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Estimation of phenolic and flavonoid content and *in vitro* antioxidant and antibacterial activity of *Memecylon umbellatum* Burm

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Abstract
Infectious diseases are the striking causes of about 50% mortality worldwide. Phytochemical constituents of the plant, particularly secondary metabolites, play major role in human defense against infections. In the current study the leaves of *Memecylon umbellatum* Burm *F*, one of the medicinally important plants, was analyzed for its antibacterial, phytochemical and antioxidant properties. Among the six bacterial strains tested, the methanol extract of the leaves exhibited high antibacterial activity against *Bacillus* sp. and *Vibrio* sp. with zones of inhibition of 17mm and 16mm respectively. The phytochemical analysis of the plant leaf extract obtained by employing several solvents showed the presence of tannins, coumarin, sterols, flavonoids, alkaloids, quinones, terpenes and proteins. Further their total phenol content was high in methanol extract which was estimated to be 79.05 mg/ml and flavonoid as 173.3 mg/ml. They also act as a potent free radical scavenger and Nitric Oxide scavenger with an IC_{50} values of 45.53 µg/ml and 55.66 µg/ml. This implies that *Memecylon umbellatum* Burm *F* has potent antibacterial and antioxidant properties with high levels of phytochemicals packed. Thus the current findings may remain as a pre-requisite for the development of plant-based herbal formulations with enhanced efficacy against several deadly diseases.

Keywords: *Memecylon umbellatum*, Gudiyam forest, antibacterial, phytochemical, antioxidants

Introduction
Bacterial infections are one of the notable causes of major mortality in humans. Particularly infectious diseases exhibited a noteworthy impact in children and young adults. Infectious diseases nowadays are life threatening due to the antibiotic resistance properties of bacteria, emergence of new disease and lack of vaccines for emerging diseases, bacterial mutations, nosocomial infections [1], international travels and migration of mass population [2]. Human body has its own defense mechanisms to combat these microbes. Several drugs are used to enhance this defense mechanism. With the use of commercial antibiotics the microorganisms became resistant to most of the commonly available antibiotics. Exhaustion of beneficial microorganisms in the body, allergic reactions, hypersensitivity is some of the aftereffects of the antibiotic usage [3]. Thus one of the alternative and safest methods is to use herbs as the antibacterial drugs.

With the increasing chronic illness and soaring healthcare costs, traditional medicine system remains as a boon to the patients and healthcare providers. Use of medicinal plants as drugs for various illnesses has been reported previously. A study by WHO reported that almost all parts of the world now rely on traditional medicine system and other countries have been developing in the herbal usage [4]. Many plants and their extracts are being used time immemorial for treating many common ailments including cold, cough, fever, skin diseases, respiratory ailments and also in the treatment of conjunctivitis, malaria, cancer, gonorrhea and several infectious diseases [5].

Free radicals such as superoxide anion, hydroxyl radicals and non-free radicals such as hydrogen peroxide are some of the forms of highly reactive activated oxygen, often termed as reactive oxygen species (ROS). These free radicals are responsible for various deleterious biochemical reactions such as protein oxidation, lipid peroxidation, oxidative DNA damage and so on which eventually end up in cellular damage and cell death [6]. Phytochemicals such as phenols, flavonoids, terpenoids, steroids, tannins, saponins, cardiac glycosides are secondary metabolites protective to humans and plants as antioxidants naturally. Thus the plants and herbs are heeded now-a-days for the use of these phytochemicals against the infectious diseases and other ailments [7].

Memecylon umbellatum Burm *F* (commonly called Iron Wood tree), is an endangered species found predominantly in peninsular India and Andaman islands.

The leaves and shrubs of this plant are widely used in the treatment fever, eye infections, bruises, inflammation, gonorrhea, leucorrhoea, liver injury, cancer, arthritis, ulcer and other viral infections. The biologically active compounds present in this plant includes ursolic acid, umbelactone, oleanoic acid, tartaric acid, maleic acid and many more. These compounds are reported to be responsible for their various pharmacological properties [8, 9].

Gudiyum forest is present in west part of Jaganathapuram village-Nagari District (AP), East part of Uttukottai-Tiruvallur District (TN), North part of Nagalapuram Village – Nagari District (AP) and South part of Poondi Village – Tiruvallur District (TN). It is located in the 13.28790 latitude and 79.80867 Longitude with an area covered with 148 Acre of Tiruvallur District in Tamil Nadu. The Climate of the District is on the whole dry, except during the North- East monsoon season. The average annual rainfall in the Gudiyum forests is 1104 mm and in summer, maximum temperature is 21.5ºC to 37.5ºC. The exploration in this region is outlined to 60 different species with medicinal importance belonging to 35 families. The most common represented families are Euphorbiaceae, Lamiaceae, Mimosaceae, etc [10]. The compulsive use of traditional use of herbs in the gudiyum forest reflects the revitalizing interest in the use of traditional medicine. The present study is designed to investigate the phytochemical constituent, antibacterial and antioxidant activity of *Memecylon umbellatum* Burm in Gudiyum forest, India.

