

In vitro Antioxidant, Antibacterial Activity of *C. aromaticus* Essential Oil against Multidrug Resistant (MDR) Urinary Tract Infected Pathogens

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ABSTRACT

The present work is aimed to the extraction of essential oil from *Coleus aromaticus* and evaluation of its antioxidant and antibacterial activity against multidrug resistant urinary tract infected pathogens. Essential oil extracted by steam distillation using Clevenger type apparatus and thus free radical scavenging ability tested with 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and Nitric oxide (NO). The antibacterial activities were conducted against urinary tract infected multi drug resistant (MDR) Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*) and Gram negative pathogens (*Escherichia coli*, *Shigella sonnei*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*) by agar diffusion, growth inhibition analysis through optical density measurement. The free radical scavenging ability of essential oil expressed in IC₅₀ (50% of inhibition) value in this for DPPH the IC₅₀ was 54.23µl/ml, whereas nitric oxide scavenging was 72. 91µl/ml. The antibacterial activity of *C. aromaticus* essential oil is dosage dependent from lower to higher (20-80µl/ml) concentration. Whereas the maximum zone of inhibition was determined in Gram positive pathogens than Gram negative at the higher concentration of 80µl/ml. The highest percentage of growth inhibition was measured in 24hr incubated culture through optical density measurement study. *C.aromaticus* essential oil possessed potent antioxidant and antibacterial activity and this *in vitro* study supports its traditional approach as a preventive therapy for the treatment of microbial diseases and replacement of artificial antioxidants.

Keywords: *Coleus aromaticus*, essential oil, urinary tract infection, zone of inhibition, free radical scavenging

INTRODUCTION

Free radicals and reactive oxygen species formation in living cells play a vital role in the origin of life and biological evaluation^(1,2). However, it has been found that those reactive oxygen species also play a key role in oxidative damage to cellular organelles which leads cell injury and death. This has been associated with pathogenesis of a variety chronic diseases such as cardiac failure, carcinomas and other health problems associated with advancing age^(3,4). Thus, to increase the antioxidant ingestion in the human diet is one important way to minimize such oxidative damage. Many plant species in different locations of the world have been screened for the antioxidant activity. However, most of them are not fit for human consumption. In this case, Lamiaceae species are considered as high importance because of their use in folk medicine, culinary, cosmetics, flavoring and production of essential oils throughout the world. The genus *coleus* comprises around 300 species distributed all over the world is among the major general belonging to Lamiaceae family. *C. aromaticus* (Benth) is a perennial succulent plant, which contains essential nutrients⁽⁵⁾, essential oils, phytochemicals as caryophyllene, patchoulene and flavonoids⁽⁶⁾. Its extracts are widely used in traditional

medicine for asthma, bronchitis, chronic coughs, sores, insect stings, pesticides and fragrance products.

Urinary tract infection (UTI) is the second most common type of infection in the body and it is more common in woman as compared to men. The consumption of antibiotics for human therapy and agricultural caused the emergence of MDR in bacteria, especially in developed countries⁽⁷⁾. It is reported that the worldwide about 150 million people suffer from urinary tract infections per year⁽⁸⁾ and it involves in the infection of kidneys, ureters and urethra caused by bacteria that may affect any part of the urinary tract. In most cases, bacteria travel to the urethra and multiply, causing kidney infection⁽⁹⁾. About 35% of healthy women suffering the symptoms of UTI and about 5% of women suffering each year with the problem of painful urination (dysuria) and frequency⁽¹⁰⁾.

Several potent antibiotics are available for the treatment of UTI, but increasing drug resistance among bacteria has made a difficulty. Bacteria encompass the genetic ability to transmit and gain resistance to drugs⁽¹¹⁾. Plant metabolites are great sources of compounds and serve as a good alternative to combat with the diseases caused by MDR organisms. Since plant secondary metabolites are natural isolates, they are safe to use as therapeutic agents.

Table 1: Antibacterial activity of *C.aromaticus* essential oil against urinary tract infecting multidrug resistant pathogens.

