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RESEARCH NOTE

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Antimicrobial Activity of an Edible Wild Plant, Apiifolia Virgin's Bower (*Clematis apiifolia* DC)

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Abstract An edible wild perennial plant with extremely potent antimicrobial activity was found and identified as apiifolia Virgin's Bower (*Clematis apiifolia* DC) which is easily found around wet wildernesses. Fresh fruit extract of *C. apiifolia* exhibited minimum inhibitory concentrations (MIC) in the vicinity of 0.1% against various yeasts and of less than or equal to 0.4% for non-lactic acid bacteria. MICs against lactic acid bacteria were about 2.0%. The antimicrobial activity of *C. apiifolia* fruit was even more potent than that of garlic which has been known for its potent antimicrobial activity. The principal antimicrobial compound of fruit extract of *C. apiifolia* was isolated and identified by high performance liquid chromatography and gas chromatography as protoanemonin (a gamma lactone of 4-hydroxy-2,4-pentadienoic acid). The antimicrobial activity of *C. apiifolia* was stable at high temperatures, and the activity was maintained after heating at 121°C for 10 min. The antimicrobial compound of *C. apiifolia* was supposed to inhibit microorganisms by reacting with sulfhydryl groups of cellular proteins.

Keywords: antimicrobial activity, apiifolia Virgin's Bower, *Clematis apiifolia*, protoanemonin

Introduction

Plants (1-3) produce biologically active antagonistic compounds to effectively defend themselves from predators. Many naturally occurring compounds found in dietary and medicinal plants, herbs, and fruit extracts have been shown to possess antimicrobial activities (3-15). Phenolic phytochemicals in plant extract have antimicrobial properties that inhibit the bacteria that cause food poisoning (8). Not only phenolic compounds but also other phytochemicals including lactones (11, 12) and derivertives of unusual amino acids (13, 14) have antimicrobial activities. Garlic, among other edible plants, is well known for its potent antimicrobial activity, exhibiting a minimum inhibitory concentration (MIC) of 0.075%(w/v) when tested against *Candida utilis* as an indicator organism (15). Other edible plants such as onion (14), cabbage (16), and various spices (5, 6, 8-10) including oregano, cranberry etc., have also been reported to possess antimicrobial activity which, however, are not as potent as that of garlic.

On the process of continuing effort to screen higher plants with potent antimicrobial activity, we collected fresh plant parts (fruits, flowers, and leaves) and tested for their antimicrobial activity at 0.1%(w/v) level on fresh weight basis against an indicator yeast, *C. utilis* ATCC 42416. We encountered a perennial climbing bush plant with extremely potent antimicrobial activity which is even more potent than fresh garlic against the indicator yeast.

A perennial wild plant with potent antimicrobial activity was screened and the antimicrobial compound of the plant was isolated and identified. The antimicrobial potency of the plant was compared with that of garlic.

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Materials and Methods

Collection and treatment of *C. apiifolia* fruits Fresh green fruits of apiifolia Virgin's Bower (*Clematis apiifolia* DC) were collected from low hilly areas near Seoul on the middle of September of 2005 and 2006. The fresh fruits were blended with 9 times water and the upper aqueous layer was used for the measurement of antimicrobial activity and for the sample of chemical analysis. A portion of the green fruits was set aside to dehydrate under shade.

Microbial strains and culture conditions *Staphylococcus aureus* B33, *Escherichia coli* B34, *Enterobacter aerogenes* B146, *Lactobacillus plantanum* LA 97, *Saccharomyces cerevisiae* ATCC 4126, *C. utilis* ATCC 42416, *Candida albicans* HY1, *C. albicans* KCTC 7121, *C. albicans* KCTC 7965, *Zygosaccharomyces rouxii* KCCM 50546 were obtained from the culture collection of our lab. They were stored at -64°C in basal media containing 16% glycerol. The basal media were tryptic soy broth (TSB; Difco Lab., Detroit, MI, USA) for non-lactic acid bacteria, MRS broth (Difco Lab.) for lactic acid bacteria, and YMPG broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose) for yeasts. For resuscitation, frozen cultures were streaked on agar medium of the identical basal medium used for growth, and an isolated colony was picked and cultivated at least twice before a 24 hr culture was used as a seed culture for bacteria and 48 hr culture for yeasts.

Determination of MIC Ten % aqueous extract of *C. apiifolia* fruits was filter-sterilized (Microfilter, 0.45 μm; Gelman Sci., Ann Arbor, MI, USA) and diluted with heat sterilized culture broth to give the desired final concentrations. Broths containing desired concentrations of *C. apiifolia* fruit extract were inoculated with microorganisms to give initial counts between 10^4-10^5 CFU/mL and incubated at 30°C for 24 and 48 hr for bacteria and yeasts, respectively. The sensitivity of the test organisms was expressed as the minimum inhibitory concentration (MIC, %). Experiments were performed triplicates, and the higher values were recorded as the MIC. A complete absence of growth based on optical observation after the incubation period was regarded as non-growth.

