Antioxidant properties of flavonoid rich fraction from the leaves of *Premna integrifolia*
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**ABSTRACT**

Leaves of *Premna integrifolia* belong to Verbanacea is an important rasayana drug in ayurvedic medicine and are considered to be useful in the treatment of variety of ailments. The present study was aimed to assess its antioxidant activities. The flavonoid rich fraction, showed antioxidant activity was evaluated using the superoxide scavenging, anti lipid peroxidation, hydroxyl radical scavenging and reducing power assays. *Premna integrifolia* flavonoid rich fraction showed significant anti-oxidant activity. The results endow with the confirmed that the studied medicinal plant flavonoid fractions are to be potent source of natural antioxidant and medicinally important compounds.

**Keywords:**

**INTRODUCTION**

In living systems, reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion radical, and singlet oxygen are known to attack polyunsaturated fatty acids (PUFAs) in cell membranes giving rise to lipid peroxidation, which is believed to be strongly associated with the aging process and carcinogenicity (Cutlar, 1984). Search for anti-oxidative agents from natural products containing DNA cleavage protector’s properties are still going on. Plants have been the source of traditional medicines all over the world for thousands of years and maintain to offer new remedies to people; an immense deal of attempt has consequently focused on using available experimental techniques to discover natural antioxidants from plants. Flavonoids are a major class of plant secondary metabolites commonly distributed in the higher plants that are present in a broad range of commonly consumed fruits and vegetables and plant-derived products such as cocoa, tea or wine. Since the pioneer work of Hertog et al. (2003), several epidemiological studies have been published showing an inverse correlation between dietary flavonoid intake and reduced incidence and mortality from cardiovascular disease, diets rich in antioxidants contribute not only cardiovascular certain types of cancer, and neurodegenerative diseases (Sampson et al., 2002).

*Premna integrifolia* is an important plant belonging to the family Verbenaceae, and is one of the most widespread large shrubs in the forests of India, usually occurring in deciduous forests. The whole plant possesses medicinal properties, useful in the treatment of cardiovascular diseases, skin diseases, inflammatory diseases, arthritis, gonorrhea, rheumatism, anorexia and jaundice. It is popularly known as “Minnal godi” in Tamil, and “Munney” in Ayurvedic system of medicine. Root forms an ingredient in well known Ayurvedic formulation “Dasamula” which is used for variety of affections (Kirtikar and Basu, 1975). Leaves are used to cure “weakness of limbs” and the leaves and leaf sap were used to alleviate headache (Sathya Bama et al., 2013). In view of these considerations, the aim of this study was to evaluate the free-radical scavenging capacity of flavonoid and its effect on linoleic acid peroxidation induced by H$_2$O$_2$ UV-photolysis.
MATERIAL AND METHODS

Collection of plant material

The leaves of *Premna integrifolia* were collected from the Sri Sairam Siddha Medical College and Research Centre, Herbal garden, Tamilnadu, India. The plant was identified and authenticated by Dr. S. Sankaranarayanan, The Head, Department of Medicinal Botany, Sri Sairam Siddha Medical College, West Tambaram, Chennai-44.

Preparation of extracts

Organic solvents (methanol) extract of the *Premna integrifolia* leaves were prepared according to the method described by Sumathy and Sankaranarayanan (2013) with little modifications. Twenty grams of *Premna integrifolia* leaves extracts were air-dried, crushed and blended into powder using an electric blender. The blended material was transferred to a beaker and soaked separately in 100 ml of the 70 % aqueous-methanol at room temperature. The mixture was extracted by agitation on a rotary shaker. The extract obtained was vacuum-dried and used for further test.

Estimation of flavanoid

Total flavanoids were estimated using the modified method of Nabavi et al (2009). To the flavanoid extract, 1.5ml, 1ml of 2% aluminium chloride and 0.5 ml of 33% acetic acid was added and mixed thoroughly. The obtained solution is allowed to stand for 30 minutes and the absorbance was measured at 414 nm using a UV-Visible Spectrophotometer. Rutin was used as a standard.

