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향나무 잎의 추출성분[1]

배영수[2] · Chuan Ling Si[2] · 김진규[2] · Joseph J. Karchesy[3]

Extractive of Juniper Needle[1]

Young Soo Bae[2], Chuan Ling Si[2], Jin Kyu Kim[2] and Joseph J. Karchesy[3]

요 약

향나무 잎을 채취하여 분쇄한 후 MeOH로 추출하고 분획한 다음 EtOAc 및 수용성 화합물을 칼럼크로마토그래피법을 사용하여 4개의 화합물 (+)-catechin (**1**), (-)-epicatechin (**2**), myricitrin (**3**) 및 hinokiflavone (**4**)을 단리하였다. 단리 화합물은 NMR 및 질량분석기를 사용하여 그 구조를 구명하였으며 DPPH radical 소거법을 이용하여 항산화 활성 검정을 시행하였다. 4가지 화합물 모두 우수한 활성을 보였으며 화합물 **1**, **2** 및 **3**은 대조구로 사용한 α-tocopherol, BHT 및 curcumin 보다 우수하였으나 화합물 **4**는 대조구보다 약간 낮은 활성을 나타내었다.

ABSTRACT

Four compounds, (+)-catechin (**1**), (-)-epicatechin (**2**), myricitrin (**3**), and hinokiflavone (**4**), were isolated from the needles of *Juniperus occidentalis* Hook. The structures of the isolated compounds were established by NMR and MS spectrometer. The antioxidant activity of the isolated compounds was determined by measuring free radical scavenging activity with DPPH and the results indicated that compound **1**, **2**, and **3** showed better activity than the controls, while compound **4** had weak activity compared with α-tocopherol, BHT, and curcumin.

keywords : *Juniperus occidentalis* Hook., Column chromatography, Biflavone, Antioxidant activity

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2. College of Forest Sciences, Kangwon National University, Chunchon, 200-701, Korea.
3. Oregon State University, Corvallis, OR, 97331, U.S.A.

INTRODUCTION

Juniperus occidentalis Hook. (Cupressaceae) is a long-lived conifer that is the dominant tree species of the Pacific Northwest, USA (Fowells, 1965; Franklin and Dyrness, 1988). Tissues obtained from the *Juniperus* are popularly known as folk medicine for a variety of pharmacological effects (Schepetkin, *et al.*, 2005). Biological studies showed that the extractive from *J. occidentalis* exhibits anti-inflammatory (Moreno, *et al.*, 1998), hypoglycemic (Sanchez, *et al.*, 1994), antibacterial (Johnston, *et al.*, 2001), and anti-tumor activity (Ali, *et al.*, 1996). Several researches have isolated oil terpene (Rudloff, *et al.*, 1980), phenol glucosides, biflavones, and diterpene (Nakanishi, *et al.*, 2002) from the needles of *J. occidentalis*. However, the detail chemical compositions of *J. occidentalis* are still subjected to be investigated.

MATERIALS AND METHODS

1. Plant material

Fresh juniper needles were collected in central Oregon in May 2004, air dried for three weeks and ground to fine particles to be extracted. A part of the ground needles (2.5 kg) was extracted three times with methanol (4 *l*) at room temperature to give enough crude extractive.

2. Extraction and purification

After filtration and concentration under reduced pressure, the aqueous residue was sequentially fractionated with *n*-hexane, chloroform and ethyl acetate, then freeze dried.

A portion of aqueous fraction (25 g) was applied to a sephadex LH-20 column using MeOH-H_2O (1 : 1, v/v) as an eluent to give five fractions and labeled JNW-1, 2, 3, 4, and 5. Fraction JNW-2, 3, and 4 was combined together and retreated with MeOH-H_2O (1 : 4, v/v) to get (+)-catechin (**1**)(70 mg), (-)-epicatechin (**2**)(220 mg), and myricetin- 3-O-*α*-L-rhamnopyranoside (**3**)(224 mg). Ethyl acetate soluble powder (18 g) was also chromatographed on a sephadex LH-20 column using 95% EtOH as an eluent. Four main fractions were collected and named JNE-1, 2, 3, and 4. From JNE-4, hinokiflavone (**4**)(100 mg), a apigenin dimer, was obtained.

