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Biological Activities of *Larix kaempferi* Needles

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ABSTRACT : The needles of *L. kaempferi* was extracted with 95% ethanol and successively partitioned with *n*-hexane, CH_2Cl_2 and EtOAc. Repeated column chromatography on the EtOAc and H_2O soluble fractions gave three flavan-3-ols, one flavone glycoside, six flavonol glycosides and one lignan derivative. Their structures were elucidated on the basis of chemical and spectroscopic evidences. The antioxidant activities of the isolated compounds were evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method. Flavan compounds indicated good antioxidative potentials compared with BHT (butylated hydroxytoluene) and α-tocopherol as controls. In the anti-inflammatory test on most of the isolated compounds, NO (nitric oxide) assay against the RAW 264.7 (Mouse Macrophage) showed similar inhibitory potentials to NO production of the control. The cytotoxicity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and most of the isolated compounds indicated no toxicity in various concentration.

Keywords : *Larix kaempferi* Carr., Needles, Antioxidant activity, Anti-inflammatory activity, Cytotoxicity

INTRODUCTION

There are two larch species in Korea. : one is *Larix kaempferi* Carr. brought in Japan in 1904 and another is *Larix gmelini* var. *principisruprechtii* which naturally grows in Korea and China. *L. kaempferi* which grows up to 30 m high and 1 m diameter is one of major softwood tree species in Korea (Kim, 1994; Lee, 1996; Lee, 1980). Recently there is a great concern on the utilization of softwood needle extractive to study medicinal or functional supplementary food using bioactive natural resources. Many softwood needles contain a large amount of flavonoids and their glycosides which are useful to formulate some bioactive products.

Some studies on the chemical compositions of *L. kaempferi* sapwood (Takehara and Sasaya, 1979a, 1979b), inner bark (Miki and Sasaya, 1979) and heartwood (Miki and Sasaya, 1980) have been reported. We have previously been investigated the chemical composition of *L. kaempferi* green needles (Kim *et al*., 1997) and fallen needles (Kwon *et al*., 2006). This study was carried out to investigate the biological activities such as antioxidant, anti-inflammatory and cytotoxicity of the isolated compounds from *L. kaempferi* needles.

MATERIALS AND METHODS

1. Plant materials

The green needles (4.6 kg) and fallen needles (4.6 kg) of *Larix kaempfperi* Carr. were collected from the research forest of Kangwon National University in October and November of 2002, air-dried for 2 weeks at room temperature and ground to be extracted.

2. Equipments

Chromatographic column was packed with Sephadex LH-20 to be washed with aqueous methanol and EtOH-hexane mixture as eluting solvents. Eluents were collected using a Gilson FC 204 fraction collector.

TLC was performed on a precoated cellulose plate (25 DC-Plastikfolien Celulose F, Merck) and developed with *t*-BuOH-HOAc-H_2O (3:1:1, v/v/v) or HOAc-H_2O (3:47, v/v).

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Visualization was done by illuminating ultraviolet light (UV, 254 and 365 nm) and by spraying 1% $FeCl_3$ (in EtOH) or vanillin-HCl-EtOH (60:0.15:6, w/v/v) followed by heating.

^{1}H (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using a Bruker Avance DPX 400 NMR spectrometer in Central Laboratory of Kangwon National University. EI-MS and positive FAB-MS were recorded with a Micromass Autospec M363 spectrometer for molecular weight determination.

3. Extraction, Fractionation and Isolation

The ground samples were extracted with 95% EtOH three times at room temperature. Then the extractive were combined together, filtered and evaporated under the reduced pressure. The mixture was sequentially partitioned with *n*-hexane, CH_2Cl_2, EtOAc and H_2O using a separatory funnel. Each fraction was concentrated and freeze dried to give *n*-hexane soluble (53.3 g), CH_2Cl_2 soluble (30.1 g), EtOAc soluble (24.9 g), water soluble (189.7 g) from green needles and also to give *n*-hexane soluble (12.3 g), CH_2Cl_2 soluble (3.0 g), EtOAc soluble (44.4 g), water soluble (340.1 g) from fallen needles.