Pathogens	Zone of inhibition in dm			
	20µl	40 µl	60 µl	80 µl
<i>G(+)</i> ve				
<i>B. subtilis</i>	17±0.5	18±1.1	20±0.3	22±1.0
<i>S. aureus</i>	12±1.0	14±0.5	16±0.5	18±0.5
<i>E. faecalis</i>	12±0.5	15±0.7	18±1.0	21±1.2
<i>G(-)</i> ve				
<i>E. coli</i>	10±1.0	11±1.2	11± 0.5	13±1.0
<i>S. Sonnei</i>	11±1.1	12±1.1	18±1.2	20±1.1
<i>S. Sonnei</i>	13±0.5	15±1.0	16±1.0	17±1.3
<i>K.pneumoniae</i>	13±1.2	13±1.3	17±1.0	19±0.5
<i>P.vulgaris</i>	15±0.5	16±1.0	18±0.7	18±0.6

All Values are as Mean ± SEM

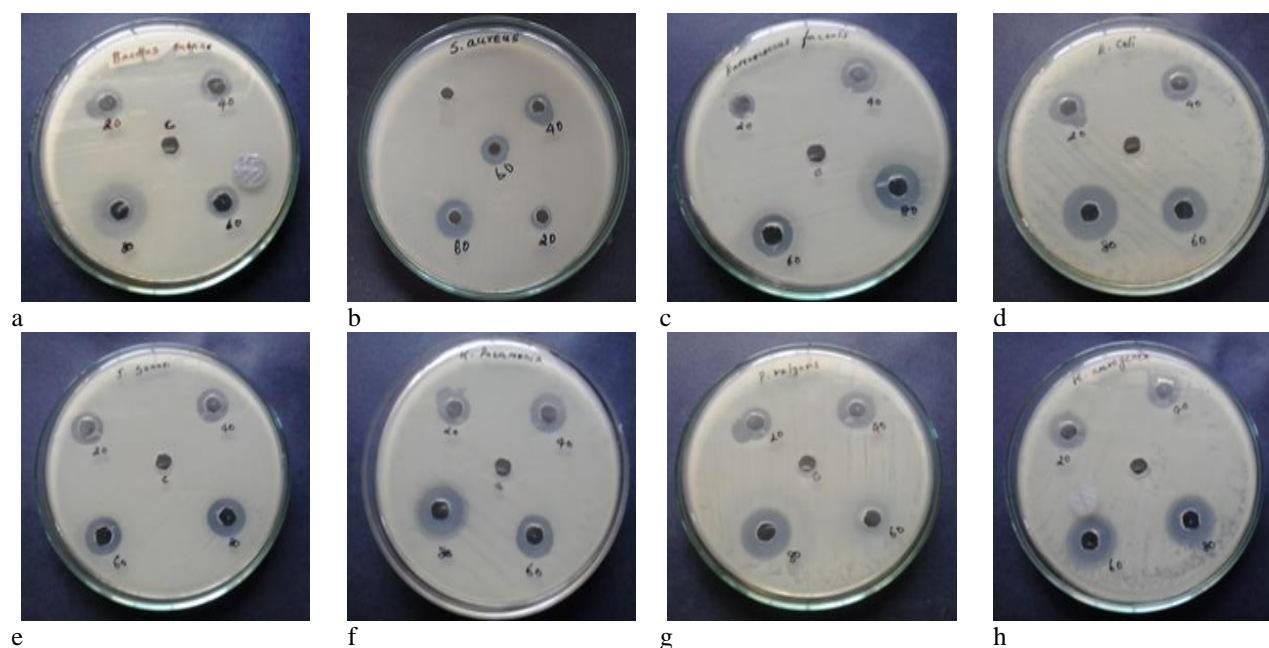


Fig. 2: Inhibition zone of Gram positive: (a) *B. subtilis*, (b) *S. aureus*, (c) *E. faecalis* and Gram negative: (d) *E.coli*, (e) *S.sonnei*, (f) *K.pneumoniae*, (g) *P.vulgaris*, (h) *P.aeruginosa* by agar diffusion method.

Table 2: IC₅₀ values for both Gram positive and negative pathogens.

