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Inhibitory Effect of the Essential Oil from *Chamaecyparis obtusa* on the Growth of Food-Borne Pathogens

Mi-Jin Park[1], Won-Sil Choi[1], Ha-Young Kang[1], Ki-Seob Gwak[2], Geun-Shik Lee[3], Eui-Bae Jeung[3], and In-Gyu Choi[2,4]*

[1]Division of Green Business Management, Department of Forest Resources Utilization, Korea Forest Research Institute, Seoul 130-712, Republic of Korea
[2]Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea
[3]College of Veterinary Medicine, Chungbuk National University, Chungbuk 361-763, Republic of Korea
[4]Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea

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In this study, the antibacterial activity of essential oil from *Chamaecyparis obtusa* (Sieb. et Zucc) leaves and twigs was investigated. The test strains were *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Legionella pneumophila*, and Methicilline-resistant *Staphylococcus aureus*. Antibacterial activity was estimated by measuring bacterial growth inhibition. Histopathological examination was also performed. *C. obtusa* oil distinctly inhibited the growth of all test strains and exhibited the strongest antibacterial activity against *L. monocytogenes*. It was chromatographically divided into several fractions. The fractions were further tested against antibacterial activity and their chemical compositions were analyzed. The fraction containing terpinen-4-ol (TA) showed high antibacterial activity toward all strains tested. Tests with authentic samples showed that TA played a major role in the antibacterial activity of *C. obtusa* oil, and in a mice test, the oil actively minimized inflammation by *S. aureus*.

***Keywords*:** *C. obtusa*, essential oil, antibacterial activity, terpinen-4-ol

Food-borne illnesses are caused mainly by food contaminated with pathogenic bacteria. According to the statistical data of the Korea Food and Drug Administration, the outbreak of food-borne illnesses in 2001-2005 was caused primarily by pathogenic bacteria such as *Salmonella* sp., *Vibrio* sp., *Staphylococcus* sp., and norovirus (http://fm.kfda.go.kr). In 1996, food poisoning cases occurred in Japan because of *Salmonella* sp., *Vibrio* sp., pathogenic *Coliform bacilli*, *Camphylobacter jejuni*, and *Staphylococcus aureus*. In 2005, the outbreak rate of food poisoning by *Camphylobacter jejuni* was two times higher compared with that by other fungi (http://fm.kfda.go.kr). Unfortunately, the increase in meal provision facilities such as those found in schools brought about an increase in food poisoning outbreak. Various methods are needed in order to reduce such epidemics. In general, natural products that can inhibit pathogenic bacterial growth and extend food shelf life have been developed and used since ancient times. As a natural product, essential oils have been regarded as a potential material for inhibition of pathogenic microbial growth due to its unique fragrance and antimicrobial properties.

Chamaecyparis obtusa, called Japanese cypress in Japan and Taiwan, is a coniferous tree used for construction and furniture due to its good structural properties and natural scent. This species was introduced in the central and southern region of Korea in the beginning of the 20th century. *C. obtusa* oil, the essential oil extracted from pruned leaves and twigs of the *C. obtusa* tree, has been commercially used in soap, toothpaste, and cosmetics as a functional additive with good fragrance. However, scientific studies are insufficient when it comes to the biological functionality of these products. Several studies on the chemical composition and biological activities of *C. obtusa* oil reveal that it contains α-pinene, sabinene, myrcene, limonene, γ-terpinene, terpinen-4-ol, α-terpinyl acetate, and elemol (Hong *et al*., 2004), all of which are chemically classified as terpenoids. Physiologically, *C. obtusa* oil affects the nervous system similar to the way a sedative does, as proven by an experiment on mice (Na *et al*., 1999). Moreover, it has an alleviative effect on stress (Na *et al*., 1998). As an antimicrobial material, *C. obtusa* oil has high antifungal activity against wood rot fungi and plant pathogenic fungi (Na *et al*., 1998) and exhibits antibacterial effects against *Staphylococcus epidermidis*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Candida albicans* (Lee *et al*., 2001), *Aeromonas* sp., *Edwardsiella tarda* ECK-1, and *Streptococcus* sp. (Lee, 1999). Its insecticidal and allelopathic effects were reported by Park *et al*. (2003) and Kil (2000), respectively.

As mentioned above, the antifungal and antibacterial activities exhibited by *C. obtusa* oil have already been demonstrated. However, quantitative data related to its antibacterial activity against pathogenic bacteria are lacking. Moreover, the efficacy of *C. obtusa* oil antibacterial compounds against food-borne pathogens has not been reported to date.

