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Antimicrobial activity of *Eucalyptus citriodora* essential oil

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Abstract

The antimicrobial activity of *Eucalyptus citriodora* essential oil against pathogenic fungi, bacteria and drug-resistant mutants of *Candida albicans*, *Escherichia coli* and *Mycobacterium smegmatis* was evaluated following agar disc diffusion and broth dilution assay procedures both qualitatively and quantitatively. The essential oil of *E. citriodora* was found to be active against *Trichophyton rubrum* followed by *Histoplasma capsulatum*, *Candida albicans* (MTCC) and *Cryptococcus neoformans*. Similarly, it was found active toward Gram-positive bacteria compared to Gram-negative and showed activity towards drug-resistant mutants of *C. albicans* and *E. coli*. The findings of our pilot study suggest that characterization and isolation of the active phytoceutical(s) from the *E. citriodora* oil may provide a valuable antimicrobial agent for counteracting fungal and drug-resistant infections.

Key words: *Eucalyptus citriodora*, antimicrobial, bacteria, fungi, drug resistant mutants

Introduction

The Myrtaceae family represents an important source of essential oils with diverse biological activities including bacteriostatic, fungistatic and anti-inflammatory effects. Various Myrtaceae species possess strong antimicrobial potential and their volatile oils are used as antimicrobial and antifungal agents in creams, soaps and toothpastes [1-3]. Within the family, the Eucalyptus genus has been cultivated and exploited on a large scale for many years [4-7]. Several species of eucalyptus are used in folk medicine as an antiseptic and against infections of the upper respiratory tract, such as cold, influenza and sinus congestion [8]. Antimicrobial, analgesic and anti-inflammatory properties of *E. citriodora*, *E. globulus* and *E. teretcorni* have been reported from different parts of the world [9-11]. The leaves of *E. citriodora* contain about 1.36% essential oil that is predominately citronellal (57%) followed by citronellol (15.89%), citronellyl acetate (15.33%) and other compounds [12, 13]. This essential oil showed a wide spectrum of antimicrobial [14-17], antifungal [18, 19], anticandidal [20], antibacterial [21, 22], expectorant and cough stimulant activity [23]. Due to its disinfectant action, the essential oil is used externally, applied to cuts and skin infections but it has deleterious effect on the body in high doses [24, 25]. Beside antimicrobial activity, the essential oil and its constituents have also been used for their herbicidal [26, 27], insecticidal [28, 29], antihelmintic [30], anti-tumour [31] and anti-leech [32] properties, as well as in integrated disease management against phytopathogenic fungi [18], nonspecific skin infections [33] and mastitis in animals [34, 35]. To the best of our knowledge there are no previous reports on the antimicrobial activity of *E. citriodora* essential oil on drug-resistant mutants. Therefore as a part of our bioactivity prospection of medicinal and aromatic plants, we performed a pilot study on the antimicrobial activity of the essential oil from *E. citriodora* against pathogenic fungi, bacteria and their efficacy was also evaluated against some drug-resistant mutants of *Candida albicans*, *Escherichia coli* and *Mycobacterium smegmatis*.

Materials and methods

Collection of plant material and extraction of essential oils

Leaves of *Eucalyptus citriodora* L. (Myrtaceae) were collected by Dr. Alok Kalra from the Research farm of the Central Institute of Medicinal and Aromatic Plants (CSIR), Lucknow. The leaves were shade dried and a

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voucher specimen was deposited at CIMAP herbarium (CIMAP-7661) Gyan Surabhi of CIMAP Lucknow, India. The dried leaves were subjected to steam distillation for 3-4 h using a Clevenger-type apparatus [36]. The essential oils were collected after decantation and were tested for antimicrobial activity against pathogenic fungi, bacteria and drug-resistant mutants using agar disc diffusion and broth dilution assays.

Microorganisms used in present study

The microorganisms used in the present pilot study were the same as reported previously [37].

Pathogenic fungi:

- *Candida albicans* (AIIMS and MTCC 1637)
- *Aspergillus niger,*
- *Aspergillus flavus,*
- *Sporothrix schenckii*
- *Trichophyton rubrum*
- *Cryptococcus neoformans*
- *Microsporum gypseum*
- *Histoplasma capsulatum*.

(All India Institute of Medical Sciences, New Delhi).

