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Antimicrobial Activity of Lime Essential Oil Against Food-borne Pathogens Isolated from Cream-filled Cakes and Pastries

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ABSTRACT

The volatile oil from *Citrus aurantifolia* (Christim) Swingle (lime) fruit peel is abundantly used as flavoring agent in food industries. In this study chemical composition and antimicrobial activity of essential oil *Citrus aurantifolia* against food-borne pathogens was determined to investigate its potential in reducing microbial population of cream-filled baked goods. Fifty components were identified in *Citrus aurantifolia* essential oil by GC-MS analysis and limonene, α-terpineol and γ-terpinen were the most abundant constituents. The results of bioburden determination showed that cream-filled cakes and pastries were mainly contaminated with *Staphylococcus epidermidis* and *Bacillus subtilis*. Lime essential oil showed potent antibacterial activity against spoilage bacteria. MICs (Minimum Inhibitory Concentration) of lime essential oil against *S. epidermidis* and *B. subtilis* were determined 4 and 8 μL $disc^{-1}$, respectively. By using 16 and 32 μL mL^{-1} of essential oil, more than 99.9% reduction in *S. epidermidis* and *B. subtilis* counts were observed, respectively. The use of *Citrus aurantifolia* essential oil in concentrations higher than MIC value can improve shelf life of cream-filled cakes and pastries. According to our results, lime oil can increase the time needed for the spoilage bacteria to reach concentrations able to produce a perceivable spoilage and it may consequently reduce the risk of diseases associated with consumption of contaminated products.

Key words: Lime, essential oil, *Citrus aurantifolia,* cream-filled cakes, antimicrobial activity, preservative

INTRODUCTION

Poisoning by cream-filled cakes and pastries is one of the most widespread food poisoning in humans, particularly in summer (Smith *et al*., 2004; Riemann and Cliver, 2006). The cream filling in cream-filled goods is usually contaminated and provides good nutritive media for foodborne

pathogens, particularly *Staphylococcus aureus* (Stewart *et al*., 2003). Therefore, introduction of reasonable methods for inhibition of bacterial growth in cream-filled baked goods seems to be necessary. Today, application of antimicrobial preservatives is important method in protecting the food supply. Chemical food preservatives such as salt, nitrites and sulfites have been used since many years ago for reduction of microbial contamination in susceptible food products, whereas the uncertain safety aspects of synthetic preservatives induce growing consumer demand for natural ones (Davidson *et al*., 2002; Meyer *et al*., 2002).

Since ancient times people have known antimicrobial properties of herbs and used them as food preservatives. The essential oil fractions commonly possess the major antimicrobial constituents of plant materials (Meyer *et al*., 2002; Joseph and Sujatha, 2011). Essential oil, also defined as essence, volatile oil, etheric oil or aetheroleum, is a complex mixture of structurally different volatile chemicals. They may comprise volatile compounds of terpenoid or non-terpenoid derivation. They may consist of alcohols, acids, esters, epoxides, aldehydes, ketones, amines, sulphides, etc. (Baser and Demirci, 2007).

The genus *Citrus*, containing 12 known species, is widely spread all over the world. *Citrus aurantifolia* (Christim) Swingle, commonly known as lime, is an important species of this genus. It is a flowering plant from Rutaceae family and it is native to Southern Asia and cultivated in the West Indies, semi-tropic areas of the U.S. and Central America (Gruenwald *et al*., 2000). Lime is also abundantly cultivated in south parts and south and southeast coasts of Iran including Bandar Abbas, Minab, Jahrom and Shiraz (Ghahreman, 1887). Literature existing on the antimicrobial activities of lime oil states its potent antibacterial (Aibinu *et al*., 2007) and antifungal effects (Barrera-Necha *et al*., 2009; Razzaghi-Abyaneh *et al*., 2009). In addition to antimicrobial activities, lime essential oil has several medicinal properties and potential health benefits which make it a good candidate as a natural antimicrobial preservative in food products. In the present study, chemical composition and antimicrobial activity of essential oil *Citrus aurantifolia* against food-borne pathogens was determined to investigate its potential in reducing microbial population of cream-filled cakes and pastries.

