Antimicrobial Activity of the Volatile Constituents of *Perilla frutescens* and Its Synergistic Effects with Polygodial

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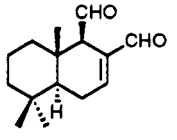
The steam distillate of the green leaves of *Perilla frutescens* has broad antimicrobial activity assumed from its use as food and food additives. The steam distillate mainly consists of perillaldehyde, limonene, β-caryophyllene, α-bergamotene, and linalool. The most abundant, perillaldehyde, inhibits moderately a broad range of both bacteria and fungi. Perillaldehyde works in synergy with polygodial not only against fungi but also against both Gram-positive and Gram-negative bacteria. Synergistic effects are evaluated by the fractional inhibition concentration method.

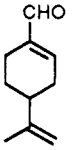
INTRODUCTION

The leaves as well as seeds of *Perilla frutescens* (Labiatae) are part of the popular and traditional Chinese herb medicines, which is prescribed for colds, coughs, and promoting digestion (Duke, 1988). This fast-growing herb is used in a wide variety of applications including in foods, food coloring, and flavoring and as a sweetening agent. The edible green leaves of *P. frutescens*, Japanese general name "shiso" or "aoziso", in particular, are commonly used in the preparation of raw fish and shell fish in Japanese dishes such as "sushi" and "sashimi" which have become popular worldwide.

Many of the medical properties including antidermatophytic properties of *P. frutescens* have been reported (Honda et al., 1984; Terao et al., 1991; Hirose et al., 1990; Duke, 1988). However, the antimicrobial activity being assumed from its use as a food or food additive has never been clearly established. The present work reports on the antimicrobial activity of the volatile constituents from the leaves of *P. frutescens* harvested in Oxnard, CA, and distributed in the United States. In addition, the synergistic effect of a main constituent, perillaldehyde, combined with polygodial, which is a constituent of the



polygodial (1)



perillaldehyde (2)

red bud leaves of *Polygonum hydropiper*, "akame" in Japanese, popularly eaten together with *P. frutescens*, will be discussed.

MATERIALS AND METHODS

Chemicals. Polygodial was a gift from Takasago International Corp. (Tokyo). Perillaldehyde, perillyl alcohol, benzaldehyde, α-caryophyllene, β-caryophyllene, limonene, linalool, and β-pinene were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Plant Material. *P. frutescens* was harvested and collected at a farm in Oxnard, CA, and transported without drying for extraction.

Extraction. The methanol extract (8.6%) was obtained from the partially dried leaves of *P. frutescens*. A steam distillate was obtained from partially dried *P. frutescens* leaves using simultaneous steam distillation/extraction. Finely macerated leaves (39.2 g) were combined with approximately 600 mL of distilled water in a round-bottom flask. The flask was then connected to a Likens-Nickerson distillation/extraction head. Pentane was used as the extraction solvent, and the distillation was allowed to proceed for approximately 90 min. This process yielded 0.3 g (0.77%) of an aroma concentrate possessing a characteristic "shiso" aroma.

GC-MS Analysis. The steam distillate was injected into a 60-m DB-1 capillary column (0.25 mm o.d.; J&W Scientific, Folsom, CA). The oven was programmed from 50 to 240 °C with a 2-min hold at 50 °C and He flow of 2 mL/min. The GC-MS spectrometer, JEOL JMS DX303HF coupled with a Hewlett-Packard GC 5890A, was used in the electron ionization mode (70 eV) with an ion source temperature of 220 °C.

Microorganism. All microorganisms were purchased freeze-dried from American Type Culture Collection and included *Actinomyces naeslundii* ATCC 19039, *Aspergillus niger* ATCC 16404, *Bacillus subtilus* ATCC 9372, *Candida albicans* ATCC 18804, *Candida utilis* ATCC 9226, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 9637, *Mucor mucedo* ATCC 20094, *Penicillium chrysogenum* ATCC 10106, *Porphyromonas gingivalis* ATCC 33277, *Propionibacterium acnes* ATCC 11827, *Pseudomonas aeruginosa* ATCC 25619, *Saccharomyces cerevisiae* ATCC 7754, *Salmonella choleraesuis* ATCC 10708, *Staphylococcus aureus* ATCC 6538, and *Streptococcus mutans* ATCC 25175. Culture conditions for anaerobic bacteria were 37 °C anaerobically in *Actinomyces* broth (BBL), 30 °C aerobically for *S. mutans* in 3.7% brain heart infusion broth (Difco), fungi in 1.5% malt extract broth (Difco), and all the others in a mixture consisting of 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), and 0.1% glucose.

