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Antinocicepetive Effects of 3,4-Dicaffeoyl Quinic Acid of *Ligularia fischeri* var. *spiciformis*

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Abstract - The plant *Ligularia fischeri* var. *spiciformis* (Compositae) is a candidate for available functional foods. It has been used to treat diabetes mellitus and rheumatoid arthritis. We have reported the isolation of a new eremophilanolide named 6-oxoeremophilenolide and cytotoxic intermedeol together with the isolation of hydrophilic constituents, chlorogenic acid, 3,4-di-O-caffeoylquinic acid (3), and 5-O-[1-butyl]-3,4-di-O-caffeoylquinic acid. Compound 3 was again isolated by combination of silica gel- and ODS column chromatography for the anti-nociceptive action. Compound 3 and 4 were assayed in hot plate- and writhing tests in the rat. Although the three derivatives of caffeic acid exhibited significant anti-nociceptive effects at 10 mg/kg dose (i.p.), (activity potency: 4 > 3). These results suggest that compound 3 is responsible for at least rheumatoid arthritis, and caffeic acid moiety is the active moiety of dicaffeoylquinic acid.

Key words - *Ligularia fischeri* var *spiciformis*, Compositae, 3,4-dicaffeoylquinic acid, Caffeic acid methyl ester, Anti-nociceptive

Introduction

In the course of screening on antinociceptive medicinal herbs, we found the herbs of *Ligularia fischeri* var. *spicformis* has a significant action. Therefore, we fractionated the MeOH extract of this plant and the fractions were assayed by hot plate- and writhing method for the antinociceptive action. EtOAc fraction with significant antinociceptive action was subjected to column chromatography for the isolation of active components and afforded caffeoylquinic acids. A new caffeoyl quinic acid, 5-O-[1-butyl]-3,4-di-O-caffeoylquinic acid, along with chlorogenic acid, 3,4-di-O-caffeoylquinic acid were isolated and the structures were determined on the basis of spectroscopic data.

It is very well known that caffeoylquinic acid has a variety of bioactivity including. Since dicaffeoylquinic acid has two dicafferoyl moieties, the caffeoyl group may be responsible for the antinociceptive action. Therefore, we tested not only isolated components but also caffeic acid and its methyl ester for the antinociceptive action.

The plant of *Ligularia fischeri* (Ledebour) Turcz. var. *spiciformis* Nakai (Compositae) is used for mountain-edible plants as an endemic perrenial herbal species in Korea (Nakai *et al.*, 1943). This vegetable has been also known to be effective in the diseases of jaundice, scarlet fever, rheumatoidal arthritis and hepatic function failure (Choi *et al.*, 1991). Ligularia species, in general, contains sesquiterpenes (Zhao et al., 1994; Gao *et al.*, 1997; Jia *et al.*, 1993; Bohlmann 1980), phenylpropanoids (Zhao and Ji *et al.*, 1994; Ma *et al.*, 1997) and pyrrolizidine-type alkaloids (Asada *et al.*, 1984). We have previously reported the isolation of intermedeol and 6-oxoeremophilenolide from diethyl ether fraction and the cytotoxicity of intermedeol and diethylether fraction (Park *et al.*, 2000). We have also reported the inductive effect of intermedeol on HL-60 cell differentiation and its biochemical mechanism (Jeong *et al.*, 2002).

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Materials and Methods

General Experimental Procedure

IR spectra were recorded on a Hitachi were recorded on a Hitachi 260-01 spectrometer in KBr cells (neat). EI-MS (ionization voltage, 70 eV) was measured with JEOL JMS DX-300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with TMS as an internal standard. 2D NMR spectra (^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC) were measured by the use of JEOL standard pulse sequences.

