Natural Product Sciences
17(4) : 296-302 (2011)

Activities of Essential Oils from *Perilla frutescens* var. *acuta* against Antibiotic-Susceptible and -Resistant *Vibrio* and *Salmonella* Species

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Abstract – We determined the inhibitory activity of the essential oil fraction obtained by steam distillation from the fresh and dried leaves of *Perilla frutescens* var. *acuta* against some pathogenic *Salmonella* and *Vibrio* spp. The activities of compounds isolated from the essential oils, apiol and myristicin, were also tested and the results were compared with those of the essential oil fraction. The *Perilla* essential oil fraction and its main components showed significant inhibition against antibiotic-susceptive and antibiotic-resistant strains of the tested *Salmonella* and *Vibrio* strains. Synergistic or additive effects were identified by combing the oils with ampicillin by checkerboard-titer tests. We conclude that essential oils from *P. frutescens* can be useful in the treatment of *Salmonella* and *Vibrio* infections and as safe additives to food materials for the prevention of contamination of food by these bacteria. This is especially important because of the rapid increase in antibiotic-resistant strains, which could cause severe symptoms in humans.
Keywords – *Perilla frutescens* var. *acuta,* essential oil, apiol, myristicin, *Salmonella, Vibrio*

Introduction

The development of resistance of both human and animal bacterial pathogens has been associated with the extensive therapeutic use of antimicrobials and with their administration as growth promoters in livestock feed (Shin, 2005). *Salmonella* and *Vibrio* species are among the common pathogens causing food-borne diseases. There has been a rapid increase in the emergence of antibiotic resistant strains in recent times (Varma *et al.,* 2006; Hald *et al.,* 2007; Andrysiak *et al.,* 2008; Rahim *et al.,* 2010; Haldar *et al.,* 2011). This is thought to have largely resulted from the consumption of processed food and agricultural products that have been in contact with antibiotics (Baker-Austin *et al.,* 2008; Devi *et al.,* 2009; Roig *et al.,* 2009; Kitiyodom *et al.,* 2010; Kitaoka *et al.,* 2011, Oh *et al.,* 2011).

Perilla frutescens var. *acuta* is an annual herb cultivated in Korea, mainly in the southern district, and also in Japan and China. It is the main source of Perillae Herba, which has been used in oriental traditional medicine to disperse colds and treat various gastrointestinal symptoms (Shin, 1986; Zhu, 1998; Liu *et al.,* 1999; Son *et al.,* 2010). In particular, it is used for prevention and treatment of seafood poisoning by fishes or crabs contaminated with bacteria that produce various toxins (Kim *et al.,* 2004). The leaves of this plant are edible and used as food additives for various purposes (Lee *et al.,* 1999; Choi *et al.,* 2010).

In this study we evaluated the inhibitory activity of the essential oil fraction obtained by steam distillation from the fresh and dried leaves of *P. frutescens* var. *acuta* against some pathogenic antibiotic-susceptible and antibiotic-resistant *Salmonella* and *Vibrio* spp. The essential oils of this plant were analyzed by GC-MS and the main components were isolated by column chromatography. The activities of the isolated main single components were also tested and the results were compared with those of the essential oil fraction.

Experimental

Extraction of essential oils from *P. frutescence* var. *acuta* – The essential oils (2.4 g) from the fresh leaves (1 kg) of *P. frutescens* var. *acuta* (cultivated in Wando) were obtained by steam distillation for 6 hr using a spontaneous distillation and extraction apparatus. A portion of the fresh leaves were dried at 40 and their essential oils extracted by the same method.

Analysis of the essential oil fraction by gas chromatography-mass spectrometry (GC-MS) – The

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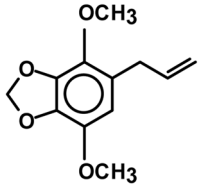
essential oil fraction was analyzed using a Hewlett-Packard 6890 GC, Hewlett-Packard 5973 MSD apparatus (Agilent 5973 network mass selective detector, 280ºC) with an HP-5MS capillary column (30 m × 250 μm × 0.25 μm). The injector was adjusted to 260 ºC and the oven temperature was programmed as follows: initial temperature: 50 ºC for 5 min, 2 ºC/ min up to 180 ºC, and then 3 ºC/min up to 280 ºC; the final temperature was 260 ºC with a hold time of 10 min.

