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Antioxidant and antimicrobial activities of methanol leaf extract of *Aegle Tamilnadensis* Abdul Kader

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Abstract

The present investigation was aimed to evaluate the phytochemical constituents, free radical scavenging activities, antioxidant properties and antimicrobial activities of the methanol leaf extract of *Aegle tamilnadensis* Abdul Kader. The phytochemicals present in the methanol leaf extract of *A. tamilnadensis* Abdul Kader were determined qualitatively and quantitatively using standard procedures. The antioxidant activities were carried out by DPPH free radical scavenging assay, $OH^{\bullet}$ radical scavenging assay, $NO^{\bullet}$ radical scavenging assay, Fe^{3+} reducing power assay, and phosphomolybdenum reduction assay methods. The antimicrobial activity was carried out by well diffusion method. The methanol leaf extract of *A. tamilnadensis* Abdul Kadershowed good free radical scavenging as well as reducing power activities which were found to increase with the increasing concentration of the extract. The study revealed the presence of major phytochemicals such as phenols and flavonoids at the concentration of 211.0 mg/g at 100 µg and 52.91 mg/g at 100µg/mL respectively. The present study revealed that the methanol leaf extract of *A. tamilnadensis* Abdul Kader possess significant antioxidant and antimicrobial activities.

Keywords: Aegle tamilnadensis, phenols, flavonoids, antioxidant, antimicrobial.

Introduction

The genus *Aegle* Correa (Rutaceae) contains 3 species *viz*., *Aegle marmelos* (L.) Correa (= *Crataeva marmelos* L., *Aegle marmelos* var. *mahurensis*), *A. decandra* F. villar (= *A. glutinosa* Blanco Merr. and *A. barteri* Hook. f. ex Oliv. (= *Afraegle paniculata* Schum & Thonn. Among these, only *A. marmelos* (commonly called 'Vilvam' in Tamil) is found growing in India, including Tamil Nadu [1]. This new *Aegle* species was reported by Abdul Kader in 2012 and 2015 which is found in the campus of Govt. Siddha Medical College, Arumbakkam, and Chennai. It is an evergreen thorny tree. The leaves are pinnately trifoliate, leaflets ovate-elliptic (sometimes lanceolate), petiolulate, cordate at base, emarginate at tip, crenate at margin and having very pungent smell (when crushed, the leaves emit a strong pungent smell similar to *Citrus medica*). The flowers are very fragrant, greenish-white, borne in long axillary and terminal 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 [2].

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases [19]. Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [5, 39]. The hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system and contributes to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the low intensity of red colour [21]. Nitric oxide (NO·) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use. The plants have various phytochemicals some of which act as antioxidants which react with free radicals. Since *A. tamilnadensis* is a recently discovered one, only very few studies were carried out regarding its antioxidant potential so far [3]. Hence, we have undertaken the present study to evaluate the antioxidant potential and antimicrobial activities of methanolic leaf extract of *A. tamilnadensis* Abdul Kader.

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Materials and Methods

Collection of Plant material

Leaves of *Aegle tamilnadensis* Abdul Kader (Figure.1) were collected from Govt. Siddha Medical College campus, Chennai during the month of September 2014. The collected leaves were shade dried for 20 days, powdered mechanically and stored in airtight container for further analysis.

Figure.1. Habitat of *Aegle tamilnadensis* Abdul Kader

Preparation of extract

The extract was obtained from the powdered leaves of *A. tamilnadensis* using methanol as a solvent by maceration method. Initially leaves coarse powder was soaking in methanol for 72 h. Then the supernatant was filtered through filter paper and concentrated using rotary evaporator at 50ºC which gave a greenish-black coloured sticky residue.

Qualitative phytochemical screening

The methanol leaf extract of *A. tamilnadensis* was subjected to preliminary phytochemical screening using standard methods. The phytoconstituents such as alkaloids, terpenoids, flavonoids and phenolic compounds, steroids, glycosides, carbohydrates and saponins [4-6, 16].

Estimation of total phenols

Total phenol content was estimated by Folin-Ciocalteau reagent method [7] 0.1 mL of methanol leaf extract (1mg/mL) was mixed with 0.9 mL of methanol and 1 mL of Folin-Ciocalteau reagent (1:10 dilution). After 5 minutes, 1 mL of aqueous Na_2CO_3 (20%) solution was added. The mixture was then incubated at room temperature in dark for 30 minutes. The quantification of phenolic compounds was performed by measuring the absorbance in UV-Vis spectrophotometer at 765 nm. The total phenolic content was expressed, in terms of Gallic acid equivalent (mg/g of dry mass of extract), which is a common reference compound.

