Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*

Javad Safaei-Ghomi, Atefeh Abbasi Ahd

*Essential Oils Research Institute, University of Kashan, Kashan, I. R. Iran*

Submitted: 16-02-2010 Revised: 07-03-2010 Published: 30-07-2010

**ABSTRACT**

This study was conducted to evaluate the *in vitro* antimicrobial properties of essential oil, its major component, 1,8-cineole, and extracts of two *Eucalyptus* species, *Eucalyptus intertexta* and *Eucalyptus largiflorens*. Minimum inhibitory concentration (MIC) of the extracts was calculated by broth dilution method and the zone of inhibition was studied by agar disk diffusion method. Gentamicin (10 μg/disk) and rifampin (5 μg/disk) were used as reference controls for antibacterial studies and nystatin (100 μg/disk) for antifungal studies. The results of MIC study revealed that the essential oil has a stronger activity and broader spectrum than those of methanol extracts. It is interesting to point out that the oils had even greater potential of antimicrobial activities than those of 1,8-cineole as their main component.

**Key words:** Antimicrobial activity, *Eucalyptus largiflorens*, *Eucalyptus intertexta*, minimum inhibitory concentration, zone of inhibition

**INTRODUCTION**

The genus *Eucalyptus* is known for its rich source of bioactive compounds. It is a source for several unique secondary metabolites, which show a variety of biological activities, such as those of antioxidants, antibacterials, HIV inhibitors, attachment inhibitors, and others. Although reports on the essential oil composition of different *Eucalyptus* species are relatively common, investigations on their biological activities are still scarce. *Eucalyptus intertexta* and *Eucalyptus largiflorens* are two cultivated and adapted *Eucalyptus* species in warm regions of Iran, Kashan. To the best of our knowledge, the chemical composition of their essential oil is previously reported, but there is no report on antimicrobial profiles of these two species. Thus, in this study, the *in vitro* antimicrobial activities of their essential oil, its main component and extracts, were evaluated against a set of 11 microorganisms. Their activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and minimum inhibitory concentration (MIC) values.

**MATERIALS AND METHODS**

**Plant material**

Leaves of *E. largiflorens* and *E. intertexta* were collected from cultivated sample in Kashan Botanical Garden (Isfahan Province, Iran) at an altitude of ca. 1000 m in December 2006. The voucher specimen of the plant has been placed in the herbarium of Kashan Research Botanical Garden, Research Institute of Forests and Rangelands, Kashan, Iran.

**Drugs and chemicals**

1,8 Cineol was obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Gentamicin, rifampin and nystatin were purchased from Himedia (Mumbai, India). Analytical grade methanol, dimethyl sulfoxide (DMSO), HPLC grade chloroform, anhydrous sodium sulfate, tween 40, and all culture media were obtained from Merck (Darmstadt, Germany). Ultrapure water was used for the experiments.

**Isolation of the essential oil**

A portion (100 g) of dried and finally ground plant material was subjected, for 3.5 h, to water distillation using a Clevenger-type apparatus as recommended by European Pharmacopoeia. The obtained essential oil was dried over anhydrous sodium sulfate and, after filtration, stored in amber vial at 4°C until analysis. The yield based on dry weight of the sample was calculated.
Preparation of methanol extracts
A portion (20 g) of the powdered plant material was soxhlet-extracted with methanol for 8 h, at a temperature not exceeding the boiling point of the solvent. The extracts were concentrated using a rotary evaporator at 50°C to get crude extracts. Dried extracts were suspended in water and partitioned with chloroform to obtain polar (MW) and non-polar (MC) fractions. All the extracts were dried and kept in the dark at 4°C prior to use.

Antimicrobial activity

Microorganisms
The essential oil was tested against 11 microorganisms including Aspergillus niger ATCC 16404, Candida albicans ATCC 10231, Pseudomonas aeruginosa ATCC 27853, Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 29737, Escherichia coli ATCC 10536, Klebsiella pneumoniae ATCC 10031, Staphylococcus epidermidis ATCC 12228, Shigella dysenteriae PTCC 1188, Proteus vulgaris PTCC 1182 and Salmonella paratyphi-A serotype E. coli. All were provided by the Microbiology Department of the Pasteur Institute of Iran. Bacterial strains were cultured overnight at 37°C in nutrient agar (NA) and fungi were cultured overnight at 37°C in sabouraud dextrose agar (SDA).