Materials and Methods

Collection and authentication of plant sample

Leaf samples of *Memecylon umbellatum* were collected from Gudiyam forest, Tiruvallur district, Tamil Nadu, India. The authenticity of the collected plant samples were identified by Raw Material Herbarium and Museum (reference number: NISCAIR/RHMD/Consult/2016/3003-30), New Delhi, India.

Preparation of plant leaf extract

The collected plant leaves were shade dried and powdered using 50g of the powdered plant leaves were extracted in soxhlet apparatus with Ethanol, Methanol, Ethyl acetate, Benzene and Chloroform sequentially. It was extracted with solvent for 6 hours by soxhelation. The obtained extracts were evaporated at room temperature to set a crude dried extract. The weight of the concentrated yield was determined and stored in air tight container until further use to prevent the loss of biological activity [11].

Assessment of Antibacterial activity of leaf extracts

The antibacterial activity of the plant was assessed with the stored leaf sample by agar well diffusion method [12]. The antibacterial efficacy of the leaf extracts were screened against 6 human pathogenic bacterial strains *viz., E. coli, Pseudomonas, Klebsiella, Vibrio, Bacillus* and *Staphylococcus* species. 10ml of the 18 to 24 hours bacterial suspension culture was added to the nutrient agar well of 6 mm. Kanamycin was used as the positive control. The plates were incubated at 37º C overnight. The result was determined by measuring the zones of inhibition.

Qualitative Phytochemical analysis of leaf extract

All leaf extracts were subjected to qualitative phytochemical analysis to detect the presence of various phyto constituents viz., Carbohydrate, Amino acid, protein, Vitamin-C, iron, Tannis, Phenol, Flavonoid, Alkaloids and Steroids by following standard phytochemical tests methods [13].

Determination of total phenolic and total flavonoid contents

Total phenolic content of methanolic leaves extract was analysed using the Folin – Ciocalteu colorimetric method [14] and total flavonoid content was determined using the Aluminium chloride colorimetric method [15].

Determination of antioxidant activity

DPPH radical scavenging activity

The free radical scavenging capacity of the aqueous and ethanol extracts of *M. umbellatum* was determined using DPPH method [16]. DPPH solution (0.0045w/v) was prepared in 95% methanol. The concentration of the extract solution was 10mg/ml. From stock solution 0.2-1.0 ml of solution were taken in five test tubes and serially diluted to concentrations such as 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml respectively. Freshly prepared DPPH solution was incubated with test drug and the absorbance was read spectrophotometrically at 517 nm after 10 min. of incubation at room temperature. Ascorbic acid was used as the standard reference and dissolved in distilled water to make the stock solution of the same concentration as the test (10mg/100ml or 100µg/ml).

Percentage of scavenging of the DPPH free radical was measured using the following equation.

$$\text{DPPH scavenging activity (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

($A_{control}$ - absorbance of the control reaction, A_{test} - absorbance of the test extracts).

The antioxidant activity of the leaf extract was expressed as IC_{50} and compared with activity of the standard. The IC_{50} value is defined as the concentration of extract that inhibited the formation of DPPH radicals by 50%.

Reducing power assay

The reduction potential of the plant leaf extracts were assessed by reduction power assay [17]. Substances which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. 1ml of methanolic plant extract solution was mixed with 2.5ml phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferricyanide [$K_3Fe(CN_6)$] (10g/l). The mixture was incubated at 50˚C for 20 min. To this 2.5ml of Trichloroacetic acid (100g/l) was added and centrifuged at 3000 rpm for 10 min. Finally, 2.5ml of the supernatant solution was mixed with 2.5ml of distilled water and 0.5ml $FeCl_3$ (1g/l) and the absorbance was measured at 700 nm in UV-Visible spectrophotometer. Ascorbic acid was used as standard and phosphate buffer as a blank solution. Increased absorbance of the reaction indicates stronger reducing power.

$$\text{\% increase in reducing power} = \frac{A_{test}}{A_{Blank}} \times 100$$

(A_{test} - absorbance of test solution; A_{blank} - absorbance of blank)

