while those compounds isolated from water soluble extracts, glycoside and monosaccharide components displayed significant antioxidant and low antimicrobial activity.

The antioxidant potential of different solvent extracts of three different locally grown citrus varieties; grape fruit, lemon and mussambi, was assessed using some antioxidant assays like estimation of total phenolic contents(TPC), total flavonoids contents(TFC), percentage inhibition of linoleic acid oxidation and DPPH radical scavenging capacity. Based on the results it could be concluded that methanol was considered as an efficient solvent for the extraction of antioxidant components from dry citrus peels. (Shahzad *et al*., 2011)

Study based on methanolic extracts of peel, pulp and seed of banana fruit were investigated for *in vitro* antioxidant activity using DPPH radical scavenging capacity, reducing power, CUPRAC (Cupric Reducing Antioxidant capacity) and total antioxidant capacity. Results of the study indicate that, seed has strong *in vitro* antioxidant activity than that of the peel and pulp. (Preeti *et al.*,2011). Recent studies by Baskar *et al* (2011) in our laboratory have established the antioxidant potential of peel of nine different varieties of locally available banana.

The antibacterial and antioxidant activities of different parts of local seeded banana fruit were investigated *in vitro* (Preeti *et al.,* 2011). Dried peels, pulps and seeds of the fruit were extracted with hexane, ethyl acetate and ethanol. Antibacterial property of the extracts was evaluated against 4 gram positive and gram negative bacteria using this diffusion technique. Based on the results, it was concluded that peels, pulps and seeds of local seedy banana fruits possesses significant antibacterial and antioxidant activity.

2.7 Antioxidant capacity assay

2.7.1 DPPH radical scavenging assay

The 1, 1-diphenyl-2-picryl hydroxyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical scavenged by antioxidants through the donation of proton forming the reduced DPPH. When DPPH is placed in an assay system containing free radical scavengers such as flavonoid, the color vanishes. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition.

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. It was found that the radical scavenging activities of all the extracts were increased with increasing concentration. According to Kamran *et al.,* (2009), there were no correlation between the total phenolic and/or flavonoids contents and antioxidant activity in tissues and/or peels.

In the case of dragon fruit (*Hylocereus polyrhizus*), DPPH radical scavenging assay was performed and the results indicated that the extract contained phenolic contents

comparable to standard antioxidant agents like vitamin C and Catechin. (Rebecca *et al.*,2010). It has also been indicated that in terms of function, as a primary antioxidant or chain-breaking antioxidant, the pulps of the two species in *Hylocerus* showed equal antiradical power. By taking the function as secondary antioxidant or metal chelator, the pulp of H. *undatus* was much better than the pulp of H. *polyrhizus*. The contribution of phenolics and ascorbic acids to each sample's antioxidant activity varied from one another. This study also showed that the peel of both H. *polyrhizus* and H. *undatus* have antioxidant potential. (Wee and Wee 2011)

2.7.2 Ferric reducing Antioxidant Potential Assay (FRAP)

The reducing capacity was investigated by measuring Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activities of putative antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction and radical scavenging.

2.7.3 ABTS Radical Scavenging Assay

In this improved version, ABTS.-, the oxidant is generated by persulfate oxidation of 2, 2-azinobis (3-ethylbenzoline-6-sulfonic acid)- (ABTS2-) [15]. ABTS radical cation was produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture were allowed to stand in dark at room temperature for 12-16 hrs before use. For the study, different concentrations (50-250 µg/ml) of methanolic extract (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with ethanol to make 1.0 ml. The absorbance was read at 745 nm and the percentage inhibition calculated.

2.7.4 Lipid Peroxidation Inhibition Assay

Initiation of lipid peroxidation by ferrous sulphate takes place either through hydroxyl radical by Fenton's reaction. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxyl radical or the superoxide radicals or by changing the Fe^{3+} / Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or chelating the iron itself.

Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological

molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane. Lipid hydroperoxides can be decomposed to produce apoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage generation of cancer and aging related disease. Thus, decrease in MDA level with increasing in the concentration of the extracts indicates the role of the extracts as an antioxidant.

2.8 Natural sources of antioxidants

A number of vitamins and substances found in natural foods provide these antioxidant benefits.

2.8.1 Vitamin A

According to the National Cancer Institute, diseases caused by free radicals include various cancers. They advise to eat foods rich in vitamins, minerals and other substances that act as antioxidants. One of these recommended vitamins, vitamin A, can be found in liver, sweet potatoes, mozzarella cheese, egg yolks, milk and carrots. The International Food Information Council Foundation also lists fish as a vitamin A source.

2.8.2 Vitamin C

The Cleveland Clinic's Miller Family Heart and Vascular Institute suggest vitamin C as an effective antioxidant. The Institute lists a large variety of foods rich in vitamin C, including papaya, mango, pineapple, guava, citrus fruits and juices, cantaloupe, red, green and yellow peppers, tomatoes and tomato juice, berries, and dark green vegetables such as spinach, broccoli and other greens. The National Cancer Institute expands on this list by also citing certain cereals, beef, poultry and fish as providing large amounts of vitamin C.

2.8.3 Vitamin E

Vitamin E is also endorsed as a beneficial antioxidant. The National Cancer Institute, the International Food Information Council Foundation and the Miller Family Heart and Vascular Institute recommend consuming vegetable oils, nuts, seeds, cereals, whole grains, wheat, rice, oats, mangoes, legumes, soybeans, dark green vegetables and sweet potatoes to receive high levels of vitamin E.

2.8.4 Selenium

Selenium is a mineral that acts as a natural antioxidant, according to the Miller Family Heart and Vascular Institute. Selenium sources include Brazil nuts, yeast, oats, brown rice, eggs, chicken, dairy, most vegetables, whole grains, wheat, seafood, onions, garlic and molasses.

2.8.5 Carotenoids

The International Food Information Council Foundation states that all carotenoids are natural antioxidants. Carotenoids include beta carotene, lutein and lycopene. To add carotenoids to your diet, eat eggs and orange, red, yellow and green vegetables and fruits. These include kale, broccoli, spinach, tomatoes, mangoes, cantaloupe and apricots.

2.8.6 Flavonoids

Flavonoids are another beneficial antioxidant. Flavonoids such as anthocyanidins, flavanols, flavanones and proanthocyanidins can be found in an abundant variety of foods, including onions, apples, tea, broccoli, citrus fruits, cocoa, cherries, grapes, berries, wine, peanuts and cinnamon.

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. Flavanoids exhibit several biological effects such as anti-inflammatory, anti-hepatotoxicand anti-ulcer actions. Free radicals are molecules that are present in the environment and also occur in the body as a natural part of physiological functions. They can cause cell damage, however, and when present in high levels, may contribute to chronic conditions such as cancer, diabetes, heart disease. It's impossible to avoid free radicals, but antioxidants can minimize their effect. One of the best sources of antioxidants is a diet rich in deeply colored fruits and vegetables: the pigment in these foods in responsible for the antioxidant activity.

