Antioxidant activity and total flavonoids content of different parts of Azadirachta indica A. Juss

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Reactive oxygen species are known to play a key role in many pathological conditions, and antioxidants from plant sources have been of great contribution in modulating this effect. Different parts of Azadirachta indica (AI) A. Juss were studied for their antioxidant capacity. We investigated antioxidant properties and total flavonoid contents of successive solvent extracts of different parts of AI. Total antioxidant assay was performed using the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant potential (FRAP) methods. Total flavonoid contents were measured using colourimetric method. Root bark methanolic extract was also subjected to lipid peroxidation inhibitory activity. Methanolic extract of root bark was found to exhibit highest IC50 value of 13.83 μg/ml by ABTS total antioxidant assay, 420.04 μg/ml by lipid peroxidation inhibition and EC50 value of 503.53 μg/ml by FRAP assay. Content of total flavonoids were expressed in quercetin equivalents ranging from 65.06 to 72.96 μg/ml in the leaf, 18.99 to 93.03 μg/ml in the seed and 55.70 to 98.00 μg/ml in the root bark. The present study evaluated the antioxidant activities and total flavonoids content of different parts of Azadirachta indica A. Juss, and root bark methanolic extract showed significant antioxidant activities due to free radical scavenging potential, which was comparable with its total flavonoids content in Azadirachta indica A. Juss.

Key words: Azadirachta indica, antioxidant activity, total flavonoids content, root bark extract.

INTRODUCTION

There are many speculations that the generation of free radicals in the body results in the cellular changes and development of cancer and other diseases, and this could be neutralized by the antioxidants from different medicinal plants (Sathisha et al., 2011; Ames et al., 1993). In recent years, much attention has been devoted to natural antioxidants and their role in health care system (Amous et al., 2001). Several studies have shown that plant derived antioxidant neutraceuticals scavenge free radicals, and modulate oxidative stress related. This protection can be explained by the capacity of the antioxidants of plants to scavenge free radicals (Argalo et al., 2004; Tere et al., 2005). Investigation of natural products as antioxidants is a research field with great potential, and is especially important in countries possessing great biodiversity, like India.

The ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) assay is based on the ABTS radical cation decolourisation which is applicable for both lipophilic and hydrophilic antioxidants (Maria et al., 2010; Maryam et al., 2011). The FRAP (ferric reducing antioxidant potential) assay evaluates total antioxidant power, and is chosen to assess the presumable effects of medicinal plants (Szollosi and Varga, 2002). Lipid peroxidation has gained more importance today because of its involvement in pathogenesis of many diseases like atherosclerosis, cancer, diabetes mellitus, myocardial infarction and also ageing. Free radicals or reactive oxygen species (ROS) are produced in vivo from various sources and can damage cellular molecules resulting in the generation of free radicals.

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biochemical reactions, and are the main agents in lipid peroxidation (Cheesman and Scater, 2003). The neem tree (Azadirachta indica A. Juss., Meliaceae family) is called ‘arista’ in Sanskrit, a word that means ‘perfect, complete and imperishable’ (Neem Conference, 2006; Sateesh, 1998). Neem tree is the most researched tree in the world, and is said to be the most promising tree of 21st century (Brahmachari, 2004). Since time immemorial, many continents are aware of the medicinal properties of neem (Subapiya and Nagini, 2005). Good antioxidant activity was observed with neem leaf aqueous extract, ethanolic extracts of flower and stem bark (Sithisarn et al., 2005).

In the present study, the successive solvent extracts and hydro alcoholic extracts of the leaves, seeds and root barks of AI were screened for their antioxidant capacities using in vitro standard procedures and total flavonoid contents, so as to assess the medicinal potential of this plant and thus justify its folklore use as thorough investigation and comparison between parts of the plant, and extraction capacity of solvents were not yet reported.

MATERIALS AND METHODS

Collection of plant materials

Young leaves, dried seeds, and matured root barks (around ten years of age) of AI were collected from the Institutional medicinal garden. The plant sample was authenticated at Botany division, Osmania University, and the voucher specimen was deposited at the same place (0125).

Preparation of plant extracts

Plant material was extracted successively with petroleum ether, chloroform, ethylacetate and methanol in the increasing order of their polarity, using soxhlet apparatus, and solvent was recovered, and the remaining trace amounts of solvent was removed under reduced pressure, to obtain fluid extracts. Hydroalcoholic extracts of leaves, seeds and root barks were also prepared and yield% of each extract was recorded. All the chemicals and solvents used were of analytical grade. Qualitative chemical analysis was conducted for all the extracts, to identify the presence of active constituents responsible for antioxidant activity such as flavonoids, phenolics, tannins and terpenoids (Khndelwal, 2003).

