Food Sci Biotechnol (2018) 27(3):789–797
https://doi.org/10.1007/s10068-018-0315-3

Isolation and identification of antimicrobial substances from Korean Lettuce (*Youngia sonchifolia* M.)

Tae Yean Hwang[1] · **Chang Ki Huh**[1]

Received: 11 August 2017 / Revised: 28 December 2017 / Accepted: 9 January 2018 / Published online: 16 January 2018

Abstract The aim of this study was to identify of antimicrobial substances from Korean Lettuce (*Youngia sonchifolia* M.). Water and ethanol extracts of *Youngia sonchifolia* M. exhibited antimicrobial activities against the microorganisms tested. Ethanol extract showed strong antimicrobial activities against most Gram positive and Gram negative microorganisms, whereas no lactic acid bacteria and yeasts were not affected. The antimicrobial compound G-6 was isolated from the ethyl acetate fraction obtained by silica gel column chromatography and HPLC and was confirmed as stable against heat treatment. Molecular weight of G-6 was calculated as 154 kDa based on information in the MS spectrum. G-6 was identified as 2-nonynoic acid ($C_9H_{14}O_2$) by UV, LC-EI/MS, and LC-CI/MS. In support of this, the chromatogram of G-6 was consistent with that of the 2-nonynoic acid standard. Antimicrobial activity of 2-nonynoic acid was identified in all samples, with a significant difference based on concentratio.

Keywords *Youngia sonchifolia* M. · Antimicrobial activity · Ethylacetate extract fraction · HPLC–MS · GC chromatogram · 2-nonynoic acid

✉ Chang Ki Huh
hck1008@sunchon.ac.kr

[1] Department of Food Science and Technology, Sunchon National University, 255, Jungang-ro, Suncheon, Jeollanam-do 57922, Korea

Introduction

Korean lettuce (*Youngia sonchifolia* M., YSM), an annual or biennial plant belonging to the composite family, is native to the fields and mountains of South Korea and sometimes cultivated on farms. YSM is characterized by a height of approximately 80 cm, a straight stem with many sprigs, and a tinge of reddish purple color. Its leaves are oval-shaped, 2.5–5.0 cm in length, 1.4–1.7 cm in width, without any petiole, and split into a comb shape. The front side of its leaves is green while the back is green tinged with gray, and there is no hair on either side [1, 2].

According to the shape and classification system, Korean lettuce native to the fields and mountains of South Korea is classified into nine types [3, 4]: *Youngia sonchifolia* M., *Youngia denticulate*, *Youngia denticulate*, *Youngia chelidoniifolia* KITAMURA, *Crepidiastrum koidzumianum* (Kitam.), *Lactuca triangulata* Maxim, *Lactuca indica* var. *laciniata* (O. Kuntze), *Lactuca indica* var. *laciniata* (Houtt.) H. Hara, *Lactuca indica* var. *dracoglossa*. Of these plants, *Youngia sonchifolia* M., *Youngia denticulate*, and *Lactuca indica* var. *laciniata* (O. Kuntze) are used as food [5]. Plants belonging to the same subfamily are *Lactuca sativa*, *Hieracium umbellatum*, *Taraxacum platycarpum*, chicory (*Cichorium intybus*), and *Ixeridium dentatum* (Thunb.) Tzvelev [6].

YSM has long been eaten an herb in springtime or made into kimchi in autumn in South Korea. Nowadays, in some regions of the southern part of the country, it is made into kimchi and eaten during all four seasons. In the southern regions of South Korea, including Gurye, Gwangyang, Suncheon, and Jinju, it is still widely consumed in the form of kimchi, and currently cultivated in order to meet the demands of large cities. A, large amount of YSM is

supplied to other regions such as Seoul, and its consumption has gradually expanded to all regions of South Korea [7].

YSM has been reported to improve blood circulation and have anti-inflammatory effects [8–12]. In a study on the components and biological activation of YSM, Park [13] reported its amino acid composition, identified chlorogenic acid, and tested the anti-cancer effects of YSM extract. Bae et al. [14] applied YSM extract to the body parts of rats with induced liver toxicity and observed inhibited generation of lipid peroxides. Young et al. [15] reported reduction of the serum cholesterol level upon intra peritoneal injection of YSM extract into mice. Hwang et al. [16] reported that YSM kimchi affects protein digestibility. However, no research has yet evaluated the antimicrobial activity and active substances of YSM.

Therefore, the aim of this research was to obtain basic data for raw material standardization based on evaluation of the antimicrobial activity and antimicrobial substances of YSM by analyzing general components according to collection periods in order to verify its functionality.

