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Research Article

Biochemical Characterization of Selected Plant Species and Investigation of Phytochemicals for *In-Vitro* Antioxidant Activity

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ABSTRACT

The phytochemical analysis of the plants *Anethum graveolens*, Betel leaf (*Piper betel*), Coriander (*Coriandrum sativum*), cinnamon (*Cinnamomum zeylanicum*), Wheat grass andJack fruit leaf (*Artocarpus heterophyllus*)was carried out to evaluate the antioxidant potential. Alcoholic extracts of the leaves was subjected to *in vitro* antioxidant activity screening models such as inhibition of lipid peroxidation, nitric oxide and superoxide radical scavenging activity, reduction of ferric ions, metal ion chelating activity, hydrogen peroxide and hydroxyl radical scavenging activity. Ascorbic acid was used as the standard for superoxide anion radical scavenging, reduction of ferric ions and hydrogen peroxide scavenging activities. EthyleneDiamineTetraAcetic acid (EDTA) is used as a standard for metal ion chelating activity. Butylated Hydroxy Toluene (BHT) is used as standard for anti-lipid peroxidation activity and for nitric oxide scavenging activity Gallic acid was used as standard. In all the models studied, the extracts showed potent antioxidant activity, thereby indicating its potential to treat or prevent certain medical conditions.

Key words: *Anethum graveolens*, *Piper betel*, *Coriandrum sativum*, *Cinnamomum zeylanicum*, *Artocarpus heterophyllus*, lipid peroxidation, free radical scavenging

INTRODUCTION

Many plants have been known to produce biologically active substances, some of which are related to special flavour or taste and others are found to be useful as antioxidants and/or antimicrobial agents. Methanolic extract of various plant samples can be used to carry out several biochemical studies. Methanol is an efficient solvent system for obtaining various plant extracts. Medicinal Plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments[4].Approximately 80% of the world population depends exclusively on plants for their healthand healing[12]. In the developed world, reliance on surgery and pharmaceutical medicine ismore usual, but in recent years, more and more people are complementing their treatmentwith natural supplements obtained from medicinal plants [11].

The plant kingdom holds many species of plants containing substancesof medicinal interest which are yet to be investigated. Large numbers of plant are constantlybeing screened for their chemical and pharmacological properties[15]. By the application of modern techniques of isolation and pharmacological evaluation,many new plant drugs find their way to medicine as purified substances [13]. A recent aspect of interest in plant drug research is the new concept of a nonspecifically increased resistance of an animal to diseases attributable to other substances,besides the active principles responsible for specific biological activity[17]. This will probablyjustify the use of many of the plant drugs as household remedies by indigenous people of manycountries from ancient times and hence warrants their evaluation in more detail.

The fact that onlyvery few percent of the six lakhs species of plants on the planet has been investigated, indicatesthe opportunity provided and challenges thrown to phytochemists[18]. The surge in the number of publications on the investigation of anti-oxidant and anti-inflammatory properties of various plants and methods of finding those properties [19]clearly indicates the importance of the antioxidants. Oxygen is necessary for the survival of all on this earth. During the process of oxygenutilization in normal physiological and metabolic processes approximately 5% of oxygen gets
univalently reduced to oxygen derived free radicals. Free radicals are types of Reactiveoxygen species (ROS), which include all highly reactive, oxygen-containing molecules.The main endogenous (originates within the organism) sources of most of the oxidants producedby cells in different ways, including normal aerobic respiration. Exogenous (coming from outside) sources of free radicals include tobacco smoke,ionizing radiation, certain pollutants, organic solvents and pesticides[23].

Cell damage caused by free radicalsappears to be a major contributor to aging and degenerative diseases of aging such as cancer,cardiovascular disease, cataracts, immune system decline, liver diseases, diabetes mellitus,inflammation, renal failure, brain dysfunction and stress among others. To protect the cellsand organ systems of the body against reactive oxygen species, humans have evolved ahighly sophisticated and complex antioxidant protection system, that functions interactivelyand

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Table 1: Phytochemical constituents of the methanolic plant extracts