MATERIALS AND METHODS

Essential oil preparation and analysis: The edible oil of lime fruit peel was prepared from Zardband Company, Tehran, Iran in May 2009 and dried over anhydrous sodium sulfate and kept at 4°C in the sealed brown vials until required.

Analytical gas chromatography was carried out using a Termoquest 2000 GC with capillary column DB-5 (30 m. 0.25 mm i.d., 0.25 μm film Thickness); carrier gas, He; split ratio, 1:25 and using a flame ionization detector. The column temperature was programmed at 50°C for 1 min and then heated to 265°C at a rate of 2.5°C min^{-1} and then kept constant at 265°C for 20 min. GC-MS was performed on a Thermoquest 2000 with a quadruple detector, on capillary column DB-5 (GC); carrier gas, He; flow rate, 1.5 mL min^{-1}. The column was held at 50°C for 1 min and programmed up to 265°C at rate of 2.5°C min^{-1}, then kept constant at 256°C for 20 min. The MS operated at 70 eV ionization energy. Retention indices were calculated by using retention times of n-alkanes that were injected after the oil at the same chromatographic conditions.

Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the oils were identified by comparison of their mass spectra and retention indicates with Wiley library and those published in the literature (Sandra and Bicchi, 1987; Adams, 2004).

Sample preparation and bioburden determination: Forty Samples of cream-filled cakes and pastries were collected from local confectionaries (Tehran, Iran, April to July 2010) and transported to the laboratory within 1 h of purchase.

The cream part of each sample was separately added to sterile 0.1% peptone, homogenized in a stomacher for 2 min and diluted serially in 0.1% peptone solution. One hundred microliters aliquots of serial dilutions were spread-plated in triplicate on the surface of Tryptic Soy Agar (TSA; Merck, Germany) incubated at 35°C for total bacterial count and Sabouraud Dextrose Agar (SDA; Merck, Germany) incubated at 25°C for total fungal count. After 48 to 72 h, the average number of visible colonies obtained from plate counts were determined and transformed to log 10 values. Afterwards, single colonies were isolated using streak plate method and identified by cultivating in differential culture media and performing suitable biochemical tests.

Antibacterial activity of lime essential oil: Subsequently, antimicrobial potential of lime essential oil was screened against isolated microorganisms by disc diffusion method (CLSI, 2009a; Ali *et al*., 2010). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the essential oil against contaminating bacteria were also determined by broth microdilution method (CLSI, 2009b; Sivapriya *et al*., 2011) by using 96 U-shaped wells plates.

For the disc diffusion assay, Petri dishes with 25 mL of Mueller-Hinton agar were seeded with test strain suspension (1.5×10^8 CFU mL^{-1}) using a sterile cotton swab. Then, filter paper discs (6 mm in diameter) were impregnated with 1, 2, 4, 8, 16, 32 μL of lime oil and placed on the inoculated plates which were incubated at 37°C for 24 h. Inhibition was detected by measuring clear zones around discs in millimeters. The lowest concentration of essential oil showing a clear zone of growth inhibition around disc was taken as Minimum Inhibitory Concentration (MIC).

For MIC determination by microdilution method, a stock concentration of 10 μL mL^{-1} from essential oil was prepared in Mueller-Hinton broth (MHB, Merck Co. Germany) by using 10% v/v DMSO and 1% Tween 80. Then two-fold serial dilution of the stock solution of each oil (100 μL) was prepared by using MHB (100 μL) in ten wells. The stock microbial suspension with twofold test inoculum was prepared in MHB from a 24 h old culture. Then aliquot of 100 μL of twofold test strain inoculum was added to each well to reach the final inoculum size of 5×10^5 CFU mL^{-1}. After 24 incubation at 37°C the microdilution plates were tested for the absence or presence of visible growth in comparison with that of the growth in essential-free control well. The endpoint MIC is the lowest concentration of the essential oil at which the test strain does not demonstrate visible growth. The MBC was determined by quantitative subculture of 100 μL from each clear well onto MH agar plate. Plates were incubated at 37°C for 48 h. The MBC is defined as the lowest of essential oil that results in more than 99.9% killing of the bacteria being tested.