A portion of the EtOAc and H_2O soluble fractions from both needles were chromatographed on a Sephadex LH-20 using MeOH-H_2O (3:1, 1:1, 1:3, 1:4 etc., v/v) and EtOH-hexane (4:1, 4:3, 3:1, 3:2, 2:1 etc., v/v) as eluting solvents. Each fraction was reapplied to a column for further purification and gave 11 compounds from *L. kaempferi* needles.

4. Antioxidant activity (DPPH assay)

The antioxidative activity was done on the basis of the scavenging activity of the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical method firstly introduced by M. S. Blois (Blois, 1958) with slight modification. Samples of different concentrations (10~80 μg/ml) were added to a solution of 1 ml DPPH (0.15 mM) in 4 ml MeOH. After mixing gently and standing at room temperature for 30 min, the optical density was measured at 517 nm with a UV-visible spectrophotometer (Libra S32, Biochrom LTD). The results were calculated by taking the mean of all triplicated values. IC_{50} values were obtained through extrapolation from concentration of sample necessary to scavenge 50% of the DPPH free radicals. BHT (butylated hydroxytoluene) and α-tocopherol were used as controls.

5. Anti-inflammatory activity (NO assay)

NO (nitric oxide) inhibition activity was measured in the culture medium by Griess reaction (Green *et al*., 1982). RAW 264.7 (Mouse macrophage) cells were incubated at 37℃ in DMEM (Dulbecco's modified Eagle's medium) medium supplemented with 10% FBS (fetal bovine serum), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 with LPS (Lipoplysaccaride) 0.1 μg/ml (Lee *et al*., 2003). Cells were seeded in the growth medium (1 ml) into 96-well plates (1×10^6 cells per each well) and incubated for 24 hr. The test samples were dissolved in DMSO and adjusted to sample concentrations 50 and 100 μg/ml by diluting with the growth medium. 50 uM of positive control (NG-monomethyl-L-arginin (NMMA)) was treated with same method. After 24 hr of incubation at 37℃, 50 μl of cell culture medium was mixed with 50 μl of Griess reagent (equal volumes of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine-HCl) and incubated for 10 min, and then the absorbance at 540 nm was measured in ELISA reader.

6. Cytotoxicity activity (MTT assay)

Cytotoxic activity was measured by a modified Microculture Tetrazolium (MTT) assay (Mosmann, 1983). HaCaT (Immortalized Human Keratinocyte) human skin cells were maintained in DMEM medium supplemented with 10% FBS, penicillin (100 U/mll) and streptomycin (100 μg/ml) in a humidified 37℃ incubator gassed with 5% CO_2. Cells were seeded in the growth medium (100 μl) into 96-well plates (1×10^5 cells per each well) and incubated for 24 hr.

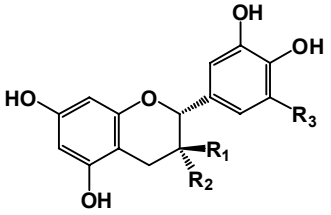
The test samples were dissolved in DMSO and adjusted to sample concentrations 50, 100 and 200 μg/ml by diluting with the growth medium. The culture plates were incubated for 24 hr, after which 20 μl of MTT solution (5 mg MTT/ml PBS) was added (final concentration 1 mg/ml), and further incubated at 37℃ for 4 hr. 100 μl acid iso-propanol (0.04N HCl in iso-propanol) added each well, the plates was centrifuged for 30 min at 1500 rpm and then the absorbance at 570 nm was measured in ELISA reader.

RESULTS AND DISCUSSION

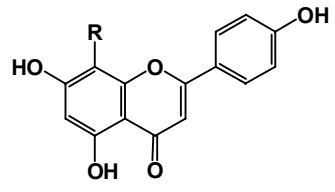
1. Isolation of compounds

Three flavans: (+)-catechin (1), (-)-epicatechin (2) and (+)-gallocatechin (3), the flavone glycoside; 2"-*O*-rhamnosylvitexsin (4); the six flavonol glycosides: juglanin (kaempferol-3-*O*-*α*-L-arabinofuranoside) (5), astragalin (kaempferol-3-*O*-*β*-D-glucopyranoside) (6), afzelin (kaempferol-3-*α*-L-rhamnopyranoside) (7), hirsutrin (quercetin-3-*O*-*β*-D-glucopyranoside) (8), isorhamnetin-3-*O*-*β*-D-glucopyranoside (9); and laricitrin-3-*O*-*β*-D-glucopyranoside (10), and one lignan derivative: cedrusin (11) were isolated from *L. kaempferi* needles (Fig. 1).

