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Evaluation of Antioxidant Activity of Two Indian Medicinal Plants

M. Padmaja, M. Sravanthi , K.P.J. Hemalatha*

Department of Biochemistry, College of Science and Technology,Andhra University,Visakhapatnam-530 003, Andhra Pradesh, India

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***Corresponding Author**

Tel : +91-8912-844688

Email:
hemalathakpj @ gmail.com

Summary

Adhatoda vasica Nees and *Sesbania grandiflora* (L.) Pers are the two important medicinal plants native to India. The aqueous leaf extracts of these two plants have been analysed for their free radical-scavenging activity in different *in vitro* systems, e.g. DPPH radical-scavenging activity, hydroxyl radical-scavenging activity in Fe^{3+}/ascorbate/EDTA/H_2O_2 system, inhibition of lipid peroxidation induced by $FeSO_4$ in egg yolk, metal chelating activity. The free radical scavenging activities were compared with standard antioxidants like butylated hydroxy toluene (BHT), ascorbic acid and EDTA. Total antioxidant activity was measured, based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acid pH and reducing power by Fe^{3+} - Fe^{2+} transformation in the presence of extracts. The content of total phenolics (expressed as mg of gallic acid equivalents/gm) and total flavonoids (expressed as mg of quercetin equivalent/gm) and ascorbic acid were determined along with antioxidant enzymes. The results indicated that *A. vasica* and *S. grandiflora* showed significant antioxidant activity *in vitro*. The enzymatic and non enzymatic antioxidants in *A. vasica* were found to be more than that of *S. grandiflora*, similarly the antioxidant and radical scavenging activities of *A. vasica* were found to be more significant than *S. grandiflora*.

Key Words: *Adhatoda vasica*, Free radical, Lipid peroxidation, *Sesbania grandiflora*

Introduction

Plants are the source of energy for the animal kingdom. In addition, plants can synthesize a large variety of chemical substances that are of physiological importance [1]. Medicinal, herbal and aromatic plants constitute a large segment of the flora, which provide raw materials for use by pharmaceutical, cosmetic, fragrance and flavour industries. They have been used in the country for a long time for their medicinal properties. Many plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite [2].

Several studies have demonstrated a converse relationship between the consumption of antioxidant rich plants or vegetables and the incidence of human diseases [3]. Medicinal plants are commonly used in treating or preventing specific ailments or diseases and are considered to play a beneficial role in health care. Therefore, the study of plants as a resource of medicine has become more important in the context of present global trade scenario where oxidative stress is found to be one of the major causes of health hazards. India is considered as a treasure house of valuable medicinal and aromatic plant species. The Indian systems of medicine, popularly known as Ayurveda, Yunani and Siddha have identified 1500 medicinal plants of which 500 species are mostly used as healing agents for various disorders. *Adhatoda vasica* Nees and *Sesbania grandiflora* (L.) Pers are the two important medicinal plants native to India and were used in Indian medicine since ancient times.

Adhatoda vasica Nees (family Acanthaceae) also known as *Justicia adhatoda* L, is an evergreen shrub found all over India. It is used as expectorant, antispasmodic, bronchodilator, anti-histaminic, uterine stimulant, used in the treatment of menstrual disorders, eye infections, skin diseases, sore throat, bleeding diarrhoea and has sedative properties [4]. The leaves collected during flowering of the plant are medicinally important and are rich source of vitamin C. Leaves show hypoglycemic and antiulcer activities [5,6]. Alkaloids like vasicine, l-vasicinone, maiontone, vasicinone and vasicinol from leaves and roots, flavonoids like apigenin, astragalin, kaempferol etc, and triterpenes like daucosterol from flowers have been isolated [7,8,9]. The bronchodilatory, thrombopoeitic, antihistaminic and hypotensive, uterotonic, anti-inflammatory activities have been attributed to vasicine [10]. Pretreatment of rats with *A. vasica* crude extract can be protective against cadmium-induced genotoxicity and oxidative stress [11].