Disk diffusion assay
The in vitro antimicrobial activity of samples was evaluated by the disk diffusion method (NCCLS). The dried plant extracts were dissolved in DMSO to a final concentration of 30 mg/mL and filtered using 0.45 μm millipore filters for sterilization. Antimicrobial tests were carried out using the disk diffusion method and employing 100 μL of suspension containing 10⁶ CFU/mL of bacteria, 10⁵ CFU/mL of yeast and 10⁴ spore/mL of fungi spread on the NA, SDA and potato dextrose (PD) agar mediums, respectively. The disks (6 mm in diameter) impregnated with 10 μL of the essential oil, a commercial sample of 1,8-cineole or the extract solutions (300 μg/disk) and DMSO (as negative control) were placed on the inoculated agar. The inoculated plates were incubated for 24 h at 37°C for bacterial strains and for 48 and 72 h at 30°C for yeast and mold isolates, respectively. Gentamicin (10 μg/disk) and rifampin (5 μg/disk) were used as positive controls for bacteria and nystatin (100 IU) for fungi. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated two times.

Microwell dilution assay
MIC values were measured by microwell dilution assay method. The inocula of the microbial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The samples were dissolved in 10% DMSO and diluted to the highest concentration (500 μg/mL) to be tested, and then serial twofold dilutions were made to a concentration ranging from 7.8 to 500 μg/mL in 10 mL sterile test tubes containing brain heart infusion (BHI) broth for bacterial strains and sabouraud dextrose (SD) broth for yeast. The 96-well plates were prepared by dispensing 95 μL of the culture media and 5 μL of the inoculum into each well. A 100-μL aliquot from the stock solutions of the plant extracts initially prepared at the concentration of 500 μg/mL was added into the first well. Then, 100 μL from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μL of the culture media without the test materials and 5 μL of the inoculum on each strip was used as the negative control. The final volume in each well was 200 μL. Gentamicin and rifampin for bacteria and nystatin for yeast were used as standard drugs for positive control in conditions identical to tests materials. The plates were covered with sterile plate sealers. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 μL samples from clear wells on NA medium. The MIC value was defined as the lowest concentration of the plant extracts required for inhibiting the growth of microorganisms. All tests were performed in duplicate.

Minimum inhibitory concentration agar dilution assay
MIC values of 1,8-cineole for the fungus isolate sensitive to it were evaluated based on the agar dilution method. Appropriate amount of this compound was added aseptically to sterile, melted SDA medium containing tween 20 (0.5%, v/v) to produce a concentration range of 7.8–500 μg/mL. The resulting SDA agar solutions were immediately mixed and poured into petri plates. The plates were spot inoculated with 5 μL (10⁴ spore/mL) of fungus isolate. Nystatin was used as reference antifungal drug and the inoculated plates were incubated at 30°C for 72 h. At the end of the incubation period, the plates were evaluated for the presence or absence of growth. The MIC was defined as the lowest concentration of the compound needed to inhibit the growth of microorganisms. Each test was repeated at least twice.

RESULTS AND DISCUSSION

Hydrodistillation of aerial parts of E. largiflorens and E. intertexta yields, respectively, 1.85 and 1.5% (w/w) of light yellowish oil.

According to the results given in Table 1, the essential oil of E. intertexta had great potential of antimicrobial activities...
## Table 1: Antimicrobial activity of the essential oil, its major components (1,8-cineole) and methanol fractions of E. intertexta and E. largiflorens

| Test microorganisms | Essential oil | E. intertexta | E. largiflorens | E. intertexta | E. largiflorens |
|---------------------|---------------|---------------|----------------|---------------|----------------|
|                     | Mic | DD | Mic | DD | Mic | DD | Mic | DD | Mic | DD |
| Ps. aeruginosa       | 28  | 500 | 28  | 500 | 28  | 500 | 28  | 500 | 28  | 500 |
| B. subtilis          | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| E. coli             | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| St. aureus          | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| St. pneumoniae      | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| Sh. dysenteriae     | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| Pr. vulgaris        | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| Sa. paratyphi-A      | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| C. albicans        | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |
| A. niger            | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  | 12  | 1  |

- *DDa* (disk diffusion method), inhibition zones in diameter (mm) around the impregnated disks, *a* indicates no antimicrobial activity, OD (disk diffusion method), inhibition zones in diameter (mm) around the impregnated disks, *b* (minimum inhibitory concentrations as µg/mL, NA (not applicable).