The flavonoid content in the pulp and peel extracts was expressed in terms of catechin equivalent (Mohammad *et al.,* 2009). The results showed that sour summer pulp cultivar had the most antioxidant effect with significant difference with the other cultivar ($p<0.05$) which can be introduced as a potent source of natural antioxidants, and the peel of three cultivars (sweet saveh malas. Sour summer and black peel) as a suitable source for extraction and purification of phenolic and flavonoid compound. The antioxidant capacity of pomegranate peel extract is 10 times higher than the pulp extract.

Apple represents an important source of natural antioxidants, and the considerable antioxidant power of this fruit is thought to be related to the high polyphenolic content, particularly flavonoids. The polyphenol content and the antioxidant properties of "Annurca" apple have been investigated, in both peel and flesh during the different phases of its peculiar ripening process. (Stefania *et al.,* 2007)

According to a study conducted by Suganya *et al.,* (2007**)**, the methanolic extract of thai guava leaves has high antioxidant capacity. The methanolic extracts of Guava showed a scavenging activity towards ABTS free radical decolourization assay and Ferric Reducing power assay (FRAP). It has been identified that the most active compound was found to be quercetin along with two flavonoid compounds, quercetin-3-O- glucopyrinosyl and morin.

In the case of Mandarin (Citrus *reticulate*), as other citrus fruits, has nutritional importance due to its particular composition. Flavonoids, especially polymethoxy flavons and flavonons are identified in citrus pulp as well as in peel (Vesna *et al.,* 2010). Based on the results, they suggest that mandarin peel powders can be used as substitutes for synthetic antioxidants, to increase the shelf life of food products containing fats and oils, imparting health benefits to the consumers.

Studies shows that tropical fruits such as guava, star fruit and papaya have high primary antioxidant potential when compared to orange (Lim *et al*., 2007). Banana though weaker than orange, is a primary antioxidant is, however, a powerful secondary antioxidant.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Flavonoids are one of the most diverse and widespread group of natural compounds and are one of the most important natural phenolics. These compounds possess a broad spectrum of activities including radical scavenging properties.

Recycling of fruit waste is one of the most important means of utilizing it in a number of innovative ways yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry. The work by Neeraj *et al.,*(2011**)** has identified the antibacterial activity against the test organisms

and phytochemical constituents in *Citrus limon* and *Citrus sinensis* peels extracts obtained from different solvents.

The activity of the ethyl acetate, chloroform and methanol extracts were compared favorably with that of standard antibiotic Streptomycin . The minimum inhibitory concentration showed that methanol and etyl acetate extracts had the lowest MIC value against *C. albicans*, indicating higher potency (Idris *et al*.,2009). Preliminary phytochemical screening revealed the presence of flavonoids, saponins, tannins, steroids, alkaloids and terpenoids.

The effects of aqueous seed extract of *Persia americana* on blood pressures, plasma and tissue lipids of a albino rats were investigated (Imafidon and Amaechina. 2010). The different dose of *P.americana* aqueous extract, significantly reduced blood pressures of the hypertensive rats.

The *in vitro* antibacterial and antioxidant properties of hydromethanolic extract of peel from *Citrus sinensis* (Sweet orange) was investigated (Dayanand *et al*.,2011). In this study the antibacterial activity of *Citrus sinensis* peel extract against different gram positive and gram negative bacteria by disc diffusion method and antioxidant activity was undertaken and concluded that hydromethanolic extract of sweet lime has good antioxidant activity.

The radical scavenging activity of the avocado epicarp extract and α-tocopherol were measured by the thiocyanate method with a linolinic acid system.In addition Naoko *et al*.,2006 concluded that avocado epicarp extract containing polyphnols such as epicatechin showed high radical scavenging and antioxidative activities that were stable to heating.

The aqueous and methanolic extracts of carambola,guava,kiwi,papaya and strawberry were evaluated for their antimicrobial,antioxidant and chemopreventive potential by Mishra *et al*.,2010.Antioxidant capacity and chemopreventive ability of these extracts was also assayed in which kiwi fruit was best among all the selected fruits .Natural components from these fruits can be further extracted and used as an alternative to synthetic drugs.

The fruits of *Carica papaya* L.(Caricaceae) are valuble as food and are also used in traditional medicine. The present study was designed to access the antioxidant juices of 3

papaya cultivars (PCJ):Sunrise Solo,Red Lady, and Tainung. The antioxidant capacity of PCJ obtained from fully ripened fruit was determined by the following methods: scavenging of the free radical 1,1-diphenyl-2-picrylh ydrazyl (DPPH),reducing power assay, scavenging of superoxide radicals,2-deoxyribose oxidation assay, and thiocyanate assay.Total phenolic content (TPC) of PCJ was determined by the folin-Ciocalteu reagent method(Aysun *et al.*,2011).This study demonstrated that different papaya cultivars have different antioxidant capacities and TPC amounts. Significant correlations were found between antioxidant capacity and TPC, indicating that phenols contribute to antioxidant capacity.

Nine tropical fruits were analyzed for total phenol contents, ascorbic acid contents and antioxidant activities. The antioxidant activities were evaluated based on the ability of the fruits to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), reduce ferric to ferrous ions (Lim *et al.*,2005).It was found that guava, papaya, and star fruit have higher primary antioxidant potential, as measured by scavenging DPPH and iron reducing assays. Banana, star fruit, water apple, langsat and papaya have higher secondary antioxidant potential as measured by the iron chelating experiment.

The antioxidant activities of the ethanol, petroleum ether, ethyl acetate,*n*-butanol and water extract fractions from the seeds of papaya were evaluated by Kaibing *et al.,(2011)*in this study. The ethyl acetate fraction showed the strongest DPPH and hydroxyl free radical-scavenging activities, and it's activities were stronger than those of ascorbic acid and sodium benzoate, respectively.

The antioxidant activity of the fruits available in the Aizawl market of Mizoram, India was estimated.A total of 20 fruits were evaluated for their antioxidant activity based on the ability of the fruit extracts to scavenge 1,1-diphenyl-2-picrylhydrozyl free radicals, to reduce ferric ions determined by ferric reducing antioxidant potential assay ant total phenolic content determination (Ayub *et al.*,2010).Among the fruits used in the present investigation, the highest antioxidant activity was observed in Amla and least in coconut water.

MATERIALS AND METHODS

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

1,1- Diphenyl-2-Picryl hydrazyl (DPPH), methanol, Ammonium persulphate, 2,2-azobis-3-ethylbenzthiazoline-6-sufonic acid (ABTS), acetone, ethanol, Ammonium molybdate, disodium hydrogen phosphate, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Ferrous sulphate, acetic acid, sulphuric acid, hydrochloric acid, Sulphuric acid, Ammonium molybdate, Disodium Hydrogen Phosphate, Ferric Chloride, 2,4,6-Tripyridyl- 5 –Triazine (TPTZ), Hydrochloric acid, Acetate buffer. All reagents used were of the analytical grade.