Estimation of total flavonoids

Aluminium chloride colorimetric method was used to determine the total flavonoids [8] with slight modifications. 0.5 mL of methanol leaf extract (1mg/mL) of *A. tamilnadensis* was mixed with 0.5 mL of methanol with 0.5 mL of 10% aluminium chloride, 0.5 mL of 1 M potassium acetate and 0.5 mL of distilled water and incubated at room temperature for 30 minutes. The absorbance of the reaction mixture was measured by UV-Vis spectrophotometer at 415 nm. The total flavonoid content was expressed in terms of quercetin equivalent, which is a common reference standard.

In vitro Antioxidant Assay

DPPH radical scavenging activity

The stable DPPH radical was used for determination of free radical scavenging activity of *A. tamilnadensis* methanolic leaf extract (Brand-Williams *et al.* [9].) The 0.1 mM solution of DPPH was freshly prepared in methanol. Various concentrations (20-120 µg/mL) of 1 mL of methanolic leaf extract was mixed with 0.1 mM of 1 mL of DPPH solution, and kept at room temperature in the dark.

After 30 minutes, the decrease in absorbance was measured at 517 nm using UV-Vis spectrophotometer. DPPH radical scavenging activity was calculated by using the following formula:

$$\% \text{ of DPPH radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Hydroxyl ($OH^{\bullet}$) radical scavenging activity

Hydroxyl radical scavenging assay was carried out according to Klenin *et al.* [10]. Various concentrations (5-30 µg/mL) of methanol leaf extract of *A. tamilnadensis* were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of DMSO (0.85% v/v) in 0.1 M phosphate buffer (pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 90 C for 15 minutes in a water bath. After incubation the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% w/v). 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and kept at room temperature for 15 minutes. The reaction mixture without sample was used as control. The intensity of colour developed was measured at 412 nm using UV-Vis spectrophotometer against reagent blank. The percentage of hydroxyl radical scavenging activity was calculated by using the following formula:

$$\% \text{ of OH radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Nitric oxide ($NO^{\bullet}$) radical scavenging activity

Different concentrations of 1 mL methanol leaf extract of *A. tamilnadensis* with nitric oxide were assessed by the nitrite detection method as described by Sreejayan and Rao [11]. Nitric oxide was generated from sodium nitroprusside previously bubbled with nitrogen and measured by the Greiss reaction. 0.25 mL of sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) was mixed with 0.25 mL of different concentrations (5-30µg/mL) of extracts and incubated at 25 C in the dark for 150 minutes. The control was run as above but the sample was replaced with the same amount of water. After the incubation period, 0.25 mL of Griess reagent a (1% sulphanilamide in 5% phosphoric acid) was added, and kept at 30 C for 10 minutes. Later, 0.25 mL of Griess reagent B (0.1% *N*-1-naphthylethylene diamine dihydrochloride) was added, thoroughly mixed, and incubated at 30 C for 20 minutes. The absorbance of the chromophore formed was read at 546 nm using UV – Vis Spectrophotometer. The percentage of NO radical scavenging activity was calculated using the following formula:

$$\% \text{ of NO radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of methanol leaf extract of *A. tamilnadensis* was determined by slightly modified method of Oyaizu [12]. One mL each of methanolic leaf extract concentration (20 - 120 µg/mL) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were then incubated at 50 C for 20 minutes. One mL of trichloroacetic acid (10 %) was added to each mixture, which were then centrifuged for 10 minutes at 1036 x g. The upper layer of the solutions (1 mL) were mixed separately with distilled water (1 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance were measured at 700 nm using UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference.

Phosphomolybdenum reduction activity

The phosphomolybdenum reduction activity of the methanol leaf extract of *A. tamilnadensis* was assessed as described by Prieto *et al.* [13]. The methanol leaf extract of *A. tamilnadensis* in dilution from 20 - 120 µg/mL was combined with the reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 95ºC for 90 minutes. The absorbance of the coloured complex was measured at 695 nm UV-Vis spectrophotometer. Ascorbic acid was used as standard reference.

Antimicrobial Activity

Test organisms

The subculture organisms Gram-positive bacterium *Staphylococcus aureus* (ATCC 12600) and Gram-negative bacterium *Escherichia coli* (ATCC 11775) was available in the laboratory Centre for Advanced Studies in Botany, University of Madras, Guindy Campus**,** is stored in a refrigerator and used for the antimicrobial study.

Reference and Control

Tetracycline was chosen as the reference compound.