Pathogenic bacteria:

- *Streptococcus mutans* (SM) MTCC 890
- *Enterococcus faecalis* (EF) MTCC 439
- *Mycobacterium smegmatis* (MS) ATCC 10231
- *Bacillus subtilis* (BS) MTCC121
- *Staphylococcus aureus* (SA) MTCC 96
- *Staphylococcus epidermidis* (SE) MTCC 435
- *Klebsiella pneumoniae* (KP) MTCC 109
- *Pseudomonas aeruginosa* (PA) MTCC 741
- *Salmonella typhi* (ST) MTCC 733
- *Salmonella typhimurium* (STm) MTCC 98
- *Escherichia coli* (EC) MTCC 723
- *Enterobacter aerogenes* (EA) MTCC111
- *Yersinia enterocolitica* (YE) MTCC 861.

Drug resistant mutants:

The sensitive (wild type) and drug-resistant mutants of *Candida albicans*, *Escherichia coli* and *Mycobacterium smegmatis* used in the present study are shown in Table 1.

Standard antifungal and antibacterial agents used

Clotrimazole (10 mg/ml), amphotericin B (10 mg/ml), streptomycin (10 mg/ml) and nalidixic acid (10 mg/ml) were used as positive controls while DMSO was used as a negative control.

Qualitative analysis: disc diffusion assay

Antifungal and antibacterial disc diffusion assays were carried out following the method as described by Bauer et al [38]. Fungal and bacterial inoculums were prepared from overnight cultures (24 h) in Luria broth and Sabouraud Dextrose broth (Hi Media, India), respectively, and the turbidity was adjusted equivalent to 0.5 McFarland standards (approximately 1.5×10^8 CFU/ml). Aliquots (100 µl) of inoculums were spread over the surface of agar plate with a sterile glass spreader. Five µl of oil was put on the paper disc (5 mm diameter, Whatman filter paper no.3); air-dried and then placed on the pre-made fungal and bacterial growths. The plates were then incubated for 16-24 h at 37°C and the zone of complete growth inhibition was measured in millimetres (mm). The values reported are mean of three experiments in replicate.

Quantitative analysis: Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and Minimum Bactericidal Concentration (MBC) determination

The MIC of the essential oils extracted from *Eucalyptus citriodora* against pathogenic fungi, bacteria and drug resistant mutants of *Candida albicans*, *Escherichia coli* and *Mycobacterium smegmatis* was determined by two-fold serial dilution broth assay as described by Petersdorf and Sherris [39], Jorgenson et al. [40], National Committee for Clinical Laboratory Standards (NCCLS) [41] and Zentz et al [42]. The oil was diluted into a final concentration of 10 to 0.625 mg/ml. The micro titre plates were inoculated with 10 µl of diluted 24 h grown culture of test organism with a titre equivalent to 0.5 McFarland standards. The inoculated microtitre plates were then incubated at 37°C for 16-24 hours and the growth was recorded spectrophotometrically at 600 nm using a Spectramax 190-microplate reader (Molecular Devices, CA, and USA). The MIC (IC_{80}) value was detected from the turbidimetric data as the lowest concentration of oil showing growth inhibition equal to or greater than 80% as compared to oil-free control. The MFC and MBC values were also detected from the turbidimetric data as the lowest concentration of oil where 99% of killing was observed. The MIC, MFC and MBC values reported are a mean of three experiments in replicate.

Results and discussion

The essential oil obtained from *E. citriodora* L. were tested

Table 1. Wild type and drug resistant mutants of *C. albicans*, *E. coli* and *M. smegmatis*.

mutants	drug resistant property	reference(s)
C. albicans AI & MTCC, Clo 31, C 6R Clo GMC 128, CETR Amp 2R, Amp 45, D 1R, cAmp 8R, Amp 8R, KGMC 1, KGMC 3	wild type (sensitive to polyenes & azoles) and resistant mutants of clotrimazole, amphotericin B and clinical isolates resistant to both amphotericin B and clotrimazole	Luqman et al., 2007 [37]; Gupta, 2005 [77]
E. coli CA 8000, DH5α, NK 5819, ET 8000	wild type (sensitive to quinolones & fluoroquinolones) and resistant mutants of nalidixic acid	Kumar, 1976 [78]; Luqman et al., 2005 [79]; Santha et al., 2000 [80]
M. smegmatis MC^2 155, MSR 101, $CSMC^2105$, $CSLMC^2205$	wild type (sensitive to quinolones & fluoroquinolones) and resistant mutants of fluoroquinolones	Snapper et al., 1988 [81]; Sinha, 2003 [82]; Srivastava, 2002 [83]; Luqman et al., 2005 [79]

