

Protease Inhibitory Activity of Potato Peel Exerted by Antioxidants



A Thesis Submitted

By

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Examination Roll: Ms-170702

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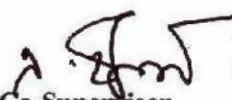
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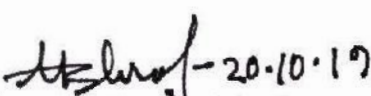
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DECLARATION

I hereby declare that this thesis report is original work except as otherwise stated. The materials presented have not been submitted in a whole or in a part for a degree from Khulna University or any other institutions. This thesis work will not be published anywhere without the concern of supervisor.

Md. Abdullah Al Munna

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Dedicated to
My beloved mother and father



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The Author

ABSTRACT

In vitro antioxidant potential of methanolic and water extracts of potato peels was evaluated. Total flavonoid, Total phenolics, tannin content, antibacterial activity and protease inhibitory activity of potato peels were also evaluated. Water and methanolic extract potato peel showed the free radical scavenging activity with IC₅₀ 181.70 µg/ml and 248.68µg/ml respectively. Ferric reducing antioxidant power was 351.81±0.016 (µM Fe (II)/g) and 301.89 ± 0.018 (µM Fe (II)/g), respectively in methanolic and water extract. Total phenol content was 85.13 ± 0.006 (mgGAE/g of methanolic extract) and 56 ± 0.003 (mgGAE/g of water extract). Total Flavonoid Content of methanolic and water extracts was 33.17 ± 0.002 (mgQE/g of extract) and 16.5 ± 0.002 (mgQE/g of extract), respectively. Total Tannin Content was 57.04 ± 0.007 (mgGAE/g of extract) and 43.29 ± 0.004 (mgGAE/g of extract), respectively in methanolic and water extract. In case of Antimicrobial activity of methanolic extract of potato peel showed maximum zone of inhibition against *Salmonella typhi* and *Vibrio cholera*. Water extract of potato peel showed maximum zone of inhibition against *Micrococcus* spp and *Salmonella paratyphi*. In protease inhibition assay methanolic extract showed protease inhibition activity with IC₅₀ 534.67 µg/ml and water extract extract showed protease inhibition activity with IC₅₀ 735.10 µg/ml. Column chromatography for enrichment of flavonoid content of methanolic fractions showed high amount of flavonoid content and high protease inhibitory activity than crud methanolic polar fractions. Thus, methanolic extract of potato peel is more potential than water extract.

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HAPTER 1

Introduction

1. Introduction

Natural sources are boundless inspiration for novel drug development because of the availability of promising drug candidates (Newman *et al.*, 2012). According to Food and Drug Administration (FDA), about 50% of approved drugs are derivatives of natural sources (Kingston, 2011; Chin *et al.*, 2006). Among these sources “phytochemicals” (derived from plants) have been the single most productive source of leads for the development of drugs. Plants synthesize these chemicals for their particular needs, especially for their defense and they may become a base and natural blueprint for the development of new drugs. These biologically active compounds would provide selective ligands for disease-related targets and influence the disease-related pathways and eventually shift the biological network from diseased condition to the healthy status (Clardy *et al.*, 2004; Jiangyong *et al.*, 2013). Chemical diversity may provide the core scaffolds for future drugs. During the last few decades, scientists paid more attention in the use of phytochemicals and their role as a basis for drug development. Plants have been used for health care the Sumerians. In India, these dates back several thousand years to the Rig-Veda, there are some of the earliest available documents detailing the medical knowledge that formed the basis of the Ayurveda system. Ancient China is also a source of information about the uses of plants as a medicine. Unani medicine with its Muslim and Greek roots is another widely practiced traditional medicine. Western medicine can be traced back to the Greek physician Hippocrates (460-377 BC). In the nineteenth century, scientists began purifying the active extracts from medicinal plants. Continuing this progress for many accounts such as population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases.

Today, leading pharmaceutical industries, research institutions and universities are searching for natural diversities for medically useful compounds. According to World Health Organization (WHO), 70-80% population relies on traditional plant based medicines for their primary health care needs and thus reduces the financial burden. More than two thirds of the world's plant species - at least 35,000 of which are estimated to have medicinal value come from the developing countries. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants. Researchers identified 122 compounds and widely used in modern medicine today (Interactive European Network, 2002-2005). There are various examples of development

of new drugs from the plant sources. Acetyldigoxin (*Digitalis lanata*) used as cardiogenic, Vinblastine and Vincristine (*Catharanthus roseus*) both used as antitumor and antileukemic agent, Glasiovine (*Ocotea glaziovii*) as antidepressant, Nordihydroguaiaretic acid (*Larrea divaricata*) as antioxidant and so many. During the years 2005–2007, 13 natural product related drugs were approved (Butler, 2008), such as Elliptinium for cancer, Galantamine and Huperzine-A for Alzheimer's disease. In 2008, clinical trials are ongoing on more than 100 natural product derived drugs (Harvey, 2008).

The potato is a starchy, tuberous crop from the perennial nightshade *Solanum tuberosum*. In many contexts, potato refers to the edible tuber. Potatoes were introduced to Europe in the second half of the 16th century by the Spanish. Today they are a staple food in many parts of the world and an integral part of much of the world's food supply. As of 2014, potatoes were the world's fourth-largest food crop after maize (corn), wheat, and rice. A raw potato is 79% water, 17% carbohydrates (88% is starch), 2% protein, and contains negligible fat. In an amount measuring 100 grams (3.5 oz), raw potato provides 322 kilojoules (77 kilocalories) of energy and is a rich source of vitamin B6 and vitamin C (23% and 24% of the Daily Value, respectively), with no other vitamins or minerals in significant amount. The potato is rarely eaten raw because raw potato starch is poorly digested by humans (Beazell *et al.*, 1993). When a potato is baked, its contents of vitamin B6 and vitamin C decline notably while there is little significant change in the amount of other nutrients (Nutritiondata.com).

Potatoes are often broadly classified as having a high glycemic index (GI) and so are often excluded from the diets of individuals trying to follow a low-GI diet. The GI of potatoes can vary considerably depending on the cultivar or cultivar category (such as "red", russet, "white", or King Edward), growing conditions and storage, preparation methods (by cooking method, whether it is eaten hot or cold, whether it is mashed or cubed or consumed whole), and accompanying foods consumed (especially the addition of various high-fat or high-protein toppings) (Fernandes *et al.*, 2005). Potato skins, along with honey, are a folk remedy for burns in India. Burn centers in India have experimented with the use of the thin outer skin layer to protect burns while healing (Mark Leyner and Billy Goldberg, 2005). Potato skins also contain B vitamins, calcium and other nutrients. And because potato skins contain no fat, no cholesterol

and no sodium, they can be part of a healthy weight loss diet; two potato skin halves contain just 114 calories.

Potato skins are a rich source of iron. In addition to transporting oxygen, iron helps make neurotransmitters and supports your immune system by regulating the growth of cells that fight pathogens. Potassium and magnesium carry electrical charges that stimulate muscle contraction and nerve transmission. Potassium maintains a regular heartbeat, while magnesium helps lower blood pressure by relaxing muscles in blood vessel walls.

Potato is a popular vegetable in Bangladesh. In many countries potato is cultivate instead of rice and wheat to meet the requirement of food and nutrition and also as a source of high carbohydrate but in Bangladesh Potato is cultivate as a general vegetable. About 40 developed countries potato is considered as main diet. To fulfill calories and food security potato is a cheap and available crop. In our country 102.15 lakh metric tons potato was produced in financial year of 2016-2017. According to the ministry of agriculture yearly demand of potato in our country is 70-77 lakh tons. In most of the countries potato peel is considered as a waste material. People consumed potato without its peel.

Therefore, the broad objective of this present study is to investigate the scientific basis of the medicinal uses and subsequently evaluating biological potential of potato peel. The following objectives were considered:

- ❖ Determination of antioxidant properties of potato peel using methanolic and water extracts.
- ❖ Investigation of antimicrobial activity of the potato peel against gram positive and gram negative bacteria by Disc diffusion method.
- ❖ Investigation of protease inhibition activity of the potato peel against trypsin enzyme.

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HAPTER 2

Literature Review



2.1 Literature Review of Potato

2.1.1 General Information

A potato is a root vegetable, the *Solanum tuberosum* is a small plant with large leaves. The part of the potato that people eat is a tuber that grows under the ground. A potato contains a lot of starch and other carbohydrates. The potato is originally from the high and cool areas of the Andes Mountains. It was grown as a food crop thousands of years ago. When Spanish conquistadores came to South America in the 1500s they took potatoes back to Europe.

2.1.1.1 Taxonomy of potato

Kingdom: Plantae – Plants

Phylum: Anthophyta – Flowering Plants

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Asteridae

Order: Solanales

Family: Solanaceae – Potato family

Genus: *Solanum* – Nightshade

Species: *Solanum tuberosum*

2.1.1.2 Common Name of Potato

Potato, Tater, Spud, Tuber

2.1.1.3 Geographical Distribution of potato:

The potato was first domesticated in the region of modern-day southern Peru and extreme northwestern Bolivia (Spooner et al., 2005) between 8000 and 5000 BC (Office of International Affairs, 1989). It has since spread around the world and become a staple crop in many countries. The earliest archaeologically verified potato tuber remains have been found at the coastal site of Ancon (central Peru), dating to 2500 BC. The most widely cultivated variety, *Solanum tuberosum*, is indigenous to the Chiloé Archipelago, and has been cultivated by the local indigenous people since before the Spanish conquest.

2.1.1.4 Use of the Plant

Potato tubers are a staple food source in temperate regions and are eaten after cooking. They may be cut or sliced and made into potato chips or fries. Potatoes can also be processed into starch, alcohol or flour.

2.1.1.5 Medicinal uses

Potato is a plant. The fleshy part of the root (potato) is commonly eaten as a vegetable. Potato is also used to make medicine. People take raw potato juice for stomach disorders and water retention (edema). A purified protein powder made from potato is mixed with water and used to control appetite for weight loss. Some people put raw potato directly on the affected area for arthritis, infections, boils, burns, and sore eyes.

In foods, potato is eaten, used as a source of starch, and fermented into alcohol. The Nutrition Facts Label says it all. Potatoes are:

- An excellent source of vitamin C.
- A good source of potassium (more than a banana).
- A good source of vitamin B6.
- Fat-, sodium- and cholesterol-free.

Potatoes are an excellent source of vitamin C (30% of the DV). Vitamin C is a water-soluble vitamin that acts as an antioxidant stabilizing free radicals, thus helping prevent cellular damage. It aids in collagen production; assists with iron absorption; and helps heal wounds and keep your gums healthy. Vitamin C may help support the body's immune system.

One medium potato with the skin contributes 2 grams of fiber or 7% of the daily value per serving. Dietary fiber is a complex carbohydrate and is the part of the plant material that cannot be digested and absorbed in the bloodstream. Soluble fiber may help with weight loss as it makes you feel full longer, and research has shown it also may help lower blood cholesterol.

Potatoes are a good source of vitamin B6 with one medium potato providing 10% of the recommended daily value. Vitamin B6 is a water-soluble vitamin that plays important roles in carbohydrate and protein metabolism. It helps the body make nonessential amino acids needed to make various body proteins; it is a cofactor for several co-enzymes involved in energy metabolism; and is required for the synthesis of hemoglobin – an essential component of red blood cells.

One medium potato provides 6% of the recommended daily value of iron. One medium potato with skin provides 620 milligrams or 15% of the recommended daily value (DV) of potassium per serving and is considered one of the best foods with potassium. Potatoes rank highest for foods with potassium and are among the top 20 most frequently consumed raw vegetables and fruits. Potassium is a mineral that is part of every body cell. It helps regulate fluids and mineral balance in and out of cells and in doing so, helps maintain normal blood pressure. Potassium is also vital for transmitting nerve impulses or signals, and in helping muscles contract. Potassium is a powerful dietary factor that may help lower blood pressure. Unfortunately, few Americans are getting the recommended 4700 milligrams per day of potassium they need. Potatoes are naturally fat free, cholesterol free, and low in sodium. In addition, potatoes are an excellent source of vitamin C, and those eaten with the skin are a good source of potassium. Foods that are good sources of potassium and low in sodium, such as potatoes, may reduce the risk of high blood pressure and stroke.

All varieties of potatoes are nutritious and, while both the type and amounts of nutrients may vary slightly depending on the variety, the differences are minimal. So minimal in fact, the FDA nutrition label for potatoes represents a composite of varieties (“market-basket approach”) based on typical US consumption patterns (i.e., 70% Russet, 18% white and 12% reds). Based on the FDA label the following claims can be made for the potato:

- An excellent source (> 20% of the DV) of vitamin C and potassium with skin

- A source (> 8% of the DV) of fiber with skin
- A good source (> 10% of the DV) of vitamin B6
- Low in sodium (< 140 mg/serving) and cholesterol
- Fat free (< .5 g fat/serving)

Processed potatoes (such as dehydrated and frozen potatoes) deliver the same nutrients as fresh potatoes, (such as potassium, vitamin C and fiber), but the amounts will vary depending on the form of potato.

Both sweet and white potatoes provide similar amounts of key nutrients including protein (2g and 3g respectively), potassium and vitamin B6, all of which contribute to a well-balanced, nutrient-dense diet.

Table 2.1: Nutrient content of Potato.

Nutrient	Russet (Baked w/skin)	Potato Skin (raw)
Calories	110	22
Total carbohydrate (g)	26	5
Fiber (g)	2	1
Fat (g)	0	0
Protein (g)	3	1
Vitamin C (mg)	27	4
Potassium (mg)	620	157

2.1.2 Biological and Chemical Investigation of potato peel

2.1.2.1 Bioassay related work

Singh and Rajini, (2004) worked on Inhibition of lipid peroxidation by potato peel extract, DPPH radical scavenging activity of potato peel, Reducing power of potato peel extract, Scavenging of superoxide radical by PPE, Hydroxyl radical scavenging activity of PPE, Ferrous ion-chelating effect of PPE. Nara *et al.* (2006) published a research paper on Ant oxidative activity of bound-form phenolic in potato peel.

Singh and Saldaña, (2011) investigated the extraction of eight phenolic compounds (Gallic acid, GAC; Chlorogenic acid, CGA; Caffeic acid, CFA; Protocatechuic acid, PCA; Syringic acid, SGA; p-hydroxyl benzoic acid, PBA; Ferulic acid, FRA and Coumaric acid,

CMA) from potato peel using subcritical water. Experiments were performed in a batch stainless steel reactor at 6 MPa, 2 mL/min and temperatures ranging from 100°C to 240°C at residence time of 30 to 120 min. High recoveries of phenolic compounds (81.83 mg/100 g; wet basis wb) were obtained at 180 °C and extraction time of 30 min compared to 3h extraction with methanol (46.59 mg/100 g wb). CGA (14.59 mg/100 g wb) and GAC (29.56 mg/100 g wb) were the main phenolic compounds obtained from potato peel at 180 °C. Subcritical water at 160 to 180 °C, 6 MPa and 60 min might be a good substitute to organic solvents such as methanol and ethanol to obtain phenolic compounds from potato peel.

De Sotillo *et al.*, (1994) work on extraction, identification and degradation phenolic in aqueous potato peel extract. According to their investigation phenolic were extracted from potato peel waste using water or methanol. Phenolic acids in the extracts were quantified by HPLC. The greatest amounts of phenolic acids resulted when potato homogenate was refluxed with water for 30 min. yielding a total concentration of 48 mg/100g. Four phenolic acids (chlorogenic, gallic, protocatechuic, and caffeic) were characterized as major components. Aqueous extracts were stored 20 days and after 7 days at 25°C exposed to light; chlorogenic acid had degraded to caffeic acid.

De Sotillo *et al.*, (1994) in another work they investigated stability and antioxidant activity of a freeze dried extract. They reported that, High performance liquid chromatography (HPLC) of the freeze dried extracts revealed that chlorogenic (50.31%), gallic (41.67%), protocatechuic (7.81%), and caffeic (0.21%) acids were the major phenolics. During 15 days storage of the freeze dried extract, no degradation of phenolic occurred. After 4 days storage at 63°C, 5.00g of sunflower oil containing either the freeze dried extract (200 ppm) or butylated hydroxyanisole (BHA) (200 ppm) reached peroxide values (PV) of 37.38 and 37.47 meq kg⁻¹ respectively. L-ascorbic acid-6-palmitate was the best antioxidant (PV= 10.65 meq kg⁻¹) but the freeze dried extract was as good as BHA.

De Sotillo *et al.*, (1994) published a paper about antimicrobial activity of potato peel. This paper reported that, Freeze dried water extract of potato peels (pp), containing chlorogenic, caffeic, gallic, and protocatechuic acids, was tested for effectiveness as a natural antioxidant. The pp was prepared from peel waste of Russet Burbank potatoes. Safety concerns regarding the use of pp in foods were investigated. The pp was examined for mutagenic activity using the in vitro Salmonella typhimurium- Escherichia coli microsome assay and found to be nonmutagenic. Plate

count of pp revealed <10 CFU/g. The antibacterial activity of the pp was species dependent. It was effective only at high concentration against gram negative and one gram positive bacteria but it was bacteriostatic.

Al-Saikhan *et al.*, (1995) reported Antioxidant activity and total phenolic in different genotypes of potato (*Solanum tuberosum*, L.). They reported that Antioxidant activity of potato compared with that of broccoli, onion, carrot and bell peppers was higher than all except broccoli. Patatin appeared to be the major water-soluble compound that showed antioxidant activity. The activity varied among potato cultivars, but was not related to flesh color or total phenolics. Antioxidant activity was evenly distributed within tuber parts and/or sections, except for skin tissue which had the greatest antioxidant activity and total phenolic content. Total phenolics varied among cultivars, with some containing twofold higher concentrations than other cultivars. Phenolic content differences were genotype dependent and not related to flesh color.

Onyeneho and Hettiarachchy, (1993) worked on Antioxidant activity, fatty acids and phenolic acids compositions of potato peels. They reported that the effects of freeze dried extracts from the peels of six potato varieties as antioxidants in pure soy oil were investigated using the active oxygen method (AOM) procedure, carotene bleaching and linoleic acid oxidation experiments on thin layer chromatography (TLC) plates. At 9 h under AOM conditions, 20 g soy oil treated with 0.05 g of these extracts attained lower peroxide values (PV, 22.0- 28.0 meq kg⁻¹) than the control oil sample (PV, 109.0 meq kg⁻¹) indicating very strong antioxidant activities. Oils treated with the same amount of tertiary butylhydroquinone, butylated hydroxyanisole-butylated hydroxytoluene mixture and rosemary extract attained PV of 10, 19.0 and 16.0 meq kg⁻¹, respectively. Results of the carotene bleaching and linoleic acid oxidation experiments on TLC plates indicated that the antioxidant activities of these extracts were due to the presence of phenolic acids. Among the phenolic acids separated and identified by both high-performance liquid chromatography and TLC, chlorogenic, proto-catechuic and caffeic acids were predominant and appeared to be mainly responsible for the strong antioxidant activities of the extracts. Peels from the red potatoes contained more polyphenols than those from the brown skinned varieties, but their oil contents were similar.

Habib and Shah, (2004) worked on Utilization of potato peels extract as a natural antioxidant in soy bean oil. They reported that Potato peel extract, as natural antioxidant was

evaluated during 60 days storage of refined soy bean oil at 25 and 45 °C. Free fatty acids (FFA), peroxide values (POV) and iodine values (IV) were used as criteria to assess the antioxidant activity of potato peel extract. Different organic solvents, including ethanol, methanol, acetone, hexane, petroleum ether and diethyl ether, were used to prepare extracts of potato peels. Maximum amount of extract (21%) was obtained with petroleum ether, followed by diethyl ether (15.25%) and methanol (14.75%). After 60 days' storage at 45 °C, soy bean oil, containing 1600 and 2400 ppm of petroleum ether extract of potato peels, showed lower values of FFA (0.120, 0.109%) and POVs (10.0, 9.0 meq/kg) than the control samples (FFA 0.320%, POV 59 meq/kg). Soy bean oil containing 200 ppm of BHA and BHT showed FFA values of 0.102 and 0.078%, whereas POVs were 8.0 and 6.0 meq/kg, respectively, after 60 days, storage at 45 °C. Similarly, after 60 days, storage at 45 °C, iodine values of soy bean oil containing 1600 and 2400 ppm of potato peel extract were 71 and 77, respectively, which were higher than the control samples of oil (58). However, iodine values for soy bean oil treated with 200 ppm of BHA and BHT were 80 and 84, respectively, after 60 days' storage at 45 °C. These results illustrate that potato peel extract, at various concentrations exhibited very strong antioxidant activity which was almost equal to synthetic antioxidants (BHA & BHT). Therefore, potato peel extract in oils, fats and other food products can safely be used as natural antioxidant to suppress lipid oxidation.