3.1.2 Fruit samples

Fresh fruits were purchased from the local market. The samples used were:

1. Sweet lime
2. Avocado
3. Papaya
4. Sweet lime + Avocado
5. Sweet lime + Papaya
6. Avocado + Papaya
7. Sweet lime + Avocado + Papaya

3.2 Methods

3.2.1 Phytochemical Analysis

Identification of Carbohydrates

Molisch's Test

To 2 ml of aqueous extract add few drops of 5% α-naphthol in ethyl alcohol. Then add about 1 ml of concentrated sulphuric acid along the sides of the tube. Appearance of reddish-violet ring at the junction of two layers indicates the presence of carbohydrates.

Fehling's Test

Add 1 ml of Fehling's reagent (copper sulphate in alkaline conditions) to the filtrate of the extract in distilled water and heat in a steam bath. Brick red precipitate indicates the presence of carbohydrates.

Test for Starch

To 1 ml of the extract add few drops of iodine solution. Formation of blue colour indicates the presence of starch.

Test for Cellulose

To 1 ml of the extract add 2-3 drops of iodine solution followed by 2 drops of sulphuric acid. Appearance of dark/deep brown/cherry red colour indicates the presence of cellulose.

Identification of Amino Acids and Proteins

Millon's Test

To 2 ml of the filtrate add 5-6 drops of Millon's reagent. Formation of red precipitate indicates the presence of proteins and free amino acids.

Biuret Test

To the ammoniated alkaline filtrate, add 2-3 drops of 0.02% copper sulphate solution and formation of red colour indicates the presence of proteins and free amino acids.

Ninhydrin Test

To the filtrate add lead acetate solution to precipitate tannins. Filter, spot the filtrate on a paper chromatogram, spray with ninhydrin reagent and dry at 110°C for 5 minutes. Appearance of violet spots indicates the presence of proteins and free aminoacids.

Bradford's Test

To 1 ml of the extract add few drops of Bradford's reagent (Coomassie Brilliant Blue G 250) and formation of blue colour product indicates the presence of proteins.

Identification of Phenols

Ferric Chloride Test

To 2 ml of the extract, add 2 ml of ferric chloride solution ($FeCl_3$) and formation of a deep bluish green solution indicates the presence of phenols.

Phosphomolybdic Test

To the ethanolic extract add phosphomolybdic acid reagent and liquor ammonia. Appearance of blue colour indicates the presence of phenols.

Identification of Catechol

To 2 ml of the test solution, add Erhlich's reagent and few drops of concentrated HCl. Appearance of brown or black colour indicates the presence of catechol.

Identification of Sterol and Steroids

Libermann-Buchard Test

To the ethanolic extract add 2 ml of chloroform followed by 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. Appearance of rose red colour which quickly changes through blue to green indicates the presence of cholesterol.

Salkowski Test

Dissolve the ethanolic extract in chloroform and shake with an equal volume of concentrated sulphuric acid. Appearance of red colour in the chloroform layer and green fluorescence in the acid layer indicates the presence of cholesterol.

Identification of Glycosides

Kellar-Killani Test

Dissolve the ethanolic extract in glacial acetic acid containing a trace of ferric chloride. Add same amount of $FeCl_3$ dissolved in concentrated sulphuric acid along the sides of the test tube to settle at the bottom. Appearance of a reddish brown colour changing to bluish green colour at the junction of two reagents within 2-5 minutes spreading slowly into the acetic acid layer confirms the presence of cardiac glycosides.

Legal's Test

Dissolve a few ml of ethanolic extract in few drops of pyridine. To this add a drop of 2%w/v sodium nitroprusside solution and a drop of 20%NaOH solution. Appearance of a pink or deep red colour indicates the presence of glycosides.

Raymond's Test

Dissolve a small quantity of the ethanolic extract in 1 ml of 50% ethanol. Add to it 0.1 ml of Raymond's reagent and 2-3 drops of 20% NaOH solution. Appearance of violet colour slowly changing to blue gives an affirmative test.

Xanthydrol Test

Add to the ethanolic extract, 0.5 ml of Xanthydrol solution (for deoxysugars only) and development of red colour indicates the presence of glycosides.

Antimony Trichloride Test

To the ethanolic extract add a solution of antimony trichloride and trichloroacetic acid and then heat the mixture. Appearance of a blue or violet colour shows the presence of cardiac glycosides.

Kadde's Test

Treat the ethanolic extract with a small amount of Kadde's Reagent and development of blue or violet colour that fades out in 1 to 2 hours shows the presence of cardiac glycosides.

Identification of Saponins/Saponin Glycosides

Froth Test

Weigh 1 g of the sample in a conical flask. Add 10 ml of sterile distilled water and boil for 5 min. filter the mixture and add 2.5 ml of the filtrate to 10 ml of sterile distilled water in a test tube. Stopper the test tube and shake vigorously for about 30 sec. Allow to stand for half an hour and honeycomb froth indicates the presence of saponins. Mix the froth with 3 drops of olive oil and shake vigorously. Formation of emulsion indicates the presence of saponins.

Sodium bicarbonate Test

To few ml of the ethanolic extract add few drops of sodium bicarbonate and shake well. Formation of honey comb indicates the presence of saponins.

Identification of Quinones/Anthraquinones

Chloroform-Ammonia Test

Boil 0.5 g of the plant sample with 10 ml of 5% sulphuric acid and filter. To the hot filtrate add 5 ml of chloroform and heat in a boiling water bath. To 2 ml of the chloroform extract, add 1 ml of diluted 10% ammonia and shake the mixture. Pink-red colour in the ammoniacal layer shows the presence of anthracene derivatives.

Borntrager's Test

Heat about 50 ml of the extract with 10% ferric chloride solution and 1 ml of concentrated HCl. Cool the extract, filter and shake the filtrate with diethyl ether. Further extract the ether extract with strong ammonia. Pink or deep red coloration of aqueous layer indicates the presence of anthroquinone.

Hydrogen peroxide Test

Filter the ethanolic extract. To 1 ml of the filtrate add 10 ml of dichloromethane. Separate the aqueous and organic layers. To 5 ml of the aqueous layer add 1 ml of 20% H2O2 and 1 ml of 50% H2SO4. Heat in a boiling water bath. Then add 5 ml of toluene and 1 ml of 5% NaOH. Separate the aqueous and toluene phase. Red colour in toluene phase indicates the presence of quinines.

Identification of Alkaloids

To 1 g of the sample add 10 ml of 5% HCl and heat in a boiling water bath for 10 minutes and filter. To the filtrate add 5 ml dilute ammonia and 5 ml of chloroform. Use the aqueous layer for the following tests:

Mayer's Test

Add a few drops of Mayer's reagent to the aqueous layer and formation of creamy layer indicates the presence of alkaloids.

Dragendorff's Test

To 1 ml of the extract add 1 ml of Dragendorff's reagent. Appearance of orange precipitate indicates the presence of alkaloids.

Hager's Test

Treat 1 ml of acid extract with 1 ml of Hager's reagent. Orange precipitate indicates the presence of alkaloids.

Wagner's Test

Treat the acid extract with few ml of Wagner's reagent. Reddish brown precipitate indicates the presence of alkaloids.

