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Characterization of Contact Lens Associated Bacteria and Their Responses to Botanical Essential Oils

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Abstract

Study was conducted to evaluate antimicrobial potential of some essential oils (EOs) against contact lens associated bacteria. Bacteria were isolated from used contact lens (CLs) solutions, identified by routine biochemical procedures, their antibiotic resistance and responses to EOs were worked out. A total of 85 bacterial isolates were identified from 56 samples. All isolates were sensitive to Quinolone group of antibiotics and resistant against Oxacillin (penicillin group) and Cefixime (cephalosporin group). Twenty strains including *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Aeromonas hydrophila*, *Escherichia coli*, *Proteus penneri*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus* spp. and *Bacillus* spp. were screened for their responses to EOs from *Trachyspermum ammi* Linn. (Ajowan), *Eugenia caryophyllata* Thunb.(Clove), *Eucalyptus globulus* Labill. (Blue gum) and *Citrus sinensis* L. (Orange). The minimum inhibitory concentration (MIC) was worked out for *E. coli* and *S. aureus*. Ajowan EO displayed greater antibacterial properties followed by clove, eucalyptus and orange EOs. MIC of ajowan oil was 128 arbitrary units (AU) for both *E. coli* and *S. aureus*. In the light of antibacterial spectra of oils, it is suggested that ajowan oil or its components may be further explored as antiseptic in lens cleaning solutions. © 2014 Friends Science Publishers

Keywords: *Trachyspermum ammi* essential oil; Antibacterial; Antibiotic resistance profiling; Contact lens solution; CL associated bacteria

Introduction

The conventional spectacles are being replaced by plastic corrective contact lenses (CLs) worn over the cornea to improve vision and solve the problem of inconvenient wearing (Emina and Idu, 2011). However, adverse responses can occur as a result of bacterial colonization in the lenses (Willcox *et al.,* 2001). The major risk factors associated with long time wearing of CLs can be corneal hypoxia, infection, edema, and corneal vascularization (Morgan *et al.,* 2005). Multiple species of bacteria, including *Staphylococcus epidermidis, S. aureus,* Enterobacter spp. and *Pseudomonas* spp. have been reported on surface of contact lenses from healthy persons (Sankaridurg *et al.,* 2000). *Pseudomonas aeruginosa* is considered main cause of ocular infections in contact lens wearers (Willcox, 2007).

Ocular infections like other bacterial infections are normally treated with antibiotics, whereas for sterility the contact lenses are kept in lens care solutions which contain antiseptics like polyaminopropyle binguanide or polyhexamethylene biguanide etc. The inappropriate and indiscriminate use of antibiotics in various bacterial morbidities has led to emergence of resistant strains of bacteria worldwide that has headed towards inefficiency of antiseptic and antimicrobial treatments (Ang *et al.,* 2004). There has been considerable interest in the search of new antimicrobial substances from multiple sources including medicinal plants (Izadi *et al.,* 2010; Toroglu *et al.,* 2012). The natural products are documented as source of advanced therapeutic agents for various infectious and non-infectious diseases (Clardy and Walsh, 2004). Essential oils (EOs), volatile complex compounds of aromatic plants, are among such compounds. In plants, EOs play important role as antibacterial, antivirals, antifungals, insecticidal and herbicidals. They have the potential to be utilized as alternatives to synthetic chemical products to protect the ecological equilibrium (Masotti *et al.,* 2003). Additionally, in comparison to synthetic antibiotics, EOs are believed to limit the antibiotic resistance (Deans and Ritchie, 1987; Högberg *et al.,* 2010). They are documented to exhibit considerable inhibitory effects against many bacteria including hospital isolates, ATCC strains and food spoilage microbes (Dorman and Deans, 2000; Takarada *et al.,* 2004). However, these have not been tested against contact lens associated bacteria. The lens associated bacteria might respond differently because of their special characteristics that make it more resistant to the action of antimicrobials (Wilson *et al.,* 1990). The present study was intended to evaluate the antibacterial potential of EOs from

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Trachyspermum ammi (Ajowan), *Eugenia caryophyllata* (Clove), *Eucalyptus globulus* (Blue gum) and *Citrus sinensis* (Orange) against contact lens associated isolates.