The antioxidant activity of the extract was expressed as IC_{50} and compared with activity of the standard.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of the plant extract was assessed by measuring the amount of nitric oxide, generated from sodium nitroprusside (SNP), scavenged [18]. The reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25˚C for 180 min. in front of a visible polychromatic light source (25W tungsten lamp). The nitric oxide radical thus generated interact with oxygen to produce the nitrite ion (NO) which was assayed at 30 minutes interval by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene-diaminedihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylene-diaminedihydrochloride was measured at 546 nm and referred to the absorbance of ascorbic acid, used as a positive control treated in the same way with Griess reagent.

$$\text{Nitric Oxide scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

($A_{control}$ - absorbance of control reaction; A_{test} - absorbance in the presence of the samples of extracts)

Statistical analysis

Each experiment was performed in triplicates. The mean difference in the levels of various biochemical parameters between the test samples and standard were tested for statistical significance using Students t-test. The results are expressed as mean + SD (Standard Deviation).

Results and Discussion

Assessment of Anti-Bacterial activity of leaf extracts

To assess the antibacterial activity of *Memecylon umbellatum* leaf extracts two gram positive bacterial pathogens *Bacillus* and *Staphylococcus aureus* and four gram negative bacterial pathogens *E.coli, Klebsiella, Vibrio* and *Pseudomonas* were selected. The antibacterial activity was found to be both solvent and strain specific. Methanol extracts of *M. umbellatum* showed highest inhibitory activity against all the six bacterial strains tested when compared to other extracts *viz*., ethanol, ethyl acetate, benzene, chloroform and aqueous extracts. Of all the solvents tested, highest zone of inhibition (17 mm) was observed with methanol extract of the test sample against *Bacillus* while the lowest zone of inhibition (10 mm) was observed with ethanol extract of the test sample against the bacterium *Pseudomonas aeruginosa* (Table 1). Benzene extract exhibited activity only against *Vibrio* and Chloroform extract exhibited activity against *Vibrio* and *Pseudomonas aeruginosa*. But ethyl acetate and aqueous extracts showed no activity against any of the tested bacterial strains which exhibited a high resistance to these extracts. Earlier studies [19] also reported that methanol extract of *Memecylon* sp. leaves has shown significant activity against both gram (+) and gram (-) bacteria and fungi. These results infer that methanolic leaf extract of *Memecylon umbellatum* exhibited a broad spectrum of inhibition activity against the pathogens. Of all the six strains tested, *Staphylococcus aureus* was highly resistant to the plant leaf extract.

Table 1: Antibacterial activity of various extracts on six different bacterial strains

Different Extracts	*E.coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Vibrio sp*	*Bacillus sp*	*Staphylococcus aureus*
Ethanol	-	10 mm	-	-	12 mm	-
Methanol	15 mm	13 mm	14 mm	16mm	17 mm	11 mm
Ethyl Acetate	-	-	-	-	-	-
Benzene	-	-	-	13 mm	-	-
Chloroform	-	16 mm	-	13 mm	-	-
Aqueous	-	-	-	-	-	-

'-'indicates no activity

Qualitative phytochemical analysis of leaf extract

The solvents of varying polarities were used to extract the active components from *M. umbellatum* plant leaves. The Phytochemical analysis of leaf powder of *Memecylon umbellatum* with various chemical reagents indicated the presence of various class of molecules in different extracts. From the data obtained it is observed that the *Memecylon umbellatum* leaf powder showed the presence of tannins, coumarin, sterols, flavonoids, alkaloids, quinones, terpenes and proteins. Terpenes were present in hexane, chloroform and ethanol extracts. Whereas, coumarins were present in all extracts except hexane; sterols were present in all extracts except water; tannins were present in all extracts except chloroform extract; flavonoids, alkaloids and proteins were present in ethyl acetate, ethanol and water extract; quinones were present in hexane, ethanol and water extract [5]. Reported that methanolic extract showed the significant presence of diverse class of molecules including terpenoids, flavonoids and tannins and moderate amount of phenols and glycosides, whereas chloroform extract possessed a good amount of flavonoids and steroids. The petroleum ether extract showed the presence of smaller amount of steroids and flavonoids.