Identification of Flavanoids

Decolourization Test

Reduce the water extract of the sample to dryness in a boiling water bath. Treat the residue with dilute NaOH followed by addition of dilute HCl. A yellow solution with NaOH which turns colourless with dilute HCl confirms the presence of flavonoids.

Shinoda Test

To 5 g of the sample add 10 ml of ethanol and heat in a boiling water bath. To the ethanolic extract add concentrated HCl (8-9 drops) and some magnesium filings. Allow it to stand for 10-15 minutes at room temperature. Formation of red colour indicates the presence of flavonoids.

Ammonia Test

Dip the filter paper strips in the aqueous and alcoholic extract and expose to ammonia vapours. The change of the colour of the filter paper to yellow indicates the presence of flavonoids. Add 10 ml of the concentrated sulphuric acid to the above yellow coloured filter paper. Disappearance of the yellow colour indicates the presence of flavonoids.

Pew Test

Add a piece of metallic magnesium/zinc to 1 ml of the extract, followed by addition of 2 drops of concentrated HCl. Formation of brown colour confirms the presence of flavonoids.

Lead acetate Test

To a few ml of ethanolic extract, add equal volume of 0.5% acetic acid and filter. To this filtrate add 1.0 ml of 1% lead acetate. Flocculent white precipitate indicates the presence of flavonoids.

Aluminium chloride Test

Add 2 drops of 1% aluminium chloride to 1 ml of the aqueous extract. Yellow colouration indicates the presence of flavonoids.

Identification of Tannins

Preparation Of Extract

Suspend the plant material in methanol and allow it to stand overnight. Reflux it for 4 hours. Filter it and wash the residue with methanol. Allow the filtrate to cool down. Observe for any modification and use an aliquot of this to assay tannin.

Braemer's Test

Add 10ml of water to 0.5g of methanolic/ethanolic extract. Boil it and then filter. Add few drops of 10% ferric chloride to the filtrate. A dark green, blue or brown colour indicates the presence of tannin.

Identification of Hydrolysable Tannins

Shake 4 ml of the extract in a test tube and add 4 ml of 10% ammonia solution. Formation of an emulsion on shaking indicates the presence of hydrolysable tannins.

Identification of Anthocyanin

Boil few ml of aqueous extract for 5 minutes and filter. To the 2 ml of filtrate add 1 ml of NaOH. A colour reaction indicates the presence of primary amine. Add 1 ml of HCl to another 2 ml of the filtrate. Different colouration indicates the presence of secondary amine. Colouration for the primary and secondary amine indicates the presence of anthocyanin.

Identification of Volatile Oils

Shake 2 ml of extract solution with 0.1 ml dilute sodium hydroxide and a small quantity of dilute HCl. Formation of white precipitate indicates the presence of volatile oils.

Identification of Lignin

Spot Test

Dip the filter paper place one drop of phloroglucinol reagent on the filter paper dipped in the extract and appearance of red/purple spots indicates the presence of lignins.

Test for Terpenoids

Add 2 ml of chloroform to 0.5 g of the sample. To this add 5 ml of concentrated sulphuric acid along the sides of the test tube. A reddish brown colouration in the interphase indicates the presence of Terpenoids.

3.2.2 Preparation of fruit peel extracts

5 g of the shade dried and powdered sample, mixed with 50 ml of ethanol. Then it was kept in orbital shaker for 24 hours at 37◦C, after which the solution was filtered and the filtrate was dried completely. The dried extract was then scrapped off and weighed (Kalpana *et al.*, 2011). This was used for further assays.

3.2.3 Assays

Determination of Total Antioxidant Capacity

(Mohammad *et al.*, 2011)

Principle

This assay is based on the principle of reduction of Molybdenum (VI) to Molybdenum (V) by the extract and the subsequent formation of green phosphate / molybdenum complex at acid pH.

Reagents

1. Reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate)
2. Ascorbic acid

Procedure

- The working solutions (10 to 100µg/ml) of samples were prepared by dissolving the extracts in water.
- 0.1ml of the extracts was mixed with 1ml of reagent solution.
- The tubes were capped with aluminum foil and incubated at 95˚C for 90 minutes.
- The tubes were then cooled to room temperature and the absorbance was measured at 695 nm against a blank.
- Ascorbic acid was used as standard.
- The total antioxidant capacity was expressed as ascorbic acid equivalent.

DPPH Radical scavenging Assay

Principle

DPPH scavenging activity was measured by the slightly modified spectrophotometric method. 1,1-diphenyl1-2-picryl-hydrazyl radical is scavenged by antioxidants through the donation of protons forming reduced DPPH. The electrons become paired off and the solution loses color depending on the number of electrons taken up. The color changes from purple to yellow after reduction, which is quantified by the decrease of absorbance at 517 nm.

Reagents

1. Methanol
2. DPPH in methanol (0.004%)

Procedure

- The fruit peel extracts were dissolved in methanol. A solution of DPPH in methanol (0.2mM) was prepared freshly.
- 3ml of this solution was mixed with 1ml of the samples of varying concentrations (100-1000µg/ml).
- The solution in the test tubes were mixed well and incubated in dark for 30 minutes at room temperature and absorbance was measured at 517 nm.
- The control had equal volume of DPPH in methanol instead of extract. 5ml of methanol was taken as blank.

- The percentage inhibition of the radicals due to the antioxidant property of the extract was calculated.

Ferric Reducing Antioxidant Power Assay (FRAP)

(Stefania *et al.*, 2007)

Principle

The total antioxidant potential of sample was determined using ferric reducing ability of plasma FRAP assay as a measure of antioxidants power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe II-tripyridyl triazine compound from colourless oxidized Fe III form by the action of electron donating antioxidants.

Reagents

1. 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ)
2. 40 mM HCl
3. 20 mm Ferric chloride
4. 0.3 M Acetate buffer, pH 3.6
5. Trolox
6. FRAP reagent : It contains 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It were freshly prepared and warmed to 37℃.

Procedure

- The stock solution of 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared.
- The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer.
- It were freshly prepared and warmed to 37℃, 900 µl FRAP reagent were mixed with 90 µl water and 30 µl test sample/methanol/distilled water/standard antioxidant solution.
- The reaction mixture was then incubated at 37℃ for 30 minutes and the absorbance was recorded at 595 nm.

- An intense blue color complex were formed when ferric tripyridyl triazine (Fe^{3+} - TPTZ) complex were reduced to ferrous (Fe^{2+}) form.
- The absorption at 595 nm was recorded. The calibration curve were plotted with absorbance at 595 nm vs concentration of ferrous sulphate in the range 0.1 mM both aqueous and methanol solutions).
- The concentrations of $FeSO_4$ were in turn plotted against concentration of standard antioxidants L-ascorbic acid or Trolox.

ABTS Cation Radical Scavenging Assay

(Rakholiya *et al.*, 2011)

Principle

The ABTS (2,2-Azobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) assay is based on the inhibition of the absorbance of ABTS radical cation, which has a characteristics long wavelength absorption spectrum.

