



Original Research Article

Ocular infection – Bacterial isolates and their sensitivity to essential oils of selected herbals

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ABSTRACT

Keywords

Ocular infection,
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Ocular microbiology remains an applied science. Many pathogens and opportunistic pathogenic agents are increasingly encountered in ocular infections. Bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *Proteus mirabilis* isolated from ocular infections cases were tested for their sensitivity to essential oils from *Cymbopogon citratus*, *Cymbopogon martinii*, *Cinnamomum zeylanicum*, *Rosmarinus officinalis*, *Mentha piperita*, *Pelarogonium graveolens*, and *Vitexnegundo*. In agar well diffusion method the selected essential oils were effective against both gram positive as well as gram negative organisms. For e.g. *C. citratus* was highly active against *S. pneumoniae* and least against *S. aureus*. *C. martinii* was highly active against *S. typhi* and least against *S. aureus*. *C. zeylanicum* was highly active against *S. pneumoniae* and least against *P. mirabilis*. *R. officinalis* was highly active against *P. mirabilis* and least against *P. aeruginosa*. The minimal inhibitory concentration (MIC) of essential oil of *C. zeylanicum* against all test organisms was best (0.25 mg/ml) and it was quite comparable with the reference drug Ciprofloxacin (0.25 mg/ml). The results for all other essential oils were moderate, when compared with the reference drug. The results for minimal bactericidal concentration (MBC) were similar to minimal inhibitory concentration results. As no organism was found to be resistant to the tested essential oil, the results indicated that those essential oils could be used (aromatherapy) in different forms for the prevention, control and treatment of opportunistic bacterial infections caused by those organisms isolated from ocular infections cases.

Introduction

Infection of the eye leads to conjunctivitis, keratitis, endophthalmitis and other infections which are responsible for increased incidence of morbidity and blindness worldwide (Chirambo *et al.*, 1986; Juarez-Verdayes *et al.*, 2006). Suppurative keratitis can cause corneal opacity and perforation,

which leads to severe visual loss and is the second most common cause for blindness in developing countries (Upadhyay *et al.*, 1991; Ashaye and Aimola, 2008). The etiological cause for suppurative keratitis may vary at different geographical locations (Leck *et al.*, 2002). Different types of fungi

that are one of the important etiological agents also affect cornea orbit and other ocular structures. Fungal infection is a life threatening condition which needs early diagnosis and treatment to save the patients' eye. In some cases when medical treatment fails early surgical debridement is resorted (Thomas, 2003). The study has been conducted to detect various types of eye infections and the different trends of bacterial as well as its fungal etiology.

The conjunctival sac is colonized by bacteria at birth remains so throughout life with changes in the flora due to various factors. Microbial flora mainly consists of *Staphylococcus epidermidis*, *S. aureus*, *Corynebacterium* *sps*, and *Propionibacterium acnes* and with increasing age, Gram negative bacteria also become part of the flora. Microbial adherence to epithelial surface occurs due to molecular interactions between bacterial surface proteins and protein receptors (integrins) on the cell surfaces. Pili or fimbriae of gram negative bacteria such as *Pseudomonas aeruginosa* play an important role for adhesion to the cell surface. Bacteria colonizing conjunctival sac produce bacteriocins and inhibitory products such as lactic and acetic acids when help them the necessary competitive advantage to survive and prevent establishment of pathogenic micro-organism. Prolonged usage of topical antibiotics may result in change of microbial flora with implantation of fungal and antibiotic resistant bacteria.

Plants can resist parasitic attacks using several defense mechanisms. One of such is the synthesis of antimicrobial compounds which elicit defense substances called phytoalexins. Plant defense substances belong to a wide range of different chemical classes including flavonoids, terpenoids, alkaloids, steroidal saponins, tannins, phenolic acids, lactones, quinones essential

oil, and polyphenols (Cowan, 1999).

Herbal and alternative medicines are popular in the general population worldwide. A great number of modern drugs are still derived from herbs (Cooper, 2005). In recent years there has been an increasing interest in the use of natural substances, and some questions concerning the safety of synthetic compounds have encouraged more detailed studies of plant resources. Essential oils, odors and volatile products of plant secondary metabolism, have a wide application in folk medicine as well as in fragrance industries. Essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants by hydro- or steam-distillation. The main constituents of essential oils, for example, monoterpenes and sesquiterpenes and phenylpropanoids including carbohydrates, alcohols, ethers, aldehydes and ketones, are responsible for the fragrant and biological properties of aromatic and medicinal plants. Various essential oils and their components possess pharmacological effects, demonstrating anti-inflammatory, antioxidant and anti-carcinogenic properties (Golab *et al.*, 2005; Naser *et al.*, 2005; Ito *et al.*, 2008). In addition to inducing resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immunosuppression and allergic reactions (Ahmad *et al.*, 1998). Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases (Berahou *et al.*, 2007; Salomao *et al.*, 2008).