Hayase and Kato, (1984) published a paper on Antioxidative components of sweet potatoes. They reported the antioxidative activity of a 70% methanol extract of sweet potatoes was estimated in a linoleic acid=aqueous system. The extract had a markedly strong antioxidative activity. Major phenolic components contained in the 7% methanol extract were identified as chlorogenic acid and isochlorogenic acid-1, -2 and -3 by using high-performance liquid chromatography. The other minor free phenolics were identified, or tentatively identified, as caffeic acid and 4-o-caffeoylquinic acid. Chlorogenic acid and/or isochlorogenic acids, however, had only slight antioxidative activity. From the results of the addition of chlorogenic acid, isochlorogenic acids and the other coexisting components contained in the sweet potato extract, the effective antioxidant activity of the sweet potato extract was proposed to be mainly based on the synergistic effect of phenolic compounds with amino acids.

Koduvayur *et al.*, (2010) reported Antioxidant activity of potato peel extracts in a fish-rapeseed oil mixture and in oil in water emulsions. Their objectives were (a) to extract the

phenolic fraction from the peels of two Danish varieties of potatoes, viz. Sava and Bintje, and examine their antioxidant capacity in in-vitro systems (b) to evaluate the effect of these extracts on the storage stability of a fish-rape seed oil mixture and oil in water emulsions. Multiple antioxidant activity of the potato peel extracts was evident from in-vitro systems as they showed strong reducing power, radical scavenging ability, ferrous ion chelating activity and prevented oxidation in a liposome model system. The Sava variety, which showed strong antioxidant activity in in-vitro systems, was tested in oil and oil in water emulsions. Ethanolic extracts of Sava ($\geq 1,600$ mg/kg) prevented lipid oxidation in emulsions and in oil. Water extracts showed no antioxidant activity in oil whereas it showed pro-oxidant activity in emulsions. Thus, the results of the present study show the possibility of utilizing waste potato peel as a promising source of natural antioxidants for retarding lipid oxidation.

Kähkönen *et al.*, (1999) worked on Antioxidant activity of plant extracts containing phenolic compounds and the found Potato peel (*Solanum tuberosum*, *Rosamunda*) 4.3 ± 0.2 , potato, peel (*Solanum tuberosum*, *Matilda*) 2.5 ± 0.1 total phenolics (mg of GAE/g dw).

Kanatt *et al.*, (2005) investigated natural antioxidant from Potato peel extract for retarding lipid peroxidation in radiation processed lamb meat. They reported that Potato peel extract (PPE) exhibited high phenolic content (70.82 mg of catechin equivalent/100 g), chlorogenic acid (27.56 mg/100 g of sample) being the major component. The yield of total phenolics and chlorogenic acid increased by 26 and 60%, respectively, when the extract was prepared from γ irradiated (150 Gy) potatoes. PPE showed excellent antioxidant activity as determined by β -carotene bleaching and radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The suitability of PPE for controlling lipid oxidation of radiation processed lamb meat was also investigated. PPE (0.04%) when added to meat before radiation processing was found to retard lipid peroxidation of irradiated meat as measured by TBA number and carbonyl content. The antioxidant activity of PPE was found to be comparable to butylated hydroxytoluene (BHT).

Farvin *et al.*, (2012) examine the utilisation of potato peel, a waste material, as a source of natural antioxidants for retarding lipid and protein oxidation in minced mackerel. Mackerel mince with two different concentrations (2.4 or 4.8 g/kg) of water or ethanol extracts of potato peel and a control with no added extracts were prepared. The samples were stored at 5

°C for 96 h and the sampling was done at time points 0, 24, 48 and 96 h. The ethanol extracts, which contained high amounts of phenolic compounds, was found to be very effective in retarding lipid and protein oxidation as it resulted in low levels of peroxide value, volatiles, carbonyl compounds and protected against the loss of α -tocopherol and tryptophan and tyrosine residues. Water extracts was less efficient especially at higher concentrations, which might be due to lower phenolic content or due to the pro-oxidative nature of some of the phenolic acids/co-extracted compounds.

Mohdaly *et al.*, (2010) investigated Antioxidant properties of various solvent extracts of potato peel, sugar beet pulp and sesame cake. They found among the six solvents tested, methanol gave the highest extract yield of potato peel and sugar beet pulp, while diethyl ether gave the highest extract yield of sesame cake. Methanol exhibited the highest extraction ability for phenolic compounds, with total phenolics amounting to 2.91, 1.79 and 0.81 mg gallic acid equivalent g⁻¹ dry weight in potato peel, sugar beet pulp and sesame cake extracts respectively, and also showed the strongest antioxidant capacity in the three assays used. All three methods proved that potato peel extract had the highest antioxidant activity owing to its high content of phenolic compounds and flavonoids.

Amado *et al.*, (2014) worked on Optimization of antioxidant extraction from *Solanum tuberosum* potato peel waste by surface response methodology. This study reports the optimized conditions (temperature, ethanol concentration and processing-time) for antioxidant extraction from potato peel (Agria variety) waste. At short extraction times (34 min), optimal yields of phenolic (TP) and flavonoid (Fv) compounds were reached at 89.9 °C and ethanol concentrations of 71.2% and 38.6%, respectively. The main phenolic compounds identified in the extracts were chlorogenic (Cl) and ferulic (Fer) acids. A significant positive correlation was found between antioxidant activity and TP, Fv, Fer and Cl responses. Potato peel extracts were able to stabilize soybean oil under accelerated oxidation conditions, minimizing peroxide, totox and p-anisidine indices. The production of hexanal and 2-hexenal in soybean oil samples was maximal for extracts obtained at intermediate temperatures and ethanol concentrations. Our results demonstrate potato peel waste is a good source of antioxidants able to effectively limit oil oxidation, while contributing to the revalorization of these agro food by-products.

Kanatt *et al.*, (2005) investigate the ability of PPE to protect erythrocytes against oxidative damage, *in vitro*. The protection rendered by PPE in erythrocytes was studied in terms of resistance to oxidative damage, morphological alterations as well as membrane structural alterations. The total polyphenolic content in PPE was found to be 3.93 mg/g powder. The major phenolic acids present in PPE were predominantly: gallic acid, caffeic acid, chlorogenic acid and protocatechuic acid. We chose the experimental prooxidant system: FeSO₄ and ascorbic acid to induce lipid peroxidation in rat RBCs and human RBC membranes. PPE was found to inhibit lipid peroxidation with similar effectiveness in both the systems (about 80–85% inhibition by PPE at 2.5 mg/ml). While PPE *per se* did not cause any morphological alteration in the erythrocytes, under the experimental conditions, PPE significantly inhibited the H₂O₂-induced morphological alterations in rat RBCs as revealed by scanning electron microscopy. Further, PPE was found to offer significant protection to human erythrocyte membrane proteins from oxidative damage induced by ferrous–ascorbate. In conclusion, our results indicate that PPE is capable of protecting erythrocytes against oxidative damage probably by acting as a strong antioxidant.

Singh *et al.*, (2008) reported Protective effect of potato peel extract against carbon tetrachloride-induced liver injury in rats. They investigate its potential to offer protection against acute liver injury in rats. Rats pretreated with PPE (oral, 100 mg/kg b.w./day for 7 days) were administered a single oral dose carbon tetrachloride (CCl₄, 3 ml/kg b.w., 1:1 in groundnut oil) and sacrificed 8 h of post-treatment. Hepatic damage was assessed by employing biochemical parameters (transaminase enzyme levels in plasma and liver (AST—aspartate transaminase; ALT—alanine transaminase, LDH—lactate dehydrogenase)). Further, markers of hepatic oxidative damage were measured in terms of malondialdehyde (MDA), enzymic antioxidants (CAT, SOD, GST, GPX) and GSH (reduced glutathione) levels. In addition, the CCl₄-induced pathological changes in liver were evaluated by histopathological studies. Our results demonstrated that pretreatment of rats with PPE significantly prevented the increased activities of AST and ALT in serum prevented the elevation of hepatic MDA formation as well as protected the liver from GSH depletion. PPE pretreatment also restored CCl₄-induced altered antioxidant enzyme activities to control levels. The protective effect of PPE was further evident through the decreased histological alterations in liver. Our findings provide evidences to

demonstrate that PPE pretreatment significantly offsets CCl₄-induced liver injury in rats, which may be attributable to its strong antioxidant propensity.

Lewis *et al.*, (1998) determine anthocyanins, flavonoids and phenolic acids in potatoes. They reported that the major anthocyanins, flavonoids and phenolic acids in the tubers (skin and flesh), flowers and leaves of 26 cultivars of *Solanum tuberosum* L with coloured skins and/or flesh have been identified and quantified using analytical HPLC. Red tubers contained mostly pelargonidin-3-(p-coumaroyl-rutinoside)-5-glucoside (200–2000 µg g⁻¹ FW) plus lesser amounts of peonidin-3-(p-coumaroyl-rutinoside)-5-glucoside (20–400 µg g⁻¹ FW). Light to medium purple tubers contained petunidin-3-(p-coumaroyl-rutinoside)-5-glucoside (1000–2000 µg g⁻¹ FW) plus small amounts of malvidin-3-(p-coumaroyl-rutinoside)-5-glucoside (20–200 µg g⁻¹ FW) whilst dark purple–black tubers contained similar levels of petunidin-3-(p-coumaroyl-rutinoside)-5-glucoside together with much higher concentrations of malvidin-3-(p-coumaroyl-rutinoside)-5-glucoside (2000–5000 µg g⁻¹ FW). Tuber flesh also contained chlorogenic acid (30–900 µg g⁻¹ FW) and other phenolic acids plus low concentrations of flavonoids (0–30 µg g⁻¹ FW). Tuber skins showed much higher levels (1000–4000 µg g⁻¹ FW) of chlorogenic acid. The major anthocyanins in flowers were present as the rutinosides or other glycosides of pelargonidin, petunidin and malvidin whilst glycosides of cyanidin and delphinidin were found in some flowers, together with many of the same phenolic acids as found in tubers. The commonest flavonoids included rutin, kaempferol-3-rutinoside and two quercetin-rhamnose-glucosides. Flowers and leaves contained higher concentrations of flavonoids which fell into two patterns, with some cultivars containing high concentrations of quercetin-3-glycosides, whilst others had much lower concentrations.

Wu *et al.*, (2012) work on Isolation, identification and quantification of unsaturated fatty acids, amides, phenolic compounds and glycoalkaloids from potato peel. Eleven compounds were isolated from potato peels and identified. Their structures were determined by interpretation of UV, MS, 1D, and 2D NMR spectral data and by comparison with reported data. The main components of the potato peels were found to be chlorogenic acid and other phenolic compounds, accompanied by 2 glycoalkaloids, 3 low-molecular-weight amide compounds, and 2 unsaturated fatty acids, including an omega-3 fatty acid. The potato peels showed more potent

radical scavenging activity than the flesh. The quantification of the 11 components indicated that the potato peels contained a higher amount of phenolic compounds than the flesh.

2.1.2.2 Compound isolation related work

Compounds isolated and described from various varieties of potatoes are summarized in table 2.2

Table 2.2: Compounds isolated and described from various variety of potatoes.

Extraction System	Analytical Technique	Potato Cultivar	Described Phenolic Compounds	References
Solid-liquid extraction	HPLC-DAD	Kufri	Chlorogenic acid, caffeic	Kanatt <i>et al.</i> ,
	HPLC UV-Vis	9 italian cultivars (Agata, Primura, Arinda, Merit, Marabel, Jelli, Frinka, Sponta, Agria)	Chlorogenic acid	Finotti <i>et al.</i> , (2006)
	HPLC-MS	Ranger Russet, Norkotah Russet	Neochlorogenic acid, chlorogenic acid, caffeic acid, quercetin-3-o-glu-rut, rutin, kaempferol-3-o rutinoside, cryptochlorogenic acid, quinic acid	Shakya and Navarre, (2006)
	HPLC-DAD, HPLC-MS, HPLC-FLD	23 Native Andean cultivars	Chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, caffeic acid, protocatechuic acid, vanillic acid, ferulic acid, petanin, rutin, kaempferol-3-o-rutinoside	Andre <i>et al.</i> , (2007)

	HPLC-DAD	320 specialty potato genotypes	Chlorogenic acid, caffeic acid, gallic acid, catechin	Külen <i>et al.</i> , (2013)
	Not cited	'Russet Burbank'	Chlorogenic acid, ferulic acid, vanillic acid, caffeic acid, benzoic acid	Yang and Bernards, (2007)
Solid-liquid extraction	HPLC-MS	Jasim, Atlantic, Jawan, Superior, Jopung	Chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, trans-cinnamic acid	Im <i>et al.</i> , (2008)
	HPLC-DAD	Nicola, Sieglinde F, Isci 4052, Isci 67	Chlorogenic acid, caffeic acid, ferulic acid, catechin	Leo <i>et al.</i> , (2008)
	HPLC	Not cited (Indian cultivar)	Gallic acid, caffeic acid, chlorogenic acid, protocatechuic acid	Singh and Rajini, (2008)
	HPLC-DAD	13 native Andean genotypes	Neochlorogenic acid, cryptochlorogenic acid, chlorogenic acid, kaempferol-3-o-rutinoside, quercetin	André <i>et al.</i> , (2009)
	HPLC	Karlana	Gallic acid, neochlorogenic acid, protocatechuic acid, catechin, cryptochlorogenic acid, chlorogenic acid, vanillic acid, caffeic acid, ferulic acid, p-coumaric acid	Mäder <i>et al.</i> , (2009)
	HPLC UV-Vis	Siecle, Purple Majesty, Dakota pearl, FL 1533, Vivaldi, Yukon gold	Chlorogenic acid, caffeic acid	Al-Weshahy and Venket Rao, (2009)

Solid-liquid extraction	HPLC-DAD, HPLC-MS	Goldrosh, Nordonna, Dakota Pearl, Norkotah, Red Nordland, Sangre, Viking, Dark Red Nordland	Chlorogenic acid, caffeic acid, gallic acid, ferulic acid, catechin, p-coumaric acid, o-coumaric acid	Xu <i>et al.</i> , (2009)
	HPLC-DAD	8 cultivars	Chlorogenic acid, caffeic acid, epicatechin, p-coumaric acid, vanillic acid, quercetin	Blessington <i>et al.</i> , (2010)
	HPLC-DAD	Sava, Bintje	Protocatechuic acid, gentisic acid, gallic acid, chlorogenic acid, salicylic acid, caffeic acid, ferulic acid, p-coumaric acid	Koduvayur Habeebullah <i>et al.</i> , (2010)
	HPLC-DAD-MS	Bintje, Piccolo, Purple Majesty	Chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, kaempferol rutinose, rutin	Navarre <i>et al.</i> , (2010)
	HPLC-DAD/APCI-MS	16 cultivars	Chlorogenic acid, caffeic acid, 3-o-caffeoylquinic acid, 1-o-caffeoylquinic acid	Garcia-Salas <i>et al.</i> , (2010)
Solid-liquid extraction	HPLC-DAD-MS	13 Italian cultivars	5-o-caffeoylquinic acid, 4-o-caffeoylquinic acid, 3-o-caffeoylquinic acid, ferulic acid, anthocyanins	Ieri <i>et al.</i> , (2011)
	UPLC-MS	Purple Majesty, Yukon gold, Atlantic	Chlorogenic acid, caffeic acid, ferulic acid, sinapic acid	Madiwale <i>et al.</i> , (2011)

	HPLC-DAD-MS	50 cultivars	Chlorogenic acid, rutin, kaempferol-3-rutinoside	Navarre <i>et al.</i> , (2011)
	UPLC-DAD	Vitelotte, Luminella, Charlotte, Bintje	Chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, syringic acid, vanillic acid, catechin, rutin, kaempferol-3-o-rutinoside	Deußer <i>et al.</i> , (2012)
	HPLC-DAD	Sava	Gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, vanillic acid, syringic acid, caffeic acid, salicylic acid, p-coumaric acid, ferulic acid	Habeebullah <i>et al.</i> , (2012)
	HPLC-DAD	Not cited	Chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, coumaric acid, genistin, quercetin-3- β -D-galactoside, naringin, naringenin, luteolin, genistein, kaempferol, flavan-3-ol	Wallis <i>et al.</i> , (2012)
Solid-liquid extraction	UPLC-MS	Not cited	chlorogenic acid, quinic acid, caffeic acid, methyl caffeate	Wu <i>et al.</i> , (2012)
	HPLC-DAD-MS	15 Colombian cultivars	Chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, caffeic acid	Mulinacci <i>et al.</i> , (2008)
	HPLC UV	Agria	Chlorogenic acid, ferulic acid, gallic acid	Amado <i>et al.</i> , (2014)

	HPLC UV	Valfi, Blaue Elise, Bore Valley, Blue Cango	Chlorogenic acid, caffeic acid, ferulic acid, coumaric acid, cryptochlorogenic acid, neochlorogenic acid, p-coumaric acid	Rytel <i>et al.</i> , (2014)
Ultrasound- assisted extraction	HPLC-DAD	Nicola, Timo, Siikli, Rosamund, Van Gogh	Chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, vanillic acid, syringic acid	Mattila and Hellström, (2007)
Ultrasound- assisted extraction	HPLC-DAD	Agria	Protocatechuic acid, chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid	Barba <i>et al.</i> , (2008)
	HPLC-DAD	20 potato cultivars	Chlorogenic acid, petunidin- 3-glucoside chloride, pelargonidin-3 glucopyranoside	Ji <i>et al.</i> , (2012)
	HPLC-MS	Purple, Innovator, Russet, Yellow	Chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid	Albishi <i>et al.</i> , (2013)
	HPLC-DAD	Penta, Marcy	Chlorogenic acid, caffeic acid, gallic acid, p-coumaric acid, ferulic acid	Al-Weshahy <i>et al.</i> , (2013)
	HPLC-DAD	Diamond	Chlorogenic acid, caffeic, 4- hydroxybenzoic, p coumaric, and trans-o- hydroxycinnamicacids	Mohdaly <i>et al.</i> , (2013)
	HPLC- DAD-MS	Blue Bell, Melody	Chlorogenic acid, caffeic acid, quinic acid, ferulic acid, cryptochlorogenic acid, rutin	López-Cobo <i>et al.</i> , (2014)

	HPLC-MS	Russet	Chlorogenic acid, caffeic acid, neochlorogenic acid	Sánchez Maldonado <i>et al.</i> , (2014)
Ultrasound-assisted extraction	RP-HPLC UV-DAD	BP1	Chlorogenic acid, caffeic acid, ferulic acid	Ngadze <i>et al.</i> , (2014)
	HPLC-DAD	Netherlands #7	Gallic acid, protocatechuic acid, chlorogenic acid	Wang <i>et al.</i> , (2015)
Microwave-assisted extraction	HPLC-UV	Red	Chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid, syringic acid, ferulic acid, coumaric acid	Singh and Saldaña, (2011)
	HPLC-DAD	Calwhite	Chlorogenic acid, caffeic acid, neochlorogenic acid, cryptochlorogenic acid, ferulic acid, p-coumaric acid	Wu <i>et al.</i> , (2012)
Pressurized liquid extraction (PLE) + solid-liquid extraction	HPLC-DAD	Lady Claire	Caffeic acid	Wijngaard <i>et al.</i> , (2012)
	HPLC-UV	Red	Gallic, chlorogenic and syringic acid	Alvarez <i>et al.</i> , (2014)

Here, HPLC: High Performance Liquid Chromatography; UPLC: Ultra Performance Liquid Chromatography; DAD: Diode Array Detector; UV: Ultraviolet detector; MS: mass spectrometer.