Reagents

1. ABTS (7mM)
2. Ammonium persulfate (2.45mM)
3. Methanol

Procedure

- ABTS radical was produced by reacting ABTS solution (7mM) with ammonium per sulphate (2.45mM) and the mixture was allowed to stand in the dark at room temperature for 12-16 hours to give a dark colored solution.
- The absorbance was measured at 745 nm. The initial absorbance was found to be around 2.99.
- This stock solution was diluted with methanol to give a final absorbance value around 0.7(±0.2) and equilibrated at 30˚C.
- Different concentrations of the sample (100-1000µg/ml) were prepared by dissolving the extracts in water.
- About 0.3ml of the sample was mixed with 3ml of ABTS working standard in a micro cuvette.

- The decrease in the absorbance was measured after mixing the solution in 1 minute intervals up to 6min.
- The final absorbance was noted.
- A solution of ABTS working and 0.3ml of methanol was used as the control. About 3ml of methanol was used as blank.
- The percentage inhibition was calculated.

Lipid peroxidation inhibition Assay

(Ohkawa *et al.*, 1979)

Principle

Initiation of lipid peroxidation by ferrous sulphate takes place through the hydroxyl radical formation by Fenton's reaction. These produce malondiaaldehyde (MDA), which reacts with TBA to for a pink chromogen. The inhibition of lipid peroxidation could take place due to the scavenging of the hydroxyl radicals/ superoxide radicals or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself.

Reagents

1. Phosphate buffer saline(pH 7.4)
2. 0.07M Ferrous sulphate
3. 20% acetic acid (pH 3..5)
4. 0.8%TBA in 1.1% SDS
5. 20% TCAButan-1-ol

Procedure

- Goat liver was washed thoroughly in cold phosphate buffered saline (pH 7.4).
- This was then minced in a mortar and pestle with a measured volume of cold buffer in ice.
- The minced liver was then homogenized in a homogenizer to give a 10% homogenate.
- The homogenate was filtered using cheese cloth to remove unwanted residue.
- The filtrate was then centrifuged at 10000 rpm for 10 minutes in refrigerated centrifuge.
- The supernatant was used for assay.

- To 0.5ml of supernatant 0.5 ml of extracts of varying concentrations in water was added.
- The volume was made up to 1ml with distilled water. To this, 0.05ml of 0.07M ferrous sulphate was added.
- The solution was incubated at room temperature for 30minutes.
- To the incubated solution, 1.5ml of 20% acetic acid (pH 3.5), 1.5ml of 0.8% TBA (in 1.1% SDS) and 0.05ml of 20% TCA were added.
- The tubes were vortexed to ensure appropriate mixing.
- Then the tubes were incubated at 100˚C for 1 hour.
- The tubes were then cooled to room temperature the absorbance was read at 532nm.
- The control contained PBS instead of the sample.
- The percentage inhibition was calculated

3.2.4 Statistical analysis

The experimental results are expressed as mean± SD of three replicates. The data were subjected to two-way ANOVA and significance of difference between the sample means were calculated by DMRT using IRRISTAT version 3.1. p values<0.05 were regarded as significant.

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Yield Estimation

Table 4.1 Yield of the friut peel extracts in terms of percentage

Fruit peel extracts	Yield in %
Sweet Lime	35.86 ± 0.46
Papaya	9.90 ± 0.5
Avocado	17.90 ± 0.25
Sweet Lime + Papaya	8.20 ± 0.07
Sweet Lime + Avocado	16.23 ± 0.18
Avocado + Papaya	12.00 ± 0.3
Sweet Lime + Papaya + Avocado	11.73 ± 0.41

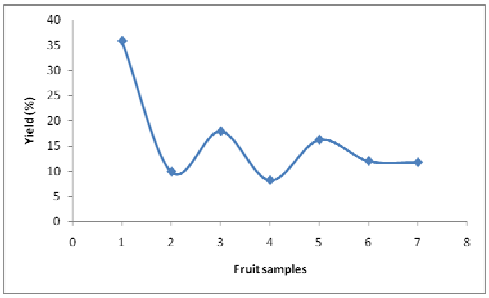

Figure 4.1 Yield % of ethanolic extracts of fruit samples

From the above graph, it has been found that the sweet lime peel extract contained the highest amount of yield, i.e. 35.86%, followed by avocado- 17.90% and then the sweet lime+avocado combination which is 16.23%. The papaya+avocado sample extract was found to be 12%, the combination of the three fruit peel extracts contained 11.73%, papaya-9.9%

and then the sweet lime+papaya sample extract was weighed and found to be 8.2%. Though sweet lime peel extract exhibits higher yield % than avocado, the antioxidant activity exhibited is higher in avocado compared to the other peel extracts.

4.2 *In vitro* Antioxidant activity

4.2.1 Total Antioxidant Capacity Activity

The total antioxidant activities of the fruit peels extracts are depicted in Table 4.2. The total antioxidant assay gives an estimate of the overall antioxidant potential of the fruit peels. There is a formation of phosphomolybdenum complex, the intensity of which indicates the potential of the peel as a scavenger of free radicals (Mohammad *et al*., 2011). The total antioxidant capacity of fruit peel extracts were expressed as number of equivalents of ascorbic acid. It has been shown that avocado fruit peel extract contained the maximum ascorbic acid equivalents of 17.25±0.33 mM/g.

Table 4.2 Total antioxidant activity of ethanolic extracts of fruit peels expressed as ascorbic acid equivalents.

Extracts	Ascorbic acid Equivalent (AAE) μM g^{-1}
Sweet lime	5.04^{a} ± 0.11
Papaya	5.12^{b} ± 0.15
Avocado	$\mathbf{17.25}^{d}$ ± 0.33
Sweet lime + Papaya	12.98^{c} ± 0.09
Sweet lime + Avocado	7.24^{b} ± 0.03
Papaya + Avocado	4.78^{a} ± 0.07
Sweet lime + Papaya + Avocado	7.60^{b} ± 0.04

Values represent mean ± SD of 3 replicates.

Means followed by a common letter are not significantly different at the 5% level by DMRT.

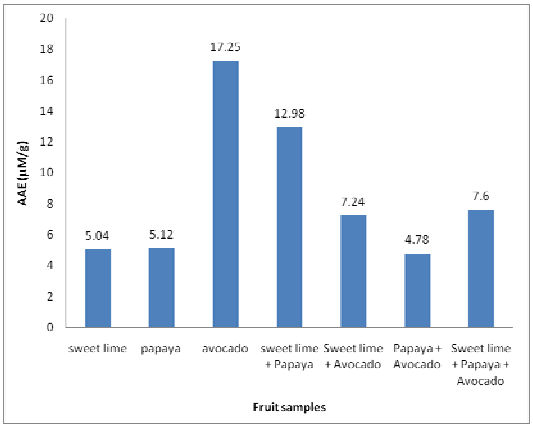

Figure 4.2 Total antioxidant activity in ethanolic extracts of fruit peel samples

4.2.2 DPPH Radical Scavenging Activity

DPPH is nitrogen centered free radical that shows strong absorbance at 517 nm (Arazo *et al*., 2011). Deep violet coloured methanolic DPPH solution changes to yellow colour in presence of DPPH radical scavengers. DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colour. The antioxidant activity was compared with Trolox as standard.

