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Characterization of *in vitro* antioxidant potential of *Azadirachta indica* and *Abutilon indicum* by different assay methods

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ABSTRACT

The free radicals are implicated for many diseases like diabetes mellitus, arthritis, cancer, etc. Hence, antioxidant therapy has gained utmost importance in the treatment of such diseases linked to free radicals. Plants are considered enduring sources for antioxidant molecules for a long time in the history of mankind. Quite a large number of research studies have been carried out across the Globe relating to the antioxidant activity of numerous plant extracts and powders *Azadirachta indica* (A. Juss.) and *Abutilon indicum* (Linn.) were two important medicinal plant used in wide range of therapy. The present study aimed at studying the *in vitro* antioxidant activity of flowers and leaves of *A. indica* and *A. indicum*, respectively using different assay methods. Methanol extracts of flowers and leaves of *A. indica* and *A. indicum*, respectively were obtained and studied for their *in vitro* antioxidant activity using different models *viz.* DPPH radical scavenging assay, phosphomolybdenum assay, hydroxyl radical scavenging activity, metal chelating activity and superoxide anion radical scavenging activity. The extracts from *A. indica* flowers were found to scavenge the radicals (DPPH, HRSA and phosphomolybdenum), chelate metal ions and possess superoxide anion radical scavenging activity. *A. indica* flower extract was found to possess better antioxidant activity compared to *A. indicum* leaf extracts. Initial phytochemical screenings of *A. indica* flower extract have concluded that the antioxidant property may be related to the phenolics and flavonoids present in the extract. Thus, the present study ascertained the antioxidant potential of *A. indica* flower extract through various methods and found the chemical nature of the extract.

Keywords: *Azadirachta indica, Abutilon indicum,* antioxidant activity, assay methods, phenolics, flavonoids

INTRODUCTION

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms [1]. However, the excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous diseases and accelerate ageing. In the past decade, a number of epidemiological studies have confirmed that intake of exogenous antioxidants is effective in preventing or suppressing such diseases [2,3]. Several commercially available synthetic antioxidants such as butylated hydroxyanisole, Butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are currently in use but their possible toxic properties for human health and environment are inevitable [4]. Hence the development of alternative antioxidants from natural origin is the need of the hour. Among dietary antioxidants, phenolic compounds, the secondary metabolites from plants, are the most abundant natural antioxidants, which act as reducing agents, hydrogen donators, free radicals scavengers, and singlet oxygen quenchers and therefore, as cell saviours [5].

Neem (*Azadirachta indica* (A. Juss.)) is the most useful traditional medicinal plant in India. Each part of the neem tree has some medicinal property and is thus commercially exploitable. Neem tree, named as 'Arishtha' (reliever of sickness) in Sanskrit has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine. Its importance has been recognized by the US National Academy of Sciences, which published a report in 1992 entitled 'Neem – a tree for solving global problems' [6]. The non-wood products of neem like flowers, fruits, seeds, leaves, bark and gum also find various uses such as antifungal, antibacterial, insecticidal and other versatile biological activities. Moreover, Nimbidin, an active component extracted from neem seed oil is said to possess a number of medicinal properties like, anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, spermicidal, antifungal, antibacterial and diuretic [7].

Abutilon indicum (Linn.) commonly known as Indian Mallow is a climbing, annual spreading herb, distributed throughout plains and wetlands of India [8]. Traditionally, the plant is used for inflammation, piles, gonorrhea treatment and as an immune stimulant. In general, its root and bark are used as aphrodisiac, anti-diabetic, nervine tonic, and diuretic; its seeds are used in urinary disorders and as a laxative in piles and in the treatment of cough. More importantly, the juice from its leaves has been used to formulate an ointment for quick ulcer healing [9]. Furthermore, the plant is reportedly used in folk medicine for treating fever, cough, lung disease, urine output, deafness, ringing in the ears, mumps and pulmonary tuberculosis.

Though both the plants were explored for various medicinal properties, the present study utilized the solvent extracts from flowers of *A. indica* and leaves of *A. indicum* to find their potential in exhibiting antioxidant activities through various *in vitro* assay methods. Moreover the active component for antioxidant activity of the plant extract was also characterized.