3. Instrumentation

^{1}H-NMR, ^{13}C-NMR were performed using a Bruker Avance DPX 400 ㎒ NMR spectrometer. Positive FAB-MS data were obtained using a Micromass Autospec M363 spectrometer at the Central Laboratory of Kangwon National University. CD_3OD and DMSO-d_6 were used as NMR solvent.

4. Isolation of compounds

A portion of ethyl acetate soluble powder (18 g) and aqueous powder (25 g) were applied on a Sephadex LH-20 columns using MeOH-H_2O (3 : 1, 1 : 1, 1 : 4, v/v) as eluents to give four compounds which were elucidated as (+)-catechin, (-)-epicatechin, myricetin-3-*O*-*α*-L-rhamnopyranoside (myricitrin), and hinokiflavone. The spectral data were listed below.

4.1 (+)-catechin

^{1}H-NMR (400 ㎒, δ, MeOH-d_4) : 2.50 (1H, *dd*, J = 8.2 Hz and J = 16.1 Hz, H-4ax), 2.84 (1H, *dd*, J = 5.5 Hz and J = 16.1 Hz, H-4eq), 3.98 (1H, *m*, H-3), 4.56 (1H, *d*, J = 7.5 Hz, H-2), 5.85 (1H, *d*, J

= 2.2 Hz, H-6), 5.92 (1H, *d*, *J* = 2.3 Hz, H-8), 6.71 (1H, *dd*, *J* = 1.9 Hz and *J* = 8.1 Hz, H-6'), 6.76 (1H, *d*, *J* = 8.1 Hz, H-5'), 6.83 (1H, *d*, *J* = 1.8 Hz, H-2').

^{13}C-NMR (100 ㎒, δ, MeOH-d_4) : 28.55 (C-4), 68.84 (C-3), 82.88 (C-2), 95.53 (C-8), 96.32 (C-6), 100.85 (C-10), 115.28 (C-2'), 116.12 (C-5'), 120.08 (C-6'), 132.24 (C-1'), 146.26 (C-3'), 146.28 (C-4'), 156.95 (C-9), 157.61 (C-5), 157.86 (C-7).

4.2 (-)-epicatechin

^{1}H-NMR (400 ㎒, δ, MeOH-d_4) : 2.73 (1H, *dd*, *J* = 2.8 Hz and *J* = 16.8 Hz, H-4ax), 2.86 (1H, *dd*, *J* = 4.6 Hz and *J* = 16.8 Hz, H-4eq), 4.17 (1H, *br s*, H-3), 4.81 (1H, *s*, H-2), 5.92 (1H, *d*, *J* = 2.2 Hz, H-6), 5.94 (1H, *d*, *J* = 2.2 Hz, H-8), 6.78 (2H, *m*, H-5', 6'), 6.97 (1H, *d*, *J* = 1.7 Hz, H-2').

^{13}C-NMR (100 ㎒, δ, MeOH-d_4) : 29.31 (C-4), 67.54 (C-3), 79.93 (C-2), 95.93 (C-8), 96.43 (C-6), 100.11 (C-10), 115.37 (C-2'), 115.93 (C-5'), 119.43 (C-6'), 132.33 (C-1'), 145.83 (C-3'), 145.99 (C-4'), 157.41 (C-9), 157.72 (C-5), 158.05 (C-7).

4.3 Myricetin-3-*O*-*α*-L-rhamnopyranoside

FAB-MS : $[M+H]^+$ m/z 465.

^{1}H-NMR (400 ㎒, δ, MeOH-d_4) : 0.97 (3H, *d*, *J* = 6.2 Hz, H-6''), 3.30~4.23 (4H, *m*, H-2'', 3'', 4'', 5''), 5.31 (1H, *br s*, H-1''), 6.02 (1H, *d*, *J* = 1.8 Hz, H-6), 6.36 (1H, *d*, *J* = 1.8 Hz, H-8), 6.95 (2H, *br s*, H-2', 6').