Materials and Methods

Plant Material and Extraction of EOs

Plant materials [peel of *C. sinensis* (orange), seeds of *E. caryophyllata* (clove), seeds of *T. ammi* (Ajowan) and leaves of *E. globulus* (Blue gum)] were collected from local market and identified by Professor Dr. Ghazala Naseem (Institute of Agricultural Sciences, University of Punjab, Lahore). EOs were extracted by hydro-steam distillation for 5 h using the Clevenger type apparatus (PYREX 250 mL). The extracted EOs were kept in air tight sealed glass vials at 4°C and protected from light by aluminum foil**.**

Sample Collection and Bacterial Cultures

Contact lens cases containing used lenses, dipped in lens care solution, were collected from female university students (age 21-24) with their written consents. Lenses were wiped up in the same solution with help of cotton swab. A total of 100 µL of this solution was spread on the nutrient agar plate and incubated for 24 h at 37°C. Bacterial isolates were purified on nutrient agar after several rounds of streaking and finally they were preserved in mixture of brain heart infusion broth and glycerol (1:1) at -20ºC till further use.

Identification of Contact Lens Associated Isolates

Gram positive bacteria were identified by gram staining and biochemical test including catalase, oxidase, glucose oxidation-fermentation and coagulase tests following Gerhardt *et al*. (1994). Gram negative bacterial isolates were identified by the API-20E identification system (BioMerieux, Inc. Canada). BioMérieux's API® identification products are test kits for identification of bacteria and yeast to species level.

Antibiotic Resistance Screening

Antibiotic resistance profiling was performed by Kirby-Bauer disc diffusion susceptibility test (Bauer *et al.,* 1966). Antibiotic discs (OXOID) of fourteen commonly employed antibiotics belonging to seven functional groups (Macrolid, penicillin, tetracycline, quinolones, aminoglycosides, cephalosporins and polypeptide) were used in the study. These included clarithromycin (15 µg/disc), erythromycin (15 µg/disc), amoxycillin (25 µg/disc), oxacillin (10 µg/disc), tetracycline (30 µg/disc), ciprofloxacin (5 µg/disc), levofloxacin (5 µg/disc), kanamycin (10 µg/disc), gentamycin (10 µg/disc), cefixime (5 µg/disc), ceftriaxone (30 µg/disc), nitrofurantoin (300 µg/disc), chloramphenicol (30 µg/disc) and bacitracin (30 µg/disc).

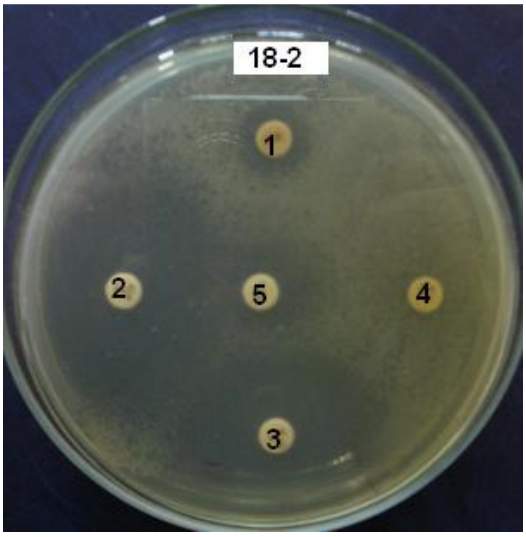

Fig. 1: Antibacterial activity Tetracycline (1), Ciprofloxacin (2), Levofloxacin (3), Nitrofurantoin (4) and Chloramphenicol (5) against *Pseudomonas aeruginosa*