2.2 Literature Review of tested procedure

2.2.1 Anti-oxidant

Oxidation is a type of chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals that are highly active to react with other molecules. These free radicals can start chain reactions (Sies & Helmut, 1997). These radicals are important part of life, which are produced during cellular metabolism and functional activities and these radicals have important roles in cell signaling, apoptosis, gene expression and ion transportation. Although oxidation reactions are crucial for life, they can also be damaging (Boonchoong *et al.*, 2004). Excessive free radicals attack bases in nucleic acids, amino acid side chains in proteins and double bonds in unsaturated fatty acids, cause oxidative stress, which can damage DNA, RNA, proteins and lipids resulting in an increased risk for cardiovascular disease, cancer, autism and other diseases. Antioxidant is a molecule that inhibits the oxidation of other molecules. Cells are normally able to defend themselves against free radicals damage through the use of intracellular enzymes to keep the homeostasis of free radicals at a low level (Figure 3.1). However, during times of environmental stress and cell dysfunction, free radicals levels can increase dramatically, causes significant cellular damage in the body. In order to prevent or reduce the free radicals-induced oxidative damage, the human body and other organisms have developed an antioxidant defense system that includes enzymatic, metal-chelating and free radical-scavenging activities to neutralize these radicals after they have formed. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body (Giles *et al.*, 2002).

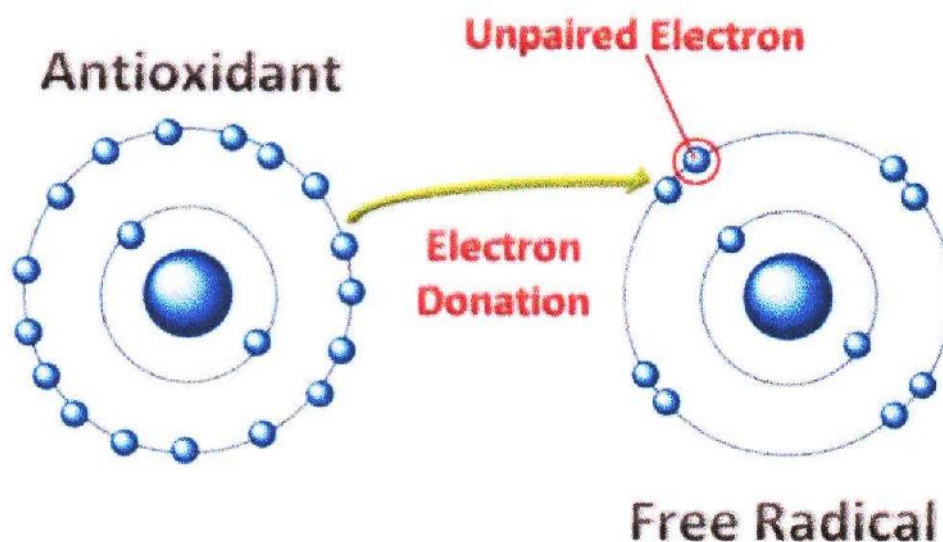


Figure 3.1: Mechanism and action of antioxidants.

Antioxidants can neutralize free radicals by accepting or donating electrons to eliminate the unpaired condition of the radical. The antioxidant molecules may directly react with the reactive radicals and destroy them, while they may become new free radicals which are less active, longer-lived and less dangerous than those radicals they have neutralized. They may be neutralized by other antioxidants or other mechanisms to terminate their radical status. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. Many antioxidants have aromatic ring structures and are able to delocalize the unpaired electron. For example, Vitamin C ($\text{AscH}^\cdot$) in the aqueous phase and vitamin E (TOH) in the lipid phase will directly react with or neutralize hydroxyl, alkoxyl ($\text{LO}^\cdot$) and lipid peroxy ($\text{ROO}^\cdot$) radicals and form H_2O , alcohol and lipid hydroperoxides, (Figure 3.2) respectively. Vitamin E itself becomes a phenyl radical ($\text{TO}^\cdot$) and vitamin C turns to a very stable radical ($\text{Asc}^{\cdot-}$), due to its delocalized structure. Furthermore, vitamin C can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical and regenerate these antioxidants. Vitamin C itself is readily regenerated from $\text{Asc}^{\cdot-}$ with NADH or NADPH-dependent reductases (Hossain *et al.*, 1985). Many antioxidants may directly react

with free radical intermediates induced by reactive oxygen species and terminate the chain reaction, thereby stopping the free radical-induced damage (DeFeudis *et al.*, 2003).

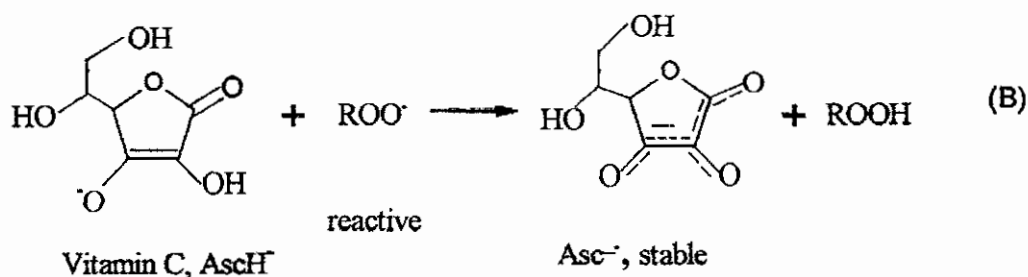
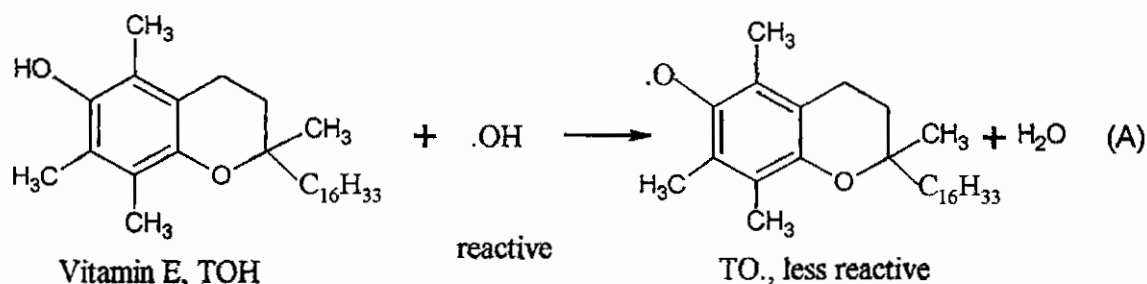


Figure 3.2: Direct reactions of vitamin E (TOH) with hydroxyl radical (A) and vitamin C (AscH⁺) with lipid peroxyl radical (ROO⁺) (B), and regeneration of vitamin E from vitamin C (C).

Small molecules such as vitamin C, vitamin E, uric acid and glutathione play important roles as cellular antioxidants. Synthetic antioxidants such as tert-butylhydroxyl-toluene, tert-butylhydroxyanisole and tert-butylhydroquinone have been widely used in the food industry to retard lipid oxidation. However, such synthetic antioxidants are not preferred for pharmacologic use due to toxicological concerns. Thus, more and more interests have focused on identifying

plant extracts to use as dietary antioxidant supplements (Rababah *et al.*, 2004). Many research models have been established in chemical or biological systems for the studies of antioxidant activity as well as identification of new antioxidants especially from natural substances. Generally, the antioxidant ability was measured and presented as 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging activity, total antioxidant activity, total antioxidant potentials, ferric chloride reducing power, trolox equivalent antioxidant capacity, total radical absorption potentials and oxygen radical absorption capacity. These methods are based on either a single-electron transfer reaction or a hydrogen atom transfer reaction from an antioxidant or oxidant to a free radical. The change of optical absorbance of either antioxidant or oxidant is measured for the antioxidant capability.

2.2.1.1 DPPH Free Radical Scavenging Assay

2.2.1.2 Principle

A simple, rapid and inexpensive method to measure antioxidant capacity of sample involves the use of free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sharma & Bhat, 2009). It is a dark-colored crystalline powder composed of stable free-radical molecules and a trap ("scavenger") for other radicals. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. Antioxidants reduce 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound). The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured (Figure 3.3) (Cuvelier *et al.*, 1992).

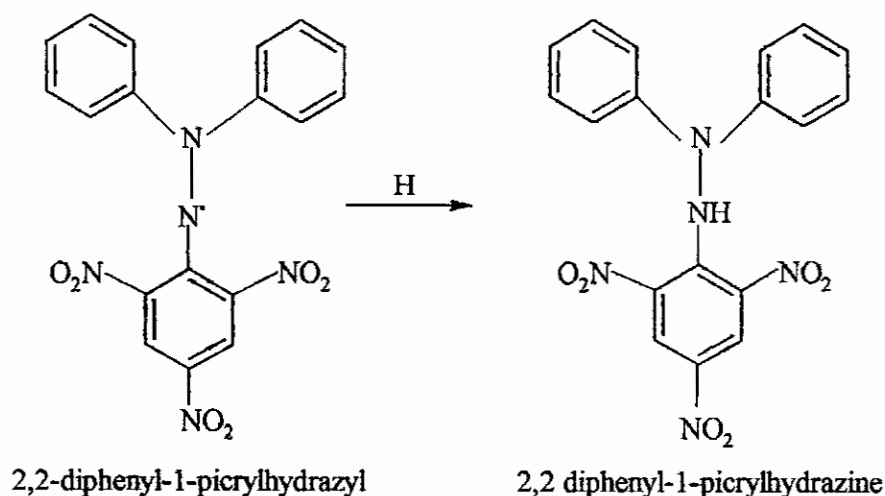


Figure 3.3: Reaction between DPPH and antioxidant.

2.2.2 FRAP (Ferric ion Reducing Antioxidant Power) Assay

Ferric ion reducing antioxidant power (FRAP, also Ferric reducing ability of plasma) is a simple test to determine the total antioxidant power of sample. The FRAP assay was first performed by Iris Benzie and J. J. Strain of the Human Nutrition Research Group at the University of Ulster, Coleraine. It is often used to measure the antioxidant capacity of plant extract, foods, beverages and nutritional supplements containing polyphenols (Benzie *et al.*, 1996).

2.2.2.1 Principle

The FRAP assay (ferric ion reducing antioxidant power) evaluates total antioxidant power and is chosen to assess the presumable effects of medicinal plants (Szollosi *et al.*, 2002). FRAP assay depends upon the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by the action of electron donating antioxidants at low pH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm. The FRAP assay was determined according to the method of Benzie and Strain (1996) (Benzie *et al.*, 1996).

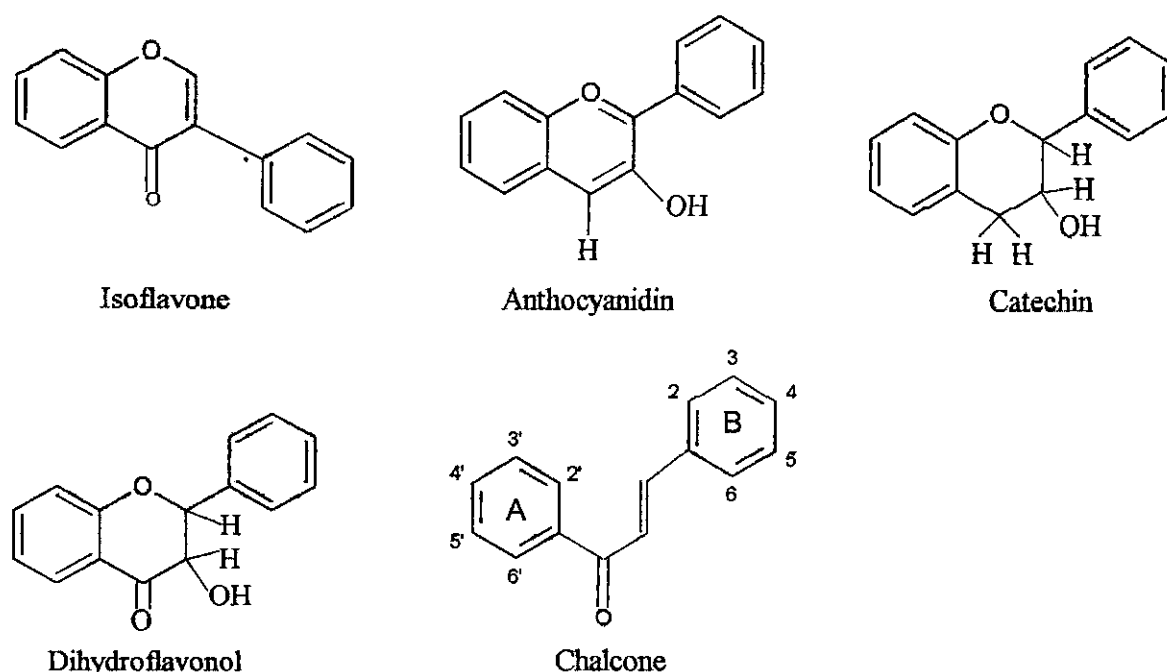


Figure 3.5: Chemical structure of different types of flavonoids (Cook *et al.*, 1996).

In plants, flavonoids are often present as O-glycosides or C-glycosides. The O-glycosides possess sugar substituent bound to $-OH$ of aglycone, usually at position 3 or 7, whereas, C-glycosides possess sugar groups bound to Carbon of aglycone usually 6-C or 8-C (Buhler & Miranda, 2000).

Flavonoids are phenolic substances isolated from a wide range of plants, with over 8000 individual compounds known. They act in plants as photoreceptors, visual attractors, feeding repellants and for light screening (Pietta, 2000). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. Many studies have suggested that flavonoids exhibit biological activities, including antibacterial, antiallergenic, antiviral, anti-inflammatory (Cushnie *et al.*, 2005, Cook *et al.*, 1996, Murray, 1998), cytotoxic, antitumour, treatment of neurodegenerative diseases and vasodilating actions (Murray, 1998, Williams *et al.*, 2004, Tsuchiya *et al.*, 2010, Chebil *et al.*, 2006). In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation (Cook *et al.*, 1996, Chebil *et al.*, 2006,

Middleton *et al.*, 2000). These are also reported to inhibit variety of enzymes like hydrolases, hyaluronidase, alkaline phosphatase, arylsulphatase, cAMP phosphodiesterase, lipase, α -glucosidase, kinase (Narayana *et al.*, 2001). However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers (Pal *et al.*, 2009). This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available “H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure (Tripoli *et al.*, 2007). Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction (Heim *et al.*, 2002) as shown in fig. 3.6:

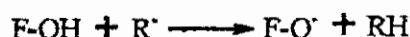


Figure 3.6: Scavenging capacity of free radical (R.)

Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical as in fig (V), thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain (Cook *et al.*, 1996, Ferreira *et al.*, 2010).

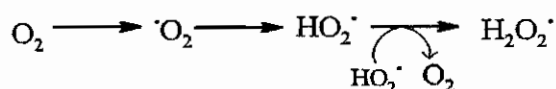


Figure 3.7: Formation of peroxy radical

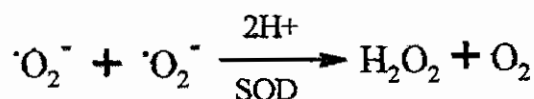


Figure 3.8: Mechanism catalysed by SOD

According to kinetic studies of aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is linked to its three structural groups as shown in fig.

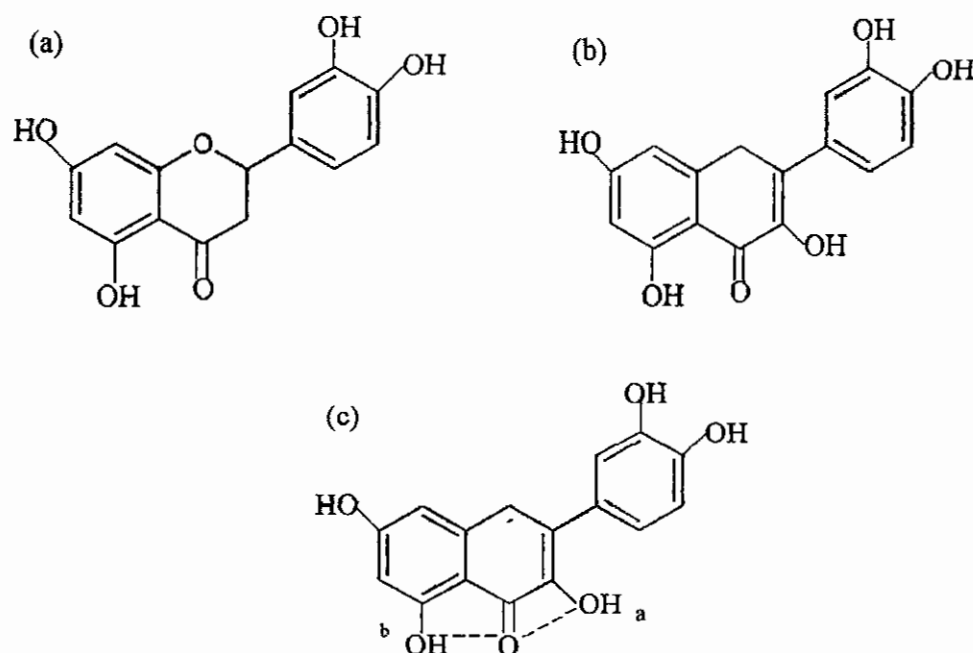


Figure 3.9: Structural groups responsible for antioxidant activity (Tripoli *et al.*, 2007).

1. The ortho-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in electron dislocation.
2. The 2,3-double bond, in conjugation with a 4-oxo function, responsible for electron dislocation from the B-ring.
3. The presence of both 3-(a)-and 5-(b)-hydroxyl groups (Fig.VII(c)).

3',4'-catechol structure in B-ring strongly enhances lipid peroxide inhibition and this arrangement is an important characteristic of most potent scavengers of peroxyl, superoxide and peroxynitrite radicals (Heim *et al.*, 2002) and its absence decreases antioxidant activity. The absence of the hydroxyl group at position 3 in flavanones and flavones decreases their antioxidant ability (Tripoli *et al.*, 2007). The content of flavonoids in the examined plant extracts

was determined using spectrophotometric method. Total flavonoids were determined following the procedure by Dewanto, Wu, Adom, and Liu, (2002) (Dewanto *et al.*, 2002).

2.2.4.1 Principle

Flavonoids in the sample extracts react with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm (Tsuchiya *et al.*, 2010).

2.2.5 Total Tannin Content

Tannins are a complex group of polyphenolic compounds found in a wide range of plant species as secondary metabolites which have been known and used by man for centuries. Their name comes from the French '*tan*' meaning the bark of the holm oak and other trees used in tanning (Harborne, 1999, Schofield, 2001). Tannins are a heterogeneous group of high molecular weight phenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals etc (Figure 3.10 and 3.11) (McLeod, 1974, Mole *et al.*, 1987, Mangan, 1988, Mueller *et al.*, 1992, Van soest, 1994, Giner, 1996, Schofield, 2001). Tannins play an important role in plant function and evolution (Cates & Rhoades, 1977, Zucker, 1983). Tannins found in plants are divided into two major classes: the condensed and the hydrolysable tannins.

Hydrolyzable tannins (HT) are further grouped into gallotannins and ellagitannins that are composed of gallic acid or hexahydroxydiphenic acid esters, respectively, linked to a sugar moiety. More complex hydrolyzable tannins can form by oxidative transformations which can yield macrocyclic ellagitannins.