for antimicrobial activity against pathogenic fungi and bacteria; their efficacy was also evaluated against some of the drug-resistant mutants of *C. albicans*, *E. coli* and *M. smegmatis* following agar disc diffusion and broth dilution assay procedures. Results were recorded in terms of zone of inhibition, minimum inhibitory concentration, minimum fungicidal concentration and minimum bactericidal concentration. The essential oil from *E. citriodora* was found active against all the tested non-filamentous, filamentous and dermatophytic pathogenic fungi. Interestingly, activity was found more towards drug resistant mutants of *C. albicans* followed by *E. coli* in comparison to wild types (Figures 1, 2 and 4). Similarly, the oil was found active against Gram-positive pathogenic bacteria, whilst little activity was observed against Gram-negative bacteria (Figure 3). The observed antifungal, antibacterial and resistant modifying activity of the essential oil from *E. citriodora* in terms of zone of inhibition against pathogenic fungi, bacteria, and drug-resistant mutants of *C. albicans*, *E. coli* and *M. smegmatis* was quantified using the broth dilution assay by recording the MIC, MFC and MBC respectively. The MIC, MFC and MBC of *E. citriodora* essential oil ranged from 1.25 mg/ml to 10 mg/ml against pathogenic fungi, 1.25 mg/ml to 5.0 mg/ml against drug resistant mutants of *C. albicans*, 10 mg/ml to more than 10 mg/ml against human pathogenic bacteria and 1.25 mg/ml to more than 10 mg/ml in drug resistant mutants of *E. coli* and *M. smegmatis* (Tables 2-5).

The present study was undertaken with the objective of

Table 2. MIC and MFC of essential oil from *Eucalyptus citriodora* and antifungal agents against pathogenic fungi.

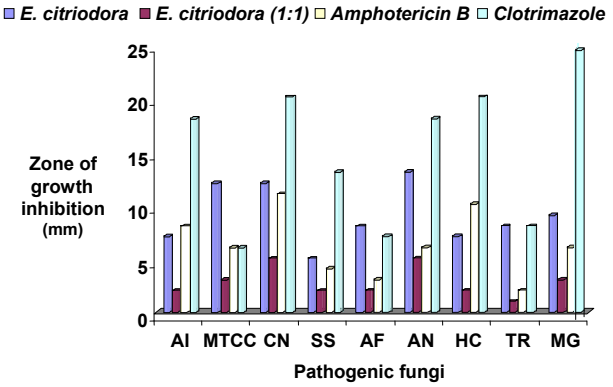
fungal strains	MIC and (MFC) of *E. citriodora* mg/ml	MIC and (MFC) of amphotericin B µg/ml	MIC and (MFC) of clotrimazole µg/ml
C. albicans (AI)	5.0 (5.0)	1.56 (3.125)	0.39 (0.78)
C. albicans MTCC	2.5 (5.0)	1.56 (3.125)	1.56 (3.125)
C. neoformis	5 (10)	1.56 (3.125)	0.39 (0.78)
T. rubrum	1.25 (1.25)	12.5 (12.5)	6.25 (12.5)
H. capsulatum	1.25 (2.50)	0.78 (1.56)	0.195 (0.39)
S. schenckii	5.0 (5.0)	3.125 (6.25)	1.56 (3.125)
A. flavus	10 (>10)	3.125 (6.25)	3.125 (6.25)
A. niger	10 (10)	1.56 (3.125)	0.39 (1.56)

Table 3. MIC and MFC of essential oil from *Eucalyptus citriodora* and antifungal agents against drug resistant mutants of *Candida albicans*.

fungal strains	MIC and (MFC) of *E. citriodora* mg/ml	MIC and (MFC) of amphotericin B µg/ml	MIC and (MFC) of clotrimazole µg/ml
KGMC 1	2.5 (5.0)	6.25 (12.5)	0.095 (0.195)
KGMC 3	5.0 (10.0)	6.25 (12.5)	0.095 (0.195)
Clo 31	2.5 (5.0)	3.125 (6.25)	6.25 (12.5)
C 6R	2.5 (2.5)	6.25 (12.5)	3.125 (6.25)
Clo GMC128	1.25 (2.5)	6.25 (6.25)	3.125 (6.25)
CETR Amp 2R	2.5 (5.0)	3.125 (3.125)	6.25 (6.25)
Amp 45	1.25 (2.5)	3.125 (6.25)	0.195 (0.39)
D 1R	2.5 (10.0)	6.25 (12.5)	3.125 (6.25)
cAmp 8R	5.0 (10.0)	6.25 (12.5)	3.125 (6.25)
Amp 8R	1.25 (2.5)	6.25 (6.25)	0.195 (0.39)