ABTS assay

ABTS was dissolved in water to a 7 mM concentration. ABTS radical ion (ABTS•−) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. Due to ABTS and potassium persulfate reacting stoichiometrically at a ratio of 1:0.5, this results in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until after 6 h had elapsed. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of compounds, the ABTS solution was diluted with ethanol to get an absorbance of 0.70 to 0.80 at 30°C stock solutions. After addition of 1.0 ml of diluted ABTS solution, appropriate solvent blanks were run in each assay, and ascorbic acid was used as standard (Arnao et al., 2001). Each sample was assayed in triplicate, and the average IC50 value was calculated.

FRAP assay

The Ferric reducing antioxidant power (FRAP) was performed based on the method of Benzie and Strain (1999). The assay mixture contained 2.5 ml of 300 mM acetate buffer at pH 3.6, 0.25 ml of 10 mM TPTZ (tripyridyltriazine) solution in 40 mM HCl, 0.25 ml of 20 mM FeCl3 and test substance in 0.1 ml of methanol. The absorbance was measured after 30 min of incubation at 593 nm. Standard graphs were constructed using known concentrations of ferrous salts in methanol to replace FeCl3. All tests were run in triplicate, and mean values were used to calculate EC50 values. EC50 is defined as concentration of an antioxidant having a ferric reducing ability equivalent to that of 1 mM ferrous salt (Benzie et al., 1999). The efficacy of the test substance was compared with the known antioxidant vitamin C.

Lipid peroxidation inhibition assay

Preparation of egg lecithin involves the separation of the egg yolk and washing it with acetone until the yellow colour disappears. The creamy white powder thus obtained is used for the procedure, by dissolving in phosphate buffer pH 7.4 (3 mg/ml). The reaction mixture containing egg lecithin (1 ml), ferric chloride (0.02 ml), ascorbic acid (0.02 ml) and extract or standard (0.1 ml) in DMSO, at various concentrations, was kept for incubation for 1 h, at 37°C. After incubation, 2 ml of 15% trichloro acetic acid and 2 ml of 0.37% thio barbituric acid were added. Then, the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm (Sultana et al., 2007). Each sample was assayed in triplicate and the average IC50 value was calculated.

Determination of total flavonoid content

Total flavonoid content was determined according to Chang et al. (2002) method, with little modification. Briefly, an aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 1% aluminum chloride and 0.1 ml of potassium acetate solution (1 M). In the mixture, 2.8 ml of distilled water was added to make the total volume of 5 ml. The mixture was vortexed, and absorbance was measured at 415 nm and calculated. Quercetin was used as standard (Chia Chichang et al., 2002; Akbay et al., 2003).

Statistical analysis

One-way ANOVA was carried out, and statistical analysis among trials was performed with Dunnet’s test, using a statistical package programme (SPCC, 17.0). p < 0.05 was considered as significant for all statistical analysis in this study. Correlation coefficient (R^2) values for ABTS assay were calculated from dose response curves.

RESULTS

Qualitative chemical analysis of extracts showed the presence of biologically active constituents and the
Table 1. Qualitative chemical analysis of different extracts of *Azadirachta indica*.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Leaf</th>
<th>Seed</th>
<th>Root bark</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃</td>
<td>EtOAc</td>
<td>MeOH</td>
<td>HA</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

CHCl₃ = Chloroform, EtOAc = Ethyl acetate, MeOH = Methanol, HA = hydroalcoholic (80% ethanol), +++ = Three chemical tests are positive, ++ = Two chemical tests are positive, + = One chemical test is positive, - = not responded.

Table 2. ABTS radical scavenging activity, inhibition (%) and correlation coefficient values of *Azadirachta indica* in different solvents at concentrations ranging from 5 to 100 µg/ml.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Leaf</th>
<th>Seed</th>
<th>Root bark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition (%)</td>
<td>R² value</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Chloroform</td>
<td>39.16</td>
<td>0.9866</td>
<td>17.96</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>30.57</td>
<td>0.9969</td>
<td>25.77</td>
</tr>
<tr>
<td>Methanol</td>
<td>45.31</td>
<td>1.0000</td>
<td>41.82</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>46.18</td>
<td>0.9909</td>
<td>16.04</td>
</tr>
<tr>
<td>Ascorbic acid (Standard)</td>
<td>45.45</td>
<td>0.9923</td>
<td></td>
</tr>
</tbody>
</table>

R² values were obtained from dose-response curve.

results are given in Table 1. Yields of the extracts and their DPPH activity were reported elsewhere. Inhibition (%) of ABTS free radicals and their dose dependant correlation are shown in Table 2. ABTS, FRAP assay results and total flavonoid contents are shown in Table 3, the highest activity was observed in the root bark methanolic extract as determined by total antioxidant assays (13.83, 503.53 µg/ml for ABTS, FRAP, respectively), followed by leaf hydroalcoholic (16.51 µg/ml, ABTS radical ion scavenging) and leaf methanol (38.61 µg/ml). Remaining extracts showed moderate to low antioxidant activity. Our data revealed that the root bark showed a good inhibition of lipid peroxidation (420 µg/ml) (Figure 1). Comparison between IC₅₀ values of methanolic extracts of all three parts of AI and their total flavonoid contents are shown in Figure 2.

