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ANTIMICROBIAL ACTIVITY OF AROMA CHEMICALS AGAINST UROPATHOGENS

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Abstract

The purpose of the study was to evaluate the antimicrobial activity of some aroma chemicals, namely, β- citronellol, citronellyl formate, trans-geraniol and l- linalool, which are also known to be the major components of *P.graveolens* essential oil. The antimicrobial activity of chemicals was determined against both antibiotic sensitive and resistant uropathogens using the disc diffusion method. Minimum inhibitory concentration and minimum lethal concentration was also determined by broth microdilution method. The anti-swarming effects in *Proteus mirabilis* isolates were also determined in the terms of inhibition of swarm fronts. The antimicrobial activity was determined to be concentration dependent; the best inhibitory activity was shown by trans-geraniol, followed by citronellyl formate and l-linalool. Trans-geraniol also highly inhibited the diameter of both the swarm fronts. The inhibitory effects of these aroma chemicals indicate their promising candidature for developing newer anti-microbial agents/drugs.

Keywords: antimicrobial activity, citronellol, citronellyl formate, geraniol, linalool, swarming

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Introduction

Aromatic chemicals or aroma chemicals are cyclic compound containing at least one benzene ring and characterized by the presence of alternating double bonds within the ring (Belitz *et al*., 2009). Fragrant chemicals are either synthetically synthesized or are contained in the essential oils (EOs) extracted from the vegetal parts of aromatic plants. EOs are chemically a cocktail of different terpenes, terpenoids, aromatic and aliphatic constituents (Singh and Malik, 2008; Bassole and Juliani, 2012). In addition to the pleasing aroma, these odourous chemicals have beneficial health properties; hence have been used in the aromatherapy (Edris, 2007; Singh and Malik,,2008). In the recent past, EOs have also been introduced for their application in control of plant and postharvest pathogens (Koul *et al*., 2008). Some of these chemicals are permissible to be used as a flavour or fragrance in food items, considered as GRAS (Generally Recognized As Safe) by Food and Drug Administration, United States (FDA, US) (Hyldgaard *et al*., 2012). Nowadays, a number of aroma chemicals are commercially available; impart flavours and fragrances to the food items, are also used in feed, cosmetic and pharmaceutical industries; constitutes a large fraction of global market, accounts for approx 7 billion US$ a year (Dubal *et al*., 2008).

Aroma chemicals are not only evaluated for their olfactory quality by the professional perfumers, but some of these have also been tested for their antimicrobial activity against different microorganisms (Morris *et al*.,1979; Kotan *et al*., 2007).

These chemicals are usually characterized by high C/N ratios; low pH values and hence is inhibitory for most of the microorganisms (Belleti *et al*., 2007).

Pelargonium graveolens L'Herit, commonly known as 'geranium', an aromatic herb, is cultivated for its rosy odoured essential oil (geranium oil) (El-Wahab *et al*., 2009). Geranium essential oil has been used in perfumery (Douglas, 1968), confectionery (Leung, 1980), cosmetics (Lis-Balchin, 2005) and medicines (Ranade, 1988). The main compounds present in the *P.graveolens* essential oil have been determined to be citronellol, citronellyl formate, geraniol and linalool (Rana, 2002; Džamić *et al*., 2014). Antimicrobial potential of *P.graveolens* essential oil has been previously authenticated by a number of researchers (Lis-Balchin *et al.,*1998; Malik *et al*., 2008; Malik *et al*., 2011; Džamić *et al*., 2014). However, its constituents have not been explored much for their antimicrobial activity. Therefore, the purpose of the present study was to evaluate the antimicrobial activity of β-Citronellol, citronellyl formate, trans-geraniol and l- linalool.

MATERIAL AND METHODS:-

CHEMICALS: The following aroma chemicals were purchased from Sigma-Aldrich, USA:

i. β-Citronellol

ii. Citronellyl formate

iii. trans-Geraniol

iv. l- Linalool

MICROORGANISMS

Microorganisms which are used in this study were previously isolated from the urine samples of Urinary Tract infections (UTIs) patients. Ten bacterial and a yeast isolate were used in the present study, previously screened and interpreted to be antibiotic sensitive/resistant on the basis of antibiotic sensitivity testing. The antibiotics used were: Ampicillin (A, 10mcg), Amikacin (Ak, 30mcg), Ceftazidime (Ca, 30mcg), Cefotaxime (Ce, 30mcg), Ceftriaxone (Ci, 30 mcg), Cephalothin (Ch, 30mcg), Ciprofloxacin (Cf, 50mcg), Chloramphenicol (C, 30mcg), Co-trimoxazole (Co, 25mcg), Erythromycin (E,15mcg), Gentamicin (G, 10 mcg), Kanamycin (K, 30mcg), Nalidixic acid (Na, 30mcg), Nitrofurantoin (Nf, 300mcg), Streptomycin (S, 25mcg) and Tetracycline (T, 30mcg)}. Accordingly, the microorganisms used were described as follows:

i. *Escherichia coli* ET1 (sensitive)

ii. *E.coli* (resistant to K,A, E)

iii. *Pseudomonas aeruginosa* PT2(sensitive)

iv. *P. aeruginosa* PT3(resistant to C,K,T,A)

v. *Klebsiella pneumoniae* KT2 (sensitive)

vi. *K. pneumoniae* KT6 (resistant to K,T, Co)

vii. *Proteus mirabilis* PRT3 (sensitive)

viii. *P. mirabilis* PRT7 (resistant to K,S, E, Co)

ix. Staphylococcus aureus ST2 (sensitive)

x. *S. aureus* ST2 (resistant to K,A, Cf)

xi. *Candida albicans* CT1(sensitive to amphotericin)

(Malik and Singh, 2010).

Screening of antimicrobial activity of purified components of *P. graveolens* essential oil

The components were screened for antimicrobial activity by disc diffusion test. In brief, Mueller Hinton Agar plates were inoculated by the inoculum (~ 10^6 cfu/ml), sterile filter paper discs impregnated with 10 µl of the chemical to be tested. The plates were observed for the zone of inhibition after incubation (37°C) of 18h/48h for bacteria and yeast respectively. The inhibition for each disc was expressed as the average of three readings for diameter of zone of inhibition (Malik and Singh, 2010).

Determination of Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

The determination of Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) was carried out by micrbroth dilution methods, as described previously (Eloff *et al*., 1998; Malik and Singh, 2010). Briefly, the stock solution of the chemicals was prepared in appropriate solvent. 95µl of sterile

mueller hinton broth was dispensed in all the wells, subsequently 5µl of inoculum were added in all the wells except first well which served as a negative control. The chemical was serially diluted in wells of a row, except the last one which served as positive control. The plate was incubated for overnight at 37°C. After incubation, 20µl of resazurin dye was added and plate was again incubated at 37°C for 4 h, the colour change (blue/purple/pink/ colourless) was noted. The last concentration showing the lack of turbidity/blue colour indicated the killing was considered as MIC. A loopful of broth from each well was subcultured on fresh Trypticase Soy Agar medium. The last concentration showing the absence of growth was considered as MBC**.**

Effect of aroma-chemicals on swarming parameters in *P. mirabilis* isolate

The inhibition of swarming effects of aroma-chemicals was determined in *P. mirabilis* PRT3 isolate. Briefly, 5µl of the overnight culture of *P. mirabilis* was inoculated in the centre of the dried Luria Bertani (LB) agar (1.5%) plates, having different concentrations of chemicals. The plates were incubated at 37 ºC and the swarming inhibition was determined in terms of diameter of 1st swarm and last swarm fronts (Echeverrigaray *et al.,* 2008).

Statistical analysis

The results of disc diffusion experiments were statistically analyzed. The means of inhibition zone were analyzed by one way analysis of variance (ANOVA) followed by post hoc 'Least Significant Difference' (LSD) test at 5% level of significance, using SPSS software package version for windows. A set of critical difference (CD) values were determined for all the experiments.

RESULTS AND DISCUSSION:-

Antimicrobial activity of different components of *P. graveolens* essential oil varied with their concentration and kind of uropathogen. Disc diffusion experiments showed that the best inhibition was exhibited by trans-geraniol against most of the isolates, followed by citronellyl formate and l-linalool (Table 1). The results of micrbroth dilution studies of four components of *P. graveolens* essential oil, namely β-citronellol, citronellyl formate, trans-geraniol and l-linalool showed that trans-geraniol exhibited best inhibitory activity, showed lowest values of MIC and MBC (Table 2 & Table 3). Trans-geraniol, citronellyl formate and l-linalool also inhibited the swarming fronts of *P. mirabilis*; maximum inhibition of both swarm fronts was found at 4% of trans-geraniol (Fig 1 & Fig 2).

