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Isolation and Identification of Antifungal Fatty Acids from the Extract of Common Purslane(*Portulaca oleracea* L.)

Jong Seong Park*, Shoyo Nishimura, Shingo Marumo** and Masato Katayama****

* *College of Agriculture, Chungnam National University, Tajon 300-31, Korea*
** *Faculty of Agriculture, Nagoya University, Nagoya 464, Japan*

쇠비름 汁液에서 얻은 抗菌性 脂肪酸의 分離 및 同定

朴鍾聲* · 西村正暘** · 丸茂晋吾** · 片山正人**

* 忠南大學校 農科大學
** 名古屋大學 農學部

ABSTRACT

Five antifungal substances were isolated from the long-term storaged extract of common purslane, and identified as isobutyric, butyric, isovaleric, valeric and caproic acids belonging to short-chain fatty acids (C4-C6). Each of these fatty acids showed more or less antifungal potency against spore germination and mycelial growth of *Alternaria alternata* Japanese pear pathotype *in vitro*. Antifungal potency of each fatty acid against spore germination was greater than that against the mycelial growth. No one of these fatty acids completely inhibited the mycelial growth at concentration lower than 200 ppm, while 50 ppm of caproic acid and 200 ppm of valeric acid completely inhibited the spore germination. The results of bioassay also suggested that chain-length of the fatty acids might be related with the antifungal potency, since fatty acids with longer chain showed higher antifungal potency.

Key words: fatty acids, *Portulaca oleraces*, antifungal effect.

要　　約

오래 저장해 둔 쇠비름 汁液에서 5種의 抗菌性 物質이 分離되었으며 이들 物質이 isobutyric acid, butyric acid, isovaleric acid, valeric acid 그리고 caproic acid 등과 같은 C4-C6의 低級脂肪酸으로 同定되었다. 各各의 脂肪酸은 室內實驗에서 多少間에 배나무 검은무늬病菌(*Alternaria alternata* Japanese pear pathotype)의 胞子發芽나 菌糸伸長을 다같이 阻害하였다. 胞子發芽에 대한 各各 脂肪酸의 抗菌力은 例外없이 菌糸伸長에 대한 그것보다 컸다. 例컨대 200ppm 以下의 濃度에서 菌糸生長을 完全히 阻害하는 抗菌性 脂肪酸은 하나도 없었지만 caproic acid의 50ppm과 valeric acid의 200 ppm은 胞子發芽를 完全히 抑制하였다. 또한 生物檢定의 結果에서 이는 脂肪酸의 炭素사슬의 길이가 길수록 더욱 높은 抗菌力을 보여주었으므로 炭素사슬의 길이가 抗菌力과 關係가 있는 것으로 推定되었다.

INTRODUCTION

It has been known that some of apple growers have used the common purslane extract as a therapeutant for control of apple canker caused by *Valsa ceratosperma* in Korea since 1973(7). Practical trials to use the common purslane extract for the disease control were carried out by some workers of Horticultural Experiment Station, Suwon Korea for three years from 1980 to 1982, and the results from the field trials presented some informations that the extract apparently showed antifungal efficacy for control of apple canker and moreover it was superior to the other chemicals recommended by the government(2). One of the authors and his coworkers that MeOH-soluble fraction of the extract inhibited mycelial growth and spore germination of phytopathogenic fungi such as *Valsa ceratosperma*, *Alternaria alternata* Japanese pear pathotype and *Pyricularia oryzae in vitro*, and its active principle was regarded as a kind of lipid(7).

There are some reports on the antimicrobial activities of fatty acids and their derivatives(5,6,10, 12,13). Some of N-substituted amides of fatty acids, fatty esters and aliphatic acids which inhibit mycelial growth and spore germination of some phytopathogenic microbes were reported by some workers. However, none of fatty acids obtained from natural plant products was reported to possess antimicrobial potency. On the other hand, some of fats and fatty acid derivatives are known as growth stimulants and carbon sources for some phytopathogenic fungi(1,4). Investigations for practical use of antifungal fatty acids obtained from natural plant products must be an important task in the respect of future environmental problems to reduce several hazards.

In the present work, we have isolated the antifungal fatty acids inhibiting both mycelial growth and spore germination of *Alternaria alternata* Japanese pear pathotype from extract of common purslane by the selective use of organic solvents, silica gel column chromatography, thin layer chromatography and gas chromatography, identified the chemical structure of each fatty acid by gas chromatography and mass spectroscopy, and confirmed the chemical identification of each fatty acid by comparison of gas chromatogram and mass spectrum of the corresponding authentic sample obtained commercially.