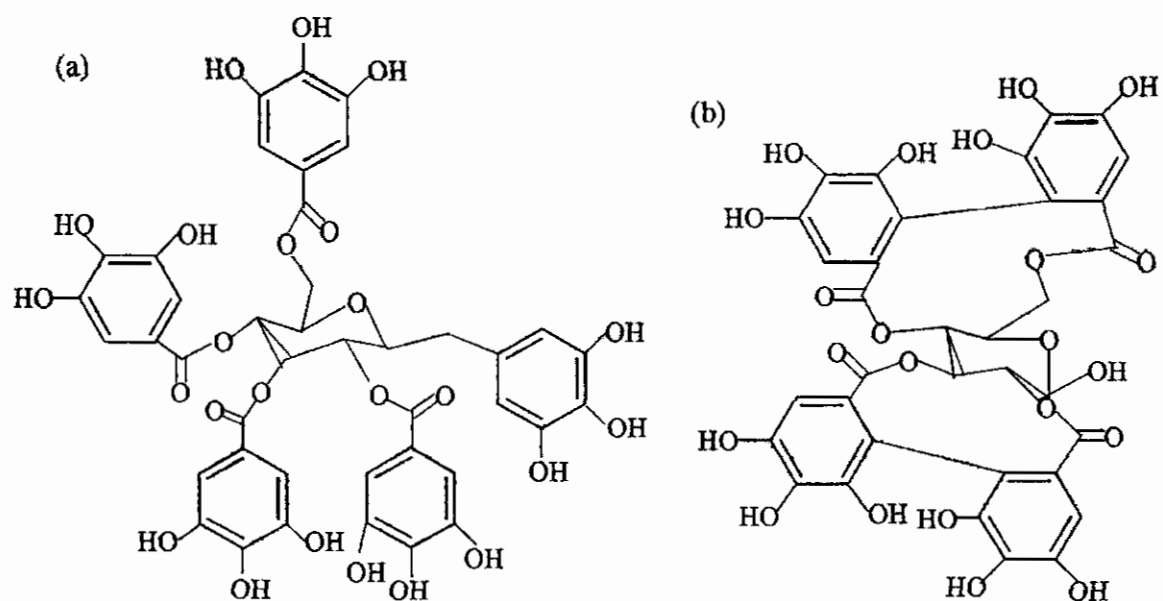


Figure 3.10: Hydrolyzable tannins (a) a simple gallotannin and (b) a simple ellagitannin.

Condensed tannins (CT) also referred to as proanthocyanidins are polymers of three-ring flavanols joined with C-C bonds. The monomer units that make up CT are distinguished by the number of OH groups on the B-ring: procyanidins (PC) have a di-hydroxy B-ring while prodelphinidins (PD) have a tri-hydroxy B-ring. The monomer units may also have either *cis* or *trans* C2–C3 stereochemistry (Okuda *et al.*, 2000).

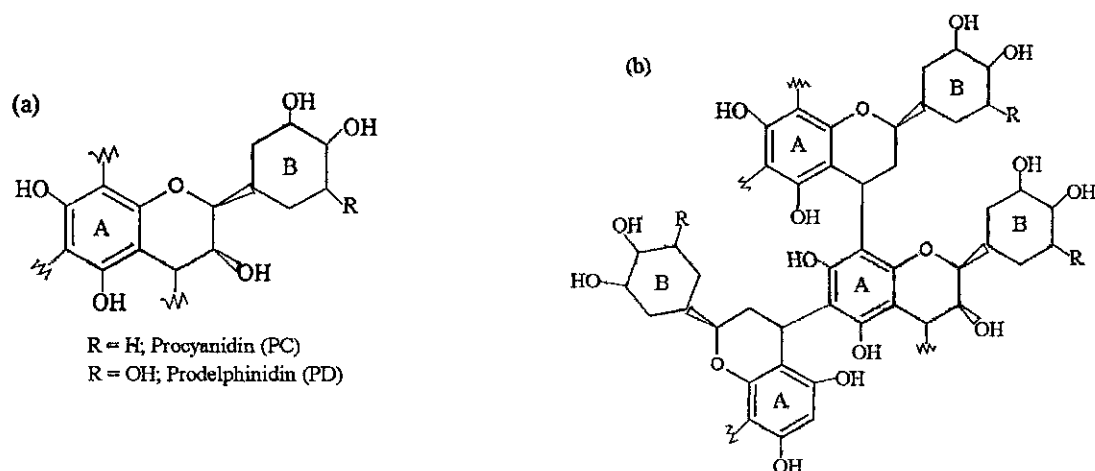


Figure 3.11: (a) a condensed tannin trans monomer unit, (b) a condensed tannin trimer showing different intermonomer linkages (C4–C8 and C4–C6) and different *cis/trans* monomers.

The tannins of different plant species have different physical and chemical properties (Mangan, 1988) and therefore they have very diverse biological properties (Zucker, 1983). Gymnosperms and monocots produce only CT, dicots can produce either CT or HT or a mixture of both tannin types (Bate-Smith, 1977; Haslam, 1988). It is important to note that even within CT and HT there is a wide range of structural variation (Okuda *et al.* 2000; Porter 1989; Porter 1992). Differences between individual tannins arise from variations in chain length, number of OH groups, position of intermonomer linkages (e.g., C4–C6 or C4–C8 linkages in CT, C–C or ether bonds in HT), stereochemistry, branching extent, glycosylation and substitutions with aliphatic or carbohydrate moieties. Tannins composed of both procyanidins and ellagitannins are also known (Okuda *et al.*, 2000; Porter, 1992). Thus there is an enormous diversity in tannins, and the larger the polymer the greater the probability of variation (Behrens, 2003). Most plants produce a variety of tannin structures (Polshettiwar, 2007), suggesting that within individual plants there are distinctive roles for specific tannin compounds (Hagerman, 1998, Zucker, 1983). Content of tannins in plant extract was determined by Folin-Denis method (Polshettiwar *et al.*, 2007).

2.2.5.1 Principle

The method is based on the oxidation of molecule containing a $-OH$ groups. The tannin and tannin like compound reduce Phosphotungstomolybdic acid in alkaline solution to produce a highly blue colored solution (Cetkovic *et al.*, 2008, Jain & Dixit, 2004).

2.3 Evaluation of Antibacterial Activity

There is constant interaction between microbes and humans in all walk of human life. The vast majority of the microbes in the body are rendered harmless by the protective effects of the immune system and few are health beneficial. Microbes have been the causes of many diseases in the human being and other animals. Infectious disease development involves the complex interactions between the microbe and the host. Microbes produce disease by killing host cells or by liberating toxins that can cause tissue damage and functional derangements even without extensive colonization of host tissues. So, we need to protect ourselves from these to lead a healthy life. The capacity of the substances to kill or inhibit microbial growth is called antimicrobial activity and the substances that possess this activity are called antimicrobials. The history of antimicrobials began with the observation of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. In the age of time various types of antimicrobials like antibiotics, antivirals, antifungals, anti-parasitics have been developed against diseases caused by corresponding microorganisms.

Advances of modern medicine, many infectious diseases once considered incurable and lethal are now amenable to treatment with antimicrobial agents. The remarkably powerful and specific activity of antimicrobial drugs is due to their selectively for highly specific targets that are either unique to microorganisms. Among these targets are specific bacterial and fungal cell wall-synthesizing enzymes that required for nucleotide synthesis and DNA replication of microbes (Bertram & Susan, 2009). Nowadays multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. For this reason, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientist to search for new antimicrobial substances. Given the alarming incidence of

antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. Antimicrobial properties of medicinal plants are being increasingly reported from different part of the world. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant material effects of plant materials typically result from the combination of secondary products present in the plant which are capable of producing definite physiological action on body.

In vitro antimicrobial of plants can be detected by observing the growth response of various microorganisms to those plant extracts or their solvent fractions that are placed in contact with them. This can be done in three ways (diffusion, dilution and bioautographic methods) and a number of modifications have been made in the technique in order to obtain better results. Since some factors (culture medium composition, microorganisms tested, extractive method, pH, solubility of the sample in the culture medium etc.) can change results, it is difficult to standardize a procedure for the study of antimicrobial plants. Diffusion and dilution methods have been employed to study the antimicrobial activity of different plants. Bioautography is another method for studying antimicrobial activity (Myran & Mendoza, 1998). Disc diffusion technique is widely acceptable for the preliminary screening of antimicrobial activity. If the test material has any antimicrobial activity, it will inhibit the growth of microorganism giving a clear distinct zone called "zone of inhibition". The antimicrobial activity of the test agent is determined by measuring the diameter of the zone of inhibition in term of millimeter. Antimicrobial activity of the crude extract was determined by disc diffusion method. However, no distinction between bacteriostatic and bacteriocidal activity can be demonstrated by this method (Reiner, 1982).

2.3.1 Test of Antimicrobial Activity by Disc Diffusion Assay

2.3.1.1 Principle

In this method measured amount of test sample were dissolved in definite amount of solvent to get a solution of known concentration ($\mu\text{g/ml}$). Then sterile filter paper discs were impregnated with known amount of test substances using micropipette and dried. Standard antibiotic discs were used as positive control and disc impregnated with solvent was used as negative control. These discs were then placed in Petri dishes containing agar medium seeded with the test organisms of log phase. The test material diffuses from the discs to the surrounding medium. The seeded Petri dishes containing test and control discs were then placed into the incubator for 12 to 18 hours at 37°C to allow the growth of microorganisms. If the test material had any antimicrobial activity, it would inhibit the growth of microorganism giving a clear distinct zone called "Zone of Inhibition". The antimicrobial activity of the sample was determined by measuring the diameter of the zone of inhibition in terms of millimeter.

2.4 Serine Protease Inhibition Assay

Proteolytic enzymes play a crucial role in living systems. So far, polypeptides that has proteolytic activity reach about 140,000 chains (Rawlings *et al.*, 2008). Many diseases such as infections, heart diseases, cystic fibrosis, cancers are related with protease or malfunction of protease. It is reported that there are 80 genetic diseases in human caused by mutation of protease genes; metastasis of tumor has also resulted from proteinase malfunction (Sabeh *et al.*, 2009). The proteolytic enzymes are also actively participating in the process of inflammation in human beings. The high reaction of inflammation may cause tissues damage, necrosis (Armstrong, 2006, Segel *et al.*, 2010, Sharony *et al.*, 2010).

Protein degradation ability of protease is magnificently high, thus nature adapts many level of regulations: gene regulation, secretion, activation, or inhibition. Plants possess efficient mechanisms against pathogen and pathogenic proteases enzyme system. These mechanisms have

included the secondary metabolism with polyphenol compounds and their derivatives. Polyphenol is well-known as the antioxidant agent, and research has shown that polyphenol derivatives protect protein and genetic materials against free radical (Gharass, 2009). Polyphenol, specifically the flavonoid may interact to protein/enzyme in different ways which reduce cell damage, tumor formation and metastasis (Ren *et al.*, 2003).

The high molecular polyphenol so call tannin can be unspecifically attached to protease resulting in precipitation and denaturation of protein (Chung *et al.*, 1998, Segel *et al.*, 2010). Other compounds can mimic the substrate then act as a specific inhibitor, these inhibitors show high potential for pharmaceutical application with a lower side effects than the synthesis compounds.

2.4.1 The mechanism of serine proteases

The availability of relatively large amounts of serine proteases from a variety of sources has provided numerous research groups with a useful means to undertake detailed studies of their physicochemical properties and catalytic specificity against a wide range of both endogenous protein/peptide and synthetic substrates. Many of the most valuable insights into structure, function and specificity have been gained through the use of site-directed mutagenesis studies (extensively reviewed in (Perona and Craik, 1995).

Serine proteases are characterized chiefly by the presence of an active site serine residue (Ser 195 (chymotrypsin numbering)), the γ hydroxyl group of which acts as a nucleophile during the hydrolytic process. Two other amino acid residues are directly involved in the catalytic mechanism, in addition to this serine residue. These are His 57, a general base/acid catalyst, and Asp 102, which is believed to orientate the catalytic histidine. In addition to this so-called catalytic triad, the enzyme possesses an oxyanion binding site. This is made from the backbone amide NH groups of Ser 195 and Gly 193. The mechanism is outlined in figure 3.12.

After noncovalent binding of the substrate to the enzyme active-site region, the OH group of Ser 195 attacks the carbonyl carbon of the scissile bond via general base catalysis by His 57. This action leads to formation of the tetrahedral intermediate. The structure of the tetrahedral

intermediate is stabilized through the newly formed oxyanion component by forming hydrogen bonds to the backbone NH groups of Ser 195 and Gly 193 residues.

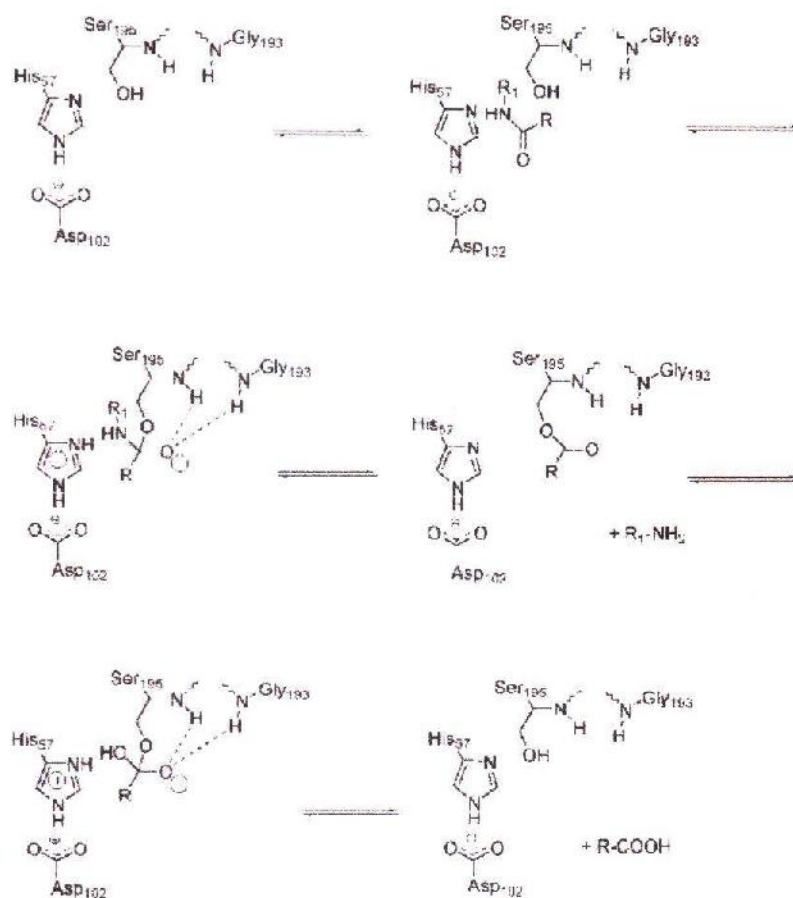


Figure 3.12: The mechanism of action of the serine proteases.

His 57, acting now as a general acid, transfers a proton to the amine of the tetrahedral intermediate; this subsequently breaks down, a product amine leaves and the covalent acyl enzyme intermediate is formed.

The acyl enzyme complex is attacked by a water molecule to generate a new tetrahedral intermediate. This intermediate collapses, assisted by general acid catalysis from His 57, resulting in the formation of product acid and the regenerated Ser 195 OH leaving group.

Whilst Barrett and Rawlings have revolutionized the nomenclature for the classification of all hydrolytic enzymes, describing species in so-called families and clans (Barrett and Rawlings,

For the purposes of this review we shall simply distinguish serine proteases on their differences in substrate specificity. The topology of the enzyme active site discriminates between enzymes with defined amino acids sequences, particularly at the P1 specificity subsite (Schechter and Berger, 1968). Serine proteases are generally classified according to their broad substrate specificity and while there are notable exceptions (such as granzyme B, which cleaves after Asp), there are three main categories: trypsin-like (cleaving after basic amino acids), chymotrypsin-like (cleaving after bulky hydrophobic residues) and elastase-like (cleaving after small, aliphatic residues). Almost all synthetic inhibitor development has been targeted against serine proteases with these broad substrate characteristics.

Protease inhibitor assay was performed against trypsin following the Kunitz method with some modifications (Kunitz, 1947).

2.5 Column Chromatography

2.5.1 Chromatography:

'Chromatography' is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analyzed. There are many types of chromatography e.g., liquid chromatography, gas chromatography, ion-exchange chromatography, affinity chromatography, but all of these employ the same basic principles.

Chromatography has been developed into a new method of separation of mixture of substances mainly when they are available in small amounts. This method is very useful when the components of a mixture have almost the same physical and chemical properties and hence can't be separated by other usual methods of separations. The term chromatography means writing in colour (in Greek: Khromatos-colour, and graphos-written). It was discovered by Mikhail Tswett in 1906.

The methods of separation in chromatography are based on the distribution of the components in a mixture between a fixed (stationary) and a moving (mobile) phase. The stationary phase may

the mobile phase moves on. The mobile phase may be a liquid or a gas. When a solid stationary phase is taken as a column it is known as column chromatography.

2.5.2 Column Chromatography:

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent.

The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the activity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only a poor separation, i.e., the components separated is not 100% pure.

The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as elution. That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called is called eluent. The weakly adsorbed component will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components. Intermolecular forces, which vary in strength according to their type, make organic molecules to bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column.

C

HAPTER 3

Materials and Methods

3.0 Materials and Methods

3.1 Plant materials

3.1.1 Collection of the sample:

Peels of Potato (*Solanum tuberosum*) were collected from hotels located beside Khan Jahan Ali Hall, Khulna University, Khulna, Bangladesh.

3.1.2 Preparation of Extract

3.1.2.1 Drying and Grinding

The peels were carefully cleaned and separated from undesired materials. The peels were dried under sunshade and air for few days. After completion of drying the peels, they were ground into fine powder with a grinder. The powder of peels were weighted and stored in airtight plastic bags in a dark cool and dry place for further use.

3.1.2.2 Extraction with solvent

The powder of potato peels were taken into two clean, flat-bottomed glass jars and soaked into water and methanol. They were then sealed and kept for a period of 7 days in a dark room accompanying occasional shaking and stirring. The mixtures filtered coarsely using white cotton material.

3.1.2.3 Evaporation and storage

Filtrates obtained from different solvent systems were evaporated using rotary evaporator and finally dried at room temperature with an electric fan. The crude extracts were weighed and kept in refrigerator for further experiments.

3.2 Evaluation of Antioxidant Activity

3.2.1 DPPH Free Radical Scavenging Assay

3.2.1.1 Chemical Requirements

The chemicals were ethanol, distilled water, 0.004% DPPH ethanolic solution, Quercetin (as positive control).

3.2.1.2 Equipment

The equipment used for this experiment was test tubes, beakers, digital balance, vortex machine, refrigerator, marker, Micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), spoon, test tube stand, aluminum foil and UV spectrophotometer.

3.2.1.3 Methodology of DPPH free radical scavenging assay:

1. At first 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and 9 test tubes were taken to make aliquots of 9 concentration (1.57, 3.13, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.
2. Sample extract and quercetin were weighed and dissolved in ethanol to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
3. DPPH was also weighed and dissolved in ethanol to make 0.004% (w/v) solution.
4. After making the desired concentrations, 2ml of 0.004% DPPH solution was applied on each test tube containing 1ml of solution by pipette. The final volume of the solution was 3ml. This was done for both the quercetin and sample extract.
5. Then the test tubes were kept for 30 minutes in dark to complete the reaction.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
7. After 30 minutes, absorbance of each test tube was determined by UV spectrophotometer at 517 nm.
8. Percentage of inhibition was calculated as-

$$\% \text{ Inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of sample}} \times 100$$

9. IC₅₀ value is the concentration of sample required to scavenge 50% of DPPH free radical and was calculated from the plot of % inhibition against the log concentration of sample extract.

3.2.2 FRAP (Ferric ion Reducing Antioxidant Power) Assay

3.2.2.1 Chemicals required

The chemicals were acetate buffer (300 mM; pH 3.6), TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$).

3.2.2.2 Equipment

Test tubes, beakers, digital balance, vortex machine, water bath, micropipette (20 μl , 200 μl and 1000 μl), pipettes (5ml), UV spectrophotometer, marker and aluminum foil.

3.2.2.3 Acetate buffer (300 mM; pH 3.6) preparation

3.1gm sodium acetate trihydrate was weighed and added into 16 ml of glacial acetic acid and make the volume to 1L with distilled water.