Sesbania grandiflora (L.) Pers (family Fabaceae) an ornamental tree, is a folk remedy for bruises, catarrh, dysentry, fevers, headaches, rheumatism, small pox and stomatitis. According to Ayurveda fruits are used for the treatment of anaemia, bronchitis and tumors; flowers are used for gout treatment and in Yunani medicine the plant is considered to be useful in biliousness [12]. The plant is one of the richest natural sources of vitamin A. Anticonvulsant and anxiolytic, hepatoprotective, antiurolithiatic, chemoprotective activities have been reported in the plant [13,14,15]. Oleanolic acid and its methyl ester and kaemferol-3-rutinoside in flower, two anthocyanadins leucocyanidin and cyanidin, a saponin sesbanimide in seeds, two alpha-glucosidase inhibitors SGF60 and SGF90 and chemopreventive protein SF2 in flowers have been reported [16,15]. The preliminary phytochemical screening of the flowers showed the presence of flavonoids, tannins, alkaloids, triterpenes, gums and mucilages [17].

The aim of the present study is to investigate the antioxidant potential and radical scavenging activity of aqueous leaf extracts of *A. vasica, S. grandiflora* using different *in vitro* assays, along with the determination of various enzymatic and non enzymatic antioxidants.

Materials and Methods

Plant material collection and preparation of the extracts

The leaves of the two plants *Adhatoda vasica* and *Sesbania grandiflora* were collected from Andhra university campus, Visakhapatnam and washed with distilled water. One gram of each fresh leaf tissue was weighed and ground in a chilled mortar and pestle with 10 ml buffer solution containing Tris HCl 0.05 M, pH 7.0 consisting of 3 mM $MgCl_2$ and 1 mM EDTA. The extract was centrifuged at 4°C for 10 min at 5000 rpm and the supernatant obtained was used for the determination of enzymatic and non enzymatic antioxidants and for the determination of antioxidant potential.

Chemicals

Diphenyl picryl hydrazyl (DPPH), thiobarbituric acid (TBA), and ferrozine were obtained from Himedia laboratories pvt.Limited, Mumbai. Gallic acid and potassium ferricyanide were obtained from Qualigens, Mumbai. O-dianisidine, nitro blue tetrazolium (NBT), hydrogen peroxide were obtained from Merck Limited, Mumbai, India. All other chemicals used were of analytical grade obtained from commercial source.

Enzymatic antioxidants

Assay of Superoxide dismutase

Assay of Superoxide dismutase was carried according to the method of Beauchamp and Fedovich [18]. To 0.5 ml of plant extract, 1 ml of 125 mM sodium carbonate, 0.4 ml of 25 μM NBT and 0.2 ml of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 ml of 1 mM Hydroxylamine hydrochloride and the absorbance was read at 560 nm using spectrophotometer (Hitachi) at 5 min intervals. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

Assay of Catalase

Catalase activity was determined by the titrimetric method described [19]. To 1ml plant extract, 5 ml of 300 μM phosphate buffer (pH 6.8) containing 100 μM hydrogen peroxide (H_2O_2) was added and left at 25°C for 1 min. The reaction was arrested by adding 10 ml of 2% sulphuric acid, and residual H_2O_2 was titrated with potassium permanganate (0.01N) till pink colour was obtained. Enzyme activity was estimated by calculating the decomposition of μM H_2O_2 per min per mg protein.

Assay of Peroxidase

Assay of Peroxidase was carried out according to the method of Malik and Singh [20]. To 3.5 ml of phosphate buffer (pH 6.5), 0.2 ml of plant extract and 0.1 ml of O- dianisidine solution were added. The reaction was initiated by adding 0.2 ml of 0.2 mM H_2O_2 and the absorbance was read at every 30 sec intervals upto 3 min. The peroxidase activity was calculated using an extinction coefficient of oxidized O-dianisidine and the enzyme activity was expressed as units per mg of protein.

Assay of Ascorbic acid Oxidase

Assay of Ascorbic acid oxidase was carried out according to the method of Oberbacher and Vines [21]. To 3 ml of ascorbic acid solution, 0.1 ml of plant extract was added and the change in absorbance at 265 nm was measured at 30 sec intervals for 5 min. One enzyme unit was expressed as to 0.01 OD change per min per mg protein.

Determination of Non enzymatic antioxidants

Determination of Total phenols

The amount of total phenolics in extracts was determined according to the Folin- ciocalteu procedure [22]. Samples (200 μl) were introduced into test tubes. One milliliter of Folin-ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram tissue as calculated from standard gallic acid graph.