Sl.No.	Test	Artocarpus heterophyllus	Anethum graveolens	Cinnamomum zeylanicum	Coriandrum sativum	Wheat grass	Piper betel
1	Alkaloids	+	-	+	-	-	+
2	Anthraquinones	+	-	+	-	-	+
3	Flavonoids	-	+	-	+	+	-
4	Saponins	+	-	+	+		+
5	Steroids	-	-	-	-	-	-
6	Tannins	+	+	+	-	+	-
7	Acidic Compounds	-	+	+	+	+	+
8	Cardiac glycoside	+	+	+	+	+	+
9	Terpenoids	+	+	+	+	+	+

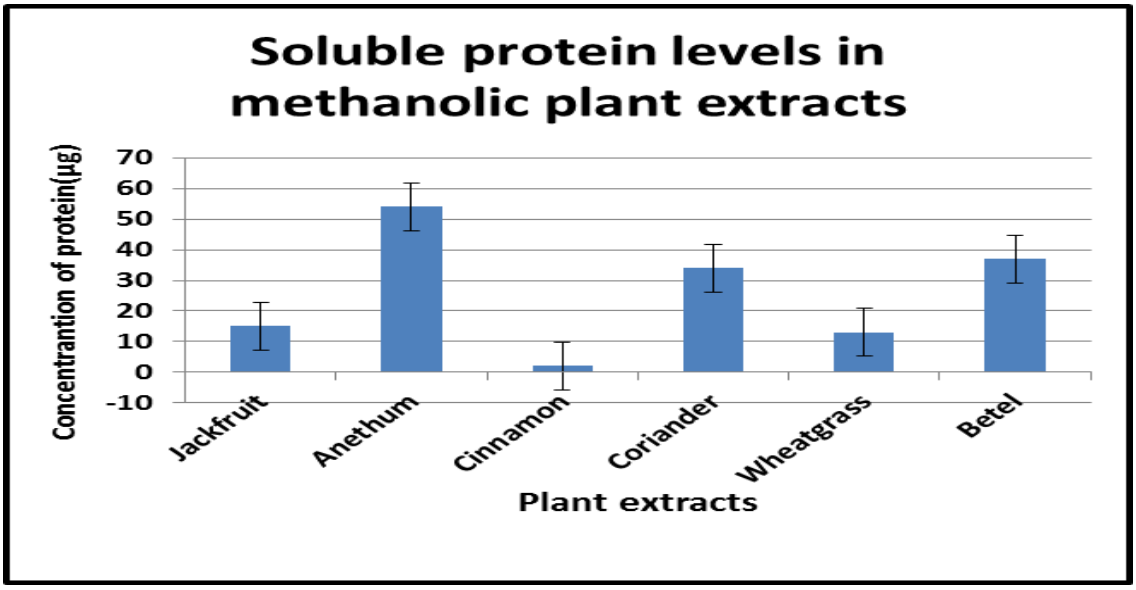

Figure 1 Concentration of soluble protein (µg/g of extract)

synergistically to neutralize free radicals[5]. Antioxidants are absolutely criticalfor maintaining optimal cellular and systemic health and well-being [27].

Antioxidant based drugs and formulations for the prevention and treatment ofcomplex diseases like Alzheimer's disease and cancer have appeared during last threedecades [1]. Recent studies have shown that a number of phytochemicals includingflavonoids, polyphenols, terpenes and various plant extracts exerted an antioxidant action[21].There is also a considerable amount of evidence revealing an association betweenindividuals who have diet rich in fresh fruits and vegetables and the decreased risk ofcardiovascular diseases and certain forms of cancer [14]. There is currently immense

interest in natural antioxidants and their role in human health and nutrition [3].Considerable amount of data have been generated on antioxidant properties of food plantsaround the globe [8]. Several medicinalplants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda)system of medicine for the treatment of number of diseases [9]. Some of these plants haveshown potent antioxidant activity [16]. However, majority of plants have not yet beenscreened for such activity.The aim of this study was to carry out the biochemical characterization of the selected plant species and determine its antioxidant and free radical scavenging properties.

MATERIALS AND METHODS

Source of material: Leaves of *Anethum graveolens*, *Piper betel*, *Coriandrum sativum*, *Artocarpus heterophyllus*,Wheat grassand bark of*Cinnamomum zeylanicum*were collected locally and used for the isolation of bioactive compounds. The present study was conducted at Research Centre, Mount Carmel College, Bangalore.

