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Research Article

IN VITRO FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS AND METHANOLIC LEAF EXTRACTS OF *AEGLE TAMILNADENSIS* ABDUL KADER (RUTACEAE)

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ABSTRACT

Objective: The present study was aimed to find out antioxidant property of methanolic and aqueous extracts of *Aegle tamilnadensis* Abdul Kader (Rutaceae) leaf.

Methods: The phenolic and flavanoid contents of methanolic and aqueous extract were determined. The antioxidant potential of the leaf samples was evaluated by using five *in vitro* assays such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical, superoxide radical, reducing power and metal ion chelating assays.

Results: The phenol and flavanoid contents were found to present in considerable quantities. The methanolic leaf extract showed better ability to scavenge superoxide radical (IC_{50} value of 50.50±0.84 µg /ml) followed by hydroxyl radical (IC_{50} value of 232.95±1.10 µg/ml). It also showed good reducing power ability. In contrast, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals were effectively scavenged by aqueous leaf extract (IC_{50} value of 82.05±1.02 µg/ml). Both the methanolic and aqueous leaf extracts of *A. tamilnadensis* leaves at 1000 µg/ml showed maximum scavenging activity (91.01% and 95.67%) in quenching metal ions.

Conclusions: The results obtained from the present study indicated that the *A. tamilnadensis* leaf extract is a potent source of natural antioxidants and both the aqueous and methanolic leaf extracts possessed antioxidant property. However methanolic leaf extract showed better antioxidant activity than the aqueous leaf extract.

Keywords: *Aegle tamilnadensis,* Antioxidant, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Hydroxyl radical, Superoxide radical.

INTRODUCTION

Living cells generate free radicals and other reactive oxygen species (ROS) as by-products of various physiological and biochemical processes. ROS are highly reactive molecules which includes free radicals such as, superoxide ions (O^{2-}), hydroxyl radicals (OH^-), nitric oxide radical (NO), singlet molecular oxygen, peroxynitrite radicals and hydrogen peroxide (H_2O_2). ROS play a major role in cell metabolism including energy production, phagocytosis and intercellular signaling *in vivo* [1]. However, these ROS produced by endogenous sources and exogenous sources can lead to a wide variety of pathological effects such as DNA damage, carcinogenesis and various degenerative disorders and neurodegenerative diseases [2-4]. The most effective way to get rid of free radicals which cause the oxidative stress is with the help of antioxidants. It has been reported earlier that the antioxidants have the ability to prevent oxidative damage induced by reactive oxygen species as it can interfere with the oxidation process by inhibiting the initiation or propagation of oxidizing chain reactions [5].

The human body uses an antioxidant defense system to neutralize the reactive oxygen species. The system which consists of enzymatic and non enzymatic antioxidants and some antioxidant enzymes in order to provide a protection against ROS are superoxide dismutases, catalases, and glutathione peroxidases [6] and the non enzymatic molecules include glutathione, tocopherol (vitamin E), vitamin C, β-carotene, and selenium [7,8]. The antioxidants may be either natural or synthetic. Natural antioxidants such as α-tocopherol and ascorbic acid are widely used because they are regarded as safer and causing fewer adverse reactions. While synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and tertiary butyl-hydroquinone (TBHQ) are known to amend oxidative damages, but they have restricted use in foods as they are carcinogenic [9]. Natural antioxidants are considered as more safer and cause fewer adverse health effects than synthetic antioxidants [10]. Therefore, considerable interests in development of antioxidants of natural origin have received much attention in recent years, especially within biological, medical, nutritional and in agrochemical areas [11].

Aegle tamilnadensis Abdul Kader (Rutaceae) is a recently discovered tree species found in the campus of Govt. Siddha Medical College, Arumbakkam, Chennai [12]. It is an evergreen thorny tree, found in the forests of Tamil Nadu. The leaves are pinnately trifoliately compound with ovate-elliptic (sometimes lanceolate) petiolulate leaflets having cordate base, emarginate tip, crenate margin and having very pungent smell (than *A. marmelos*). The flowers are very fragrant, greenish-white, borne in long axillary and terminal cymose panicles. The calyx is tubular with triangular lobes. The petals are typically 5 and recurved. The fruits are very large, pear-shaped, depressed at tip, 13- celled, containing many ovate hairy seeds. Since the species is a newly reported one, no studies were carried out regarding antioxidant potential so far. Hence, we have undertaken the present study to evaluate the antioxidant potential of methanolic and aqueous leaf extracts of *A. tamilnadensis* Abdul Kader.