Determination of Total Flavonoids

Total flavonoid content of the buffer extracts was determined according to a modified colorimetric method of Bao *et al* [23]. Plant extract (1.0 ml) was mixed with 1ml of distilled water and 75 μl of a 5% $NaNO_2$ solution. After 5 min, 75 μl of 10% $AlCl_3.H_2O$ solution was added. After 5 min, 0.5 ml of 1M Sodium hydroxide was added. The solution was mixed well and kept for 15 min. The increase in absorbance was measured at 510 nm using a UV-Visible spectrophotometer. The total flavonoid content was calculated using standard quercetin calibration curve. The results were expressed as milligrams of quercetin equivalents (QE) per gram tissue.

Estimation of Ascorbic acid

Ascorbic acid content was determined by the procedure given by Sadasivam and Theymoli Balasubramanian [24]. To 5 ml of ascorbic acid solution 10 ml of 4% oxalic acid was added and titrated against the di chloro phenol indophenol solution. The amount of dye consumed is equivalent to the amount of ascorbic acid consumed. Similarly 5 ml of plant extract was titrated against the dye.

Antioxidant ability assays

Total antioxidant activity

The assay was based on the reduction of Mo(VI)-Mo(V) by the extracts and subsequent formation of a green phosphate/Mo(V) complex at acidic pH [25]. The extract (0.1 ml) was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95ºC for 90 min. The mixture was cooled to room temperature, then the absorbance of the solution was measured at 695 nm against blank. The total antioxidant activity was expressed as ascorbic acid equivalents (AAE) in milligrams per gram of the extract.

Reducing power assay

The reducing power was determined by the Fe^{3+} - Fe^{2+} transformation in the presence of the extracts as described in the literature [26]. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the plant extract, 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. Supernatant (2.5 ml) was diluted with 2.5 ml of water and shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. The reducing power of the extracts was expressed as Vitamin E equivalents (Vit EE) in mg per gm sample. Increased absorbance of the reaction mixture indicated greater reducing power. BHT was used as a positive control.

DPPH Radical scavenging assay

DPPH stable free radical scavenging activity was determined by the method of Blois [27]. Plant extract 3 ml was added to 1ml of 0.1 mM solution of DPPH in methanol. After 30 min incubation at 37°C absorbance was measured at 517 nm against control using a spectrophotometer (Hitachi). Ascorbic acid and BHT were used as the reference materials. The percentage of inhibition was calculated by comparing the absorbance values of the test samples with those of the controls (not treated with extract). The inhibition percentage (I) was calculated as radical scavenging activity as follows

$I = (Abs_{control} – Abs_{sample}) / Abs_{control} \times 100$

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system [28]. The attack of the hydroxyl radical on deoxyribose leads to TBARS (thiobarbituric acid reactive substances) formation, which was measured by the method of Ohkawa *et al*. Each extract, 0.1ml was added to the reaction mixture containing 3.0 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM EDTA, 0.1 mM ascorbic acid, 1 mM H_2O_2 and 20 mM phosphate buffer (pH 7.4), making upto a final volume of 3.0 ml. The reaction mixture was incubated at 37ºC for 1 hr. One millilitre of thiobarbituric acid (1%) and 1.0 ml trichloroacetic acid (2.8%) were added to test tubes and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Inhibition of deoxyribose degradation in percent (I) was calculated using the formula

$I = (Abs_{control} – Abs_{sample}) / Abs_{control} \times 100$

Inhibition of Lipid peroxide Formation

A modified thiobarbituric acid reactive species (TABRS) assay was used to measure the lipid peroxide formed using the egg yolk homogenate as lipid rich media [29]. Malondialdehyde, a secondary end product of oxidation of polyunsaturated fatty acids reacts with two molecules of thiobarbituric acid (TBA) yielding a pinkish red chromogen with absorbance maximum at 532 nm. Egg homogenate (0.5 ml of 10% v/v) and 0.1 ml of plant extracts were added to a test tube and made up to 1 ml with distilled water and peroxidation was induced by adding 0.05 ml of 0.07 M $FeSO_4$. The reaction mixture was then incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.8 % (w/v) TBA in 1.1% SDS and 20% TCA were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the upper organic layer was measured at 532 nm. The percentage of inhibition (I) was calculated by the formula

$I = (Abs_{control} – Abs_{sample}) / Abs_{control} \times 100$

Metal chelating assay

To determine metal chelating ability the protocol according to Eric Decker and Welch was used [30]. Plant extract, 5 ml was added to a solution of 0.1 ml of 2 mM $FeCl_2$. This was followed by the addition of 0.2 ml of 5 mM ferrozine solution, which was left to react at room temperature for 10 min under shaking conditions before determining the absorbance of the solution at 562 nm. The percentage inhibition of Ferrozine–Fe^{2+} complex formation was calculated using the formula

$I = (Abs_{control} – Abs_{sample}) / Abs_{control} \times 100$

EDTA was used as a positive control.