Determination of total phenolic content

The amount of total phenolic content was determined by Folin-Ciocalteau reagent method (Gutfinger, 1981). The different concentration of flavanoid extracts and 1 ml of 50% Folin-Ciocalteau reagent was mixed and the mixture was incubated at room temperature for 15 mins. Then 2.5 ml of sodium carbonate solution was added and further incubated for 30 mins at room temperature and the absorbance was measured at 760 nm. Total phenol values are expressed in terms of catechin equivalent (mg/g of extracted compound).

Reducing power determination of *Premna integrifolia* leaves flavanoid extract

The reducing power of the Flavaonoid extracts was determined by spectrophotometric method of Yen and Chen (1995). The crude extracts (25-100 µl) was mixed with 2.5 ml of 0.2 M Potassium phosphate buffer (pH-6.6) and 2.5 ml of 15mM Potassium ferricyanide [K₃Fe(CN)₆]. The mixture was incubated at 50°C for 20 minutes, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 5000 rpm for 3 minutes. An aliquot (2.5ml) of supernatant was diluted with distilled water (2.5ml) and 0.5 ml of 1% Ferric chloride was added and allowed to stand for 10 minutes. The absorbance was read spectrophotometrically at 700 nm. Increased absorbance indicates increased reducing power. Vitamin C was used as positive control.

Antioxidant activity in a hemoglobin induced linoleic acid of *Premna integrifolia* leaves flavanoid extract

The antioxidant activity in a hemoglobin induced linoleic acid of flavanoid extracts was carried out by method of Song-Hwan and Hyung-Joo, (2007). The crude extracts (25-100 µl) was mixed with 1 ml of 1 mmoi/l of Potassium phosphate buffer (pH-6.5) followed by the addition of 20 µl of 0.0016% hemoglobin was shaken vigorously. The mixed solution was incubated at 37°C for 45 minutes. After incubation, 2.5 ml of 0.6% HCl in ethanol was added and mixed thoroughly to stop the lipid peroxidation. Then, 100 µl of 0.02 mol/l FeCl₃ and 100 µl of ammonium thiocyanate (15g/50ml) was added and vortexed thoroughly. The total antioxidant activity determination was performed in triplicate using the thiocyanate method by reading the absorbance at 480 nm.

Superoxide radical scavenging assay of *Premna integrifolia* leaves flavanoid extract

Superoxide radicals were generated via an enzymatic reaction. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 milli-Intl. Units (mIU) xanthine oxidase, 1 mL of 12 mM diethylenetriaminepentaacetic acid, 1 mL of 186 mM nitro blue tetrazolium, and 1 mL of the extracts (final concentration of the phenolics in the assay medium was 50 or 100 ppm phenolics as catechin equivalents). Catechin was used as the reference antioxidant. All solutions were prepared in a 0.1 M phosphate buffer (pH 7.4) solution. The absorbance values of the mixtures were read at 560 nm. The following equation was used to calculate superoxide radical scavenging capacity (Nishimiki et al., 1972).

$$\text{Superoxide radical scavenging capacity (\%)} = \frac{\text{Absorbance of medium containing the additive}}{\text{Absorbance of the control medium}} \times 100$$
Metal chelating assay

The chelation of ferrous ions of flavonoid rich fraction was estimated by the method (Velickovic Ana, 2008). The different concentration of flavanoid rich fraction (25-100 μl/ml) was mixed with 0.05 ml of 2 mM FeCl₃ followed by the addition of 0.2 ml of 5 mM Ferrozine. The mixture was then shaken vigorously and left standing at room temperature for 10 minutes. Absorbance levels of the solutions were measured using spectrophotometer at 562 nm. The percentage of inhibition of ferrozine-Fe²⁺ complex formation was calculated using the formula given below:

\[
\% \text{ Inhibition} = \left( \frac{A₀ - A_S}{A_S} \right) \times 100
\]

Hydroxyl radical-scavenging activity:

Hydroxyl radical scavenging activity of extract was measured according to the method of Halliwell et al. (1987). One milliliter of the final reaction solution consisted of aliquots (500 μL) of various concentrations of the extract, 1 mM FeCl₃, 1 mM EDTA, 20 mM H₂O₂, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37 °C, and further heated in a boiling water bath for 15 min after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2-thiobarbituric acid. The color development was measured of 532 nm against a blank containing phosphate buffer.