Extraction: Leaves of the selected plant specieswere obtained and washed with distilled water. They wereshade dried and used for extraction. The crude extracts were obtained by extracting 10 g of dried plant powder in 100 ml methanol and was kept on a rotary shaker for 24 h. The extract was filtered, centrifuged at 5000 g for 15 minutes and was thereafter concentrated by evaporation in a rota-vacuum distillator.One gram of the extracts was dissolved in 100 ml of its own mother

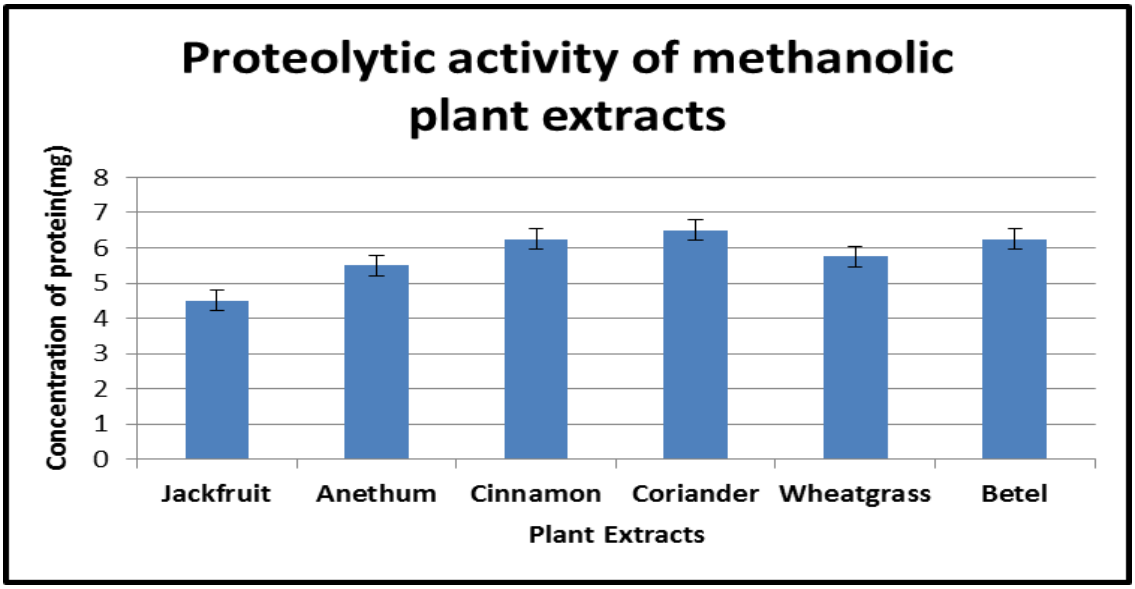

Figure 2 Determination of Proteolytic activity (mg/g of extract)

Table 2 Estimation of Lycopenes and β- Carotene

PLANT SAMPLES	Jack fruit leaf	Anethum	Cinnamon	Coriander	Wheat grass	Betel leaf
LYCOPENES (mg/100mg of extract)	0.1536	0.036	0.033	0.0891	0.1441	0.0134
β- CAROTENE (mg/100mg of extract)	1.5435	0.5011	0.0172	0.9123	1.5838	0.7498

solvent to obtain a stock of concentration 1% (W/V). Qualitative analysis of
the crudeextract was further carried out to test the presence of phytochemicals such as alkaloid, anthraquinones, flavonoids, terpenoids, sterols, tannins, etc.Thin layer chromatographic examination of the extracts: Thin layer chromatographic plate (5 X 20 cm) 0.5mm thickness was prepared by usual method using silica gel G. the samples of 0.1% of all the extracts were dissolved in methanol separately and spotted manually using a capillary tube. The plate was developed in n-hexane: ethyl acetate (9:1) as solvent system. After development, the chromatogram was observed for the presence of spots under U.V. light.

Preliminary Phytochemical Screening: Test for alkaloids: 2 ml of the extracts were diluted separately to 10ml with acid alcohol, boiled and filtered. To 5ml of the filtrate 2ml of dilute ammonia was added. 5ml of chloroform was added and shaken gently to extract the alkaloid base. The chloroform layer was extracted with 10ml of acetic acid. Few drops of Wagner's solution added to chloroform solution, reddish brown precipitate indicates the presence of alkaloids.

Test for anthraquinones:2 ml of the extracts were taken into a dry test tube and 5 ml of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red colour in the ammoniacal layer indicates presence of anthraquinones.