Antimicrobial Assay. The sample is dissolved in an appropriate amount of dimethylformamide (DMF) to give a concentration equivalent to 100 mg/mL. Five or more twofold serial dilutions are made from this concentrate by adding an aliquot to an equal volume of DMF. Thirty microliters of each concentration is added to 3 mL of the appropriate media followed by 60 μL (or a small piece in the case of filamentous fungi) of a 2-day-old culture. Therefore, each tube contained 1% DMF which did not affect the growth of the microorganism. The tubes are incubated for 48 h at their appropriate conditions. Growth after 48 h is decided as an increase in turbidity read at 660 nm. The minimum inhibition concentration (MIC) is determined as the lowest concentration with no growth. Combination mixtures were prepared by using aliquots of appropriate concentrations and by using DMF as the diluent to correct for the final concentrations before inoculation into media. The experiments were generally carried out in duplicate.

RESULTS AND DISCUSSION

Antimicrobial Activity of the Extracts. The steam distillate and methanol extracts of *P. frutescens* were tested against microbes using a broth dilution method

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Table I. Antimicrobial Activity of the Steam Distillate of *P. frutescens* and Its Constituents[a]

	MIC against microbes, μg/mL									
organism	SD	2	3	4	5	6	7	8	9	10
A. naeslundii	250	500	31.2	125	500	>1000	250	>1000	250	125
B. subtilis	500	500	>1000	>1000	1000	>1000	>1000	1000	>1000	>1000
P. gingivalis	500	1000	125	>1000	>1000	>1000	1000	>1000	250	125
P. acnes	31.2	250	250	7.8	500	>1000	125	250	125	15.6
S. aureus	125	1000	125	>1000	>1000	>1000	125	1000	500	125
S. mutans	1000	500	125	>1000	1000	>1000	125	500	500	>1000
E. aerogenes	>1000	500	>1000	>1000	>1000	>1000	>1000	500	500	>1000
E. coli	1000	500	>1000	>1000	>1000	>1000	>1000	500	500	>1000
P. aeruginosa	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
S. choleraesuis	500	1000	>1000	>1000	1000	1000	1000	500	500	>1000
A. niger	500	250	>1000	>1000	1000	250	>1000	500	500	>1000
C. albicans	500	500	>1000	>1000	>1000	>1000	2000	1000	500	>1000
C. utilis	250	500	62.5	>1000	500	1000	1000	500	>1000	>1000
M. mucedo	62.5	250	>1000	>1000	500	250	1000	250	250	>1000
P. chrysogenum	62.5	250	>1000	>1000	500	500	125	500	250	>1000
S. cerevisiae	250	500	62.5	>1000	1000	1000	250	500	500	>1000

[a] SD, steam distillate; 2, perillaldehyde; 3, limonene; 4, β-caryophyllene; 5, linalool; 6, benzaldehyde; 7, β-pinene; 8, perillyl alcohol; 9, isoeugenol; 10, α-caryophyllene.

Table II. Constituents of Steam Distillate from the Leaves of *P. frutescens*

compd	ratio	compd	ratio
2-hexanol	0.23[a]	pulegone	0.90
ocimene	0.45[a]	perillaldehyde	74.00
benzaldehyde	1.60	perillyl alcohol	0.26
sabinene	0.40	α-terpinyl acetate	0.13[a]
β-pinene	0.60	isoeugenol	0.25
myrcene	tr	β-caryophyllene	3.80
pseudolimonene	0.20	α-caryophyllene	0.13
limonene	12.80	α-bergamotene	3.50[a]
terpinolene	0.10	farnesene	0.10[a]
linalool	2.60	aromadendrene	0.30[a]
limonene oxide	0.10[a]		

[a] Identification is tentative.

(Taniguchi and Satomura, 1972). This broth dilution method used to test antimicrobial activity is extremely suitable for less polar and less diffusible compounds compared to an agar plate diffusion method. The methanol extract did not remarkably inhibit any microbes at 500 μg/mL; instead, the crude steam distillate exhibits broad spectrum activity in the range 125–1000 μg/mL for both Gram-positive and Gram-negative bacteria as well as fungi (Table I). It is particularly active against filament fungi, with MIC values for *M. mucedo* and *P. chrysogenum* as low as 62.5 μg/mL. More interestingly, the steam distillate inhibits the Gram-negative *S. choleraesuis*, which is one of the major bacteria that causes food poisoning from eating raw foods such as fish and eggs. These results encouraged us to investigate the volatile constituents of *P. frutescens*.

