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Antibacterial effect of naturally occurring unsaturated fatty acids from *Prunus japonica* against *Propionibacterium acnes*

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SUMMARY

The antibacterial activity-guided fractionation of the MeOH extract of seeds of traditional medicinal plant *Prunus japonica* resulted in the isolation of linoleic acid and cis-11-eicosenoic acids, and their methyl esters. Linoleic acid inhibited the growth of *Propionibacterium acnes*, the acne-causing anaerobic bacterium, but cis-11-eicosenoic acid, methyl linoleate, and cis-11-eicosenoate were found to be inactive. Together with isolated linoleic acid, authentic saturated and unsaturated fatty acids were also tested against *P. acnes* with other bacteria and fungi. Most of the unsaturated fatty acids possessed anti-acne (MIC 16-128 μg/mL) and antimicrobial properties.

Key words: *Propionibacterium acnes*; *Prunus japonica*; Rosaceae; fatty acids; antibacterial; antifungal

INTRODUCTION

Acne vulgaris is the most common skin disease that is characterized by open and closed comedones, papules, pustules, and nodules (Burkhart *et al*., 1999). It has been recognized that *Propionibacterium acnes*, an anaerobic non-spore-forming gram-positive bacillus, is thought to play an important role for the inflammation of acnes (Leyden, 1995). Inhibition of *P. acnes* growth could be one of the effective strategies for the prevention of acne vulgaries. Benzoyl peroxide, isotretinoin, tetracycline, erythromycin, and clindamycine are topically-applicable and/or systemically-administrable agents for effective treatments of acnes (Bershad, 2001). However, indiscriminating use of antibiotics against *P. acnes* is often causing the bacterium to develop drug-resistance (Eady *et al*., 2003; Swanson, 2003). Hence, plant extracts or plant-derived compounds (herbal remedies) would be alternatives for acne treatments as antiseptics and antimicrobial agents (Eady *et al*., 2003; Barbour *et al*., 2004; Chomnawang *et al*., 2005; Weckesser *et al*., 2007).

In search for antibacterial compounds against *P. acnes* from Korean medicinal plants (Moon *et al*., 2004; Sultan *et al*., 2008), methanolic extracts of over 200 plants were tested using disc diffusion assay method against *P. acnes*. Around 10% of the tested plants such as *Salvia miltorrhiza* (Labiatae), *Echinops setifer* (Compositae), *Pinus densiflora* (Pinaceae), and *Asarum sieboldii* (Aristolochiaceae) were found to be active at 500 μg/disc. Among the plants tested, the methanolic extracts of the seeds of bush berry, *Prunus japonica Thunb.* var. nakaii (Lev.) Rehder (Rosaceae) has been selected for isolation of active ingredients. This plant is an about 1 m long deciduous shrub, indigenous at the mountainous and hilly area of Republic of Korea, China and

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Japan. It flowers pale pink in May and ripens in late July. The fruits are a bit bitter and the seeds 12 mm long in a rusty oval shape. The seeds of *P. japonica* are used in Republic of Korea as traditional folk medicine against constipation, child fever, pinworms and teeth problems, and as carminative, diuretic, laxative (Bae, 2000).

This paper describes the characterization of linoleic acid, cis-11-eicosenoic acid, and their methyl esters from the seeds of *P. japonica*, and their antibacterial activities against *P. acnes*. It also reports antibacterial activities of the isolates and a number of authentic saturated and unsaturated fatty acids against aerobic and anaerobic bacteria including *P. acnes*, and fungi.

MATERIALS AND METHODS

General

1H- and 13C-NMR spectra were recorded on a Varian Mercury 400 Spectrometer (Varian Inc.; Palo Alto, CA, USA), with standard pulse sequences operating at 400 MHz for 1H NMR and 100 MHz for 13C spectra. The chemical shifts were given in ppm and were referenced to the residual peaks of $CDCl_3$ at 7.26 and 77.24 for 1 h and 13C NMR, respectively. Normal phase column chromatography was carried out on silica gel 60 (70 - 230 mesh, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel 60, F254, 20 × 20 cm, 0.25 mm thick, Merck, Darmstadt, Germany). Spots were visualized under UV light at 254 and 365 nm or by dipping in a methanolic solution of p-anisaldehyde-sulfuric acid (3:1) followed by heating. Preparative HPLC was performed on a Sykam system (model S 2100) (Sykam GmbH, Eresing, Germany) with a diode array detector (S 3210 UV/ Visible detector) and a reverse phased C18 column (Senshu Pak, Pegasil ODS, 20 id x 250 mm) at a flow rate of 7 mL/min at 214 nm.

