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Studies on the Antioxidant Components of Korean Ginseng (III)

Identification of Phenolic Acids

Byung Hoon Han, Myung Hwan Park and Yong Nam Han

*Natural Products Research Institute, Seoul National University, Seoul 110, and *Department of Chemistry, College of Natural Science, Kangweon National University, Chuncheon 200, Korea*

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Abstract□The effective components of Korean ginseng showing the lipid-peroxide depressing activity were isolated. From the ether-soluble acidic fraction of fresh ginseng three phenolic acids were obtained. Salicylic acid and vanillic acid exhibited the potent antioxidant activity, whereas *p*-hydroxycinnamic acid did not.

Keywords: Korean ginseng, Anti-oxidant, Inhibition of lipid peroxidation, Phenolic acids

In our previous paper[1], it was reported that a ether-soluble acidic fraction of Korean ginseng exhibited the potent antioxidant activity against lipid peroxidation in liver from ethanol-intoxicated mice, and also that maltol (3-hydroxy-2-methyl-γ-pyrone) was isolated as one of the active principles of the fraction of red ginseng.

This paper describes that the purification of fraction of fresh ginseng is carried out by tracing the antioxidant activity with the animal experiments[1] to yield three phenolic acids in crystalline states. They were identified by chemical and spectrometric methods to be salicylic acid, vanillic acid and *p*-hydroxycinnamic acid. Salicylic acid and vanillic acid showed very potent antioxidant activity, whereas *p*-hydroxycinnamic acid did not. The contents of the three phenolic acids were also assayed.

EXPERIMENTAL

Materials

Vanillic acid, acetyl vanillic acid and acetyl vanillic acid methylester used for the authentic standards were synthetized from vanillin by the usual processes of acetylation (pyridine/Ac_2O), oxidation ($KMnO_4$) and methylation (diazomethane).

Instrumental Analysis

All melting points were taken on a heat block apparatus and given uncorrected values. Recording spectrometer, Schimazu Model RV-50, was used for the measuremetns of UV-visible absorption spectra and color density. Mass spectra were taken at 75 eV on JEOL-O1-SG-2 spectrometer. PMR(100MHz) and CMR(25.15MHz) spectra were obtained in $CDCl_3$, methanol-d_4 and acetone-d_6, and DMSO-d_6 solution using TMS as internal standard on JEOL-PFT-100 NMR-spectrometer and recorded by δ ppm.

Solvent Syetems for Chromatography

The following solvent systems were used for column chromatography using silica-gel and thin layer chromatography (TLC) on silica-gel plates (G. Type 60; E. Merck)

Solvent A; n-hexane/ethyl acetate/acetic acid (6 : 1 : 0.2)

Solvent B ; n-hexane/ethyl acetate/acetic aicd (5 : 1 : 0.2)

Solvent C; n-hexane/chloroform/methanol/acetic acid (6 : 5 : 1 : 0.2)

Solvent D; n-hexane/chloroform/methanol/acetic acid (5 : 5 : 1 : 0.2)

Solvent E; n-hexane/ethyiacetate (6 : 1)

Solvent F; n-hexane/ethyl acetate (1: 1)

Solvent G; benzene/acetone (6 : 1)

Assay of Lipid-peroxide content;

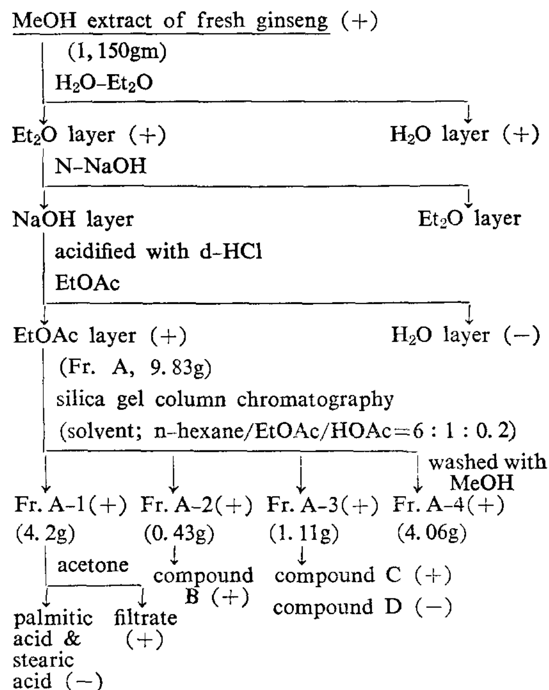
The lipid-peroxide contents in liver from ethanol-intoxicated mice were assayed by F. Masugi's thiobarbituric acid (TBA) method[1] with some modification, as described previously,[2] and expressed as TBA value (absorbancy at 532 nm per gm wet weight of liver).