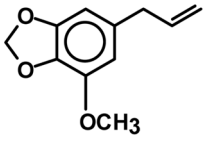
Isolation of the main components from the essential oil fraction – The essential oil fraction (1 g) was subjected to silica gel column chromatography and eluted with hexane-dichloromethane (9.5 : 0.5). Fractions 14-17 afforded compound **1** (myristicin, 47 mg). After removal of solvent, fractions 21 - 42 were subjected to rechromatography on a silica gel column with hexane-ethyl acetate (9 : 1, then 2 : 1) as solvent to give compound **2** (apiol, 95 mg). The spectral data of the isolated compounds were identical to published data (Benevides *et al.,* 1999).

Compound 1. Colorless oil. EI-MS: *m/z*: 192 (M^+, 100%), 177 , 165, 147, 131, 119, 103, 91, 77; UV λ_{max} ($CHCl_3$): 211, 277; IR (KBr, ν_{max}, cm^{-1}): 3076, 2894, 1845, 1632, 1508, 1432, 1357, 1317; ^{1}H-NMR ($CDCl_3$) δ: 6.37 (1H, *d*, *J* = 1.4 Hz, H-2), 6.33 (1H, *d*, *J* =1.4 Hz, H-6), 5.91 (2H, *s*, -OCH_2O-), 5.89 (1H, m, H-8), 5.08 (1H, *dd*, *J* = 1.8: 17.2 Hz, H-9b), 5.02 (1H, *dd*, *J* = 1.8: 8.1, Hz, H-9a), 3.87 (3H, *s*, -OCH_3), 3.27 (2H, *d*, H-7); ^{13}C-NMR ($CDCl_3$) δ: 148.8 (C-4), 143.4 (C-5), 137.3 (C-3) 134.6 (C-8), 133.4 (C-1), 108.6 (C-2), 102.6 (C-6), 101.2 (-OCH_2O-), 56.5 (-OCH_3), 40.2 (C-7).

Compound 2. Oil. EI-MS *m/z*: 222 (M^+, 100%), 207, 177, 165, 161, 149, 121, 106, 91, 77; UV λ_{max} ($CHCl_3$): 209, 287; IR (KBr, ν_{max}, cm^{-1}): 3077, 2938 1837, 1626, 1499, 1480, 1463, 1415, 1375, 1280; ^{1}H-NMR ($CDCl_3$) δ: 6.33 (1H, *s*, H-6), 5.89 (2H, *s*, -OCH_2O-), 5.85 (1H, *m*, H-8), 5.03 (1H, *dd*, *J* = 1.5; 9.0 Hz, H-9a), 5.00 (1H, *dd*, *J* = 1.5; 9.0 Hz, H-9b), 3.99 (3H, *s*, -OCH_3-5) and 3.74 (3H, *s*, -OCH_3-2), 3.29 (2H, *dd*, *J* = 1.5; 6.6 Hz, H-7); ^{13}C-NMR (80 MHz, $CDCl_3$) δ: 144.6 (C-4), 144.3 (C-5), 137.6 (C-3), 137.4 (C-8), 135.9(C-2), 126.0 (C-1), 115.5 (C-9), 101.1 (-OCH_2O-), 61.2 (-OCH_3, C-5), 59.9 (-OCH_3, C-2), 33.9 (C-7)



Apiol



Myristicin

Bacterial strains – Antibiotic-susceptible and antibiotic-resistant strains of *Samonella enteritidis, V. harveyl*, *V. parahaemolyticus,* and *V. vulnificus* were obtained from the Korean Culture Center of Microorganism (KCCM) and Culture Collection of Antibiotic Resistant Microbes (CCARM).

