Antifungal Activity of Fistulosides, Steroidal Saponins, from *Allium fistulosum* L.

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Allium fistulosum L. (Welsh onion) is a perennial herb that is widely cultivated throughout the world, especially in China, Japan and Korea. Although, various activities were reported, little is known about antimicrobial activity of *A. fistulosum* L.. In this study, strong antimicrobial substances, fistuloside A, B, and C were isolated from the edible parts of *A. fistulosum* L. and their antimicrobial activity was evaluated with pathogenic- or food-spoilage microorganism based on disk-diffusion assay, minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) determination. Fistuloside A and fistuloside C showed strong antifungal activity and anti-*Proteus* activity, while fistuloside B is effective to only fungi. The fistuloside C showed a prominent antifungal activity with 3.1~6.2 μg/ml of MIC and MFC. Our results indicated that fistuloside C has a prominent antifungal activity and support the use of *A. fistulosum* to treat microbial infection.

Key words – *Allium fistulosum* L., antifungal activity, fistuloside, fungicidal activity, steroidal saponins

During the last few years, antimicrobial properties of plant extract and natural products have been intensively investigated as the demand for safe drugs which has increased due to the misuse of antibiotics and an increase in immuno-deficiency[1,6,10,15]. Plants of the genus *Allium* have been recognized as rich sources of secondary metabolites endowed with interesting biological activities[7,17]. The genus *Allium* represented by about 50 bulbous species and many of them contains various bioactive substances, such as steroidal saponin[4], flavonoid glycosides[2,5], antifungal protein[9], and unsaturated fatty acid monoglyceride[12] showing anti-thrombotic, anti-hyperlipemic and anti-atherosclerosis.

Allium fistulosum L. (Welsh onion) is a perennial herb that is widely cultivated throughout the world, especially in China, Japan and Korea. In Korea, *A. fistulosum* L. is an important vegetable crop and its annual production reaches 500,000 tons. This vegetable is characterized by a specific flavor and both the bulbs and the leaves are edible. The roots and bulbs have been used for the treatment of febrile disease, headache, abdominal pain, diarrhea, snakebite, ocular disorders, and habitual abortion, as well as having antifungal and antibacterial effects[8,14]. Recently, numerous studies have indicated that *A. fistulosum* L has antifungal activity[11,12,17] as well as human platelet anti-aggregation activity[2,3,5], antioxidative and anti-hypersensitive activity[13,16,18] and anti low-density lipoprotein oxidation and nitric oxide production[18].

Until now, little is known in antimicrobial activity of *A. fistulosum* L., although it has been used as antimicrobial plant in Asia. Yin and Tsao reported antimicrobial activity of water-soluble extract from *A. fistulosum* against *Aspergillus niger*, *A. flavus* and *A. fumigatus*[17]. Fistulosin (octadecyl 3-hydroxyindole) isolated from the roots and glycerol-mono-8,11,12-trihydroxy-9-octadecenoate isolated from the seeds showed high antifungal activity against *Fusarium oxysporum* and *Phytophtohora capsici*, respectively [11,12].

In this study, antimicrobial substances were isolated from the edible parts of *A. fistulosum* L. and their antimicrobial activity was evaluated using pathogenic- or food-spoilage microorganism. Our results showed that fistuloside C, a steroidal saponin from *A. fistulosum* L., has a prominent antifungal activity, and support the use of *A. fistulosum* to treat microbial infection as a traditional medicine.

Materials and Methods

Plant material, extraction and isolation

The bulbs and roots of *A. fistulosum* L. were collected in Kyungpook, Korea in the summer season of 2003. A voucher specimen was deposited at the College of

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Pharmacy, Yeungnam University, Korea. The dried chopped bulbs and roots (10 kg) were refluxed with hot methanol, three times. After evaporation of methanol in vacuo, the residue was suspended in water and then extracted successively with chloroform and *n*-butanol. The *n*-butanol fraction was subjected to silica gel (Merck Co., USA) column chromatography eluted with ethylacetate saturated with H_2O/MeOH (gradient 0 to 10%) to give six fractions. The third fraction was chromatographed over silica gel with $CHCl_3$-MeOH-H_2O (7:3:1) to give compound 1. The fifth fraction was subjected to silica gel column chromatography eluted with ethylacetate saturated with H_2O/MeOH (gradient 0 to 8%), as eluent to yield compound 2 and 3.