* For correspondence. E-mail: cingyu@snu.ac.kr; Tel: +82-2-880-4785; Fax: +82-2-873-2318

Therefore, this study was undertaken to investigate the antibacterial effects of *C. obtusa* oil against *Klebsiellla pneumoniae*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Legionella pneumophila*, and Methicillin-resistant *Staphylococcus aureus.* In relation to this, this study aims to identify the antibacterial compounds of *C. obtusa* oil.

Materials and Methods

Materials and microorganisms

The *C. obtusa* oil used in this study was provided by Enbita Co., Ltd., Korea and produced through steam distillation of pruned twigs and leaves of the *C. obtusa* tree. Terpinen-4-ol (TA, 99%, w/w) and Mueller Hinton agar were purchased from Sigma-Aldrich (Korea) and Fluka BioChemika (Buchs, Switzerland), respectively and used for the antibacterial assay.

Test strains used were *K. pneumoniae* (KCTC 2241), *L. monocytogenes* (KCTC 3569), *S. typhimurium* (KCTC 12401), *S. aureus* (KCCM 11764), *E. coli* O157:H7 (KCCM 40406), *L. pneumophila* (ATCC 33216), and Methicillin-resistant *S. aureus* (MRSA, CCARM 3089). The Center of Biological Resources of the Korea Research Institute of Bioscience and Biotechnology provided the *K. pneumoniae*, *L. monocytogenes*, and *S. typhimurium* strains. The Korea Culture Center of Microorganisms supplied the *S. aureus*, *E. coli* O157:H7, and *L. pneumophila* strains, while the MRSA was obtained from the Culture Collection of Antimicrobial Resistant Microbes. The bacterial cultures were maintained at 37°C in different media as shown in Table 1.

Essential oil fractionation

The *C. obtusa* oil was divided into seven fractions (Fr. A-Fr. G) according to a previous method (Choi *et al*., 2006). Briefly, the essential oil was divided into seven fractions by silica-gel column chromatography with n-hexane-ethyl acetate mixture elution based on TLC classification and concentration.

In vitro antimicrobial assay

Antibacterial activity was evaluated via paper disc diffusion and agar dilution methods. In the paper disc diffusion method, paper discs (8 mm in diameter) were impregnated with test samples (50 μl for *C. obtusa* oil, 20 μl for the fractions and TA) and placed on the agar plates (780 mm in diameter) spread with 200 μl cell suspension of bacteria, respectively. For the preparation of the inoculation, tested bacteria were cultured at 37°C for 24 h. Cell suspensions were collected by scrapping and the suspensions were standardized to the final concentration of 1×10^8 CFU/ml with 0.9% sterile saline solution (w/w) by microscopic examination. The inoculated plates were incubated at 37°C for 24 h. The antibacterial activities were evaluated by measuring the diameter of the halo zone, which presented inhibition against the test organisms. All treatments were replicated three times.

Minimum inhibitory concentration (MIC) of the tested essential oils was determined using the agar dilution method (Oussalah *et al*., 2007). Briefly, Petri plates of culture media containing various concentrations of essential oil were inoculated with the cell suspension solution of each bacterial strain. All treatments were replicated three times. Plates were evaluated for the presence or absence of colonies after 48 h of incubation.

Table 1. Culture media according to each microorganism

Microorganism		Culture medium
Gram-negative	*K. pneumoniae*	Nutrient agar
	S. typhimurium	Nutrient agar
	E. coli O157:H7	Trypticase soy agar
Gram-positive	*L. monocytogenes*	Brain heart infusion agar
	S. aureus	Nutrient agar
	L. pneumophila	Charcoal yeast extract buffered medium
	MRSA	Nutrient agar

In vivo test of terpinen-4-ol

Animals

A total of 36 female ICR mice weighing 15 to 20 g were obtained from Dae Han Biolink Co., Ltd. (Korea). The mice were housed in an environmentally controlled room (temperature: 20±3°C, relative humidity: 30±10%, frequent ventilation, and 12 h light cycle) and were fed the standard laboratory food and water *ad libitum*. All experimental and animal procedures were approved by the Ethics Committee of Chungbuk National University.

Bacteria preparation for infection

S. aureus was used for evaluating the antibacterial effect of TA. The organism was cultured in Mueller Hinton broth at 37°C for 24 h. It was harvested and the culture medium was washed out with saline. Before the bacterial fluid was used, it was first diluted to a final concentration of 10^8 CFU/ml.