Antimicrobial Constituents. The constituents of the essential oils of *P. frutescens* as well as their genetic control have also been extensively studied (Ito, 1970; Koezuka et al., 1986). They indicate that the constituents of *P. frutescens* are widely different depending on the variety of strains and the cultivated location. In addition, the constituents of the steam distillate of *P. frutescens* harvested and distributed in the United States have not been reported. Therefore, we first analyzed the steam distillates by GC–MS to determine the antimicrobial principals.

The GC–MS data (Table II) showed that the steam distillate of the leaves of *P. frutescens* harvested in California mainly consists of perillaldehyde (**2**) (74%), β-caryophyllene, limonene, and α-bergamotene. These are slightly different from other samples harvested in Japan (Ito, 1970) or from callus tissues (Nabeta et al., 1985). In particular, the extremely high concentration of perillaldehyde compared to that of other steam distillates of *P. frutescens* reported, the existence of α-bergamotene, and the ratio of β-caryophyllene and perillaldehyde are significantly different. Although a seasonal variation of the constituents was observed, its details will be published elsewhere. Most constituents of the steam distillate were obtained as pure compounds, but α-bergamotene was not obtained in sufficient quantity for the assay.

The most abundant component, perillaldehyde, has moderate and broad-spectra activity against all microbes tested. It has an MIC between 125 and 1000 μg/mL, close to that of the steam distillate. In the case of Gram-positive bacteria and fungi, the activity is generally weaker when compared to that of the steam distillate while the activity is generally the same for Gram-negative bacteria. This would indicate that the high concentration of perillaldehyde (**2**) is the major compound responsible for the activity of the crude distillate. The second major compound, limonene, inhibits Gram-positive anaerobes, particularly *A. naeslundii*, and yeast. The other minor components, perillyl alcohol, isoeugenol, and linalool, show weak but broad-spectrum activity, with isoeugenol as the most active and linalool as the least active of the three. β-Caryophyllene shows activity against Gram-positive anaerobic bacteria, especially against *P. acnes* (MIC of 7.8 μg/mL), while β-pinene shows activity against both Gram-positive bacteria and fungi.

Synergistic Effects. The leaves of *P. frutescens* are commonly served with the red bud leaves of *P. hydropiper* containing a hot taste constituent, polygodial (**1**). This tradition suggests an interplay between the constituents of both herbs, and it would be assumed that they would not interact in a negative way. In fact, none of the combinations are antagonistic. Since polygodial exhibits synergy with both actinomycin D (Kubo, 1988) and anethole (Kubo and Himejima, 1991) against fungi, we explored the possible synergy between polygodial and perillaldehyde (**2**). The individual MIC values of the two compounds are shown in Table III while the synergistic effects of combining these two compounds are clearly shown in Table IV.

The amount of polygodial needed to inhibit the yeast *C. utilis* and *S. cerevisiae* is reduced to one-fourth of the uncombined MIC at the same time the concentration of perillaldehyde is reduced 4-fold from their uncombined MIC value. A similar but more dramatic combination effect occurs against *C. albicans* and *M. mucedo*, where a 4-fold decrease from the uncombined MIC of polygodial at 1.95 μg/mL reduces the amount of perillaldehyde

Table III. Antimicrobial Activity of Polygodial (1) and Perillaldehyde (2)

organism	MIC against microbes, μg/mL 1	2
B. subtilis	125	500
S. choleraesuis	2000	1000
P. aeruginosa	>1000	>1000
C. albicans	3.9	500
C. utilis	7.8	500
M. mucedo	1.95	250
P. chrysogenum	7.8	250
S. cerevisiae	1.95	500

Table IV. Synergistic Effects against Microbes of Polygodial (1) and Perillaldehyde (2) with Individual FIC and Combined FIC Values