Identification of compound 1, 2 and 3.

Melting points were taken on a Yanaco apparatus and are uncorrected. Optical rotations were measured on a Rudolph autopol Ⅲ. IR (Infra Red) spectra were determined in KBr on a Mattson Polaris TM (FT-IR) spectrophotometer. Elemental analysis was performed on a Perkin-Elmer 240C instrument. Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker AM-300 (300 MHz for ^1H NMR and 75.5 MHz for ^{13}C NMR) spectrometer using tetramethylsilane as an internal standard and measured at room temperature.

Antibacterial activity assay

Bacterial strains used were *Proteus vulgaris* KCTC 2433, *Pseudomonas aeruginosa* KACC 10186, *Escherichia coli O157* ATCC 43895, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus mutans* JC-2, and *Staphylococcus aureus* KCTC 1916. The antibacterial activity was evaluated by disk-diffusion assay determined by growth inhibition zone[11,17]. The bacteria to be tested were grown in nutrient broth (Difco Co. USA) at 37℃ for 24 h, and collected by centrifugation. After spreading of each microorganism ($2x10^6$ cells) on nutrient agar (Difco Co., USA), disk of Whatman No. 2 filter paper (diameter of 6 mm) containing fistulosides (70 ㎍) was applied, and the growth inhibition was measured after 24 hr. Fistulosides were dissolved in dimethylsulfoxide. Dimethylsulfoxide (0.5%) was used as solvent controls, and ampicillin (1 ㎍/disk) was used as positive control. All data are presented as the mean values of triplicates for each microorganism.

Antifungal activity assay

Fungal strains used were *Candida albicans* ATCC 10231 as a representative fungus of Candidiosis and *Saccharomyces cerevisiae* IFO 0233 as a control strain. The antifungal activity was evaluated by disk-diffusion assay, and minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) using Sabouraud dextrose broth (Difco Co., USA), as previously reported[11,15,17]. For MIC determination, the fungi were grown in Sabouraud dextrose broth at 30℃ for 24 h, and then 0.1 ml of cell culture was inoculated with 0.9 ml of fresh medium with different concentrations of fistulosides (0. 1.5, 3.1, 6.2, 12.5, 25.0, 50.0, 70.0, and 100 ㎍/ml), which were dissolved in dimethylsulfoxide. The MIC was defined as the lowest concentration able to inhibit any visible microorganism growth and was determined by measure of cell growth OD after 48 h. The MFC was determined by culturing 0.1 ml of the vortexed broth from all tubes in the MIC assay on Sabouraud dextrose agar plates at 30℃ for 48 h. The MFC was defined as the lowest concentration which growth of fungal colony was completely inhibited. Dimethylsulfoxide (0.5%) was used as solvent controls, and miconazole (1.0, 1.5, and 2.0 ㎍/ml) were used as positive control, respectively. All data are presented as the mean values of triplicates for each microorganism.

Results and Discussion

The analysis of physicochemical and spectral data of isolated compound 1, 2 and 3 revealed that compound 1 is yuccagenin 3-*O*-α-L-rhamnopyranosyl-β-D-galactopyranoside (fistuloside A), compound 2 is yuccagenin 3-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-D-galactopyranoside (fistuloside B), and compound 3 is yuccagenin 3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl-β-D-galactopyranoside (fistuloside C) (data not shown). The compounds were also identified by direct comparison with authentic fistuloside A, B, and C obtained from Do et al. [4], and the structures of fistuloside A, B, and C were shown in Fig. 1.

Antimicrobial activity of fistulosides was evaluated against various pathogenic or food spoilage microorganisms based on disk-diffusion assay (Table 1). Ampicillin showed high activity against gram positive-, and gram negative- bacteria, except *Pseudomonas aeruginosa*, while miconazole was active to fungi. Fistuloside A and C showed antifungal and anti-*Proteus* activity, but fistuloside B is active to only fungi. Fistuloside C has more effective