Pathogens	50% of inhibitory concentration (IC ₅₀)	
	12h	24h
<i>G(+)</i> ve		
<i>B. subtilis</i>	65.62	30.81
<i>S. aureus</i>	52.62	24.72
<i>E. faecalis</i>	59.42	26.57
<i>G(-)</i> ve		
<i>E. coli</i>	122.62	39.90
<i>S. Sonnei</i>	74.58	33.18
<i>S. Sonnei</i>	119.1	30.15
<i>K.pneumoniae</i>	90.09	28.09
<i>P.vulgaris</i>	80.63	30.19
<i>P.aeruginosa</i>		

In India medicinal plant parts and their extracts are in use from very ancient time. World Health Organization (WHO) reported that the majority of Indian populations depend on plants and their extracts for primary health care (12). A number of studies have been conducted on different plant parts to confirm their antimicrobial efficacy (13, 14, 15). Plant extracts are a mixture of many small and large

compounds of different nature, therefore the chance of bacterial resistance is minimized.

Essential Oils (EO) are the volatile liquids of the secondary metabolites of plants and have shown to exhibit antimicrobial properties (16, 17). It is most likely reported that the antimicrobial effects associated with EOs are governed by a multitude of antimicrobial mechanisms (18, 19). Fresh leaves of *C. aromaticus* were also found to be active against UTI causing bacteria. Present investigation focuses on antioxidant activity and antibacterial activity of *C. aromaticus* essential oil against multidrug resistant urinary tract infecting Gram positive (*B.subtilis*, *S.aureus*, *E. faecalis*) and Gram negative (*E.coli*, *S.sonnei*, *P.aeruginosa*, *K.pneumoniae* and *P.vulgaris*) pathogens.

MATERIALS AND METHODS

Plant material and essential oil isolation: The leaves of *C. aromaticus* were collected from the garden of Periyar university, Salem, Tamil Nadu during the vegetative stage of the plant. The samples were dried in the shade away from light at room temperature. After drying, the sample was ground to a fine powder and used for the isolation of essential oil. The dried leaves were subjected to steam

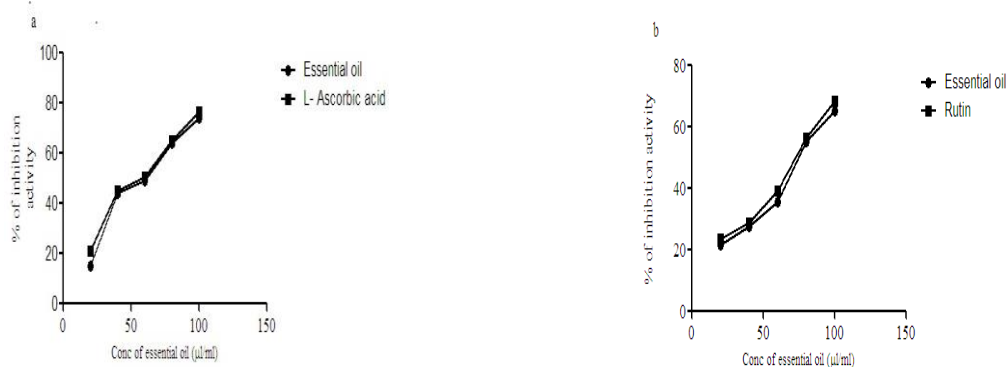


Fig. 1: Free radical scavenging activity: (a) DPPH radical scavenging activity, (b) Nitric oxide scavenging activity.

distillation using a Clevenger type apparatus. Briefly, the powdered leaves completely immersed in water and heated to boiling, after which the essential oil was evaporated together with water vapor and finally collected after decantation. The distillate was isolated and dried in a Rota vapor to giving brownish yellow oil. The oil was stored at 4°C until the completion of assays.