MATERIALS AND METHODS

Preparation of common purslane extract. Fresh common purslane collected from upland fields was cut and ground in a mortar with addition of small amount of water, strained through cheese-cloth and squeezed in a hand press to obtain the crude extract. It was pooled in a storage jar and naturally fermented under comparatively high temperature for about two weeks. Thereafter it has been preserved more than one year at room temperature. To set up the isolation of antifungal substance, it was autoclaved and filtered through filter paper.

Isolation of antifungal substances. The autoclaved and filtered extract (1 ℓ) was concentrated by evaporation under vacuum at 50°C and the concentrate (100 ml) was extracted with MeOH (2x100 ml) by shaking. The precipitated layer was discarded and the MeOH-soluble layer was concentrated under vacuum at 35°C to prepare the same volume of aqueous solution by addition of distilled water. The aqueous solution adjusted to pH 3.5 with HCl was extracted with EtOAc three times and the EtOAc layer was concentrated by evaporation under vacuum. The residue was extracted with n-Hexane and the n-Hexane layer was concentrated to dryness under vacuum after it was pooled. The residue was chromatographed on a silica gel column (Wakogel C-200). The column was developed stepwise with varying proportions of n-Hexane and EtOAc. The n-Hexane/EtOAc (8:2, v/v) eluate, colorless liquid, was evaporated to dryness and then subjected to silica gel TLC with a solvent system of n-Hexane/EtOAc (8:2, v/v). The

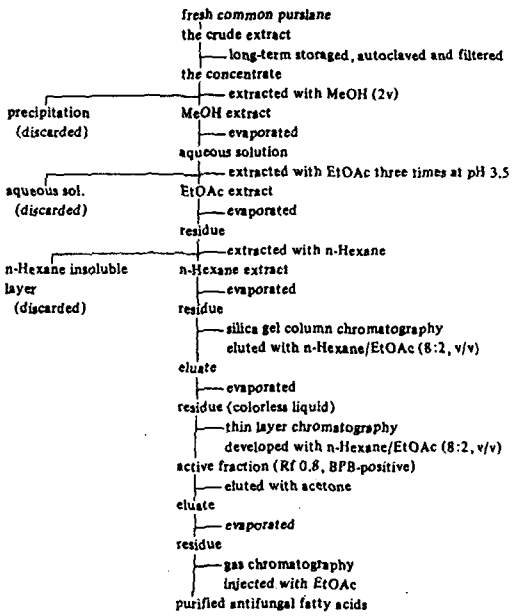

Fig. 1. Isolation procedure of antifungal fatty acids from common purslane.

plates were scraped into several zones different in Rf value and each zone was eluted with acetone for bioassay and chemical analysis. The purified antifungal fatty acids were obtained from the last TLC at Rf 0.8 on the separated zones. The isolation procedures are summarized in Fig. 1. During the isolation procedure, antifungal potency of active fractions was monitored by biological activities against conidia germination of the test fungus. The active fraction obtained from the last TLC was further chromatographed with a JEOL JGC gas chromatograph (GC) as follows: A solution of the sample in ethyl acetate were injected into a JEOL JGC-1100 equipped with the gass column (3mm ID x 2m) which was packed with 5% SP-1200 on 80-100 mesh Uniport S. Carrier gas was nitrogen with a flow rate of 45 ml/min. and column temperature was 100°C.

Identification of antifungal substances. For identification of each compound resolved by gas chromatographic isolation, gas chromatography-mass spectroscopy(GC-MS) was carried out as follows: A solution of the sample in ethyl acetate were on-column injected into a JEOL GCG05 ga chromatograph connected to a JEOL DX 300 mas spectrometer through a two-stage all-glass je separator. The glass column (2mm ID x 2m) wa packed with 5% SP-1200 on 80-100 mesh Unipor S. Carrier gas was helium with a flow rate of 40 ml, min and column temperature was 100°C. The elec tron impact mass spectra were obtained at 70 eV Each of the authentic samples was used as the check in the gas chromatographic isolation and mass spectroscopic identification.