RESULTS AND DISCUSSION

The essential oil of the fruit peel of *Citrus aurantifolia* had yellow color with a distinct sharp odor. As shown in Table 1, 50 components were detected in the essential oil of *Citrus aurantifolia* representing 98.31% of the total oil. The major constituents of the oil with known antimicrobial effects, were limonene (53.53%), α-terpineol (9.41%) and γ-terpinen (6.26%) (Amiri, 2007; Talei and Meshkatalsadat, 2007). In particular, monoterpenes hydrocarbons were the most abundant compounds in the essential oil (76.21%). Cyclic terpene hydrocarbons like α-pinene together with β-pinene, limonene and terpinolene, were shown that they have toxic effects on microorganisms (Sikkema *et al*., 1995). As result of the lipophilic character, they accumulate in the lipid structure

Table 1: Chemical composition of the fruits of *Citrus aurantifolia* essential oil

Compound	RT	RRI	Percent
Unknown	1.59	-	0.52
Unknown	2.10	-	0.03
Isopentyl alcohol	3.47	722	0.01
n-nonane	8.47	928	0.04
α-pinene	9.62	957	2.48
Camphene	10.04	967	0.66
β-pinene	11.32	999	4.7
Myrcene	12.00	1013	1.31
α-phellandrene	12.42	1021	0.25
1,4-cineole	12.81	1030	0.18
α-terpinene	12.99	1033	0.29
p-cymene	13.16	1036	2.02
Limonene	14.32	1060	53.53
(Z)-β-ocimene	14.34	1062	0.16
(E)-β-ocimene	14.67	1069	0.48
γ-terpinen	15.23	1079	6.26
M-cymenene	16.04	1097	0.2
Terpinolene	16.42	1103	2.47
Linalool	16.82	1111	0.65
Endo-fenchol	17.25	1119	1.32
1-terpineol	17.93	1133	Trace
Cis-β-terpineol	18.50	1144	0.41
Borneol	19.51	1163	0.91
Terpinen-4-ol	20.17	1176	1.83
α-terpineol	21.05	1193	9.41
γ-terpineol	21.15	1195	0.2
Trans- carveol	21.88	1209	Trace
Geraniol	23.67	1245	0.08
N-decanol	24.67	1265	0.2
δ-elemene	27.70	1337	0.19
Neryl acetate	28.40	1358	0.44
Geranyl acetate	29.20	1381	0.51
β-elemene	29.94	1402	0.2
(E)-caryophyllene	31.07	1420	1.69
γ-elemen	31.60	1429	Trace
Trans-α-bergamotene	31.99	1435	2.19
α-humulene	32.36	1441	0.24
(E)-β-farnesene	32.67	1447	0.36
Unknown	33.27	1456	0.21
Germacrene D	33.39	1458	0.22
β-selinene	33.57	1461	0.19
Unknown	33.66	1463	0.12
γ-selinene	33.84	1465	0.29
α-selinene	34.00	1468	0.17
(Z)-α-bisabolene	34.43	1475	0.33
Unknown	34.83	1482	0.78
β-bisabolene	34.88	1483	0.32
(Z)-γ-bisabolene	34.95	1484	Trace
δ-cadinene	35.15	1487	Trace

Table 1: Continued

Compound	RT	RRI	Percent
(E)-γ-bisabolene	35.60	1493	0.19
selina-3,7(11)-diene	35.84	1498	Trace
germacrene B	36.30	1511	0.41
caryophyllene oxide	36.94	1530	0.16
α-cadinol	39.63	1606	Trace
Unknown	40.84	1630	0.03
α-bisabolol	40.94	1631	0.16
Oxygenated monoterpenes			14.54
Monoterpene hydrocarbons			76.21
Oxygenated sesquiterpenes			0.32
Sesquiterpene hydrocarbons			6.99
Non-terpenes			0.25
Total identified compounds			98.31

