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Flavonoid Glycosides from Needles of *Larix leptolepis*(Pinaceae)*1

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요 약

일본잎갈나무잎을 채취하여 아세톤 : 물(7 : 3, v/v)의 혼합용액으로 추출한 후, 에틸아세테이트용성 화합물과 수용성 화합물로 분리하였으며 Sephadex LH-20으로 충진한 칼럼을 사용하여 화합물을 단리 하였다. 단리 화합물을 확인하기 위하여 셀룰로오스 박층 크로마토그래피(TLC)를 실시한 후, 자외선 램프 하에서 관찰하였다. 바닐린 발색제를 분무하여 정색반응을 조사하고 R_f 값을 구하였다. 단리된 화합물들의 구조는 ^{1}H-NMR과 ^{13}C-NMR 스펙트럼을 이용하여 그 구조를 규명하였으며 에틸아세테이트용성 화합물에서는 (+)-catechin, (-)-epicatechin, kaempferol-3-O-arabinofuranoside와 kaempferol-3-O-arabinopyranoside, 수용성 화합물에서는 apigenin-8-C-rhamnosyl-(1‴→2″)-glucoside(2″-O-rhamnosylvitexin)을 단리 하였다.

Keywords : *Larix leptolepis*, needles, extractives, flavonoid glycosides, column chromatography

1. INTRODUCTION

There are two larch species in Korea. One is Larix leptolepis brought in Japan in 1904 and another is *Larix gmelini* var. *principisruprechtii* which naturally grows in Korea and China. Larix leptolepis which grows up to 30m high and 1m diameter is one of major softwood tree species implanted by the artificial reforestation in this country(Kim, 1994; Lee, 1996; Lee, 1980). However, there is a serious problem for the proper utilization of softwood thinning logs including this tree species and still no alternative to solve such a problem.

Recently, there is a great concern on the utilization of extractives of softwood needles to develop a medicine or a functional supplementary food using bioactive materials. In general,

most of softwood needles contain a large amount of flavonoids and their glycosides which can play an important role to formulate a bioactive medicine. This study was carried out to isolate any flavonoid compounds from the extractives of needles of Larix leptolepis and to obtain any basic useful information on the isolated compounds through structure identification for chemical utilization of the needles.

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2. METERIALS & METHODS

2.1 Equipments

^{1}H-NMR and ^{13}C-NMR spectra were obtained from a Varian Gemini 200 NMR spectrometer with samples dissolved in methanol-d_4 and acetone-d_6 and chemical shifts are given in δ values.

Chromatographic columns were packed with Sephadex LH-20 and eluents were collected using a Gilson FC 204 fraction collector. Analytical thin layer chromatography(TLC) was performed on cellulose plates(Merk DC-plastikfolien cellulose F) and developed with *t*-butanol-acetic acid-water(3 : 1 : 1, v/v/v, solvent A) and 6% acetic acid-water(3 : 47, v/v), solvent B). Visualization was done by illuminating ultraviolet light or by spraying vanillin-hydrochloric acid-ethanol(60 : 0.15 : 6, w/v/v) followed by heating.

2.2 Extraction and purification

Needles of larix leptolepis, which were air-dried in laboratory for three weeks after collection at Experimental Forests of Kangwon National University in March 1996, were prepared to fine powder by a grinding mill. The powder (1.15kg) was soaked in acetone-water(7 : 3, v/v) at room temperature for three days and then the acetone-water solution was decanted, filtered to give a crude extract. Each batch of powder was extracted three times. The combined extractants were then concentrated on a rotary evaporator under reduced pressure. The mixture was extracted with chloroform to remove chlorophyll and its derivatives and then treated with hexane to remove waxy materials. The residue was separated into ethylacetate and water soluble fractions to give 9.9g and 76.5g of freeze dried powder, respectively.

