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Full Length Research Paper

Biocidal activity of citrus peel essential oils against some food spoilage bacteria

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Antibacterial activity of peel essential oils of various Pakistani *Citrus* species viz. fewtrell's early [*Citrus reticulata* (L.) var. Tangerine], orange [*C. reticulata* (L.) var. Mandarin], mousami [*Citrus sinensis* (L.) var. Mousami], malta [*C. sinensis* (L.) var. Malta], grapefruit (*Citrus paradisi* Macf.) and Eureka lemon (*Citrus limon* L.) were evaluated against food-borne bacteria namely *Escherchia coli, Salmonella typhii, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas fluorescence, Proteus myxofaciens, Staphylococcus epidermidis* and *Streptococcus* sp. The peel essential oil yield of the *C. reticulata* var. Mandarin, *C. reticulata* var. Tangerine, *C. sinensis* var. Malta, *C. sinensis* var. Mousami, *C. limon* and *C. paradisi* were 0.32, 0.24, 0.22, 1.21, 0.05 and 0.21%, respectively. The disc diffusion method was used to evaluate the inhibition zones of bacterial growth at two concentrations (5 and 10 ul) of the oil and results were compared with cefipime, a commercial antibacterial agent. Essential oils of *C. reticulata* var. Mandarin showed highest antibacterial activity followed by essential oils of *C. lemon*. Essential oils of *C. paradise* and *C. sinensis* var. malta exhibited least antibacterial activity. Among the various bacterial species, *S. typhii* and *P. myxofaciens* were found most susceptible to various *Citrus* peel essential oils. Present study concludes that essential oils of *C. reticulate* var. Mandarin have a broad spectrum antibacterial activity against food spoilage bacteria.

Key words: Antibacterial, *Citrus* spp, essential oils, food spoilage bacteria.

INTRODUCTION

Pakistan is one of the largest citrus producing countries of the world. Citrus is cultivated on an area of 197,000 ha with annual production of 1816,000 tones (Anonymous, 2004). Oranges, mandarins, lime, lemons and grape fruits are commonly grown verities of citrus in the country. Processing of citrus fruit results in peels and membrane residue from the juice extractor as a primary waste fraction; amounting approximately to 40 to 50% of wet fruit mass. Part of the peel is employed for a variety of uses, such as fodder at fisheries, activated carbon, raw materials for traditional paper, cosmetic materials and bio-ethanol (Kim et al., 2007; Sharma et al., 2007; Kim et al., 2008). Nevertheless, the peels are a potential source of essential oil in balloon shaped oil sacs of flavedo (Braddock, 1999) and yield oil in the range of 0.5 to 3.0 kg tone^{-1} of fruit (Sattar and Mahmud, 1986). Citrus essential oils contain large amounts of terpens, aliphatic sesquiterpene, oxygenated derivatives and aromatic hydrocarbons (Merle et al., 2004). The composition of the terpenic mix varies depending on the examined citrus species to which it owns. The mix of each species is in different proportion, made of: limonene, α-pinene, β-pinene, myrcene, linalool and terpinen (Ahmad et al., 2006; Hérent et al., 2007; Mohamed et al., 2010). Chemically, these oils are distinct from edible oils, because they are not esters of glycerides (Anonymous, 2005).

Versatile citrus essential oil is widely exploited in alcoholic beverages, confectioneries, soft drink, perfuming, soap, cosmetics and household products owing to its aromatic flavor. It also serves as masking agent in pharmaceutical products in abundant quantity (Lis-Balchin and Hart, 1999; Njoroge et al., 2005). While its ability to improve the shelf life and the safety of minimally processed fruits (Lanciotti et al., 2004), skim milk and low-fat milk (Dabbah et al., 1970) cannot be overlooked. Besides, citrus peel essential oil has been identified to exhibit antibacterial activity (Ayoola et al.,

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2008; Palakawong et al., 2010; Upadhyay et al., 2010). Bacteria related food poisoning is the most common, but fewer than 20 different bacteria actually are the culprits (Soković et al., 2007). Previous reports on the antimicrobial potency of peel essential oils of various citrus species against food spoilage bacteria suggest their utilization in food safety (Friedman et al., 2002). The exploitation of citrus peel essential oil as antibacterial agents oil could also provide substitution of the high cost antibiotics which have resulted in increased morbidity and mortality of pathogens (Lewis and Ausubel, 2006). Thus, the current investigation was aimed to determine the antibacterial activity of essential oils of six *Citrus* spp. namely *Citrus reticulata* var. Tangerine, *C. reticulata* var. Mandarin, *Citrus sinensis* var. Mousami, *C. sinensis* var. Malta, *Citrus paradisi* and *Citrus limon* against eight food spoilage bacterial species.