Experimental design

Before the experiment, hair on both flanks of all the mice were widely clipped and all 36 mice were randomly divided into two groups, infection group and non-infection group, with the latter as the negative control. Each group was divided into three subgroups: non-TA treated, TA treated once, and treated twice a day. Bacterial infection was performed through subcutaneous injection of 100 μl diluted bacterial fluid (10^7 CFU). Treatment was performed through topical application of 10 μl TA. Animals were sacrificed at 4 and 7 days after bacterial infection. The skin lesions were collected for evaluation through histopathological examinations.

Histopathological examination

Skin samples were fixed in 10% buffered neutral formalin and then paraffin block processed. The inoculated lesions were selected and stained with hematoxylin-eosin. Microscopic evaluation was performed to characterize the antibacterial effect of TA.

GC-MS analysis

Essential oils were analyzed by gas chromatography/mass spectrometry (GC-MS) to identify their chemical compositions. The GC (HP 6890, USA) was equipped with an HP-5 MS column (length: 30 m, diameter: 0.25 mm, film thickness: 0.25 μm, Hewlett Packard, USA). Inlet and MSD transfer line heater temperatures were maintained at 250°C and 280°C, respectively. Helium was employed as the carrier gas at 1 ml/min flow rate. The initial oven temperature was maintained at 60°C for 10 min and then increased to 280°C at a rate of 5°C/min. The temperature was held for 10 min. Mass spectrometry (HP 5973) was used at EI mode under the following conditions: ionization voltage, 70 eV; scan range, 35-600 m/z ion; source temperature, 200°C. The chemical structure of each constituent was identified by comparing the mass data of their peaks with the standard library data, Wiley7N (Agilent Technologies, USA).

Table 2. Antibacterial activities of *C. obtusa* oil

Test bacteria		*C. obtusa* oil	
		Inhibition zone (mm)[a]	MIC (%)[b]
Gram-negative	*K. pneumoniae*	11.9±1.5	>0.8
	S. typhimurium	12.4±1.0	>0.8
	E. coli O157:H7	11.2±0.3	0.8
Gram-positive	*L. monocytogenes*	21.9±4.3	>0.8
	S. aureus	11.1±0.6	>0.8
	L. pneumophila	12.2±0.6	>0.8
	MRSA	12.8±0.7	>0.8

[a] Diameter of inhibition zone including disc diameter of 8 mm
[b] MIC, minimum inhibitory concentration (v/v, solution concentration)

Results

Antibacterial activities of *C. obtusa* oil

The inhibition zone diameter and MIC were measured to investigate the antibacterial activities of *C. obtusa* oil against pathogenic bacteria. As shown in Table 2, *C. obtusa* oil inhibited the growth of all pathogenic bacteria used in this study. For the Gram-negative bacteria, *K. pneumonia*, *S. typhimurium*, and *E. coli* O157:H7, the inhibition zone diameters were 11.9, 12.4, and 11.2 mm, respectively. For the Gram-positive bacteria, *L. monocytogenes*, *S. aureus*, *L. pneumophila*, and MRSA, the inhibition zones were 21.9, 11.1, 12.2, and 12.8 mm, respectively. *C. obtusa* oil exhibited the highest antibacterial activity against *L. monocytogenes*.

The MIC of *C. obtusa* oil against *E. coli* O157:H7 was 0.8% by volume, while in the other strains, MICs were more than 0.8%.

Antibacterial activities of *C. obtusa* oil fractions

To investigate the active antibacterial constituent, *C. obtusa* oil was divided into seven fractions. The antibacterial activity of each fraction was examined via the same method used in crude *C. obtusa* oil. The results are shown in Table 3.

Three Gram-negative bacteria were more resistant than the Gram-positive strains in most fractions, while fraction D was active for all Gram-negative bacteria and showed higher activity than that of crude *C. obtusa* oil.

Antibacterial activity toward *S. typhimurium* was shown only in fraction D (16.8 mm) and toward *K. pneumoniae* in fraction A (13.2 mm) and D (15.7 mm). Fractions A (13.3 mm), B (10.8 mm), D (23.5 mm), and G (9.2 mm) exhibited antibacterial activity against *E. coli* 0157:H7. All fractions demonstrated antibacterial activity against *L. monocytogenes*, *L. pneumophila*, and MRSA. Among the tested fractions, fraction D showed the highest activity toward *K. pneumonia*, *S. typhimurium*, *E. coli* O157:H7, *L. pneumophila*, and MRSA. Fractions B and F demonstrated the highest antibacterial activity against *S. aureus* and *L. monocytogenes*, respectively.