Many plant materials have been investigated for their antimicrobial activity. The addition of raisins to the formulation of beef jerky had a marked inhibitory effect on pathogenic bacteria (Bower *et al.*, 2003). Rosemary extract has demonstrated antimicrobial activity against a number of food borne pathogenic bacteria (Del Campo *et al.*, 2000).

The present study was carried out to identify the effectiveness of seven essential oils against bacterial pathogens isolated from ocular infection cases, because of the lesser works done in the area.

Materials and Methods

Essential oils

Seven essential oils such as Lemongrass oil (*Cymbopogon citratus*-Graminae), Palmarosa oil (*Cymbopogon martini*-Graminae), Cinnamon bark oil (*Cinnamomum zeylanicum*-Lauraceae), Rosemary oil (*Rosmarinus officinalis*-Labiatae), Geranium oil (*Pelargonium graveolens*-Geraniaceae), Peppermint oil (*Mentha piperita*-Labiatae), and Chaste tree leaf oil (*Vitexnegundo*-Lamiaceae) were obtained from Aromax Trading Co, India (commercial producers of plant essential oils and aromatic substances) were used in this study. Quality of the oils was ascertained to be more than 98% pure. The oil was stored in the dark at 4°C until used within a maximum period of one week.

Collection of specimens

The sample collection was made by the ophthalmologist based on the procedures for diagnosis of ocular infections by K. Lilytherese and H.N. Madhavan, L&T Microbiology Research centre, Vision research foundation, Chennai.

Eyelid margin specimen

For this no topical anesthetic is needed. Sterile cotton tipped swab or calcium alginate swab moistened in HBSS/brain heart infusion broth (BHIB) was rubbed over the lid margin. Swab was inoculated directly on Blood agar (BA) preferably a single Petri dish/Chocolate agar (CA)

medium was used for each specimen, *Brucella* blood agar (BBA) from each eyelid. If pus is present, swabs were used for its collection and BA, CA, MA, BBA and BHIB were directly inoculated and at least three smears were made using fresh sterile swab.

Collection of conjunctival material (Conjunctival Swab)

Sterile moistened cotton swab or calcium alginate swab was used. Bacterial culture medium such as BHIB or normal saline was used for moistening the swab. Patient was requested to look up, the lower eye lid was pulled down using thumb with an absorbing tissue paper and moistened swab was rubbed over the lower conjunctival sac from medial to lateral side and back again. The procedure was often slightly painful. Sterile plastic (soft) bacteriological loop was used for collection of material. Collection of tears alone was avoided. Swab was directly inoculated onto blood agar (aerobic incubation) chocolate agar (5–10% CO₂) and BBA (anaerobic). On solid media main inoculum only was made and further streaking was done in the laboratory.

Antibacterial assay

Agar well diffusion method

In this study standard agar well diffusion method was followed (Erdemoglu *et al.*, 2003). Each bacterial isolate was suspended in Brain Heart Infusion (BHI) (Himedia, India) broth and diluted to approximately 10⁵ colony forming unit (CFU) per mL. They were “flood inoculated” onto the surface of BHI agar and then dried. Five-millimeter in diameter wells were cut from the agar using a sterile cork-borer, and 100µl of the samples solutions were delivered into the wells. The plates were incubated for 18 h

at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Ethanol was used as solvent control. Ciprofloxacin was used as reference antibacterial agent. The tests were carried out in duplicates.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The estimation of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were carried out by the broth dilution method (Van der Berghe and Vlietinck, 1991). Dilutions of essential oil from 2.0 to 0.075 mg/ml were used. Test bacterial culture was used at the concentration of 10^5 CFU/ml. MIC values were taken as the lowest essential oil concentration that prevents visible bacterial growth after 24 h of incubation at 37°C, and MBC as the lowest concentration that completely inhibited bacterial growth. Chloramphenicol was used as reference and appropriate controls with no essential oil were used. Each experiment was done in triplicate.

Statistical analysis

Data were analyzed using Least Significant Difference (LSD) test following –way analysis of variance (ANOVA) using SPSS 10.0 computer software package. Difference on statistical analysis of data were considered significant at $p < 0.05$.