MATERIALS AND METHODS

Collection of plant materials

Fruits of six citrus varieties namely orange, mousami, malta and grapefruit were collected from local market, while lemon and tangerine were obtained at the mature stage from local fields of University of the Punjab Lahore, Pakistan.

Extraction procedure

One kilogram of fresh citrus peels of each of the six species were subjected to hydrodistillation for 4 h to obtain essential oil. The citrus peel residue was removed by filtration through filter paper. The essential oils were dried over anhydrous sodium sulfate and stored in sealed vials. Yields of essential oils obtained were calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of extract recovered}}{\text{Weight of fresh citrus peel}} \times 100$$

Source of microorganisms

Cultures of eight bacterial species namely *Escherchia coli, Salmonella typhii, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas fluorescence, Proteus myxofaciens, Staphylococcus epidermidis* and *Stretococcus* sp. were procured from Pathology Laboratory of Ittefaq Hospital and Fungal Culture Bank of Pakistan (FCBP), Institute of Plant Pathology, University of the Punjab, Lahore, Pakistan. Each bacterial strain was cultivated on Luria Broth (2% w/v), constant at 37°C for 18 h. One mililiter of culture was transferred to 9 ml of broth medium and incubated at 37°C for another 15 h; cell concentration was then adjusted to obtain final concentration of 10^6 cfu ml^{-1} using Luria Broth (Chanthaphon et al., 2008).

Evaluation of antibacterial activity

All hydrodistilled essential oils of citrus peels were tested for antibacterial activity against eight food spoilage bacterial pathogen through disc diffusion method (Kim et al., 1995). Stock solution of each variety of citrus peels oil was prepared at concentration of 500 µg ml^{-1} in dimethylsulfoxide (DMSO). From this known volume, that is, 5 and 10 µl of each essential oil was coated on separate sterile Whatman No. 1 filter paper discs measuring 6 mm in diameter. These oil-impregnated discs were made dry under laminar flow cabinet. one hundred microliters of each bacterial suspension was spread on nutrient agar medium with sterile L-shaped glass spreader and essential oil coated discs were positioned in the centre of inoculated agar plates. Each treatment was replicated thrice. Sterile distilled water was used as negative control, while antibiotic cefipime at concentration of 500 µg ml^{-1} as used as positive control for obtaining comparative results. All the plates were incubated for 24 h at 37°C and size of inhibition zone diameters surrounding filter paper disc was measured.

Statistical analysis

The data were statistically analyzed by applying ANOVA to know the significant difference in antimicrobial susceptibility of antibiotics, and various oils.

RESULTS AND DISCUSSION

Analysis of variance revealed that the effect of bacterial species, bactericides (essential oils and antibiotic), dose of the bactericides as well as their various interactions was significant (P≤ 0.001) for inhibition zone formation (Table 1). The peel essential oil yields of the *C. reticulata* var. Mandarin, *C. reticulata* var. Tangerine, *C. sinensis* var. Malta, *C. sinensis* var. Mousami, *C. limon* and *C. paradisi* were 0.32, 0.24, 0.22, 1.21, 0.05 and 0.21%, respectively.