Minimum inhibitory concentrations (MICs) – MIC values of the oils were determined using the broth dilution method. A range of two-fold dilutions (16 down to 0.125 mg/ml) of essential oils in medium containing 2% Tween-80 was prepared. One hundred μl of the suspensions were taken and added to the wells on 96 well plates. The following procedure was used to inoculate 100 μl of the prepared broth culture of strains cultivated at 37 ºC. The MIC was determined by reading the turbidity of the wells after 24 hours incubation at 37 ºC.

Checkerboard microtiter tests – For checkerboard titer tests, 50 μl aliquots (64 to 1 mg/ml) of each dilution of *P. frutescens* var. *acuta* essential oil fraction or apiol were added to the wells of 96-well plates in a vertical orientation, and 50 μl aliquots (64 to 1 μg/ml) of antibiotics added in a horizontal orientation, so that the plate contained various combinations of the two compounds. A 100 μl suspension of the two *Vibrio* strains was added to each well, and cultured at 36 ºC for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of the *Perilla* essential oil fraction or apiol and antibiotics, divided by the MIC of the oil or oxacillin alone. The FIC index, obtained by adding both FIC values, was interpreted as a synergistic effect at ≤ 0.5, additive or indifferent at > 0.5 and ≤ 2.0, and antagonistic at > 2.0 (Davidson and Parish, 1989). An isobologram was constructed from the checkerboard data to depict the synergism of apiol or the essential oil fraction of *P. frutescens* var. *acuta* with antibiotics against the corresponding bacterial strain. DMSO and Tween-80 solvents were used at concentrations equivalent to those in the test solutions to certify that these vehicles did not affect bacterial growth.

Inhibitory activity of *Perilla* essential oils on DNA – One microgram of plasmid vector pBR322 (Takarabio, Otsu, Shiga, Japan) or λ DNA (Takarabio, Otsu, Shiga, Japan) was treated with 3 mg of the Perilla essential oil fraction or apiol at 37 ºC for 4 h. After DNA was electrophoresed on a 1% Tris-acetate/EDTA (TAE) agarose gel, DNA fragments were stained with ethidium bromide. The electrophoresis pattern of fragmented DNA was compared with that of untreated DNA.

Data analysis – MIC values were determined in triplicate and re-examined where appropriate.

Table 1. Compounds identified in the essential oil fraction of the fresh and dried leaves of *P. frutescens* var. *acuta*

Compound	*RT* (min)	*RI*	Area (%)	
			Fresh	Dried
2-Hexenal	3.87	841	0.16	-
α-Pinene	6.00	913	-	0.01
β-Pinene	7.53	800	-	0.01
Benzaldehyde	8.13	986	0.06	0.01
dl-Limonene	9.52	1027	0.14	0.36
Linalool	12.27	1104	-	0.08
Camphor	13.55	1143	-	0.01
l-Menthone	13.94	1155	-	0.05
endo-Borneol	14.30	1166	-	0.05
Borneol	14.32	1166	0.02	0.01
Menthol	14.61	1175	-	0.09
α-Terpinolene	15.26	1195	-	0.03
P-Allylanisole	15.71	1209	-	0.06
Pulegone	16.75	1243	-	0.02
Perilla aldehyde	17.78	1277	-	1.89
P-tert-Butylphenol	18.67	1307	-	0.88
Perilla aldehyde	19.18	1325	0.01	-
Camphene	19.48	1336	0.03	-
α-Copaene	20.55	1374	0.01	0.03
β-Bourbonene	20.80	1383	0.01	0.05
β-Damascenone	20.91	1387	-	0.02
α-Copaene	20.97	1389	0.00	-
β-Elemene	21.04	1391	0.02	0.05
trans-Caryophyllene	21.80	1420	-	5.21
β-Caryophyllene	21.81	1420	6.63	-
Calarene	22.01	1429	-	0.02
α-Gurjunene	22.40	1444	0.01	-
Longifolene	22.54	1449	0.01	-
α-Humulene	22.65	1454	0.58	0.48
trans-β-Farnesene	22.83	1461	1.71	0.76
Isocaryophillene	23.27	1478	0.01	-
α-Amorphene	23.29	1479	-	0.01
Germacrene	23.40	1483	0.24	-
β-Cubebene	23.40	1483	-	0.17
γ-Cadinene	23.52	1488	-	0.01
β-Ionone	23.63	1492	-	0.08
Valencene	23.68	1494	0.02	-
α-Farnesene	23.88	1502	6.35	4.36
δ-Cadinene	24.52	1527	0.04	0.04
Myristicin	24.69	1534	0.71	27.05
Germacrene B	25.33	1559	0.02	-
Elemicin	25.57	1568	-	6.55
E-Farnesol	25.60	1570	0.32	-
Spathulenol	25.90	1581	0.07	0.54
Caryophyllene oxide	26.00	1585	0.15	2.12
Valencene	26.23	1594	0.03	-
Apiole	27.53	1649	69.55	29.29
Caryophyllenol	28.22	1678	-	0.24