Bioassay of active fractions and identified compounds. During the isolation procedures, antifungal potency of each fraction against conidia germination of *Alternaria alternata* Japanese pathotype was monitored by the filter paper disc agar diffusion method. Conidia and mycelial mats of the fungus were also used to investigate antifungal potency of each fatty acid finally identified. The inocula were prepared as follows: The mycelial mats were obtained from the 5 to 7 day-old cultures on potato sucrose agar (PSA) at 26°C unless otherwise noted. The conidia were harvested by blushing and suspending the 5 to 7 day-old cultures on PSA in distilled water, the conidial suspension was then sieved through a few of layers of cheese cloth and adjusted to give the final concentration which was determined microscopically to observe 20-30 spores/field at 100 x. The culture media were prepered as follows: Water agar (2%) for the conidia germination and PSA for the mycelial growth were prepared respectively. No attempt was made to control pH of each medium. Each medium was finally prepared to contain 5% acetone as a dispersant of each compound in it. The conidial suspension prepared as the inoculum was sprayed on the water agar containing a given concentration of each compound by a glass atomizer, each plate was incubated at 26°C for 24 hr and the germination rate and germ tube elongation were microscopically observed. On the other hand, approximately 4mm disc of the mycelial mats prepared as the inoculum was seeded on PSA containing a given

concentration of each compound, each plate was incubated at 26°C for 5 days and the mycelial growth was observed. Concentrations of each compound were ranged from 50 to 400 ppm at intervals of 50 ppm.

RESULTS AND DISCUSSIONS

Isolation and Identification of antifungal substances. During the isolation procedures as shown in Fig. 1., the active fractions succeedingly obtained from MeOH extract, EtOAc extract, n-Hexane extract, eluate (colorless liquid) separated from silica gel column chromatography with a solvent system of n-Hexane/EtOAc (8:2, v/v) and acetone eluate of the Rf. 0.8 zone on TLC developed with the same solvent system by the bioassay. The acetone eluate was subjected to gas-chromatographic isolation were clearly resolved into five fractions showing the respective peak at 5.8, 7.6, 10.9 15.3 and 31.2 min. of retention times as shown in Fig. 2. For identification of the resolved fractions GC-MS analysis were succeedingly carried out. Mass spectra of 1st fraction (retention time 5.8 min.) showed, m/e 88(M+), 73(M+, CH_3) and 43 corresponding to isobutyric acid ($C_4H_8O_2$) as shown in Fig. 3. Mass spectra of 2nd fraction (retention time 7.6 min.) showed, m/e 88(M+), 73(M+, CH_3) and 60 corresponding to butyric acid ($C_4H_8O_2$) as shown in Fig. 4. Mass spectra of 3rd fraction (retention time 10.9 min.) showed, m/e 102(M+), 87(M+, CH_3), 74(M+, CO) and 60 corresponding to isovaleric acid ($C_5H_{10}O_2$) as shown in Fig. 5. Mass spectra of 4th fraction (retention time 15.3 min.) showed, m/e 102(M+), 73(m+, C_2H_5) and 60 corresponding to valeric acid ($C_5H_{10}O_2$) as shown in Fig. 6. Mass spectra of 5th fraction(retention time 31.2 min.) showed, m/e 116(M+), 87(M+ C_2H_5), 73(M+, C_3H_7) and 60

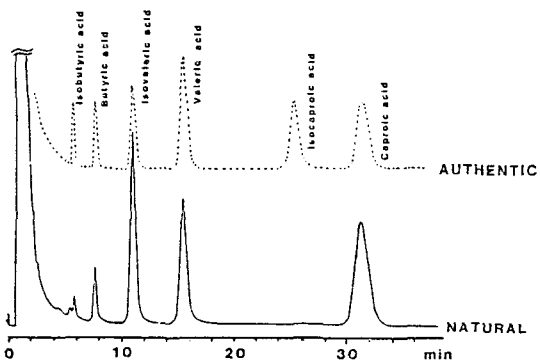

Fig. 2. GC analysis of the active fraction obtained from the extract of common purslane (*Portulaca oleracea*).

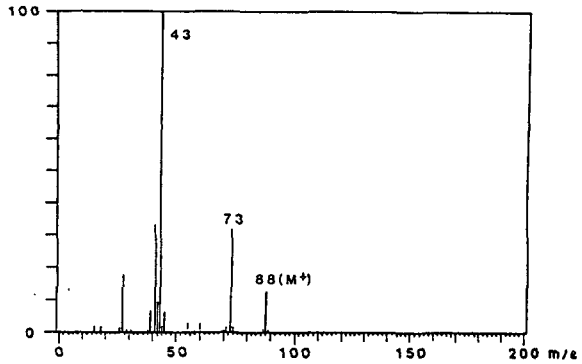

Fig. 3. Mass spectra of isobutyric acid from the extract of common purslane.