^{13}C-NMR (100 ㎒, δ, MeOH-d_4) : 17.70 (C-6''), 71.91 (C-5''), 72.07 (C-3''), 72.13 (C-2''), 73.36 (C-4''), 94.71 (C-8), 99.82 (C-6), 103.65 (C-1''), 103.89 (C-10), 109.59 (C-2', 6'), 121.93 (C-1'), 136.34 (C-3), 137.92 (C-4'), 146.16 (C-3', 5'), 158.52 (C-9), 159.47 (C-2), 163.22 (C-5), 165.88 (C-7), 179.69 (C-4).

4.4 Hinokiflavone (apigenin(4'→6'')apigenin)

^{1}H-NMR (400 ㎒, δ, DMSO-d_6) : 6.25 (1H, *d*, *J* = 1.6 Hz, H-6), 6.53 (1H, *d*, *J* = 1.6 Hz, H-8), 6.78 (1H, *s*, H-8''), 6.89 (1H, *s*, H-3), 6.89 (1H, *s*, H-3''), 7.00 (2H, *d*, *J* = 8.6 Hz, H-3''', 5'''), 7.09 (2H, *d*, *J* = 8.8 Hz, H-3', 5'), 8.00 (2H, *d*, *J* = 8.6 Hz, H-2''', 6'''), 8.06 (2H, *d*, *J* = 8.8 Hz, H-2', 6').

^{13}C-NMR (100 ㎒, δ, DMSO-d_6) : 94.63 (C-8''), 95.22 (C-8), 99.51 (C-6), 103.12 (C-3''), 104.35 (C-10), 104.48 (C-3), 104.57 (C-10''), 115.89 (C-3', 5'), 116.59 (C-3''', 5'''), 121.69 (C-1'''), 124.76 (C-1'), 125.29 (C-6''), 128.93 (C-6'), 1129.16 (C-2'''), 153.65 (C-5''), 154.38 (C-9''), 157.94 (C-7''), 158.05 (C-9), 161.25 (C-4''') 161.87 (C-4'), 162.02 (C-5), 163.74 (C-2), 164.72 (C-2''), 164.86 (C-7), 182.35 (C-4''), 182.63 (C-4).

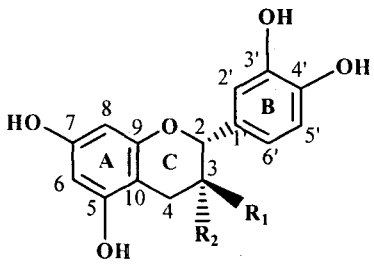
5. DPPH free radical scavenging assay

Free radical scavenging activity of the extractives was examined with the method described by Yoshida *et al.* (1989) with some modification. Samples having different concentrations in 4 ml were added separately to the solution of DPPH (0.15 mM, 1 ml) in methanol. The mixture was shaken and left to stand at room temperature for 30 min. Absorbance of the resulting solution was measured spectrophotometrically at 517 nm. BHT, α-tochopherol, and curcumin were used as controls. IC_{50} values were obtained through extrapolation from the concentration of sample needed to scavenge 50% of the DPPH free radicals.