Fractionation of Ether-soluble Acidic Fraction

Fresh Korean ginseng (10kg) was extracted with boiling methanol under nitrogen stream and concentrated in vacuo to give a syrupy extract (yield 1,150g). The extract was fractionated by solvent partitioning process as shown in Scheme 1.

The ether-soluble fraction was extracted with N-NaOH solution. The alkaline layer was acidified by dil-HCl and extracted with ethyl acetate.

The ethyl acetate layer was washed with water, dried over anhydrous sodium sulfate, and concentrated under vacuum to yield



Scheme 1. Fractionation of fresh ginseng extract.

9.83 gm of the ether-soluble acidic fraction (Fr. A).

With tracing the anti-oxidant activity, Fr. A was fractionated by silica-gel (300gm) column chromatography using the solvent system A as eluent, to give three sub-fractions of Fr. A-1, Fr. A-2 and Fr. A-3. Fr. A-4 was obtained by washing the column with methanol. The yields of the four sub-fractions were also shown in Scheme 1.

Isolation of Compound B(salicylic acid) from Fr. A-2

Fr. A-2 was preparatively chromatographed on a thin-layer silica-gel plate (thickness 0.3mm) using the solvent B. A main component (R*f* 0.58), giving red-purple spot on TLC by $FeCl_3$ spray, was isolated in a pure state, and crystallized from chloroform to give fine

needles of 34mg, mp 124°, and designated it as compound B.

Isolation of Compound C(vanillic acid) and Compound D(p-hydroxycinnamic acid) from Fr. A-3

Fr. A-3 was further fractionated by column chromatography using silica-gel and the solvent C. Two main components were isolated in chromatographically pure states on a thin layer silica-gel plate developing with the solvent C.

Compound C (R*f* value 0.30, yield 0.55g) gave brownish-yellow by $FeCl_3$, yellowish-red by diazotized sulfanilic acid solution (Pauly's reagent) and a positive reaction by $FeCl_3$ plus $K_3Fe(CN)_6$. compound D(Rf value 0.21, yield 0.26g) gave yellowish-red by Pauly's reagent.

Acetylation Procedure

Compound C(0.55g) and D(0.26g) were dissolved in pyridine-acetic anhydride (1 : 1) mixture solution to make 50mg/ml respectively, and kept at room temperature over night. Excess reagents were removed under a nitrogen flow. Acetyl derivative of compound C was purified by silica-gel column chromatography using the solvent G as eluent and obtained a chromatographically pure state on TLC using the solvent G (130mg yield), mp 172–4°. And purification of acetyl-derivative of compound D was carried out by silica-gel column chromatography using the solvent F as eluent to yield rod-crystal (27mg), mp 168 (sublime).

Methylation Procedure

Acetyl drivative of compound C (130mg) was dissolved in 35 ml of ether-acetone (6 : 1) and methylated by excess of diazomethane at 4°C for 6 hours. The methylated product was purified by column chromatography using silica-gel (30g) and the solvent E as eluent and yielded in needle crystal (14mg), mp 76–77°.

Assay of Phenolic Acids by Preparative TLC Method

1) test sample solution ; The ether-soluble acidic fraction (Fr. A) was quantitatively prepared from 20 gm of air-dried ginseng powder, as illustrated in Scheme 1. Fr. A was dissolved in 2 ml of methanol-acetone (3 : 1) to use as the test sample solution.

2) standards solutions; Salicylic acid, vanillic acid and *p*-hydroxycinnamic aicd were dissolved in methanol to give 2mg/ml, respectively.

3) preparative TLC; Silica-gel plates (20 × 5cm) of 0.3 mm thickness were use. Fifty μl of test sample and standards solutions were applied to form homogenous bands, respectively, and chromatographed 15 cm by the solvent D. After drying, the chromatograms were exposed under UV-lamp. The bands of test sample corresponding to the three standards and those of the standards were positioned, collected quantitatively in 50 ml beaker and extracted with 5 ml of methanol. Absorbancies of the extracted solutions were measured at 307 (salicylic acid), 261 (vanillic acid) and 305 nm (*p*-hydroxycinnamic acid). From standard calibration curves made previously by the same process as the above method, the concentration of each solution was determined.