From the results, fraction D was estimated to have a wide antibacterial spectrum and prominent bacteriostatic activity, even if fractions B and F were also considered.

Major constituents of the active fraction

Based on the results of the fractions' antibacterial assay, the major constituents of fraction D were identified by GC-MS analysis. Results are provided in Table 4. Fraction D consisted of monoterpenes and sesquiterpenes. The monoterpenes were linalool, TA, nerolidol, and α-cedrol, while the sesquiterpenes were δ-selinene, δ-cadinene, and α-bisabolol. Terpinen-4-ol content was 53% in fraction D and amounted to 1% of the crude *C. obtusa* oil. Fraction D also contained 9.6% δ-cadinene, 6.33% δ-selinene, 5.1% α-cedrol, 4.3% α-bisabolol, 3.81% nerolidol, and 2.5% linalool.

Antibacterial activity of terpinen-4-ol

Terpinen-4-ol was evaluated as a major component of the D-fraction, having the highest antibacterial activity among the seven fractions. It was tested for antibacterial activities to investigate the role of TA in *C. obtusa* oil.

As shown in Table 5, TA had a similar antibacterial spectrum pattern with that of the D-fraction while showing the highest activity of 33.8 mm clear zone diameter against *S. typhimurium* compared with the D-fraction against *E. coli* O157:H7. TA also showed antibacterial activities with clear zone diameters of 25.0 mm for *K. pneumonia*, 20.0 mm for MRSA, 18.8 mm for *E. coli* O157:H7, 15.6 mm for *L. monocytogenes,* and 14.6 mm for *S. aureus*.

For TA antibacterial activities, the Gram-positive bacteria were inclined to be more resistant than the Gram-negative

Table 3. Antibacterial activities of subfractions of *C. obtusa* oil

Test bacteria		Inhibition zone diameter[a]						
		Fr. A	Fr. B	Fr. C	Fr. D	Fr. E	Fr. F	Fr. G
Yield (percent, w/w) on chromatographic fractionation[b]		70.9	9.5	1.6	1.8	5.3	0.9	10.0
Gram-negative	*K. pneumonia*	13.2±0.7	n[c]	n	15.7±0.3	n	n	n
	S. typhimurium	n	n	n	16.8±0.1	n	n	n
	E. coli O157:H7	13.0±0.4	10.8±1.1	n	23.5±0.4	n	n	9.2±0.1
Gram-positive	*L. monocytogenes*	10.2±0.3	10.7±0.1	10.5±0.1	17.5±0.4	17.4±1.0	22.8±0.1	15.5±1.0
	S. aureus	14.3±0.3	17.6±0.2	n	12.9±0.4	10.7±0.1	11.6±0.4	14.7±0.2
	L. pneumophila	8.8±0.2	8.8±0.3	9.7±0.5	13.8±0.3	11.0±0.3	10.4±0.5	10.8±0.4
	MRSA	12.0±0.7	12.3±1.0	9.5±0.5	19.9±1.5	16.4±1.3	13.0±0.4	12.1±2.6

[a] Diameter of inhibition zone including disc diameter of 8 mm.
[b] based on single running
[c] n, no inhibition observed

Table 4. Major constituents of active antibacterial subfraction D of *C. obtusa* oil identified by GC-MS

Fraction	Constituent	Area (%)[a]	Mass spectral data[b]
	Linalool	2.5	71(BP), 93, 43, 55, 80, 121
	Terpinen-4-ol	53.08	71(BP), 93, 111, 43
	Nerolidol	3.81	69(BP), 41, 93, 107, 136
Fr. D	α-Cedrol	5.1	95(BP), 150, 43, 81, 107, 135
	delta-selinene	6.33	189(BP), 161, 204, 133, 91, 59
	delta-cadinene	9.55	161(BP), 204, 43, 105, 134
	α-bisabolol	4.32	109(BP), 119, 69, 43, 204

[a] Percentage of peak area is the ratio of each peak area to total peak area on the basis of TIC values of GC-MS
[b] Major fragmentation ions, base peak (BP) and other ions in decreasing order of relative abundance

bacteria, and *L. monocytogenes* and *S. aureus* were more resistant compared with other strains.

