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Studies on the Anti-inflammatory Activity of *Aralia continentalis* (III)

Anti-inflammatory Activity of (-)-Kaur-16-en-19-oic Acid

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Abstract □ Besides (−)-pimara-8(14), 15-dien-19-oic acid [I] which had already been isolated as an active anti-inflammtory principle of *Aralia continentalis*, (−)-kaur-16-en-19-oic acid [II] was separated as another active component of the plant, by tracing albumin stabilizing activity. IC_{50} of [II] for the protein stabilizing activity was 0.026 mg/3 ml, when those of [I] and phenylbutazone were 0.032 and 0.32 mg/3 ml, respectively. Being investigated employing carrageenin-induced edema test in rat hind paw, the anti-inflammatory activity of [II] administered s.c. was slightly lower than that of phenylbutazone, whereas the activity of [II] administered p.o. was three times greater than that of phenylbutazone. These results of [II] were contrary to those of [I] in the aspect of administration routes.
Keywords □ Anti-inflammatory activity, (−)-kaur-16-en-19-oic acid, diterpenic acid, *Aralia continentalis*, albumin stabilizing activity.

The root of *Aralia continentalis* K. (Korean name "Dokwhal") has been used as a folk medicine for anti-rheumatic and anti-inflammation. In our previous report[1], its methanol extract was screened for the isolation of active principles, by tracing the stabilizing activity against heat denaturation of albumin, which is known to be the simple screening method for determining an anti-inflammatory activity of non-steroidal compound *in vitro*, developed by Mizushima[2]. We had isolated (-)-pimara-8(14), 15-dien-19-oic acid [I] from the ether-soluble acidic fraction, and it was proved of [I] to be a strongly potent anti-inflammatory component as being determined by carrageenin-induced edema test in rat hind paw,

COOH [I] COOH [II]

Besides [I], (-)-kaur-16-en-19-oic acid [II] was already isolated by Shibata S. *et al*, from *A. cordata*, *A. continentalis* and *A. racemosa*. The present study was undertaken to investigate the anti-inflammatory activity of [II]. For the isolation of [II], we re-examined the extraction procedure of our previous paper[1], in which the roots of *A. continentalis* were percolated with n-hexane to exclude oils or fatty acids that are known to give false positive reactions in Mizushima's method; this was turned to result in loss of a large amount of [I] and [II]. In this study, therefore, the roots were directly extracted with refluxing methanol, and then the methanol extracts were further fractionated on tracing the protein stabilizing activity. Both of [I] and [II] were found in the ether-soluble acidic fraction and were crystallized in fairly good yields.

IC_{50} of [II] for the protein stabilizing activity was 26 μg/3 ml, of which potency was more strong than that of [I] (32 μg/3 ml), and more

twelve times than that of phenylbutazone. On carrageenin-induced edema test, the anti-inflammatory activity of [II] administered *p.o.* was three times more potent than that of phenybutazone, whereas it *s.c.* was slightly lower. This paper described the studies on the isolation and identification of [II] and on its anti-inflammatory characteristics.

EXPERIMENTAL METHODS

Materials

Roots of *A. continentalis* were purchased from a market, Chongno Street, Seoul. Bovine serum albumin (Cohn fraction V) and carrageenin (λ type) were purchased from Sigma Chemical Co. Phenybutazone was grade of USP.

Protein Stabilizing Activity

The protein stabilizing activity of a sample was assayed by its ability to inhibit heat denaturation of bovine serum albumin, as described previously[1]. An insoluble sample in water was thoroughly dissolved in 0.3N NaOH and then neutralized pH 8 to 9 with 0.3N HCl. The sample solution was serially diluted with 0.1 M phosphate-saline solution(pH 5.3). One unit of the protein stabilizing activity was defined as the amount of sample inhibiting 50% the heat denaturation of albumin. Specific activity was expressed as protein stabilizing activity units per gram of sample.