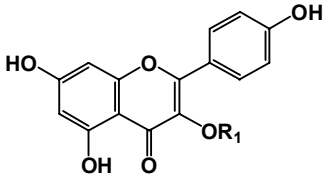
Six compounds (1, 2, 3, 4, 6, 9) were isolated from green needles and eight compounds (1, 2, 4, 5, 7, 8, 10, 11) from fallen needles of *L. kaempferi*. There was no positive difference in the chemical constituents between green and fallen needles. Compound (1), (2), (4) were isolated from both and supposed to main components of *L. kaempferi* needles.



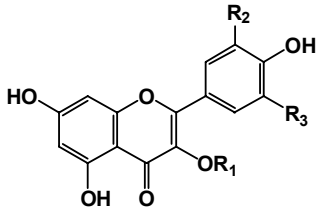
R_1 = OH, R_2 = R_3 = H : (+)-catechin (1)
R_1 = R_3 = H, R_2 = OH : (-)-epicatechin (2)
R_1 = R_3 = OH, R_2 = H : (+)-gallocatechin (3)



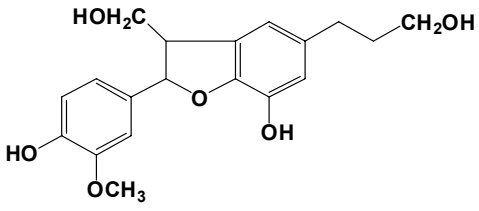
R = rhamnosyl-(1→2)-glucoside :
2"-*O*-rhamnosylvitexin (4)



R_1 = arabinose : juglanin (5)
R_1 = glucose : astragalin (6)
R_1 = rhamnose : afzelin (7)



R_1 = glucose, R_2 = OH, R_3 = H : hirsutrin (8)
R_1 = glucose, R_2 = OCH_3, R_3 = H :
isorhamnetin-3-*O*-glucopyranoside (9)
R_1 = glucose, R_2 = OCH_3, R_3 = OH :
laricitrin-3-*O*-glucopyranoside (10)



cedrusin (11)

Fig. 1. Structures of the isolated compounds from *L. kaempferi* needles.

2. Antioxidant activity of the isolated compounds

The DPPH radical scavenging activities (IC_{50} values) on the isolates were showed in Table 1. Flavan 3-ol compounds were indicated the highest activity. Among the flavonol glycosides (5~10), hirsutrin and laricitrin-3-*O*-glucopyranoside containing the catechol B-ring were more active than juglanin, astragalin afzelin and isorhamnetin-3-*O*-glucopyranoside. The catechol B-ring indicated higher antioxidant active than a single OH group in the B-ring (Rice-evans *et al*., 1996). Substitution with the methoxy group in the 3' position of the B-ring of isorhamnetin-3-*O*-glucopyranoside showed significantly low activity compared with hirsutrin.

3. Anti-inflammatory activity of the isolated compounds

Nitric oxide is a well-known signal in physical and pathological reaction, especially in acute inflammatory response (Surh *et al*., 2001). It is known that activation of inducible nitric oxide synthase (iNOS) by pro-inflammatory agent such as LPS can significantly increase nitric oxide (NO) production in macrophages (Kojima *et al*., 2000). In this study, a LPS-stimulated RAW 264.7 cell assay was employed to evaluate the NO inhibition activity of the compounds isolated from *L. kaempferi* needles. The results indicated that most of the isolated compounds were effective in NO production inhibition (Fig. 2). (+)-gallo-