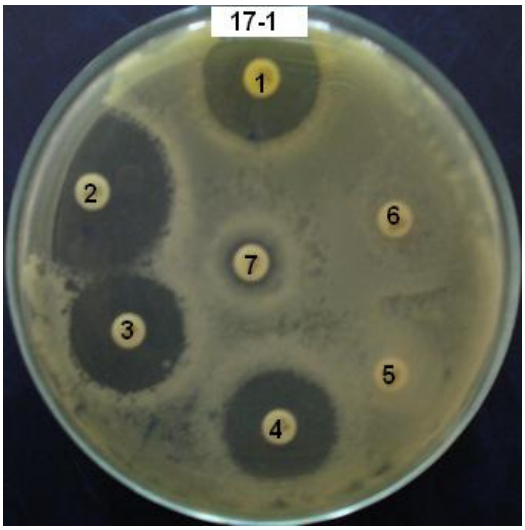

Fig. 2: Antibacterial activity of Nitrofurantoin (1), Chloramphenicol (2), Kanamycin (3), Gentamicin (4) , Cefixime (5), Ceftriaxone (6) and Bacitracin (7) on *Bacillus* spp

A fresh culture of each bacterium was used in all tests. Initially the optical density of cultures was adjusted to 1.00±0.05 at 600 nm corresponding to 10^8 CFU/mL. The cultures were plated on Mueller Hinton agar plates with the help of sterile cotton swabs. Antibiotic containing discs were placed aseptically on the plates. The plates were incubated for 18-24 h at 37°C and observed for the presence of zone of inhibition (ZI) that was measured in terms of mm including disc (Fig. 1 and 2). The ZI were interpreted as sensitive, intermediate or resistant (Jorgensen and Turnidge, 2003).

Determination of Bactericidal Activity of EOs

A total of 20 bacterial isolates (10 Gram positive and 10 Gram negative) including *P. aeruginosa* (n=4), *Acinetobacter* spp. (n=2), *Aeromonas hydrophila* (n=1), *Escherichia coli* (n=2), *Proteus penneri* (n=1), *S. aureus* (n=4), *S. epidermidis* (n=1), *Streptococcus* spp. (n= 3) and *Bacillus* spp. (n=2) were selected on the basis of their abundance and used to assess the antibacterial potential of EOs.

Sensitivity of CL isolates to various EOs was determined by disc diffusion assay using empty Sensi-Discs. Inoculum was prepared as mentioned in previous section and plated on Mueller Hinton agar. The Sensi-Discs were loaded with 15 µL of essential oil separately and placed on the bacterial lawn. Following incubation at 37°C for 24 h, all plates were examined for inhibition zones. Levofloxacin was used as positive control while discs soaked in distilled water were used as negative control. Zones of inhibition of Levofloxacin ≤ 12 mm were considered resistant, 13-15 mm intermediate and ≥ 16 mm sensitive (Lacy *et al.,* 2004). The bacteria with a clear ZI of ≥ 11 mm were considered to be sensitive for EOs.

Minimum Inhibitory Concentration (MIC) of EOs

MIC of EOs was determined by micro dilution assay with slight modification. A series of two-fold dilutions of oil in absolute ethanol was prepared in microtiter plate. Sensi-Disc impregnated with 15 µL of different dilutions of oil was placed aseptically on bacterial lawn. Levofloxacin (5 µg/disc) and sensi-disc impregnated with ethyl alcohol was used as positive and negative controls respectively. Disc diffusion assay was performed with different concentrations of oils to obtain ZI at smallest concentrations of oil. The antimicrobial activity was expressed in arbitrary units (AU) per ml following (Mayr-Harting *et al.,* 1972). One AU was defined as the reciprocal of the highest serial twofold dilution producing a ZI of ≥11 mm on the indicator lawn (i.e., comparable to positive control).

Statistical Analysis

Data were analyzed using SPSS 14.0 (SPSS Inc. Chicago IL USA). The p value < 0.05 was considered significant.

Results

A total of 85 bacterial strains were isolated from 56 lens samples. Gram positive bacteria were identified as *S. epidermidis* (18.80%), *S. aureus* (9.40%), *Bacillus* spp. (7%) and *Streptococcus* spp. (9.40%). While Gram negative bacteria included *P. aeruginosa* (14.11%), *E. coli* (11.76%), *Klebsiella pneumoniae* (10.58%), *Acinetobacter* spp. (3.52%), *A. hydrophila* (3.52%), *Citrobacter* spp. (3.52%), *Pasteurella* spp. (2.35%), *Serratia rubidaea* (2.35), *Burkholderia cepacia* (2.35%) and *P. penneri* (1.17%).