Statistical analysis

For all the experiments three samples were analyzed and all the assays were carried out in triplicate. The results were expressed as mean±standard deviation.

Results and Discussion

Complex antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species.

These include both enzymatic and non enzymatic antioxidants.

The enzymatic antioxidants are represented in Table 1. High superoxide dismutase (8.876±0.696 units/mg protein) and catalase (87.393±1.248 units/mg protein), and ascorbic acid oxidase (1.553±0.11 units/mg protein) activities were detected in *A. vasica* as compared to *S. grandiflora* (7.423±0.846 units/mg protein, 76.06±1.483 units/mg protein and 0.374±0.007 units/mg protein of superoxide dismutase, catalase and ascorbic acid oxidase activities respectively). High peroxidase activity was reported in *S.grandiflora* (0.693±0.046 units/mg protein) than *A. vasica* (0.302±0.013 units/mg protein). The high ascorbic acid oxidase activity *A. vasica* can be related to high ascorbic acid content, as the enzyme is reported of participating mainly in oxidation reduction reactions involving ascorbic acid. Ascorbic acid provides first line of defence against oxidative stress [31].

Table 1. Levels of Enzymatic antioxidants

Name of the plant	SOD Units/mg protein	Catalase µMH_2O_2 decomposed /min/mg protein	Peroxidase Units/mg protein	Ascorbic acid oxidase Units/mg protein
A. vasica	8.876±0.696	87.393±1.248	0.302±0.013	1.553±0.11
S. grandiflora	7.423±0.846	76.06±1.483	0.693±0.046	0.374±0.007

The values are the average of three determinations and are expressed as mean ± S.D

The phytochemical screening of the extracts showed the presence of high amount of total phenols (100.66±1.154 GAE/gm), flavonoids (25.83±1.44 QE/gm) and ascorbic in *A. vasica* compared to *S. grandiflora* (31.34±0.577 GAE/gm, 5.2±0.721 QE/gm, of total phenols and flavonoids respectively) (Fig 1). The amount of ascorbic acid was also found to be high in *A. vasica* (224.15±0.989 mg/gm) compared to *S. grandiflora* (128.85±3.08 mg/gm) (Fig. 2).

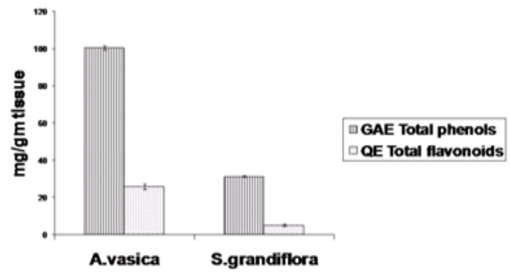

Fig. 1 Comparision of total phenols and flavonoids
i. Total phenols are expressed as mg of gallic acid equivalents/gm tissue and total flavonoids as mg of quercetin equivalents/gm tissue. ii. The values are the average of three determinations and are expressed as mean ± S.D

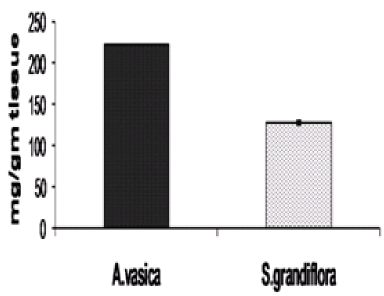

Fig. 2 Ascorbic acid
Ascorbic acid is expressed in mg/gm tissue and the values are the average of three determinations and are expressed as mean ± S.D

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [32]. The total antioxidant and reducing power activity in *A.vasica* were found to be 83.7±1.76 AAE/gm and 40.3±0.288 Vit E E/gm respectively, whereas in *S. grandiflora* they were found to be 46.7±1.44 AAE/gm and 35.3±1.154 Vit E E/gm respectively (Fig 3). Reducing power of *A.vasica*, *S. grandiflora* and standard compounds with increasing concentrations followed the order, BHT> *A. vasica* > *S. grandiflora* (Fig 4).