Similar studies by Sun *et al*.,(2011) showed the DPPH radical scavenging activity of the fruit peel extracts under different concentrations.

Table 4.3 DPPH Radical Scavenging of Fruit Peel Samples in Terms of Trolox Equivalents.

Extracts	Trolox Equivalent µM g^{-1}
Sweet lime	$9.76^{c} \pm 0.14$
Papaya	$5.13^{a} \pm 0.08$
Avocado	**16.87^{f}** ± 0.42
Sweet lime + Papaya	$6.41^{b} \pm 0.07$
Sweet lime + Avocado	$7.00^{b} \pm 0.15$
Papaya + Avocado	$7.80^{c} \pm 0.46$
Sweet lime + Papaya + Avocado	$8.76^{b} \pm 0.33$

Values represent mean ± SD of 3 replicates.

Means followeed by a common letter are not significantly different at the 5% level by DMRT.

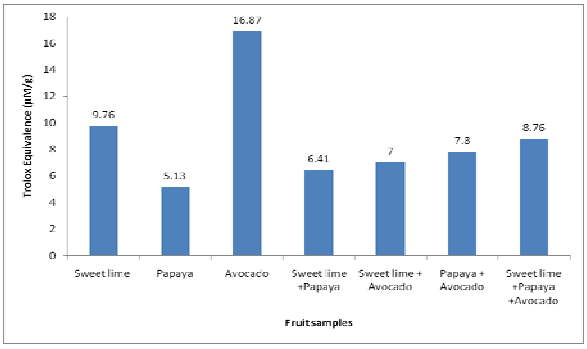

Figure 4.3 DPPH Radical Scavenging of Fruit Peel Samples in terms of Trolox Equivalents.

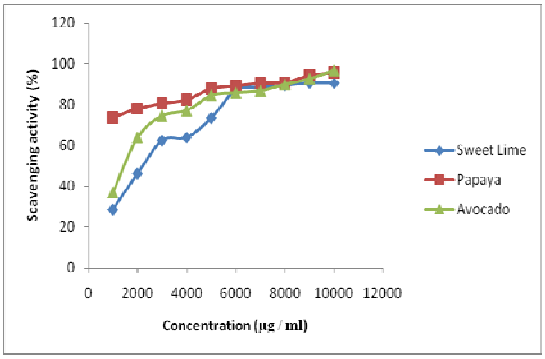

Figure 4.4 Scavenging Activity (%) on DPPH by ethanolic extracts of three fruit peels

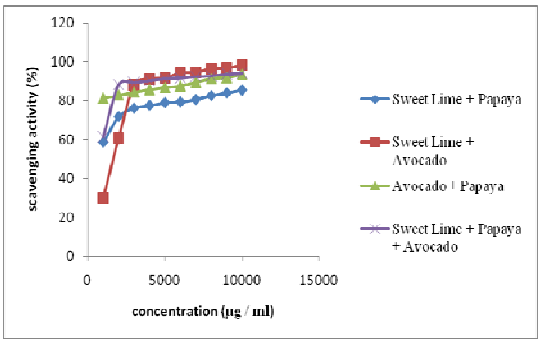

Figure 4.5 Scavenging Activity (%) on DPPH of ethanolic extracts of mixed fruit peels

Free radical scavenging potential of the ethanolic extracts of the fruit peel samples is shown in Figure 4.4 and 4.5 which increases with increase in concentration.

At 1 – 10 mg ml^{-1}, the ethanolic extract of Sweet Lime, Papaya, Avocado, Sweet Lime + Papaya, Sweet Lime + Avocado, Avocado + Papaya and Papaya + Sweet Lime + Avocado showed the scavenging activity of 26.60 – 90.56%, 73.66 – 95.57%, 36.85 – 96.86%, 58.46 – 85.32%, 30.01 – 98.39%, 81.55 – 91.81% and 61.40 – 94.23% respectively. However, at 10mg ml^{-1}, Sweet Lime + Avocado peel extract exhibited highest DPPH scavenging activity.

4.2.3 FERRIC REDUCING ANTIOXIDANT POTENTIAL ACTIVITY

The reducing capacity of a compound may serve as a significant indicator of the extracts potential antioxidant activity. To determine the reducing power of the extract, we measured Fe^{3+} - Fe^{2+} transformation in the presence of the peel extracts. Table 4.5 and Figure 4.6 shows the reducing power of the peel extracts as function of their concentration. The presence of antioxidants causes the reduction of Fe^{3+} / ferricyanide complex to the ferrous form was measured at 595 nm. The different peel extracts exhibited a dose dependent reducing power activity at various concentrations.

Table 4.5 Ferric Reducing Antioxidant Potential Activity of Fruit Peel Samples in Terms of Trolox Equivalents.

Extracts	Trolox Equivalent µM g^{-1}
Sweet lime	$371.5^{c} \pm 0.25$
Papaya	$164.52^{a} \pm 0.41$
Avocado	**569.94^{d}** ± 0.31
Sweet lime + Papaya	$275.76^{b} \pm 0.08$
Sweet lime + Avocado	$244.12^{b} \pm 0.33$
Papaya + Avocado	$183.56^{a} \pm 0.27$
Sweet lime + Papaya + Avocado	$256.21^{b} \pm 0.44$

Values represent mean ± SD of 3 replicates.

Means followed by a common letter are not significantly different at the 5% level by DMRT.

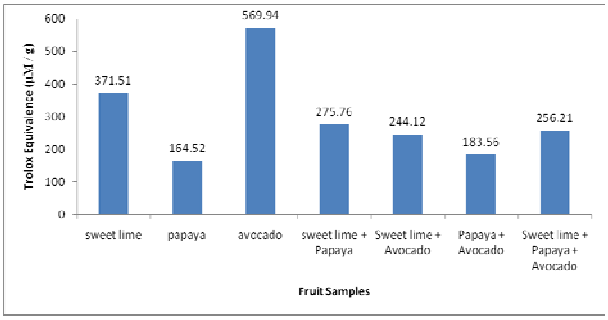

Figure 4.6 Ferric Reducing Antioxidant Potential Activity of Fruit Peel Sample in terms of Trolox Equivalents.