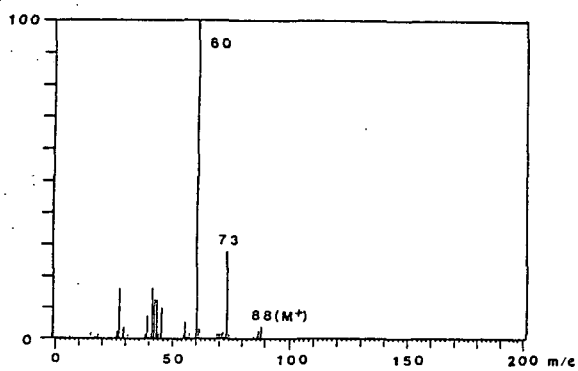

Fig. 4. Mass spectra of butyric acid from the extract of common purslane.

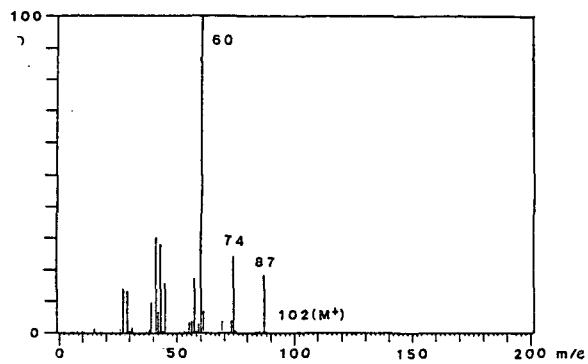

Fig. 5. Mass spectra of isovaleric acid from the extract of common purslane.

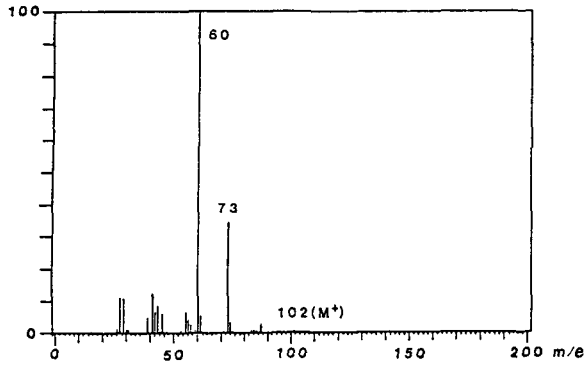

Fig. 6. Mass spectra of valeric acid from the extract of common purslane.

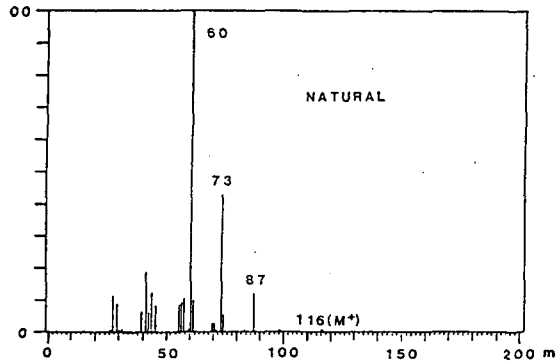

Fig. 7. Mass spectra of natural and authentic caproic acid

corresponding to caproic acid ($C_6H_{12}O_2$) as shown in Fig. 7. GC-MS analysis of the active fraction also gave the reconstructed ion chromatogram shown in Fig. 8. That showed the presence of isobutyric, butyric, isovaleric, valeric and caproic acids. Chemical identification of each fatty acid was confirmed by comparison of its gas chromatogram and mass spectrum with those of the authentic sample as shown in Fig. 2, 3, 4, 5, 6 and 7. The results of bioassay showed that each of fatty acids possess more or less antifungal potency inhibiting spore germination of the test fungus. As previously suggested by one of the authors and his co-worker (7), those of antifungal substances obtained from extract of common purslane are well-known short-chain fatty acids (C_4-C_6) as natural or synthetic products, although they are not yet listed in fungitoxic substances. Recently, another report in addition to our previous report(8) on the antifungal fatty acids was presented by Schmidt E.L.(12).

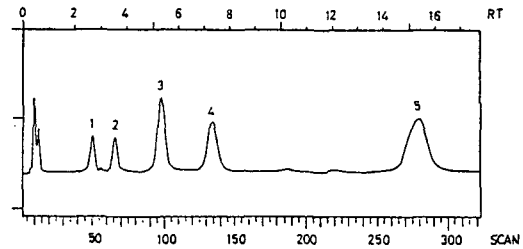

Fig. 8. Reconstructed ion chromatogram of the active fraction obtained from the extract of common purslane (*Portulaca oleracea*). (1) isobutyric acid, (2) butyric acid, (3) isovaleric acid, (4) valeric acid, (5) caproic acid.