Test for flavonoids: 2ml of the extracts were dissolved in the methanol, to this a small piece of magnesium ribbon was added and 1 ml of concentrated Hydrochloric added from the side of the test tube. A magenta colour indicates the presence of flavonoids.

Test for saponins: To 5ml of the extracts, 5ml of distilled water was added and shaken for the formation of froth which confirms the presence of saponins.

Test for steroids: 1ml of extracts was taken to which 10ml of chloroform was added. After this 10ml of concentrated sulphuric acid was added along the sides of the test tubes. A colour change from violet to blue/green confirms the presence of steroids in the samples

Test for tannins:0.5ml of extracts was boiled in 10ml of water for 5-10 minutes and filtered. Ferric chloride (0.1%) was added to this and a brownish green or blue black colouration formed confirms the presence of tannins.

Test for acidic compounds: Sodium bicarbonate solution was added to the extracts and checked for the appearance of effervescence. This confirms the presence of acidic compounds in the sample.

Test for cardiac glycosides: 2mlof extracts was treated with 2 ml of glacial acetic acid containing a drop of $FeCl_3$ solution. This was under layered with 1 ml of concentrated H_2SO_4. A brown ring obtained at the interface indicates the presence of de-oxy sugar characteristics of cardenolide.

Test for terpenoids:2 ml of extracts was treated with 2 ml of chloroform and concentrated $H_2SO_4$was carefully added to form a layer. A reddish brown colour formation at the interface confirms the presence of terpenoids.

Extraction and Determination of Soluble Protein: Samples of the leaves and bark were diluted in a solution of sodium phosphate buffer 0.1 mol /L pH 7.0, shaken during 1 h at 4ºC, and then centrifuged at 12,000 x g for 15 min at 4ºC. The precipitate was discharged and the supernatant was denominated crude extract. Protein concentration wasdetermined using BSA as standard[6].

Determination of Proteolytic Activity: Proteolytic activity was determined by incubation of 0.1 ml of crude extract with 0.5 ml of caseinsolution 1% (w/v), prepared in phosphate buffer 0.1 mol/L pH 7.6 at 37ºC for 20 min. Reaction wasterminated by the addition of 1.5 ml of solution of TCA 5% (v/v). After centrifugation at 2,000 x g, the supernatant were read at 280 nm using a spectrophotometer [2]. One enzyme unit(EU) was defined as the amount of enzyme necessary to produce the elevation of 0.1 in opticaldensity (OD) after 20 min[24].

Determination of Protease inhibitor activity: Samples of crude extracts were incubated with 0.1 ml trypsin solution 1.0 mg /ml and phosphatebuffer 0.1 mol /L pH 7.6 at 37ºC for 10 min. After that 0.5 ml of a casein solution 1% (w/v) wasadded to the solution and the reaction mixture was incubated for 10 min. The reaction was interruptedby adding of 1.5 ml of TCA solution 5% (v/v) [2].