3.2.2.4 TPTZ (2, 4, 6-tripyridyl-s- triazine) preparation

0.031gm TPTZ dissolve in 10ml of 40mM HCl and kept at 50°C in water bath for 20minutes.

3.2.2.5 Ferric Chloride $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM) preparation

0.054gm $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolves in 10ml distilled water.



3.2.2.6 FRAP reagent preparation

The working FRAP reagent was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution, then warmed at 37°C before using.

3.2.2.7 Protocol for FRAP assay

1. Sample extract and Quercetin were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for plant extract and 5 test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for Quercetin which was taken as positive control.
3. Another five test tubes were taken with 0.2ml extract and quercetin solution.
4. Then 3ml FRAP reagent was applied on each test tube.
5. The mixture was kept at 37°C in water bath for 30 minutes.
6. After cooling, the absorbance was measured at 593nm.

3.2.3 Total Phenolic Content Assay

3.2.3.1 Chemicals requirement

Sodium Carbonate (7% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

3.2.3.2 Equipment

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.3.3 Methodology for Total Phenolic Content

1. Gallic acid was used as standard. 250µg/ml stock standard solution of gallic acid was prepared by dissolving 0.0025 gm in 10 ml distilled water and then diluted to five concentration (50, 100, 150, 200 and 250 µg/ml).
2. The extract was prepared at concentration of 100µg/ml.
3. 9 ml distilled water and 1 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 10 ml of 7% sodium carbonate was added to the mixture and mixed gently.
6. 25 ml adjust with distilled water.
7. After standing at room temperature for 60 minutes, the absorbance was measured at 750nm using spectrophotometer.

The standard calibration curve of gallic acid was plotted.

3.2.4 Total Flavonoid Content

3.2.4.1 Chemicals requirement

5% Sodium nitrite (5% NaNO₂): 5gm in 100ml distilled water.

10% Aluminum chloride (10% AlCl₃): 10gm in 100ml distilled water.

1 M Sodium hydroxide (1 M NaOH): 4gm NaOH in 100ml distilled water

3.2.4.2 Equipment

Test tubes, beakers, volumetric flask, digital balance, vortex machine, micropipette (200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.4.3 Methodology for Total Flavonoid Content

1. Quercetin was used as standard and were weighed and dissolved in distilled water to make homogeneously stock solution with the highest concentration i.e. 400 µg/ml, with the help of vortex and sonicator.
2. Five test tubes were taken to make aliquots of 5 concentrations (25, 50, 100, 200 and 400 µg/ml) by serial dilution technique for Quercetin.
3. The extract was prepared at concentration of 100µg/ml.
4. 5 ml distilled water and 0.3 ml of 5% NaNO₂ was added to each diluted Quercetin and 1ml of extract solution.
5. After 5 minutes 0.6 ml of 10% AlCl₃ was added.
6. After 5 minutes 2 ml of 1M NaOH.
7. Then the mixture was allowed to stand at room temperature for 5 minutes.
8. After standing at room temperature, the absorbance was measured at 510 nm using spectrophotometer.

The standard calibration curve of Quercetin was plotted.

3.2.5 Total Tannin Content

3.2.5.1 Chemicals requirement

Sodium Carbonate (35% w/v), Folin-Ciocalteu (FC) reagent (diluted 10-fold with distilled water), Gallic acid (standard).

3.2.5.2 Equipment

Test tubes, beakers, digital balance, vortex machine, micropipette (20µl, 200µl and 1000µl), pipettes (5ml, 10ml), UV spectrophotometer, marker and aluminum foil.

3.2.5.3 Methodology for Total Tannin Content

1. Gallic acid was used as standard. 100µg/ml stock standard solution of gallic acid was prepared by dissolving 0.001 gm in 10 ml distilled water and then diluted to five concentrations (6.25, 12.5, 25, 50 and 100 µg/ml).
2. The extract was prepared at concentration of 100µg/ml.
3. 7.5 ml distilled water and 0.5 ml FC reagent was added to each diluted gallic acid and extract solution.
4. The mixture was allowed to stand at room temperature for 5 minutes.
5. Then, 1 ml of 35% sodium carbonate was added to the mixture and mixed gently.
6. 10 ml adjust with distilled water.
7. After standing at room temperature for 30 minutes, the absorbance was measured at 725nm using spectrophotometer. The standard calibration curve of gallic acid was plotted.

3.3 Evaluation of Antibacterial Activity

3.3.1 Test of Antimicrobial Activity by Disc Diffusion Assay

3.3.1.1 Materials for test

3.3.1.1.1 Test material:

Crude Water and Methanolic extract of Potato peels.

3.3.1.1.2 Positive control:

Antibiotic disc Azithromycin (100µg/disc)

3.3.1.1.3 Equipment:

Aluminum foil, autoclave, cotton, digital slid calipers, electronic balance, filter paper discs, laminar air flow cabinet, marker, micropipette, nose mask, apron and gloves, Petri dishes, pipette, punch machine, refrigerator, spirit lamp, sterile forceps, sterile inoculating loop and test tubes.

3.3.1.1.4 Reagents:

Distilled Water, Methanol, nutrient agar medium and nutrient broth.

3.3.1.2 Bacteria used for the activity test

Both Gram-positive and Gram-negative bacterial strains were taken for the test (Table 3.1). The bacterial strains used for this investigation are listed in following table. These organisms are preserved in Microbiology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh.

Table 3.1: List of bacteria used for the evaluation of antimicrobial activity

Gram-positive	Diseases	Gram-negative	Diseases
<i>Staphylococcus aureus</i>	Skin infections, respiratory disease and food poisoning.	<i>Pseudomona aurigonisa</i>	Infection in pulmonary tract, urinary tract, burns and wounds.
<i>Micrococcus</i>	Skin infections	<i>Vibrio cholerae</i>	Cholera
<i>Bacillus megaterium</i>	Non-pathogenic	<i>Salmonella typhi</i>	Typhoid fever
		<i>Salmonella paratyphi</i>	Typhoid fever
		<i>Escherichia coli</i>	Food poisoning

3.3.1.3 Culture Media

A mixture of nutrient used in the laboratory to support growth and multiplication of a culture (a population of microorganisms) is called culture medium (Pelczar *et al.*, 1996). The

nutritional requirements of bacteria vary widely so there are great differences in the chemical compositions of the media used in the laboratory. Some microorganism can grow in medium containing only inorganic compounds, whereas others require a medium containing organic compounds (amino acids, sugars, purines or pyrimidines, vitamins or coenzymes). Some require complex natural substances (peptone, autolysate, blood cells or blood serum) and some cannot as yet be grown in an artificial laboratory medium and can be propagated only in a living host or living cells. Each kind of organism also requires specific physical conditions for growth. For example, some bacteria cannot grow below 40°C, some cannot grow above 20°C and some require a temperature close to that of the human body (i.e., 37°C). Light may be another important physical condition. The successful cultivation of bacteria requires an awareness of all of these factors. Each kind of microorganism grows in a characteristic manner. For example, growth in a liquid medium may be abundant or sparse; it may be evenly dispersed throughout the medium or it may occur only as sediment at the bottom or only as a thin film or pellicle at the top. On solid media, microbes grow as colonies distinct, compact masses of cells that are macroscopically visible. Colonies are characterized by their size, shape, texture, consistency, color and other notable features (Pelczar *et al.*, 1993). Chemically defined media are needed for the cultivation of autotrophs and are also useful for defining the nutritional requirements of heterotrophs. However, for the routine cultivation of heterotrophs, chemically defined media are not generally used. Instead, certain complex raw materials such as peptones, beef extract and yeast extract are used and the resulting media support the growth of a wide variety of heterotrophic bacteria. Agar is included as a nonnutritive solidifying agent when a solid medium is desired (Pelczar *et al.*, 1993). The following media are used normally to demonstrate the antibacterial activity and to make subculture of the test organisms:

- Nutrient Agar Media
- Nutrient Broth Media
- Mueller-Hinton Agar Media
- Tryptic Soy Broth (TSP)

Among these, the first one is most frequently used which was also used for the present antibacterial screening.

3.3.1.4 Media Composition:

For 100 ml

Ingredients	Amount
Distilled water	100ml
Peptone	0.5gm
Beef extract	0.3gm
Agar	1.5gm

3.3.1.5 Methodology:

3.3.1.5.1 Preparation of media:

3.3.1.5.2 Nutrient agar media:

Nutrient agar media was prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps were done to do this:

- 1) For 100ml nutrient agar media, 0.5gm peptone, 0.3gm beef extract and 1.5gm agar were taken in the screw capped conical flask.
- 2) So 100ml distill water was added to that amount of powder.
- 3) Then the media was subjected to sterilization in the autoclave.

3.3.1.6.3 Nutrient broth media:

Nutrient agar media was prepared by mixing commercially available ingredients in powdered form with recommended amount of water. Following steps were done to do this:

- 1) For 100ml nutrient agar media, 0.5gm peptone and 0.3gm beef extract were taken in the screw capped conical flask.
- 2) So 100ml distill water was added to that amount of powder.
- 3) Then the mixture was shaken well and then it was subjected to sterilization in the autoclave.

3.3.1.5.4 Sterilization:

Both the culture media, Petri dishes, micropipette, test tubes, forceps, screw capped vial, screw capped conical flasks, pipette were sterilized by autoclaving at a temperature of 121°C and a pressure of 15lbs/sq inch for 15 minutes. Blank disc were subjected to dry heat sterilization at 110°C for 20 minutes. The Laminar air flow cabinet was disinfected by ethanol with spray bottle. The UV light in the laminar air flow cabinet was switched on before one hour to avoid accidental contamination.

3.3.1.5.5 Preparation of Subculture:

This was done by culturing pure microbial population in the agar media. To obtain better growth of bacterial colony tow generation of subculture were prepared. Following steps was performed for preparation of first generation of subculture:

- 1) At first prepared culture media was poured into Petri dishes which were marked for desired strains of microorganisms.
- 2) At least two subculture plates were made for each strain.
- 3) The Petri dishes were allowed to cool in refrigerator for 15 minutes.
- 4) Then the inoculating loop (sterilized by spirit lamp) was dipped into the pure culture of bacteria and streaking was done in the Petri dishes marked for those strains of bacteria.
- 5) Then the Petri dishes were allowed to incubate for 12-18 hours at 37°C in the incubator.
- 6) After 12-18 hours bacterial colony was observed in the culture media.
- 7) Then the prepared plates of first generation subculture were kept in refrigerator which can be used for 2 months.

8) 3.3.1.5.6 Preparation of Seeded Test Plates:

For the evaluation of antimicrobial activity by disc diffusion assay seeded test plate of microbes is necessary to made. It was done by two steps:

In the first step-

- 2 ml of nutrient broth was taken in each screw capped vial where all of them were marked according to their strains. Two vials were taken for each strain.

- Then each vials containing nutrient broth were inoculated by the inoculating loop which looped single bacterial colony from the sub culture. These tasks were done in the laminar air flow cabinet.
- Then all the vials were allowed to incubate at 3-4 hours at 37°C. This was done to get the bacteria in their log phase at which the growth rate is very high.

In the second step-

- The sterilized agar medium was poured into sterilized Petri dishes in such a way as to give a uniform layer of depth of approximately 4 mm. After the medium became cooled at room temperature, it was stored in a refrigerator (4°C).
- Incubated vials log phase were removed from the incubation.
- Test tubes were shaken by rotation to get a uniform suspension of bacteria.
- Then 5µl nutrient broth containing bacteria was taken into corresponding solidified medium containing Petri dishes.
- The Petri dishes were rotated several times, first clock wise then anticlockwise to assure homogenous distribution of the test organisms. Thus the seeded plates were ready for disc diffusion assay. These tasks were done in the laminar air flow cabinet. All tasks were done in the laminar air flow cabinet.

3.3.1.5.7 Preparation of Test Sample

0.2 gm each of water and methanolic extract of potato peel were accurately measured by using the electronic balance and taken into screw capped vials (vials were nullified on the electronic balance by auto zero). Then 2 ml of water and methanol was added respectively in the vial to get the sample of 100 mg/ml.

3.3.1.5.8 Preparation of Discs:

Three types of discs were used for the antimicrobial screening:

- a) Sample discs
- b) Standard discs and
- c) Blank discs

★ 3.3.1.5.9 Sample discs:

Filter papers previously sterilized were perforated by punch machine thus tiny discs of 5 mm diameter were obtained. Then the discs were taken in a blank Petri dish. 20µl and 40µl of sample solution were applied on the discs with the help of micropipette in an aseptic condition from the stock solution. Then the discs were allowed to dry for a few minutes under the laminar to remove the solvent ethanol as it has some antimicrobial activity which can bias the evaluation.

3.3.1.5.10 Standard discs:

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antibacterial agent with that produced by test samples. In this investigation Azithromycin (0.1 mg/ml) standard disc were used as the reference.

✎ 3.3.1.5.11 Blank discs:

Blank discs were used as a negative control. They ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

3.3.1.5.12 Application of Discs

Sample impregnated discs, standard antibiotic discs and negative control discs were placed gently on the solidified agar plates, freshly seeded with the test organisms with the help of a sterile forceps to assure complete contact with medium surface. The spatial arrangement of the discs was such that the discs were not closer than 15 mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition. Finally the plates were incubated upside down at 37°C for 12-18 hours.

3.3.1.5.13 Determination of Antimicrobial Activity:

✎ After proper incubation, the antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition in term of millimeter with a transparent scale.

3.4 Serine Protease Inhibition Assay

3.4.1 Chemicals requirement

- 0.1M phosphate buffer pH 7.6
- 0.0025M HCl
- Trypsin (0.5 mg/mL prepared in 0.1 M phosphate buffer pH 7.6)
- 1% casein (prepared in 0.0025M HCl)
- 5 % trichloroacetic acid

3.4.2 Methodology for Serine Protease Inhibition Assay

1. Standard (Quercetine) and Sample was dissolved in ethanol to make 400µg/ml concentration. Then each of them was serially diluted in 5 concentrations (400µg/ml, 200µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml). From then 1 ml of Standard (Quercetine) or Sample was taken in 14 ml tube.
2. Then 1ml of Trypsin (previously heated at 37°C for 15 minutes before used) was added to the tube.
3. Then, 2 mL of 1% casein was added and the obtained mixture was allowed to stand for 30 min at 37°C.
4. Finally, 3 mL of 5% trichloroacetic acid (TCA) solution was added to stop the reaction.
5. After centrifugation of the reaction mixture (12,000 rpm, 15 min), the absorbance was measured at 280 nm.
6. % Inhibition was calculated by using following formula

$$\% \text{ Inhibition} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of blank}} \times 100$$

3.5 Column Chromatography

3.5.1 Methodology for column chromatography:

1. Place the column in a ring stand in a vertical position and plug of glass wool is pushed down to the bottom of the column.
2. Place some amount of washed sand into the column.
3. Prepare slurry of silica gel with n-hexan & pour gently into the column.
4. Open the stop cock & allow some solvent to drain out.
5. Again Place some amount of washed sand into the column.
6. To wash the column pour gently 100 ml of n-hexan into the column and drain it out.
7. Dissolve the methanolic extract of potato peel in a minimum amount of methanol.
8. Remove the solvent by adding silica gel and place it to the column.
9. Pass the stationary phase (different percentage of n-hexan, ethayl acetate and methanol) carefully and collect the different fraction in beaker.
10. Dried the fraction by using air.

C

HAPTER 4

Results and Discussion

4.0 Results and Discussion

4.1 Antioxidant Assay

4.1.1 DPPH Free Radical Scavenging Assay

The DPPH antioxidant assay is a rapid, direct and reliable method for the antioxidant screening of plant extracts. A number of methods are available for the determination of free radical scavenging activity but the assay employing the stable 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience. This test is based on the ability of DPPH, a stable nitrogen-centered free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the color of DPPH changes from violet to yellow, which can be quantitatively measured from the changes in absorbance. The purple color of DPPH fades due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The DPPH radical scavenging activities of sample increased gradually as the concentration increased. Decrease in absorbance of DPPH solution depends on intrinsic antioxidant activity of samples as well as on speed of reaction between DPPH and antioxidant. Quercetin was chosen as the reference antioxidant for this test.

In this study, antioxidant activity of water and methanolic extracts of potato (*Solanum tuberosum*) peel were examined and analyze their graphical representation.

4.1.1.1Methanolic extract of potato peel

Graphical analysis of Absorbance vs. Log Concentration, the absorbance of the extract decreased gradually as the log concentration increased. In figure 4.1 showed the absorbance of methanolic extract of potato peel and standard quercetin at different log concentration.

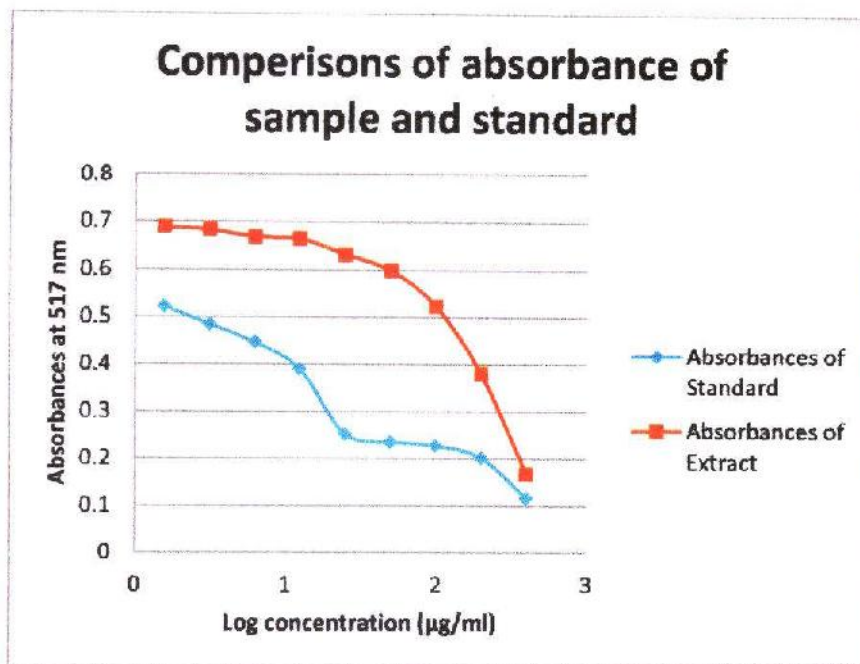


Figure 4.1: DPPH Scavenging assay of potato peel methanolic extract (Absorbance vs Log Concentration).

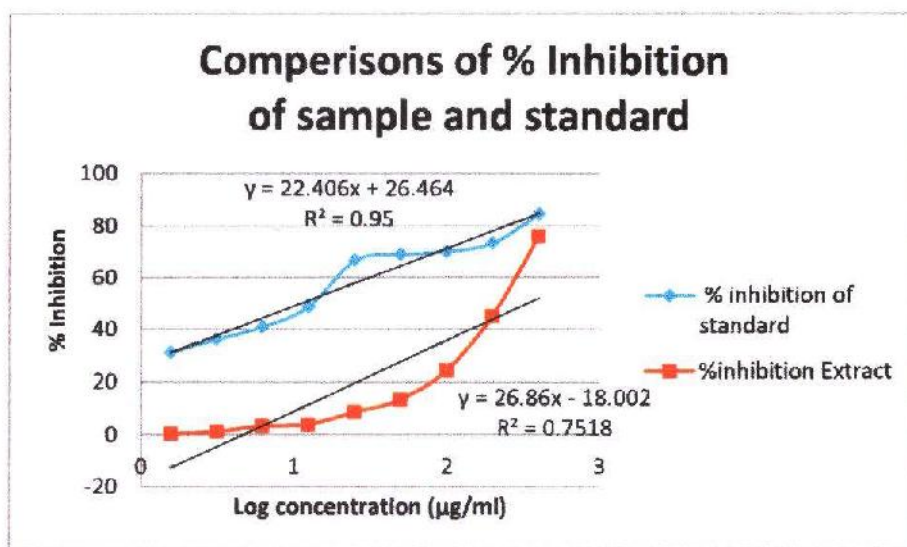


Figure 4.2: DPPH Scavenging assay of potato peel methanolic extract (% inhibition vs Log Concentration).