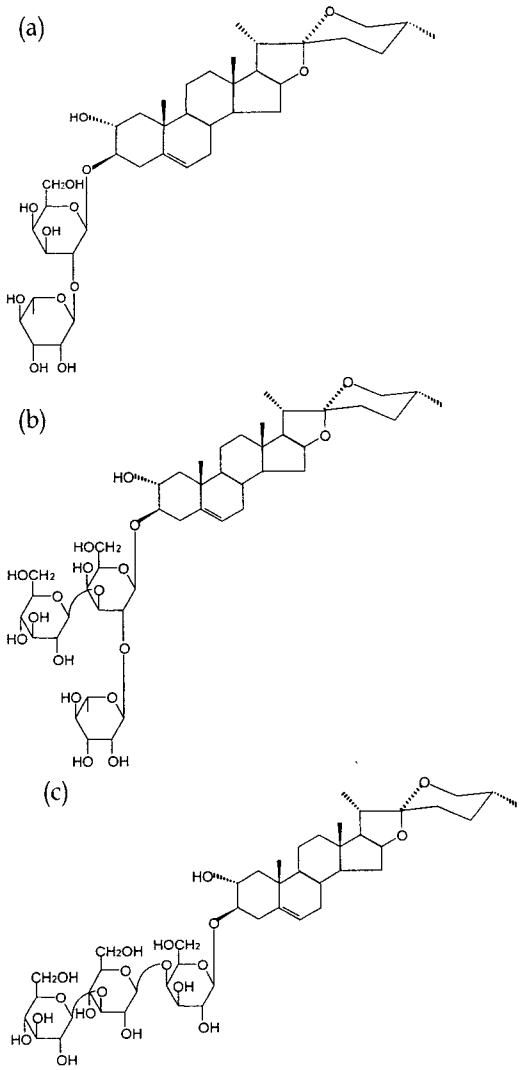

Fig. 1. The structure of (a) fistuloside A, (b) fistuloside B, and (c) fistuloside C, steroidal saponins from *A. fistulosum* L.

Table 1. Evaluation of antimicrobial activities of fistuloside A, B, and C against different bacteria and fungi

Compounds	Diameter of growth inhibition zone (mm)							
	Fungi		Gram negative bacteria			Gram positive bacteria		
	CA[1]	SC[1]	PV[1]	PA[1]	EC[1]	SE[1]	SM[1]	SA[1]
Ampicillin	-[2]	-	13	-	13	13	15	30
Miconazole	8	12	-	-	-	-	-	-
Fistuloside A	12.5	12	12	-	-	-	-	-
Fistuloside B	13	10	-	-	-	-	-	-
Fistuloside C	13	20	15	-	-	-	-	-

70 μg of samples (fistuloside A, B, and C), and 1 μg of ampicillin or 1 μg of miconazole were used per disk, respectively. [1]CA: *Candida albicans*, SC: *Saccharomyces cerevisiae*, PV: *Proteus vulgaris*, PA: *Pseudomonas aeruginosa*, EC: *Escherichia coli* O157, SE: *Staphylococcus epidermidis*, SM: *Streptococcus mutans*, SA: *Staphylococcus aureus*. [2]- : No inhibition.

against *S. cerevisiae* than *C. albicans*.

The MICs and MFCs of fistuloside A, B, and C against fungi were determined (Table 2). The miconazole at the concentration of 1.5 μg/ml completely inhibited fungal growth and prevented fungal colony formation. Fistuloside A, B, and C against *C. albicans* and *S. cerevisiae* were showed strong antifungal activity with 3.1~50 μg/ml of MIC. The slight differences of glycosylation in yuccagenin moiety resulted in big changes of antifungal activity. The strongest activity was found in fistuloside C with 3.1~6.2 μg/ml of MIC. Although fistuloside B has very similar structure with fistuloside C, the MICs of fistuloside B were at concentration of 25~50 μg/ml. The prominent activity of fistuloside C was comparable to MIC of fistulosin (octadecyl 3-hydroxyindole) against *Fusarium* sp. [11]. The MFCs of fistuloside A, B, and C were 3.1~100 μg/ml, and the values were similar to that's of MICs, suggested that the cell growth inhibition is mediated by fungicidal action. The strong fungicidal activity was also found in fistuloside C. As shown in Table 1, the higher sensitivity of *S. cerevisiae* to fistuloside C was observed in determination of MIC and MFC. The effect of fistuloside C on the growth of the fungi was investigated (Fig. 2). After treatment of different concentrations of fistuloside C to culture (10^6 cell/ml), the viable cell was counted using Sabouraud dextrose agar plates every 3 h. The suppressive effects of fistuloside C were evident and showed concentration dependent manner. At treated concentrations of 12.5 and 25 μg /ml, the cell viability was rapidly decreased showing first-order kinetics. The cell viability was rapidly decreased by treatment of miconazole for first 3 h, and then maintained for following 9 h. These results indicate that fistuloside C, a steroidal saponin from *A. fistulosum* L., has a prominent fungicidal activity, and support the use of *A. fistulosum* to treat microbial infection as a traditional medicine.