MATERIALS AND METHODS

Collection of plant material

The flowers of *Azadirachta indica* and leaves of *Abutilon indicum* were collected and authenticated by Dr. M. Kumar, Taxonomist, MK University, India, for the study. The collected plant parts were cleaned and shade-dried

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for seven days and powdered. 20 g of each powdered samples were extracted with 200 mL of methanol under shaking condition [10]. The extracts were decanted into pre-weighed glass flasks. The process was repeated thrice to exhaustively extract the plant material and combined extracts were concentrated in a rotary evaporator (REMI, Mumbai, India). The condensate were weighed and stored for further analysis. The methanolic extracts of *A. indica* flowers were designated as AFE while the methanolic extracts of *A. indicum* as ALE.

Antioxidant activity of the plant extracts

i. Free radical scavenging activity on DPPH

The antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH**,** according to Chang [11]. Sample extract at various concentrations was taken and the volume was adjusted to 100 µL with methanol. 5 mL of methanolic solution of DPPH (0.1 mM) was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27ºC. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% DPPH radical scavenging activity = (Control OD – Sample OD /Control OD) × 100.

ii.Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto [12]. An aliquot of 100 µL of sample solution was combined with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 mL vial. The vials were capped and incubated in a water bath at 95ºC for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. Ascorbic acid (10 mg/mL in DMSO) was used as standard. The results were reported as % phosphomolybdenum reduction potential.

iii.Super oxide anion radical scavenging activity

The superoxide anion radical scavenging ability was assessed using the method of Nishikimi [13]. 1 mL of NBT solution, 1 mL of NADH solution, 0.1 mL of plant extract (10 mg in 0.1 mL DMSO and 0.9 mL PO_4 buffer) and 0.1 mL of PMS solution were added together and incubated at 25°C for 5 min. After 5 min, the absorbance was read at 560 nm. The results were reported as % SOD activity.

iv.Hydroxyl radical scavenging activity

The scavenging activity of the methanol extracts on hydroxyl radical was measured according to the method of Klein [14]. Various concentrations (250, 500, 750 and 1000 µg/mL) of extracts were added with 1.0 mL of iron-EDTA solution, 0.5 mL of EDTA solution, and 1.0 mL of dimethyl sulphoxide (DMSO). The reaction was initiated by adding 0.5 mL of ascorbic acid and incubated at 80–90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0 mL of ice-cold TCA. 3 mL of Nash reagent was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity (HRSA) is calculated by the following formula:

$\%\ HRSA = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

$Abs_{control}$ is the absorbance of the control; Abs_{sample} is the absorbance of the extract/standard.

v.Metal chelating activity

The chelating effect of ferrous ions by the extracts was estimated by the method of Dinis [15]. 100 µL of the extract was added to 0.05 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 mL of ferrozine (5 mM) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as % metal chelating activity. The ratio of inhibition of ferrozine Fe^{2+} complex was calculated as follows:

% inhibition= (Control OD – Sample OD /Control OD) × 100.

Quantification of phytochemical compounds

The phytochemical compounds were quantified using the following methods,

i. Total Phenolics

Total phenolic constituents of plant extracts were estimated by Folin-Ciocalteau's method using Folin-Ciocalteau reagent [16]. The estimation was done spectrometrically at 760 nm and the results were expressed as gallic acid equivalents (GAE).

ii. Estimation of Total Flavonoids- Aluminium Chloride Method

Aluminium chloride method was employed to quantify the total flavonoid content in the plant extracts. The results were expressed as quercetin equivalents (QE) [17].

iii. Estimation of Alkaloids

Total alkaloid content of the plant extracts were determined using the method specified by Sutharsingh [18]. 5 g of the sample was filtered and concentrated to one quarter of the original volume on a water bath after treatment with 200 mL of 10% acetic acid in ethanol. Con. NH_4OH was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH, filtered and weighed.

iv. Estimation of Saponins

20 g of powdered sample was treated with 100 mL of 20% aqueous ethanol, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re-extracted. The combined extracts were reduced to 40 mL over water bath at about 90°C and the concentrate was transferred into a separating funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added to the combined extracts and washed twice with 10 ml of 5% aqueous NaCl. The remaining solution was heated in a water bath, dried in an oven to a constant weight and the saponin content was calculated as percentage [18].

RESULTS

Free radical scavenging activity on DPPH

Table 1 shows the results of free radical (DPPH) scavenging activity of AFE and ALE, expressing the activity in percentage inhibition. The result revealed that the methanol fraction of AFE exhibited the highest radical scavenging activity with 73.36% at the concentration of 200 µg followed by ALE with 60.76% at the same concentration. In both AFE and ALE, the radical scavenging activity increased with increase in concentration of the extract. At the concentration of 10 mg/mL the standard (a- tocopherol) exhibited 84.24% inhibition.