Figure 4.2 showed that the comparison of percentage inhibition of potato peel methanolic extract and standard quercetin at different concentrations. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by extrapolation. By using the equation $y = mx + c$ (where 'c' is intercept and 'm' is slope); IC_{50} value of extract was found at the concentration of 248.68 $\mu\text{g/ml}$ and standard quercetin was 17.12 $\mu\text{g/ml}$. Here the concentration of the extract required for 50% inhibition is much greater than that of control.

4.1.1.2 Water extract of potato peel

Analysis of potato peel water extract, the absorbance of the extract decreased gradually as the log concentration increased. Absorbance at different log concentration of extract and standard quercetin is given in figure 4.3.

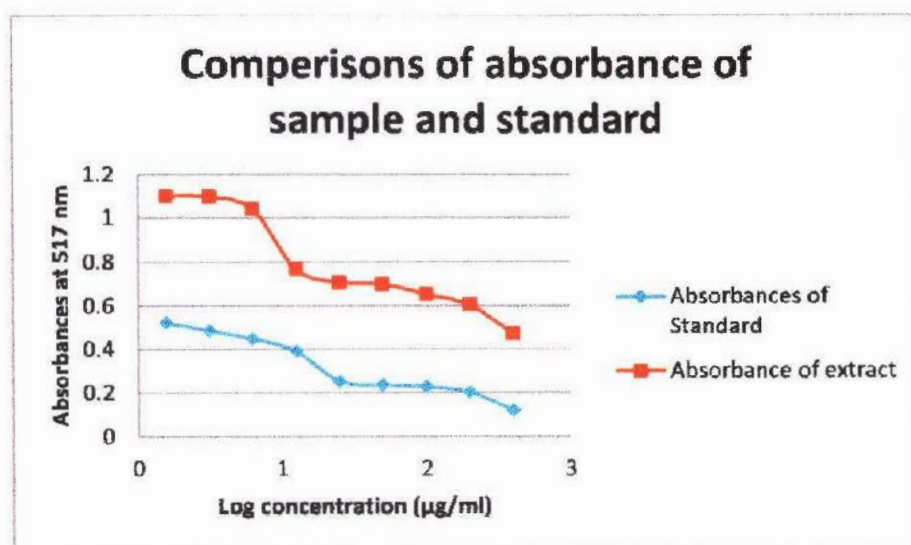


Figure 4.3: DPPH Scavenging assay of potato peel water extract (Absorbance vs Log Concentration).

Comparison of percentage inhibition of water extract of potato peel and standard quercetin at different log concentrations is given in fig. 4.4. The concentration at which 50% inhibition (IC_{50}) of DPPH occurred were obtained by using the equation $y = mx + c$. IC_{50} value

of extract was found at the concentration of 181.7 µg/ml and standard quercetin was 17.12 µg/ml. So we observed that the concentration of the extract required for 50% inhibition is greater than that of control.

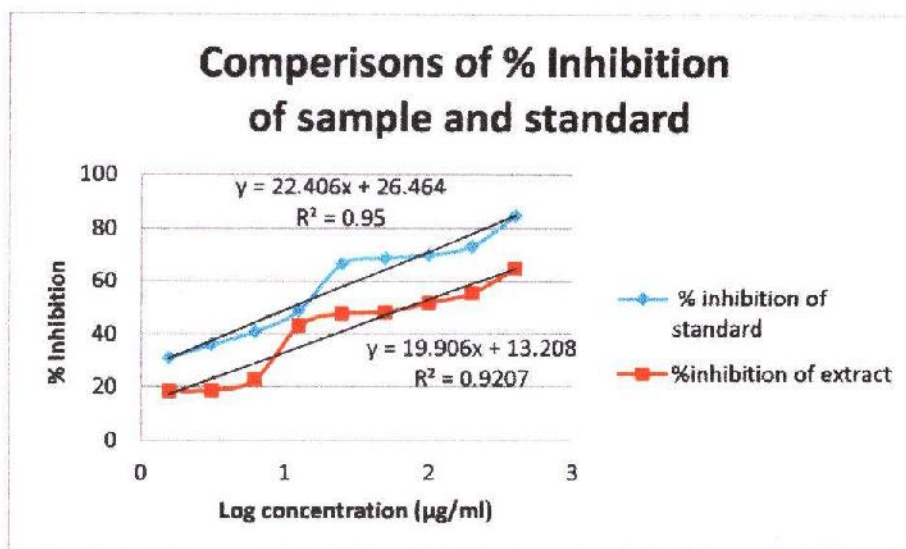


Figure 4.4: DPPH Scavenging assay of potato peel water extract (% inhibition vs Log Concentration).

4.1.1.3 Discussion

Antioxidant activity of plants extract were examined by DPPH free radical scavenging assay where plant extracts exhibited radical scavenging effect of DPPH in concentration dependent manner. In this study, waste part of a common and widely used vegetable, potato peel was selected for knowing their medicinal properties. On the basis of their ethnobotanical information we emphasized to analyze their antioxidant properties in this research.

Methanolic extract of potato peel exhibited 50% radical inhibitions at 248.68 µg/ml concentration and water extract showed at the concentration of 181.7 µg/ml. Whereas, standard quercetin exhibited 50% inhibition at a concentration of 17.12 µg/ml. Figure 4.2 shows the comparison of percentage inhibition between methanolic extract of *C. crista* and standard quercetin. According to this figure the scavenging activity of extract did not increased likely to the standard. So methanolic extract of potato peel have smaller antioxidant potentiality. This is

for the presence of less amount of polar compound in this extract. Figure 4.4 shows the comparison between percentage inhibition of water extract of potato peel and standard quercetin. This figure represents that the scavenging activity of extract is not so nearer to the standard. It indicates that antioxidant property of potato peel water extract is not so good but its antioxidant potentiality is higher than methanolic extract. As we know that water is more polar than methanol because oxygen in methanol is satisfied with electron releasing methyl group as well as oxygen is bonded with more carbon which is more electronegative than hydrogen. So charge separation of between the atoms is less. For this reason water extract showed more antioxidant potentiality than methanol. This antioxidant activity may be due to the presence of different phenolic acid and fatty acid (De Sotillo *et al.*, 1994, Al-Saikhan *et al.*, 1995, Onyeneho and Hettiarachchy, 1993).

4.1.2 FRAP (ferric reducing antioxidant power) Assay

The total antioxidant potential of sample was determined using FRAP assay as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored FeII-tripyridyltriazine compound from colorless oxidized FeIII form by the action of electron donating antioxidants. In this research work, the total antioxidant potential of potato peel was determined by using FRAP reagent with standard Quercetin. Standard curve was prepared using different concentrations (400- 25) $\mu\text{g/ml}$. In the FRAP assay the antioxidant efficiency of the antioxidant under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were expressed in $\mu\text{M Fe (II)}/100\text{g}$ dried plant material using the equation $y = 0.0044x + 0.1297$, coefficient $R^2 = 0.9974$, where y is absorbance at 593 nm and x is the total antioxidant potential of different extracts. Absorbance of FeSO_4 in different concentration is given in table 4.1 and from which calibration curve plotted in figure 4.5.

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (593 nm)	Absorbance $\pm$ SD
400	1.87	1.87 ± 0.002
200	1.07	1.07 ± 0.011
100	0.57	0.57 ± 0.009
50	0.35	0.35 ± 0.007
25	0.21	0.21 ± 0.003

Table 4.1: Absorbance of standard quercetin at different concentration

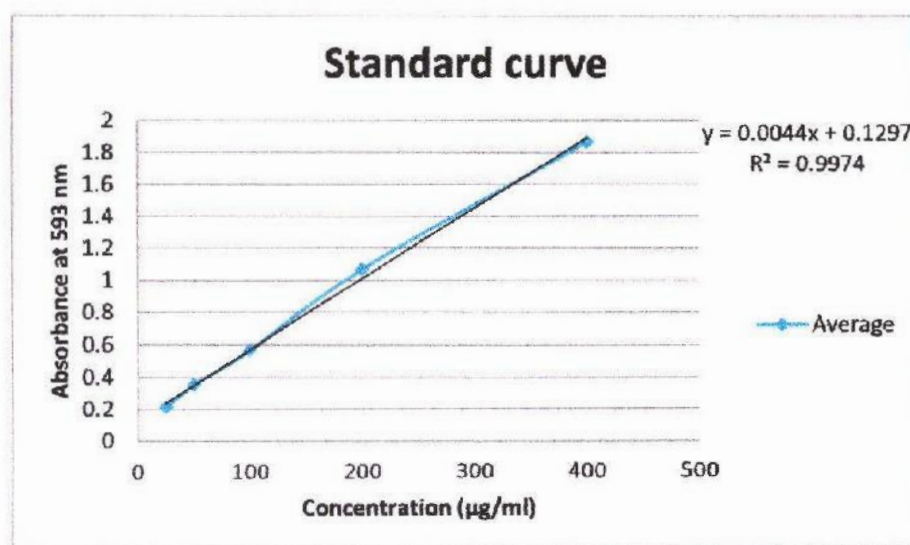


Figure 4.5: Standard curve of FeSO_4 for determination of FRAP assay.

4.1.2.1 Total antioxidant capacity of Ascorbic acid and potato peel

In this research, using standard calibration curve, total antioxidant power of Ascorbic acid and potato peel water and methanolic extract was determined. Table 4.2 shows the absorbance and antioxidant power of Ascorbic acid and potato peel water and methanolic extract. Total antioxidant power of Ascorbic acid (standard) exhibited 575.83 $\mu\text{M Fe (II)}$ /100g of dried plant material. Methanolic extract showed 351.81 $\mu\text{M Fe (II)}$ /100g of dried plant material and Water extract showed 301.89 $\mu\text{M Fe (II)}$ /100g of dried plant material.

Table 4.2: Antioxidant power of Ascorbic acid and potato peel.

Sample	Absorbance	Ferric reducing antioxidant power ($\mu\text{M Fe (II)/g}$) $\pm$ SD
Ascorbic acid	2.66	575.83 ± 0.113
Methanol extract	1.68	351.81 ± 0.016
Water extract	1.46	301.89 ± 0.018

4.1.2.3 Discussion

FRAP assay measure the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+} -TPTZ) ((Guo *et al.*, 2003)). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction

The reducing capacity of an extracts may serve as a significant indicator of its potential antioxidant activity. FRAP assay showed variability between various extracts. In this research using FRAP assay the range of antioxidant capacity of extracts was 301.89 to 351.81 $\mu\text{M Fe (II)/100g}$ of dried plant material. From the quantitative estimation of total antioxidant potential of potato peel, methanolic and water extract of potato peel exhibited 351.81 and 301.89 $\mu\text{M Fe (II)/100g}$ of dried plant material respectively. Methanolic extract of potato peel showed significant antioxidant power. Investigating the results of the present study, potato peel water extract showed the slightest antioxidant capacity.

In this research work, previous assays also showed that the methanolic extract had a greater antioxidant potentiality than the water extract. Methanolic extract of potato peel found as the moderate scavenger (fig. 4.2) in DPPH assay and also strong reducing power capability in reducing power assay. Water extract showed moderate capacity in these tests.

4.1.3 Total Phenolic Content

The amount of total phenol was determined with the Folin-Ciocalteu assay using Gallic acid as a standard phenolic compound. This method is a fast and simple method and can be useful in characterizing and standardizing botanical samples. Folin-Ciocalteu method is based on oxidation of phenolics by a molybdotungstate in Folin-Ciocalteu reagent to yield a colored product with λ_{max} 745 – 750 nm (Javanmardi *et al.*, 2003).

A linear calibration curve of Gallic acid, in the range of 25–250 µg/ml with coefficient of determination (R^2) value of 0.968 was obtained. Absorbance of gallic acid in different concentration is given in table 4.3 and from which calibration curve plotted in figure 4.6. Using this calibration curve, the total amount of phenolic content of plant extract was determined using the equation $y = 0.002x + 0.036$, where y is the absorbance at 750 nm and x is total phenolic content in the different plant extracts.

Table 4.3: Absorbance of standard gallic acid at different concentration

Concentration (µg/ml)	Avg. of Absorbance (750 nm)	Absorbance $\pm$ SD
Blank	0.0656	
250	0.4876	0.4876 ± 0.012
200	0.444	0.444 ± 0.016
150	0.3693	0.3693 ± 0.005
100	0.253	0.253 ± 0.006
50	0.133	0.133 ± 0.011
25	0.0526	0.0526 ± 0.008

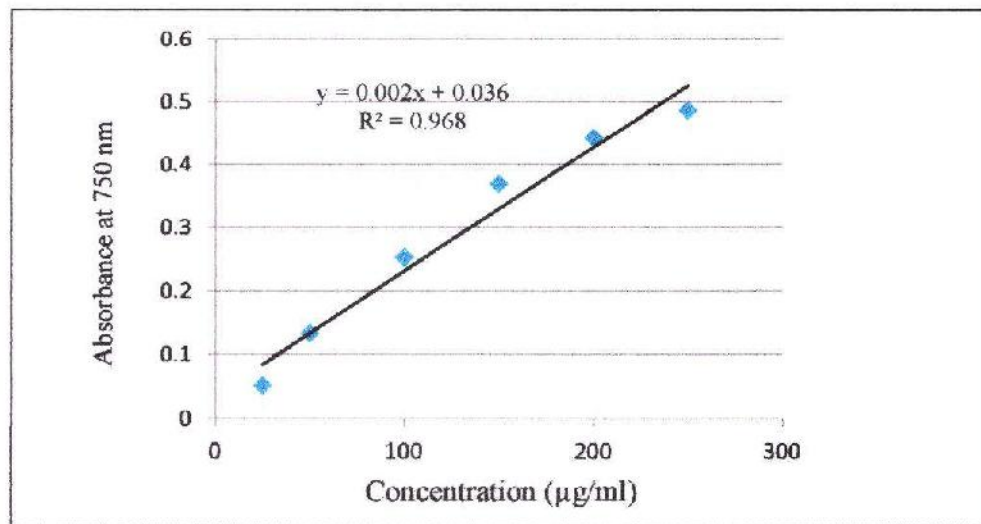


Figure 4.6: Calibration curve of standard Gallic acid for determination of total phenolic content.

4.1.3.1 Total phenolic content of potato peel

In this work, total phenolic content of two different extract of potato peel was determined using the standard calibration curve. Table 4.4 represents the amount of total phenolic content in

Methanolic and water extract of potato peel. Methanolic extract shows 85.13 ± 0.006 mg GAE/g of extract and water extract shows 56 ± 0.003 mg GAE/g of extract respectively.

Table 4.4: Total phenolic content of potato peel.

Extract	Conc. ($\mu\text{g/ml}$)	Absorbance (750 nm)	Total Phenolic Content (mgGAE/g of extract) $\pm$ SD
Methanol	100	85.13	85.13 ± 0.006
Water	100	56	56 ± 0.003

4.1.3.2 Discussion

Phenolic or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolic compounds have been shown to contain high levels of antioxidant activities (Razali *et al.*, 2008). The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation and dismutation of radicals. In fact, in some studies, theoretical methods have been proposed to estimate the antioxidant activities of phenolic substances. Their structure activity relationships are examined for this purpose. Phenols are compounds that have the ability to destroy radicals because they contain hydroxyl groups (Das and Pereira, 1990; Younes, 1981). These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and from stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts (De Gaulejac *et al.*, 1999).

Investigating the results of the present study, the quantitative estimation of total phenolic content of methanolic extract of potato peel exhibited 85.13 ± 0.006 mg GAE/g of extract. Water extract of potato peel showed the amount of total phenolic content is 56 ± 0.003 mg GAE/g of extract. So this poor amount of phenolic content in potato peel extract could be related to the higher IC₅₀ values (248.64 $\mu\text{g/ml}$ and 181.7 $\mu\text{g/ml}$ respectively) means weaker scavengers.

4.1.4 Total Flavonoid Content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo- γ -pyrone structure. These 105 compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Total flavonoid contents can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm (Bakar *et al.*, 2009). A linear calibration curve of Quercetin, in the range of 25–400 $\mu\text{g/ml}$ with coefficient of determination (R^2) value of 0.984 was obtained. Absorbance of quercetin in different concentration is given in table 4.5 and from which calibration curve plotted in figure 4.7. Using this calibration curve, the total amount of flavonoid content of plant extract was determined using the equation $y = 0.001x + 0.023$, where y is the absorbance at 510 nm and x is total flavonoid content in the different plant extracts.

Table 4.5: Absorbance of standard quercetin at different concentration

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (510 nm)	Absorbance $\pm$ SD
Blank	0.076	
400	0.718	0.718 ± 0.001
200	0.369	0.369 ± 0.013
100	0.258	0.258 ± 0.002
50	0.093	0.093 ± 0.003
25	0.04	0.04 ± 0.006

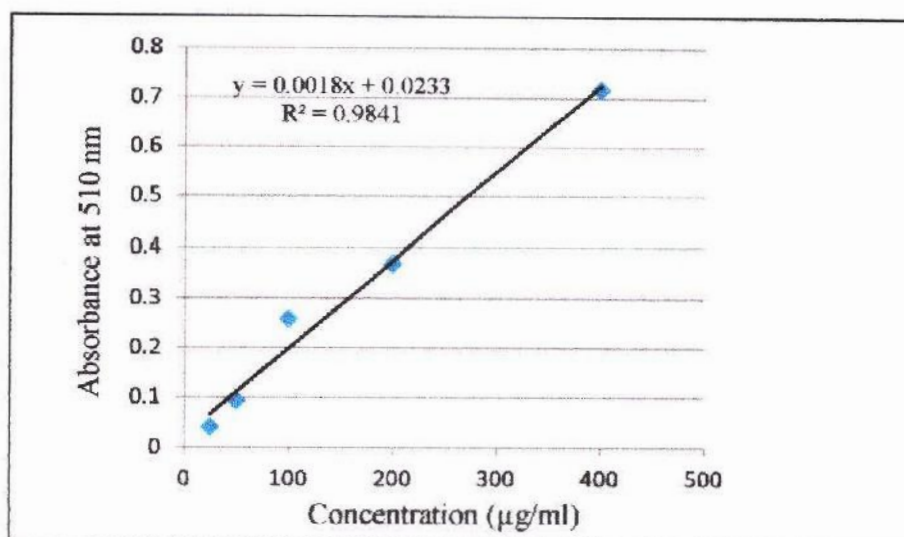


Figure 4.7: Calibration curve of standard Quercetin for determination of total flavonoid content.

4.1.4.1 Total flavonoid content of potato peel

In this work, total flavonoid content of potato peel was determined using the standard calibration curve. Table 4.6 represents the amount of total flavonoid content in methanolic and water extract of potato peel. Methanolic extract shows 33.17 ± 0.002 mg QE/g of extract and water extract shows 16.5 ± 0.002 mg QE/g of extract respectively.

Table 4.6: Total flavonoid content of potato peel

Extract	Conc. (µg/ml)	Absorbance (510 nm)	Total Flavonoid Content (mgQE/g of extract) $\pm$ SD
Methanol	100	0.083	33.17 ± 0.002
Water	100	0.053	16.5 ± 0.002

4.1.4.2 Discussion

Flavonoids constitute a wide array of biological active compounds that are found abundantly in plant kingdom and dietary intake (Cushnie *et al.*, 2005). They are gaining interest due to their wide variants and number of members. These are reported to be effective in pathogenesis of majority of diseases. Antioxidant activity is the foundation of many actions

which lead to its beneficial effects in majority of the diseases. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers (Pal *et al.*, 2009). This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available “H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure (Tripoli *et al.*, 2007). Flavonoids inhibit lipid peroxidation in vitro at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain (Cook *et al.*, 1996; Ferreira *et al.*, 2010). The flavonols having ortho or para hydroxyl group in the 2-phenyl ring are known to have strong antioxidant properties, while free hydroxyl at the 5, 7- positions proved to have a pro-oxidant effect (Heim *et al.*, 2002). A positive correlation between the content of flavonoids and the antioxidant capacity in plant extract has been found (Nagarajan *et al.*, 2008). For that reason, determination of the quantity of flavonoids compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total flavonoid content of methanolic extract of potato peel exhibited 33.17 ± 0.002 mg QE/g of extract. This poor amount of flavonoid content in methanolic extract could be related to the weaker scavenger in DPPH assay (fig. 4.2). Water extract of potato peel showed the amount of total flavonoid content is 16.5 ± 0.002 mg QE/g of extract. In this research work, we found methanolic extract of potato peel showed a quite good amount of flavonoid content and also showed a good antioxidant potentiality in FRAP assay, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of 248.64 μ g/ml in DPPH assay along with moderate phenol content. This could be due to the fact that the free radical scavenging assay is not only specific to the presence of polyphenols or flavonoids (Singleton *et al.*, 1965). But we also found in the literature, polyphenols or flavonoids are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants (Okawa *et al.*, 2001).