Ethylacetate soluble freeze dried powder(9.9g) was applied on a Sephadex LH-20 column(3× 10cm). The column was washed with methanol-water(3 : 1, v/v) as an eluent and three main fractions were collected and labeled E1, E2 and E3.

A portion of water soluble powder(30g) was also chromatographed on a Sephadex LH-20 column(3× 40cm) using methanol-water(1 : 1, v/v) as an eluting solvent and four major fractions were collected and labeled W1, W2, W3 and W4. Each fraction was reapplied to a column for further purification.

2.2.1 (+)-catechin

Fraction E2 was rechromatographed using methanol-water(1 : 2 and 1 : 3, v/v) and ethanol-hexane(1 : 4 and 1 : 2, v/v) as eluting solvents to give 260mg of a white browm powder. The powder gave a red spot on a cellulose sheet when was visualized with the spraying reagent. R_f 0.53(solvent A) and 0.40(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.5(1H, dd, J = 8.2㎐, J = 16.1㎐, H-4ax), 2.85(1H, dd, J = 5.4㎐, J = 16.2㎐, H-4eq), 3.99(1H, m, H-3), 4.57(1H, d, J = 7.42㎐, H-2), 5.85(1H, d, J = 2.3㎐, H-6), 5.93(1H, d, J = 1.3㎐, H-8), 6.69~6.88(3H, m, H-2', 5', 6').

^{13}C-NMR(ppm, Acetone-d_6) : 82.9(C-2), 68.9 (C-3), 28.5(C-4), 157.8(C-5), 96.4(C-6), 157.1 (C-7), 95.6(C-8), 158.0(C-9), 100.9(C-10), 132.4(C-1'), 115.4(C-2'), 146.4(C-3' and 4'), 116.2(C-5'), 120.2(C-6').

2.2.2 (-)-epicatechin

Fraction E2 also gave 16mg of a brown powder by column chromatography using the same solvent as mentioned the above. R_f 0.41(solvent A) and 0.36(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.69~2.93(2H, m, H-4eq, H-4ax), 4.19(1H, s, H-3), 4.82(H, s, H-2)), 5.94(2H, m, H-6,8), 6.78~6.99 (3H, m, H-2', 5', 6').

^{13}C-NMR(ppm, MeOH-d_4) : 79.73(C-2), 67.33(C-3), 29.07(C-4), 157.97(C-5), 96.30(C-6), 157.62(C-7), 95.80(C-8), 157.35(C-9), 99.99 (C-10), 132.24(C-1'), 115.24(C-2'), 145.72(C-3'), 145.89(C-4'), 115.84(C-5'), 119.35(C-6').

2.2.3 Kaempferol-3-O-arabinofuranoside

Fraction E3 was rechromatographed with ethanol-hexane(1 : 1, 2 : 1 and 4 : 1, v/v) and etanol-water(1 : 9, v/v) to give 12mg of a yellow powder. R_f 0.73(solvent A) and 0.29(solvent B).

^{1}H-NMR(δ, Acetone-d_6) : 3.47(2H, d, J = 4.06, H-5"), 3.89(1H, m, H-3"), 3.98(1H, m, H-2"), 4.32(1H, d, J = 1.73, H-4"), 5.47(1H, s, H-1"), 6.25(1H, d, J = 1.86, H-6), 6.49(1H, d, J = 2.14, H-8), 6.98(2H, d, J = 8.89, H-3', 5'), 8.03(2H, d, J=8.76, H-2', 6').

^{13}C-NMR(ppm, Acetone-d_6) : 158.51(C-2), 134.54 (C-3), 179.59(C-4), 162.50(C-5), 99.63(C-6), 165.60(C-7), 94.71(C-8), 158.04(C-9), 105.14 (C-10), 122.16(C-1'), 131.97(C-2' and 6'), 116.43(C-3' and 5'), 161.26(C-4'), 109.30(1"), 82.43(C-2"), 78.19(C-3"), 88.42(C-4"), 61.98(C-5").