Gross examination

All mice with scar lesions on the TA-applied skin surface were analyzed by gross examination. In addition, a hard tactile of the scarred skin on topical TA treatment was increased depending on the days and times of application. Terpinen-4-ol was implied to have irritated the treated lesion. After four days of bacterial infection, the mice showed similar-sized abscesses, where no boundary was found between the abscess and the surrounding tissue. However, after seven days of infection, the infected mice exhibited large subcutaneous abscess, further proving the adhesion phenomenon between the abscess and the surrounding tissue.

Histopathological examination

Histopathological examination was performed to identify any pathological changes in the infected subcutaneous lesions (Fig. 1). In the daily single-TA-treated group with no bacterial infection, the dermoepidermal junction was distinctive and the inflammatory lesion was observed. Interestingly, in the daily twice-TA-treated mice after non-infection, the thickness of the epidermis markedly increased compared to that of the non-TA-treated mice. Parallel with the above gross examination, hyperkeratosis shown as a scar was observed in this histopathological test. The data suggest that TA induced the skin irritation. In the non-TA-treated group after bacterial infection, non-bordered subcutaneous inflammatory lesions and fistulas were observed. In day 7, however, the inflammatory reactions of the single and twice-TA-treated mice after bacteria injection exhibited a boundary that contrasted with that of the non-TA-treated group after infection.

Discussion

Various compounds, including terpenoids, phenolics, and alkaloids, exist in plant. They are mostly secondary metabolites that contribute to antibacterial activity jointly or independently. In particular, the antimicrobial properties of essential oils have been known for many centuries and much effort has been focused on determining the nature and practical uses of plant essential oils. In this study, the antibacterial activity of *C. obtusa* oil was investigated against several pathogenic bacteria, and an active constituent was identified by *in vitro* and *in vivo* antibacterial assay.

As shown in the Table 2, the essential oil from *C. obtusa* exhibited antibacterial activity against most kinds of bacteria tested. Above all, the antibacterial assay by agar disc diffusion method showed that *C. obtusa* oil effectively inhibited the growth of *L. monocytogenes*. Yang *et al*. (2007) also reported that *C. obtusa* oil showed strong antibacterial activity against *L. monocytogenes*. Antimicrobial activities of *C. obtusa* oils against pathogenic bacteria have been reported by many researchers, and *C. obtusa* oil showed strong antibacterial activity against Gram-positive bacteria in previous studies (Lee *et al*., 2002, 2009; Hong *et al*., 2004; Yang *et al*., 2007). However, our results were significantly different from those of the previously mentioned studies. According to Hong *et al*. (2004) and Yang *et al*. (2007), *C. obtusa* oil showed strong antibacterial activities against *L. monocytogenes* and *S. aureus*, but exhibited no antibacterial activities against *K. pneumonia*

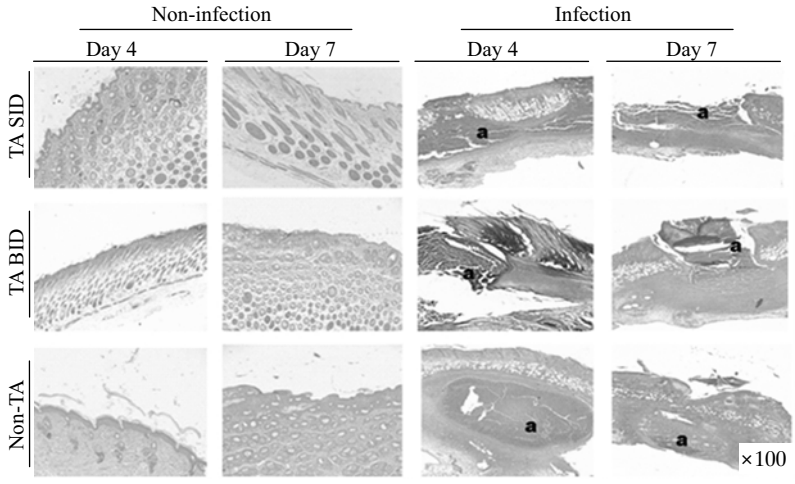

Fig. 1. Histopathological examination of the antibacterial effect of terpinen-4-ol (TA) using animal models. TA treatment and bacterial infection were described in the 'Materials and Methods'. SID, single a day; BID, twice a day; a, infiltrated white blood cell forming abscess.

and *S. typhymurium*. In contrast, our investigation revealed that *C. obtusa* oil could effectively inhibit not only *L. monocytogenes* and *S. aureus* growth but also that of *K. pneumonia* and *S. typhymurium*.