Table 2: Phytochemical analysis of various solvent extracts to screen the presence of phytochemicals

Phytochemicals	Ethanol	Methanol	Ethyl Acetate	Benzene	Chloroform	Aquaous
Carbohydrate	-	+	-	-	-	-
Amino Acid	-	-	-	-	-	-
Protein	-	-	-	-	-	-
Vitamin-C	-	-	-	-	-	-
Iron	-	-	-	-	-	-
Tannis	+	+	-	-	-	-

Phenol	+	+	-	-	-	-
Flavonoid	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Steroids	+	+	+	+	+	+

'+' indicates presence of the compound; '-'indicates absence of the compound

Determination of total phenolic content

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: y = 7.026x – 0.0191, r2 = 0.999). The total phenolic content in plant leaf extracts of *Memecylon umbellatum* was 79.05 mg/l. The total phenolic contents in the examined extracts ranged from 1.25 to 640 mg /ml with the absorbance ranging from 0.59 to 1.91. The highest concentration of phenols was measured in methanolic extracts.

Determination of total flavonoid content

The concentration of flavonoids in various plant extracts of the species *Memecylon umbellatum* was determined using colorimetric method with aluminum chloride. The content of flavonoids was expressed in terms of rutin equivalent (the standard curve equation: y = 17.231x – 0.0591, r2 = 0.999), mg of RU/g of extract. The concentration of flavonoids in plant extracts from *Memecylon umbellatum* was 173.3 mg/l. A similar study [20] reported that methanolic extracts of *Saussurea medusa*, a traditional medicinal plant, contains the highest flavonoid concentration. The lowest flavonoid concentration was measured in petroleum ether and water extract. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation.

DPPH radical scavenging activity

The activated and highly reactive free radicals cause several infectious diseases, autoimmune disorders, inflammatory diseases and also result in various biochemical reactions causing damage to cell and ends up in cell death [21]. Thus the DPPH free radical scavenging property of the different solvent extracts of *Memecylon umbellatum* was determined by the method of [16] by incubating the test samples with a stable free radical, DPPH. The free radical scavenging activity was visibly observed by the change of colour from blue to yellow. The IC_{50} value of the test and standard samples were calculated using the formula and it was found to be 45.53 µg/ml as the required concentration of extracts required to scavenge 50% free radicals**.** But IC_{50} of the methanol extract of the test sample was found to be higher than the IC_{50} of the ascorbic acid (33.28 µg/ml) which is used as the standard (Table 3). 22. Jamuna *et al*. [22] reported a similar significant value in the leaf extract of *Hypochaeris radicata* of ~90% inhibition which was high compared with the standard. These *in vitro* free radical scavenging test results infer that the phytochemicals present in the leaf of *Memecylon* possess a high degree of free radical scavenging property.

Table 3: Free radical scavenging activity of methanolic extract of *Memecylon umbellatum*

Concentration	Methanol Extract		Standard*	
	% of Inhibition	IC_{50} Value µg/ml	% of Inhibition	IC_{50} Value µg/ml
20	30.64±1.8	45.53	39.92±1.6	33.28
40	45.88±1.5		50.40±1.1	
60	53.68±1.9		63.31±3.9	
80	59.67±1.3		72.19±1.7	
100	80.08±2.3		81..98±1.3	

*-DPPH

Reducing power assay:

All the concentrations of methanol extracts of *Memecylon umbellatum* showed significantly higher reducing power activities when compared to standard ascorbic acid. An IC50 of 42.23 µg/ml was identified as the required concentration of the test sample to quench the ferric radicals in the reaction mixture which was 37.2 µg/ml for the standard (Table 4). The phytochemicals present in the ethanolic extract of leaves and seeds of the plant *Rumex crispus*, which are used in Turkish medicine, exhibited enhanced reducing power which is shown by high level of absorbance [17]. This deduces that like free radical scavenging activity, the methanolic extracts of the plant leaf have high reducing power potential.

Table 4: Reducing power efficiency of methanolic extract of *Memecylon umbellatum*

Concentration	Methanol Extract		Standard*	
	% of Inhibition	IC_{50} Value µg/ml	% of Inhibition	IC_{50} Value µg/ml
20	26.36±2.4	42.23	30.71±1.6	37.2
40	42.17±1.0		43.17±1.6	
60	64.55±3.4		71.63±2.0	
80	74.18±3.3		86.37±0.8	
100	87.86±2.6		93.06±2.1	

***-** Ascorbic acid

Nitric oxide scavenging activity

The results of NO scavenging activity of the selected plant extracts are shown as percent of NO scavenged. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with super oxides, such as NO_2, N_2O_4, N_3O_4, NO_3 and NO_2 are very reactive. These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 250° C for 2 h resulted in linear time-dependent nitrite production, which is

reduced by increasing concentrations of the methanolic extracts of *Memecylon umbellatum* leaves. It showed 24.69%, 32.32%, 50.19%, 63.04% and 75.47% of inhibition with an IC_{50} value of 55.66 µg/ml compared to standard, ascorbic acid (45.7 µg/ml) respectively (Table 5). An enhanced nitric oxide scavenging ability of *Memecylon* from tropical dry evergreen forest has been reported [23]. This may be due to the antioxidant compounds in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The nitrogen scavenging ability was comparatively higher than the free radical scavenging and reducing potential of the methanolic leaf extract of *Memecylon umbellatum* which is depicted in the Fig 1.