2.2.4 Kaempferol-3-O-arabinopyranoside

Fraction E3 was retreated with ethanol-hexane(1 : 1 and 2 : 1, v/v) and methanolwater (1 : 3, v/v) to give 14mg of a yellow freeze dried material. R_f 0.57(solvent A) and 0.43(solvent B).

^{1}H-NMR(δ, Acetone-d_6) : 3.3-3.9(4H, m, H-2", 3", 4", 5"), 5.14(1H, d, J = 6.42, H-1"), 6.2(1H, d, J=1.31, H-6), 6.39(1H, s, H-8), 6.89(2H, d, J = 8.96, H-3', 5'), 8.07(2H, d, J = 8.75, H-2',6').

^{13}C-NMR(ppm, Acetone-d_6) : 158.14(C-2), 135.10 (C-3), 179.11(C-4), 162.40(C-5), 99.77(C-6), 165.93 (C-7), 94.73(C-8), 157.92(C-9), 105.03 (C-10), 121.95(C-1'), 132.06(C-2' and 6'), 116.21(C-3' and 5'), 161.29(C-4'), 103.59(C-1"), 73.20(C-2"), 72.08(C-3"), 67.85(C-4"), 65.96(C-5").

2.2.5 Apigenin-8-C-rhamnosyl-(1"' →2")-glucoside(2"-O-rhamnosylvitexin)

W2, water soluble fraction, was reapplied to a column with methanol-water(1 : 1 and 3 : 7, v/v) and ethanol-hexane(2 : 1, v/v) to give 68mg of a yellow powder. R_f 0.69(solvent A) and 0.61 (solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 0.63(3H, d, J = 6.14, H-6"' (rha)), 3.0~4.0(9H, m, sugar protons), 4.27(1H, d, J = 9.17, H-2" (glc), 5.03(1H, d, J = 9.93, H-1"' (glc)), 5.09(1H, s, H-1"' (rha)), 6.27(1H, s, H-3), 6.60(1H, s, H-6), 6.93(2H, d, J = 8.56, H-3', 5'), 7.82(2H, d, J = 8.55, H-2', 6').

^{13}C-NMR(ppm, Acetone-d_6) : 166.71(C-2), 103.52(C-3), 184.18(C-4), 162.75(C-4' and C-5), 99.74(C-6), 164.20(C-7), 105.58(C-8), 157.90(C-9), 123.47(C-1'), 130.08(C-2' and C-6'), 116.92(C-3' and 5'), 73.34(C-1" and 3"'), 77.99(C-2"), 81.48(C-3"), 71.97(C-4", 2"' and 4"'), 82.74(C-5"), 62.88(C-6"), 102.43(C-1"'), 69.82(C-5"'), 17.85(C-6"').

3. RESULTS & DISCUSSION

Three flavonoid glycosides, kaempferol-3-O-arabinofuranoside, kaempferol-3-O-arabinopyranoside and apigenin-8-C-rhamnosyl-(1"' →2")-glucoside(2"-O-rhamnosylvitexin), were isolated on repeated column chromatography using Sephadex LH-20 from the extractives of *Larix leptolepis* needles and characterized by two dimensional TLC(Fig. 1) and NMR spectroscopy.

The applied eluting solvents for column chromatography were methanol, ethanol, aqueous alcohols and ethanol-hexane mixture. Two flavan compounds, (+)-catechin and (-)-epicatechin, were also successfully purified on a

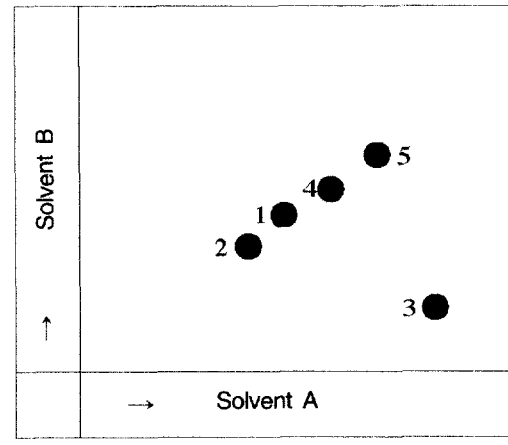


Fig. 1. Two dimensional thin layer chromatogram of the isolated compounds.