Extraction and Isolation

Roots of *A. continentalis*(9 Kg) were continuously extracted and concentrated in an apparatus of a large scale consisting of an extractor (200 *l* volume) and a concentrator (50 *l* volume), which was recently installed in our Institute. A syrupy methanol-extract was removed to a rotary evaporator and further concentrated to dryness *in vacuo*(1.45 kg). Solvent fractionation of the methanol extract was carried out by tracing the protein stabilizing activity as previously described.[1]

The ether-soluble acidic fraction (290 g) exhibiting the major activity was chromatographed on a silica gel column, using the elution solvent of hexane/ethylacetate/acetic acid (20:10:1) to give three fractions. (Fig. 1). Fraction 1 (201 g) showed a single spot at Rf 0.6 on TLC, and was crytallized from methanol to give (-)-pimara-8(14), 15-dien-19-oic acid [I] (colorless needles, 6 g), mp 158°, $[\alpha]_D^{25}$-147.5 ($CHCl_3$). The filtrate was concentrated to dryness *in vacuo*, and the residue was crystallized from n-hexane to yield compound II (colorless plates, 19 g). mp: 176~8°C; $[\alpha]_D^{20}$: -101.2 ($CHCl_3$); IRν_{max}^{KBr} cm^{-1}: 3000~2500(broad), 3060, 1690, 1650, 875; MS: m/z 302 (M^+, 40.6), 287(M^+-15, 29.6), 91(100); PMR δ_{ppm}^{TMS}: 0.95(3H, s), 1.24(3H, s), 2.65 (1H, m), 4.75(2H, d, J=3Hz), 11.4(1H, br. s.); CMR δ_{ppm}^{TMS}: 15.66 (C-20), 18.53(C-11), 19.24(C-2), 21.96(C-6), 29.01(C-18), 33.20(C-12), 37.96(C-3), 39.84 (C-14), 39.84(C-10), 40.91, 41,48, (C-1, C-7), 43.94(C-4), 43.94(C-13), 44.37(C-8), 49.17(C-15), 55.42(C-9), 57.35(C-5), 103.21 (C-17), 155.56(C-16), 184.86(C-19).

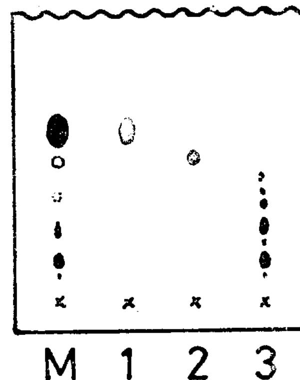

Fig. 1. Thin layer chromatograms of ether-soluble acidic fraction and its sub-fractions on a silica gel plate. Solvent : Hexane/ethylacetate/acetic acid (20 : 10 : 1). Visualized by d-H_2SO_4. 1, 2, 3 : Fr. 1, 2 and 3

Methylation of 〔*II*〕

Methyl ester of 〔II〕 was obtained by treatment of diazomethane in ethyl ether and recrystallized from methanol. mp: 86~8°C; IR ν_{max}^{KBr} cm^{-1}: 3050, 1725, 1650, 870; MS: m/z 316 (M^+, 4.7), 273(20.9) 257(44.2), 94(100); PMR δ_{ppm}^{TMS}: 0.85(3H, s), 1.18(3H, s), 2.60(1H,m), 3.62(3H, s), 4.72(2H, br. s.)

GC/MS Analysis

GC/MS analysis of the ether-soluble acidic fraction after treatment of diazomethane was carried out under the conditions; OV-1 fused capillary column (0.2mm i.d.×250m, temperture 1: 180°C, time 1: 2 min, rate: 4°C/min, temperature 2: 320°C), carrier gas: helium ($\bar{\mu}$ =20cm/sec), scanning mass range: m/z 40~600. Four main peaks were obtained. (Fig. 2)

Peak 1: m/z 270(M^+, 2.9), 239(2.3), 227(4.7), 213(1.0), 199(2.0), 185(2.9), 171(2.6), 157(1.4), 143(13.5), 129(6.4), 74(100).

Peak 2: m/z 294(M^+, 1.2), 263(2.0), 220(1.4), 205(0.8), 191(1.2), 178(2.4), 164(5.9), 150(8.8), 136(10.4), 135(11.4), 123(1.36), 109(25.7), 95(60.7), 81(75.6), 67(96.1), 55(100).

Peak 3: m/z 316(M^+, 1.9), 301(1.2), 273(0.2), 257(7.6), 241(9.8), 216(1.9), 188(7.9), 180(18.2), 159(5.6), 121(100).