MATERIALS AND METHODS

Plant material

A. tamilnadensis leaves were collected from the campus of Govternment Siddha Medical College, Arumbakkam, Chennai during October 2012. The leaves were air dried under shade and coarsely powdered. The powdered samples were stored in airtight bottles for further use.

Chemicals

Nitro blue tetrazolium (NBT), riboflavin, 2.2 Diphenyl -1-picryl hydrazyl (DPPH), deoxyribose, ferrozine, potassium ferricyanide and the solvents were purchased from HiMedia Laboratories Pvt. Limited, Mumbai, India. Trichloroacetic acid (TCA), ethylene diamine tetra acetic acid (EDTA) and ascorbic acid were purchased from Sisco Research Laboratories, Mumbai, India. Thiobarbituric acid (TBA) was purchased from Rolex Chemical Industries, Mumbai, India. All the other chemicals and reagents used were of analytical grade and were prepared in deionized water.

Fluorescence analysis

The fluorescence characteristics of the powdered leaf were studied under long UV (315 to 400nm), short UV (100 to 280nm) and visible lights (390 to 700nm) after treating with different solvents such as ethanol, methanol, ethyl acetate, acetone, distilled water and petroleum ether [13].

Antioxidant assays

Preparation of extracts

The powdered leaf samples (30g) were subjected to cold extraction using methanol and water (250 ml each) for 72 hours. Extracts were filtered using a Whatman No 1 filter paper and was concentrated under reduced pressure at 40°C using a rotary evaporator (IKA RV 10 digital). The dried extracts were then stored at 4°C for further assays.

Determination of total phenolic content

The total phenolic content of aqueous and methanolic leaf extracts was determined according to the method described by Malik and Singh [14]. Aliquots of the extracts were made up to 3 ml with distilled water. Then 0.5 ml folin ciocalteau reagent (1:1 with water) and 2 ml Na_2CO_3 (20%) were added sequentially. The tubes containing the above reaction mixture were warmed for 1 minute, and cooled subsequently for measuring at 760 nm. The concentration of phenol in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample.

Determination of flavonoid content

Total flavonoid content was determined by aluminium chloride method [15]. 1ml sample and 4 ml of water were added to a volumetric flask. 0.3 ml of 5 % sodium nitrite, 0.3 ml of 10% aluminium chloride was added after 5 min. Then after 6 min incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture and immediately made up to 10 ml with distilled water. The absorbance was read at 510 nm and the results were expressed as catechin equivalents (mg catechin/g dried extract).

DPPH free radical scavenging assay

The DPPH free radical scavenging activity of *Aegle tamilnadensis* leaf extracts at different concentrations were measured from bleaching of the purple colour of 2.2 Diphenyl -1-picryl hydrazyl [16]. A 0.1 ml solution of different concentration of extract was added to 1.4 ml of DPPH and kept in dark for 30 min. The absorbance was measured at 517 nm and the percentage inhibition was calculated by using the following Equation.

Percentage inhibition (%) = (A0 –A1) / A0) × 100 (Equation I)

where: A0 is the Absorbance of control and A1 Absorbance of test. The result is expressed in terms of IC_{50} value which is the effective concentration at which the antioxidant activity is 50%.

Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by a Fenton reaction (Fe3+-ascorbate-EDTA-H_2O_2 system), and the scavenging capacity towards the hydroxyl radicals was measured by using deoxyribose method [17]. The reaction mixture contained 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), ferric chloride (20 µM), EDTA (100 µM), hydrogen peroxide (500 µM), ascorbic acid (100 µM) and various concentrations (10-1000 µg/ml) of the test sample in a final volume of 1 ml. The mixture was incubated for 1 h at 37 °C. After the incubation an aliquot of the reaction mixture (0.8 ml) was added to 2.8% TCA solution (1.5 ml), followed by TBA solution (1% in 50 mM sodium hydroxide, 1 ml) and sodium dodecyl sulphate (0.2ml). The mixture was then heated (20 min at 90 °C) to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicates. The percentage of inhibition was expressed, according to the following equation: (%) = [A0 − (A1 − A2)]/A0 × 100, where: A0 is the absorbance of the control without a sample, A1 is the absorbance in the presence of the sample and deoxyribose and A*2* is the absorbance of the sample without deoxyribose.