Table 1. IC_{50} values of antioxidant activity of the isolated compounds

Samples		IC_{50} (μg/ml)
Controls	BHT	30
	α-tocopherol	26
Isolated Compounds	(+)-catechin (1)	6
	(-)-epicatechin (2)	10
	(+)-gallocatechin (3)	11
	2"-*O*-rhamnosylvitexin (4)	> 100
	juglanin (5)	64
	astragalin (6)	> 100
	afzelin (7)	> 100
	hirsutrin (8)	17
	isorhamnetin-3-*O*-glucopyranoside (9)	> 100
	laricitrin-3-*O*-glucopyranoside (10)	22
	cedrusin (11)	58

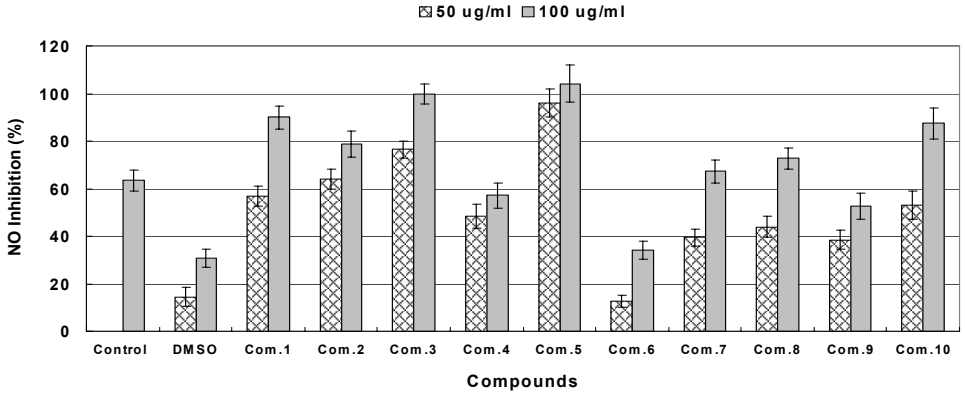


Fig. 2. NO inhibition effects of the isolated compounds from *L. kaempferi* at 50 and 100 μg/ml on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. Data are the means±S.E. of values from three independent experiments.

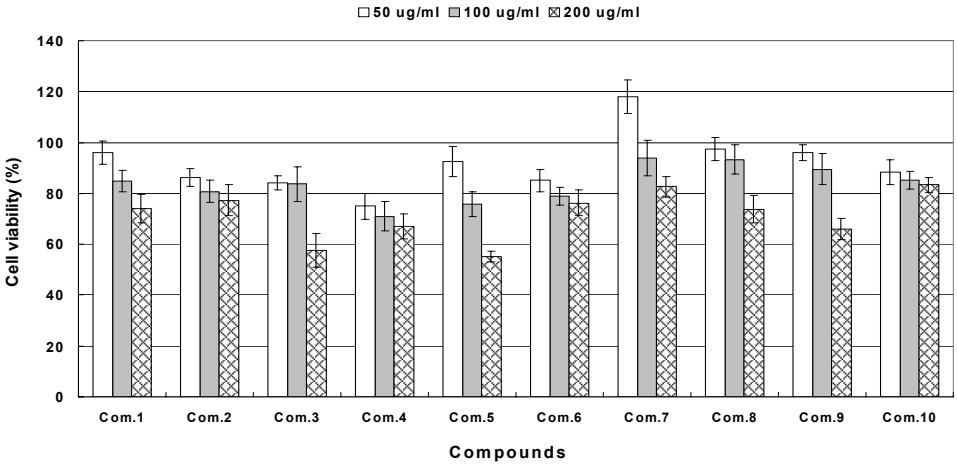


Fig. 3. Effect of the isolated compounds from *L. kaempferi* on cell viability of HaCaT cells. Cell viability was determined by MTT assay. Data are the means±S.E. of values from three independent experiments.

catechin (3) and juglanin (5) indicated inhibition activity above 90% of the NO production at a concentration of 100 μg/ml.

4. Cytotoxic activity of the isolated compounds

The isolated compounds (1~10) were evaluated for their cytotoxic activity against HaCaT normal skin cell lines in vitro by MTT assay method. The results are shown in Fig. 3. Most of the compounds indicated considerable cytotoxicity against the normal cell lines a dose-dependent manner, and showed almost no cytotoxicity, whereas (+)-gallocatechin (3) and juglanin (5) showed only about 50% cytotoxicity at concentration of 200 μg/ml.

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