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Antimicrobial properties of yuzu and lime oils and their storage stabilities in inclusion complex with cyclodextrin and oil-in-water emulsion

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ABSTRACT

We examined the antimicrobial properties of two essential oils obtained from yuzu (*Citrus junos*) and lime (*C. aurantifolia*) against ten microorganisms. We also kinetically examined the storage stability of the inclusion complex with β-cyclodextrin and oil-in-water emulsion prepared using the oils by measuring remaining limonene in each system. Both yuzu and lime oils effectively inhibited the growth of bacilli; however, there was the difference in their antimicrobial activities against fungi. We estimated the rate constant *k* for the disappearance of limonene in each oil and found that in comparison with bulk system, the inclusion complex had high stability, whereas the emulsion had low stability. Limonene in yuzu oil had lower *k* values than that in lime oil in the inclusion complex but not in the emulsion. We suggest that more compounds with polar groups (such as terpene alcohol) in lime oil can lead to differences in antimicrobial properties between the two oils and influence the stability of the inclusion complex and emulsion.

Keywords: antimicrobial activity; inclusion complex; lime oil; oil-in-water emulsion; yuzu oil

1. INTRODUCTION

Citruses are usually not suitable for raw consumption; thus, the juice extracted from these fruits is used for making processed products. Their residues, such as peel and seed, after extraction possess unique flavors and physiological components. Essential oils extracted from these residues contain these volatile and functional compounds. Therefore, essential oils are lipophilic and aromatic and used as a flavoring agent for food, beverages, and cosmetics (Fancello et al., 2016; Yorgancioglu and Bayramoglu, 2013). The appearance, taste, and smell of these essential oils have been investigated for many years along with their role in the maintenance and promotion of good health for elucidating the availability and mechanism underlying the development of products comprising functional oil components (Kuraya et al., 2017; Wua et al., 2013). A major component of essential oil is monoterpene, the oxidation of which leads to off-flavor in and deterioration of the product. In particular, limonene, a monocyclic monoterpene abundantly present in essential oils from citruses, produces odor and coloration upon oxidation due to the double bond in its molecular structure and its polymerization. This is one of the important problems for long-term storage of essential oils and its related products. In addition, the usage of these oils and their components is limited due to their strong

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lipophilicity. Cyclodextrin, a cyclic oligosaccharide, contains a minimum of six D-(+)-glycopyranose units attached by β-1,4-linkages (Bekers et al., 1991); the outside of this structure is hydrophilic and inside is hydrophobic. An inclusion complex is formed when a molecule fits entirely or partially into the cavity. Hydrophobic molecules, rather than hydrophilic ones, have a higher affinity to the cavity. Thus, the cyclodextrin complex is stabilized by various intermolecular forces such as hydrophobic interactions, van der Waals forces, hydrogen bonding, the release of strain energy in the macromolecular cyclodextrin ring, and the release of high energy water molecules during complex formation. The solubilization and stabilization of lipophilic compounds such as lipids and vitamin E have been studied by their inclusion in cyclodextrin (Kim et al., 2000; Regiert, 2005). This may also be effective for the storage and use of essential oils. Oil-in-water (O/W) emulsion (where oil and liposoluble components are dispersed into water phase) is a thermodynamically metastable disperse system that results in phase separation after creaming, flocculation, and coalescence due to high interfacial tension between the aqueous and oil phases. It may not be suitable for long-term storage but is useful as a form of essential oil in aqueous system.

Many essential oils, such as lime (*C. aurantifolia*) oil, are known to have antimicrobial ability (Burt, 2004; Calo et al., 2015; Jafari et al., 2011; Martos et al., 2008; Massilia et al., 2008; Simas et al., 2017), but the antimicrobial property of yuzu (*Citrus junos*) oil has not been fully investigated. In this study, we evaluate the antimicrobial properties of essential oils from yuzu and lime against ten microorganisms by disc diffusion test on solid medium and the measurement of the minimum inhibition concentration in liquid culture. Furthermore, we kinetically examined the storage stability of the inclusion complex with cyclodextrin and O/W emulsion (prepared using the oils) by measuring the remaining amount of limonene.