In his study, more active capry(C8), pelargonic(C9) and capric(C10) acids than valeric(C5) and caproic (C6) are listed as spore germination inhibitors of some wood-decay fungi.

Antifungal potency of the identified fatty acids. The further studies to evaluate antifungal potency of each fatty acid were carried out using *Alternaria alternata* Japanese pear pathotype as the test fungus. The results showed that each fatty acid inhibited not only conidia germination but also mycelial growth of the test fungus even though their antifungal potency were different from each other as shown in Table 1 and 2. None of these fatty acids except caproic acid completely inhibited the conidia germination at lower concentration than 150 ppm, but caproic acid completely inhibited the

Table 1. Effects of fatty acids in common purslane on conidia germination of *Alternaria alternata* Japanese pear pathotype incubated for 24 hr. at 26°C on the water agar[a]

Fatty acid	Concentration(ppm)						
	0	50	100	150	200	250	300
isobutyric acid	++[b]	++	+	+	+	+	–
butyric acid	++	++	+	+	+	–	–
isovaleric acid	++	++	+	+	+	–	–
valeric acid	++	+	+	+	–	–	–
caproic acid	++	–	–	–	–	–	–

[a] The medium containing 5% of acetone.

[b] ++ = normal germination, + = germination delayed and germ tube elongation retarded, – = completely inhibited germination.

Table 2. Effects of fatty acids in common purslane mycelial growth of *Alternaria alternata* Japanese pear pathotype incubated for 5 days at 26°C on the PSA[a].

Fatty acid	Concentration(ppm)						
	0	150	200	250	300	350	400
isobutyric acid	++[b]	++	+	+	+	+	+
butyric acid	++	+	+	+	+	–	–
isovaleric acid	++	+	+	+	–	–	–
valeric acid	++	+	+	–	–	–	–
caproic acid	++	+	+	–	–	–	–

[a] The medium containing 5% of acetone.
[b] ++ = normal growth, + = mycelial growth significantly retarded, – = no growth.

conidia germination at 50 ppm. In general, 25 ppm of caproic acid, 50 ppm of valeric acid and 100 ppm of the other three fatty acids inhibited normal germination of the conidia, and minimal inhibitory concentration (MIC) of each fatty acid against the condia germination appeared to be in the range of 25 to 50 ppm for caproic acid, 150 to 200 ppm for valeric acid, 200 to 250 ppm for isovaleric acid and butyric acid and 250 to 300 ppm for isobutyric acid as shown in Table 1. The conidia on fatty acid-amended water agar in which their germination was completely inhibited germination were obviously deteriorated by vacuolation and fragmentation of cytoplasm soon or later. The pattern of cytoplasm deterioration of conidia on fatty-acid amended water agar resembled that encountered in earlier reports by some workers(11, 12). One of recent works on the antifungal fatty acids presented that fatty acids of chain-lengh C8-C10 such as caprylic (C8), pelargonic (C9) and capric (C10) prevented spore germination and killed spores of some wood-decay fungi at concentration of 20 to 50 ppm in media, whereas other acids such as valeric (C5), caproic (C6), and enanthic (C7) tested were less active, and 1000 ppm of valeric acid, 100 ppm of caproic acid and 100 ppm of enanthic acid completely inhibited spore germination of the tested fungi. Our results suggested that chain-length of each fatty acid tested might be correlated with the antifungal potency, since the fatty acids with longer chain-length showed higher antifungal potency as shown in Table 1. No one of the antifungal fatty acids completely inhibited the mycelial growth at concentration lower than 200 ppm. Of antifungal fatty acids tested, 250 ppm of caproic and valeric acids, 300 ppm of isovaleric acid and 350 ppm of butyric acid completely inhibited the mycelial growth, while isobutyric acid showed only significant retardation of the mycelial growth even at 400 ppm, as shown in Table 2. The results also showed that MIC of each fatty acid against the mycelial growth was in the range of 200 to 250 ppm for caproic acid and valeric acid, 250 to 300 ppm for isovaleric acid, for 300 to 350 ppm, and higher than 400 ppm for isobutyric acid. MIC of each fatty acid against the mycelial growth was more or less higher than that against conidial germination. It is not certain whether such difference is due to genetic character of the test fungus of due to the media used. It might be originated from sugar in PSA for the mycelial culture(3). The relationship between chain-length of the fatty acids and their antifungal potency against the mycelial growth was similar to that of the conidia germination. No one of noticeable side effects on the test fungus by addition of 5% of acetone in the media used was observed in this study as previously reported(9).

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