RT: retention time *RI*: retention indices. RIs were calculated against C_9 to C_{24} *n*-alkanes on an HP-5MS column.

Results and Discussion

Many varieties, cultivars and chemotypes of *P. frutescens* were reported which showed marked variation in essential oil composition (Chen *et al.,* 2004; Ohk and Chae, 2004; Zhang *et al.,* 2009; Huang *et al.,* 2011). In addition there could be a series of other factors (condition of cultivation, collection time, processing of the plant, etc.) that affect their composition and that might cause the differences in their activities (Lee *et al*., 1997; Choi *et al*., 2010; Laureati *et al*., 2010).

To determine the composition of *P. frutescens* var. acuta cultivated oils from the fresh and dried leaves for the activity tests, GC-MS analysis was performed using an HP-5MS column. The components identified in this essential oil fraction are listed in Table 1. A Wiley 275 library search using GC-MS data, and GC analysis with standard compounds led to the identification of 26 and 36 compounds from the two tested *P. frutescens* essential oils respectively. The oils from the fresh and dried leaves showed quite different patterns. In both of the oils, the prominent component was apiol; however, the oil from the fresh leaves showed much a higher apiol content (69.55%) than the oil from the dried leaves (29.29%). On the other hand, the content of myristicin was higher in the oil from the dried leaves (27.05%). Accordingly, these main components may contribute significantly to the antibacterial activity of the essential oil of *P. frutescens* var. *acuta* leaves. These main components were isolated from the essential oil fraction by silica gel column

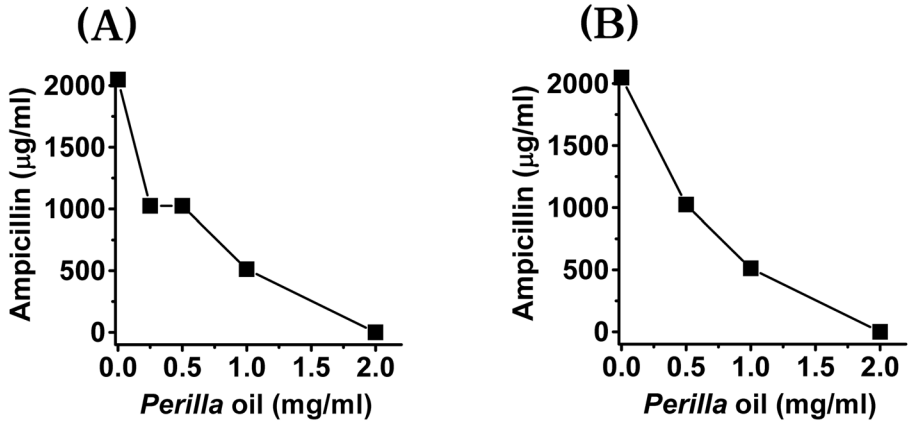


Fig. 1. Isobologram of *P. frutescens* var. *acuta* essential oil combined with ampicillin against *V. harveyl* CCARM 0111 (A) and *V. parahaemolyticus* CCARM 7001 (B).

Table 2. MICs of the essential oil of *P. frutescens* var. *acuta* against strains of *Salmonella* and *Vibrio* spp.