Statistical analysis

Statistical analysis was done by using INTA Software. Data were analysed by one way analysis of variance (ANOVA) followed by Dunnet’s test. Results were presented as Mean± SEM. Values of *p<0.05, **p<0.01 were regarded as statistically significant.

RESULT AND DISCUSSION

Flavonoid screening and total phenolic content of Premna integrifolia

The screening of Flavonoid in leaves of Premna integrifolia showed the presence of flavonoids in both test alkali reagent and ammonia. The phenolics content yield from the leaves of Premna integrifolia ranged from 12.2% to 20.6% (w/w). Therefore, the total phenolic contents were reported as catechin equivalents. In addition, catechin was used as a reference antioxidant in other experiments.

The Partial characterization of methanol petal extract of Premna integrifolia by TLC

The methanol flavonoid extract of Premna integrifolia loaded on Pre-coated TLC plates (60 F₂₅₄ Merck) and developed with a solvent system of hexane, chloroform and methanol in the ratio of 1:0.5:0.1 was efficient to extract the antibacterial compound, and it is used for further studies. The developed plate was viewed under UV 240nm and 360nm. The Rf value of flavonoids compound shown in Table-1 and Fig-1.

<table>
<thead>
<tr>
<th>Table-1 Partial characterization of methanol leaves extracts of Premna integrifolia by TLC</th>
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<tbody>
<tr>
<td><strong>Flavonoid extract of Premna integrifolia</strong></td>
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<tr>
<td><strong>Component No.</strong></td>
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<tr>
<td>1</td>
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<tr>
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<td>3</td>
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Reducing power determinations of flavonoid extract from the leaves *Premna integrifolia*

The reducing power of the flavonoid extract of *Premna integrifolia* leaves was determined. The flavonoid extract reduced Fe$^{3+}$ to Fe$^{2+}$. The formation of perls Prussian blue was measured at 700nm. The increasing in absorbance showed the increase in reduction. The increases in concentration of the flavonoid extract of *Premna integrifolia* leaves ranges from 25 to 100 µl/ml. The increase in concentration of the flavonoid extract showed increase in reducing property. The maximum reducing property was found at 100 µl/ml flavonoid extract (Graph-1). Present data was consistent with the previous study of Wang et al. (2010). It was confirmed that the flavonoid fraction might contains hydroxy groups at C-3’ and C-4’ of the B-ring to be more active in reducing iron concentration (Moran et al., 1997).

**Antioxidant activity in a hemoglobin induced linoleic acid of *Premna integrifolia* leaves flavonoid extract**

The antioxidant activity of flavonoid extract of *Premna integrifolia* leaves was determined by using the hemoglobin induced linoleic acid system. The flavonoid extract act as electron donors and react with radical and stabilize the radical to terminate radical chain reaction. The maximum inhibitory activity was 88 % in 100 µl/ml concentration of flavonoid extract (Graph-2). Antihemolytic activity and the relationship between iron ion chelating activity and defensive activity against oxidative injure to erythrocyte membrane by flavonoid compound quercetin have been reported previously (Ferrali et al., 1997). It seems that high total phenol and flavonoid contents in the extract led to its potent antihemolytic activity.
Superoxide scavenging assay of *Premna integrifolia* flavonoid extract on O$_2^\bullet$ expressed as percentage of inhibition of NADH oxidation.

*Premna integrifolia* flavonoid extracts exhibited potent scavenging activity for superoxide radicals in a concentration dependent manner. Increased concentration of Flavonoid extract of *Premna integrifolia* were decrease the formazan dye which then indicated the consumption of the generated superoxide anion in the reaction (Table-2). Min Zhang et al., 2012 have been reported that the total flavonoid contents in leaves, stems, rachis and roots of *D. erythrosora* ethanol extracts showed strong superoxide anion scavengers abilities. Similarly, maximum superoxide anion inhibitory activity was found in 20µl/ml of flavanoid rich fraction of *Hibiscus rosa sinensis* than *M. oleifera* (Sumathy and Sankaranarayanayanan, 2013).

### Table-2. Superoxide anion scavenging activity of *Premna integrifolia* flavonoid extract observed with a riboflavin-light–NBT system.