Peak 4: m/z 316(M^+, 3.3), 301(2.7), 273(10.5), 257(42.3), 241(37.5), 213(20.0), 91(100).

Instrumental Analysis

All melting points were taken on a Mitamura heat block apparatus and given uncorrected values. A UV/visible spectrophotometer, Gilford type 2600 was used for the measurements of UV absorption spectra and the turbidity of heat denaturated albumin solution. Proton and ^{13}C NMR spectra were obtained in $CDCl_3$ solution using TMS as an internal standard on a Varian FT 80-A spectrometer. IR spectra were determined in KBr pellets on a Perkin-Elmer type 283 B spectrophotometer. Gas-liquid chromatograms were obtained on a Hewlett Packard type 5840 A chromatography under the conditions:

Column; 5% SE-30, size 4mm×2m; N_2, flow rate 40ml/min, temperature; column 200°, detection 240° (FID).

Mass spectra were obtained on a Hewlett Packard GC/Mass Spectrometer (type 5985B) using a electron impact method.

Carrageenin-Induced Edema Test

Carrageenin-induced edema test was performed using male albino rats (Splague Dawley strain) weighing 170±10g, according to the method of Winter *et al.*[5] Five or six rats per group were given compound 11 orally or *s.c.*, twice,six and one hour befor the injection of 0.05ml of 1% carrageenin saline solution into the plantar surface of the right hind paw. The edema volume was measured with a plethysmometer.

RESULTS AND DISCUSSION

Purification of (−)−*Kaur-16-en-19-oic Acid* 〔*II*〕

The results on screening for the isolation of active principles and on the protein stabilizing activities are summarized in Table I.

Almost all the activities in the methanol extracts of *A. continentalis* were coucentrated on the ether-soluble acidic fraction. When a small portion of this fraction was methylated with diazomethane and subjected to GC/MS, it gave four major peaks as shown in Fig. 2.

Peaks 1 and 2 were turned to be methyl esters of palmitic acid and linoleic acid, respectively. The mass spectrum of peak 4 coincided with that of(−)-pimara-8(14), -15-dien-19-oic acid methyl ester, by comparing its authentic sample. Peak 3 was postulated to be methyl ester of 〔II〕 as referring to the data of literature[6].

The ether-soluble acidic fraction was chromat-

Table I: Summary of Purification of Compound-I and -II

	Total amounts (g)	Total* activity (units)	Specific* activity (units/g)	Activity yield (%)
A. continentalis	9,000	—	—	—
Methanol extract	1,450	7.84×10^{6}	5.1×10^{3}	100
Solvent fractionation				
1. ether fr.	620	7.40×10^{5}	1.19×10^{4}	94.4
2. butanol fr.	50	9.45×10^{3}	189	0.1
3. ether-soluble acidic fr.	290	7.28×10^{5}	2.51×10^{4}	92.9
Silica gel chromatography				
Fr. 1	201	5.68×10^{5}	2.83×10^{4}	72.4
Fr. 2	14	3.28×10^{5}	2.34×10^{4}	4.2
Fr. 3	35	—	—	—
Crystallization from Fr. 1				
Compound I (from methanol)	6	1.88×10^{5}	3.13×10^{4}	2.4
Compound II (from hexane)	19	7.31×10^{4}	3.85×10^{4}	9.3

* One unit of protein stabilizing activity was defined as the amount of sample required to give 50% inhibition on heat denaturation of albumin.

** Protein stabilizing activity units per gram of sample.

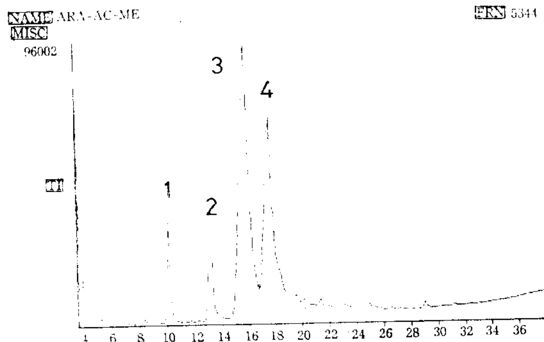

Fig. 2. Total ion chromatogram of ether-soluble acidic fraction on a Hewlett Packard GC/Mass spectrometer (type 5985B) using a electron impact method. Scanning mass range : m/z 40-600. GC conditions : OV-1 fused capillary column (0.2mm i.d. ×25m); temp. 1 : 180°C, time 1 : 2 min, rate : 4°C/min, temp. 2 : 320°C, carrier gas : helium (μ=20cm/sec).