Samples	*Se10*	*Se11*	*Vh11*	*Vp01*	*Vp64*	*Vv65*
Essential oil fraction of *P. frutescence* var. *acuta*	2	2	2	2	2	2
Apiol	2	4	2	2	4	4
Myristicin	4	8	8	8	8	4
Ampicillin	>32 (r)	>32(r)	>32 (r)	>32(r)	2(s)	2(s)
Kanamycin	16(s)	8(s)	2(s)	2(s)	16(s)	8(s)
Norfloxacin	0.5(s)	1(s)	0.5(s)	<0.5(s)	<0.5(s)	<0.5(s)
Oxacillin	4(r)	0.5(s)	≥ 4(r)	≥ 4(r)	4(r)	4(r)
T/S*	0.25/4.75(s)	0.25/4.75(s)	0.25/4.75(s)	0.25/4.75(s)	0.12/2.37(s)	0.06/1.18(s)

* T/S: Trimethoprim/sulfamethoxazole.

R; resistant, i; intermediate and s; susceptible to the corresponding antibiotics discriminated by the criteria of the Clinical and Laboratory Standards Institute, USA (2007). Ampicillin: ≤ 8(s), 16(i), ≥ 32(r); kanamycin: ≤ 16(s), 32(i), ≥ 64(r), T/S: ≤ 2/38 (s), ≥ 4/76 (r), norfloxacin: ≤ 4(s), 8(i), ≥ 16(r), oxacillin: ≤ 2(s), ≥ 4(r), T/S: ≤ 2/38 (s), ≥ 4/76 (r).

P. frutescence var. *acuta* and its main component, apiol are shown in mg/ml, the values for ampicillin, oxacillin and penicillin G are shown in g/ml. *Se10*: *S. enteritidis* CCARM8010, *Se11*: *S. enteritidis* CCARM8011, *Vh11*: *V. harveyl* CCARM0111, *Vp01*: *V. parahaemolyticus* CCARM7001, *Vp64*: *V. parahaemolyticus* KCCM41164, *Vv65*: *V. vulnificus* KCCM41165.

Table 3. Fractional inhibiting concentrations (FICs) and fractional inhibiting concentration indices (FICIs) of the essential oil fraction of *P. frutescens* var. *acuta* and apiol against *Vibrio* spp.

Combination	Vh11		Vp01	
	FIC	FICI	FIC	FICI
P. frutescens essential oil fraction-ampicillin	0.25 0.12	0.37	0.25 0.25	0.50
Apiol-ampicillin	1.00 1.00	2.00	1.00 1.00	2.00
P. frutescens essential oil fraction-T/S	0.50 0.12	0.62	0.50 0.25	0.75
Apiol-T/S	0.50 0.25	0.75	0.50 0.25	0.75

FIC: MIC of the combined samples / MIC of the sample alone), FICI: Sum of the FICs of combined samples.

chromatography. And their structures were elucidated by ^{1}H- and ^{13}C-NMR (Fig. 1) and identified as apiol and myristicin.

As listed in Table 2, the susceptibility of *Salmonella* and *Vibrio* to *P. frutescens* essential oil or apiol alone differed slightly depending upon the species; however, there were no distinct differences in their MICs (2 - 4 mg/ml). Myristicin, the second most prominent compound of the oil from dried leaves, showed mostly milder inhibiting activity against the tested bacteria. Both of the tested *S. enteritidis* strains showed resistance to ampicillin. Among *Vibrio* species, *V. harveyl* CCARM 0111 and *V. parahaemolyticus* CCARM7001 were resistant, while *V. parahaemolyticus* KCCM 41664 and *V. vulnificus* KCCM 11665 were susceptible to ampicillin. All of the tested strains of *Vibrio* and *Salmonella* showed susceptibility to T/S with MICs of 0.06/1.18~0.25/4.75 ug/ml.