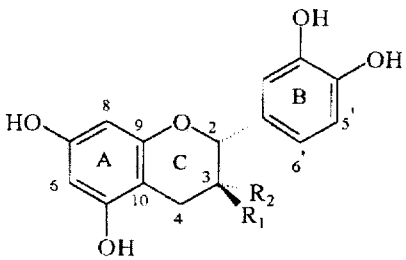
Sephadex LH-20 column using ethanol-hexane mixture(2 : 1 and 4 : 1, v/v) as eluents. (+)-catechin (compound 1) and (-)-epicatechin(compound 2) gave dark red spots on a cellulose plate with the vanillin-HCl spray and their Rf values also were very similar to authentic samples reported by several researchers(Bae *et al.*, 1994; Foo *et al.*, 1983; Foo & Karchesy, 1989; Foo, 1987; Ham & Bae, 1995). ^{1}H and ^{13}C-NMR spectra on these flavans also showed typical resonances corresponding to phloroglucinol A-ring, catechol B-ring and heterocyclic C-ring(Clark-Lewis, 1968; Foo & Karchesy, 1989).

3.1 Kaempferol-3-O-arabinofuranoside

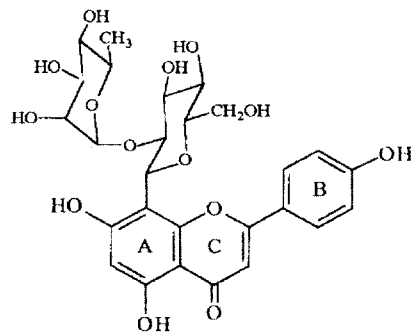
Compound 3 gave a yellow spot on a cellulose sheet when sprayed with the detecting reagent and R_f was 0.73(solvent A) and 0.29(solvent B).

^{1}H-NMR spectrum gave typical phloroglucinolic A- and phenolic B-ring signals at δ6.25 and δ6.49 for H-6 and H-8, respectively and at δ6.98 and δ8.03 for H-3' or H-5' and H-2' or H-6', respectively.

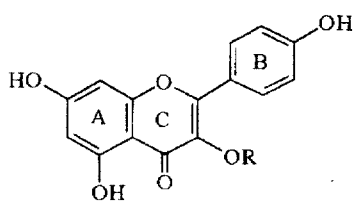
The sugar also showed five proton peaks at δ 3.47, δ3.89, δ3.98, δ4.32 and δ5.47 and these signals were similar to α-L-arabinofuranose (Agrawal, 1992; Harbone & Mabry, 1982; Markham, 1992). However, these proton signals might not

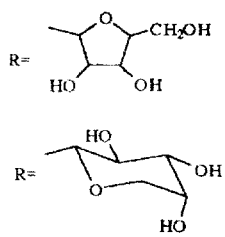


(1) R_1 = OH, R_2 = H, (+)-catechin
(2) R_1 = H, R_2 = OH, (-)-epicatechin



(5) apigenin-8-C-rhamnopyranosyl(1‴→2″)-glucopyranoside(2″-O-rhamnosylvitexin)





(3) R = α-L-arabinofuranosyl, kaempferol-3-o-α-L-arabinofuranoside
(4) R = α-L-arabinopyranosyl, kaempferol-3-o-α-L-arabinopyranoside

be helpful to identify correct the structure of the sugar moiety.