Phosphomolybdenum assay

The antioxidant capacity of AFE and ALE were determined by the formation

of phospho complexes. The phosphomolybdenum reduction potential (PRP) of AFE and ALE were 70.43 and 62.4%, respectively. The standard (ascorbic acid) showed PRP of 87.13%. The results in Table 2 indicate that AFE has powerful antioxidant activity than ALE.

Super oxide anion radical scavenging activity

Results of superoxide anion scavenging activities of AFE and ALE are shown in Table 3. The extracts demonstrate a dose-response inhibition of the superoxide anion radicals. AFE exhibit good superoxide anion radical scavenging activity at 10 mg/mL. The percentage inhibition of superoxide, generated by AFE and ALE was found to be 53.31 and 39.73%, respectively at 10 mg/mL. On the other hand, at the same concentration, the standard (a-tocopherol) showed inhibition of 66.58%.

Hydroxyl radical scavenging activity

Table 4 shows the hydroxyl radical scavenging effects of AFE and ALE at different dose levels (25, 50, 100, 150 µg/mL). Both the extracts registered good hydroxyl radical scavenging activity in a concentration dependent manner. Among them, AFE showed the highest hydroxyl scavenging potential (54.11% at 100 µg/mL concentration). The standard (a-tocopherol) exhibited 62.5% at 100 µg/mL concentration.

3.5 Metal chelating activity

The metal chelating activity of AFE and ALE at 10 mg/mL is shown in Table 5. At the same concentration, EDTA (standard) showed metal chelating activity of 72.9%, followed by AFE (69%) and ALE (59%).

3.6 Phytochemical estimation of the extracts

Methanol extracts of AFE registered for high levels of total free phenolics (350±0.97 mg GAE/g) followed by total alkaloids (32±2.46 mg/g); total saponins (1.426±1.05 mg/g) and total flavonoids (0.355±1.35 mg QE/g) (Table 6).

Table 1. DPPH radical scavenging activity of AFE and ALE

Concentration (µg)	% inhibition		
	Standard[a]	AFE	ALE
20	18.01	16.62	13.83
40	35.12	23.45	14.75
60	47.36	33.02	16.84
80	50.47	33.68	19.06
100	55.25	39.05	19.58
120	59.45	54.17	20.1
140	66.03	66.48	27.02
160	72.13	62.14	30.02
180	74.55	65.53	54.83
200	84.24	73.36	60.76

[a]a-Tocopherol (10 mg/ml); All the values are mean of triplicate determination;

Table 2. Phosphomolybdenum reducing potential of AFE and ALE

Sample	Phosphomolybdenum activity (%)
Standard	87.13
AFE	70.43
ALE	62.40

Ascorbic acid at 10 mg/ml was used as a standard.

Table 3. Super oxide anion radical scavenging activity of AFE and ALE

Sample	SOD activity (%)
Standard	66.58
AFE	53.31
ALE	39.73

a-Tocopherol at 10 mg/ml was used as a standard.

Table 4. Hydroxyl radical scavenging activity (HRSA) of AFE and ALE

Concentration (µg)	% HRSA		
	Standard	AFE	ALE
25	22.5	21.56	11.78
50	39.16	37.55	19.17
75	58.75	43.83	36.43
100	62.5	54.11	40.27

a-Tocopherol at 10 mg/ml was used as a standard.

Table 5.Metal chelating activity of AFE and ALE

Sample	Metal chelating activity (%)
Standard	72.9
AFE	69
ALE	59

EDTA at 10 mg/ml was used as a standard

Table 6. Quantitative phytochemical estimation of AFE

Phytochemicals	Concentration
Total alkaloids	32±2.46[a]
Total flavonoids	0.355±1.35[b]
Total free phenolics	350±0.97[c]
Total saponins	1.426±1.05[d]

[a]Alkaloids expressed in mg per gram; [b]Flavonoids expressed as mg quercetin equivalent per gram; [c]Phenols expressed as mg gallic acid equivalent per gram; [d]saponins expressed in mg per gram; All the values are mean of triplicate determination.

DISCUSSION

Oxidative stress represents the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders [19]. Many enzymes and secondary compounds of higher plants have been demonstrated in *in vitro* experiments to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. A great number of research studies have been carried out relating to the antioxidant activity of numerous plant extracts and powders [20]. Thus, many plants and their parts are explored continuously for antioxidant molecules.