4.1.5 Total Tannin Content

Tannin compounds are polyphenol compounds that are very complex and widely distributed in almost all plant species (Okuda, 2005; Makkar and Becker, 1998). These complex polyphenol compounds are soluble in water and organic solvents (Haslam, 1996). In this research work, Tannin content in each sample was determined using Folin-Denis method and Gallic acid as a standard (Polshettiwar *et al.*, 2007). The tannin and tannin like compound reduce Phosphotungstomolybdic acid in alkaline solution to produce a highly blue colored solution with λ_{max} 725 nm (Cetkovic *et al.*, 2008, Jain & Dixit, 2004). A linear calibration curve of Gallic acid, in the range of 6.25–100 $\mu\text{g/ml}$ with coefficient of determination (R^2) value of 0.988 was obtained. Absorbance of Gallic acid in different concentration is given in table 4.20 and from which calibration curve plotted in figure 4.8. Using this calibration curve, the total amount of tannin content of plant extract was determined using the equation $y = 0.005x + 0.037$, where y is the absorbance at 725 nm and x is total tannin content in the different plant extracts.

Table 4.7: Absorbance of standard Gallic acid at different concentration

Concentration ($\mu\text{g/ml}$)	Avg. of Absorbance (725 nm)	Absorbance $\pm$ SD
Blank	0.058	
100	0.586	0.586 ± 0.016
50	0.354	0.354 ± 0.038
25	0.196	0.196 ± 0.002
12.5	0.095	0.095 ± 0.004
6.25	0.057	0.057 ± 0.002

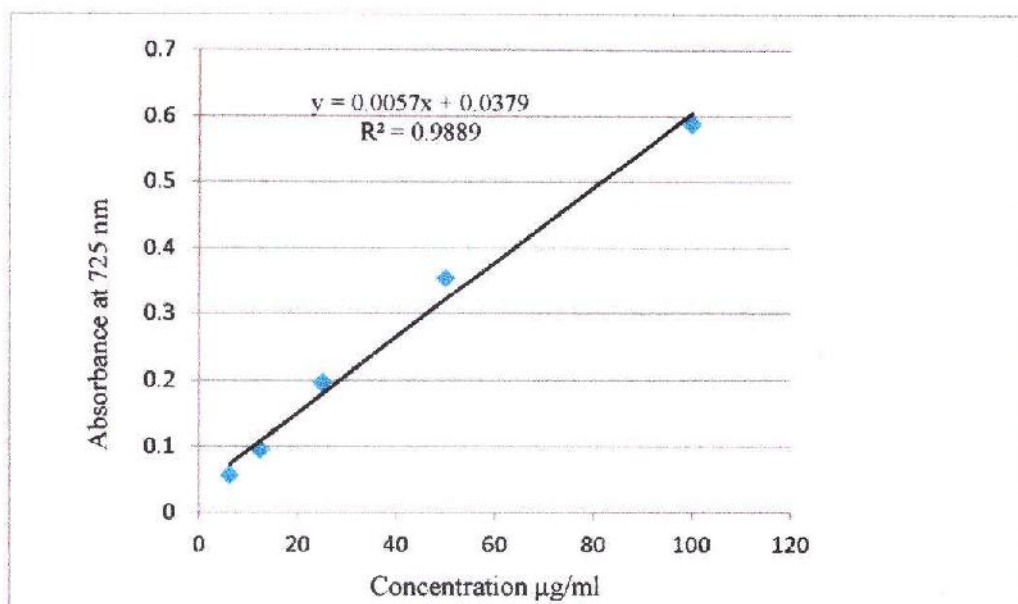


Figure 4.8: Calibration curve of standard Gallic acid for determination of total tannin content.

4.1.5.1 Total tannin content of potato peel

In this work, total tannin content of two extract of potato peel was determined using the standard calibration curve. Table 4.8 represents the amount of total tannin content in methanolic and water extract of potato peel. Methanolic extract shows 57.04 ± 0.007 mg GAE/g of extract and water extract shows 43.29 ± 0.004 mg GAE/g of extract respectively.

Table 4.8: Total tannin content of Potato peel

Extract	Conc.	Absorbance (725)	Total Tannin Content (mgGAE/g of extract) $\pm$ SD
Methanolic	100	0.363	57.04 ± 0.007
Water	100	0.285	43.29 ± 0.004

4.1.5.2 Discussion

Tannins are very complex polyphenolic compounds and are widely distributed in almost all plant species (Tamilselvi *et al.*, 2012). Tannins have been reported to exhibit a wide range of activities and prevent the attack of free radicals on the human body. Therefore, determination of the quantity of tannin compounds is very important in order to determine the antioxidant capacity of plant extracts.

Investigating the results of the present study, the quantitative estimation of total tannin content of water extract of potato peel exhibited 43.29 ± 0.004 mg GAE/g of extract. This poor amount of tannin content in *C. crista* extract could be related to the weaker scavenger in DPPH assay (fig. 4.2). Methanolic extract of potato peel showed the amount of total tannin content is 57.04 ± 0.007 mg GAE/g of extract. In this research work, we found methanolic extract of potato peel showed a quite good amount of tannin content, a good antioxidant potentiality in FRAP assay and quite good flavonoid content, but that did not seem to be contributed towards a good antioxidant property as we found the IC_{50} value at a concentration of 248.64 μ g/ml in DPPH assay along with good phenol content. This could be due to the fact that the free radical scavenging assay is not only specific to the presence of polyphenols or tannins (Singleton *et al.*, 1965). But we also found in the literature, polyphenols or tannins are an important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants (Okawa *et al.*, 2001).

4.2 Antibacterial activity Related Assay

The type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions, and post-harvest processing. For example, the relatively high temperatures that can be generated during tissue grinding can denature chemical constituents and the extraction solvent, time period, and temperature can affect the level and composition of secondary metabolites extracted from plant tissues. Petroleum ether, chloroform, methanol, ethanol and acetone separately or mixed with water are commonly used to extract bioactive compounds from plant materials, depending on the intended use of the extract. In this study methanol and water were chosen as extraction solvents because of their availability. In the present study an attempt has been made to screen potato peel, known for their medicinal properties, especially antimicrobial activity against a number of both gram positive and gram negative bacteria such as *S. aureus* (skin flora), *S. epidermis* (skin infection), *B. megaterium* (Non-pathogenic), *E. coli* (Food poisonous), *P. aurigona* (pulmonary infection), *V. cholera* (Cholera) and *S. typhi* (Typhoid fever). Disc diffusion method was used to investigate antimicrobial activity of methanolic and water extract of potato peel. Standard antibiotic discs of Azithromycin (100 µg/disc) were used for comparison purpose and it was found that the extracts showed responses in varying levels. The result of the antibacterial activity of the methanolic and water extracts was measured from the diameters from the zone of inhibition expressed as mm. The extracts exhibited varying levels of activities against the bacteria.

4.2.1 Disc Diffusion assay

4.2.1.1 Methanolic extract of potato peel

Methanolic extract of potato peel showed its maximum inhibition against *V. cholera* with average zone of inhibition (13.63 ± 0.53 mm) and *S. typhi* with average zone of inhibition (13.25 ± 0.35 mm) for 100 mg/disc at concentration in compared to standard Azithromycin (100µg/disc) as well. The diameters of zone of inhibition of Methanolic extract of potato peel against all tested microorganisms are shown in table 4.9 and illustrative appearance is represented in fig. 4.9.

Table 4.9: Antimicrobial activity of Methanolic extract of potato peel and positive control Azithromycin

Test microorganisms	Diameter of zone of inhibition			Negative control
	(mm ± SD) (n = 4)			
	Methanolic		Positive control	
	(100 mg/disc)	(50 mg/disc)	Azithromycin	
Gram-positive bacteria				
<i>Bacillus megaterium</i>	9.38 ± 0.18	7.38 ± 0.18	24.75 ± 0.35	n.d
<i>Staphylococcus aureus</i>	11.25 ± 0.35	7.88 ± 0.18	18.75 ± 0.35	n.d
<i>Micrococcus spp</i>	9.88 ± 0.18	6.83 ± 0.11	18.25 ± 0.35	n.d
Gram-negative bacteria				
<i>Escherichia coli</i>	10.38 ± 0.18	7.38 ± 0.18	16.25 ± 0.35	n.d
<i>Pseudomona</i>	7.38 ± 0.18	7.08 ± 0.25	17.13 ± 0.53	n.d
<i>Salmonella typhi</i>	13.25 ± 0.35	8.38 ± 0.18	22.75 ± 0.35	n.d
<i>Vibrio cholera</i>	13.63 ± 0.53	9.75 ± 0.35	22.75 ± 0.35	n.d
<i>Salmonella paratyphi</i>	9.5 ± 0	6.88 ± 0.18	21.44 ± 0.53	

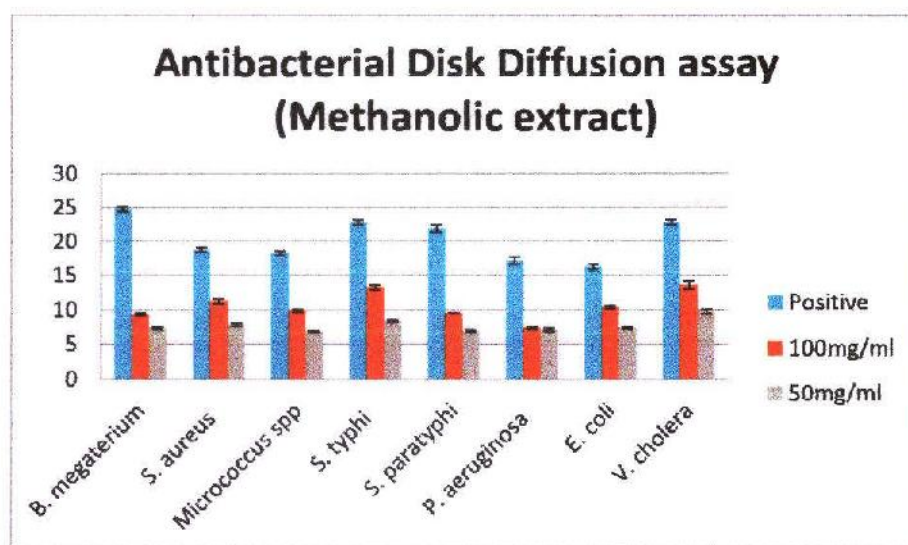


Figure 4.9: Evaluation of antimicrobial activity of potato peel methanolic extract (Microorganisms vs Zone of inhibition).

4.2.1.2 Water extract of Potato peel

Water extract of Potato peel showed its maximum inhibition against *Micrococcus spp* with average zone of inhibition (12.75 ± 0.35 mm) for 100 mg/disc at concentration in compared to standard Azithromycin (100µg/disc) with average zone of inhibition (20.45 ± 0.64 mm) as well. The diameters of zone of inhibition of Water extract of Potato peel against all tested microorganisms are shown in table 4.10 and illustrative appearance is represented in fig. 4.10.

Table 4.10: Antimicrobial activity of water extract of potato peel and positive control Azithromycin

Test microorganisms	Diameter of zone of inhibition			Negative control
	(mm ± SD) (n = 4)			
			Positive control	
	Water			
	(100 mg/disc)	(50 mg/disc)	Azithromycin	
Gram-positive bacteria				
<i>Bacillus megaterium</i>	9 ± 0.71	6.38 ± 0.53	25.75 ± 0.35	n.d
<i>Staphylococcus aureus</i>	10.75 ± 0.35	9.38 ± 0.53	19.85 ± 0.21	n.d
<i>Micrococcus spp</i>	12.75 ± 0.35	9.13 ± 0.53	20.45 ± 0.64	n.d
Gram-negative bacteria				
<i>Escherichia coli</i>	11.25 ± 0.35	6.75 ± 0.35	17.80 ± 0.28	n.d
<i>Pseudomona</i>	10.75 ± 0.35	6.25 ± 0.35	19.65 ± 0.92	n.d
<i>Salmonella typhi</i>	8.63 ± 0.53	7.10 ± 0.14	21.75 ± 0.35	n.d
<i>Vibrio cholera</i>	8.63 ± 0.53	9.13 ± 0.53	24.70 ± 0.99	n.d
<i>Salmonella paratyphi</i>	11.88 ± 0.18	6.50 ± 0.71	22.75 ± 0.35	n.d

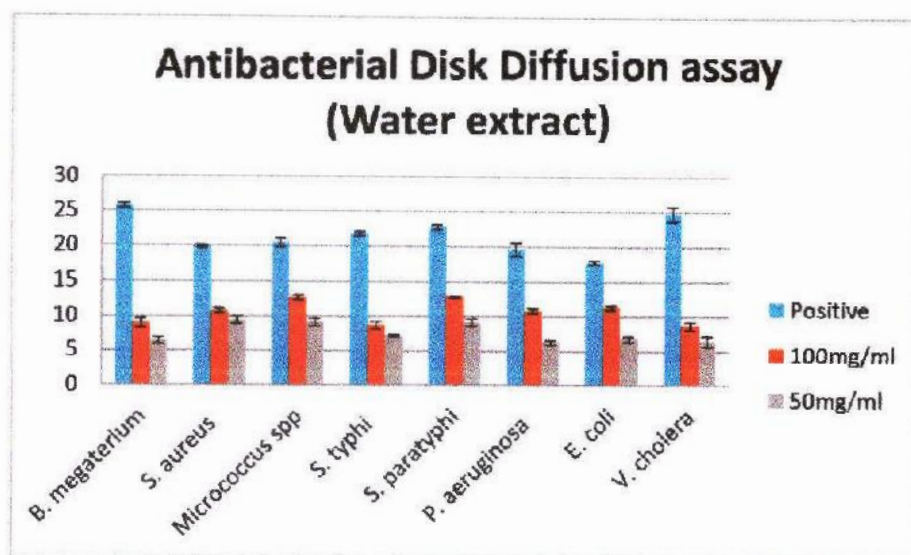


Figure 4.10: Evaluation of antimicrobial activity of potato peel water extract (Microorganisms vs Zone of inhibition).

4.2.1.3 Discussion

Advances of modern medicine, many infectious diseases once considered incurable and lethal are now amenable to treatment with antimicrobial agents. The remarkably powerful and specific activity of antimicrobial drugs is due to their selectively for highly specific targets that are either unique to microorganisms. Nowadays multiple drug resistance has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. Antimicrobial properties of medicinal plants are being increasingly reported from different part of the world. The beneficial medicinal effects of plant materials typically results from the combination of secondary products present in the plant.

In this point of view, in this research we have selected potato peel and evaluate antibacterial activity of crude methanolic and water extracts of potato peel by using disc diffusion method. A number of gram positive bacteria (*B.megaterium*, *S. aureus*, *Micrococcus*

spp) and a number of gram negative bacteria (*E. coli*, *P. aurigonisa*, *S. typhi*, *S. paratyphi* and *V. cholera*) were used in this test. The results of the present study clearly indicated that both the extract inhibited the growth of all the tested pathogens. The antimicrobial activities of the crude extracts were increased with increasing the concentration. Now here we discuss about the activity of all plant extract against individual microorganism.

Methanolic extract of potato peel showed its maximum inhibition against *V. cholera* with average zone of inhibition (13.63 ± 0.53 mm) and *S. typhi* with average zone of inhibition (13.25 ± 0.35 mm) for 100 mg/disc at concentration in compared to standard Azithromycin (100µg/disc) as well. So, Methanolic extract of potato peel showed maximum activity against gram negative bacteria. Both bacteria are responsible for food borne diseases. In this research lower activity was observed against Gram-positive bacteria. This can be explained because the outer membrane of Gram-positive bacteria is known to present a barrier to the penetration of numerous antibiotic molecules and the periplasmic space contains enzymes which are able of breaking down foreign molecules introduced from the outside (Duffy and Power 2001; ; Schaechter *et al.*, 1999).

Water extract of Potato peel showed its maximum inhibition against *Micrococcus spp* with average zone of inhibition (12.75 ± 0.35 mm) for 100 mg/disc at concentration in compared to standard Azithromycin (100µg/disc) with average zone of inhibition (20.45 ± 0.64 mm) as well. So, Water extract of Potato peel showed maximum activity against gram positive bacteria. This bacterium is responsible for skin diseases. Again we found, lower activity was observed against Gram-negative bacteria.

4.3 Serine Protease Inhibition Assay

Serine protease inhibition assay was performed according to Kunitz, (1947) with some modifications. In this method, sample donate electron in trypsin casein reaction. Sample inhibited trypsin to react with casein. Trichloroacetic acid was used to stop the reaction. And finally the reaction mixture was centrifuge at 12,000 rpm, 15 min and the supernatant was measured at 280 nm. In this method Quercetin was used as standard.

In this study, Serine protease inhibition activity of methanolic and water extracts of potato peels were examined and analyze their graphical representation.

4.3.1 Methanolic extract of potato peel

Analysis of potato peel methanolic extract, the absorbance of the extract increased gradually according to concentration increased. Absorbance at different log concentration of extract and standard quercetin is given in figure 4.11.

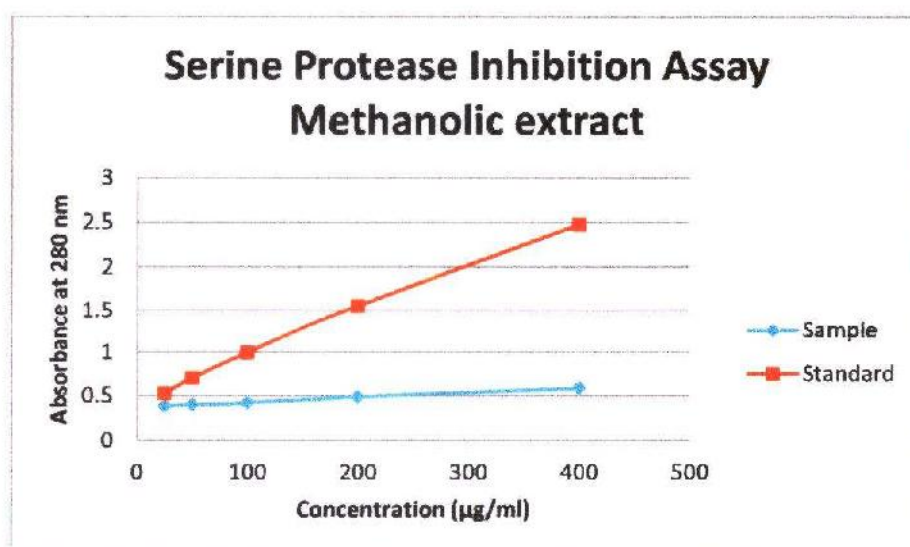


Figure 4.11: Serine protease inhibition assay of potato peel methanolic extract (Absorbance vs concentration).

Comparison of percentage inhibition of methanolic extract of potato peels and standard quercetin at different log concentrations is given in fig. 4.12. The concentration at which 50% inhibition (IC_{50}) of trypsin occurred were obtained by using the equation $y = mx + c$. IC_{50} value of extract was found at the concentration of 534.67 µg/ml and standard quercetin was 77.64 µg/ml. So we observed that the concentration of the extract required for 50% inhibition is greater than that of control.