Results and Discussion

Agar well diffusion method

In agar well diffusion method the selected essential oils were effective against both gram positive as well as gram negative

organisms. *C. citratus* was highly active against *Streptococcus pyogenes* and least against *S. aureus*. *C. martinii* was highly active against *S. typhi* and least against *S. aureus*. *C. zeylanicum* was highly active against *S. pneumoniae* and least against *P. mirabilis*. *R. officinalis* was highly active against *P. mirabilis* and least against *P. aeruginosa*. *M. piperita* was highly active against *E. coli* and least against *Micrococcus luteus*. *P. graveolens* was highly active against *P. mirabilis* and least against *S. pneumoniae*. No organism was found to be resistant to the test oils. The results are shown in Table 1 and Figure 1.

Results for minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of essential oils

The results are shown in Table 2 and Figure 2. The results of MIC of *C. citratus*, *C. martinii*, *R. officinalis*, *M. piperita* and *V. negundo* were moderate when compared with the reference drug ciprofloxacin (0.25 mg/ml). The results of MIC of *C. zeylanicum* against all test organisms were similar and it was 0.25 mg/ml and it was quite comparable with the reference drug. The results of MIC of *P. graveolens* against all test organisms were similar and it was 2.00 mg/ml and it was very high dose when compared to the reference drug.

The results for Minimum Bactericidal Concentration (MBC) were similar to Minimum Inhibitory Concentration (MIC) results, but in MBC confirmation was made by the presence and absence of culture. The results are shown in Table 3 and Figure 3.

All the selected essential oils were found to be active against both gram positive and gram negative bacterial isolates from ocular infection cases. The isolate may be a

pathogen or an opportunistic pathogen depending upon condition of the patient's immune system, which has not been focused in this study. Cinnamon oil was more active compared to other oils among the 7 oils tested. Lemongrasses, palmarosa, rosemary were found to be more than the moderate range. The others such as geranium, peppermint and chaste tree leaf oil. As far as the chemical analysis, and antibacterial study, the following studies were comparable to the results with the present study. Lemongrass (*C. citratus*) was found to be as effective in a 2.5% cream as four other commercial creams against ringworm and clinical isolates of four dermatophytes in vitro (Wannissorn *et al.*, 1996). Each of the commercial creams had clotrimazole, isoconazole nitrate, ketoconazole, benzoic acid, and salicylic acid as their main active ingredients.

Onawunmi and Ogunlana, (1986) found lemongrass effective against *E. coli* and *Bacillus subtilis* in both broth dilution and agar diffusion tests. Lemongrass essential oil had an activity comparable to the standard antibiotic disks in the study, thus indicating that lemongrass is a viable option against certain pathogens (Hmamouch *et al.*, 1990). Gachkar *et al.*, (2007), reported the chemical composition, antibacterial, antioxidative and radical-scavenging properties of the essential oils of *R. officinalis* obtained by steam distillation (Santoyo *et al.*, (2005), attributed the antimicrobial property of the essential oil of *R. officinalis* to the presence of -pinene, 1,8-cineole, camphor, verbinone and borneol with borneol being the most potent followed by camphor and verbinone.

The quantities of these compounds were very high in our oils. The volatile oils of *R. officinalis* were screened against two Gram-positive (*S. aureus*, and *B. subtilis*) and two Gram-negative (*E. coli* and *K. pneumoniae*)

bacteria strains.

However, there is evidence that essential oils are more strongly antimicrobial than is accounted for by the additive effect of their major antimicrobial components; minor components appear, therefore, to play a significant role (Lataoui and Tantaoui-Elaraki, 1994).

Many naturally occurring extracts like essential oils from edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and could serve as a source for antimicrobial agents against food spoilage and pathogens (Bagamboula *et al.*, 2003). More particularly, essential oils and their components are known to be active against a wide variety of microorganisms, including Gram negative bacteria (Helander *et al.*, 1998). The findings of the chemical analysis of the present study were comparable to the results of the following. The antimicrobial activity of essential oils is assigned to a number of small terpenoids and phenolic compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity (Conner, 1993).

The antibacterial properties of these compounds are in part associated with their lipophilic character, leading to accumulation in membranes and to subsequent membrane-associated events such as energy depletion (Conner, 1993). Chemical analysis of these oils have shown that the principal active compounds of these oils are principally carvacrol, thymol, citral, eugenol, 1–8 cineole, limonene, pinene, linalool and their precursors (Demetzos and Perdetzoglou, 2001). However, there are often large differences in the reported antibacterial activity of oils from the same essence.