Traditional Indian medicine is utilizing many herbs and trees for various medicinal properties including antioxidant activity. For the past few decades research studies were intensified to scientifically validate the traditionally used medicinal plants for their active compound and mechanisms of action. The juice from the leaves of *A. Indicum* has been used in formulation of an ointment for quick ulcer healing [9]. Nimbidin, an active component extracted from neem seed oil possesses various medicinal properties like anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antiulcer, spermicidal and antifungal [7].

Being a stable free radical, DPPH is frequently used to determine radical scavenging activity of natural compounds. In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form, DPPH-H [21]. Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution. Unlike other free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain metal ion chelation and enzyme inhibition. The extracts of *A. indica* and *A. indicum* showed free radical

scavenging activity property at all the concentrations studied. There was a reduction in the concentration of DPPH due to the scavenging ability of the extracts of *A. indica* and *A. indicum.* Also, many researchers have reported positive control between free radical scavenging activity and total phenolic compound. Oki *et al*, observed that the radical scavenging activity increased with the increase of phenolic compound content [22]. The two studies conducted by Lu and Foo [23] and Siriwardhana *et al*, [24] reported a high correlation between DPPH radical scavenging potential and total phenolic content. These results indicate that the extracts with high total phenolic contents presented high radical scavenging activities which could be related to the inherent nature of phenolic compounds, thus contributing to their electron transfer or hydrogen donating ability [25]. High correlation between radical scavenging and phenolic content has been reported in cereal [26], fruits [27], beverages [28] and culinary herbs [29]. AFE appeared to be as potent as a-tocopherol with a maximum inhibition of 73.36±0.98% at 200µg which is comparable to 84.24±0.7 for a-tocopherol at the same concentration. From the result, it is inferred that AFE was found to be the best in scavenging free radicals, compared to ALE and the increase in free radical scavenging capacity of AFE can be explained by the presence of higher phenolic content.

The phosphomolybdenum method is based on the reduction of molybdenum by the antioxidants and the formation of green molybdenum (V) complex, which has absorption of 695 nm. It is known that hydrogen and electron transfer from antioxidant analytes to DPPH- and Mo (VI) complex occur in the DPPH and phosphomolybdenum assay methods, respectively. The transfer occurs at different redox potentials in these two assays and also depends on the structure of the antioxidant. DPPH scavenging assay detects antioxidant such as flavonoids and polyphenols, whereas phosphomolybdenum assay usually detects antioxidant such as ascorbic acid, some phenolics, a-tocopherol and carotenoids [12]. Due to this reason, antioxidant potential may sometimes differ in these two types of assays. However, the results obtained in phosphomolybdenum assay confirm high reducing potency of AFE towards the transition metal ions. The reducing power was comparable to the recognized hydrophilic antioxidant ascorbic acid.

Superoxide can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the breakdown of superoxide radical [30]. Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species such as hydroxyl radical, hydrogen peroxide and singlet oxygen [31]. Thus the study of the scavenging of this radical is important. Superoxide radicals were generated in a PMS-NADH system and assayed by the reduction of NBT [32]. In this study, AFE showed comparatively higher superoxide anion activity than ALE. Scavenging of hydroxyl radical is an important antioxidant activity because of its very high reactivity, which can easily cross the cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death. Thus, removing the hydroxyl radical is very important for the protection of living systems [33]. Neem bark extracts showed strong scavenging ability on hydroxyl radicals [34]. The ability of AFE to quench hydroxyl radicals seems to be directly related to the prevention of propagation of lipid peroxidation. Also, AFE seems to be good scavengers of active oxygen species thus reducing the rate of chain reaction.

Transition metal has played a pivotal role in the generation of oxygen free radicals in living organisms. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes [35]. Transition elements like iron and copper are powerful catalysts of oxidation reactions because they contain one or more unpaired electrons that can enable to participate in electron transfer reactions [36]. They can participate in the conversion of H2O2 to OH in the Fenton reaction and in the decomposition of alkyl peroxides to the heavy reactive alkoxyl and hydroxyl radicals [37]. Due to this property, transition metal chelation to form low redox potential complexes can be an important antioxidant property [38]. The metal chelating property of AFE and ALE may be attributed to their endogenous chelating agents, mainly phenolic compounds that have properly oriented functional groups, which can chelate metal ions. Thus, the present study characterized the antioxidant activity of AFE and ALE through various *in vitro* methods in a comprehensive fashion. The active ingredient in the crude AFE which showed remarkable antioxidant activity was found to be phenolics. This study also creates a lead to further research work to validate these extracts in *in vivo* animal model for mechanisms of action and possible therapy options.

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