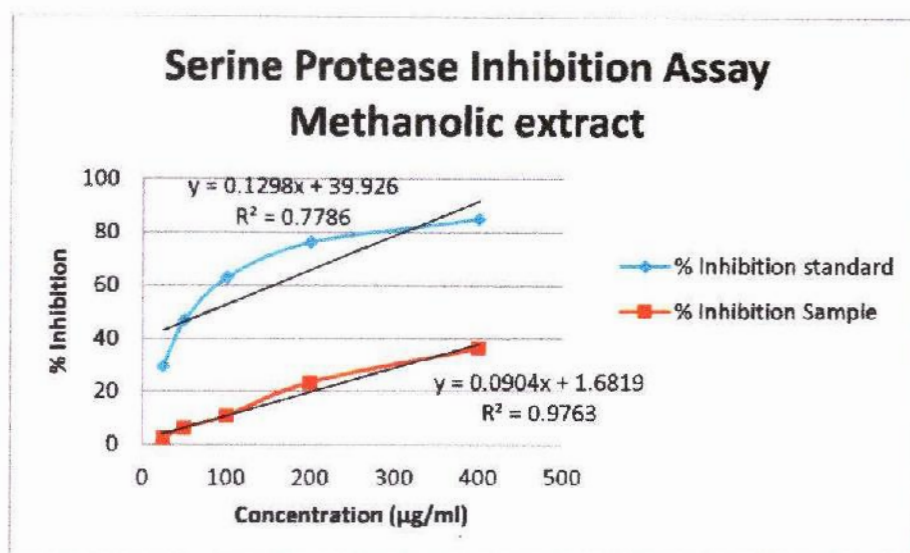


Figure 4.12: Serine protease inhibition assay of potato peel methanolic extract (% inhibition vs Concentration).

4.3.2 Water extract of potato peel

Analysis of potato peel water extract, the absorbance of the extract increased gradually according to concentration increased. Absorbance at different log concentration of extract and standard quercetin is given in figure 4.13.

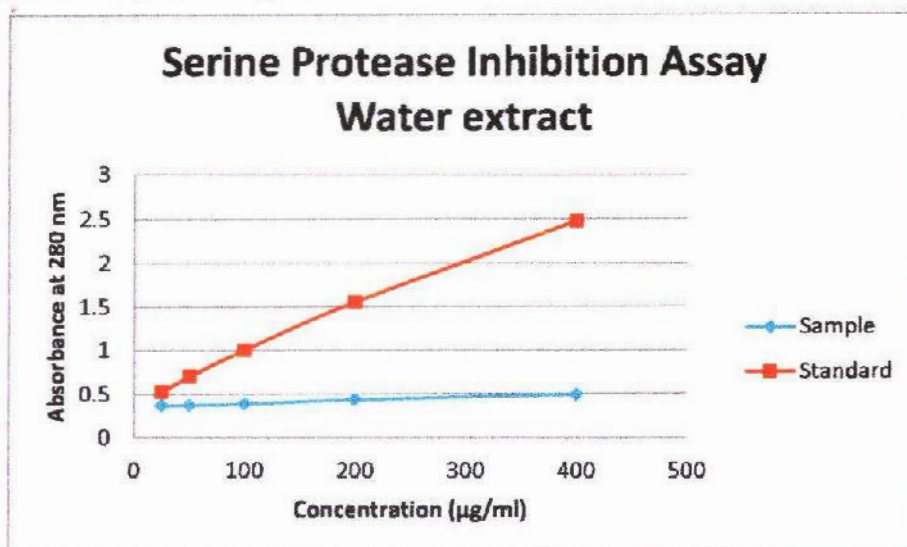


Figure 4.13: Serine protease inhibition assay of potato peel water extract (Absorbance vs concentration).

Comparison of percentage inhibition of water extract of potato peel and standard quercetin at different log concentrations is given in fig. 4.14. The concentration at which 50% inhibition (IC_{50}) of trypsin occurred were obtained by using the equation $y = mx + c$. IC_{50} value of extract was found at the concentration of 735.10 $\mu\text{g/ml}$ and standard quercetin was 77.64 $\mu\text{g/ml}$. So we observed that the concentration of the extract required for 50% inhibition is greater than that of control.

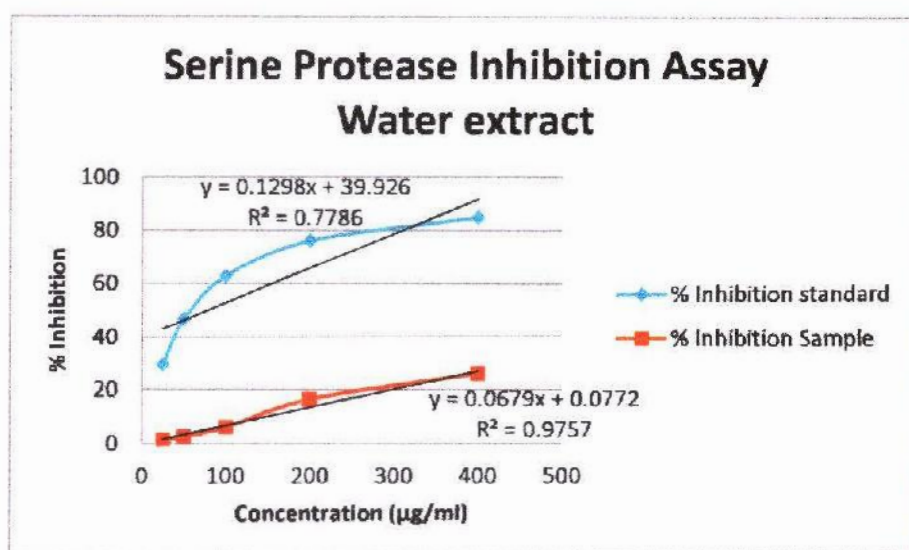


Figure 4.14: Serine protease inhibition assay of potato peel water extract (% inhibition vs Concentration).

4.3.3 Discussion

Serine protease inhibition activity was determined by the method of Kunitz, (1947) with some modifications. According to literature we found that *Solanaceae* family has potential protease inhibition activity. For this reason we select potato peel for our experimental sample. Methanolic extract of potato peel exhibited 50% trypsin inhibition at 534.67 $\mu\text{g/ml}$ and standard Quercetin exhibited 50% trypsin inhibition at 77.64 $\mu\text{g/ml}$. % inhibition of standard Quercetin at highest 400 $\mu\text{g/ml}$ concentration is 85.01 and % inhibition of sample at highest 400 $\mu\text{g/ml}$ concentration is 36.18. This methanolic extract of potato peel has moderate protease inhibition activity. This is due to the presence of phenolic acid, flavonoid etc. This result support our previous DPPH free radical scavenging assay, FRAP assay, phenolic content, flavonoid content and Tanin content.

Water extract of potato peel exhibited 50% trypsin inhibition at 735.10 $\mu\text{g/ml}$ and standard Quercetin exhibited 50% trypsin inhibition at 77.64 $\mu\text{g/ml}$. % inhibition of standard Quercetin at highest 400 $\mu\text{g/ml}$ concentration is 85.01 and % inhibition of sample at highest 400 $\mu\text{g/ml}$ concentration is 26.13. This water extract of potato peel has less protease inhibition activity. This is due to the presence of less phenolic and flavonoid content.

4.4 Column chromatography

Column chromatography technique is used for the isolation and separation of single compound from a mixture of compound. In our methanolic extract of potato peel different polarity compound was present. Our goal was to separate compound of similar polarity. For this purpose we used three type of solvent non polar, medium polar and polar in the mobile phase. We use n-hexane as non-polar, ethyl acetate as medium polar and methanol as polar solvent. When these solvents of different polarity pass the stationary phase they elute same type of compounds. For example, n-hexane elute non polar compounds, ethyl acetate elute medium polar compounds and methanol elute polar compounds. As we know phenolics and flavonoids are polar compounds so our target was to separate the phenolics and flavonoids from crude extract. Different percentage of solvents we used given in table 4.11.

Table 4.11: Number of different fraction and their solvent percentages

Fraction number	n-hexane (%)	Ethyl acetate (%)	Methanol (%)
F1	100	0	0
F2	90	10	0
F3	80	20	0
F4	70	30	0
F5	60	40	0
F6	50	50	0
F7	40	60	0
F8	30	70	0
F9	20	80	0
F10	10	90	0
F11	0	100	0
F12	0	95	5
F13	0	90	10
F14	0	85	15
F15	0	80	20

F16	0	75	25
F17	0	70	30
F18	0	65	35
F19	0	60	40
F20	0	55	45
F21	0	50	50
F22	0	45	55
F23	0	40	60
F24	0	35	65
F25	0	30	70
F26	0	25	75
F27	0	20	80
F28	0	15	85
F29	0	10	90
F30	0	5	95
F31	0	0	100

Flavonoid content of different fraction is given in table 4.12

Table 4.12: Flavonoid content of different fraction

Fraction number	Total Flavonoid Content (mgQE/g of extract) $\pm$ SD
F25	36.68 ± 0.002
F26	48.39 ± 0.003
F27	48.91 ± 0.0006
F28	37.43 ± 0.0006
F29	39.09 ± 0.002
F30	68.91 ± 0.004
F31	42.24 ± 0.003

From the above table we found that in all the fractions we tasted for flavonoid content, amount of total flavonoid content increase than the crude methanolic extract. The maximum number of total flavonoid content found in F30 (68.91 ± 0.004 mgQE/g of extract). Again in some fraction like F25, F28, F29 amount of total flavonoid content do not increase much. This may occur because all flavonoids present in potato peel are not in same polarity. So due to the presence of different polarity flavonoid all fractions do not contain high amount of total flavonoid content.

Next we checked the bioactivity of some fractions which contain high amount of total flavonoids among the fractions.

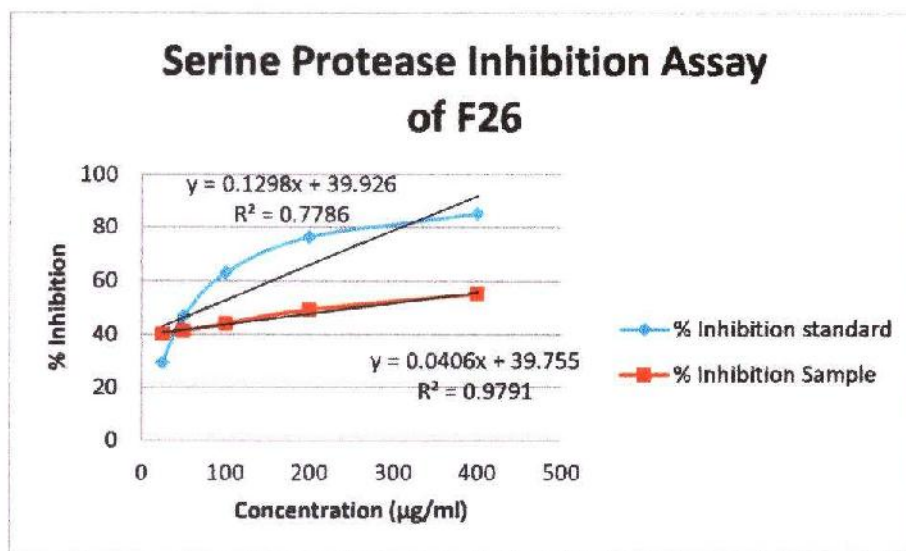


Figure 4.15: Serine protease inhibition assay of Fraction 26 (% inhibition vs Concentration).

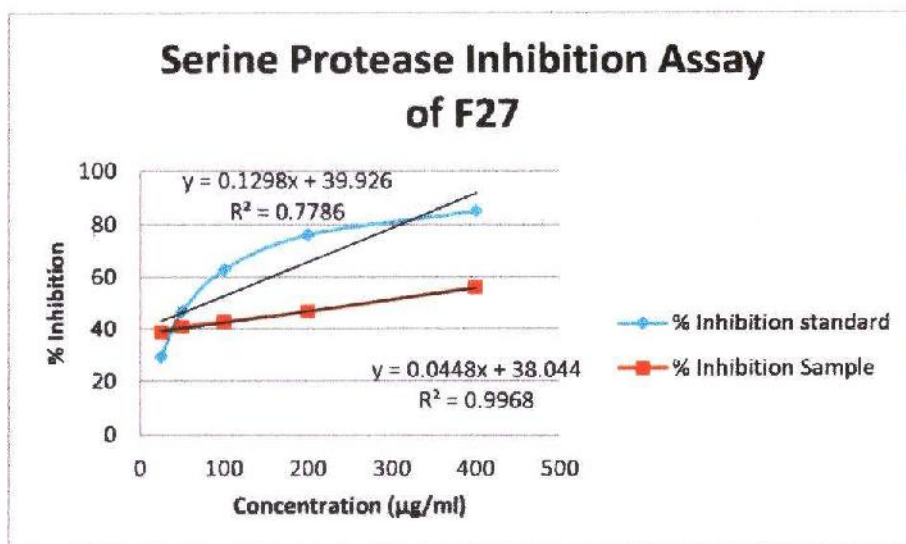


Figure 4.16: Serine protease inhibition assay of Fraction 27 (% inhibition vs Concentration).

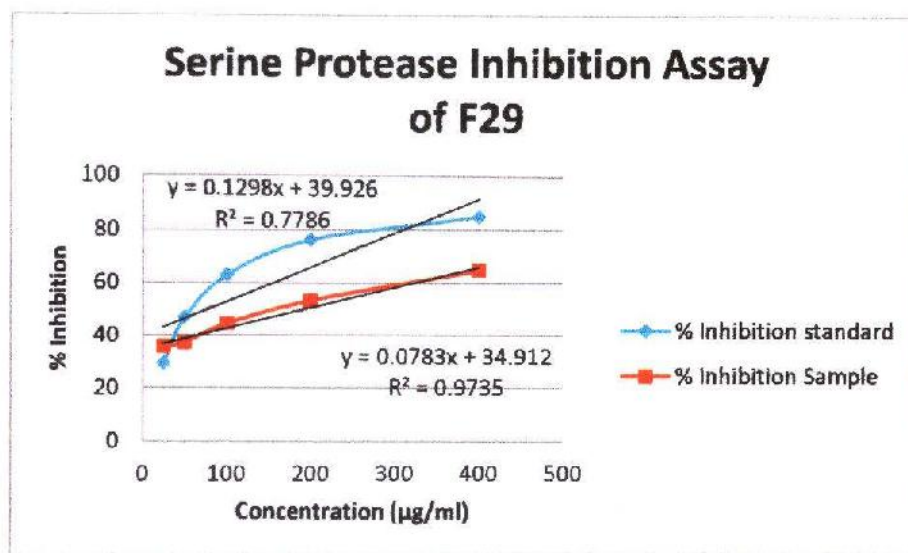


Figure 4.17: Serine protease inhibition assay of Fraction 29 (% inhibition vs Concentration).

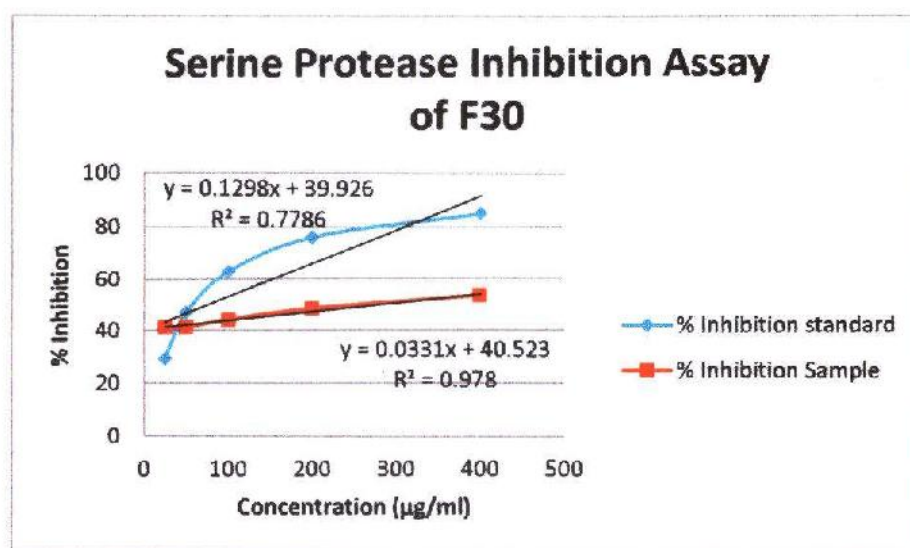


Figure 4.18: Serine protease inhibition assay of Fraction 30 (% inhibition vs Concentration).

Comparisons of serine protease inhibition activity is described in table 4.27

Table 4.13: Comparisons of serine protease inhibition activity.

Concentration	Standard	Crude methanolic extract	F26	F27	F29	F30
400	85.01	36.18	55.29	55.95	64.80	53.26
200	76.15	23.33	49.37	46.81	53.19	48.27
100	62.88	10.66	44.04	42.74	44.34	44.08
50	46.89	6.05	41.34	40.76	37.13	41.34
25	29.27	2.23	40.22	38.63	35.78	41.34
IC50	77.64	534.67	252.24	267.17	192.69	286.12

From the comparative analysis of the serine protease inhibition activity of tested fraction we found highest inhibition in fraction 29. It showed 64.80 % inhibition at highest concentration, which is much higher than crude methanolic extract. Other Fractions also showed remarkable inhibition in highest concentration. Again in terms of IC50, in fraction F29 decrease at a good rate than crude extract. From these findings we can conclude that, compounds responsible for protease inhibitory activity are highly polar (Dissolved in 90% methanol) and their polarity is very close to one another.

C

HAPTER 5

Conclusion

5.0 Conclusion

In the present study, we investigate some bioactivity of potato peel such as its antioxidant properties, antibacterial properties and its protease inhibitory activity. Again we have performed column chromatography technique to enrich the potentiality of potato peel. We use two solvent systems viz, water and methanol. These solvents were choose because literature recommended that methanol extract show more activity than other solvents. In addition cooking process, water is used, so that a clear idea could be obtained regarding the differences between the two solvent systems. Oxidative stress is a condition in which free radicals are generated extra- or intracellularly, which can exert their toxic effects to the cells. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant-derived agents may help to reduce it. Currently, there has been an increased interest globally to identify antioxidant compounds from plant sources which are pharmacologically potent and have low or no side effects for use in protective medicine. Research findings revealed that potato peel have some amount of vitamin C and different polyphenols. These are responsible for the antioxidant activity of potato peel. In our study we found more antioxidant activity in water extract than methanolic extract In DPPH free radical scavenging assay. Water is more polar than methanol. So in water extract more polar compound present and the donate electrons in these assay. So IC₅₀ become less in water extract than methanolic extract. In other antioxidant related assay like FRAP assay, total phenolics content, total flavonoid content and total tannin content methanolic extract showed high result that support previous literatures.

Plants represent an unlimited source of phytochemicals. In the present study an attempt has been made to screen antibacterial activity of potato peel of water and methanolic extract. Several common pathogenic bacteria like *S. aureus*, *S. epidermis*, *E. coli*, *V. cholera*, *S. typhi* and *P. aurigonisa* were used in the present investigation. In antimicrobial activity, both water and methanolic extract showed antimicrobial activity against gram positive and gram negative bacteria. Water extract showed good antibacterial activity against gram positive bacteria and methanolic extract showed good antibacterial activity against gram negative bacteria.

Protease inhibition is one of the mechanisms to kill bacteria. Different protease enzymes present in bacterial cell wall are inhibited by protease inhibitors, so bacteria cannot grow in presence of

protease inhibitors. In our study, we investigated protease inhibition activity of water and methanolic extract. Methanolic extract showed more inhibitory activity than water extracts. This is for the presence of more phenolics and flavonoids in methanolic extract. Due to these reason methanolic extract showed more antibacterial activity than water extract.

In our study, we also performed column chromatography to separate bioactive compound. Our target was to enrich flavonoids than the crud methanolic extract. For these purpose we used polarity gradient solvent as mobile phase. After fractionation we checked flavonoid content and protease inhibitory activity of the fractions. We found that flavonoid content increased in higher polarity fractions and these fractions showed almost double protease inhibitory activity than crud methanolic extract.

Further studies are required to identify the bioactive compounds present in in the fraction which are responsible for protease inhibitory activity.

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HAPTER 6

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6. References

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