Antioxidant activities

DPPH radical-scavenging assay: The scavenging effect of DPPH radical was determined by the spectrophotometric method. 3ml of 0.1mM ethanol dissolved DPPH was added with different concentrations (20, 40, 60, 80 and 100µl/ml) of essential oil. Equal volume of DPPH alone used as blank (Control). The mixture was shaken vigorously and left to stand for 30 min in the dark room temperature. The amount of DPPH remaining after each time period was measured at 517nm and all the measurement was performed in triplicate. L-ascorbic acid was taken as standard. The radical scavenging activity was calculated as % of inhibition from the following equation:

$$\% \text{ of inhibition} = ((OD_{\text{control}} - OD_{\text{sample}}) / OD_{\text{control}}) \times 100$$

Nitric oxide radical scavenging assay: Sodium nitroprusside generated nitric oxide was measured by Griess reaction. 5.5 mM sodium nitroprusside in standard phosphate buffer solution was incubated with different concentrations (20, 40, 60, 80 and 100 µl/ml) diluted essential oil with phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the essential oil, but with equivalent amounts of buffer were conducted in an identical manner. After 5 hours, 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthalene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylene diamine was read at 546 nm. Rutin was taken as standard.

Antimicrobial activity study

Microorganisms: The selected UT infected Gram positive *B. subtilis*, *S. aureus*, *E. faecalis* and Gram negative *E. coli*, *S. sonnei*, *P. aeruginosa*, *K. pneumoniae* and *P. vulgaris* were collected from clinical laboratory. The collected cultures were cultured overnight in Luria broth (LB) to reach the stationary phase of growth.

Agar diffusion method: The agar diffusion method described by Mitscher⁽²⁰⁾ was followed for the evaluation of antimicrobial activity of *C. aromaticus* extracted essential oil. Extracted oil from the leaves was evaluated at the concentrations of 20, 40, 60 and 80 µl/ml in dimethyl sulfoxide (DMSO) by dilution. Inoculums for the screening assay were prepared by growing overnight cultures of bacteria in Luria broth. The antimicrobial activity of the essential oil was evaluated using the standardized agar diffusion method. Briefly, 100 ml of a suspension containing approximately 107 colony-forming units (CFU)/ml were spread on nutrient agar. The diluted essential oil (20, 40, 60 and 80 µl/ml) was placed on agar plates containing well and DMSO considered as control, the diameter of inhibition zones (DIZ) was measured after 24h of incubation at 37°C and the tests were performed in triplicate.

Growth inhibition measurement by optical density: Increasing volumes (20 to 80µl) of essential oil were added in the liquid culture medium LB study the sensitivity of the microbial strain with respect to the increasing concentrations of oil. Into tubes containing 3ml liquid medium, various quantities of essential oil (20, 40, 60 and 80µl) were introduced under sterile condition, after a good agitation, into each tube we introduced 107 CFU of a pure culture. Incubation was carried out at 37°C up to 24 hours. After incubation, measurements of growth density in each tube were carried out twice (12th hr and 24th hr) against a blank (DMSO). The reading was made spectrophotometer at 625 nm. The tube without turbidity was determined as the Minimum inhibitory concentration (MIC) value and the tests were performed in triplicate. The percentage of microbial growth inhibition for each concentration was measured by the following formula:

$$\text{Inhibition\%} = (A_{\text{control}} - A_{\text{test}}) / A_{\text{control}} \times 100$$

RESULTS AND DISCUSSION

DPPH radical scavenging assay: In DPPH radical scavenging assay employing the stable 2, 2- diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention paid to its ease of use and convenience. The results are expressed as percentage (%) of inhibition exhibited by the test substances and the standard drug. The essential oil of *C. aromaticus* exhibited DPPH scavenging activity in the tested concentrations. It was observed that,