Antibiotic Resistance profile of Contact Lens associated Isolates

S. aureus and *P. aeruginosa* were among the most abundant bacteria and exhibited high resistance (78.50% and 75% respectively) to 14 antibiotics (Fig. 3). Isolates were sensitive to Quinolones group of antibiotic particularly, Levofloxacin which was found to be highly effective against contact lens associated bacteria, only 6.4% isolates displayed resistance against it (Table 1).

Antibacterial Activity of Essential Oils

EOs extracted from *C. sinensis* (orange), *E. caryophyllata*, (clove), *T. ammi* (ajowan) and *E. globules* (Blue gum) produced ZI of different sizes against different bacterial species. Activities of Eucalyptus, Clove and Orange oil were found to be moderate. Ajowan oil expressed high antimicrobial potential for all gram negative bacteria except

Table 1: Antibiotic resistance level in contact lens associated bacteria

Antibiotic group	Antibiotic	Isolates Resistant to Antibiotic (%)
Macrolid	Clarithromycin	54.8
	Erythromycin	51.6
Penicillin	Amoxycillin	87
	Oxacillin	100
Tetracycline	Tetracycline	77.4
Quinolones	Ciprofloxacin	9.6
	Levofloxacin	6.4
Aminoglycoside	Kanamycin	22.5
	Gentamycin	12.9
Cephalosporin	Cefixime	100
	Ceftriaxone	90.3
Others	Nitrofurantoin	58
	Chloramphenicol	16.1
Polypeptide	Bacitracin	77.4

Table 2: Antimicrobial activity of four essential oils against contact lens isolates, results are in terms of size of zone of inhibition (mm)

Bacterial strains	*Trachyspermum ammi*	*Eugenia caryophyllata*	*Eucalyptus globulus*	*Citrus sinensis*
Acinetobacter spp. (n=2)	48.5	21.5	14.5	23
Aeromonas hydrophila (n=1)	56	24	28	13
Bacillus spp. (n=2)	47	21.5	11.5	12
Escherichia coli (n=2)	30.8	13.5	11.5	9
Proteus penneri (n=1)	26	14	12	8
Pseudomonas aeruginosa (n=4)	12	8	0	0
Staphylococcus aureus (n=4)	55.4	22.7	25.1	19.5
Staphylococcus epidermidius (n=1)	15	18	11	14
Streptococcus spp.(n= 3)	54	27.3	20	17

for *P. aeruginosa* in which only 12 mm zones of inhibition was observed while for gram positive isolates, it produced ZI up to 55.4 mm (Table 2). Gram positive bacteria were observed to be more sensitive as compare to gram negative bacteria for ajowan, clove and orange oil, whereas responses of both types of bacteria for eucalyptus oil were not statistically different (Table 3).

Table 3: Comparison of antimicrobial activity of essential oils against gram positive and gram negative bacteria. Data are in terms of size of zone of inhibition in mm (Mean±SE)

Essential oils	Gram negative			Gram positive			P value of t-test
Trachyspermum ammi	32.88	±	5.90	49.26	±	4.68	0.048
Eugenia caryophyllata	12.83	±	2.64	23.4	±	1.36	0.003
Eucalyptus globulus	10.33	±	2.68	19.44	±	3.80	0.058
Citrus sinensis	8.92	±	2.65	16.7	±	1.68	0.028

Table 4: Minimum inhibitory concentration (AU) of Essential oils for *Escherichia coli* and *Staphylococcus aureus*

Essential oils	MIC (AU*)	
	E. coli	*S. aureus*
Trachyspermum ammi	128	128
Eugenia caryophyllata	4	16
Eucalyptus globulus	1	32
Citrus sinensis	1	16

*One Arbitrary Unit is defined as the reciprocal of two fold dilution that could restrict the growth of bacteria

MIC of Essential Oils

MIC of EOs was determined against *E. coli* and *S. aureus*. Ajowan oil revealed high bactericidal activity. It inhibited the growth of *E. coli* and *S. aureus* respectively at a dilution of 1:128, therefore 128 AU was considered its MIC, similarly MIC of Clove oil for *E.coli* was 04 AU. Eucalyptus and orange oils were unable to restrict the growth of *E. coli* even at 1:2 dilutions. For *S. aureus* MIC of Clove, Eucalyptus and orange oil was 16, 32 and 16 AU, respectively (Table 4).