Materials and methods

Chemicals

All solvents used for extraction procedures (hexane, ether, ethylacetate (EtOAc), and ethanol) and chloroform were obtained from Merck and Junsei, Germany and Japan. Sulfuric acid and sodium hydroxide solutions for crude fiber analyses were purchased from Daejung Chemicals & Metals, Gyeonggido, Korea. Methyl 2-nonynoate was purchased from Sigma–Aldrich (St. Louis, MO, USA). Water and ethanol for MS analyses were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Plant materials and Bacterial strains

Leaves and roots of *Youngia sonchifolia* M. (YSM) were cultivated in an open field after spraying seeds at in early June in Suncheon, South Jeolla Province, Korea. Samples were collected in the same area in early October and early December (Suncheon area weather (Data of Korea Meteorological Administration).: mean temperature/days; October → 16.13 °C/days, December → 0 °C/days, precipitation/month; October → 13.8 mm/month, December → 10.6 mm/month). The samples used for the experiments were 25–30 cm in length samples. The samples were washed and the roots and leaves were separated and stored at − 54 °C in a deep freezer (FDA5508; Ilsinbiobase, Dongducheon, Korea). Ten bacterial strains were used through-out this study: *Bacillus cereus*, *Bacillus subtilis*, *Staphylococus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens*, *Lactobacillus plantarum*, *Lactobacillus mesenteroides*, *Saccharomyces cerevisiae*, and *Hansennula anomala.*

Proximates

Moisture, crude protein, crude fat, crude ash, and crude fiber contents were determined according to the AOAC official method. Briefly, moisture content was obtained by calculating weight loss after powdered seed samples were oven-dried at 105 °C to a constant weight [17]. Crude protein and crude fat contents were analyzed using the Kjeldahl and the Soxhlet extraction methods, respectively [18, 19]. Ash content was determined gravimetrically after the samples were ignited in an oven at 600 °C [18]. Estimation of crude fiber content was based on loss upon ignition of dried residue after samples were digested with 1.25% each of sulfuric acid and sodium hydroxide solutions [19]. The following equation was used for calculation of carbohydrate contents: % carbohydrates = 100% − (% crude protein + % crude fat + % crude ash).

Preparation of YSM extracts by different solvents

Water extract of YSM (sampled December, 2014)was homogenized to a fine powder, followed by maceration of 100 g with 3 L of water at room temperature for 24 h. Ethanol, ethylacetate (EtOAc), ether, and hexane extract were also prepared by the same method used for the water extraction process, and the extracts were filtered and stored at 4 °C.

Antimicrobial activity assays

Paper disc assay [20] was used for determination of antimicrobial activities. Plates were prepared by addition of bacterial strains culture medium (10^6 viable cells per 20 mL of nutrient broth (Difco, U.S.A)) up to a concentration of 1.5% (w/v). For the paper disc assay, paper discs (diameter 8 mm; Advantec, Tokyo, Japan) on nutrient agar (Difco, U.S.A) plates were spotted with 100 µL of YSM extracts using different solvents. The plates were incubated at 30 °C for 48 h and examined for inhibition zones. Antimicrobial activity was expressed as clear zone size (mm). The above described experiment was performed in triplicate.

Minimum inhibitory concentration assay

The minimum inhibitory concentration (MIC) of ethanol extract having antibacterial activity was measured by the agar dilution method. The content of the ethanol extract

were 0, 0.25, 0.5, 1 and 1.5 mg/mL was poured into a petri dish to solidify the medium. The medium was inoculated with 0.1 mL of pre-cultured suspension and incubated at 30 °C for 20 h. The concentration at which growth of the strain did not occur was determined.

Serial solvent fractionation of ethanol extract

YSM leaves (300 g) that exhibited the best antimicrobial activity were extracted with 9 L of ethanol, and 155.8 g of extract was obtained through filtration and concentration. The method shown in Fig. 1(A) was used for solvent fractionation. Extraction was performed three times using a mixed solvent of hexane-ethanol–water (10:1:9 v/v/v) in a separatory funnel, to obtain 1 L of extract, which was concentrated to obtain the hexane fraction (39.4 g). Using the same method, solvent fractionation of the water layer was performed with ether and EtOAc, followed by concentration to 2.2 and 12.5 g, respectively. Finally, the water fractionation 99.2 g was obtained and used after being diluted to the required concentration.

Isolation of antimicrobial substances

Separation of the antimicrobial substance by silica gel (70–270 mesh, Merck, Germany) column (∅ 3 cm × 35 mm) chromatography was performed according to the following method. Each fraction was obtained through solvent fractionation of YSM leaf ethanol extract, and the antimicrobial activity of each fraction was measured. The measurements were compared, and silica gel column chromatography was performed for identification of active components from the EtOAc fraction that exhibited strong activation. After dividing the EtOAc fraction into a number of subfractions, antimicrobial substances were separated based on comparison of the antimicrobial activities of the subfractions, as shown in Fig. 1(B). Using 10 times the amount of activated silica gel as the sample, a slurry was made with chloroform, and the glass column was filled (∅ 3 cm × 50 mm). Ethanol concentration was increased from 0 to 10, 20, 30, 40, 50, 70, and 100% in a stepwise manner using chloroform-ethanol ($CHCl_3$–EOH) solvent. The flow rate was 1.0 mL/min and 10 mL per fraction was fractionated using a fraction collector (Frac 100 Pharmacia, Sweden). The fractions obtained by this method were concentrated under reduced pressure and the antimicrobial activity of each fractionation was measured and compared using the paper disc method [20].