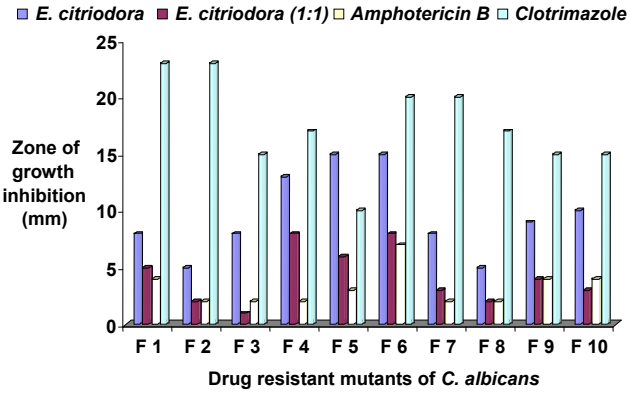
Table 4. MIC and MBC of essential oil from *Eucalyptus citriodora* and antibiotic against drug resistant mutants of *E. coli* and *M. smegmatis*.

bacterial strains	MIC and (MBC) of *E. citriodora* mg/ml	MIC and (MBC) of nalidixic acid µg/ml	MIC and (MBC) of streptomycin µg/ml
CA 8000	10 (>10)	6.25 (12.5)	1.56 (3.125)
ET 8000	1.25 (2.5)	6.25 (12.5)	6.25 (6.25)
NK 5819	10 (10)	6.25 (12.5)	50 (50)
DH5α	1.25 (2.5)	50 (100)	1.56 (3.125)
MC²155	>10	6.25 (12.5)	1.56 (3.125)
CSMC²105	>10	25 (50)	0.78 (3.125)
CSLMC²205	>10	25 (50)	0.78 (3.125)
MSR101	>10	12.5 (25)	12.5 (25)



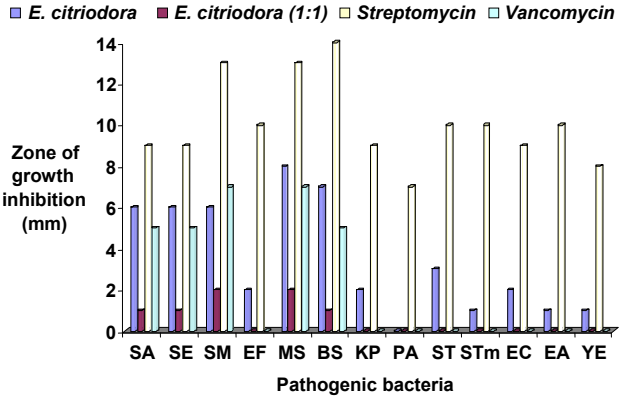
AI=*Candida albicans* (AIIMS); MTCC=*Candida albicans* (MTCC 1637); CN=*Cryptococcus neoformans*; SS=*Sporothrix schenckii*; AF=*Aspergillus flavus*; AN=*Aspergillus niger*; HC=*Histoplasma capsulatum*; TR=*Trichophyton rubrum*; MG=*Microsporum gypseum*.

Figure 1. Growth inhibitory activity of essential oil from *Eucalyptus citriodora* L. against pathogenic fungi assayed by agar disc diffusion.



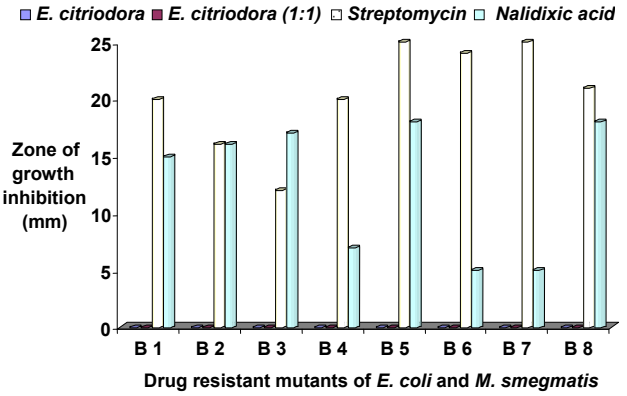
F1: KGMC 1, F2: KGMC 3, F3: Clo 31, F4: C 6R, F5: Clo GMC 128,
F6: CETR Amp 2R, F7: Amp 45, F8: D 1R, F9: cAmp 8R, F 10: Amp 8R

Figure 2. Growth inhibitory activity of essential oil from *Eucalyptus citriodora* L. against drug resistant mutants of *C. albicans* assayed by agar disc diffusion.