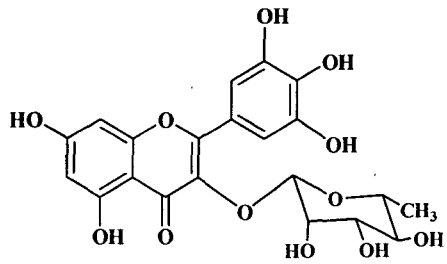
RESULTS AND DISCUSSION

1. Identification of compounds

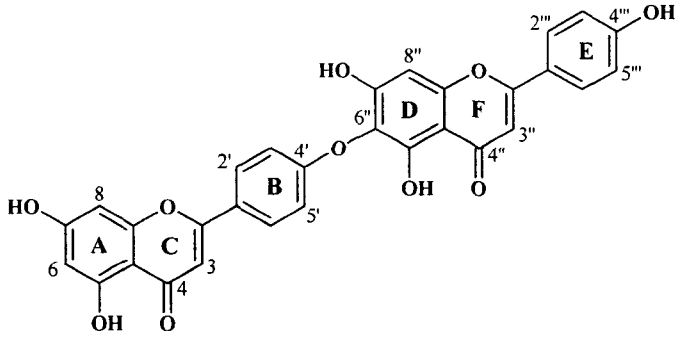
(+)-catechin (**1**), (-)-epicatechin (**2**), and myricetin-3-*O*-*α*-L-rhamnopyranoside (**3**) were obtained from the aqueous fraction and hinokiflavone (**4**) was isolated from the ethyl acetate fraction of *J.*



Compound 1 : R_1=OH, R_2=H, (+)-catechin
Compound 2 : R_1=H, R_2=OH, (-)-epicatechin



Compound 3 : myricitrin



Compound 4 : hinokiflavone

Figure 1. Compounds from *J. occidentalis* needles.

occidentalis needles.

(+)-catechin (**1**) and (-)-epicatedhin (**2**) gave strong red spots on a cellulose TLC plate when visualized with the spraying reagent. ^{1}H and ^{13}C-NMR spectra on two flavan compounds showed typical resonances corresponding to phloroglucinol A-ring, catechol B-ring and heterocyclic C-ring (Clark, 1968; Foo and karchesy, 1989).

1.1 myricitrin (myricetin-3-*O*-*a*-L-rhamnopyranoside)

The positive FAB-MS spectrum gave *m/z* 465 for $[M+H]^+$ ions indicating the molecular weight 464, which was consistent with the molecular formula $C_{21}H_{20}O_{12}$.

The ^{1}H-NMR spectrum was very informative and two *meta* coupled proton doublets (J=1.8 Hz) at δ 6.02 and δ 6.36, each integrated to one proton, were assignable to the H-6 and H-8 proton revealing the characteristic 2H AX system in the aromatic A-ring of 5,7-dihydroxyflavonol. A set of AB pattern protons overlapping at δ 6.95 as a broad singlet ascribing to H-2′ and H-6′ in the symmetric pyrogalloll B-ring. The presence of an *a* -configuration of rhamnose was evidenced by the anomeric proton resonating at δ 5.31 as a broad singlet and the methyl protons characteristically appeared at δ 0.97, together with four sugar protons between δ 3.30~4.23 (Mabry., 1970; Andary *et al.*, 1982).

In ^{13}C-NMR spectrum, the typical myricetin-3-*O*-*a*-L-rhamnopyranoside resonances were also observed. In the phloroglucinol A-ring, three oxygen-containing carbons appeared at 158.52, 163.22, and 165.88 ppm, C-6 and C-8 corresponded to 99.82 and 94.71 ppm respectively. Two pairs of symmetrical carbons, C-2′ or C-6′ and hydroxy-bearing C-3′ or C-5′, gave two strong broad singlets at 109.59 and 146.16 ppm respectively, which

corroborated the pyrogallol B-ring of aglycone. As for the heterocyclic C-ring, generally, carbons of pure myricetin give three signals at 147.1, 136.1, and 176.0 ppm for C-2, C-3 and C-4 respectively, in DMSO-d_6 (Agrawal, 1989). However, substitution at C-3 causes a slight upfield shift by 0.2 ppm for C-3 as well as a large downfield shift by 12.3 and 3.7 ppm for C-2 and C-4 respectively due to electronegativity from a substituent (Harbone and Mabry, 1982; Sen *et al.*, 1992). A signal at 17.70 ppm was a characteristic methyl group of rhamnosyl C-6″ and C-1″ linked to aglycone C-3 showed a signal at 103.65 ppm shifted downfield by 9 ppm compared to a free rhamnose (94 ppm) (Markham *et al.*, 1992). Four other rhmnosyl carbons typically resonated at 71.91~73.36 ppm.