In order to measure the effect of sulfhydryl compound (e.g., cysteine) on the antimicrobial activity of *C. apiifolia* fruits, determination of MIC was performed with *C. utilis* ATCC 42416 in YMPG broth containing cysteine (final concentrations; 0 to 0.7 mM).

Isolation and identification of antimicrobial active compound Fractions of aqueous extract of *C. apiifolia* fruit were obtained by using recycling preparative HPLC (JAI-LC-908; Japan Analytical Industry Co., Ltd., Tokyo, Japan) with both JAI UV and RI detectors. Gel permeation chromatography (GPC) column (Jaigel W252 column, 50× 2 cm i.d., Japan Analytical Industry Co., Ltd.) was used, and 50:50 water: acetonitrile was used as elution solvent at a flow rate of 3 mL/min. The injection volume of *C. apiifolia* fruit aqueous extract was 4 mL. Fractions obtained were tested for antimicrobial activity after acetonitrile was removed by rotary vacuum evaporator at 0°C.

The mass spectrum of the isolated active compound was obtained by combination gas chromatography-mass spectrometry (GC-MS; 6890N GC, Agilent Technologies, Palo-Alto, CA, USA). Two μL portions of methylene chloride extract of *C. apiifolia* fruit aqueous extract were injected for GC-MS analysis. The GC column (30 m × 0.25 mm capillary, J & W Scientific Inc., Folsom, CA, USA) was coated with DB-5MS (0.32 μm thickness). The oven temperature was programmed from 40 to 240°C at 10°C/min, respectively. The carrier gas was He (1.5 mL/min), and the split ratio was 20:1. The injector port and detector temperatures were 180°C. Electron impact ionization (70 eV potential) was used and the mass range scanned was 40 to 200 Da.

Results and Discussion

Antimicrobial plant The plant with potent antimicrobial activity was identified to be *C. apiifolia* DC belonging to the family Ranunclaceae. The plant is found anywhere near moisty hilly areas throughout Korea and is known to be used as a traditional oriental medicine (17). Young leaves and stems of *C. apiifolia* collected during early spring are known to be edible (18).

Antimicrobial activity of *C. apiifolia* Fruit extract of *C. apiifolia* did not affect the growth of the indicator yeast at 0.03%(w/v, wet weight basis) level, but lag period for growth was extended at 0.05% and growth was completely inhibited at 0.07% level (Fig. 1). The concentration for complete growth inhibition (MIC) lied between 0.05 and 0.07%. The mode of action of fruit extract of *C. apiifolia* was to be killing effect (cidal effect), judging from the declining number of live yeast cells. It was found that autoclaved *C. apiifolia* fruit for 10 min maintained the antimicrobial activity (data not shown).

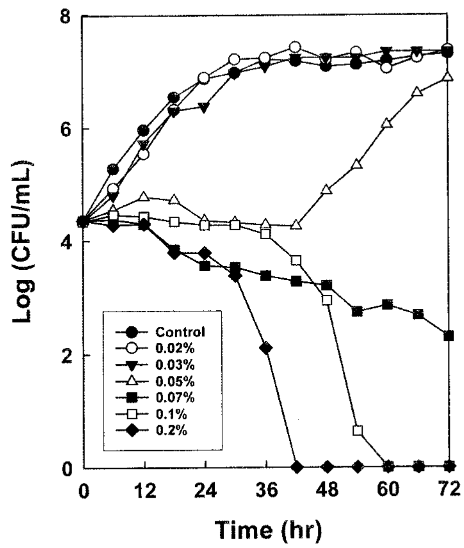

Fig. 1. Inhibitory effect of *C. apiifolia* DC fruit extract on the growth of *C. utilis* ATCC42416 in YMPG broth at 30°C.

Isolation and identification of the inhibitory compound of *C. apiifolia* Among the fractions of fruit extract of *C. apiifolia* obtained by preparative HPLC, only one fraction showed strong antimicrobial activity against *C. utilis* ATCC 42416. Efforts to concentrate the antimicrobial active fraction *in vaccuo* were not successful. When the antimicrobial fraction was concentrated 2-fold and volume-corrected, both the antimicrobial activity and active fraction were missing (data not shown), suggesting that the active compound was volatile.

The active fraction was isolated by using recycling HPLC and found to be a single compound [Fig. 2; 96(100), 68(50), 42(47), 54(29)] that matched either protoanemonin [96(100), 42(98), 68(63), 54(52)] or 4-cyclopentene-1,3-dione [96(100), 42(47), 68(42), 54(35)] based on GC-MS analysis. When commercially available 4-cyclopentene-1, 3-dione was spiked into fruit extract of *C. apiifolia*, and made a GC analysis, the 4-cyclopentene-1,3-dione peak appeared separately from that of the active compound. Responses of 4-cyclopentene-1,3-dione on UV (260 nm)- and RI-detectors were different compared with the active compound of *C. apiifolia*. Therefore 4-cyclopentene-1,3-dione was ruled out as a possible candidate for the active compound of *C. apiifolia*.