Superoxide radical scavenging assay

The superoxide radical generated from the photo reduction of riboflavin was detected by NBT reduction [18]. The reaction mixture contained EDTA (0.1 M), 0.0015% NaCN, riboflavin (0.12 mM), NBT (1.5 mM) and various concentrations of extract and phosphate buffer (67 mM, pH 7.8) in a total volume of 3 ml. The tubes were uniformly illuminated for 15 min and optical density was measured at 530 nm before and after the illumination. The percentage inhibition was calculated by using Equation (I).

Metal chelating activity assay

The ability of extracts to chelate iron (II) ions was estimated using the method described by Gulcin [19]. Different concentrations of the sample ranging from 10-1000 µg/ml were added to a solution of 2 mM iron (II) chloride (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the volume of the mixture was finally adjusted to 4 ml with methanol, shaken vigorously and left standing at room temperature for 10 min. After incubation, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition was calculated using the formula given below: (%) = [A0 − (A1 − A2)]/A0 × 100, where A0 is the absorbance of the control, containing iron (II) chloride and ferrozine only, *A1* is the absorbance in the presence of the tested sample and A2 is the absorbance of the sample under identical conditions as A1 with water instead of iron (II) chloride solution. All assays were done in triplicate and the mean values were given in the results.

Reducing power activity

The reducing power of the extracts was determined by the method described by Oyaizu [20]. An aliquot of extracts (1.0 ml) at various concentrations ranging from 10-1000 µg/ml was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 ml) and 1% potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. After adding 10% trichloroacetic acid (2.5 ml, 10%), the mixture was centrifuged at 6500 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% iron (III) chloride (0.5 ml) and the absorbance was measured at 700 nm using phosphate buffer as blank. These were done in triplicate and the mean values were given in the results. The percentage inhibition was calculated using the Equation (I).

RESULTS AND DISCUSSION

Fluorescence analysis

The leaf of *A. tamilnadensis* when treated with different solvents exhibited various colours when exposed under short UV, long UV and visible light. The results are given in Table 1. The characteristic fluorescent properties or colours recorded in fluorescent analysis could be used as a standard in the identification and authentication of the leaves of *A. tamilnadensis* in its crude form. This is helpful in the characterization of crude drugs which reveals the presence of active agents [21] in the leaf of *A. tamilnadensis*.

Table 1: Fluorescent analysis of *A. tamilnadensis* powder with different solvents

Solvents	Long UV	Short UV	Visible
Ethanol	Black	Green	Dark green
Methanol	Pink	Dark green	Dark green
Ethyl acetate	Pink	Dark green	Dark green
Acetone	Dark green	Green	Light green
Distilled water	Black	Dark green	Dark green
Petroleum ether	Pink	Dark green	Green

Determination of Phenolic and Flavanoid contents

Total phenolic content was determined by Folin Ciocalteu method and expressed as mg of catechin /1g of extract and total flavonoids was expressed in terms of catechol equivalents/1g dried extract. The flavanoid content of the *A. tamilnadensis* methanolic and aqueous

extracts were found to be 118.28±1.64mg/g and 20.75±1.13 mg/g respectively. The phenol content of the *A. tamilnadensis* methanolic and aqueous extract was found to be 20.94±1.22mg/g and 18.45±0.65mg/g. It is evident from the results that the phenolic and flavanoid contents were found in considerable amount in both the extracts. Among the two extracts tested, methanolic extract exhibited high quantity of phenolic compounds and flavanoids compared to aqueous extract. Phenolics and flavanoid compounds present in leaves have received significant importance because of their potent antioxidant properties [22]. The presence of phenolic and flavanoid component in the methanolic extract of *A. tamilnadensis* was found to be high when compared to *Aegle marmelos* [23].

***In vitro* antioxidant assays**

Antioxidants eradicate damaging chemicals in the body and protect against heart disease, arthritis, cancer and many other chronic diseases. Hence sufficient intake of antioxidants daily protects the cells from decomposition [24]. The free radicals mediated toxicity can be effectively eliminated by plant derived antioxidant compounds and many of these activities have been already reported for a wide range of plants [25].