Because the antibacterial mechanisms of essential oils appear to be substantially different from the mechanisms of currently used antibiotics, the plant essential oils can be considered a promising source of new drugs against antibiotic-resistant bacteria (Shin, 2009). However, in many cases, the antibacterial activities of plant essential oils have considerably higher MICs than commonly used antibiotics. This limits their application as therapeutic agents in clinical practice for treatment of severe symptoms. In the results of our study also, the MICs of *P. frutescens* essential oils were ca. 1000 times higher than those of the tested antibiotics. As a strategy to enhance their activity, combinatorial therapy with antibiotics were studied with T/S-sensitive and ampicillin-resistant strains of two *Vibrio* species. To evaluate the synergism, checkerboard microtiter tests were constructed in combination with *P. frutescens* essential oils or apiol and antibiotics, ampicillin or T/S, which is commonly used for evaluation of the susceptibility and resistance of *Vibrio* species to antibiotics. The fractional inhibitory concentration indices (FICIs) against the tested *Salmonella* species ranged between 0.37 and 2.00 for ampicillin combined with *P. frutescens* essential oil or apiol, indicating significant synergistic or additive effects between ampicillin and the oil samples (Table 3). Similar experiments combining the *P. frutescens essential oil* fraction or apiol with T/S resulted in FICIs between 0.62 and 0.72 indicating additive effects. Thus, the data reported in this study show that the anti-*Vibrio* effects of ampicillin can be significantly improved by the use of the *P. frutescens* essential oil fraction. There were no significant differences in FICI results between the two tested *Vibrio* species. The isobologram plots for ampicillin combined with the *P. frutescens* oil fraction against *V. harveyl* CCARM 0111 and *V. parahaemolyticus* CCARM 7001 revealed curves that distinctly deviated to the left (Fig. 1), confirming the presence of synergism or additive effects in anti-*Vibrio* activity (Davidson and Parish, 1989). The synergistic anti-*Vibrio* effects of the *P. frutescens* essential oil with ampicillin or T/S could facilitate the use of lower concentrations of this antibiotic, thus minimizing its potential side effects. This approach could also provide alternative therapies to overcome the current limitations of the use of ampicillin for the treatment of the bacterial infections.

As shown in Fig. 2, the bands of plasmid vector pBR322 or DNA were reduced after treatment with the *Perilla* essential oil fraction or its main component, apiol, indicating that they are able to interact with or damage bacterial DNA.

Thus, the results of this study indicate that *P. frutescens* var. *acuta* essential oil and its main component, apiol, may be useful agents in the treatment of *Salmonella* and

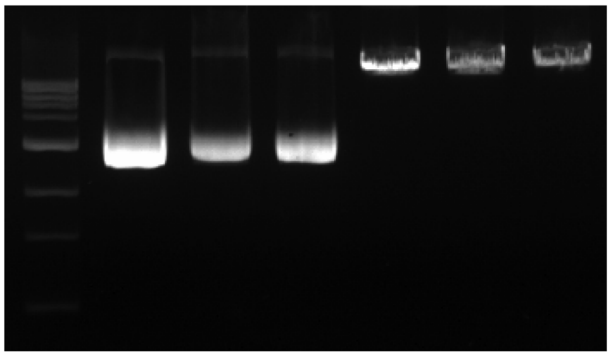


Fig. 2. Effects of the *Perilla* essential oils on pBR322 plasmid DNA and λ DNA. One μg of pBR322 or λ DNA was treated with 3 mg of the oil at 37 °C for 4 h. Then DNA was electrophoresed on a 1% agarose gel and DNA was stained with ethidium bromide.

Lane 1, 1 kb DNA marker; 2, pBR322 (1 μg); 3, pBR322 (1 μg) and *P. frutescens essential oil* fraction (3 mg); 4, pBR322 (1 μg) and apiol (3 mg): 5, λDNA (1 μg); 6, λDNA (1 μg) and *P. frutescens essential oil* fraction (3 mg); 7, λDNA (1 μg) and apiol (3 mg).

Vibrio infections, and are also as safe additives for preventing contamination of food by these bacteria, which could cause severe symptoms. However, further studies will be required.

Acknowledgements

This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (NRF-531-2008-1-E00106).

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Received August 26, 2011
Revised November 25, 2011
Accepted November 28, 2011