RESULTS AND DISCUSSION

In our previous work[1], it was confirmed that the major portion of antioxidant activity of Korean ginseng (both red and fresh ginseng) was distributed in the ether-soluble acidic fraction (Fr. A). Maltol, a phenolic acidic component was isolated form Fr. A of red ginseng.

We further fractionated the Fr. A of fresh ginseng by silica-gel column chromatography to obtain four sub-fractions, designated as Fr. A-1—4.

All of the sub-fractions exhibited the potent antioxidant activity and their chromatograms on a silica-gel plate were shown in Fig. 1.

Fr. A-1 was dissolved in acetone to give crystals of the mixtures of palmitic acid and stearic acid, not showing the anti-oxidant activity. The filtrate having the activity was not studied further.

Indentification of Compound B (salicylic acid)

Compound B, mp 124°, was isolated from Fr. A-2 by preparative TLC. It shows a strong chelating activity with ferric chloride, giving an intense red-purple color reaction. UV absorption maximum is at 307 nm (E; 3,350) and is shifted to 298 nm (E; 3260) at alkaline phase. These suggest the vicinal location of carbonyl and hydroxyl group in compound B. Mass spectrum shows molecular ion at m/e 138, M^+-H_2O at m/e 120, and M^+-HCOOH at m/e 92. In $C^{13}-$NMR spectrum compound B displays seven carbons $\delta_{ppm}^{DMSO-d_6}$; 113.61 (C-1), 162.72 (C-2), 117.79 (C-3),

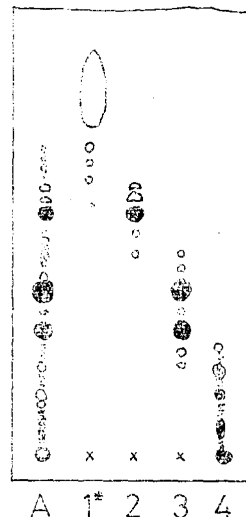


Fig. 1: Thin layer chromatograms of the ether-soluble acidic fraction and its subfraction. Chromatography was carried out on a precoated silica gel (Type G-60, F_{254}) plate, using n-hexane/chloroform/methanol/acetic acid (5:5:1:0.2) as developing solvent.
The spots were visualized with Pauly's reagent and 10% H_2SO_4(*).
A; Fr.A, 1;Fr.A-1, 2;FrA-2, 3;Fr.A-3, 4; Fr.A-4

136.17 (C-4), 117.69 (C-5), 131.21 (C-6) and 173.03 (COOH).

These properties of compound B agree well with those of salicylic acid. Additionally, it gives the identical R*f* value, blue-fluorescence under UV-lamp and red-purple color by $FeCl_3$ spray on a silica-gel TLC plate with those of salicylic acid.

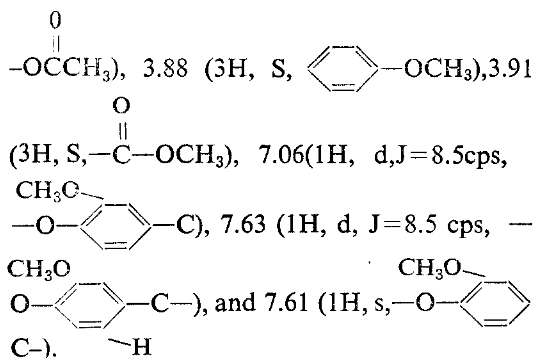
Identification of Compound C

Compound C isolated had some impurities, judging by its IR spectrum, and was further purified as the derivatives of acetate and acetyl methylester. The acetate of C, mp 172-4°, shows negative reactions by $FeCl_3$ plus K_3Fe

$(CN)_6$ solution and Pauly's reagent. IR spectrum shows absorption bands at 1750 (C=0 of acetyl), 1624, 1598 (phenyl ring), 2800-2500, 1685 (COOH), Deacetylation of the acetate of C gave the same R*f* value as that of intact compound C.

Methylation of the acetate of C with excess diazomethane gives colorless needle crystal, mp 76–77°. UV absorption maxima λ_{max}^{EtOH} are 245 (E; 8,480) and 293 (E; 3,550).