There was no inhibition zone formation in control. The reference antibiotic cefipime exhibited the highest antibacterial activity. There was 15 to 40 mm and 8 to 30 mm inhibition zones due to 10 and 5 ul treatments of cefipime against different bacterial species. *S. epidermidis* and *P. myxofaciens* were found to be the most susceptible bacterial species for this antibiotic where 40 mm inhibition zone was recorded due to 10 ul dose followed by *Streptococcus* sp. and *S. typhii* with 30 mm inhibition zone each. *P. fluorescence* was found to be the most resistant species, where only 15 mm inhibition zone was recorded due to 10 ul treatment. In case of the rest of the bacterial species, the diameter of inhibition zone was 20 mm due to the highest dose of antibiotic (Table 2). Among the various essential oil treatments, the oil of *C. reticulata* var. Mandarin exhibited the highest antibacterial activity. It was effective against all the target bacterial species. Its activity against *E. coli*, *S. typhii*, *Streptococcus* sp. and *P. fluorescence* was at par with that of reference antibiotic with 20 to 30 mm and 9 to 16 mm inhibition zones due to the 10 and 5 ul treatments, respectively. This oil also showed considerable activity against the rest of the bacterial species with 10 to 30 mm and 6 to 16 mm inhibition zones due to the 10 and 5 ul, respectively (Table 2). Essential oil of *C. lemon* showed remarkable bioactivity against most of the test bacterial species. Its activity

Table 1. Analysis of variance for the effect of different concentrations of essential oils of different *Citrus* sp. against eight bacterial species.

Sources of variation	df	SS	MS	F values
Treatments	127	32602	257	292*
Bacterial species (S)	7	3654	522	594*
Bactericides (B)	7	13137	1877	2135*
Dose (D)	1	2974	2974	3383*
S × B	49	10120	207	235*
S × D	7	272	39	44*
B × D	7	1234	176	201*
S × B × D	49	1211	25	28*
Error	256	225	0.88	
Total	384	70537		

*significant at P≤ 0.001.

Table 2. Effect of different concentrations of essential oils of different *Citrus* sp. against eight bacterial species.

Treatments	Volume (µl)	Inhibition zone (mm)							
		E. coli	***E. cloacae***	***S. typhii***	***S. epidermidis***	***Streptococcus* Sp.**	***K. pnemonie***	***P. myxofaciens***	***P. flurescence***
Control	10	0^{i}	0^{g}	0^{j}	0^{h}	0^{i}	0^{g}	0^{j}	0^{i}
	5	0^{i}	0^{g}	0^{j}	0^{h}	0^{i}	0^{g}	0^{j}	0^{i}
Cefipime (antibiotic)	10	20^{a}	20^{a}	30^{b}	40^{a}	30^{a}	20^{a}	40^{a}	15^{b}
	5	11^{c}	10^{d}	14^{g}	30^{b}	14^{f}	11^{cd}	18^{d}	8^{f}
Citrus reticulate var. Mandarin	10	20^{a}	10^{d}	30^{b}	30^{b}	22^{g}	14^{b}	25^{c}	20^{a}
	5	9^{e}	6^{f}	16^{d}	12^{d}	10^{h}	10^{de}	12^{f}	9^{e}
C. sinensis var. Mmalta	10	8^{f}	10^{d}	10^{g}	0^{h}	0^{i}	0^{g}	12^{f}	10^{d}
	5	0^{i}	6^{f}	7^{i}	0^{h}	0^{i}	0^{g}	10^{g}	6^{g}
C. sinensis var. Mousami	10	8^{f}	12^{b}	11^{f}	9^{f}	20^{d}	14^{b}	10^{g}	0^{i}
	5	6^{g}	8^{e}	8^{h}	4^{g}	15^{c}	10^{e}	6^{i}	0^{i}
C. reticulata var. Tangerin	10	4^{h}	6^{f}	40^{a}	11^{e}	21^{c}	9^{e}	26^{b}	9^{e}
	5	0^{i}	0^{g}	27^{c}	4^{g}	14^{f}	4^{f}	18^{d}	4^{h}

Table 2. Contd.

C. limon	10	16[b]	10[d]	15[d]	20[c]	25[b]	0[g]	12[f]	14[c]
	5	10[d]	0[g]	6[i]	11[e]	14[f]	0[g]	0[j]	10[d]
C. paradis	10	0[i]	11[c]	16[d]	0[h]	20[d]	11[c]	13[e]	10[d]
	5	0[i]	6[g]	8[h]	0[h]	10[h]	0[g]	9[h]	6[g]

In a column, values with different letters show significant difference as ($P \leq 0.05$) as determined by Duncan's Multiple Range Test.

against *Streptococcus* sp. and *P. fluorescence* was at par with that of reference compound with 14 to 25 mm and 10 to 14 mm inhibition zones due to the 10 and 5 ul treatments, respectively. Both the doses also exhibited significant activity against *E. coli, S. typhii* and *S. epidermidis*. This oil was comparatively less effective against *E. cloaca*, *K. pneumoniae* and *P. myxofaciens* where 5 ul treatments failed to exhibit any antibacterial activity (Table 2). Peel essential oil of *C. sinensis* var.