^{13}C-NMR spectrum showed very similar carbon resonances to kaempferol-3-O-arabinofuranoside with the hydroxylation patterns of phloroglucinol A-ring and phenolic B-ring. C-5, C-7 and C-9, three oxygen-containing carbons of A-ring, gave the signals at 162.5, 165.6 and 158.04ppm, respectively and these signals showed typical chemical shifts of kaempferol A-ring. C-6 and C-8 showed the signals at 99.63 and 94.71ppm. The B-ring signals also showed characteristic phenolic B-ring carbon shifts. C-1' appeared at 122.16ppm, the hydroxyl-containing C-4' appeared at 161.26ppm and two pairs of symmetrical carbons gave two strong signals at 131.97ppm for C-2' and C-6' and at 116.43ppm for C-3' and C-5'. In the etherocyclic C-ring, C-2 observed at 158.51ppm and the hydroxyl-containing C-3 and the carbonyl C-4 gave signals at 134.54 and 179.59ppm, respectively. C-ring carbons of the pure aglycon gives three signals at 146.8, 135.6 and 175.9ppm for C-2, C-3 and C-4, respectively (Agrawal, 1989; Harbone & Mabry, 1982; Markham, 1992). However, substitution at C-3 causes a slight upfield shift at C-3 as well as a large downfield shift at C-2 and C-4 due to a result of the increased electronegativity of the substituent(Markham, 1992). In this spectrum, C-3 was shifted upfield by about 1ppm and C-2 and C-4 were shifted downfield by 11.5 and 4ppm, respectively. Therefore, this compound must have a sugar substituent at C-3.

The sugar moiety also corresponded well to the carbon resonances of α-L-arabinofuranose. Five sugar signals observed at 61.98(C-5"), 78.19(C-3"), 82.43(C-2"), 88.42(C-4") and 109.3 (C-1")ppm. α-L-arabinofuranose substituted at C-3 of the aglycon gives signals at 60.7(C-1"), 76.8(C-3"), 81.9(C-2"), 86.2(C-4") and 107.9(C-1")ppm(Agrawal, 1992; Markham, 1992). However, the carbon signals of this compound caused a slight downfield shifts by 0.5~1.2ppm, probably due to the solvent effect. As a result of the above data, this compound was determined as kaempferol-3-O-arabinofuranoside reported by several scientists(Agrawal, 1992; Ham & Bae, 1995; Harbone *et al.*, 1972).

3.2 Kaempferol-3-O-arabinopyranoside

Compound 4 showed a yellow spot on a cellulose plate when sprayed with the detecting reagent. R_f values were 0.57(solvent A) and 0.43(solvent B). ^{1}H and ^{13}C-NMR spectra on the aglycon, kaempferol, showed the same chemical shifts as already mentioned the above.

NMR spectra on the sugar moiety were very similar to the chemical resonances of α-L-arabinopyranose. Although ^{1}H-NMR spectrum gave several signals at δ5.14 for H-1 and at δ 3.3~3.9 for the other four protons, these proton shifts also might not be useful to characterize the right structure of arabinopyranose. However, ^{13}C-NMR spectrum could be useful to identify the sugar. In general, the carbons of arabinopyranose give significant upfield shifts compare to those of arabinofuranose except C-5(Harbone & Mabry, 1982). The sugar showed five signals at 65.96, 67.85, 72.08, 73.20 and 103.59ppm for C-5, C-4, C-3, C-2 and C-1, respectively. These signals were shifted upfield by 6~20ppm compare to the arabinofuranose signals, but C-5 of arabinopyranose was shifted downfield by 4ppm. These carbon resonances corresponded well to a-L-arabinopyranose reported by researchers(Agrawal, 1992; Harbone & Mabry, 1982). Finally, this compound was identified as kaempferol-3-O-arabinopyranoside.

3.3 Apigenin-8-C-rhamnosyl-(1"' →2")-glucoside(2"-O-rhamnosylvitexin)

This compound 5 also showed a yellow spot on a cellulose sheet with the spraying solution and R_f was 0.69(solvent A) and 0.61(solvent B).