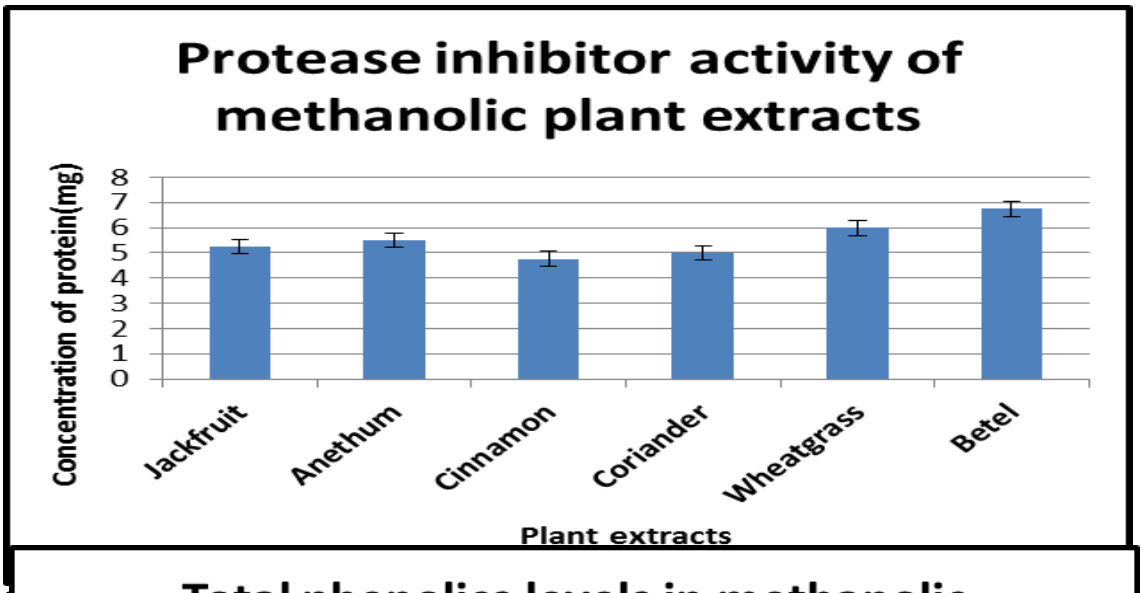

Figure 3

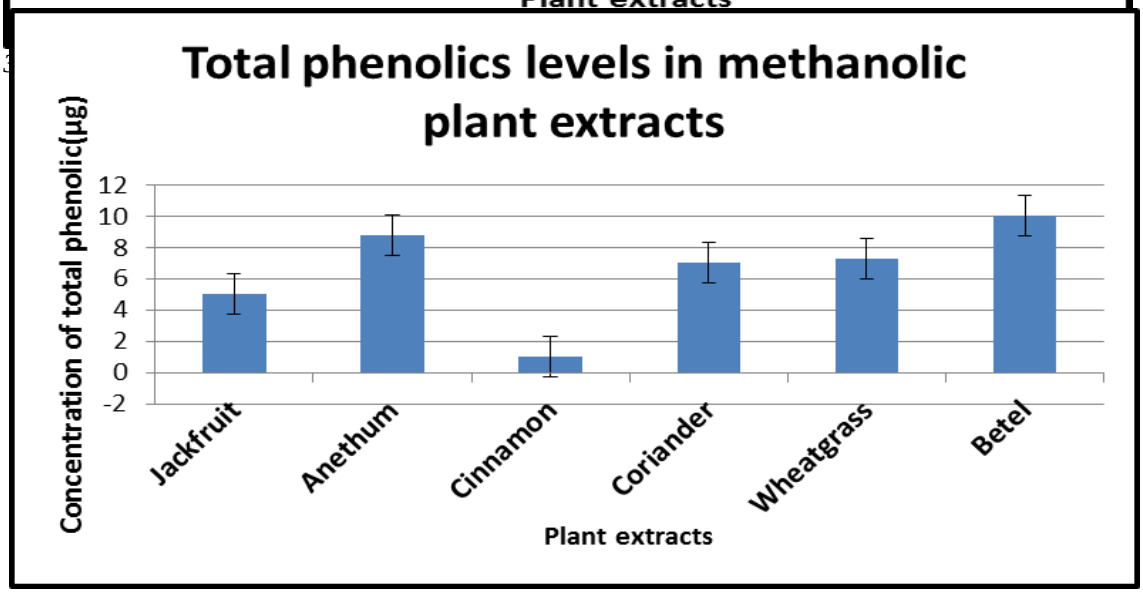

Figure 4 Estimation of total phenolics (µg/g of extract)

Quantitative Estimation ofβ-carotene and lycopene content: The dried extracts were vigorously shaken with 10ml acetone hexane mixture (4:6) for 1 minute and were filtered through Whatman No.4 filter paper. The absorbance was measured at 453nm, 505nm, 663nm. Contents of β-carotene and lycopene were calculated according to the following equations:

Lycopene (mg/100mg) = -0.0458 A663+0.372A505-0.0806 A453

β-Carotene (mg/100mg) = 0.216 A663-0.304 A505+0.452A453

Quantitative Estimation of Total Phenolics: To the

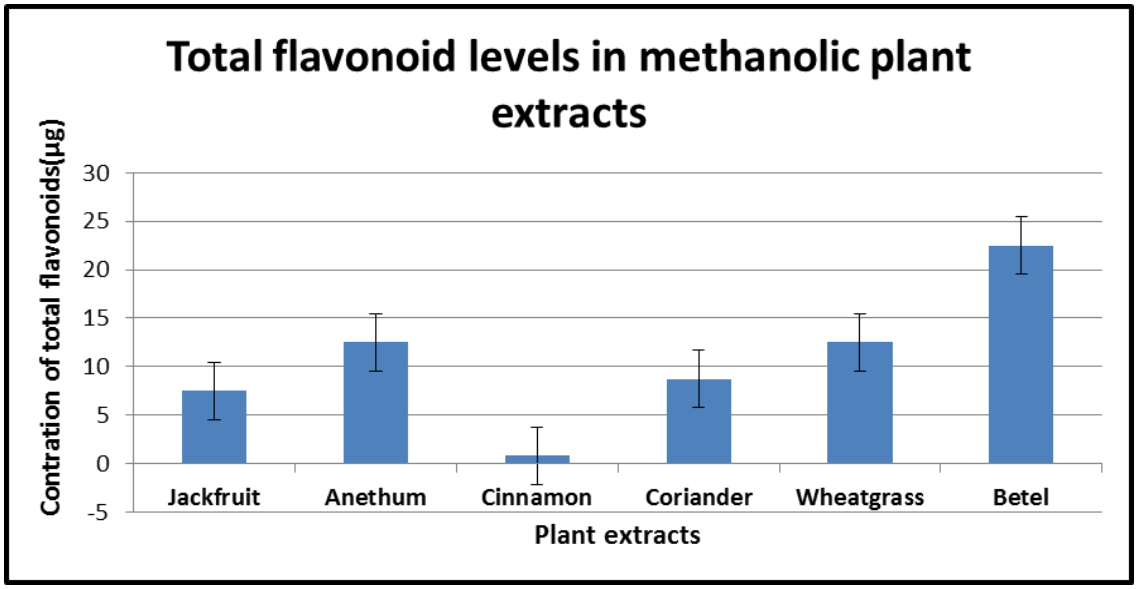

Figure 5 Estimation of total flavonoids

extracts, 20% Sodium bicarbonate was added and shaken vigorously. After 2 minutes, 1.5 ml of 1:1 diluted Folin's Reagent was added with constant shaking. The colour was allowed to develop for half an hour and absorbance at 750 nm was taken and expressed as mg/g extract[7].

Quantitative Estimation of Total Flavonoid Content: 0.5 ml of theplant extracts was mixed with 95%methanol, 10% aluminium chloride, 1M potassium acetate in distilled water. After incubation at room temperature for 30 minutes, the absorbance was measured at 415 nm along with standard Quercetin and blank. The concentration obtained by comparing with the calibration curve was prepared from a reference solution containing Quercetin[25].

In vitro anti-lipid peroxidation assay: Freshly excised goat liver was homogenized in cold Tris HCl buffer (pH 7.0) and centrifuged at 3000 rpm for 10 minutes to get a clear supernatant.

The reaction mixture containing 0.5 ml of liver homogenate, plant extracts at different concentrations, 0.15M KCl, ferrous iron (0.16mM) and 6mM ascorbic acid was incubated for 1 hour at 37°C. 10% TCA was added and centrifuged at 3000 rpm for 20minutes at 4°C. Supernatant was removed and 1 ml 0.8% TBA was added followed by heating at 90°C for 20 minutes in water bath. Absorbance was measured at 532 nm and the percentage inhibition was calculated using the formula[20]:

% =1- sample OD/blank OD x 100

Reducing Power:Reducing power of the extracts is assessed on the reduction of Fe3+ / Ferrocyanide complex to ferrous form which gives various shades of green and blue depending on the reducing ability of the plant extracts. The extracts were mixed with 2.5 ml of 1% potassium ferricyanide and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for20 minutes. Trichloroacetic acid (10%) was added to it and centrifuged at 3000 rpm for 10minutes. Then the supernatant was taken and 2.5 ml of water and FeCl3 (0.1%) was added to it. Absorbance was measured at 700 nm[10].

Metal ion chelating activity: 1mM FeSO4 solution was mixed with extracts, 1 ml Tris HCl buffer (pH 7.4) and 2,2'-bipyridly solution was added together with hydroxyl amine–HCl and ethanol, respectively. The reaction mixture was adjusted to a final volume of 5 ml with distilled water, shaken well and incubated for 10 minutes at room temperature. Absorbance was determined at 522 nm and percent chelation was calculated using the equation[26]:

% =1- sample OD/blank OD x 100

Hydrogen peroxide scavenging activity:Extracts were dissolved in phosphate buffer (0.1 mM, pH 7.4) and mixed with 600µl of hydrogen peroxide solution. Ascorbic acid was used as the reference compound. The concentration of the hydrogen peroxide was measured by reading the absorbance values of the reaction mixtures at 230 nm after 10 minutes. Hydrogen peroxide concentration was determined using molar absorptivity for hydrogen peroxide[22]. The percentage inhibition was calculated using the formula:

% =1- sample OD/blank OD x 100

RESULTS

Proteolytic activity: The plant extracts did not have the ability of proteolysis as the reaction mixture considerably showed a high protein content inferring that casein was not broken down into smaller peptides.