Table.1 Antibacterial activity of essential oils against bacteria from ocular infection cases

Essential oils	Bacterial isolates /Zone of Inhibition in mm						
	<i>Staphylococcus aureus</i>	<i>Streptococcus pneumoniae</i>	<i>Streptococcus pyogenes</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Micrococcus luteus</i>	<i>Proteus mirabilis</i>
<i>Cymbopogon citratus</i>	9.98±0.53	11.30±0.90	12.85±0.56	11.58±0.61	7.98±0.45	12.24±0.81	13.16±1.00
<i>Cymbopogon martini</i>	11.28±0.87	12.96±0.51	14.16±0.84	11.95±0.39	11.54±0.54	12.92±0.36	11.98±0.45
<i>Cinnamomum zeylanicum</i>	20.15±1.31	22.39±0.91	19.03±0.61	20.98±1.46	17.23±0.65 ^e	14.10±0.64	12.08±0.60
<i>Rosmarinus officinalis</i>	10.12±0.36	10.11±0.56	13.03±0.64	11.12±0.37	8.21±0.44	11.08±0.59	14.06±0.47
<i>Mentha piperita</i>	12.86±0.31	12.27±1.34	11.08±0.61	13.15±0.91	11.68±0.84	8.30±0.96	11.39±0.96
<i>Pelargonium graveolens</i>	7.83±0.76	13.43±0.94	10.11±0.67	8.26±1.0	11.84±0.77	13.53±0.53	16.94±0.90
<i>Vitex negundo</i>	12.15±1.01	8.91±0.85	10.16±0.82	12.85±0.75	10.86±0.75	8.92±0.65	12.27±0.70
Ciprofloxacin©	20.27±1.15	20.30±0.91	18.65±0.72	16.43±0.52	12.51±0.51	18.08±0.59	17.05±0.65

© - control antibiotic disc in 100 µg concentration; Different superscripts in the same column are significantly different at $P < 0.05$ level (Least Significance Difference) mean followed by ± S.D.

Fig.1 Antibacterial activity of essential oils against bacteria from ocular infection cases

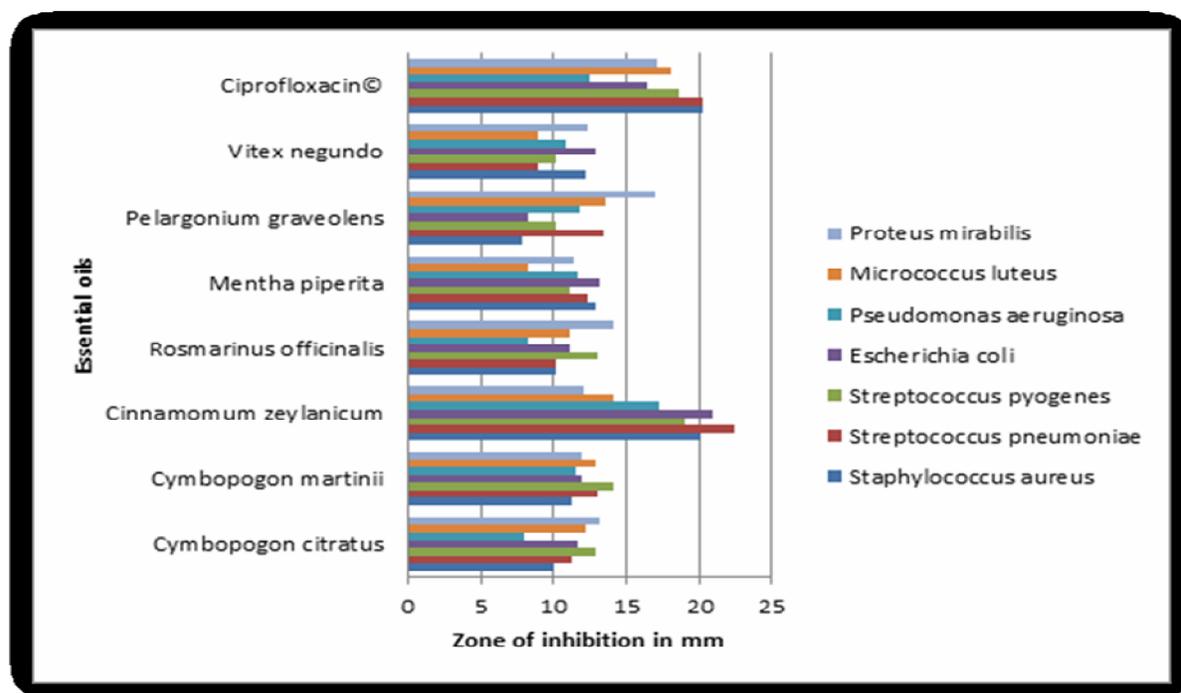


Table.2 MIC of essential oils against clinical bacterial isolates from ocular infection cases