<table>
<thead>
<tr>
<th>Concentration of flavonoid extract</th>
<th>Inhibition(^a) (%)</th>
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</thead>
<tbody>
<tr>
<td>25 µl/ml</td>
<td>15.46±5.7</td>
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<tr>
<td>50 µl/ml</td>
<td>31.95±2.3</td>
</tr>
<tr>
<td>75 µl/ml</td>
<td>54.63±2.08</td>
</tr>
<tr>
<td>100 µl/ml</td>
<td>77.31±4.08</td>
</tr>
</tbody>
</table>

All the observations in different groups showed significant (P<0.01) relationship between the concentration and percentage inhibition (Pearson’s correlation analysis)\(^a\)Mean ± SD.

**Fe$^{2+}$** chelating activity of flavonoid extract of *Premna integrifolia*

Fe$^{2+}$ chelating activity was increased with increase in concentration. The absorbance of the Fe$^{2+}$-ferrozine complex decreased dose-dependently, i.e. the activity was increased on increasing concentration from 25 to 100 µl/ml. Metal chelating capacity was significant since the flavonoid extract of *Premna integrifolia* reduced the concentration of the catalyzing transition metal in lipid per oxidation. The maximum Fe$^{2+}$ chelating activity was absorbed in 100 µl/ml concentration (Table-3). This inhibition of lipid peroxidation may be either due to chelation of Fe or by trapping of the free radicals. Gokani et al., (2011) also reported similar antioxidant properties of *Premna integrifolia* methanol extract in antioxidant activity (in vitro) of the extracts was evaluated lipid peroxidation, hydroxyl radical scavenging, nitric oxide scavenging and reducing power (ferric thiocyanate method and β-carotene bleaching test) assays.

### Table-3. Fe$^{2+}$ chelating activity of flavonoid extract of *Premna integrifolia*

<table>
<thead>
<tr>
<th>Concentration of flavonoid extract</th>
<th>a Fe$^{2+}$ chelating activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl/ml</td>
<td>46.93±3.0</td>
</tr>
<tr>
<td>50 µl/ml</td>
<td>68.36±2.5</td>
</tr>
<tr>
<td>75 µl/ml</td>
<td>87.75±4.1</td>
</tr>
<tr>
<td>100 µl/ml</td>
<td>93.87±5.0</td>
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\(^a\)Results are expressed as percentage inhibiton of Fe$^{2+}$ chelating with respect to control. Each value represents the mean+SD of five experiments.

**Site specific hydroxyl radical scavenging assay**

Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a brownish pink chromogen upon heating with TBA at low pH. When the test extracts were added to the reaction mixture, they remove hydroxyl radical from the sugar and prevented their degradation. *Premna integrifolia* flavonoid extract 100 µg/ml was found to be the most potent hydroxyl radical scavenger and 25 µg/ml fraction was the least potent hydroxyl scavenger (Table-4). The high antioxidant activity of flavonoids can be attributed to hydroxy groups in the A- and B-rings in rutin, and the greater the number of hydroxy groups, the higher is the capacity to scavenge free radicals (Kao and Chen, 2006).

### Table-4. Hydroxyl radical scavenging activity of *Premna integrifolia* flavonoid extract

<table>
<thead>
<tr>
<th>Concentration of flavonoid extract</th>
<th>Inhibition(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl/ml</td>
<td>6.17±1.15</td>
</tr>
<tr>
<td>50 µl/ml</td>
<td>18.51±1.52</td>
</tr>
<tr>
<td>75 µl/ml</td>
<td>29.62±3.0</td>
</tr>
<tr>
<td>100 µl/ml</td>
<td>55.55±4.0</td>
</tr>
</tbody>
</table>

All the observations in different groups showed significant (P<0.01) relationship between the concentration and percentage inhibition (Pearson’s correlation analysis)\(^a\)Mean ± SD.
Conclusion

In conclusion, the present study provides evidence that *Premna integrifolia* flavonoid extract exhibit interesting antioxidant properties, expressed either by their capacity to scavenge free radicals of lipid peroxidation, hydroxyl radical and superoxide activity. These effects may be useful in the treatment of pathologies in which free radical production plays a key role.

Reference