Full Length Article

In vitro antioxidant activity of leaf extracts of Alangium salvifolium (L.f.) Wang (Alangiaceae)

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ABSTRACT

In vitro antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of leaf of Alangium salvifolium have been tested using various antioxidant model system viz, DPPH, hydroxyl, superoxide, ABTS and reducing power. The methanol extract of leaf showed potent DPPH, ABTS radical cation scavenging activities. Ethanol extract of leaf showed strong hydroxyl, superoxide radical scavenging activities. Methanol extract of Alangium salvifolium showed the highest reducing ability. This study indicates significant free radical scavenging potential of Alangium salvifolium which can be exploited for the treatment of various free radical mediated ailments.

Key words: In vitro antioxidant activity, Alangium salvifolium, flavonoid, DPPH, ABTS

INTRODUCTION

Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide play a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and Parkinson’s disease. The free radicals in the human body are generated through aerobic respiration or from exogenous sources (Narayanaswamy and Balakrishnan, 2011). Many oxidative stress related diseases are as an outcome of accumulation of free radicals in the body. Antioxidant compounds may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quenches of singlet oxygen formation (Satheeshkumar et al., 2010). Antioxidants are considered as a promising therapeutic approach as they may be playing neuroprotective (preventing apoptosis) and neurodegenerative roles. The main characteristic of an antioxidant is its ability to trap free radicals (Mon et al., 2011).

Currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Recently, there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Many plant extracts and phytochemicals have shown to have free radical scavenging properties but generally there is still a demand to find more information concerning the antioxidant potential of plant species.

Alangium salvifolium (L.f.) Wang (Family: Alangiaceae) commonly known as Alangi in Tamil was found distributed in South India. The leaves of Alangium salvifolium are used as astringent,
laxative, refrigerant and used to treat rheumatism, leprosy, syphilis and asthma (Kijima et al., 1992). The root bark is used as purgative, astringent, anthelmintic, antipyretic, expectorant, anti-inflammatory, emetic, diaphoretic, anticancer, antimicrobial and antitumor agents (Ali et al., 1983; Rao et al., 1999 and Anonymous, 1992). The root is used as hypotensive agent, anthelmintic and used in the treatment of biliousness, inflammation and snakebite. The bark shows antitubercular activity. The fruits are used as laxative, refrigerant, emetic and antiphlegmatic agent. As there is no detailed report on in vitro antioxidant activity using different solvents in the leaves of this plant, the present study was carried out to develop in vitro antioxidant data on the leaves which is essential for its pharmacological studies.

MATERIALS AND METHODS
Collection of plant sample
Leaves of Alangium salvifolium (L.f.) Wang was collected from Kottaram, Kanyakumari District, Tamil Nadu. With the help of local flora, voucher specimens were identified and preserved in the Ethnopharmacology Unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu for further references.

Plant sample extraction
Leaf of A. salvifolium was cleaned, shade dried and pulverized to powder in a mechanical grinder. Required quantity of powder was weighed and transferred to Stoppard flask and treated with petroleum ether, benzene, ethyl acetate, methanol and ethanol until the powder is fully immersed. The flask was shaken every hour for the first six hours and then it was kept aside and again shaken after 24 hours. This process was repeated for three days and then the extract was filtered. The extract was collected and evaporated to dryness by using vacuum distillation unit. The final extracts thus obtained were used for in vitro antioxidants activity. The methanol extract was used for the estimation of total phenolic and flavonoids.

Estimation of total phenolic content
Total phenolic content was estimated using the Folin-Ciocalteu method (Lachman et al., 2000). Samples (100µL) were mixed thoroughly with 2 ml of 2% Na2CO3. After 2 min. 100 µL of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against a blank. Total phenolic content was expressed as gram of gallic equivalents per 100 gram of dry weight (g100g−1DW) of the plant samples.

Estimation of flavonoids
The flavonoids content was determined according to Eom et al., (2007). An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1ml of 10% aluminium chloride and 0.1ml of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5ml volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity
The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H (Shen et al., 2010). The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picyrylhydrazyl (DPPH) according to the previously reported method (Shen et al., 2010). Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50,100,200, 400&800µg/mL). The the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = \[ \frac{(A_0 - A_1)}{A_0} \times 100 \]

Where, \( A_0 \) is the absorbance of the control reaction, and \( A_1 \) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl radical scavenging activity
The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al. (1987). Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H2O2...
(10 mM) and Deoxyribose (10 mM), were prepared in distilled deionized water. The assay was performed by adding 0.1 mL EDTA, 0.01 mL of FeCl₃, 0.1 mL H₂O₂, 0.36 mL of deoxyribose, 1.0 mL of the extract of different concentration (50, 100, 200, 400 & 800 μg/mL) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM, pH 7.9), 0.1 mL of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 hour. 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation calculated by using the following equation:

Hydroxyl radical scavenging activity = \((\frac{A_0 - A_1}{A_0}) \times 100\)