Bacterial strains

Bacterial strains *P. acnes* (KCTC No. 3314), *S. mutans* (KCTC 3300), *S. sonnei* (KCTC 2009), and fungal strains *C. kruisii* (KCTC 7614), *C. glabrata* (KCTC 7219), *C. tropicalis* (KCTC 7212) and *T. mentagrophytes* (KCTC 6085) were purchased from the KCTC (Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea).

Materials

Caprylic acid (8:0), myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), arachidic acid (20:0), myristoleic acid (14:1, cis form), palmitoleic acid (16:1, cis form), cis-11-eicosenoic acid (20:1), methyl cis-11-eicosenoate (20:1), linoleic acid (18:2, cis form), methyl linoleate (18:2), linolenic acid (18:3, cis form), arachidonic acid (20:4, cis form), docosahexaenoic acid (22:6, cis form), tetracycline, and ketoconazole were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Oleic acid was purchased from Duksan Pharmaceutical Co., Ltd. (Kyongkido, Republic of Korea). Capric acid and caproic acid were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). BA (Brucella agar), BB (Brucella broth), BHIA (Brain Heart Infusion agar), BHI (Brain Heart Infusion broth), and PDB (Potato dextrose broth) were purchased from Becton Dickinson and Company (Sparks, MD, USA). Sterile 24-well plates (16 mm in diameter) were purchased from Nunc (Roskilde, Denmark).

Plant material

The seeds of *P. japonica* were purchased from the local herbal medicinal market at Geumsan, Chungnam, Republic of Korea in September, 2001, and were identified by Dr. Eunkyu Lim at the Busong Clinic of Medicinal Herbs (Iksan, Republic of Korea). A voucher specimen (SM 1081) has been deposited in the Natural Products Chemistry Laboratory of Kongju National University, Republic of Korea.

Extraction and isolation

The dried seeds (500.0 g) were pulverized, and soaked in MeOH with continuous shaking (80

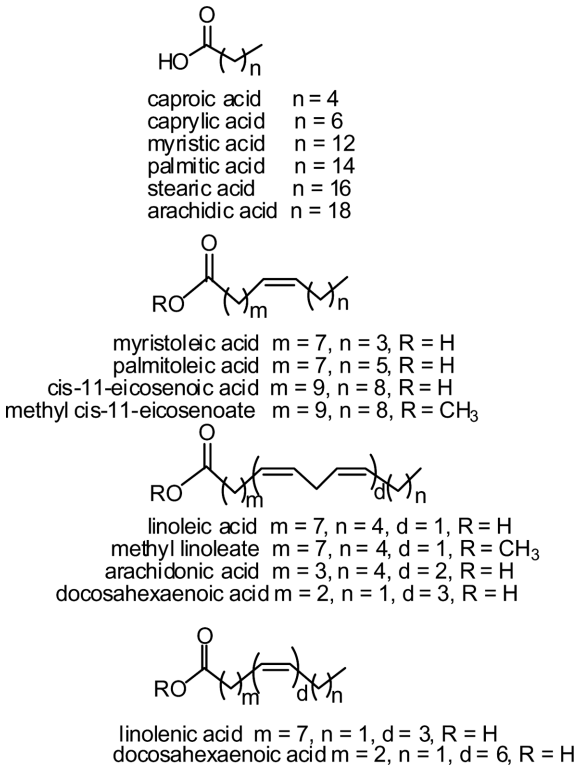

Fig. 1. Chemical structures of fatty acids.

rpm) at 27 °C for one week and extracted three times (1 L x 3). The methanolic extracts combined were evaporated under vacuum to give deep brown oily residue (13.0 g). The residue was suspended in water (500 mL) and extracted with EtOAc (500 mL x 4). The EtOAc layer was concentrated to give a brown oil (9.3 g), which was chromatographed on a silica gel column (95 id 200 mm) eluted with a mixture of hexane, EtOAc, and MeOH of increasing polarity to give six fractions. Fraction 1 (3.25 g) was subjected to silica gel column chromatography (50 id 270 mm) with gradient elution of a mixture of hexane and EtOAc to give seven sub-fractions. Antibacterial active sub-fraction 3 (1.6 g) was further purified using C18 HPLC (98% aqueous MeOH) afforded four compounds linoleic acid (110.8 mg, Rt 14.48 min), cis-11-eicosenoic acid (396.0 mg, Rt 15.47 min), methyl linoleate (66.1 mg, Rt 20.63 min), and methyl cis-11-eicosenoate (107.2 mg, Rt 24.71 min). Spectroscopic data of the isolates were in good accordance with commercially available authentic samples.