Organisms	Essential oil / MIC in mg/mL							
	<i>Cymbopogon citratus</i>	<i>Cymbopogon martinii</i>	<i>Cinnamomum zeylanicum</i>	<i>Rosmarinus officinalis</i>	<i>Mentha piperita</i>	<i>Pelargonium graveolens</i>	<i>Vitex negundo</i>	Ciprofloxacin©
<i>Staphylococcus aureus</i>	1.00	0.50	0.25	0.75	1.00	2.00	1.00	0.25
<i>Streptococcus pneumoniae</i>	1.00	0.50	0.25	0.75	1.50	2.00	1.00	0.25
<i>Streptococcus pyogenes</i>	2.00	1.50	0.25	1.00	2.00	2.00	1.50	0.25
<i>Escherichia coli</i>	2.00	1.50	0.25	1.00	2.00	2.00	1.50	0.25
<i>Pseudomonas aeruginosa</i>	2.00	2.00	0.25	1.50	2.00	2.00	1.50	0.25
<i>Micrococcus luteus</i>	2.00	2.00	0.25	2.00	2.00	2.00	2.00	0.25
<i>Proteus mirabilis</i>	2.00	1.50	0.25	1.00	1.50	2.00	2.00	0.25

© - control antibiotic disc in 100 µg concentration; MIC- Minimum Inhibitory Concentration

Fig.2 MIC of essential oils against clinical bacterial isolates from ocular infection cases

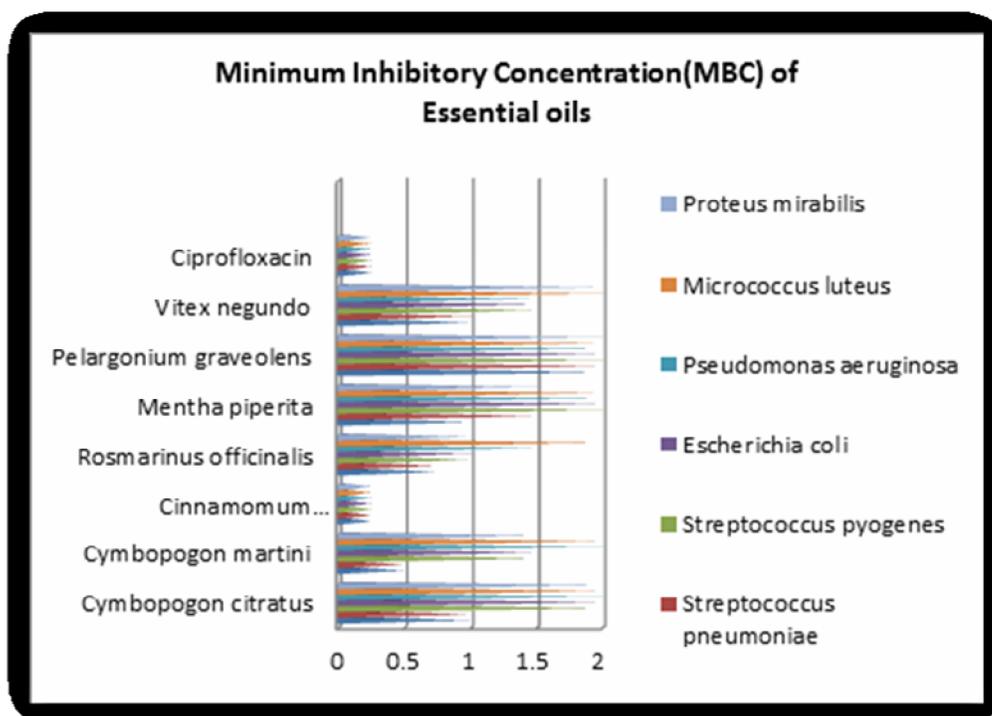
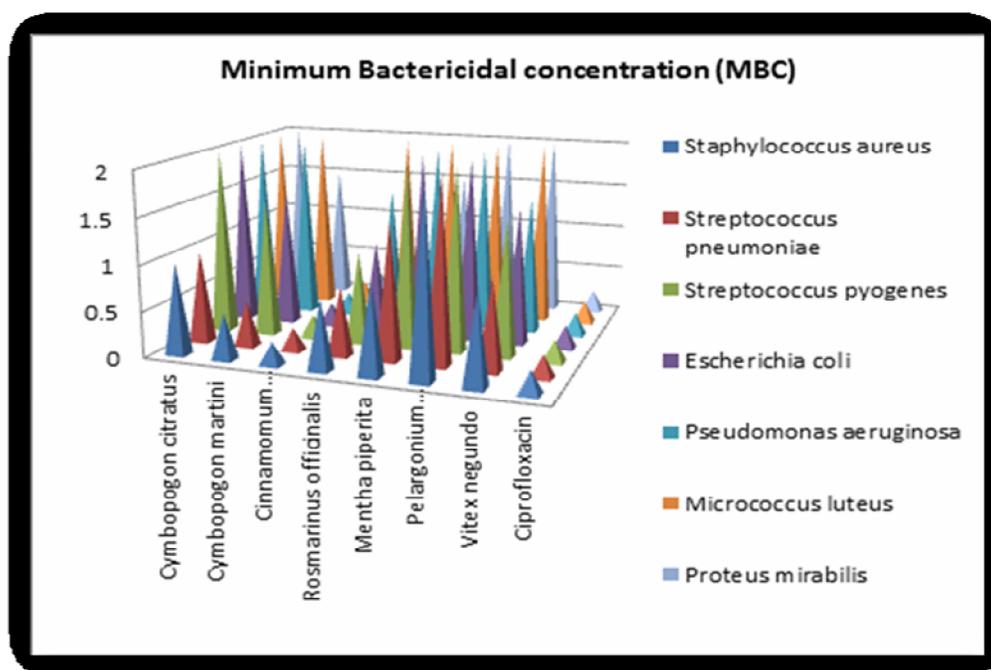