DPPH radical scavenging activity

The DPPH radical scavenging activity of the aqueous leaf extract at 1mg/ml was 71.86 % and that of the methanolic extract was 50.51% (Figure 1). The results showed that the aqueous extract exhibited strong DPPH radical scavenging ability (IC_{50} value of 82.05±1.02 µg/ml) than the methanolic extract (IC_{50} value of 987.51±1.20 µg/ml). The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability to the free radicals and reducing it to nonreactive species [26]. The antioxidant compounds reduce the purple coloured DPPH radical to yellow. Here the reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidant. When compared to DPPH scavenging ability of *Aegle marmelos* [23] the methanolic leaf extract exhibited a lower radical scavenging activity with an IC_{50} value of 28.726µg/ml (Figure 1).

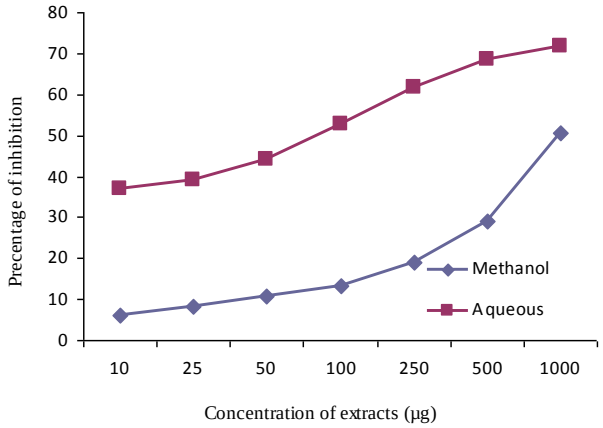

Fig. 1: DPPH radical scavenging activity of extracts.

Hydroxyl radical scavenging activity

At a concentration of 1 mg/ml, the scavenging activity of methanolic and the aqueous extract extract was found to be 54.91% and 52% respectively (Figure 2). Of these, the methanolic extract (IC_{50} value of 232.95±1.10µg/ml) was found to be more effective in quenching the hydroxyl radicals produced in the reaction mixture. The hydroxyl radical can induce oxidative damage to DNA, lipids and proteins [27]. The hydroxyl radical scavenging ability of the extracts was determined by its ability to compete with deoxyribose for hydroxyl radical. The *A. tamilnadensis* extract compete with deoxyribose and diminish chromogen formation in a dose dependant manner. In this assay, 2-deoxy-2-ribose was oxidized when exposed to hydroxyl radicals generated by Fenton-type reaction. The oxidative degradation can be detected by heating the products with TBA under acid conditions to develop a pink chromogen (thiobarbituric acid reactive species) with a maximum absorbance at 532 nm [28].

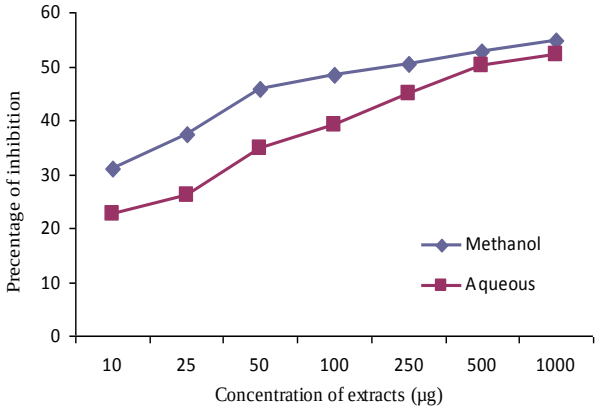

Fig. 2: Hydroxyl radical scavenging activity of extracts

Superoxide radical scavenging activity

The maximum inhibition was found to be 63.5% for methanolic and 53.4 % for aqueous extract at 1 mg/ml (Figure 3). The methanolic extract exhibited an IC_{50} value of 50.50±0.84 µg /ml. The methanolic extract was found to be an effective scavenger of superoxide radical generated by photo reduction of riboflavin. Superoxide anion radical is one of the strongest ROS among the free radicals and get converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases [29].

