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 and Jack fruit leaf (Artocarpus heterophyllus) was carried out to evaluate the antioxidant potential. Alcoholic extracts of the leaves were 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. Ethylene Diamine Tetra Acetic 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. Approximately 80% of the world population depends exclusively on plants for their health and healing. In the developed world, reliance on surgery and pharmaceutical medicine is more usual, but in recent years, more and more people are complementing their treatment with natural supplements obtained from medicinal plants. The plant kingdom holds many species of plants containing substances of medicinal interest which are yet to be investigated. Large numbers of plant are constantly being screened for their chemical and pharmacological properties. By the application of modern techniques of isolation and pharmacological evaluation, many new plant drugs find their way to medicine as purified substances. 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. This will probably justify the use of many of the plant drugs as household remedies by indigenous people of many countries from ancient times and hence warrants their evaluation in more detail. The fact that only very few percent of the six lakhs species of plants on the planet has been investigated, indicates the opportunity provided and challenges thrown to phytochemists. 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 clearly indicates the importance of the antioxidants. Oxygen is necessary for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals. Free radicals are types of Reactive Oxygen species (ROS), which include all highly reactive, oxygen-containing molecules. The main endogenous (originates within the organism) sources of most of the oxidants produced by 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. Cell damage caused by free radicals appears 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 cells and organ systems of the body against reactive oxygen species, humans have evolved highly sophisticated and complex antioxidant protection system, that functions interactivity and
synergistically to neutralize free radicals\(^5\). Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being\(^2\). Antioxidant-based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer’s disease and cancer have appeared during last three decades\(^1\). Recent studies have shown that a number of phytochemicals including flavonoids, polyphenols, terpenes and various plant extracts exerted an antioxidant action\(^2\). There is also a considerable amount of evidence revealing an association between individuals who have diet rich in fresh fruits and vegetables and the decreased risk of cardiovascular diseases and certain forms of cancer\(^1\). 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 plants around the globe\(^8\). Several medicinal plants (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 have shown potent antioxidant activity\(^1\). However, majority of plants have not yet been screened 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 grass and 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 species were obtained and washed with distilled water. They were shade 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

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Test</th>
<th>Artocarpus heterophyllus</th>
<th>Anethum graveolens</th>
<th>Cinnamomum zeylanicum</th>
<th>Coriandrum sativum</th>
<th>Wheat grass</th>
<th>Piper betel</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Anthraquinones</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Flavonoids</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Saponins</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Steroids</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>6</td>
<td>Tannins</td>
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<td>+</td>
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<td>-</td>
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<tr>
<td>7</td>
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<td>+</td>
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</tr>
<tr>
<td>8</td>
<td>Cardiac glycoside</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 1 Concentration of soluble protein (µg/g of extract)
solvent to obtain a stock of concentration 1% (W/V).

Qualitative analysis of the crude extract 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 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate 2 ml of dilute ammonia was added. 5 ml of chloroform was added and shaken gently to extract the alkaloid base. The chloroform layer was extracted with 10 ml 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: 2 ml 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 5 ml of the extracts, 5 ml of distilled water was added and shaken for the formation of froth which confirms the presence of saponins.

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

Test for tannins: 0.5 ml of extracts was boiled in 10 ml 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: 2 ml of extracts was treated with 2 ml of glacial acetic acid containing a drop of FeCl₃ solution. This was under layer with 1 ml of concentrated H₂SO₄. 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 H2SO4 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 4°C, and then centrifuged at 12,000 x g for 15 min at 4°C. The precipitate was discharged and the supernatant was denominated crude extract. Protein concentration was determined using BSA as standard.

Determination of Proteolytic Activity: Proteolytic activity was determined by incubation of 0.1 ml of crude extract with 0.5 ml of casein solution 1% (w/v), prepared in phosphate buffer 0.1 mol/L pH 7.6 at 37°C for 20 min. Reaction was terminated by the addition of 1.5 ml of TCA solution 5% (v/v). After that 0.5 ml of a casein solution 1% (w/v) was added to the solution and the reaction mixture was incubated for 10 min. The reaction was interrupted by adding of 1.5 ml of TCA solution 5% (v/v). One enzyme unit (EU) was defined as the amount of enzyme necessary to produce the elevation of 0.1 in optical density (OD) after 20 min.

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

\[
\text{Lycopene (mg/100mg)} = -0.0458 \cdot \text{A}_{663} + 0.372 \cdot \text{A}_{505} - 0.0806 \cdot \text{A}_{453}
\]

\[
\text{β-Carotene (mg/100mg)} = 0.216 \cdot \text{A}_{663} - 0.304 \cdot \text{A}_{505} + 0.452 \cdot \text{A}_{453}
\]

Quantitative Estimation of Total Phenolics: To the
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 extract7.

Quantitative Estimation of Total Flavonoid Content: 0.5 ml of the plant 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 Quercetin25.

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 37ºC. 10% TCA was added and centrifuged at 3000 rpm for 20 minutes at 4°C. Supematant 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 formula20:

\[ \% = 1 - \frac{\text{sample OD}}{\text{blank OD}} \times 100 \]

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 for 20 minutes. Trichloroacetic acid (10%) was added to it and centrifuged at 3000 rpm for 10 minutes. Then the supernatant was taken and 2.5 ml of water and FeCl₃ (0.1%) was added to it. Absorbance was measured at 700 nm10.

Metal ion chelating activity: 1mM FeSO₄ solution was mixed with extracts, 1 ml Tris HCl buffer (pH 7.4) and 2,2’-bipyridyl 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 equation26:

\[ \% = 1 - \frac{\text{sample OD}}{\text{blank OD}} \times 100 \]

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 peroxide22. The percentage inhibition was calculated using the formula:

\[ \% = 1 - \frac{\text{sample OD}}{\text{blank OD}} \times 100 \]

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 flavonolcs was found to be highest in betel leaf and lowest

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**Figure 5 Estimation of total flavonoids**
Reducing Power: The reducing capacity of a compound activity which was around 98%.

Antioxidants have been attributed to various mechanisms that may serve as a significant indicator of its potential antioxidant activity. Wheatgrass showed to have the highest reducing power and ferrocyanide complex to ferrous form. Based on the reducing capacity, 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 peroxidation was carried out indirectly by means of derivatizing MDA with TBA at high temperature and acidic 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 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. 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. 

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 peroxy and alkoyx radicals that can perpetuate the chain reaction. Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. Wheatgrass showed high iron chelating activity of 81% and cinnamon showed the least activity of 34%.

Hydrogen peroxide scavenging activity: Hydrogen peroxide scavenges free radicals and free radical intermediates which 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 toxic 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 anthraquinones, 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.

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