Plant material

The leaves of *Ligularia fischeri* var. *spiciformis* were collected in

July 1999, in Pyongchang, Kangwondo, South Korea, and the plant was identified by Dr. K. O. Yoo (Department of Biology, Kangwon National University, Chuncheon, Korea). A voucher specimen (KW-980814) is deposited in the herbarium of Department of Biology, Kangwon National University. This plant was air-dried avoiding sunlight and pulverized for the experiment.

Extraction, fractionation and isolation

The plant material (2.0kg) was extracted three times with hot MeOH under reflux and the filtered extract was evaporated on a rotatory evaporator under reduced pressure to give viscous MeOH extract (526g). This was suspended in H_2O and then partitioned with diethyl ether, EtOAc and n-BuOH to give diethyl ether fraction (25g), EtOAc fraction (63g) and n-BuOH fraction (45g).

A part (15g) of EtOAc fraction was subjected to column chromatography over silica gel (300g, 5×50cm, Merck Art 7734) with eluting solvent of CHCl3-MeOH-H2O (65:35:10, lower phase) and collected by 60ml each. These were checked on TLC by 50%-H2SO4 or under 254nm wavelength. Collected ones over 1.20 - 1.32L were evaporated and recrystallization with MeOH yielded 1. Collected bottles over retention volume of 3.3 - 3.6L) were combined and evaporated in vacuo. Purification of this material by ODS column chromatography (YMC gel ODS-A, YMC Co., LTD, Germany) {Eluent: MeOH-H2O (10:2)} gave amorphous white solid (1). The fractions over 3.72 - 5.4L was evaporated and produced compound 3 in MeOH.

Compound 3: Amorphous white powder from MeOH, IR ν (cm^{-1}): 3372 (broad, OH), 1701 (α,β-unsaturated ester), 1630, 1602, 1516, 1439 (aromatic C = C), 1285 (C - O)

FAB-MS (m/z): 591 $[M+Na]^+$, ^{1}H-NMR (500 MHz, DMSO): Table 1, ^{13}C-NMR (125 MHz, DMSO): Table 2.

Preparation of test sample solutions

Test samples (3 - 4) were first dissolved with 10% tween 80 and diluted by saline. The same volume of solvent only was administered for the normal group. Compounds 3-5 were intraperitoneally administered with 5 and 10mg/kg based on the preliminary experiments.

Table 1. ^{1}H-NMR data of caffeoylquinic acids (2, 3) isolated from *Ligularia fischerii* var. *spiciformis* (500 MHz, DMSO-d_6)

Position	2	3	△(2-3)
Quinic acid-2	1.85 (1H, d-like)	1.95 (1H, d-like)	−0.10
3	5.50 (1H, d-like)	5.50 (1H, d-like)	±0.00
4	5.05 (1H, brs)	5.04 (1H, brs)	+0.01
5	3.79 (1H, d-like)	3.86 (1H, brs)	−0.07
6α	1.75 (1H, d-like)	1.87 (1H, d-like)	−0.12
6β	1.92 (1H, d-like)	1.81 (1H, d-like)	−0.11
Caffeoyl-2'	7.01 (1H, d, J=1.8 Hz)	7.00 (1H, d, J=1.8 Hz)	+0.01
2"	7.07 (1H, d, J=1.8 Hz)	7.07 (1H, d, J=1.6 Hz)	±0.00
5"	6.71 (1H, d, J=8.2 Hz)	6.72 (1H, d, J=8.1 Hz)	−0.01
5"	6.76 (1H, d, J=8.2 Hz)	6.76 (1H, d, J=8.1 Hz)	±0.00
6'	6.90 (1H, dd, J=8.2 & 1.8 Hz)	6.91 (1H, dd, J=8.1 & 1.6 Hz)	−0.01
6"	7.01 (1H, dd, J=8.2 & 1.8 Hz)	7.00 (1H, dd, J=8.1 & 1.8 Hz)	+0.01
7'	7.39 (1H, d, J=15.8 Hz)	7.40 (1H, d, J=15.8 Hz)	−0.01
7"	7.49 (1H, d, J=15.8 Hz)	7.50 (1H, d, J=15.8 Hz)	−0.01
8'	6.14 (1H, d, J=15.8 Hz)	6.16 (1H, d, J=15.8 Hz)	−0.02
8"	6.30 (1H, d, J=15.8 Hz)	6.30 (1H, d, J=15.8 Hz)	±0.00
Butyl-1"'	3.37 (2H, t, J=6.5 Hz)		–
2"'	1.38 (2H, m)		–
3"'	1.30 (2H, m)		–
4"'	0.84 (3H, t, J=6.3)		–