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

**Superoxide radical scavenging activity**

The superoxide anion scavenging activity was measured as described by Srinivasan et al. (2007). The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract of different concentration (125, 250, 500 & 1000 μg/mL), and 0.5 mL Tris – HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation:

Superoxide radical scavenging activity = \((\frac{A_0 - A_1}{A_0}) \times 100\)

Where, \(A_0\) is the absorbance of the control reaction, and \(A_1\) is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

**Antioxidant Activity by Radical Cation (ABTS. +)**

ABTS assay was based on the slightly modified method of Huang et al (2011). ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100 μL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

**Reducing Power**

The reducing power of the extract was determined by the method of Kumar and Hemalatha (2011). 1.0 mL of solution containing 50, 100, 200, 400 & 800 μg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%). The mixture was incubated at 50°C for 20 minutes. Then 5 mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

**Statistical analysis**

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

**RESULTS**

**Total phenolic content and total flavonoid content**

The total phenolic and total flavonoid content of the methanol extract of *Alangium salvifolium* leaf were found to be 0.12 g 100 g⁻¹ and 0.54 g 100 g⁻¹.

**DPPH radical scavenging activity**

DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A. salvifolium* leaf were shown in Figure 1.
The scavenging effect increases with the concentration of standard and samples. Among the solvent tested, methanol extract exhibited highest DPPH radical scavenging activity. At 800µg/mL concentration methanol extract of *A. salvifolium* leaf possessed 104.13% scavenging activity of DPPH.
Hydroxyl radical scavenging activity
Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extract of A. salvifolium leaf were presented in figure 2. Ethanol extract showed very potent activity. At 800µg/mL concentration, ethanol extract of A. salvifolium leaf possessed 119.33% scavenging activity on hydroxyl radical.

Superoxide radical scavenging activity
The A. salvifolium leaf extracts were subjected to the superoxide radical scavenging activity and the results were shown in figure 3. It indicates that ethanol extract of A. salvifolium leaf (800µg/mL) exhibited the maximum superoxide radical scavenging activity of 103.84% which is higher than the standard ascorbic acid whose scavenging effect is 101.23%

ABTS radical cation scavenging activity
The A. salvifolium leaf extracts were subjected to ABTS radical cation scavenging activity and the results were presented in figure 4. The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800µg/mL concentration, A. salvifolium leaf extracts possessed 106.87% scavenging activity on ABTS which is slightly higher than the standard trolox whose scavenging activity is 98.34%.

Reducing power
Figure 5 showed the reducing ability of different solvent extracts of A. salvifolium leaf compared to ascorbic acid. The results clearly indicate that the reducing power of the A. salvifolium leaf extracts increased with the standard ascorbic acid. Among the solvent tested, methanol extract exhibited higher reducing activity.

IC₅₀ value
IC₅₀ values of petroleum ether extract of A. salvifolium leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide scavenging and trolox for ABTS radical cation scavenging were found to be 17.15µg/mL and 20.78µg/mL; 21.14µg/mL and 21.96µg/mL; 20.87µg/mL and 23.24µg/mL and 21.62µg/mL and 22.15µg/mL respectively. IC₅₀ values of benzene extract of A. salvifolium leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide scavenging and trolox for ABTS radical cation scavenging were found to be 16.34µg/mL and 20.78µg/mL; 17.03µg/mL and 21.96µg/mL; 18.93µg/mL and 23.24µg/mL and 18.73µg/mL and 22.15µg/mL respectively. IC₅₀ values of ethyl acetate extract of A. salvifolium leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide scavenging and trolox for ABTS radical cation scavenging were found to be 13.47µg/mL and 20.78µg/mL; 15.43µg/mL and 21.96µg/mL; 17.53µg/mL and 23.24µg/mL and 22.42µg/mL and 22.15µg/mL respectively. IC₅₀ values of methanol extract of A. salvifolium leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide scavenging and trolox for ABTS radical cation scavenging were found to be 23.63µg/mL and 20.78µg/mL; 22.83µg/mL and 21.96µg/mL; 23.94µg/mL and 23.24µg/mL and 22.98µg/mL and 22.15µg/mL respectively. IC₅₀ values of ethanol extract of A. salvifolium leaf and standard ascorbic acid for DPPH, hydroxyl, superoxide scavenging and trolox for ABTS radical cation scavenging were found to be 22.91µg/mL and 20.78µg/mL; 23.94µg/mL and 21.96µg/mL; 23.43µg/mL and 23.24µg/mL and 21.06µg/mL and 22.15µg/mL respectively (Table 1).