The results of the bioassay [Table 1] also revealed that the essential oil of *E. largiflorens* exhibited moderate to high antimicrobial activity against all the bacteria, yeast and mold tested, except three microorganisms, *Ps. aeruginosa*, *E. coli* and *Sh. dysenteriae*. The evaluation of methanol fraction indicated that polar fraction showed strong activity against 7 out of 11 microorganisms while non-polar fractions did not possess any inhibitory action against the strains evaluated except *E. coli*.

Based on these results, it is possible to conclude that the essential oil has a stronger activity and broader spectrum than those of methanol extracts.

The relatively high antimicrobial activities of essential oils are most likely due to the presence of compounds with antimicrobial properties. A number of compounds present in relatively high concentrations in the essential oils are known to have antimicrobial properties. Particularly worth noting is 1,8-cineole (eucalyptol), which accounted for approximately 70.2% (v/v) of the *E. intertexta* and 37.5% (v/v) of the *E. largiflorens* essential oil, and which has been found to possess relatively strong antimicrobial properties against many important pathogens and spoilage organisms.

These reports are further supported by our finding about 1,8-cineole which showed high inhibitory activities against *C. albicans* and *P. vulgaris* with MIC values of 31.3 and 62.5 µg/mL respectively.

However, a comparison showed that the oils have greater potential of antimicrobial activities than those of 1,8-cineole as their main component [Table 1]. Otherwise, other compounds such as limonene, α-pinene, β-cymene, and terpineol-4-ol, which have relatively strong antimicrobial activities, may be responsible for this activity. Therefore, the synergistic effects of these active chemicals with other constituents of the essential oils should be taken into consideration for the antimicrobial activity.

Moreover, as indicated in a previous report about the other *Eucalyptus* species, gram-positive bacteria are more sensitive...
REFERENCES