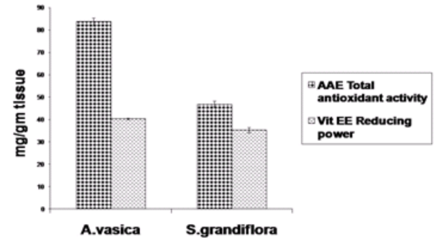

Fig.3 Antioxidant ability analysis
i. The total antioxidant activity was expressed as ascorbic acid equivalents (AAE), and reducing power as vitamin E equivalents (Vit EE). ii. The values are the average of three determinations and are expressed as mean ± S.D.

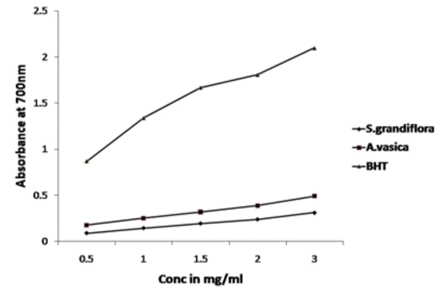

Fig. 4 Reducing power ability at different concentrations

The DPPH radical has been used widely to test the potential of the compounds as free radical scavengers of hydrogen donors and to investigate the antioxidant activity of plant extracts. The DPPH free radical scavenging activity is due to the neutralization of DPPH free radical by extract either by transfer of hydrogen or of an electron [33]. *Adhatoda vasica* (66.52±2.928%) showed high DPPH radical scavenging activity than *S. grandiflora* (43.20±0.952%) and standard antioxidants, BHT and ascorbic acid showed 78.30±1.422% and 76.71±1.830% of inhibition respectively (Table 2).

Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules by abstracting hydrogen atoms from membrane lipids and brings about peroxidation of lipids [34]. *Adhatoda vasica* (64.41±1.653%) is the highest scavenger of the hydroxyl radical compared to *S. grandiflora* (48.15±3.204%). The percentage of hydroxyl radical inhibition for BHT and ascorbic acid was found to be 75.74±5.248% and 73.46±1.626% respectively (Table 2). The inhibition of $FeSO_4$ induced lipid peroxidation was high in presence of *A. vasica* (61.12±4.809%) than in the presence of *S. grandiflora* (53.50±2.598%). It was found to be 80.90±0.606% for BHT and 67.06±1.847% for ascorbic acid (Table 2).

Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals [35]. Ferrozine can make complexes with ferrous ions. From the result it was evident that both the extracts possessed Fe^{2+} chelating activity (Table 2) and might play a protective role against oxidative damage induced by metal catalyzed decomposition reactions [36]. The metal chelating activity of positive control EDTA was found to be 93.78±1.050%. The higher chelating power of *A. vasica* (51.91±1.235%) as compared to *S. grandiflora* (24.39±0.352%) might be due to high concentration of phenolic compounds that can chelate metal ions.

Table 2. Radical scavenging activity.

Name of the plant	% of inhibition DPPH radical	Hydroxyl radical	Lipid peroxide	Ferrozine-Fe^{2+}complex
A. vasica (10mg/ml)	66.52±2.928	64.41±1.653	61.12±4.809	51.91±1.235
S. grandiflora (10mg/ml)	43.20±0.952	48.15±3.204	53.50±2.598	24.39±0.352
BHT(1mg/ml)	78.30±1.422	75.74±5.248	80.90±0.606	NT
Ascorbic acid (1mg/ml)	76.71±1.830	73.46±1.626	67.06±1.847	NT
EDTA (1mg/ml)	NT	NT	NT	93.78±1.050

The values are the average of three determinations and are expressed as mean ± S.D
NT - Not tested

Conclusion

The present study demonstrated that aqueous leaf extracts of *A. vasica* and *S. grandiflora* showed promising antioxidant and radical scavenging activities and the difference in their antioxidant activities can be attributed to their difference in phenolic content. From the observations it can be concluded that the leaves of *A. vasica* and *S. grandiflora* are the good sources of natural antioxidants and might be useful in treating the diseases associated with oxidative stress.

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