ographed on a silica gel column, and separated into its 3 fractions. Fraction 1 with the majority of activity (72.4%) exhibited a single spot on TLC (Fig. 1), but did two peaks corresponding to peaks 3 and 4 by the gas chromatography of its methyl ester. (Fig. 2)

Without any further purification of fraction 1, its methanol solution yielded compound 1. The filtrate was concentrated *in vacuo*, and the residue was dissolved in n-hexane. This n-hexane solution made easy the crystallization of compound II. And the filtrate still contained a large amout of above two compounts, which have not been completely separated each other.

Fraction 2 with a low yield except high spécific activity was turned to be the mixture of palmitic acid and linoleic acid by GC of its methyl ester. Fraction 3 without activity was not further studied.

The IR and PMR spectra of [I] were coincided with those of(−)-pimara-8 (14), 15-dien-19-oic acid previously isolated[1].

Compound II was positive in Libermann-Buchard reaction and negative in Zimmermann test and nitrogen test. Its IR spectrum showed the presences of carboxylic acid (3000∼2500, 1685 cm^{-1}) and a disubstituted vinyl group (3060, 1650, 875cm^{-1}). [II] afforded its methyl ester,

of which IR spectrum showed an absorption band at 1725cm^{-1} (ester) and did not at 4000~3100cm^{-1}, revealing the absence of ketone and hydroxyl groups. The mass spectrum of [II] showed the molecular ion at m/z 302, giving a possible molecular formula $C_{20}H_{30}O_2$. Proton NMR of [II] exhibited the presences of a carboxylic acid (11.4 ppm, 1H, diffused), a disubstituted vinyl group (4.74ppm, 2H, d-like), and two angular methyls (0.95, 1.24ppm, 2×3 H, s), which were also supported by PMR of its methyl ester; a carboxy methyl ester (3.62 ppm, s) and some up-field shifts of a vinyl group (4.62 ppm) and two methyl signals (0.85, 1.18 ppm).

The patterns of proton NMR of [II] and its methyl ester, and other spectral data mentioned above are identical with those of (−)-kaur-16-en-19-oic acid and its methyl ester, respectively, which were already isolated from *A. cordata*[3] and *Xylopia aethiopica*.[6]

The ^{13}C-NMR spectra provided conclusive assignment for the structure of [II]. Application of attached proton test of [II] yielded the data described in "EXPERIMENTAL METHODS", which were coincided with those of (−)-kaur-16-en-19-oic acid.[6]

The Contents of [I] and [II]

The contents of [I] and [II] were determined 0.9 and 2.1%, respectively, in the roots of the plant by GLC technique after methylation of its ether-soluble acidic fraction. The roots were finely powdered before methanol extraction (three times). Both methyl esters of [I] and[II] were each other used as internal references.

The Protein Stabilizing Activity of [II]

To determine the protein stabilizing activity of [II], dose-response curves were drawn as shown in Fig. 3, by plotting values for the inhibition rates of serial dilutions of [II], together with [I] and phenylbutazone as references.

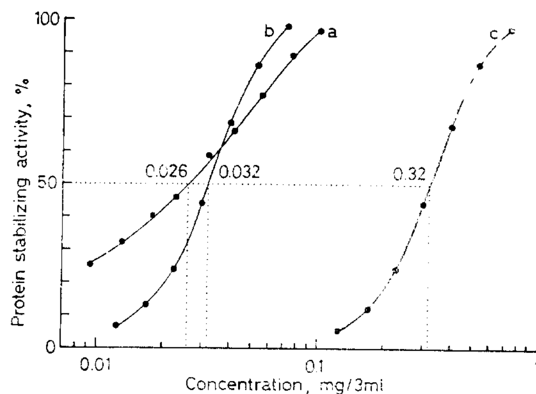

Fig. 3. Protein stabilizing potency of (−)-kaur-16-en-19-oic acid.
a : (−)-kaur-16-en-19-oic acid
b : (−)-pimara-8(14), 15-dien-19-oic acid
c : phenylbutazone

IC_{50} of [II], [I], and phenybutazone are 0.026, 0.032, and 0.32 mg/3ml, respectively.