Separation of antimicrobial active components by HPLC (Waters M510, Co. Waters, Milford, MA, USA) was performed as follows: subfractions demonstrating antimicrobial activity by silica gel column chromatography were collected and concentrated under reduced pressure, and

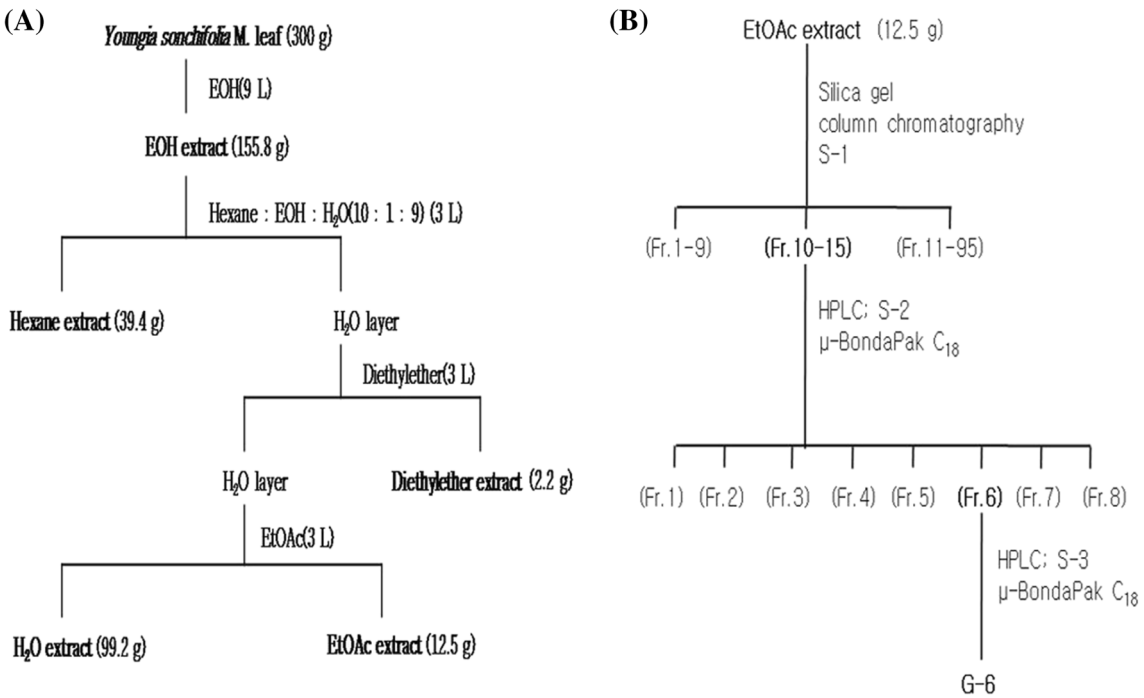

Fig. 1 Fractionation of the ethanol extract from *Youngia sonchifolia* M. (**A**) and isolation procedure of antimicrobial substances from ethylacetate extract (**B**). Solvent systems are as follows; S-1: $CHCl_3$:EOH step-wise (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 3:7, 0:10), S-2: 80% EOH, S-3: 80% EOH

active antimicrobial components were isolated from the concentrated solution using HPLC. As shown in Fig. 1(B), fractions with primarily antimicrobial activity were isolated, using HPLC and a μ-Bondapak C_{18} column for fractionation (ID 30 mm × 30 cm, Waters Co., Milford, MA, USA). The mobile phase was 70% ethanol, the flow rate was 20 mL/min, and the injection volume was 0.5 mL with detection at 275 nm in the UV spectrum. Eight fractions were obtained. After each of the eight fractions was concentrated under reduced pressure in a 50 °C water bath and diluted, the antimicrobial activity of each fractions was measured and verified using the paper disc method [20]. The fractions that exhibited antimicrobial activity were secondarily separated and concentrated under reduced pressure according to the peaks, and antimicrobial activity was verified. Finally, active antimicrobial components were isolated into a homogeneous material.

Effect of heat on antimicrobial substance (G-6)

The effects of heat on the antibacterial activity of the antimicrobial substance (G-6) was assessed. Antimicrobial substance (G-6) isolated by HPLC was heated at 10 °C for 1 h intervals from 60 °C to 100 °C and held for 15 min at 121 °C, after which growth inhibition against *B. cereus* and *E. coli* was measured using the paper disk method.

Identification of antimicrobial substance (G-6)

For identification of antimicrobial components in the UV spectrum, the antimicrobial substance (G-6), which was isolated into a homogeneous material, was dissolved in ethanol, and a light absorption spectrum was measured by scanning in the range of the UV spectrum (190–1100 nm) using a spectrometer (HP8453, Hewlett Packard Co., Germany).