Lactones constitute a large and diverse group of biologically active phytochemicals that have been identified in several plants (19). Unsaturated lactones, including protoanemonin (4-hydroxy-2,4-pentadienoic acid γ lactone), have shown to exhibit a wide range of biological activities including antimicrobial activity through the binding of the exocyclic methylene group of lactones with sulfhydryl groups of cellular protein components (12, 20). Therefore the mechanism of inhibition of microbial growth of unsaturated lactones is similar to that of allicin and other antimicrobial components of garlic (21). Chemically, lactone is a cyclic ester formed

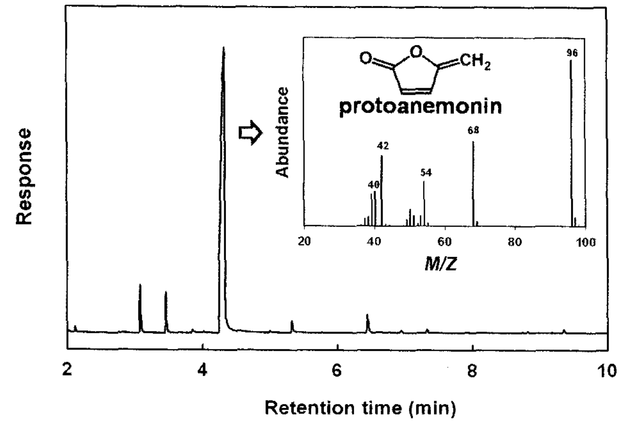

Fig. 2. GC-MS analysis of antimicrobial compound in *C. apiifolia* extract.

by condensation of an alcohol and a carboxylic acid group in the same molecule. Protoanemonin is one of the 5-membered lactone, commonly called a gamma lactone (Fig. 2). Protoanemonin has been known to be the active antimicrobial compound of plant species of *Ranunculus bulbosus* (11), *Anemone pulsatilla* (12, 22), and *Clematis dioscoreifolia* (23).

Cavallito and Heskell (20) suggested that the inhibitory effect of unsaturated lactones, such as protoanemonin, was related to the specific activity with sulfhydryl (-SH) groups of cellular proteins.

The addition of cysteine helped to overcome the inhibition by *C. apiifolia* phytochemical (data not shown). Cysteine increased the MIC values of the fruit extract of *C. apiifolia* on the dose-response manner, suggesting that the antimicrobial active compound actually react with -SH groups of cellular proteins.

Antimicrobial action against selected microorganisms The inhibitory activity of *C. apiifolia* against yeasts was almost identical to that of garlic (Table 1). *C. apiifolia* showed even more potent antibacterial activity compared with garlic, exhibiting very small MICs against the Gram-positive and negative bacteria except lactic acid bacteria.

Immature fruits with light green appearance exhibited the strongest antimicrobial activity, showing the MIC against *C. utilis* ATCC 42416 in YMPG broth of 0.06-0.07 %. Once the fruits start to get matured and attain brownish appearance, the antimicrobial activity decreased abruptly, showing MICs of larger than 0.3% or more to the same indicator yeast (data not shown). Therefore when biological activities of plants are to be investigated, it should be kept in mind that degree of maturity affects the activity. One could test a plant parts before an active compound was formed or after the active compound became inactive, if he do not pay attention to this fact.

Secondly, antimicrobial activity of *C. apiifolia* disappeared when the fruit was dried. The fruits kept in refrigerator for up to a week, however, did not lose its activity. Herz *et al.* (23) reported that the antimicrobial activity of a *Clematis* species was maintained for more than 2 months at refrigerator temperature, suggesting that protoanemonin is stable at low temperature conditions.

Table 1. Antimicrobial activity (as MIC) of fruit extract of *C. apiifolia* against various microorganisms in comparison with that of garlic

Microorganisms	MIC (%)	
	C. apiifolia	Fresh garlic[1)]
Staphylococcus aureus B 33	0.2	1.5
Escherichia coli B 34	0.2	1.0
Enterobacter aerogenes B 146	0.4	1.5
Lactobacillus plantanum LA 97	2.5	2.0
Pediococcus pentosaceus LA 3	>1.5	3.0
Saccharomyces cerevisiae ATCC 4126	0.175	0.075
Candida utilis ATCC 42416	0.07	0.075
Candida albicans HY 1	0.1	0.1
Candida albicans KCTC 7121	0.08	0.075
Candida albicans KCTC 7965	0.08	0.1
Zygosaccharomyces rouxii KCCM 50546	0.075	0.2

[1)]From Kim and Kyung (15).

Investigators of plants for biological activity usually dry plant samples to protect them from deteriorating and to transport them with ease. In this case they might miss what they want if drying process affects the stability of the active compound(s). Method of extraction including choosing extraction solvent affected the biological activity of higher plants (24).

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