2. EXPERIMENTAL PROCEDURES

2.1 Materials

Yuzu (*C. junos Sieb.ex* Tanaka) cultivated in Kochi Prefecture in Japan and Mexican lime (*C. aurantifolia S.*), whose storage periods were less than 1 month after harvest, were used for preparation of the essential oils. The peels of yuzu and lime fruits were milled, and then each oil was obtained by extraction with diethyl ether from the pericarp powder and distillation of the solvent. *Bacillus subtilis* (NBRC3134), *B. coagulans* (NBRC12583), *B. licheniformis* (NBRC12200), *B. polymyxa* (NBRC3020), *B. stearothermophilus* (NBRC 12983), *B. acidocaldarius* (NBRC15652), *Saccharomyces cerevisiae* (NBRC10217), *Staphylococcus epidermidis* (NBRC100911), *Micrococcus luteus* (NBRC3333), and *Aspergillus niger* (NBRC9455) were obtained from the National Institute of Technology and Evaluation, Tokyo, Japan. Wild type GC4468 was used as *Escherichia coli*. (R)-(+)-Limonene, β-cyclodextrin (β-CD), polypeptone, yeast extract, agar powder, and polyoxyethylene (20) sorbitan monooleate (Tween 80) were purchased from Wako Pure Chemical Industries, Osaka, Japan. The hydrophilic surfactant SY-Glyster® ML-750 (decaglycerol monolaurate) was supplied by Sakamoto Yakuhin Kogyo, Osaka. All other chemicals of analytical grade were purchased from Wako Pure Chemical Industries or Yoneyama Chemical, Osaka.

2.2 Measurement of antimicrobial activities of yuzu and lime oils

Luria–Bertani medium was prepared as follows: 10 g of polypeptone, 10 g of sodium chloride, 5 g of yeast extract, and 15 g of agar powder were dissolved in 1 L of distilled water, and the pH was adjusted to 7.2 using 5 mol/L sodium hydroxide solution. Yeast and Mold medium were prepared as

follows: 10 g of D-glucose, 5 g of polypeptone, 3 g of yeast extract, 3 g of malt extract, and 15 g of agar powder were dissolved in 1 L of distilled water without pH adjustment. Potato dextrose agar medium was prepared as follows: 20 g of D-glucose, 4 g of potato dextrose, and 20 g of agar powder were dissolved in 1 L of distilled water, and pH was adjusted to 5.6 using 1 mol/L hydrochloric acid solution. Soybean–casein digest medium was prepared as follows: 20 g of polypeptone, 5 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, 2.5 g of D-glucose, and 15 g of agar powder were dissolved in 1 L of distilled water, and the pH was adjusted to 7.3 using 5 mol/L sodium hydroxide solution. Acidic medium (AC) was prepared as follows: 1 g of yeast extract, 0.2 g of ammonium sulfate, 0.5 g of magnesium sulfate heptahydrate, 0.25 g of calcium chloride dehydrate, and 0.6 g of dipotassium hydrogen phosphate were dissolved in 500 mL of distilled water, and the pH was adjusted to ca. 3.5 using 1 mol/L hydrochloric acid solution. Subsequently, 1 g of D-glucose and 20 g of agar powder were dissolved in 500 mL of distilled water without pH adjustment. These two solutions were mixed to obtain AC medium. Media was autoclaved at 121°C for 20 min and then used for the growth of each microorganism at the culture conditions shown in Table 1.

Disc diffusion tests against 10 microorganisms were performed for evaluating the antimicrobial abilities of Vyuzu and lime oils (Watanabe et al., 2013). Each microorganism was cultivated at 1.5×10^8 CFU/mL by McFarland turbidity. The corresponding medium was autoclaved and allowed to stand at a room temperature of 50°C. One hundred microliters of the culture and 20 mL of the medium was mixed and then solidified at ambient temperature. The filter paper disc, with a diameter of 5 mm, containing 10 µL of oil or limonene was placed on the corresponding culture plate. The microorganisms were cultivated on the plate at optimal temperature for 1–2 days, and the diameter of the zone of growth inhibition was measured. Growth inhibition was measured in four identical experiments, and the mean value was evaluated. The minimum inhibition concentration (MIC) of yuzu and lime oils on the growths of fungi was measured as follows: 0.9 mL of the oil was mixed with 0.1 mL of Tween 80, and 4.5% (v/v) oil solution was prepared by mixing it with the liquid medium. Then, 2.3, 1.1, 0.56, 0.28, 0.14, 0.070, 0.035, 0.018, and 0.009% (v/v) oil solutions were prepared by two-fold serial dilution. Two microliters of the culture with microorganism was added to each oil solution and incubated for 2 days. The growth was observed by visual examination of the application of culture on agar plate. Each experiment was measured in triplicate.