**DISCUSSION**

Antioxidants present in the extract caused suppression of blue-green color of ABTS radical cation and this is proportional to antioxidant concentration.

ABTS + K₂S₂O₈ → ABTS⁺⁺
ABTS⁺⁺ + ArOH → ABTS + ArO⁻ + H⁺

where: ABTS = 2,2’-azinibis-(3-ethyl benzothiazoline-6-sulphonic acid), K₂S₂O₈ = potassium persulfate, ArOH = phenolic or flavonoid compound present in the test extract ABTS⁺⁺ = ABTS radical cation (Coruh et al., 2007).

FRAP is an electron transfer based total antioxidant assay, also called redox linked colorimetric method. There is an increase in absorbance at a pre specified wavelength as an antioxidant reacts with chromogenic reagent (Fe(II)), form charge transfer complexes with the ligand (TPTZ) (Everest and Ozturk, 2005).

Fe(TPRZ)₂³⁺ + ArOH → Fe(TPRZ)₂²⁺ + ArO⁻ + H⁺

Lipid peroxidation can be initiated by ROS such as hydroxyl radicals, by extracting a hydrogen atom from lipids and forming a conjugated lipid radical. This reacts rapidly with oxygen to form a lipid radical, until the chain reaction is terminated. The lipid peroxidation adducts may induce the oxidation of biomolecules such as DNA, proteins and other lipids, resulting in cellular damage (Resat et al., 2007; Huong et al., 1998). It is reported that phenolic and flavonoid compounds are responsible for the variation in the antioxidant activity of the plant. Plant extract may show antioxidant activity by inactivating lipid...
free radical. Flavonoid constituents are considered to be the most vital antioxidant components of herbs, and a significant correlation between concentration of plant flavonoids and the total antioxidant capacity has been reported (Miller et al., 1996; Akdemir et al., 2003; Torres et al., 2006).

It has been reported that the lipid peroxidation is one of the causes of occurrence of cardio vascular diseases and cancer. Therefore, inhibition of peroxidation by the plant extract indicates its antioxidant property. The high peroxidation scavenging effect of root bark of neem may be due to high content of flavonoids like radical scavengers involved in the extract, which can terminate the peroxidation chain reaction easily and quench reactive oxygen or nitrogen species, thereby inhibiting the oxidation of lipid and other biological molecules. Three tricyclic diterpenoids margocin, margocinin and margocilin, have been isolated from root bark of A. indica (Ara et al., 1998). Apart from these compounds, no considerable work was reported on chemical constituents of root bark.
Table 3. Antioxidant capacity of different parts of *Azadirachta indica* by ABTS (IC₅₀, µg/ml), FRAP (EC₁, µg/ml) total antioxidant assays and total flavonoids concentration in quercetin equivalent per 100 g of plant material.

| Solvent used in extraction | Leaf | | | Seed | | | Root bark | |
|---------------------------|------|------|------|------|------|------|------|------|------|
|                           | ABTS | FRAP | TFC  | ABTS | FRAP | TFC  | ABTS | FRAP | TFC  |
| Chloroform                | 100.3±2.12 | *NI | 72.96±1.22 | 99.03±1.10 | *NI | 18.99±0.07 | 100.01±0.02 | 2810.82±2.10 | 55.7±1.35 |
| Ethylacetate              | 100±0.02 | *NI | 81.0±0.16 | 98.77±2.01 | 2932±0.12 | 36.11±0.77 | 45.62±0.32 | 2334.05±1.56 | 66.33±0.45 |
| Methanol                  | 38.61±0.77 | 3000.12±7.58 | 85.05±0.12 | 49.62±2.09 | 2879.2±1.22 | 93.03±0.78 | 13.83±0.68 | 503.53±0.98 | 98±0.98 |
| 80% Ethanol               | 16.51±0.15 | *NI | 2677±2.96 | 65.06±1.07 | 100.22±1.16 | 2298.1±1.99 | 25.4±0.14 | 64.9±1.24 | 2033.83±1.02 | 56.83±0.26 |
| Ascorbic acid (Standard)  | 3.30±0.02 | 106.55±2.49 | | | | | | | |

*NI = no inhibition. Each value is expressed as the mean ± SEM (n =3), *values were significant at p < 0.05.

Conclusion

All the selected extracts, subjected to dose dependant studies to calculate IC₅₀ were from successive solvent extraction. Therefore, it could be concluded that only direct solvent extraction is not sufficient for investigating antioxidant property but every plant sample has to be successively extracted with different solvent with increasing polarities. In case of root bark methanolic extract, 50% reduction of ABTS radical was achieved at the dose of 13 µg/ml that is comparable to the standard ascorbic acid, and these results were supported by FRAP assay and lipid peroxidation inhibition assay. The results of antioxidant assays were in correlation with total flavonoid contents.

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