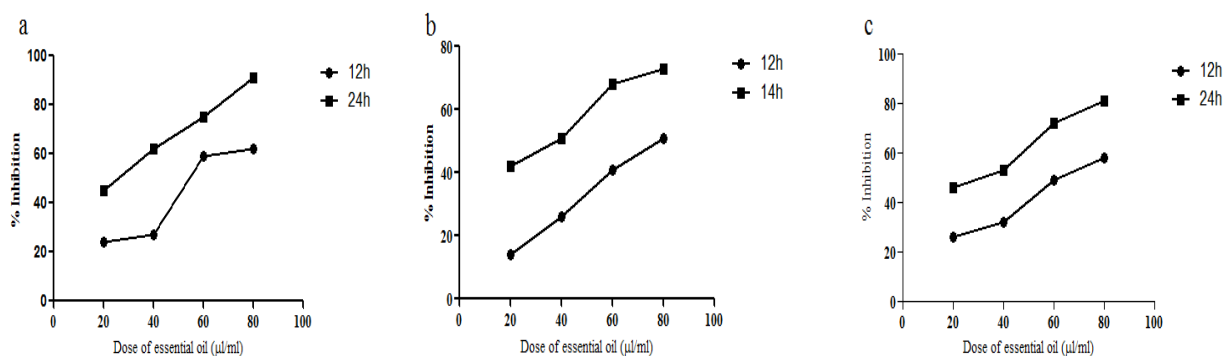


Fig. 3: Growth inhibition percentage of Gram positive strains at 12th and 24th hr. (a) *B.subtilis* (b) *S.aureus* (c) *E.fecalis*.

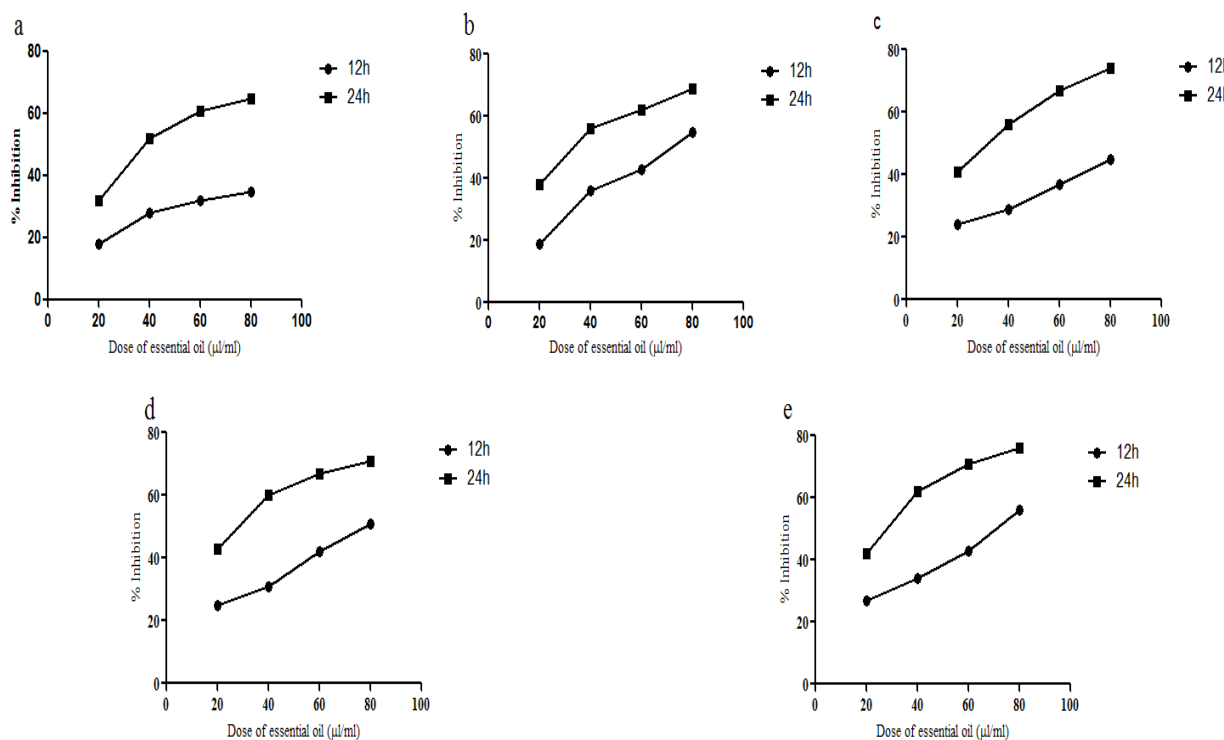


Fig. 4: Growth inhibition percentage of Gram negative strains (a) *E.coli* (b) *S.sonnei* (c) *K.pneumoniae* (d) *P.vulgaris* (e) *P.aeruginosa*.