The protein stabilizing activity of [II] is more potent to some extent than that of [I], and approximately twelve times more than that of phenylbutazone. The IC_{50} value of [I] is lower than the data described in our previous paper (0.115 mg/3ml). This was found to be an error due to its poor solubility in the buffer. In this study, the samples were thoroughly dissovled in 0.3M NaOH, neutralized to pH 8 to 9 with 0.3M HCl, and then serial dilutions were made with buffered saline (pH 5.3)

The Anti-inflammatory Activity of [II]

The anti-inflammatory effect of [II], 3, 10 and 30 mg/kg, *s.c.*; 33 and 100 mg/kg, *p.o.*, on carrageenin- induced edema in rat hind paw were investigated, and the results were compared with those of phenylbutazone, 30 mg/kg, *s.c.*and 100 mg/kg, *p.o.*, respectively, as shown in Table II and III.

On *s.c.* administration, [II], 10 and 30 mg/kg, and phenylbutazone, 30 mg/kg, suppressed significantly the edema volumes by 41.5, 48.2 and 57.6% on average 3.5 hr after the treatment with carrageenin, respectively. This sugge-

Table II: Anti-inflammatory activity of (−)-kaur-16-en-19-oic acid administered *s.c.*

Compound	Dose mg/kg *s.c.*	No. of animals	Edema increased per cent (Inhibition percent)				
			0.5 hr	1.5 hr	2.5 hr	3.5 hr	4.5 hr
Control	% CMC-Na	5	13.67±3.70	35.96±5.60	55.6±4.86	60.04±3.79	45.77±4.08
Phenylbutazone	30	5	4.93±2.26	14.18±1.29**	18.00±2.79** (67.6)	25.44±5.67** (57.6)	19.43±3.29**
[II]	30	5	3.43±0.97**	13.51±6.00*	21.35±5.01** (61.6)	31.11±4.11*** (48.2)	25.63±2.17**
[II]	10	5	15.35±4.80	23.45±3.17	34.00±6.05* (38.8)	35.15±4.65** (41.5)	23.09±2.51**
[II]	3	5	12.41±1.95	14.77±4.07*	33.23±6.01* (40.2)	42.32±7.30 (29.5)	26.18±6.56*

$^{*}p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$ when compared with control.

Table III: Anti-inflammatory activity of (−)-kaur-16-en-19-oic acid administered *p.o.*

Compound	Dose mg/kg *p.o.*	No. of animals	Edema increased percent (inhibition per cent)				
			0.5 hr	1.5 hr	2.5 hr	3.5 hr	4.5 hr
Control	1% CMC-Na	5	16.78±4.78	26.64±5.90	55.18±7.33	60.91±7.44	43.14±6.05
Phenylbutazone	100	5	7.55±2.03	15.80±1.92*	19.45±3.08** (64.8)	31.26±5.50* (48.7)	30.11±10.43
II	100	5	5.27±1.62	10.62±3.04	18.12±5.51* (67.2)	26.04±5.45* (57.2)	16.94±5.11
II	33	5	7.86±0.91**	17.53±5.97	21.89±8.09** (60.3)	30.58±6.67** (49.8)	25.87±4.80*

$^{*}p<0.05$ $^{**}p<0.01$ when compared with control.

sted that [II] administered *s.c.* showed a lower anti-inflammatory activity than phenylbutazone.

When administered *p.o.*, [II], 33 and 100 mg/kg, and phenylbutazone, 100 mg/kg, caused the significant inhibitions of 49.8, 57.2 and 48.7% on average 3.5 hour after the carrageenin treatment, respectively. By a rough estimation, [II] administered *p.o.* shows an anti-inflammatory activity of over three times of phenylbutazone. These results indicate that the *p.o.* administration of [II] is more effective. In contrast to [II], [I] previously reported was more active in case of the *s.c.* administration.

In conclusion, it can be summarized that [II] as well as [I] is a non-steroidal anti-inflammatory component, both involving the majority of activities of *A. continentalis*. And it is noteworthy that the anti-inflammatory components isolated from natural resources showed more potent actions than phenylbutazone used clinically.

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