Table.3 MBC of essential oils against clinical bacterial isolates from ocular infection cases

Organisms	Essential oil / MBC in mg/mL							
	<i>Cymbopogon citratus</i>	<i>Cymbopogon martinii</i>	<i>Cinnamomum zeylanicum</i>	<i>Rosmarinus officinalis</i>	<i>Mentha piperita</i>	<i>Pelargonium graveolens</i>	<i>Vitex Negundo</i>	Ciprofloxacin©
<i>Staphylococcus aureus</i>	1.00	0.50	0.25	0.75	1.00	2.00	1.00	0.25
<i>Streptococcus pneumoniae</i>	1.00	0.50	0.25	0.75	1.50	2.00	1.00	0.25
<i>Streptococcus pyogenes</i>	2.00	1.50	0.25	1.00	2.00	2.00	1.50	0.25
<i>Escherichia coli</i>	2.00	1.50	0.25	1.00	2.00	2.00	1.50	0.25
<i>Pseudomonas aeruginosa</i>	2.00	2.00	0.25	1.50	2.00	2.00	1.50	0.25
<i>Micrococcus luteus</i>	2.00	2.00	0.25	2.00	2.00	2.00	2.00	0.25
<i>Proteus mirabilis</i>	2.00	1.50	0.25	1.00	1.50	2.00	2.00	0.25

© - control antibiotic disc in 100 µg concentration; MBC- Minimum Bactericidal Concentration

Fig.3 MIC of essential oils against clinical bacterial isolates from ocular infection cases



The reasons for this variability can be due to the geographical sources, the harvesting seasons, the genotype, the climate, the drying and the distilled part of the plant

which are significant factors influencing the chemical composition and relative proportions of the individual constituents in the essential oils of the plant (Juliano *et*

al.,2000). Also, a number of essential oil constituents exhibit significant antimicrobial properties when tested separately (Lambert, *et al.*, 2001). In conclusion, medicinal and aromatic plants are widely used today in modern phytotherapy (Buds *et al.*, 2004). The essential oils and their components are known to be active against a wide variety of microorganisms (Vukovic, 2007).

The essential oils as antimicrobial agents present two main characters: the first is their natural origin which means more safety to the people and the environment, the second is that they have been considered at low risk for resistance development by pathogenic microorganisms. The results of this study revealed that, the essential oil can be prepared in different forms such as topical agents, eye drops and other therapeutic agents of those essential oils may be suggested as a new potential source of natural antimicrobial for the prevention, treatment and control of bacterial diseases in various patients, particularly, for ocular infection patients after further analysis on their side effects.

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