The chemical shift may be exchanged between the two caffeoyls in the same molecule.

Table 1. ^{13}C-NMR data of caffeoylquinic acids (2, 3) isolated from *Ligularia fischerii* var. *spiciformis* (125 MHz, DMSO-d_6)

Position	2	3	Chlorogenic acid[1]	△(2-3)
1	73.5	73.6	77.3	−0.1
2	37.7	37.8	37.6	−0.1
3	68.5	68.2	71.4	+0.3
4	72.6	72.5	73.0	+0.1
5	66.7	66.1	71.3	+0.6
6	37.7	39.1	37.6	+2.6
7	179.3	179.1	178.3	+0.2
1'	125.8	125.4	125.6	+0.4
1"	125.8	125.4		+0.4
2'	115.2	114.8	114.8	+0.4
2"	115.4	114.9		+0.4
3'	146.0	145.7	145.8	+0.3
3"	146.1	145.6		+0.5
4'	149.0	148.6	148.6	+0.4
4"	149.0	148.5		+0.5
5'	116.2	115.8	116.1	+0.4
5"	116.3	115.8		+0.5
6'	121.9	121.5	121.4	+0.4
6"	121.9	121.5		+0.4
7'	145.0	145.3	144.8	−0.3
7"	145.2	145.4		−0.2
8'	114.3	114.0	114.6	+0.3
8"	114.3	114.0		+0.3
9'	166.1	165.8	166.5	+0.3
9"	166.3	165.9		+0.4
1"'	60.8			
2"'	35.1			
3"'	19.1			
4"'	6.3			

The chemical shift may be exchanged between the two caffeoyls in the same molecule.

[1]Chemical shift values were cited from the literature of Arch. Pharm. Res. 14(2), 114-117 (1991).

Table 1. Effect of compounds 3 and 4 on action time in hot plate assay in mice at 10 mg/kg dose (i.p.)

Group	Action time (second)	Prolongation (%)
Control	25.4 ± 2.28	0
3 (dicaffeoylquinic acid)	53.7 ± 7.11*	111.4
4 (caffeic acid)	64.2 ± 3.52*	152.8
Aminopyrine	158.4 ± 9.16**	523.6

Values represent means ± S.D. (n = 6).

*$p<0.01$, **$p<0.001$ compared with the control.

Table 2. Effect of compounds 3, 4 and 5 on the writhing syndrome induced by acetic acid in mice at 10 mg/kg dose (i.p.)

Group	Frequency Count/10 min	Inhibition (%)
Control	40.3 ± 4.52	0
3 (dicaffeoylquinic acid)	28.0 ± 3.52*	30.5
4 (caffeic acid)	19.3 ± 1.56*	52.1
Aminopyrine	8.9 ± 1.29**	77.2

Values represent means ± S.D. (n=10).
*p<0.01, **p<0.001 compared with the control.

Animals

Both 4 week-old ICR male mice and Sprague-Dawley male rats were purchased from Korean Experimental Animal Co. and adapted them in a constant condition (temperature: 20±2℃, dampness: 40 - 60%, light/dark cycle: 12hr) for two weeks or more. Twenty-four hours before the experiment, only water was offered to the animals.

Acetic acid writhing and hot plate method

Test solution was orally or intraperitoneally administered 30min before the experiment, and further 0.1ml/10g of 0.7% acetic acid-saline was injected intraperitoneally. 10 min after the injection, the frequency of writhing in mice was counted for 10 min. Aminopyrine (100mg/kg) was used as a positive control agent.