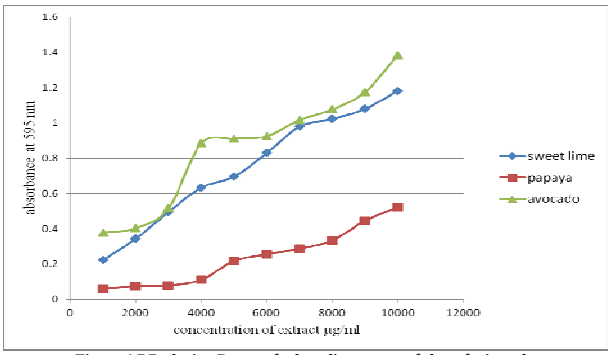

Figure 4.7 Reducing Power of ethanolic extracts of three fruit peels

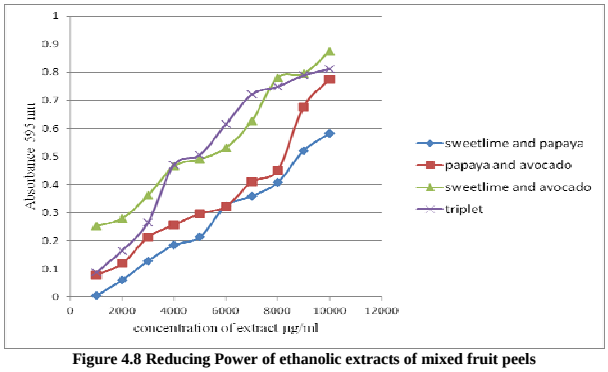

Figure 4.8 Reducing Power of ethanolic extracts of mixed fruit peels

At 1 – 10 mg ml^{-1}, the ethanolic extract of Sweet Lime, Papaya, Avocado, Sweet Lime + Papaya, Sweet Lime + Avocado, Avocado + Papaya and Papaya + Sweet Lime + Avocado showed the scavenging activity of 0.171 – 1.304%, 0.062 – 0.532%, 0.095 – 1.579%, 0.007 – 0.582%, 0.249 – 0.868%, 0.095 – 0.783% and 0.025 – 0.848% respectively.

At 10 mg ml^{-1} the ethanolic extract of Avocado peel exhibited higher reducing power activities. Significant difference in reducing power activity between the extracts was observed for ethanolic extracts.

The reducing powers of the three extracts were effective in the order Avocado > Sweet Lime > Sweet Lime + Avocado > Sweet Lime + Avocado + Papaya > Avocado + Papaya > Sweet Lime + Avocado > Papaya at 10 mg ml^{-1}.

Similar studies by Stefania *et al*. (2007) showed the ferric reducing activity potential of fruit peel extracts under different concentrations.

4.2.4 ABTS Radical Scavenging Activity

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristics long wavelength absorption spectrum.

Table 4.7 ABTS Radical Scavenging Activity of Fruit Peel Sample in terms of Trolox Equivalents.

Extracts	Trolox Equivalence µM g^{-1}
Sweet lime	285.6^{d}±0.12
Papaya	199.03^{b}±0.03
Avocado	**398.08^{b}**±0.41
Sweet lime + Papaya	272.3^{cd}±0.24
Sweet lime + Avocado	240.3^{bc}±0.45
Papaya + Avocado	199.32^{b}±0.09
Sweet lime + Papaya + Avocado	199.03^{d}±0.48

Values represent mean ± SD of 3 replicates.

Means followed by a common letter are not significantly different at the 5% level by DMRT.

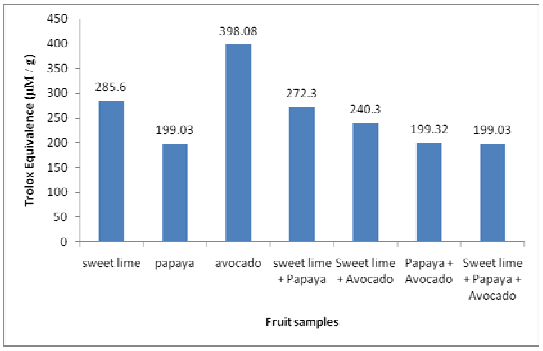

Figure 4.9 ABTS radical scavenging activity of fruit peel samples in terms of Trolox equivalents

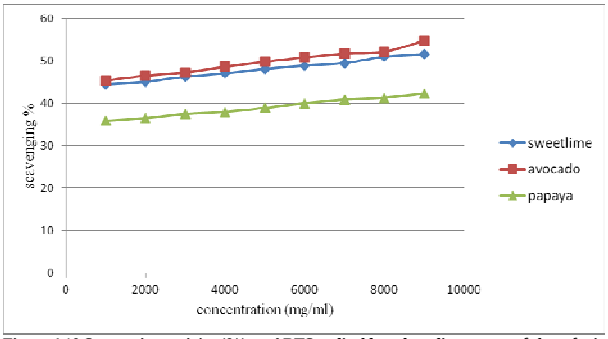

Figure 4.10 Scavenging activity (%) on ABTS radical by ethanolic extracts of three fruit peels

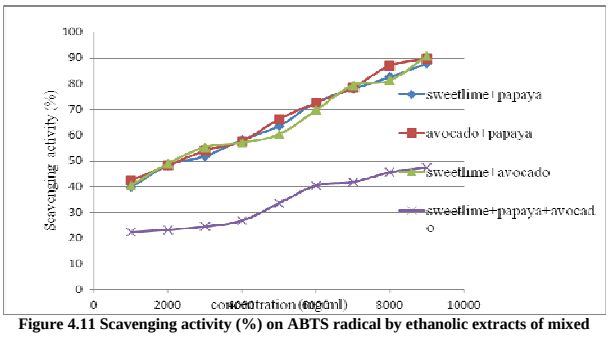

Figure 4.11 Scavenging activity (%) on ABTS radical by ethanolic extracts of mixed fruit peels

Free radical scavenging potential of the ethanolic extracts of fruit peels is shown in Figure 4.10 and 4.11 which increase with the increase in concentration.

At 1 – 10 mg ml^{-1}, the ethanolic extract of Sweet Lime, Papaya, Avocado, Sweet Lime + Papaya, Sweet Lime + Avocado, Avocado + Papaya and Papaya + Sweet Lime + Avocado shows the percentage inhibition of 44.42 – 52.46%, 36.29 – 44.63%, 45.67 – 62.1%, 41.31 – 89.5%, 40.31 – 93.93%, 43.51 – 92.81% and 21.64 – 42.51% respectively. At 10 mg ml^{-1}, Sweet Lime +Avocado peel extract exhibit highest ABTS scavenging activity.

Statistically, the scavenging activity was effective in the order of Sweet Lime + Avocado > Papaya + Avocado > Sweet Lime + Papaya > Avocado > Sweet Lime > Papaya.

Similar studies by *Ruttiros et al.*, (2010) determine the scavenging activity of ABTS radical scavenging activity.

4.2.5 Inhibition of lipid peroxidation activity

Lipid Peroxidation assay procedure for polar solvent extracts measures the malondialdehyde (MBA) formed as the split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of a lipid substrate. It is populated that the formation of MDA from fatty acids with less than three double bonds occur via the secondary oxidation of primary carbonyl compounds. The MDA is reacted with thiobarbituric acid (TBA) to form a pink pigment (TBARS) that is measured spectrophotometrically at 532 nm (Rafaela *et al.*, 2010).

From the Table 4.9 it has been observed that the highest inhibition of lipid was exhibited by ethanolic extract of avocado fruit peel with its maximum at the highest concentration when compared to the other fruit peel extracts. Those combinations with avocado peel extract also exhibited higher inhibition activity compared to the other fruit peel extracts.