SA=*Staphylococcus aureus*; SE=*Staphylococcus epidermidis*; SM=*Streptococcus mutans*; EF=*Enterococcus faecalis*; MS=*Mycobacterium smegmatis*; BS=*Bacillus subtilis*; KP=*Kleibsella pneumoniae*; PA=*Pseudomonas aeruginosa*; ST=*Salmonella typhi*; STm=*Salmonella typhimurium*; EC=*Escherichia coli*; EA=*Enterobacter aerogenes*; YE=*Yersinia enterocolitica*.

Figure 3. Growth inhibitory activity of essential oil from *Eucalyptus citriodora* L. against pathogenic bacteria assayed by agar disc diffusion.



B1: CA 8000, B2: ET 8000, B3: NK 5819, B4: DH5α, B5: MC^2 155, B6: CSMC^{2}105, B7: CSLMC^2 205, B8: MSR 101

Figure 4. Growth inhibitory activity of essential oil from *Eucalyptus citriodora* L. against drug resistant mutants of *E. coli* and *M. smegmatis* assayed by agar disc diffusion.

evaluating the antimicrobial property of the essential oil of eucalyptus and testing its efficacy against the drug-resistant mutants of *C. albicans*, *E. coli* and *M. smegmatis* in view of the emergence of resistance against the currently available antimicrobial agents [43-53]. Our observations showed that the essential oil of eucalyptus was more active towards fungi followed by bacteria, which is in agreement with previously published reports [9, 19, 23, 54-64]. The lower susceptibility of Gram-negative microorganism towards the essential oil of *E. citriodora* may perhaps be due to the presence of an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [65-68].

In the present global scenario, disease causing microbes are acquiring resistance against most of the antimicrobials used for treating antifungal and antibacterial infections [53]. The azole, polyene and quinolones/ fluoroquinolones class of antimicrobials are the last resort to treat infections; hence chances of acquiring the resistance against these antimicrobials are higher. Therefore, it is imperative to search the structurally different antimicrobial agent(s) that can kill the drug-resistant mutants with fewer side effects.

The useful observation from our pilot study, however, is that the oil was more active towards fungi, Gram-positive and drug resistant mutants (F1-F10; resistant against polyene and azole group of antifungal agents) as compared to Gram-negative and wild type microbes. The mechanism of action of eucalyptus oil has not been studied in detail due to the presence of different groups of constituents/ compounds, but it is considered that the action may be due to any of the following mechanisms reported for several essential oils activity:

- damage or degradation of cell wall
- disturbances in the cytoplasmic membrane
- depletion of proton motive force, electron flow
- leakage of cell contents
- damage to membrane proteins and active transport
- coagulation of cell contents [69-75].

Table 5. MIC and MBC of essential oil from *Eucalyptus citriodora* and antibiotic against pathogenic bacteria.

bacterial strains	MIC and (MBC) of *E. citriodora* mg/ml	MIC and (MBC) of streptomycin µg/ml
S. aureus	10(>10)	6.25 (6.25)
S. epidermidis	>10	12.5 (50)
S. mutans	>10	1.56 (3.125)
E. faecalis	>10	25 (100)
M. smegmatis	10 (10)	0.78 (1.56)
B. subtilis	>10	0.78 (3.125)
K. pneumoniae	>10	12.5 (12.5)
P. aeruginosa	>10	25 (50)
S. typhi	>10	25 (100)
S. typhimurium	>10	25 (100)
E. coli	>10	12.5 (25)
E. aerogenes	>10	12.5 (50)
E. enterocolitica	>10	12.5 (100)

In an earlier published report, it was shown that eucalyptus extract and its fraction inhibits the aflatoxin B production of *Aspergillus flavus* and showed activity against multi-drug resistant human pathogens [76]. Our finding suggests that *E. citriodora* oil is effective against the drug resistant mutants of *C. albicans* as well as *E. coli* and also that its efficacy is more towards fungi than bacteria. The present findings also suggest that characterization and isolation of the active phytoceutical (s) from *E. citriodora* oil may provide a valuable antimicrobial agent for counteracting fungal and drug resistant infections.

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