Disc diffusion assay for anaerobic *P. acnes* and aerobic bacteria and fungi

A paper-disc diffusion method was applied to measure the antimicrobial activities. Briefly, autoclaved agar media (BHIA 5.0 g and BHI 0.25 g in 100 mL water for P. acnes; BA 4.3 g in 100 mL water for aerobic bacteria; and SDA 6.5 g in 100 mL water for fungi) were poured into petridishes (diameter of 100 mm) giving a depth of 3 - 4 mm. Microbial cells pre-cultured in broth (BHI 3.7 g in 100 mL water for *P. acnes*; BB 2.8 g in 100 mL water for bacteria; PDB 2.4 g in 100 mL water for fungi) were spread over the agar plates. Filter paper discs (diameter of 8 mm) impregnated with specified concentrations of fatty acids were placed on the surface of the agar plates. *P. acnes* and *S. mutans* were incubated under anaerobic conditions at 37 °C under a mixture of 80% N_2, 10% CO_2, and 10% H_2 for 48 h. The other bacteria and fungi were incubated under aerobic conditions for 48 h (37 °C for bacteria and 26 °C for fungi). Antimicrobial activities were measured in the diameter of growth inhibition zone. Tetracycline and ketoconazole were used as positive controls for bacteria and fungi, respectively.

Determination of MIC against anaerobic and aerobic bacteria, and fungi

The MICs of the samples were determined by broth dilution method. All the fatty acids except stearic acid and arachidic acid dissolved in acetone (stearic and arachidic acids were dissolved in $CHCl_3$) were added in 24-well plates containing 1 mL of the broth described in the disc diffusion assay for each microorganism, which were then two-fold serially diluted. Overnight-precultured bacterial cells (106 CFU/mL) were inoculated in the wells followed by incubation. *P. acnes* and *S. mutans* were incubated under anaerobic conditions at 37 °C for 48 h. The other bacteria and fungi were incubated under aerobic conditions for 48 h (37 °C

for bacteria and 26 °C for fungi). Tetracycline and ketoconazole were used as positive controls for bacteria and fungi, respectively. Minimum inhibitory concentration (MIC) was defined as the lowest concentration which prevented the visible growth of microorganism. A control experiment was done in parallel to study the impact of the solvents itself (the highest concentration, 1% v/v).

RESULTS

Bioassay-guided fractionation of MeOH extract of bush berry seeds (500 g) of *P. japonica* by silica-gel column chromatography and reversed-phase C18 HPLC furnished linoleic acid (110.8 mg), cis-11-eicosenoic acid (396.0 mg), methyl linoleate (66.1 mg), and methyl cis-11-eicosenoate (107.2 mg). Their structures were characterized from spectroscopic analyses (1D and 2D NMR, IR, and MS) and by comparison with authentic samples.

Linoleic acid was found to be active at 200 μg/disc with an inhibition zone of 10 mm using disc diffusion assay and its minimum inhibitory concentration determined to be 32 μg/mL (MIC) against *P. acnes* using broth dilution methods. However, cis-11-eicosenoic acid, methyl linoleate, and methyl cis-11-eicosenoate showed no activities below 512 μg/mL (MIC). These activity profiles prompted us to test other saturated and unsaturated fatty acids from C6 - C22 such as caproic, stearic, oleic, and docosahexaenoic acids. The fatty acids were tested against anaerobic bacteria (*P. acnes* and *S. mutans*), aerobic bacterium (*Shigella sonnei*), and fungi (*Candida kruissi*, *Candida glabrata*, *Candida tropicalis*, and *Trichophyton mentagrophytes*). The activities results are shown in Table 1 and Table 2. Short chain saturated fatty acids (C6 - C10) showed no activities below 512 μg/mL (MIC) against *P.*

Table 1. Antimicrobial activities of fatty acids obtained from disc diffusion assay

Fatty Acids	Zone of inhibition in mma				
	P. acnes 200 μg	*S. sonnei* 100 μg	*C. kruissi* 100 μg	*C. tropicalis* 100 μg	*T. mentagrophytes* 200 μg
Caproic acid	-	-	10 ± 1	-	-
Caprylic Acid	-	-	15 ± 1	-	-
Capric acid	-	-	20 ± 2	-	-
Myristic acid	-	-	-	-	-
Palmitic acid	-	-	-	-	-
Stearic acid	-	-	-	-	-
Arachidic acid	-	-	-	-	-
Myristoleic acid	16 ± 2	17 ± 2	15 ± 1	16 ± 1	9 ± 1
Palmitoleic acid	14 ± 1	-	10 ± 1	14 ± 1	12 ± 2
Oleic acid	-	-	-	nt	nt
Cis-11-eicosenoic acid	-	nt	nt	nt	nt
Methyl cis-11-eicosenoate	-	nt	nt	nt	nt
Linoleic acid	10 ± 1	12 ± 2	12 ± 1	14 ± 2	10 ± 1
Methyl linoleate	-	nt	nt	nt	nt
Linolenic acid	10 ± 1	-	13 ± 2	15 ± 1	9 ± 1
Arachidonic acid	11 ± 1	nt	nt	10 ± 2	9 ± 1
Docosahexaenoic acid	10 ± 1	-	10 ± 1	9 ± 1	9 ± 1
Control[b]	25 ± 2	28 ± 2	19 ± 1	20 ± 1	12 ± 1