Table 1- Antimicrobial activity of aroma chemicals against uropathogens

Microorganisms	Diameter of inhibition zone (mean ± S.D. in mm)				
	β-Citronellol	Citronellyl formate	trans-Geraniol	l-Linalool	C.D
E.coli ET1(S)	nz	9.1±0.3	16.0±0.5	19.3±0.6	0.56
E.coli ET4(R)	nz	9.5±0.7	Nz	19.3±0.6	0.82
P.aeruginosa PT2(S)	nz	9.5±0.7	16.1±0.4	nz	0.23
P.aeruginosa PT3(R)	nz	10.9±0.5	15.2±0.7	nz	0.32
K.pneumoniae KT2(S)	nz	21.2±0.5	15.7±0.8	14.9±0.6	0.45
K.pneumoniae KT6(R)	nz	15.7±0.8	14.9±0.6	11.3±0.5	0.54
P.mirabilis PRT3(S)	16.3±0.4	20.5±0.7	23.9±0.5	16.7±0.4	0.56
P.mirabilis PRT7(R)	16.2±0.4	21.5±0.8	22.4±0.3	16.5±0.6	0.58
S.aureus ST2(S)	11.3±0.3	12.3±0.8	13.3±0.7	11.7±0.3	0.53
S.aureus ST4(R)	10.9±0.5	11.5±0.6	13.6±0.5	10.9±0.4	0.40
C.albicans CT1(S)	8.9±0.6	14.2±0.5	27.1±0.9	9.0±0.4	1.27

- **Nz: No zone of inhibition**

Table 2- MIC of aroma chemicals against uropathogens

Microorganism	MIC (µg/ml)				
	β-citronellol	Citronellyl formate	trans-Geraniol	l-Linalool	Reference antibiotic
E.coli ET1	68	35.08	4.45	8.89	4
E.coli ET4	68	35.08	4.45	8.89	>32
P.aeruginosa PT2	68	35.08	17.78	17.25	16
P.aeruginosa PT3	34	35.08	17.78	8.625	32
K.pneumoniae KT2	4.25	4.32	0.55	1.075	16
K.pneumoniae KT6	4.25	4.32	0.55	1.075	32
P.mirabilis PRT3	8.5	35.08	8.89	17.25	16
P.mirabilis PRT7	8.5	35.08	8.89	17.25	32
S.aureus ST2	34	70.16	0.55	2.155	16
S.aureus ST4	34	35.08	0.55	2.155	>32
C.albicans CT1	68	70.16	71.12	17.25	4

Table 3- MLC of aroma chemicals against uropathogens

Microorganism	MBC (µg/ml)				
	β-citronellol	Citronellyl formate	trans-Geraniol	l-Linalool	Reference antibiotic
E.coli ET1	68	35.08	4.45	8.89	4
E.coli ET4	68	35.08	4.45	8.89	>32
P.aeruginosa PT2	34	35.08	17.78	17.25	16
P.aeruginosa PT3	34	35.08	17.78	17.25	>16
K.pneumoniae KT2	8.5	4.32	1.1	2.155	16
K.pneumoniae KT6	8.5	4.32	1.1	2.155	>16
P.mirabilis PRT3	8.5	35.08	8.89	17.25	16
P.mirabilis PRT7	8.5	35.08	8.89	17.25	32
S.aureus ST2	34	70.16	1.1	4.31	16
S.aureus ST4	34	35.08	1.1	4.31	>32
C.albicans CT1	>68	70.16	71.12	17.25	4