Antimicrobial material was identified by LC-EI/MS and LC-CI/MS as follows: LC/MS was composed of the 59980B Particle Beam LC/MS Interface/5898B mass spectrometer detector (Mass range 20–1000 m/z, Hewlett Packard Co., USA); separation of the active fraction was performed using an ODS Hypersil (100 mm × 2.1 mm, i.d. 5 μm, Hewlett Packard Co. USA). The mobile phase consisted of water and ethanol 20/80 v/v at a flow rate of 0.3 mL/min, at an injection volume of 50 μL. The ionization method was selected ion monitoring (SIM) for selection and detection of specific ions; molecular weight was determined using CI (chemical ionization), and ionization was performed by EI (electron ionization); mass spectra were recorded and compared with those stored in the Wiley/NBS mass spectral library of the LC/MS data system of the apparatus. 2-nonynoic acid was identified by gas–liquid chromatography. 2-nonynoic acid was obtained by alkali hydrolysis of methyl 2-nonynoate. Gas–liquid chromatographic analyses were performed on a Hewlett-Packard (Hewlett Packard Inc., Palo Alto, CA) model 5890 instrument equipped with a flame-ionization detector and fitted with a fused-silica capillary column coated with Nukol TM (15 m, 0.53-mm diameter, 0.50-μm film thickness) or with Omega wax TM (30 m, 0.32-mm diameter, 0.25-μm film thickness) as the stationary phase at a column temperature of 200 °C. Silica gel (type 60, 70–230 mesh, Merck, Darmstadt, Germany) was used for column chromatography.

Antimicrobial activity assays of 2-nonynoic acid

The antibacterial activities of 2-nonynoic acid were assessed. The paper disks (diameter 8 mm; Advantec, Tokyo, Japan) on nutrient agar (Difco, U.S.A) plates were spotted with 100 and 150 μL of 2-nonynoic acid standard, after which growth inhibition against *B. subtilis* was measured using the paper disk method.

Statistical methods

Data were expressed as mean ± SD (standard deviation), and statistical analysis for single comparisons was performed using Duncan's multiple range test. Each experiment was repeated at least three times to yield comparable results. Values of $p < 0.05$ were considered significant.

Results and discussion

Proximates

The analysis results of the changes in the general components of roots and leaves of YSM according to collection period are shown in Table 1. Moisture contents of leaves and roots were 86.1 and 84.1%, respectively, in the test plot for October (hereafter, October) and showed slight decreases to 85.3 and 82.4%, respectively, in the test plot for December (hereafter, December). Crude ash content increased from 0.7% in October to 0.8% in December in leaves and increased from 0.6 to 1.1% in roots. Similar to crude ash content, crude protein content increased from 1.8% in October to 2.1% in December in leaves and increased from 1.8% to 2.7% in roots. Crude fat content decreased in both leaves and roots in December compared to October, whereas crude fiber increased in leaves and roots from 4.3 to 4.6% in October to 4.9 and 4.8% in December. The carbohydrate contents of both leaves and roots increased from 6.2 to 8.4% in October to 7.0 and 8.7% in December, respectively. In the content analysis of the general components of *Lactuca indica* from June to

Table 1 Proximate composition of *Youngia sonchifolia* M. (%)

Composition	Samples			
	Leaf		Root	
	A[(1)]	B	A	B
Moisture	86.1 ± 1.89[a(2)]	85.3 ± 1.53[a]	84.1 ± 1.88[b]	82.4 ± 1.76[c]
Crude ash	0.7 ± 0.04[b]	0.6 ± 0.03[b]	0.6 ± 0.03[b]	1.1 ± 0.12[a]
Crude protein	1.8 ± 0.15[c]	2.1 ± 0.28[b]	1.8 ± 0.16[c]	2.7 ± 0.53[a]
Crude fat	0.6 ± 0.03[a]	0.4 ± 0.02[b]	0.5 ± 0.02[ab]	0.3 ± 0.02[c]
Crude fibre	4.3 ± 0.89[b]	4.9 ± 1.03[a]	4.6 ± 0.81[a]	4.8 ± 0.79[a]
Carbohydrate	6.2 ± 1.23[b]	7.0 ± 1.33[b]	8.4 ± 1.62[a]	8.7 ± 1.33[a]

[(1)]A: *Youngia sonchifolia* M. sampled October, B: *Youngia sonchifolia* M. sampled December.; Each value is expressed as mean ± standard deviation, n = 3.; [(2)]Mean ± SD with different superscript within a row are significantly different ($p < 0.05$) by Duncan's multiple range test

August, Kwon et al. [21] reported that moisture content decreased while crude protein content increased, whereas no significant difference was observed in the contents of the crude fat, crude ash, and crude fiber. The results of this research were similar with regard to changes in moisture and crude protein contents but different from those of Kwon et al. [21] for contents of crude fat, crude ash, and crude fiber. These different results may be due to the fact that this research differed from that of Kwon et al. [21] in terms of the varieties used and harvesting periods from June to August versus from October to December.