Table 1 Culture conditions for the growths of various microorganisms

Microorganism	Culture Medium	Incubation Temperature (°C)
B. subtilis	LB	37
B. coagulans	LB	37
B. licheniformis	LB	30
B. polymyxa	LB	30
B. sterarothermophilus	SCD	50
B. acidocaldarius	CD	50
E. coli	LB	37
S. cerevisiae	YM	30
S. epidemidis	LB	37
M. luteus	LB	37
A. niger	PDA	30

2.3 Storage stability experiments on bulk oil, inclusion complex, and O/W emulsion systems with yuzu and lime oils

For the experiments on bulk oil system, 1 mL of yuzu or lime oil was placed in an amber glass vial with

a screw cap. The vial was stored in a mechanical convection oven at 37°C and 75°C. Ten microliters of oil was periodically taken and diluted 100 fold using 1% (v/v) methyl myristate methanol solution. The oil solution was used to measure the transient change in the amount of limonene remaining in the oils by gas chromatography (GC) analysis. The inclusion of the oil to β-CD was executed according to the previous method with a modification (Watanabe et al., 2009); 0.272 g of the oil was added to 15 mL of 70% (v/v) ethanol solution with 1.14 g β-CD and homogenized using a rotor/stator homogenizer (Ultra-Turrax T25, IKA® Japan, Nara, Japan) at 8×10^3 rpm for 30 min, with the tube immersed in ice water. After leaving for 3 h at ambient temperature, the mixture was centrifuged at 6×10^3 rpm for 15 min. The precipitate was dried in a desiccator with silica gel under reduced pressure for 1 day. The dried powder was filtered using a 100-mesh filter, and 1.5 g of the filtrate was stored in the amber vial with the cap at 37°C and 75°C. The powder (12 mg) was sampled at appropriate intervals and added to 1% (v/v) methyl myristate methanol solution. After vigorously mixing and centrifugation at 1.3×10^4 rpm for 3 min, the supernatant was applied to GC analysis of remaining limonene. The O/W emulsions with the oils were prepared using the reported procedure (Watanabe et al., 2010). Nine milliliters of 1% (w/v) ML-750 solution and 1 mL of yuzu or lime oil were added to a tube. The mixture was emulsified using the homogenizer for 5 min at 1 × 104 rpm in the tube cooled by placing in ice water to produce the coarse O/W emulsion. After pre-emulsification, coarse emulsion was circularly passed through a membrane filter with a pore size of 0.8 μm, using a peristaltic pump for 30 min at 2.0 mL/min to reduce and monodisperse the diameter of the oil droplets. The prepared emulsion was put into the capped amber vial and stored at 37°C with magnetic stirring at 200 rpm. Ten microliters of the emulsion was periodically removed from the vial and diluted by 1% (v/v) methyl myristate methanol solution. The diluted sample was used to measure the particle size distribution of the oil droplets using a centrifugal particle size analyzer (SA-CP3L, Shimadzu Co., Kyoto, Japan). The particle size distribution was measured in triplicate and mean value was calculated for investigating emulsion stability. The 100-fold diluted sample was also used for the GC analysis.

2.4 Evaluation of the stability of limonene in three systems using gas chromatography analysis

GC analysis was performed using G-3900 (Hitachi High-Tech Science Co., Tokyo) equipped with a hydrogen flame ionization detector and a Rtx-2330 capillary column (0.32 mm ϕ × 15 m, GL Science, Tokyo). Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The column temperature was increased at 20°C/min from 80 to 140°C and the temperature was held for 3 min. The injector and detector temperatures were 220°C and 240°C, respectively. Methyl myristate was used as an internal standard for the GC analysis.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial properties of yuzu and lime oils

We measured the diameter of inhibition circle in the disc diffusion test, which reflected the antimicrobial ability of the tested sample. We found that the diameter for lime oil was larger than that for yuzu oil against all the microorganisms, indicating higher antimicrobial ability of lime oil (Table 2). This difference in the inhibition diameter of the two oils depended on the type of microorganism. The diameter for lime oil was about 2-fold larger than that for yuzu oil against six microorganisms and 1.3-fold higher against *B. polymyxa*. In addition, the diameter for lime oil was 9.0 mm against *E. coli*, whereas yuzu oil did not exhibit any inhibitory activity against it. These differences could be attributed to the difference in the compositions of the two oils.