	1		2		
	MIC	lowest FIC	MIC	lowest FIC	combined* FIC[a]
B. subtilis	31.25	0.25	125	0.25	0.500
S. choleraesuis	250	0.125	250	0.25	0.375
P. aeruginosa	500	≤0.25	500	≤0.25	≤0.5
C. albicans	1.95	0.25	15.6	0.031	0.281
	0.12	0.015	250	0.5	0.515
C. utilis	1.95	0.50	125	0.25	0.750
	0.98	0.25	250	0.50	0.750
M. mucedo	1.95	0.25	7.81	0.031	0.281
	0.98	0.126	31.25	0.125	0.251
P. chrysogenum	0.48	0.246	62.5	0.25	0.496
S. cerevisiae	0.48	0.246	125	0.25	0.496

[a] Combined FIC = lowest FIC of 1 + lowest FIC of 2. lowest FIC 1 or 2 = combined MIC of 1 or 2/individual MIC of 1 or 2.

required for inhibition by as much as 32- and 16-fold, respectively. Further limited reduction in polygodial concentration will maintain the synergistic effect provided there is a corresponding increase in perillaldehyde concentration as seen with *C. utilis*, *C. albicans*, *M. mucedo*, and *P. chrysogenum*.

Polygodial has been reported to be a potentiator in combinations against fungi but has not been considered one against bacteria. Nevertheless, the antibacterial test for our curious Gram-negative bacteria *S. choleraesuis* shows it is inhibited synergistically in the combination. The MIC of polygodial is decreased 8-fold at the same time the MIC of perillaldehyde is decreased 4-fold, both at moderate concentrations of 250 μg/mL. Equally unexpected is the synergy of both at 500 μg/mL of polygodial and perillaldehyde against another Gram-negative bacteria, *P. aeruginosa*, which is one of the two most resistant bacteria selected in this study. The other resistant bacteria is also inhibited synergistically. The Gram-positive spore-forming *B. subtilis* is inhibited at a concentration 4-fold less than its uncombined MIC of both compounds.

Evaluation of Synergistic Effect. Many methods have been employed to evaluate synergistic effects. It is of increasing importance, particularly in pharmacological studies. The fractional inhibitory concentration (FIC) method for calculating synergy or antagonism (Berenbaum, 1978) is used to further analyze the data here. The FIC of each compound is defined as the MIC in combination divided by the uncombined MIC. The combined FIC values for each pair of compounds are calculated, and those values above 1.0 are considered to be antagonistic. Some workers (King et al., 1981; Norden, 1982) have suggested that, because of variation in titration, combined FIC values between 1.0 and 0.5 should indicate additive combinations or limited synergy and FIC values of 0.5 or less indicate unequivocal synergy. Using this method, there is true synergy (FIC value <0.5) for all combinations tested except *C. utilis*. The synergy works best (lowest FIC value) on *C. albicans* and *M. mucedo* from the microorganisms tested. None of the combinations demonstrated an antagonistic effect where the FIC is above 1.0 (Table IV).

Conclusion. The steam distillate of shiso has moderate and broad spectra against various microbes. The major active constituent, perillaldehyde, also having broad spectrum activity, is unequivocally synergistic with polygodial, having an FIC value of less than 0.5. No antagonism is observed for any of the microorganisms tested. Furthermore, the synergy of polygodial and perillaldehyde against bacteria is the first example of the synergistic action of polygodial against both Gram-positive and Gram-negative bacteria. The effective synergy against *S. choleraesuis* suggests that the customary addition of *P. frutescens* and *P. hydropiper* in raw seafoods such as sashimi is beneficial in the prevention of *Salmonella* poisoning. Whether this custom was started due to foresight about the antimicrobial activity of the two constituents and their synergy or whether this beneficial custom was purely coincidental is another matter for investigation.

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Registry No. 2-Hexanol, 626-93-7; ocimene, 29714-87-2; polygodial, 6754-20-7; benzaldehyde, 100-52-7; sabinene, 3387-41-5; β-pinene, 127-91-3; myrcene, 123-35-3; pseudolimonene, 499-97-8; limonene, 138-86-3; terpinolene, 586-62-9; linalool, 78-70-6; limonene oxide, 1195-92-2; pulegone, 89-82-7; perillaldehyde, 2111-75-3; perillyl alcohol, 536-59-4; α-terpinyl acetate, 80-26-2; isoeugenol, 97-54-1; β-caryophyllene, 87-44-5; α-caryophyllene, 6753-98-6; α-bergamotene, 17699-05-7; farnesene, 502-61-4; aromadendrene, 72747-25-2.