NMR spectra gave characteristic flavone signals with the hydroxylation patterns of phloroglucinol A- and phenolic B-ring.

^{1}H-NMR spectrum showed four signals on the aglycon moiety. A signal at δ6.6 was identical to H-6 of A-ring and H-3 of C-ring gave a peak at δ6.27. Two pairs of symmetrical protons in B-ring corresponded to δ7.82 for H-2' or H-6' and δ6.93 for H-3' or H-5'.

^{13}C-NMR data also gave typical flavone signals corresponding to phloroglucinol A- and phenolic B-ring. Three oxygen-bearing carbons of A-ring appeared at 157.9, 162.75 and 164.2 ppm. C-6 and C-8 corresponded to 99.74 and 105.58ppm, respectively and C-8 at 105.58ppm was shifted downfield by 7.5ppm because of a substituent attached to the aglycon by carbon-carbon linkage. In B-ring, C-1' and C-4' showed two signals at 123.47ppm and 162.75ppm, respectively. Two pairs of symmetrical carbons, C-2' or C-6' and C-3' or C-5', gave two strong signals at 116.92 and 130.08ppm. C-ring carbons also showed three sinals at 103.52, 166.71 and 184.18ppm.

As a result of the above data, this aglycon was determined as apigenin and corresponded to an authentic sample reported by researchers (Agrawal, 1989; Harbone & Mabry, 1982; Markham, 1992).

The sugar substituents gave very complex ^{1}H-NMR spectrum at δ3.0~4.0 corresponding to ten sugar protons. The signals at δ0.63 and δ5.0 were identical to H-6 and H-1 of rhamnose and this fact gave some useful information that rhamnose is a terminal unit and glucose did not be linked with the aglycon by an ether linkage.

^{13}C-NMR spectrum also gave several useful signals for structure identification of the sugar moiety. A signal at 17.85ppm was identical to rhamnosyl C-6 and a signal at 102.43ppm corresponded to rhamnosyl C-1. The other rhamnosyl carbons appeared at 69.82~73.34ppm. ^{13}C-NMR spectrum of glucose gave several signals at 62.88~82.74ppm. A signal at 73.34ppm corresponded to glucosyl C-1 and caused a significant upfield shift by 23ppm due to the steric effect from the aglycon and the hydroxyl substituent at glucosyl C-1. As already mentioned the above, glucosyl C-1 has to be linked with the aglycon by a carbon-carbon linkage.

From these data, this compound was determined as apigenin-8-C-rhamnosyl-(1'''→2'')-glucoside(2''-O-rhamnosylvitexin) and corresponded well to an authentic sample reported by a scientists(Agrawal, 1992; Harbone & Mabry, 1982).

4. CONCLUSIONS

Needles of *Larix leptolepis* were extracted with acetone-H_2O(7 : 3, v/v), and the extractives were purified by repeating column chromatography packed with Sephadex LH-20 using methanol, ethanol, aqueous alcohols and ethanol-hexane as eluents. Five compounds were isolated and characterized by two dimensional thin layer chromatography and NMR spectroscopy. The ethylacetate soluble fraction contained a large amount of flavan compounds, especially (+)-catechin and (-)-epicatechin, in addition to small amount of flavonoid glycosides, kaempferol-3-O-arabinofuranoside and kaempferol-3-O-arabinopyranoside.

Apigenin-8-C-rhamnosyl-(1'''→2'')-glucoside(2''-O-rhamnosylvitexin) was isolated from the water soluble fraction. The isolated flavonoid aglycons were composed of phloroglucinolic A-ring and phenolic B-ring and these three flavonoid glycosides have never been reported in *Larix leptolepis* species although they are already well-known compounds in the other tree species(Dictionary of Natural Products, 1994).

These sugar-containing compounds could be effectively isolated by various ethanol-hexane eluents which have not been used to purify natural phenolic compounds.

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