Table 2. MICs and MFCs of fistuloside A, B, and C, against different fungi

Compounds	*C. albicans*		*S. cerevisiae*	
	MIC[1]	MFC[2]	MIC	MFC
Miconazole	1.5	1.5	1.5	1.5
Fistuloside A	25	25	50	100
Fistuloside B	50	50	50	50
Fistuloside C	6.2	6.2	3.1	3.1

[1]MIC: Minimal Inhibitory concentration (μg/ml), [2]MFC: Minimal fungicidal concentration (μg/ml).

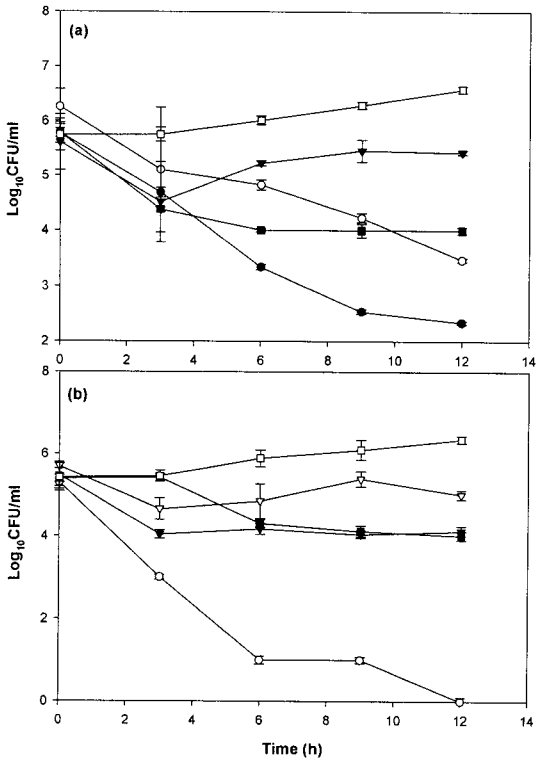

Fig. 2. The suppressive effect of fistuloside C on the growth of (a) *C. albicans* and (b) *S. cerevisiae*. □: No treatment, ▽: 3.1 μg/ml, ▼: 6.2 μg/ml, ○: 12.5 μg/ml, ●: 25 μg /ml of fistuloside C, and ■: 1 μg/ml of miconazole, respectively.

Further research on the mode of action of fistuolsides, and modification of fituloside C structure for activity increase or broad antimicrobial spectrum is necessary.