Antimicrobial activity

Measurement of antimicrobial activity was carried out by the agar diffusion method using Gram negative *E. coli* and Gram positive *B. subtilis*, and the results are shown in Table 2A. In terms of the antimicrobial activity of YSM, for October leaf extract, water, ethanol, and EtOAc extracts showed zone diameters of 12, 21, and 14 mm, respectively, with regard to *E. coli*. For roots, water, ethanol, and EtOAc extracts showed zone diameters of 11, 17, and 13 mm, respectively. For the December test plot, for leaf extract, water, ethanol, and EtOAc extracts showed zone diameters of 19, 22, and 17 mm, respectively, with regard to *E. coli*. For roots, water, ethanol, and EtOAc extracts showed zone diameters of 12, 18, and 13 mm, respectively. Thus, in comparing the different parts of YSM, higher antimicrobial activity was detected in leaves than in roots, and December samples showed higher antimicrobial activities across all collection periods. In terms of the extraction ability of each solvent, the highest extraction ability was observed for ethanol while ether and hexane were not as effective in extracting the antimicrobial substance. Park [22] reported that the antimicrobial activities of methanol extracts of *Lactuca indica L.* were all high, and antimicrobial activities of the strains were slightly different. Regarding the antimicrobial activity according to the strains, similar antimicrobial activities were detected against both *E. coli* and *B. subtilis*. Since the extracted antimicrobial material exhibited antimicrobial activity against both Gram negative and Gram positive bacteria, it may be able to inhibit food spoilage and growth of foodborne pathogens. In addition, YSM extract showed higher antimicrobial activity than sodium benzoate used as food preservative. Although suppression of initial bacterial numbers in kimchi remains a hurdle, YSM kimchi has strong antimicrobial activity and thus exhibits antimicrobial activity against foodborne pathogens and putrefactive microorganisms in kimchi. Consequently, it is thought to inhibit the growth of microorganisms harmful to the fermentation of kimchi and prevent initial rancidity of kimchi. In addition, YSM had no inhibitory effect on the growth of lactic acid bacteria involved in fermentation of kimchi, thereby allowing proper ripening to improve the flavor and storability of kimchi. This fact may indicate that YSM has no adverse effects on the quality improvement of kimchi (Table 2(B)). The minimum inhibitory concentration of YSM ethanol extract was found to be 0.25 mg/mL, and the highest antimicrobial activities of YSM ethanol extract against Gram positive *B. cereus*, *B. subtilis*, and *S. aureus* were 0.5 mg/mL, 0.5–1.0 mg/mL and 0.5–1.0 mg/mL, respectively. YSM ethanol extract showed low antimicrobial activity (1.5 mg/mL) against three Gram negative bacterial strains, *E. coli*, *S. typhimurium*, and *P. fluorescens*. These results indicate that the antimicrobial activity of YSM ethanol extract against Gram positive bacteria was higher than that against Gram negative bacteria. However, YSM ethanol extract showed no antimicrobial effects against strains such as lactic acid bacteria and yeast even at concentrations over 1.5 mg/mL.

Table 2 Antimicrobial activities of *Youngia sonchifolia* M.

Samples[1]		Clear zone on plate (mm) (50 mg/disc)									
		Water extract		Ethanol extract		Ethylacetate extract		Ether extract		n-Hexane extract	
		E.[1]	*B.*[2]	*E.*	*B.*	*E.*	*B.*	*E.*	*B.*	*E.*	*B.*
A											
Leaf	a	12 ± 0.53[3]	13 ± 0.50	21 ± 1.23	20 ± 1.32	14 ± 0.85	14 ± 0.63	–[4]	–	–	–
	b	19 ± 1.22	19 ± 1.24	22 ± 1.54	23 ± 1.36	17 ± 1.03	18 ± 1.32	–	–	–	–
Root	a	11 ± 0.13	11 ± 0.12	17 ± 1.10	16 ± 1.29	13 ± 0.52	12 ± 0.21	–	–	–	–
	b	12 ± 0.22	12 ± 0.31	18 ± 1.33	17 ± 1.22	13 ± 0.33	13 ± 0.32	–	–	–	–

Strains	Clear zone on plate (50 mg/disc)				Sodium benzoate
	Water extract		Ethanol extract		
	Leaf	Root	Leaf	Root	
B					
B. cereus	18 ± 1.23[3]	13 ± 0.71	23 ± 1.61	18 ± 1.09	17 ± 1.22
B. subtilis	17 ± 1.02	12 ± 0.68	24 ± 1.34	16 ± 1.10	15 ± 1.09
S. aureus	19 ± 1.10	12 ± 0.76	21 ± 1.23	17 ± 1.21	16 ± 1.25
E. coli	20 ± 1.23	14 ± 0.72	22 ± 1.34	18 ± 1.06	15 ± 1.16
S. typhimurium	19 ± 1.34	12 ± 0.82	22 ± 1.39	18 ± 1.16	17 ± 1.03
P. fluorescens	18 ± 1.07	12 ± 0.69	24 ± 1.65	17 ± 1.23	16 ± 1.26
L. plantarum	–	–	–	–	
L. mesenteroides	–	–	–	–	
S. cerevisiae	–	–	–	–	
H. anomala	–	–	–	–	