Hot plate made by Ugo Basile (Italy) was used for the measurement of antinociceptive effect by hot plate method. The response-time showing writhing syndrome was recorded.

Results and Discussion

Since we found the EtOAc fraction of *L. fischeri* var. *spiciformis* extract has antinociceptive action in our assay system, this was subjected to column chromatography to yielded compounds 1-3. On the basis of spectroscopic data, the structure of compound 2 was determined as 5-O-[1-butyl]-3,4-di-O-caffeoylquinic acid and the known compounds 1 and 3 were identified as chlorogenic acid and 3,4-dicaffeoyl quinic acid (Fig. 1).

The three compounds exhibited the peaks due to caffeoyl- and quinic acid moieties in ^{1}H- and ^{13}C-NMR spectra as shown in Table 1 and 2. It was found that compound 2 had an additional 1-butyl moiety than 3 from the peaks at δ_H 0.83 (3H, t, J = 6.3; δ_C 14.3), 1.30 (2H, m; δ_C 19.1), 1.38 (2H, m; δ_C 29.5) and 3.37 (2H, t, J = 6.5; δ_C 60.8). These were confirmed by examination of ^{1}H-^{1}H COSY- and ^{1}H-^{13}C COSY NMR spectra. The existence of δ_C 60.8 of C-3 and δ_H 3.37 of H-3 in the NMR spectrum indicates ethereal linkage of n-butanol. In HMBC NMR spectrum of 2, a peak of δH 3.37 long-range coupled to δC 66.7. HRFABMS exhibited the molecular fomula by m/z 568.1587 (calcd. 568.1580). Therefore, the structure of 2 was determined as 5-O-[1-butyl]-3,4-di-O-caffeoylquinic acid. This compound was isolated for the first time from a natural source.

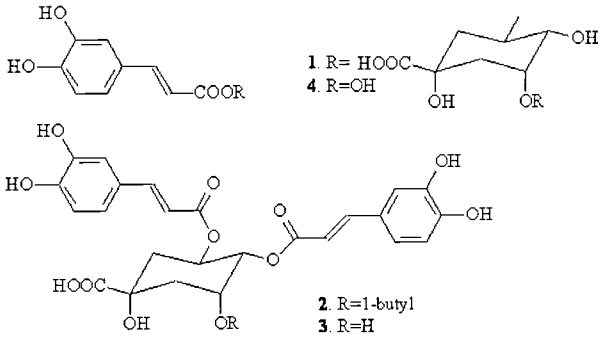

Fig. 1. Structure of caffeic acid derivatives (1 - 5).

Various biological activities on caffeoylquinic acids have been reported. Since it is believed that the caffeoyl moiety may be responsible for the antinocicpetive action, we attempted to find the activities of 3,4-dicaffeoylquinic acid (3) and caffeic acid (4). Caffeic acid methyl ester was prepared by methylation to the carboxyl in caffeic acid. The structure was confirmed by spectroscopic data (IR, ^{1}H- and ^{13}C-NMR).

Among the three compounds, methyl caffeic acid showed the significant antinociceptive activities in hot plate- and writhing assays. The potency of compound 3 was more potent than 4. A positive control showed more potent activity than other treated compounds. These results suggest that caffeoyl moiety may be responsible for the action. It is also possibly presumed that quinic

acid moiety in dicaffeoylquinic acid plays no enhancing role in antinocicpetive action. Hydrolysis of 3,4-dicaffeoylquinic acid may enhance the action than caffeic acid itself although any information on the biochemical cleavage of this compound is not reported. It could be also suggested that more hydrophobic structure of caffeic acid methyl ester than other two compounds may be characteristic physicochemical properties for the biological action. In addition, dicaffeoyl quinic acids may be the active principles of this plant for the traditional use to treat rheumatoid arthritis.

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