Discussion

Contamination of contact lenses (CLs) with microorganisms can lead to infection and inflammation during contact lens wear (Willcox, 2007). Contact lens associated bacteria have special characteristics like capsule formation, capability to adhere to hydrophobic surfaces and biofilm formation (unpublished data) which help them in resisting antimicrobial agents. Biofilm forming bacteria possessing high antibiotics resistance have been reported from contact lenses and in storage cases (McLaughlin-Borlace *et al.,* 1998). In this context exploration for new sources of antimicrobial substance has become an active area of research (Jian-Yu and Tian, 2012). The current study focused on identification of CLs associated bacteria, their antibiotic resistance and responses against EOs.

In this study 44.7% gram positive and 55.3% gram negative were isolated and identified. Some of these bacteria might be potential pathogens, gram negative bacteria particularly *Pseudomonas* spp. are principal organism of CLs associated microbial keratitis (Cheng *et al.,* 1999; Lam *et al.,* 2002), albeit multiple species from gram positive group have also been documented from CL related ocular infections (Pinna *et al.,* 2001).

Antibiotic resistance was observed in all bacterial isolates particularly in *S. aureus* and *P. aeruginosa* which were resistant to 10 out of 14 antibiotics used in the study. Present findings are in accordance with Spoering and Lewis (2001) who reported *P. aeruginosa* as antibiotic resistant biofilm forming bacteria. Other isolates also expressed resistance against Oxacillin and Cefixime. The non-biofilm forming bacteria get chances of horizontal gene transfer when entrapped in biofilms. Thus the finding of resistance to multiple antibiotics in all CL associates is not surprising. Other authors also reported presence of resistant strains from CL or lens cases (Rahim *et al.,* 2010)

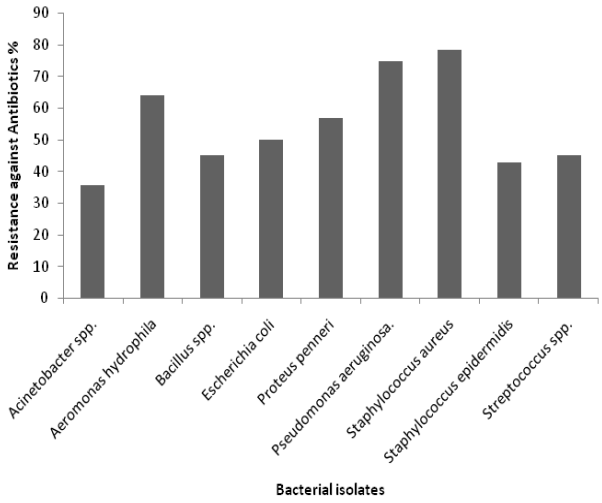

Fig. 3: Percentage Resistance of selected isolates to 14 antibiotics

Antibiotics and antiseptics limit the survival of bacteria by either disrupting their membranes, binding with enzymes, or interfering in protein and nucleic acid synthesis. To encounter that, resistant strains adopt multiple strategies like production of antimicrobial inactivating enzymes, alteration in their own metabolic pathways or formation of membrane efflux pumps (Hoffman, 2001). The responses of resistant strains to natural antimicrobials may also be different as compared to their sensitive relatives. In present research EOs from *T. ammi* (Ajowan), *E. caryophyllata* (Clove), *E. globulus* (Blue gum) and *C. sinensis* (Orange)

are used. The composition of the above mentioned oils has already been reported by different authors (Chaieb *et al.,* 2007; Cimanga *et al.,* 2002; Davazdahemami *et al.,* 2011; Delaquis *et al.,* 2002; Javed *et al.,* 2012; Tao *et al.,* 2009; Viuda-Martos *et al.,* 2008), therefore, it was not determined in this study.