the percentage inhibition was increased with the increase in concentration (Fig. 1a). IC₅₀ values for scavenging of DPPH by the essential oil of *C. aromaticus* was found to be 54.23µl/ml, while for Ascorbic acid it was 50.44 µl/ml. Nitric oxide scavenging assay: Nitric oxide is a free radical and an effector molecule in biological system as neuronal messenger, vasodilation and antimicrobial antitumor activities. NO generated from sodium nitroprusside at physiological pH reacts with oxygen to form nitrite ion. Essential oil plant inhibited nitrite formation in a concentration dependent manner. This may be due to the presence of antioxidant principles in the oil, which complete with oxygen to react with nitric oxide. The results are expressed as a percentage (%) inhibition exhibited by the test substances and the standard drug. The essential oil of *C. aromaticus* exhibited nitric oxide scavenging activity in the tested concentrations. It was observed that, the percentage inhibition was increased with the increase in concentration of the extracts (Fig.1b). IC₅₀

value for scavenging of Nitric oxide by the essential oil of *C. aromaticus* was found to be 72.91µl/ml and 67.66µl/ml for rutin. Nitric oxide is a free radical generated by endothelial cells, macrophage, neurons etc., and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals.

Antibacterial activity

Agar diffusion method: The screening of the antimicrobial activities of *C.aromaticus* essential oil against urinary tract infected gram positive and negative bacterial species were evaluated by the diameter of inhibition, the results are summarized in Table 1 and Fig.2. The results showed activities of the tested oil against all microorganisms. However, variations in the antimicrobial properties of plant essential oil according to the microorganisms were observed. The activity of oil 20µl amount was not

significantly different against all the bacteria. However, the higher amounts of the oil became more injuries to all Gram positive, negative bacterial strains. The maximum zones of inhibition were found in 80 µg /ml used oils. Comparatively, the highest inhibition zone diameter was observed in Gram positive strains *B. subtilis* (22mm), *S. aureus* (18mm), *E. faecalis* (21mm) than Gram negative pathogens *E.coli* (13 mm), *S.sonnei* (20mm), *K.pneumoniae* (17mm), *P.aeruginosa* (18mm) and *P.vulgaris* (19mm).

Growth inhibition measurement by optical density: Another complementary antimicrobial test was carried out by monitoring the different concentration of oil on the growth density of the micro-organism population. Measurements of microbial density read at 625 nm were transformed and expressed as a percentage of microbial population inhibition. Each strain was tested with 2ml of liquid broth contains 20, 40, 60 and 80µl of *C.aromaticus* essential oil individually at 12th and 24th hr of incubation time. The higher percentage of growth inhibition and quantity required for 50% of inhibition (IC₅₀ value) were recorded in 24 hr incubated Gram positive *B. subtilis* (92%), *E. faecalis* (81%) and *S. aureus* (72%) (Fig 3, Table 2). Comparatively the lower percentage of inhibition was recorded in Gram negative strain *P.aeruginosa*, *K.pneumoniae* (71%) following *S.sonnei* (69%), *P.vulgaris* (67%) and *E.coli* (65%) (Fig.4). The antibacterial properties of essential oils are associated with their lipophilic character and functional group ⁽²¹⁾. Volatile compounds interact with the phospholipid bilayer, including electron transport, ion gradients, protein translocation, phosphorylation and other enzyme dependent reactions ⁽¹⁶⁾ which result in the increase of bacterial cell membrane permeability ⁽²²⁾. It is well known that Gram negative bacteria are more resistant to essential oil compound antibacterial properties than Gram positive bacteria because they have an external lipopolysaccharide wall surrounding the peptidoglycan cell wall, which limits access of these compounds. In our results *C.aromaticus* essential oil had shown better antibacterial activity against all Gram positive and negative strains at the concentration of 80µl/ml. Thus, from the above study it can be concluded that *C.aromaticus* essential oil posses potent antioxidant and antibacterial activity the formulation of which can be explored in further study.

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