Table 2: Inhibition zone diameters of lime essential oil against isolated bacteria by disc diffusion method

	Lime oil concentration (μL $disc^{-1}$) / inhibition zone diameter (mm)				
Isolated bacteria	2	4	8	16	32
S. epidermidis	NZ[a]	9	12	14	16
B. subtilis	NZ	NZ	10	12	13

[a]NZ: no inhibition zone

Table 3: MIC and MBC of lime essential oil against isolated bacteria by microdilution method

Isolated bacteria	MIC (μL mL^{-1})	MBC (μL mL^{-1})
S. epidermidis	4	16
B. subtilis	8	32

of the cell wall which causes denaturing of proteins and loss of cell membrane integrity leading to cytoplasmic leakage and finally death of bacteria. Synergistic effects against pathogens may be resulted from the complex mixture of chemically different terpenes, oxygenated and non-oxygenated (Fisher and Phillips, 2008; Gallucci *et al*., 2009).

The results of bioburden determination showed that cream-filled samples were mainly contaminated with bacteria rather than fungi. Mean bacterial count was 2.7×10^2 CFU g^{-1} while mean fungal count was obtained lower than 10 CFU g^{-1} of tested samples. The results of Gram-staining indicated that the isolated bacteria were Gram-positive cocci and spore-forming bacilli. The Gram-positive cocci which was catalase positive, coagulase negative, sensitive to novobiocin with no fermentation of manitol was identified as *Staphylococcus epidermidis*. The spore-forming bacilli which was motile, citrate and VP positive, indole negative, fermented manitol and was sensitive to penicillin was identified as *Bacillus subtilis*.

As shown in Table 2, lime essential oil showed potent antibacterial activity against contaminating bacteria. MICs of lime essential oil against *S. epidermidis* and *B. subtilis* were determined 4 and 8 μL $disc^{-1}$ or mL^{-1} by both disc diffusion or microdilution method, respectively. In addition, by using 16 and 32 μL mL^{-1} of essential oil, more than 99.9% reduction in *S. epidermidis* and *B. subtilis* counts were observed, respectively which have been recorded as MBCs (Table 3). Hammer *et al*. (1999) showed that lime fruit oil cultivated in Western Australia inhibited *S. aureus* growth by more than 2.0 (% v/v).

Herbs are not ordinarily toxic at consumed levels and are Generally Recognized As Safe (GRAS) substances (Sunilson *et al*., 2009). Besides, plant-derived food additives have exhibited health-promoting effects such as antioxidant activities which may be beneficial to human health when they consumed regularly (Ali, 2009). Plant essential oils show great promise as natural preservatives due to their low bacteriostatic and bactericidal concentrations against some of the most important food-borne pathogens and also the growing demand for natural alternatives of artificial preservatives (Fazlara *et al*., 2008). Citrus oils have been commonly used as flavoring agent in food industries and are classified as GRAS, therefore they may be potentially ideal alternatives as starting point for the use of essential oils for antimicrobial preservation of foods. In previous studies, citrus oils showed notable results in preventing spoilage in different type of food such as fish, meat, chicken, fruit and vegetables, dairy products and confectionary (Fisher and Phillips, 2008).

Our observation indicated that the use of *Citrus aurantifolia* essential oil in concentrations higher than MIC value of 8 μL mL^{-1} in cream-filled cakes and pastries increases the time needed for the natural microflora to reach concentrations able to produce a perceivable spoilage and reduce the risk of diseases associated with consumption of contaminated products. In addition to its antimicrobial activity, lime essential oil has several medicinal properties and potential health benefits (Choi *et al*., 2000; Idu and Onyibe, 2007) which make it a good candidate as natural antimicrobial preservative in food products.

CONCLUSION

The present study introduces lime essential oil as natural antimicrobial preservative in cream-filled cakes and pastries. Present results showed that lime oil is good candidate for reducing microbial population of cream-filled cakes and pastries and it can be used to decrease the risk of food poisoning associated with consumption of these products.

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