Trachyspermum ammi (Ajowan) oil illustrated highest efficacy against all bacterial isolates including *P. aeruginosa*. The size of ZI was comparable to Levofloxacin, the positive control used in this study. *T. ammi* has already been documented to possess antiseptic, antifungal, antibacterial and antithelminitic effects (Javed *et al.,* 2012). The size of ZI for *P. aeruginosa* with Ajowan oil recorded in present study was only 12 mm as compared to 28 mm for *P. syringica* reported by Javed et al. (2012) from the same EO. The difference in size of ZI of *Pseudomonas* sp. might be due to presence of resistant strain in study. The constituents of *T. ammi* EOs include phenols majorly thymol (35 to 60%) and some carvacrol, limonine and dillapiole (Davazdahemami *et al.,* 2011).

Eugenia caryophyllata (Clove oil) produced promising results for all bacteria except *P. aeruginosa*. The antimicrobial activity of clove oil has already been documented against many bacteria including *Listeria monocytogenes, Campylobacter jejuni, Salmonella enteritidis, Bacillus cereus, E. coli* and *S. aureus* (Cressy *et al.,* 2003; Fu *et al.,* 2007; Toroğlu *et al.,* 2012). Clove oil could produce 27 mm inhibition zone against *Streptococcus* spp. whereas for *E. coli* it was only 13 mm. Higher ZI for the same essential oil are reported in literature for other gram negative bacteria (Saeed and Tariq, 2008). There results also reflect different behavior of CL associated isolates. The main constituents of the clove essential oil are phenylpropanoids such as carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb *et al.,* 2007).

Eucalyptus oil is used as antibacterial and decongestant in traditional herbal remedy. The *Eucalyptus* leave oil is also reported as powerful antiseptic (Bhatti *et al*., 2007). Antimicrobial properties of *Eucalyptus* EOs have been documented against wide range of microorganisms including *P. aerogenosa* with ZI of 16-18 mm, but it could not show such activity for *P. aeruginosa* and *S. epidermidis* included in present study. This difference may be due to difference in the responses of bacteria of same species from different environment. The major constituents of eucalyptus EOs include 1,8-cineole, and -pinene, p-cymene, myrcene, -terpinene, -terpineol and limonene as major components and neral, borneol, linalool, cinnamaldehyde, carvacrol, geraniol, myrtenal and eugenol as minor components (Cimanga *et al.,* 2002). Cimanga *et al.* (2002) further suggested that antimicrobial potential of Eucalyptus oil is due to the presence of minor components.

The activity of orange oil for gram negative organisms was also significantly lower as compared to gram positive isolates. Bactericidal activity of orange oil was high for one gram negative bacteria, the *Acinetobacter* spp. in which it presented 23 mm ZI but for all other isolates it was 0-12 mm. Espina *et al.* (2011) and Viuda-Martos *et al.* (2008) also mentioned very week activity of orange oil. Limonene is reported as the major component of orange essential oil (59–85%). Other components include myrcene (6.27%), α-farnesene (3.64%), γ-terpinene (3.34%), *α*-pinene (1.49%), sabinene (1.29%) and other minor components.

Fu *et al*. (2007) has given an extensive review on antimicrobial activity of clove oil against ATCC strains, however, much higher MIC of clove was recorded for CLs associated isolates in present study which indicates different behavior of CL associated bacteria to clove essential oil.

In culmination present findings illustrated the four EOs epitomize antibacterial activity against contact lens associated bacteria when used in pure form. However, ajowan oil exhibited highest bactericidal properties. *P. aeruginosa* showed resistance to all oils except ajowan oil. From previously reported data difference in the size of ZI and MIC were also noticed particularly for *Pseudomonas* spp. The ajowan , clove and eucalyptus oils possess some quantity of Carvacrol, which is well documented antibacterial and antiprotozoal substance (Grabensteiner *et al.,* 2007). The antimicrobial activity observed in present study might be due to this component.

In conclusion, ocular infections of CLs are potential threat for contact lens wearers. All tested bacteria are resistant to amoxycillin and oxacillin but except *P. aeruginosa* all strains are comparatively sensitive to EOs used in study. Ajowan oil (*T. ammi*) possesses high antibacterial potential followed by clove oil, Eucalyptus oil and orange oil.

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