1. Elliot WR, Jones DL. Encyclopedia of Australian plants suitable for cultivation, vol. 4, Melbourne: Lothian; 1986.
2. Ghisalberti EL, Bioactive acylphloroglucinol derivatives from *Eucalyptus* species. Phytochemistry 1996;41:7-22.
3. Yamashita N, Etoh H, Sakata K, Ina H, Ina K. New Acylated Rhaponticin Isolated from *Eucalyptus rubida* as a Repellent against the Blue Mussel Mytilus edulis. Agr Biol Chem 1989;53:2627-9.
4. Nishizawa M, Emura M, Kan Y, Yamada H, Ogawa K, Hamaanaka N. Macrocarpals: HIV-RTase inhibitors of *Eucalyptus globules*. Tetrahedron Lett 1992;33:2983-6.
5. Murata M, Yamakami H, Homma S, Arai K, Nakamura Y. Macrocarpals, Antibacterial Compounds from *Eucalyptus* leaves. Inhibit Ablode Re ductase. Biosci Biotech Biochem 1992;56:2062-3.
6. Osawa T, Namiki M, A Novel Type of Antibiotic Isolated from Leaf Wax of *Eucalyptus* leaves. Agr Biol Chem 1981;45:753-9.
7. Jovin E, Beara I, Mimica-Dukić N, Grbovic S, Bugarin D, Balog K. *Eucalyptus* species in Montenegro. Chemistry and antioxidant activity. Planta Med 2007;73:956.
8. Neghaban M, Moharrampour S. Fumigant toxicity of *Eucalyptus intertexta*, *Eucalyptus* sargentii and *Eucalyptus* camaldulensis against stored-product beetles. J Appl Entomol 2007;131:256-61.
9. Takahashi T, Kubo R, Sakaino M. Antimicrobial activities of *Eucalyptus* leaf extracts and flavonoids from *Eucalyptus maculate*. Lett Appl Microbiol 2004;39:60-4.
10. Dethier M, Nduwimana A, Cordier Y, Menut C, Lamaty G. Aromatic plants of tropical central Africa. XVI. Studies on essential oils of five *Eucalyptus* species grown in Burundi. J Essent Oil Res 1994;6:469-73.
11. Bignell CM, Dunlop PJ, Brophy JJ, Jackson JF. Volatile leaf oils of some South-western and Southern Australian species of the genus *Eucalyptus* part VI - subgenus symphyomyrtus, section adnataria. Flav Fragr J 1995;10:359-64.
12. Bignell CM, Dunlop PJ, Brophy JJ, Jackson JF. Volatile leaf oils of some South-western and Southern Australian species of the genus *Eucalyptus*. Part 7. Subgenus Symphyomyrtus, section Exsertaria. Flav Fragr J 1996;11:35-41.
13. Bignell CM, Dunlop PJ, Brophy JJ. Volatile leaf oils of some South-western and Southern Australian species of the genus *Eucalyptus* (Series I). Part XVIII. A. Subgenus Monocalyptus. B. Subgenus Symphyomyrtus: (i) section Guiffoyleanae; (ii) section Bisectaria, series Accedentes, series Occidentales, series Levispermae, series Loxophlebae, series Macrocarpae, series Orbifoliae, series Calycogonae; (iii) section Dumaria, series Incrassatae and series Ovulares. Flav Fragr J 1997;12:423-32.
14. Wirthensohn MG, Sedgley M, Jones GP. Epicuticular wax of juvenile *Eucalyptus* leaves and headspace analysis of leaf volatiles. J Essent Oil Res 2000;12:401-11.
15. Bignell CM, Dunlop PJ, Brophy JJ, Jackson JF. Volatile leaf oils of some south-western and Southern Australian species of the genus *Eucalyptus*. Part IX. Subgenus Symphyomyrtus. Section Bisectaria. (a) series Elongatae, (b) unpublished series Stricklandiae, (c) series Krusaeanae and (d) series Orbifoliae. Flav Fragr J 1996;11:95-100.
16. Sefidkon F, Assareh MH, Abravesh Z, Barazandeh MM. Chemical composition of the essential oils of four cultivated *Eucalyptus* species in Iran as medicinal plants (E-microtheca, E-spathulata, E-largiflorens and E-torquata). Iran J Pharm Res 2007;6:135-40.
17. Sefidkon F, Assareh MH, Abravesh Z, Mirza M. Chemical composition of the essential oils of five cultivated *Eucalyptus* species in Iran: *E. intertexta*, *E. platypus*, *E. leucoxylon*, *E. sargentii* and *E. camaldulensis*. J Essent Oil Bearing Plants 2006;9:245-50.
18. Assareh MH, Jaimand K, Rezaee MB. Chemical composition of the essential oils of six *Eucalyptus* species (Myrtaeae) from south west of Iran. J Essent Oil Res 2007;19:8-10.
19. Anonymous. European Pharmacopoeia. 3rd ed. Strasbourg: Council of Europe; 1996. p. 121-2.
20. Wayne PA, National Committee for Clinical Laboratory Standard. In Performance standards for antimicrobial disk susceptibility test. 6th approved standard; 1997, M2-A6.
21. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolke RH. Manual of clinical microbiology. 7th ed. Washington: ASM; 1995. p. 1773.
22. Gulluce M, Sokmen M, Sahin F, Sokmen A, Adiguzel A, Ozer H. Biological activities of the essential oil and methanolic extract of *Micromeria fruticosa* (L) Druce ssp serpyllifolia (Bieb) PH Davis plants from the eastern Anatolia region of Turkey. J Sci Food Agric 2004;84:735-41.
23. Gul HL, Ojanen T, Hanninen O. Antifungal evaluation of bis Mannich bases derived from acetophenones and their corresponding pipеридинолs and stability studies. Biol Pharm Bull 2002;25:1307-10.
24. Rosato A, Vitali C, De Laurentis N, Armenise D, Millio MA. Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. Phytomedicine 2007;14:727-32.
25. Pitarokili D, Tzakou O, Loukis A, Harvala C. Volatile metabolites of *Salvia fruticosa* as antifungal agents in soilborne pathogens. J Agric Food Chem 2003;51:3294-301.
26. Sonboli A, Babakhani B, Mehrhabian AR. Antimicrobial activity of six constituents of essential oil from Salvia. J Naturforsch C 2006;61:160-4.
27. Dorman HDJ, Deans SG. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J Appl Microbiol 2000;88:308-16.
28. Carson CF, Riley TV. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. J Appl Bacteriol 1995;78:264-9.
29. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of the essential oil and methanolic extract of *Micromeria fruticosa* as antifungal agents in soilborne pathogens. J Agric Food Chem 2003;51:3294-301.
30. Gilles M, Zhao J, An M, Agboola S. Chemical composition and antimicrobial properties of essential oils of three Australian *Eucalyptus* species. Food Chem 2010;119:731-7.