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References

1. Al-Waili, N. S. 2004. Investigating the antimicrobial activity of natural honey and its effects on the pathogenic bacterial infections of surgical wounds and conjunctiva. *J. Med. Food* **7**, 210-222.
2. Carotenuto, A., E. Fattorusso, V. Lanzotti, S. Mango, V. de Feo and C. Cicala. 1997. The flavonoids of *Allium neopolitanum*. *Phytochemistry* **44**, 949-957.
3. Chen, J.-H., H.-I. Chen, J.-S. Wang, S.-J. Tsai and C. J. Jen. 2000. Effect of welsh onion extracts on human platelet function in vitro. *Life Sci.* **66**, 1571-1579.
4. Do, J. C., K. Y. Jung and K. H. Son. 1992. Steroidal saponins from the subterranean part of *Allium fistulosum*. *J. Natural Prod.* **55**, 168-173.
5. Fattorusso, E, V. Lanzotti, O. Taglialatela-Scafati and C. Cicala. 2001. The flavonoids of leek, *Allium porrum*. *Phytochemistry* **57**, 565-569.
6. Grayer, R. J. and J. B. Harborne. 1994. Survey of antifungal compounds from higher plants. *Phytochemistry* **37**, 19-42.
7. Iwalokun, B. A., A. Ogunledun, D. O. Ogbolu, S. B. Bamiro and J. Jimi-Omojola. 2004. *In vitro* antimicrobial properties of aqueous garlic extract against multi-drug-resistant bacteria and *Candida* species from Nigeria. *J. Med. Food* **7**, 327-333.
8. Jiangsu New Medical College. 1986. *Zhong-yao-da-ci-dian*, pp. 2316-2318, In: *Zhong-yao-da-ci-dian*. Shanghai Science and Technology Publisher, Shanghai.
9. Lam, Y. W., H. X. Wang and T. B. Ng. 2000. A robust cysteine-deficient chitinase-like antifungal protein from inner shoots of the edible chive *Allium tuberosum*. *Biochem. Biophys. Res. Comm.* **279**, 74-80.
10. Negi, P. S., C. Anandharamakrishnan and G. K. Jayaprakasha, 2003. Antibacterial activity of *Aristolochia bracteata* root extracts. *J. Med. Food* **6**, 401-403.
11. Phay, N., H. Higashiyama, M. Tsuji, H. Matsuura, Y. Fukushi, A. Yokota and F. Tomita. 1999. An antifungal compound from roots of welsh onion. *Phytochemistry* **52**, 271-274.
12. Sang, S., A. Lao, Y. Wang, C.-K. Chin, R. T. Rosen and C.-T. Ho. 2002. Antifungal constituents from the seeds of *Allium fistulosum* L. *J. Agric. Food Chem.* **50**, 6318-6321.
13. Seo, G.-W., J.-Y. Cho, J.-H. Kuk, J.-H. Wee, J.-H. Moon, S.-H. Kim and K.-H. Park. 2003. Identification of antioxidative substances in *Allium fistulosum* L. by GC-MS. *Korean J. Food Sci. Technol.* **35**, 988-993.
14. Shogakukan. 1985. The Dictionary of Chinese drugs. pp. 1599-1600, In: *The Dictionary of Chinese drugs* Vol. 3. Shanghai Science and Technology Publisher and Shougakukan, Tokyo.
15. Sohn, H.-Y., K. H. Son, C.-S. Kwon, G.-S. Kwon and S. S. Kang. 2004. Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. *Phytomedicine* **11**, 666-672.
16. Yamamoto, Y., S. Aoyama, N. Hamaguchi and G.-S. Rhi. 2005. Antioxidative and antihypertensive effects of welsh onion on rats fed with a high-fat high-sucrose diet. *Biosci. Biotecnol. Biochem.* **69**, 1311-1317.
17. Yin, M.-C. and S.-M. Tsao. 1999. Inhibitory effect of seven *Allium* plants upon three *Aspergillus* species. *Int. J. Food Microbiol.* **49**, 49-56.
18. Wang, B.-S., J.-H. Chen, Y.-C. Liang and P.-D. Duh. 2005. Effects of welsh onion on oxidation of low-density lipoprotein and nitric oxide production in macrophage cell line RAW 264.7. *Food Chem.* **91**, 147-155.

초록 : 대파(*Allium fistulosum* L)로부터 fistulosides의 분리와 분리 물질의 항진균 활성

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대파(*Allium fistulosum* L)는 백합과 다년초로서 한국, 일본, 중국 등 전 세계적으로 식용으로 재배되어 왔다. 현재까지 대파의 다양한 생리활성이 보고되어 왔으나, 항균 활성에 대해서는 거의 보고되어 있지 않다. 본 연구에서는 대파 식용부위로부터 강력한 항미생물성 steroidal saponins (fistuloside A, B, 및 C)을 분리하였고, 이들의 항균력을 병원성균 및 식품부패균을 대상으로 평가하였다. Fistuloside A 및 C는 강력한 항진균 활성과 항*Proteus* 세균활성을 나타낸 반면, fistuloside B는 항진균 활성만 인정되었다. 특히 fistuloside C는 3.1~6.2 μg/ml의 MIC (minimal inhibitory concentration) 및 MFC (minimal fungicidal concentration)를 나타내어 강력한 항진균 활성이 진균사멸에 의한 항균효과임을 확인하였다. 본 연구결과는 대파가 미생물 감염증 제어용으로 사용되어 온 근거를 제시하며, 또한 fistuloside C의 항진균제로서의 이용 가능성을 제시하고 있다.