The most detected component in these two oils is limonene (55.8% and 26.6% in yuzu and lime oils, respectively) (Tachibana et al., 2011), which inhibited the growth of *B. subtilis* but not *E. coli*. Therefore, we hypothesized that the other components of the oils must also influence the growths of these microorganisms. Because disc diffusion test may be subject to the influence of the diffusivity of oil on plate, we measured the MICs of yuzu and lime oils on the growth of microorganisms (Table 3), wherein low MIC corresponds to high antimicrobial activity of the oils. MICs of lime oil were lower than those of yuzu oil for nine microorganisms, except *B. sterarothermophilus*. Thus, the antimicrobial activity of lime oil was higher than that of yuzu oil. MIC of yuzu oil could not be determined against *E. coli*, *M. luteus*, and *A. niger* at the concentration of 4.5% (v/v). Limonene also showed a tendency consistent with the results in disc diffusion test. The amount of clitoral (such as neral and geranial) in lime oil was about 13 fold that of the amount in yuzu oil (Tachibana et al., 2011). MIC of citoral against *B. subtilis* and *E. coli* was very low, indicating high antimicrobial activity of citoral (Table 3). In addition, lime oil has more terpenes and terpene alcohols with polar groups than yuzu oil (Tachibana et al., 2011). Similar to citoral, the terpenes (*p*-cymene, β-pinene, and β-bisabolene) and the terpene alcohols (β-terpineol, borneol, β-terpineol, fenchyl alcohol, terpinene-1-ol, and *p*-cymene-8-ol) more abundantly present in lime oil may function as strong antimicrobial reagents. Both oils were effective for the growth inhibition of *Bacillus* species. *Bacillus* species have a spore forming ability, and an inhibitory effect of the antibacterial compounds on the development of spores of the bacteria has been previously reported (Sugimoto et al., 1998). The components in these oils could also inhibit spore formation in bacteria. Lime oil showed antimicrobial effect against all the tested microorganisms. On the other hand, yuzu oil inhibited the growths of *Bacillus* species but had little or no antimicrobial activity against *E. coli* and fungi. Lime oil was also effective for microorganisms with different cell membrane, although the mechanism remains unclear; the abovementioned terpene alcohols, which are abundant in lime oil, could contribute to this.

Table 2 Inhibition diameters by yuzu and lime oils in disc diffusion test on the growth of microorganism

Microorganism	Inhibition diameters (mm)		
	Yuzu	Lime	Limonene
B. subtilis	9.0	18.0	6.0
B. coagulans	8.0	16.0	
B. licheniformis	8.0	16.0	
B. polymyxa	7.0	9.3	
B. sterarothermophilus	15.0	23.7	
B. acidocaldarius	14.7	22.7	
E. coli	0.0	9.0	0.0
S. cerevisiae	7.0	13.3	
S. epidemidis	8.0	15.0	
M. luteus	7.0	14.0	

Table 3 Minimum inhibition concentrations (MIC) of yuzu and lime oils on the growth of microorganism

Microorganism	MIC (% v/v)			
	Yuzu	Lime	Limonene	Citoral
B. subtilis	1.1	0.28	2.3	0.039
B. coagulans	2.3	0.28		
B. licheniformis	1.1	0.56		
B. polymyxa	0.28	0.28		
B. sterarothermophilus	0.14	0.28		
B. acidocaldarius	0.56	0.28		
E. coli	>4.5	1.1	>4.5	0.16
S. cerevisiae	0.56	0.28		
S. epidemidis	4.5	1.1		
M. luteus	>4.5	1.1		
A. niger	>4.5	2.3		

3.2 Limonene stability in inclusion complex and O/W emulsion containing yuzu or lime oils

We observed transient changes in remaining fraction of limonene in inclusion complex with β-CD and O/W emulsion containing yuzu and lime oils at 37°C and 75°C (Figure 1). Limonene in the inclusion complex was more stable than the bulk system at 37°C in both the oils. Limonene in the emulsion decreased the fastest in three systems. In the bulk and inclusion systems, the stabilities of limonene at 75°C were lower than 37°C. The stability kinetics was empirically expressed by the flexible Weibull equation, which describes deterioration kinetics (Cunha et al., 1998):

$$R = \exp [- (kt)^{n}] \quad (1)$$

where R is the fraction of remaining limonene at time t, k is the rate constant, the reverse of which is called the scale parameter, and n is the shape constant. The kinetic parameters, k and n, were evaluated by fitting the experimental results by nonlinear regression (Table 4).