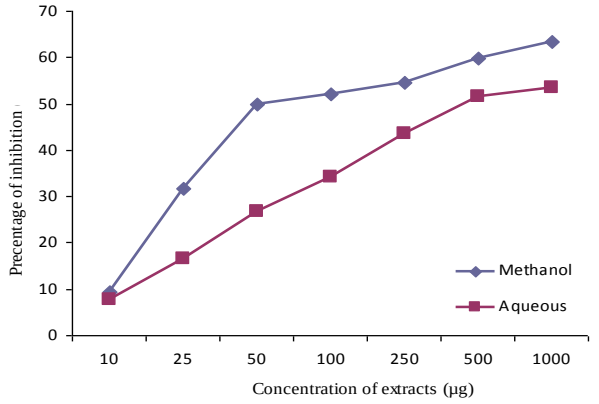

Fig. 3: Superoxide radical scavenging activity of extracts

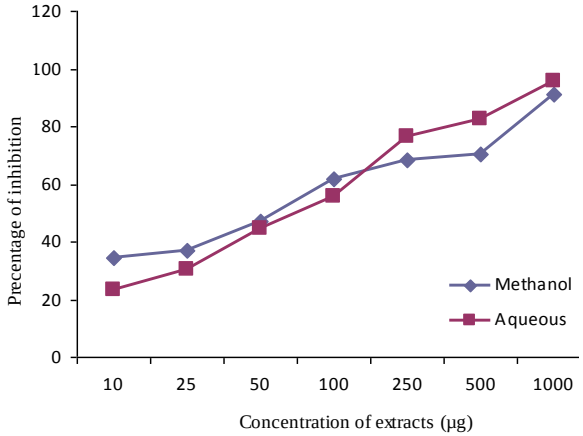

Fig. 4: Metal ion chelating activity of extracts

Metal ion chelating activity

The results showed that the aqueous and methanolic extracts at 1mg/ml have an effective capacity for ion binding with an inhibition of 95.67 % and 91.01 % respectively (Figure 4). The aqueous extract

showed a stronger scavenging activity with an IC_{50} value of 61.44±1.16µg/ml than methanolic extract (IC_{50} value of 76.73±0.82µg/ml). The chelating activity of ferrous ions by *A. tamilnadensis* was estimated using ferrozine. The chelating effects of *A. tamilnadensis* leaf extract on ferrous ions increased with increasing concentrations. The methanolic extract competes with ferrozine for ferrous ion than the aqueous extract.

Reducing power activity

Reducing power of both extracts increased with increase in concentration. The methanolic extract showed more effective reductive ability when compared to that of aqueous extract (Figure 5). In this assay, Fe (III) reduction is often used as a significant indicator of electron donating activity which is an important mechanism of phenolic antioxidant action [30] and is correlated with the presence of reductones which exhibits its antioxidant action by breaking the radical chain by donating a hydrogen atom [31]. Here, the methanolic extracts showed highest activity in a dose dependant manner which is due to the presence of these reductones.

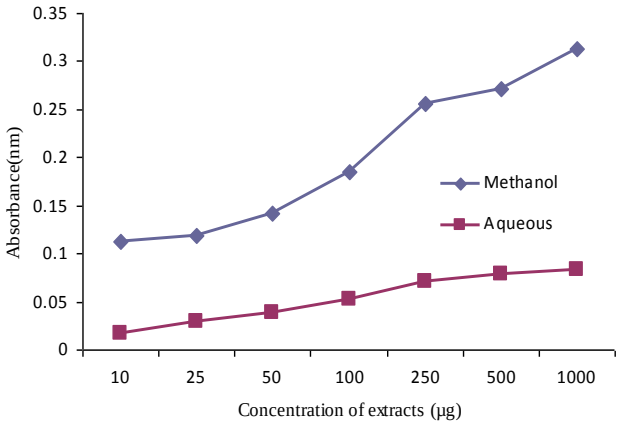

Fig. 5: Reducing power activity of extracts

CONCLUSIONS

The results of the present study revealed that *A. tamilnadensis* leaf extracts possessed potent free radical scavenging ability in methanolic and aqueous extracts. In all the assays performed, except DPPH radical scavenging assay the methanolic extract was found to be the potent radical scavenger when compared to that of aqueous extracts. The activity observed may be attributed to the presence of phenolic and flavaniod contents in the methanolic extract and further we conclude that this plant is a potential candidate for natural antioxidant.

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