Mueller Hinton Agar (Bacteria)

Mueller Hinton Agar was purchased from Hi Media (HIMEDIA-M173-500 G) to prepare the medium for bacterial culture. 3.8 g of Mueller Hinton Agar was suspended in 100 mL of distilled water in a 250 mL flask, stirred, boiled to dissolve and then autoclaved at 15 lbs and at 121ºC for 15 minutes. The pH range was between 7.0-7.5. The bacterial lawn culture was made using sterile cotton swab and labelled [14].The wells were made in the media with the help of a metallic borer with centres at least 12 mm in diameter. 125 µL, 250 µL and 375 µL concentration of the test sample was introduced in the respective wells. Other wells are supplemented with reference antibacterial drug tetracycline and incubated at 37 ^{0}C for 24 hrs. Activity was determined by measuring the diameter of zones showing complete inhibition (mm) [15]. Growth inhibition was compared with the reference drug.

Thin layer chromatography

Thin layer chromatography (TLC) was carried out on Merck TLC aluminium sheets gel 60 F_{254} (6 x 2 cm) precoated plates using TLC chamber. TLC was developed in toluene: chloroform as solvents. The methanol leaf extract of *A. tamilnadensis* was spotted to 2 mm from the edge of the sheet, immersed in beaker containing solvent and kept for 5 minutes. The chromatogram developed was then observed under UV light at 254 nm. The R_f values were calculated using the following formula:

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Results and Discussion

Qualitative phytochemical analysis

The results of Qualitative phytochemical analysis is given in Table 1

Table 1: Phytochemical constituents of methanolic leaf extract of *Aegle tamilnadensis* Abdul Kader

S. No	Phytoconstituents	Chemical reagent	Result
1	Alkaloids	Dragendroff's reagent	+
2	Terpenoids	$CHCl_3$ + conc. H_2SO_4	+
3	Flavanoids	NaOH solution	+
4	Phenols	$FeCl_3$ solution	+
5	Steroids	Acetic anhydride + conc. H_2SO_4	+
6	Glycosides	5% NaOH + Fehling's solution	+
7	Carbohydrates	-Naphthol + conc. H_2SO_4	+
8	Saponins	Foam test	+

Quantitative phytochemical estimations

The results of quantitative phytochemical analysis are given in Table 2. The total phenolic content of methanol extract of leaves of *A. tamilnadensis* was 141.1 mg/g of gallic acid equivalent. Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers [17]. The flavonoid content of methanolic leaf extract of *A. tamilnadensis* was determined by aluminium chloride method and was 4.33 mg/g of quercetin equivalent. Flavonoids demonstrate a wide range of biochemical and pharmacological effects including anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic action, and anti-allergic effects. They can inhibit enzymes such as prostaglandin synthase, lypoxygenase, and cyclooxygenase, closely related to tumorigenesis, and induce detoxifying enzyme systems such as glutathione S-transferase [18].

Table 2: Phenolic and flavanoids content in methanolic leaf of Aegle tamilnadensis Abdul kader

S. No	Components	Quantity (mg/g of extract)
1	Phenols	211.0
2	Flavonoids	52.91

In vitro antioxidant activity

DPPH radical scavenging activity

The DPPH radical scavenging activity of methanolic leaf extract of *A. tamilnadensis* was steadily increased as the concentration of the extract increased and was given in Table 3. 81.48 % at 120 µg/mL concentrion.

Table 3: DPPH radical scavenging activity of methanolic leaf extract of *A. tamilnadensis* Abdul Kader

S. No.	Concentration (µg/mL)	% of inhibition
1	20	2.08±0.14
2	40	25.19±1.76
3	60	40.30±2.82
4	80	51.40±3.59
5	100	64.15±4.49
6	120	81.48±5.70

Hydroxyl (OH•) and Nitric oxide (NO·) radical scavenging activity

The hydroxyl radical and nitric oxide radical scavenging activity of methanolic leaf extract of *A. tamilnadensis* were given in Table 4. The hydroxyl radical and nitric oxide radical scavenging activity of methanolic leaf extract were directly proportional to the concentration of the extracts.The maximum radical scavenging activity was observed at 30 µg/mL concentration.