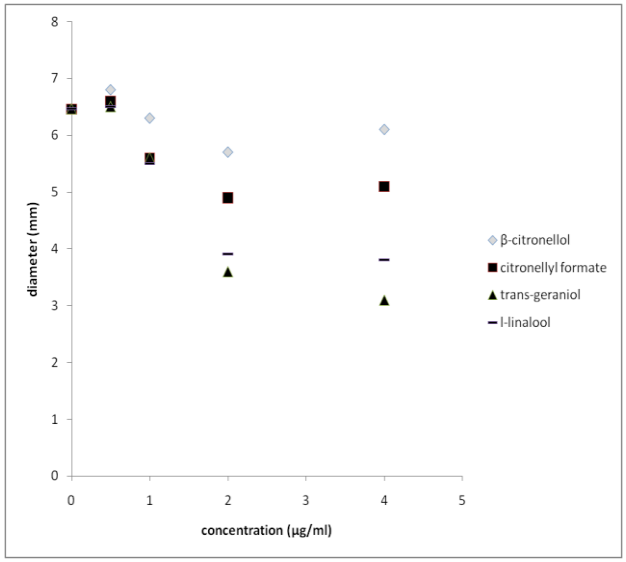

Fig 1: Inhibition of first swarm fronts in *P.mirabilis* by aroma chemicals

However, it is difficult to correlate the antimicrobial activity of essential oils with a specific compound due to their complexity and variability. Although, amongst the components of essential oils; usually alcohols, phenols and aldehydes are responsible for the cytotoxicity (Bruni *et al.*, 2003). In the present study, all the chemicals were acyclic monoterpenoids, out of which three were alcohols. Alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative microbial cells (Kotan *et al*., 2007).

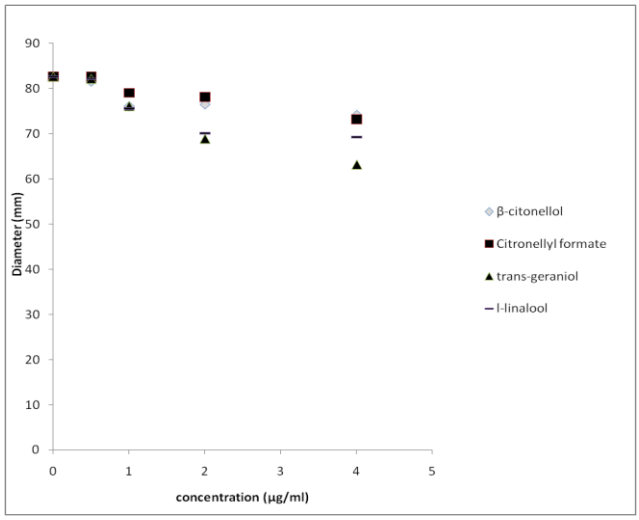

Fig 2: Inhibition of last swarm fronts in *P. mirabilis* by aroma chemicals

The bactericidal activity of the three alcohols has also been proved in the present study; l-linalool and trans-geraniol have exhibited remarkable activity against all the microorganisms as compared to β- citronellol. Weak antimicrobial activity of β-citronellol may be explained due to the presence of only one unsaturated bond in the molecule. High antimicrobial activity of trans-geraniol and l-linalool can be attributed to the presence of higher number of unsaturated bonds in the structure. The presence of both, primary alcoholic group at the end of the chain and unsaturated bonds in the chain may contribute to high inhibitory activity of trans-geraniol. The antimicrobial potential, in terms of inhibition zone diameter, MIC and MLC values were higher for citronellyl formate as compared to β- citronellol for all the test microorganisms. The presence of formate moiety in the structure appeared to increase the activity of the parent compound. The formate group acts like a hetero atom which increases the antimicrobial activity because of presence of lone pairs of electrons (Dorman and Deans, 2000).

Imelouane *et al.* (2009) and Belletti *et al*. (2004) have explained the antimicrobial activities of EOs having C10 and C15 long chain terpenes as their components, with aromatic rings and phenolic hydroxyl groups, which form hydrogen bonds with active sites of the target enzymes. The overall antimicrobial effect was attributed to other active terpenes, alcohols, aldehydes and esters.

The enantiomers of α-pinene, limonene and linalool have also been proved to be antimicrobial (Filipowicz *et al*., 2003; Koji *et al*., 2004; Tampieri *et al*., 2005).

Another interesting finding of the present study was that MIC and MLC values were similar for both the antibiotic sensitive and multidrug resistant urinary isolates. Hence, it can be concluded that *P. graveolens* essential oil and its components were not only active against the antibiotic sensitive but a high antimicrobial activity has also been observed for multidrug resistant isolates as well. The activity of essential oil and its components were independent of the level of antibiotic resistance to antibiotics. These results can be directly compared with the study by Mayaud *et al*. (2008), in which MIC of 13 different essential oils was found to be identical in both antibiotic susceptible and resistant strains.

Based on the present results, it can be concluded that β-Citronellol, citronellyl formate, trans-geraniol and l- linalool have a broad spectrum of antimicrobial activity. Hence, the aroma constituents of *P. graveolens* essential oil can be utilized for the development of natural antimicrobial agents. However, the safety and toxicity aspects of these chemicals needs to be addressed.

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