Figure 1 is based on the equation using estimated parameters. The k value for the disappearance of limonene in the inclusion systems was lower than the bulk system of both oils and temperatures, suggesting the availability of inclusion with β-CD for storage regardless of oil and temperature. On the other hand, the k values in the emulsion at 37°C were the highest for both oils. The k values in bulk and inclusion systems for both oils significantly increased with temperature. Limonene in yuzu oil had lower k values than lime oil except for the emulsion system. The k value for lime oil in the emulsion was a little higher than that for yuzu oil. As described before, lime oil has more compounds with polar groups, such as terpene alcohol, than yuzu oil. These components could affect the storage stability of limonene. For example, non-polar limonene in lime oil may be apt for being included in β-CD compared to terpene alcohols. Consequently, the slightly high stabilities in the inclusion system for lime oil were shown at all temperatures. This difference of polar compounds between two oils may also contribute to the stability in the emulsion. Figure 2 shows the transient changes in mean diameters of oil droplets in the emulsions with yuzu and lime oils at 37°C. The diameters of oil droplets increased with time in both oils, with lime oil having larger diameters indicating its low emulsifying stability of the emulsion. The presence of polar compounds in lime oil could also lead to destabilization of the emulsion. The above-mentioned high k value for lime oil in the emulsion would be ascribed to the low stability of the emulsion with lime oil. The Weibull model has characteristics of a sigmoidal pattern that can be described when $n > 1$, where the model expresses the simple first-order kinetics at $n = 1$, and this fraction steeply decreases during the early stage when $n < 1$. The significant tendency about the n values was not observed among the systems.

Table 4 Kinetic parameters of the Weibull equation for the storage stability of limonene in several system containing yuzu and lime oils

	Temperature	Yuzu		Lime	
		k [d^{-1}]	n	k [d^{-1}]	n
Bulk oil	37°C	5.37×10^{-6}	0.284	4.16×10^{-8}	0.177
Inclusion complex with β-cyclodextrin	37°C	8.41×10^{-9}	0.150	4.45×10^{-11}	0.105
Oil-in-water emulsion	37°C	2.22×10^{-2}	0.903	3.79×10^{-2}	0.599
Bulk oil	75°C	5.69×10^{-2}	1.975	4.11×10^{-2}	0.551
Inclusion complex with β-cyclodextrin	75°C	3.06×10^{-2}	1.282	3.87×10^{-4}	0.225

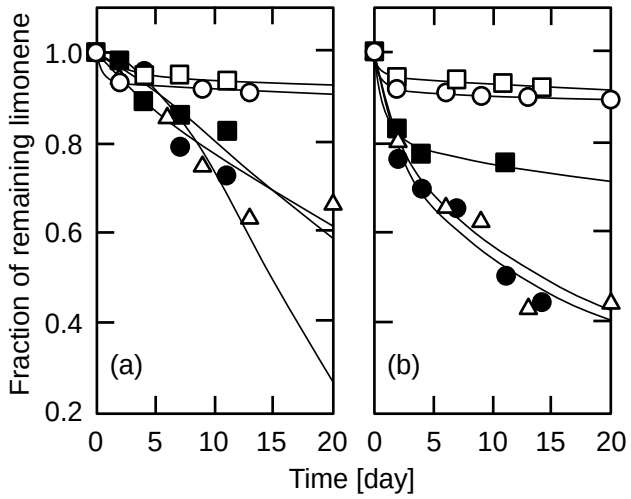

Figure 1 The transient changes in remaining fraction of limonene in (○,●) bulk, (□,■) inclusion complex with β-CD and (△) O/W emulsion systems containing (a) yuzu and (b) lime oils. Open and closed symbols represent the storage temperatures at 37°C and 75°C. The solid curves were depicted using the estimated Weibull parameters, k and n, for the storage stability of limonene.

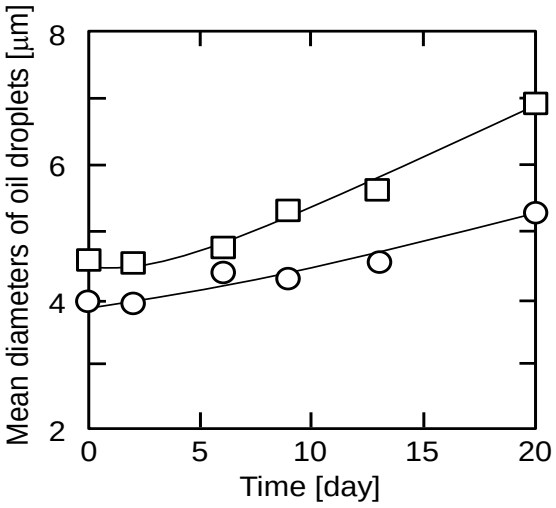

Figure 2 The transient changes in mean diameters of oil droplets in the O/W emulsions with (○) yuzu and (□) lime oils at 37°C.

4. CONCLUSION

Yuzu and lime oils showed similar antimicrobial properties and storage stabilities of limonene in three systems, but there were minor differences in the antimicrobial activity against some microorganisms, and between stabilities in the inclusion complex and the emulsion. We propose that these differences could result from the difference in the compositions of the two oils.

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