Mousami was effective against *E. cloacae*, *S. epidermidis* and *K. pneumoniae* where both doses produced considerable inhibition zones with respect to reference antibiotics. Similarly, peel essential oil of *C. paradisi* was effective against *E. cloacae*, *Streptococcus* sp. and *P. fluorescence.* Peel essential oil of *C. sinensis* var.

Malta proved to be the least effective only showing low antibacterial activity against *E. cloacae*, *S. typhii*, *P. fluorescence* and *P. myxofaciens* (Table 2). Earlier, Belletti et al. (2004) reported that industrial citrus essences from sweet orange, red orangeade, bitter orange, red orange, sicily orange, sweet lime and red orangeade had inhibitory activity against *S. cerevisiae*. It has been reported previously that the monoterpene composition of the essential oil is accountable for the antibacterial activity. These compounds could be responsible for lethal action on microbial cell through destruction of the cellular integrity of microbial cells accompanied with loss of chemiosmotic control followed by inhibiting the respiration process (Cox et al., 2000; Pavithra et al., 2009). The antibacterial activity of the essential oil may also be correlated to a synergistic effect of all the chemical components present in the oil (Dorman and Deans, 2000). However, Nedorostova et al. (2009) reported that the leakage of intracellular metabolites due to Palakawong et al. (2010), interaction of essential oil with intracellular sites alters cell protein structures and, therefore, causes death of cell. Results of the present study reveal that in general peel essential oil of *C. reticulata* var. mandarin was highly effective against various food spoilage bacterial species followed by essential oils of *C. lemon*. Peel essential oil of *C. sinensis* var. Mousami and *C. reticulata* var.

Tangerin exhibited moderate antibacterial activity while *C. sinensis* var. malta and *C. paradisi* showed the least antibacterial activity (Table 2). Variable antibacterial activity of essential oils of different *Citrus* species is in agreement with previous studies (Soković et al., 2007; Nannapanenl et al., 2008; Pandey et al., 2010; Palakawong et al., 2010). The difference in antibacterial activity among the various Pakistani *Citrus* species could be due to variation chemical compositions of essential oils.

Main constituents of mandarin oil are dl-limonene, neo-dihydrocaveol and allo-ocimene. In orange oil, the principal compounds are linalool, α-terpinolene and nonyl-aldehyde. In lemon oil, camphene, α-citral, citronellal, and limonene are among the principal components. Major constituents presented in tangerine oil are limonene, citronellal and α-terpinene (Tao et al., 2009; Mohamed et al., 2010). It has been reported that among main components (limonene, linalool, and citral) in citrus oil, limonene showed the most abundant content, followed by linalool and citral, respectively (Hérent et al., 2007; Tao et al., 2008; Palakawong et al., 2010).

Fisher and Phillips (2006) reported that limonene showed the lowest effect against microorganisms. The inhibitory effect against microorganisms resulted from linalool rather than citral or limonene. Results of the previous studies showed that greater antimicrobial potential could be ascribed to the oxygenated terpenes, especially phenolic compounds (Soković et al., 2002; Couladis et al., 2004; Soković et al., 2005, 2006).

Among the various target bacterial species, *S. typhii* and *P. myxofaciens* were found to be vulnerable to essential oils of all the tested *Citrus* species. On the other hand, *P. flurescence*, *E. cloaca* ad *Streptococcus* sp. exhibited tolerance against one and *E. coli*, *S. epidermidis* and *K. pnemonie* against two *Citrus* species peel oil (Table 2).

Differences in the susceptibility of the test

organisms to essential oil could be attributed due to variation in the rate of essential oil constituent's penetration through the cell wall and cell membrane structures (Soković et al., 2007; Palakawong et al., 2010). Results of the present study conclude that among the various Pakistani *Citrus* species, essential oils of *Citrus reticulate* var. Mandarin are highly effective against a wide range of food spoiling bacteria.

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