This disparity in results appears to be caused by the difference in plant sources. The *C. obtusa* oil tested by Hong *et al*. (2004) was prepared from leaves collected in Gyeonggi Province, but the essential oil used in this study was prepared from twigs and leaves collected in Jeonnam Province. Many studies reported that the chemical compositions of essential oils depend on geographic circumstance, harvesting period, climate, and plant age (Cheng *et al*., 2005, 2008). Thus, the differences between previous results and ours in the antibacterial activities of *C. obtusa* oil were thought to be caused by the variations in chemical composition or content of the active compound in the essential oil in relation to plant resource properties.

The MIC of *C. obtusa* oil against *E. coli* O157:H7 was observed at 0.8%, and those against other strains were more than 0.8% (Table 2). In comparison with other essential oils, the MIC of *C. obtusa* oil was similar to that of Spanish thyme (*Thymus mastichina*) oil against *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *S. aureus* (Oussalah *et al*., 2007).

In general, Gram-negative bacteria have been reported to be more resistant than the Gram-positive strains to the essential oils because of their cell wall lipopolysaccharide (Russel, 1991). This lipopolysaccharide may prevent the active compounds of essential oils from penetrating the cytoplasmic membrane of the Gram-negative bacteria (Chanegriha *et al*., 1994). However, in this study, no distinct difference in the resistance to *C. obtusa* oil was observed between Gram-negative and Gram-positive bacteria used in this study.

Among the seven fractions (fraction A-G) isolated from *C. obtusa* oil, fraction D was the most effective against the bacteria used in this study, as shown in Table 3. Several components such as TA, linalool, nerolidol, alpha-cedrol, delta-selinene, delta-cadinene, and alpha-bisabolol were found in fraction D. Among these components, TA (53% based on chromatogram peak area) was identified as the major constituent (Table 4). TA is known to constitute 4.11% of *C. obtusa* oil (Yang *et al*., 2007), and to be the primary bioactive component of tea tree oil (*Melaleuca alternifolia*) (Halcón and Milkus, 2004).

Antibacterial activities of fraction D and TA were much stronger than those of *C. obtusa* oil as shown by the antibacterial assay (Tables 3 and 5). Interestingly, both fraction D and TA showed higher activities against Gram-negative bacteria as opposed to Gram-positive bacteria. For example, fraction D exhibited the highest activity against *E. coli* O157:H7, and only TA demonstrated the highest activity against *S. typhimurium*. These results are similar to those generated by Barel *et al*. (1991) and Delaquis *et al*. (2002). In the study of Delaquis *et al*. (2002), a fraction of eucalyptus oil containing 87.9% TA displayed strong antibacterial activity against Gram-negative bacteria such as *E. coli* O157:H7 and *S. typhimurium*.

Table 5. Antibacterial activities of terpinen-4-ol

Test bacteria		Inhibition zone diameter (mm)[a]
Gram-negative	*K. pneumoniae*	25.0±1.2
	S. typhimurium	33.8±1.7
	E. coli O157:H7	18.8±1.0
Gram-positive	*L. monocytogenes*	15.6±0.9
	S. aureus	14.6±0.5
	MRSA	20.0±1.3

[a] Diameter of inhibition zone including disc diameter of 8 mm

The antibacterial activity of TA was dependent on the treatment concentration for *K. pneumonia* and *S. typhimurium*, but not for *S. aureus* and MRSA. In the case of *E. coli* O157:H7 and *L. monocytogenes*, the D-fraction containing 50% TA exhibited higher antibacterial activity than TA alone. This goes to show that other components aside from TA contributed to the activity against these specific strains. From these results, TA was considered to play an important role in the antibacterial activities of *C. obtusa* oil. In particular, compared with the other constituents, TA was the most likely antibacterial mediator in *C. obtusa* oil and the main contributor to antibacterial activity against *K. pneumonia* and *S. typhimurium*. Meanwhile, antibacterial activity against the other strains might be attributed to other components in combination with TA. In the mice test, TA was estimated to minimize inflammation but, unfortunately, induce skin irritation depending on the exposed amount and exposure period. Effects of the other constituents of sesquiterpene in *C. obtusa* oil have yet to be examined. Further studies are thus scheduled.

From the results of this study, *C. obtusa* oil was concluded to possess bacteriostatic activities that inhibit the growth of food-borne pathogenic bacteria. Owing to its antibacterial and aromatic properties, *C. obtusa* oil might be useful as a functional ingredient in flavoring agents, fragrances, preservatives, and other livelihood products. However, further research is needed, which should include available techniques toward the application of *C. obtusa* oil in commercial products.

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