Strains	Growth at various concentration (mg/mL)					MIC (mg/mL)
	0	0.25	0.5	1	1.5	
C						
B. cereus	+	−	−	−	−	0.25
B. subtilis	+	+	−	−	−	0.5
S. aureus	+	±	−	−	−	0.5
E. coli	+	+	+	+	−	1.5
S. typhimurium	+	+	+	+	−	1.5
P. fluorescens	+	+	+	+	−	1.5
L. plantarum	+	+	+	+	+	−
L. mesenteroides	+	+	+	+	+	−
S. cerevisiae	+	+	+	+	+	−
H. anomala	+	+	+	+	+	−

A: Antimicrobial activities against *E. coli* and *B. subtilis* of solvent extracts from *Youngia sonchifolia* M.; B: Antimicrobial activities of water and ethanol extracts against *Youngia sonchifolia* M; C: Minimum inhibitory concentration(MIC) of the ethanol extract of *Youngia sonchifolia* M.; a: *Youngia sonchifolia* M. sampled October; b: *Youngia sonchifolia* M. sampled December.; [1]*E. coli*; [2]*B. subtilis*; [3]Mean ± SD with different superscript within a row are significantly different ($p < 0.05$) by Duncan's multiple range test. [4]Not detected.; +, Growth; ±, Uncertain in growth; −, No growth

Isolation of antimicrobial substances

Each fraction was obtained by performing solvent fractionation of ethanol extract, and the antimicrobial activity of each fraction was measured and compared. Each fraction was then subjected to silica gel column chromatography and divided into a number of subfractions, and the antimicrobial activity of each subfraction was measured to

determine the effective ingredients of the EtOAc fraction that exhibited stronger antimicrobial activity. In other words, EtOAc fraction (12.5 g) was subjected to silica gel column chromatography in order to obtain fractions. In the chromatogram of the absorbance measurement at 270 nm for each fraction, four peaks were detected, as shown in Fig. 2(A). The strongest antimicrobial activity was observed in the third peak, which was at 60–70 nm. In comparison of the UV spectra of the fractions with strong antimicrobial activity, fractions with strong antimicrobial activity showed a strong absorption band at 270 nm. In addition, when the third peak, which is the fraction with strong antimicrobial activity, was separated by HPLC, a total of eight peaks were detected, and antimicrobial activity was observed in the sixth peak (Fig. 2(B)).

Heat stability of antimicrobial substance (G-6)

Most processed foods are produced through heat treatment. An antimicrobial substance that can be used as a preservative in foods should be stable under heat [23]. Therefore, the heat stability of the antimicrobial substance in this study was examined. Heat stability of the antimicrobial substance (G-6) isolated by HPLC is shown in Table 2(C). Antimicrobial activity of the antimicrobial substance (G-6) isolated by HPLC was not inhibited by heating at 121 °C for 15 min or heating at 100 °C for 1 h. Therefore, the antimicrobial substance (G-6) of YSM was confirmed as stable against heat treatment (no shown data).

Identification of antimicrobial substance (G-6)

The antimicrobial substance (G-6) isolated by HPLC was dissolved in ethanol, and its light absorption spectrum was obtained by scanning in the UV spectrum range of 190 ~ 1100 nm (no data shown). The antimicrobial substance showed absorption peaks at 210 nm and 260–275 nm, which indicates a combination of auxochrome with a chromophore of conjugation. The CI and EI mass spectra of the antimicrobial substance (G-6) isolated by HPLC were analyzed using the 59980B Particle Beam LC/MS Interface. Based on the Mass CI spectrum, the molecular weight of the antimicrobial substance was estimated as 154 kDa. When the EI spectrum was compared with the Wiley Database embedded in LC/MS, it was estimated as 2-nonynoic acid ($C_9H_{14}O_2$) (Fig. 3(A)). The results of the 2-nonynoic acid analysis by GC are shown in Fig. 3(B). The chromatogram of the antimicrobial