Table 5: Nitric Oxide scavenging efficiency of methanolic extract of *Memecylon umbellatum*

Concentration	Methanol Extract		Standard*	
	% of Inhibition	IC_{50} Value µg/ml	% of Inhibition	IC_{50} Value µg/ml
20	24.69±2.6	55.66	25.52±0.9	45.7
40	32.32±0.8		42.76±1.0	
60	50.19±1.1		56.73±1.5	
80	63.04±1.3		69.89±2.5	
100	75.47±1.7		81.29±0.2	

* - ascorbic acid

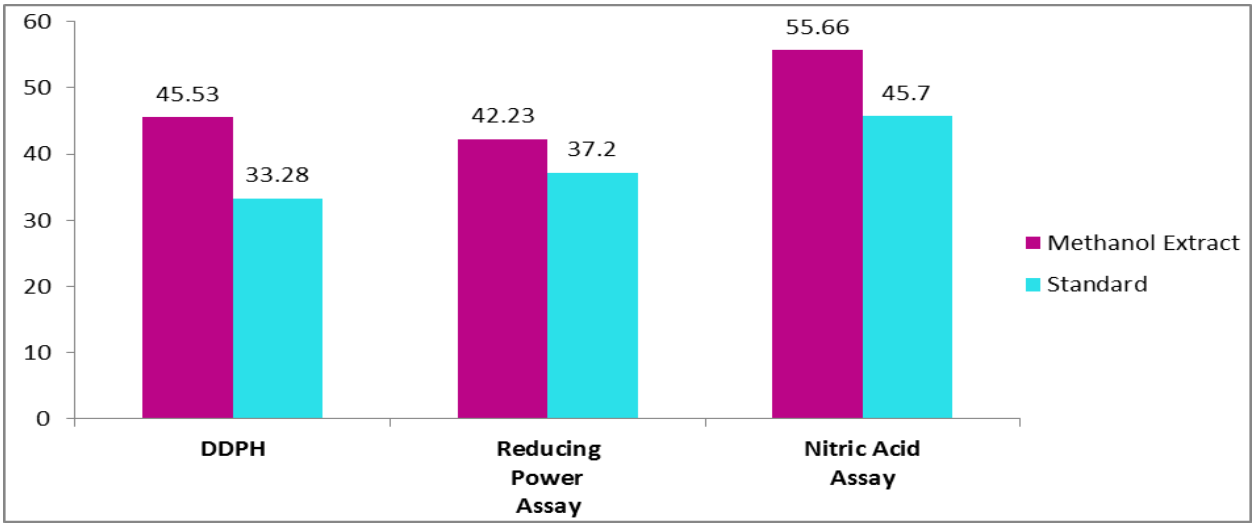

Fig 1: Comparative analysis of free radical (DPPH) scavenging activity, reducing potential and Nitric oxide scavenging activity of methanolic leaf extract of *Memecylon umbellatum* compared to their respective standards

Conclusion

The findings of the present work demonstrate various pharmacological effects of the leaf of *Memecylon umbellatum*. The bark and leaves of this plant are widely used for several diseases currently. The extracts of their leaf exhibits antibacterial effects against six tested bacterial strains. This suggests that the leaves of *Memecylon umbellatum* can be used effectively against various highly infectious human bacterial diseases. Further the screening of phytochemical constituents in the leaf extract confirms the presence of various phytochemical compounds including phenols and flavonoids, which are known to be responsible for various pharmacological properties of the plant. This was further proved by detecting the antioxidant properties of the plant by performing free radical scavenging assay, reducing power assay and nitric oxide scavenging assay. Though the plant extract shows high degree of free radical scavenging and reducing potential, the nitric oxide scavenging property was found to be in an enhanced level. This may be due to the presence of various bioactive compounds in the plant. Thus it is concluded from the study that *Memecylon umbellatum* has antibacterial property and antioxidant properties which are considered to be important pharmacological property of current health scenario. This investigation can help in further isolation, identification, structure prediction and formulation these bioactive phytochemical compounds to be used as a therapeutic drug.

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