ameasured in diameter (mm) including the paper disc (8 mm) in duplicate and the average values with standard deviation. '-' signs indicate no activity at the specified concentrations. 'nt' indicates the samples were not tested.
[b]Tetracycline (20 μg/disc) and ketoconazole (30 μg/disc) were used for bacteria and fungi, respectively.

Table 2. Minimum inhibitory concentrations (MIC) of fatty acids against bacteria and fungi determined by broth dilution method

Fatty Acids	MIC (μg/mL)[a]				
	P. acnes	*S. mutans*	*S. sonnei*	*C. kruissi*	*C. glabrata*
Caproic acid	-	-	-	64	256
Caprylic Acid	-	-	-	32	128
Capric acid	-	-	-	16	512
Myristic acid	-	-	-	-	-
Palmitic acid	-	-	-	-	-
Stearic acid	-	-	-	-	-
Arachidic acid	-	-	-	-	-
Myristoleic acid	32	128	16	32	-
Palmitoleic acid	32	16	128	64	-
Oleic acid	-	32	-	-	-
Linoleic acid	32	16	32	32	-
Linolenic acid	128	512	256	32	-
Arachidonic acid	128	-	nt	nt	nt
Docosahexaenoic acid	128	32	128	32	-
Control[b]	2	0.5	1	8	8

[a]Assay was done in duplicate at 106 CFU/mL. '-' indicates no inhibition observed up to at 512 μg/mL. 'nt' indicates the sample was not tested. [b]tetracycline was used for bacteria, and ketoconazole for fungi.

acnes as well as other bacteria but were active at 16 - 64 μg/mL (MIC) against *C. kruissi.* As in the case of linoleic acid, unsaturated fatty acids like myristoleic, palmitoleic, linolenic, arachidonic, and docosahexaenoic acids were also active against *P. acnes* with MIC range of 32 - 128 μg/mL. However, oleic acid showed no activity even at 512 μg/mL.

DISCUSSION

It was reported that linoleic acid was slightly stimulatory to *P. acne* growth at 500 μg/mL (Puhvel and Reisner, 1970) and inhibitory at 4.1 M/mL (Ko *et al*., 1978). Linoleic acid can be found in many plants such as *Helichrysum pedunculatum* (Asteraceae) (Dilika *et al*., 2000), *Portulaca oleracea* (Portulacaceae) (Palaniswamy *et al*., 2001), *Asparagus officinalis* (Liliaceae) (Jang *et al*., 2004), *Plantago major* (Plantaginaceae) (Ringbom *et al*., 2001), *Afzelia cuanzensis* (Caesalpinaceae) (Vlahov, 1996). Isolation of linoleic acid, cis-11-eicosenoic acid, and their methyl esters from *P. japonica* is reported first time. Certain types of saturated and unsaturated fatty acids are known to inhibit the growth of microorganisms (Kull *et al*., 1961; Khan and Katamay, 1969; Kabara *et al*., 1972; Gershon *et al*., 1973; Kabara *et al*., 1973; Kubo *et al*., 1994; Bergsson *et al*., 2001; Nair *et al*., 2005; Zheng *et al*., 2005). However, to the best of our knowledge, the antibacterial and antifungal activities of arachidic, myristoleic, palmitoleic, linoleic, linolenic, and docosahexaenoic acids against *S. sonnei* and *C. krusii* are established for the first time. Unsaturated fatty acids like myristoleic, palmitoleic, linolenic, and arachidonic acids are also naturally available from the plants like *Nigella sativa* (Ranunculaceae), (Cheikh-Rouhou *et al*., 2007), *Pinus pinea* (Nasir *et al*., 2005), and *Salvia miltiorrhiza* (Labiatae) (Wang *et al*., 1998).

The results suggest that traditional herbal formulations rich in linoleic acid along with other unsaturated fatty acids would be effective in the topical treatment of acne as well as other bacteria and fungal infections.

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