Similar to studies of Abhishek *et al.*, (2011) showed the lipid peroxidation inhibition activity potential of fruit peel samples under concentrations.

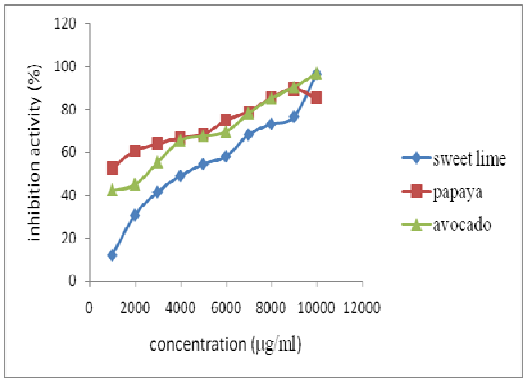

Figure 4.12 Scavenging activity (%) on lipid peroxidation by ethanolic extracts of three fruit peels

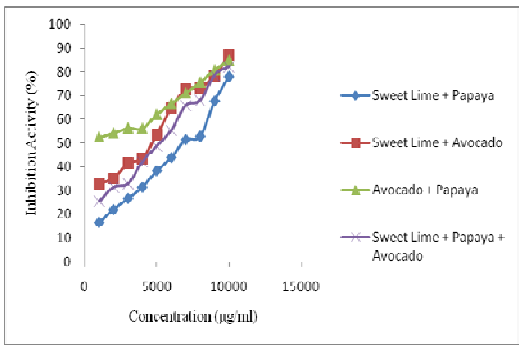

Figure 4.13 Scavenging activity (%) on lipid peroxidation by ethanolic extracts of mixed fruit peels

The avocado peel extract exhibited the highest inhibition activity as shown in the Figure 4.12.

Those combinations with avocado presence showed higher activities compared to the other peel extract combinations as revealed by Figure 4.13.

4.3 Phytochemical analysis

Preliminary phytochemical screening revealed the presence of flavanoids, saponins, tannins, steroids, alkaloids and terpenoids. The spectra of antimicrobial activities displayed by the extracts could be attributed to the presence of these phytochemicals and signifies the potential of *Persia Americana* (avocado) as a source of therapeutic agents. (Idris *et al.*, 2009)

In addition to the *in vitro* antioxidant activity assays phytochemical analysis was also carried out, where different solvent fruit peel extracts like aqueous, ethanol, ethyl acetate and chloroform were used. Tests for carbohydrates, proteins & amino acids, tannins, hydrolysable tannins, quinones, phenols, flavnoids, carotenoids, steroids / sterols, volatile oils, alkaloids and terpenoids were carried out to find the presence of these compounds in those various solvent extracts.

From Table 4.10 it is observed that the ethanolic extract of the three fruit samples showed more presence of the above compounds compared to the other solvent extracts.

Similar studies as Riyaz *et al.*, (2010) showed the phytochemical analysis of fruit peel samples in different solvents.

CONCLUSION

CHAPTER 5
CONCLUSION

Antioxidants are substrates that eliminate the free radicals that are present in the human body. These free Radicals can be eliminated by certain mechanisms which takes place with the help of antioxidants. Regular consumption of dietary foods involving antioxidants are capable of reducing the risk of several diseases. The present work has been done to evaluate the antioxidants from mixed fruit peel extracts such as *Citrus limetta* (Sweet lime), *Carica papaya* (papaya), *Persia Americana* (avocado). Several assays such as TAC, DPPH, FRAP, ABTS, LPO and Phytochemical analysis were done to study their antioxidant activities. Initially extracts involving ethanol as the solvent was considered as they showed positive results for majority of the Phytochemical analysis. The free radical scavenging ability of these fruit peel extracts was tested for various *in vitro* free radicals and the results obtained were analyzed statistically. On the whole *Persia Americana* (avocado) peel extract as singlet and in combinations showed higher antioxidant activity and the least activity was seen in *Carica papaya* (papaya) peel extract . The preliminary studies in our work confirmed the significant free radical scavenging potential of the above three fruit extracts and also the natural antioxidants present in the fruit peels. A statistical rank of ranks of the species with respect to free radical scavenging potential was carried out. The present study was mainly aimed at evaluating the antioxidant potential of mixed fruit peels. The perspective of the study is to recommend mixed fruit peel jam of the above mentioned fruits for commercial purpose.

APPENDIX

APPENDIX

Preparation of alkaline extract

Cover the plant material (0.1 – 0.5 g) with five times the volume of the so called Liquor of Prolius' [A mixture of ethyl ether – chloroform – ethanol – ammonium hydroxide (25:8:2:5:1 in volume)] and allow to stand for 24 hours. Filter and evaporate the filtrate to dryness. Treat the residue with 1% HCl favoring the dissolution of the bases with magnetic stirring, filter it and test the filtrate for phytochemicals.

Molisch's Reagent

A 5% solution of α-naphthol in alcohol.

Fehling's Reagent

It is a mixture of copper sulphate and alkaline tartaric acid. Dissolve 34.65 g of crystalline copper sulphate in distilled water and make up to 500 ml. Dissolve 125 g of potassium hydroxide and 173 g of Rochelle salt (Sodium potassium tartarate) in distilled water and make up to 500 ml. Mix these two solutions.

Millon's Reagent

A 15% solution of mercuric sulphate in 6N sulphuric acid.

Ninhydrin Reagent

A 0.1 g of Ninhydrin in 100 ml of acetone.

Biuret Reagent

Add 1.0% of copper sulphate solution drop by drop with constant stirring to 40% sodium hydroxide till the mixture assumes a deep blue colour.

Bradford's Reagent

Dissolve about 13.3 g of neutral crystalline copper acetate in 200 ml of distilled water. Filter if necessary and then add 1.8 ml of glacial acetic acid.

Raymond's Reagent

A 1% solution of m-dinitrobenzene in ethanol or methanol.

Xanthydrol Reagent

A solution of 0.125% xanthydrol in glacial acetic acid containing 1% HCl.

Kadde's Reagent

Mix equal volume of 2% solution of 3, 5-dinitrobenzoic acid in methanol and 7.5% KOH solution.

Mayer's Reagent

Prepare a solution of mercuric chloride (13.6 g) in water (600 ml) and another of potassium iodide (50 g) in 100 ml of water. Mix both solutions and make up the volume to 1000 ml with water.

Dragendorff's Reagent

Solution A

Add glacial acetic acid (10 ml) to a suspension of bismuth subnitrate (0.8 g) in water (40 ml).

Solution B

Prepare a solution of potassium iodide (20 g) in water (50 ml)

Mix solution A and B. To the mixture add 100 ml of glacial acetic acid and make up the volume to 1000 ml with water.

Hager's Reagent

Dissolve 20 g of picric acid in warm water (1000 ml). Allow the solution to cool down and use the supernatant

Wagner's Reagent

Dissolve 2 g of iodine and 6 g of potassium iodide in 100 ml of water.

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