Therefore, compound **3** was elucidated as myricetin-3-*O*-*α*-L-rhamnopyranoside and the literature data supported the result (Marco *et al.*, 1982; Agrawal, 1989).

1.2. Hinokiflavone (apigenin(4′→6″)apigenin)

In the ^{1}H-NMR spectrum, the presence of two sets of AA′BB′ system doublets at δ 7.00 (2H, J=8.6 Hz) and 8.00 (2H, J=8.6 Hz); δ 7.09 (2H, J=8.8 Hz) and 8.06 (2H, J=8.8 Hz) were attributed to eight aromatic protons of B-ring and E-ring, respectively. Two *meta* coupled proton doublets (J=1.6 Hz), each due to one proton, in the upfield aromatic region at δ 6.25 and 6.53 were ascribed to C-6 and C-8 protons of phloroglucinol A-ring. The one proton signals at δ 6.78 was assigned to H-8″ or H-6″ which implied that either C-8″ or C-6″ of D-ring had involved in the interflavonoid ether linkage. Two singlet signals overlapped to each other at δ 6.89 were attributed to H-3 and H-3″ of heterocyclic C and F-ring respectively. Thus, compound **4** was composed of two apigenin moieties with an ether linkage either between C-4′ and C-6″ or C-4′ and C-8″.

The ^{13}C-NMR chemical shifts of compound **4** appeared as duplicated apigenin carbon signals, particularly for catechol B and E-ring and heterocyclic C and F-ring, where carbon resonances showed up as twin peaks of comparable intensity. The remaining carbon signals were more or less consistent with the chemical shift values for phloroglucinol A-ring, except that C-6″ was shifted downfield to 125.29 ppm by 25 ppm while C-5″, C-7″ and C-9″ (*ortho* and *para* to the ether linked carbon of C-6″) shifted upfield to 153.65 (by 8 ppm), 153.65 (by 7 ppm), and 154.38 (by 4 ppm) respectively when compared with apigenin (Harbone and Mabry, 1982), which indicated the interflavonoid ether linkage at C-6″ (Agrawal, 1989).

Consequently, from the foregoing spectral studies, the structure of compound **4** was established as apigenin(4′→6″)apigenin (hinokiflavone), which is well coincided with the report (Chen *et al.*, 1974; Markham *et al.*, 1987).

2. Antioxidant activity

Antioxidant activities of the isolated compounds from *J. occidentalis* needles were tested by DPPH

Table 1. Antioxidative activities (IC_{50} values) of the isolated compounds from Juniper needles

	Samples	IC_{50} (μg)
Controls	α-tocopherol	12
	BHT	14
	curcumin	22
Isolated compounds	(+)-catechin	6
	(-)-epicatechin	10
	Myricetin-3-*O*-*α*-L-rhamnopyranoside	14
	Hinokiflavone	24

radical scavenging activity (Table 1). The results showed that (+)-catechin (**1**), (-)-epicatechin (**2**), and myricetin-3-*O*-*α*-L-rhamnopyranoside (**3**) exhibited significantly potent antioxidant activity, while the remaining biflavonoid, and hinokiflavone (**4**), showed weak activity compared with BHT, α-tocopherol, and curcumin. Therefore, *J. occidentalis* needles might be useful to identify a source of antioxidant components for the development of additives with appropriate protective properties in food or herbal drugs.

CONCLUSION

Four flavonoids were isolated by column chromatography using sephadex LH-20 from the needles of *J. occidentalis* and elucidated as (+)-catechin, (-)-epicatechin, myricetin-3-*O*-*α*-L-rhamnopyranoside (myricitrin), and hinokiflavone by ^{1}H and ^{13}C-NMR and positive FAB-MS spectroscopy. DPPH radical scavenging activity indicated that (+)-catechin, (-)-epicatechin, and myricetin-3-*O*-*α*-L-rhamnopyranoside exhibited strong antioxidant acitivity, while hinokiflavone was relatively less active.

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