Table 4: Hydroxyl (OH•) and Nitric oxide (NO·) radical scavenging activities of methanolic leaf extract of *A. tamilnadensis* Abdul Kader

S. No.	Concentration (µg/mL)	% of inhibition	
		OH• radical	NO• radical
1	5	14.75 ± 1.03	16.05 ± 1.12
2	10	18.85 ± 1.31	25.93 ± 1.81
3	15	28.68 ± 2.00	39.01 ± 2.73
4	20	36.89 ± 2.58	46.67 ± 3.26
5	25	42.62 ± 2.98	53.09 ± 3.71
6	30	61.48 ± 4.30	71.60 ± 5.01

Ferric (Fe^{3+}) reducing power activity

The ferric reducing powder and phosphomoloybdenum reduction activity were given in Table 5. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain. Increasing absorbance at 700 nm indicates an increase in reducing ability. The maximum absorbance of 0.52 was observed at 120 µg/mL concentration for the reduction of Fe^{3+} to Fe^{2+} by the methanolic leaf extract of *A. tamilnadensis.*

Table 5: Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction activities of methanolic leaf extract of *A. tamilnadensis* Abdul Kader

S. No.	Concentration (µg/mL)	Absorbance	
		Fe^{3+} reducing power	Phosphomolybdenum reduction
1	20	0.07±0.00	0.014±0.00
2	40	0.16±0.01	0.034±0.00
3	60	0.25±0.01	0.070±0.00
4	80	0.33±0.02	0.099±0.00
5	100	0.43±0.02	0.185±0.01
6	120	0.52±0.03	0.219±0.01

Phosphomolybdenum reduction activity

Increase in absorbance was observed in both standard and leaf extract. The maximum absorbance of 0.219 was observed at 120 µg/mL concentration for the reduction of MO (VI)-MO (V) by the methanolic leaf extract of *A. tamilnadensis.*

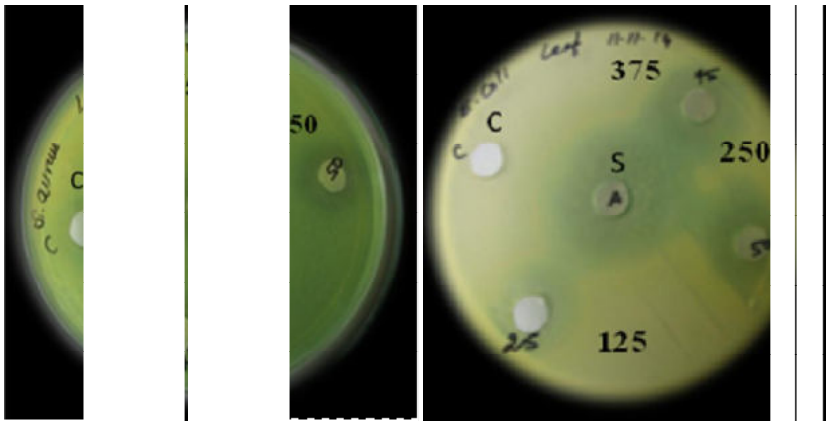
Antimicrobial activity

Antimicrobial Activity of methanolic leaf extract of *A. tamilnadensis*

The methanolic leaf extract of *A. tamilnadensis* showed maximum zone of inhibition against *Staphylococcus aureus* and *Escherichia coli* (Table 6). The activity may be due to the sensitivity of the test compounds is associated with the different cell wall structures of bacteria, while the cell walls of bacteria contain murein.

Table 6: Antimicrobial activity of methanolic leaf extract of *A. tamilnadensis* Abdul Kader

Species	Zone of inhibition (mm)			
	125 µg/mL	250 µg/mL	375 µg/mL	Standard µg/mL
Staphylococcus aureus	4mm	5mm	6mm	6mm
Escherichia coli	6mm	7mm	10mm	10mm



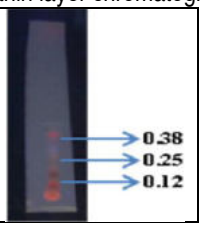
Staphylococcus aureus Escherichia coli

Thin layer chromatography (TLC)

TLC analysis was carried out for methanolic leaf extract of *A. tamilnadensis* by using toluene: chloroform (1:1 ratio) as solvent mixture. The separated bands were visualized by UV light at 254 nm. The R_f values of the separated compounds were measured and given in Table 7

Table 7: R_f values of compounds from methanolic leaf extract of *A. tamilnadensis* Abdul Kader separated by thin layer chromatography

Spots observed	R_f value (UV 254 nm)
1	0.12
2	0.25
3	0.38



Conclusion

The results of the present work indicated that the methanolic leaf extract of *A. tamilnadensis* is a potential source of natural antioxidants and could dose-dependently and significantly inhibit free radicals. The difference in the antioxidant activity may be ascribed to their different group of phenolic and flavonoids compounds. The methanolic leaf extract of *A. tamilnadensis* showed significant phenolic content contributes to the good antioxidant activity. Based on the results obtained, we conclude that *A.tamilnadensis* contains essential phytochemical constituents required for active antioxidant property. Further the antimicrobial activity of the methanolic leaf extract is effective against the bacteria Escherichia coli. Further investigations on the isolation of the active components of the extract will throw more information on the mechanism of action.

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