Table 1: IC₅₀ values of different solvent extracts of Alangium salvifolium leaf

<table>
<thead>
<tr>
<th>Different solvent extracts</th>
<th>DPPH assay</th>
<th>Hydroxyl assay</th>
<th>Superoxide dismutase activity</th>
<th>ABTS assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
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<td>17.03</td>
<td>23.43</td>
<td>18.73</td>
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<td>15.43</td>
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<tr>
<td>Methanol</td>
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<td>22.83</td>
<td>17.53</td>
<td>22.98</td>
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<tr>
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<td>23.94</td>
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<tr>
<td>Standard (Ascorbic acid)</td>
<td>20.78</td>
<td>21.96</td>
<td>20.87</td>
<td>-</td>
</tr>
<tr>
<td>Standard (Trolox)</td>
<td>-</td>
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<td>22.15</td>
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DISCUSSION: The antioxidant properties of the different solvent extracts of *A. salvifolium* were significantly corroborated by the phytochemical constituents of the extracts. Phenolic compounds are known as powerful chain breaking antioxidants and they are very important plant constituents because of their scavenging ability, which is due to their hydroxyl groups (Shahidi and Wanasundara, 1992). Total phenolic assay by using Folin-Ciocalteu reagent is a simple convenient and reducible method. It is employed routinely in studying phenolic antioxidants. Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral and anticancer activities (Umamaheswari and Chatterjee, 2008). They are capable of effectively scavenging the reactive O₂ species because of their phenolic hydroxyl groups and so they are potent antioxidants also (Cao et al., 1997). In view of their wide pharmacological and biological actions, they have a greater therapeutic potential. The presence of high phenolic and flavonoid content in the different solvent extracts of *A. salvifolium* has contributed directly to the antioxidant activity by neutralizing the free radicals. Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infection, lung damage, inflammation, reperfusion injury, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases (Jamuna et al., 2012). They are also involved in autoimmune disorder like rheumatoid arthritis, etc. Our results demonstrated that the different solvent extracts of leaf of *A. salvifolium* possess free radical scavenging activity with different *in vitro* models like DPPH, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTS radical cation scavenging activity and reducing power activity assays.

The result of DPPH scavenging activity, in this study indicates that the plant was potently active. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical’s reactivity (Dharani et al., 2011). Among five extracts, methanol extract showed maximum scavenging activity followed by ethanol extract. However this study provides a definite report about the free radical scavenging capacity of *A. salvifolium* leaf, since the antioxidant activity of a drug may depend on the free radical scavenging activity. In the present study methanol extract exhibited more DPPH radical scavenging activity with IC₅₀ value 23.63µg/mL compared to ascorbic acid (20.78µg/mL). From the result, in the present study a dose dependent relationship in the DPPH radical scavenging activity.

Hydroxyl radical is the most deleterious and reactive among the ROS and it bears the shortest half life compared with other free radicals. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe²⁺) causes degradation of deoxyribose into malodialdehyde which produces a pink chromogen with thiobarbituric acid (Halliwell, 1994). The extracts when added to the reaction mixture, scavenged the hydroxyl radicals and prevented the degradation of deoxyribose. In the present study IC₅₀ values were found to be 23.94µg/mL and 21.96µg/mL respectively for methanol extract of *A. salvifolium* leaf and ascorbic acid. The hydroxyl radical scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in *A. salvifolium* leaf extracts. Superoxide anions are the most common free radicals *in vitro* and are generated in a variety of biological systems, either by auto-oxidation processes or by enzymes. The concentration of superoxide increases under conditions of oxidative stress and related situations (Lee et al., 2002). Moreover, superoxide anions produce other kinds of cell damaging free radicals and oxidizing agents (Hu and Kitts, 2000). Therefore, *A. salvifolium* leaf was undertaken to test whether it has scavenging activity of superoxide anions. The IC₅₀ values were found to be 23.43µg/mL and 23.24µg/mL respectively for methanol extract of *A. salvifolium* leaf and ascorbic acid. The results clearly indicate that the *A. salvifolium* leaf extracts have a noticeable effect as scavenging superoxide radical.

Another screening method for antioxidant activity is the ABTS radical cation decolourisation assay. This assay is widely used to assess the antioxidant capacity. The present investigation, this method showed results the quite similar to those obtained in the DPPH reaction. Of the successively extracted *A. salvifolium* leaf with different solvents, the methanol extract possessed potent ABTS scavenging activity. The IC₅₀ values of ABTS were found to be 22.98µg/mL and 22.15µg/mL respectively for methanol extract of *A. salvifolium* leaf and trolox.
Here, the A. salviifolium leaf extracts, ABTS radical cation scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron (Yildirim et al., 2000 and Dorman et al., 2003). Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts (Duh et al., 1999 and Duh, 1998). The reducing capacity of A. salviifolium leaf is a significant indicator of this potential antioxidant activity.

Recently, much attention has been directed toward extracts and biologically active compounds isolated from plant species. The use of medicinal plants plays a vital role in covering the basic health needs in developing countries, and these plants may offer a new source of antioxidant activity. The present study suggests that the methanol and ethanol extracts of A. salviifolium could be of great importance for the treatment of radical related diseases and age associated diseases.

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