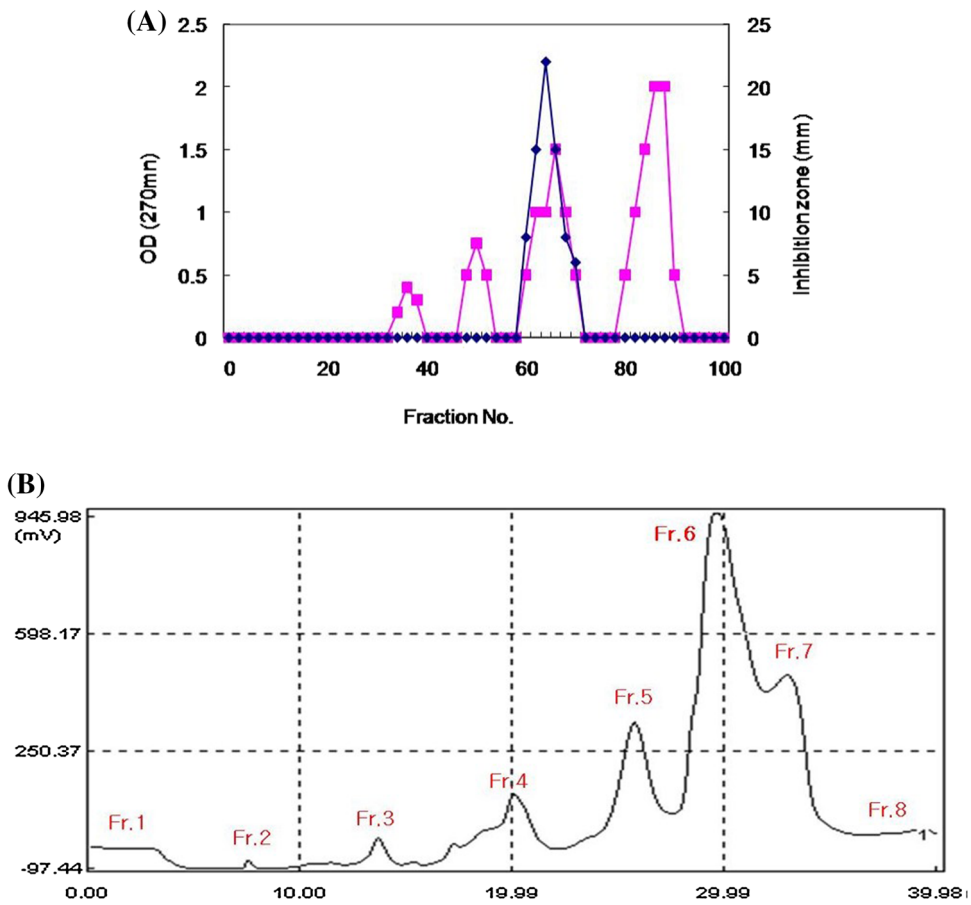

Fig. 2 Antimicrobial activities of the ethylacetate extract fractionated by the silica gel column chromatography against *E. coli*. and HPLC chromatogram of antimicrobial substance. Filled diamond inhibition zone; filled square OD(270 nm)