Protease inhibitor activity: All the six plant extracts inhibited the activity of trypsin on casein. Hence the protein concentration measured was high in all samples.

The highest concentration of lycopenes was observed in Jack fruit and wheat grass and the lowest was observed in cinnamon as indicated in table 2.

Using gallic acid as standard, concentration of total phenolics was found to be highest in betel leaf and lowest

in cinnamon extract, with 10µg/ml and 1µg/ml respectively.

Using quercetin as standard, concentration of total flavonoids was found to be highest in betel leaf and lowest in cinnamon extract, with 22.5µg/ml and 0.8µg/ml respectively.

In vitro anti-lipid peroxidation assay: Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids. If not terminated fastenough, there will be damage to the cell membrane, which consists mainly of lipids. In addition, end-products of lipid peroxidation may be mutagenic and carcinogenic.All the six plant extracts showed the same percentage of anti-lipid peroxidation activity which was around 98%.

Reducing Power: The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging.

Reducing power is the ability of the extract to reduce ferrocyanide complex to ferrous form. Based on the intensity of blue/green colour observed, cinnamon extract showed to have the highest reducing power and wheatgrass the lowest.

Metal ion chelating activity : Transition metal ions, especially iron can stimulate lipid peroxidation by the Fenton reaction and can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyses lipid peroxidation.Wheatgrass showed high iron chelating activity of 81% and cinnamon showed the least activity of 34%.

Hydrogen peroxide scavenging activity: Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe2+ and possibly Cu2+ ions to form hydroxyl radicals and this may be the origin of many of its oxide effects.Anethum showed high hydrogen scavenging activity of 29% and cinnamon the least activity of 4%.

DISCUSSION

The phytochemical experiments performed on the six plant extracts showed the presence of different constituents such as anthraquinonones, flavonoids, tannins, terpenoids, saponins steroids and acid compounds present or absent in them. These classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging or chelation. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability. The extracts have no proteolytic activity but they are shown to be potent protease inhibitors.

Phytochemical screening of the crude methanolic extract of the revealed the presence of flavonoids, alkaloid Phytochemical screening of the crude ethanolic extract of the revealed the presence of flavonoids, alkaloids, tannins, steroids, saponins and phenolic compounds. In addition, it is suggested that although the reducing power of a substance may be an indicator of its potential antioxidant activity, there is not necessarily a linear correlation between these two activities. Flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties.Determination of lipid peroxidase content was carried out indirectly by means of derivatizing MDA with TBA at high temperature andacidic condition.There was a close correlation between the antioxidant capacity and the amount of polyphenols, flavonoids, and flavonols present in the plant. Total polyphenols play a vital role in anti-oxidization as well as in the biological functions of the plant. It was observed that phenol and flavonoid content of the plants differ among them. Highest phenol and flavonoid content were noted in the extracts of the six plants. There was no correlation between total phenolic content and antioxidant activity in this study. The results of the study suggest that phytochemicals as a whole contribute the total antioxidant activity of the extracts. The result also showed that the percentage of antioxidant activity of the methanol extracts increases with increasing concentration of the extracts in 200 µl to 1000 µl in all the samples. Aluminium Chloride Colorimetric Method was the principle colorimetric method for estimation of flavonoids.

Biochemically the six plants were tested for the Proteolytic and Protease inhibitor activity. All the six plant extracts did not show proteolytic activity which suggests that the extracts cannot cleave or denature proteins. They showed good activity as protease inhibitors to proteolytic enzyme like trypsin. . In addition, it is suggested that although the reducing power of a substance may be an indicator of its potential antioxidant activity, there is not necessarily a linear correlation between these two activities.

Total polyphenols play a vital role in anti-oxidization as well as in the biological functions of the plant. Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. These tests show that the plant extracts are rich in anti-oxidants and have immense medicinal properties. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging.

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