Proton NMR gives δ_{ppm}^{CDCl3}; 2.32 (3H, S,



-OCCH$_3$), 3.88 (3H, S, —OCH$_3$),3.91 (3H, S,—C—OCH$_3$), 7.06(1H, d,J=8.5cps, —O—C), 7.63 (1H, d, J=8.5 cps, —O—C—), and 7.61 (1H, s,—O—C-).

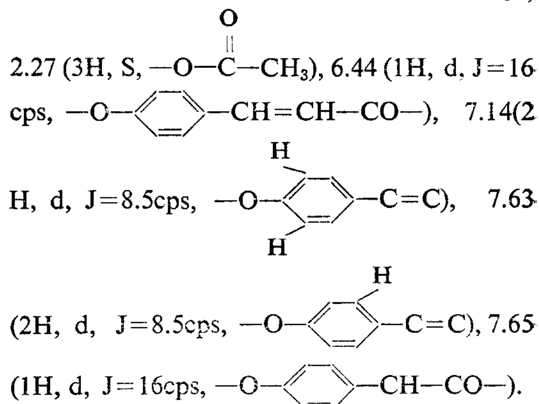
Direct comparison of acetyl C methylester with the authentic standard of acetyl vanillic acid methylester gave superimposable spectrum in proton NMR.

Alkaline hydrolysis of acetyl C methylester (5mg) gave colorless needle crystal, mp 163–5° (yield 2.7 mg), and this crystal showed the indentical R*f* value and color reaction (Pauly's) reagent and $FeCl_3$ plus $K_3Fe(CN)_6$ solution) with those of non-treated compound C and the authentic standard of vanillic acid.

Identification of Compound D (p-hydroxy cinnamic acid)

Compound D (R*f* value 0.21 on TLC by the solvent C) also had some impurities, judging by its IR spectrum and was derivatized as acetate. Acetylation of compound D gave rod-like crystal (yield 27 mg), mp 168° (sublime). UV absorption maximum λ_{max}^{EtOH} is 275 nm (E; 5580).

Proton NMR reveals $\delta_{ppm}^{CD_3OD}$ +acetone-d$_6$;



2.27 (3H, S, —O—C—CH$_3$), 6.44 (1H, d, J=16 cps, —O—CH=CH—CO—), 7.14(2 H, d, J=8.5cps, —O—C=C), 7.63 (2H, d, J=8.5cps, —O—C=C), 7.65 (1H, d, J=16cps, —O—CH—CO—).

Alkaline hydrolysate of the acetate of D gave the identical R*f* value with that of intact D and the authentic standard of *p*-hydroxy-cinnamic acid.

The contents of three phenolic acids were determined as shown in Table I, being below 10mg % in white ginseng

Salicylic acid and vanillic acid showed the

Table I: The contents of phenolic acids in various fractions.

	White ginseng	Ether extract	Fr. A
	(%)	(%)	(%)
Salicylic acid	0.0076	0.42	8.0
Vanillic acid	0.0033	0.18	3.45
p-Hydroxycinnamic acid	0.0028	0.15	2.9

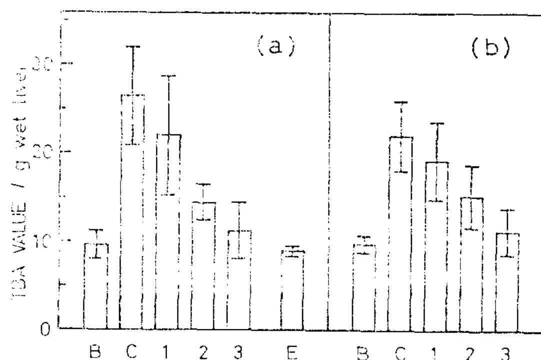


Fig. 2: Anti-oxidant activity of salicylic acid (a) and vanillic acid(b). Doses of two samples and *dl-α*-tocopherol acetate (reference, E) were 0.01mg (1), 0.1mg (2) and 1.0mg (3), and 1.0mg (E) per 30g mice body weight, respectively. TBA values are expressed as means±standard erros (n=16).
B; blank, C; control (ethanol-intoxicated only)

potent inhibition against lipid peroxidation in liver from ethanol-intoxicated mice, as illustrated in Fig. 2, whereas *p*-hydroxycinamic acid did not.

Our previous work[3] described that the addition of ferric ion during the extraction process repressed the antioxidant activity of Korean ginseng. It is probable that the depression of the antioxidant activity may be due to the interaction of ferric ion with the above phenolic acids.

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