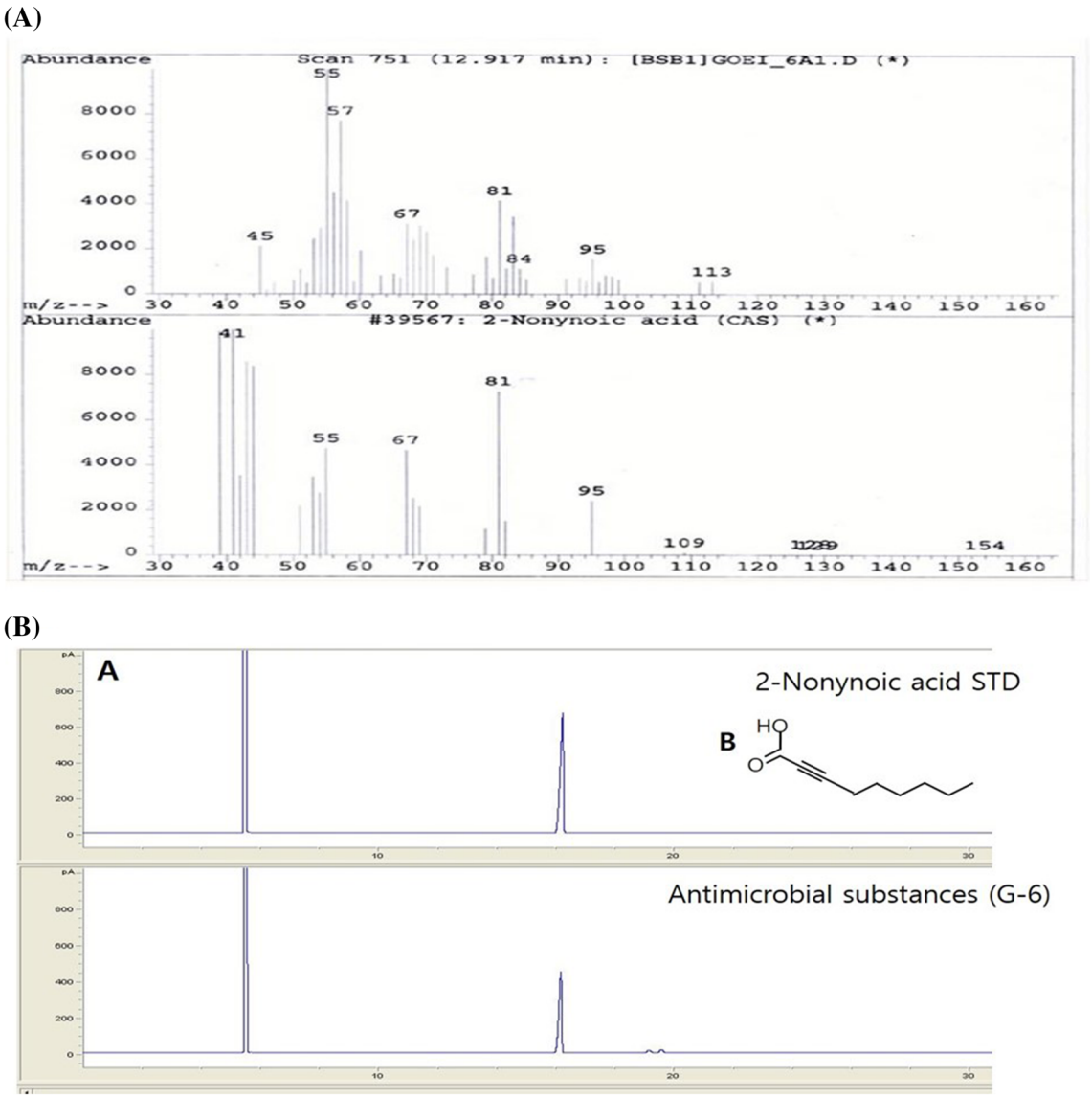

Fig. 3 Wiley data base spectrum of antimicrobial substance (G-6) and GC chromatogram and chemical structure of 2-nonynoic acid. (**A**) Chromatogram of 2-nonynoic acid, (**B**) Chemical structure of 2-nonynoic acid($C_9H_{14}O_2$)

substance (G-6) was consistent with that of the 2-nonynoic acid standard. The structural formula of 2-nonynoic acid is shown in Fig. 3(B).

Antimicrobial activity of 2-nonynoic acid

The results of the measurement of the antimicrobial activity of the 2-nonynoic acid standard using Gram-positive *B. subtilis* are shown in Fig. 4. The antimicrobial activity of 2-nonynoic acid was identified in all samples, with a significant difference according to concentration. The sample treated with 150 μL of 2-nonynoic acid standard was 19 mm and the sample treated with 100 μL was 15 mm. Therefore, the antimicrobial activity of 2-nonynoic acid in this study was confirmed. The antibacterial properties of YSM increased in the presence of 2-nonynoic acid. Antimicrobial substance from YSM was characterized based on its UV spectrum, LC/MS spectrum, and GC chromatogram, and 2-nonynoic acid was identified as the antimicrobial compound. According to R. Rieger et al. [24], 2-nonynoic acid does not exist in nature and can be produced only by synthesis. However, the results of this study are not consistent with their claim in that the antimicrobial substance in YSM was determined to be 2-nonynoic acid. 2-nonynoic acid has been used as a cosmetic raw material [24]. However, the effects of 2-nonynoic acid methyl ester on rats [25, 26] indicate that it can cause acute toxicity as well as an allergic skin reaction in humans. Thus, its use in cosmetics production is restricted to 0.2% or less by regulations.

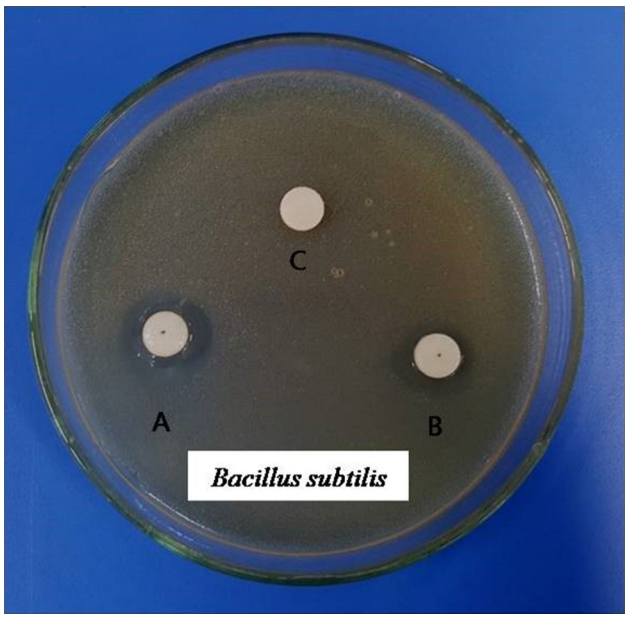

Fig. 4 Antimicrobial activities of 2-nonynoic acid standard against *B. subtilis*. (*C*) Control, (*A*) 2-nonynoic acid standard 150 μL, (*B*) 2-nonynoic acid standard 100 μL

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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