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Flavonoid Glycosides from Needles of *Taxus cuspidata*(Taxaceae)*1

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요 약

주목 잎을 채취하여 건조하고 아세톤-물(7 : 3, v/v)의 혼합액으로 추출한 후 에틸아세테이트 및 수용성 부분으로 분리하고 Sephadex-LH 20으로 충진한 칼럼을 이용하여 2개의 flavan 단량체와 2개의 후라보노이드 배당체를 단리하였다. 에틸아세테이트용성 추출물은 대부분 (+)-catechin 과 (-)-epicatechin으로 구성되어 있었으며 수용성 부분에서는 quercetin-3-0-arabinopyranosyl-(1‴→6″)-β-D-glucoside와 quercetin-3-O-rutinoside인 두 개의 탄수화물로 구성된 배당체를 분리하였으며 주목에서는 이들 화합물은 아직 보고된 바가 없다. 이들의 구조결정을 위하여 박층크로마토그래피를 실시하고 ^{1}H-NMR과 ^{13}C-NMR 스펙트럼을 기존의 스펙트럼과 비교, 분석하여 정확한 구조를 규명하였다.

Keywords : *Taxus cuspidata*, needles, extractives, flavonoid glycosides, column chromatography

1. INTRODUCTION

Taxus cuspidata S. et Z. is one of softwood tree species growing up to 17~20m high and 1m diameter at the high mountain areas in Korea, China and Japan. It has red-brown bark like a band, blade linear type needles of 1.5 to 2.5cm long and 3mm wide, and its seeds are partly enclosed by red aril(Lee, 1996; Lee, 1980). This tree species has been used for a medicinal woody plant to formulate a chinese herbal medicine and for a wood to manufacture high quality wooden furnitures or ornaments(Kim, 1994).

Most of plants, vegetables and tree species contain some amount of various flavonoids and their glycosides which can play an important role to develop medicines, functional supplementary foods and cosmetics using their bioactive function.

Recently, there is a great concern on the utilization of extracitves of woody plants. Especially, the extractives of wood, bark and needles of Taxus cuspidata have been extensively studied on the anticancer activity or bioactivity by many researchers(Hwang *et al.*, 1996).

This study was carried out to search any useful flavonoids from the extractives of Taxus

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cuspidata needles and to obtain basic structural information for manufacturing of a new functional supplementary food using the needle extractives.

2. METERIALS & METHODS

2.1 Equipments

Chromatographic glass columns were packed with Sephadex LH-20 and eluting solvents used methanol, ethanol, aqueous alcohols and ethanol-hexane. Eluents were collected using a Gilson FC 204 fraction collector.

Analytical thin layer chromatography(TLC) was performed on precoated cellulose plates(Merk DC-plastikfolien cellulose F) and developed with *t*-butylalcohol-acetic acid-water(3 : 1 : 1, v/v/v, solvent A) and 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.

^{1}H and ^{13}C-NMR spectra were obtained from a Varian Gemini 200 and a Bruker 400 NMR spectrometers with samples dissolved in methanol-d_4 and acetone-d_6 and chemical shifts are given in values.

2.2 Extraction and purification

Fresh needles of ***Taxus cuspidata*** collected at the Balwang mountain in Pyungchangkun, Kangwondo on February 1996 were dried in laboratory for three weeks and were prepared to fine particles by a grinding mill. The ground needles(953g) were extracted with acetone-water(7 : 3, v/v) three times at room temperature for three days in a 20 *l* glass jar.

The crude extractives were combined together and then concentrated on a rotary evaporator under reduced pressure. The mixture was first extracted with chloroform to remove chlorophyll and the related materials, and then hexane was used to remove some resins and waxes.

The residue was fractionated two portions, ethylacetate soluble and water soluble which were concentrated and freeze dried to give 27.6g and 171.6g of brown powder, respectively.

A portion of ethylacetate soluble powder(17g) was mounted on a Sephadex LH-20 column(3× 100cm) using methanol-water as an eluent. Three main fractions were collected and named T1, T2 and T3.

A large amount of crystals like needles were isolated by recrystallization after concentration of fraction T1 and T2. These fractions were then rechromatographed on a column using ethanol-hexane(4 : 1, v/v) for further purification and separated into six fractions: TE1 (1.6g), TE2(4.2g), TE3(2.3g), TE4(0.2g), TE5 (0.5g) and TE6(1.8g).

Water soluble fraction(67g) was also applied to a Sephadex LH-20 column using methanol-water(1 : 1, v/v) as an eluent to give five fractions and labeled TW1(52.5g), TW2(8.9g), TW3(0.9g), TW4(0.2g) and TW5(1.3g). These fractions were retreated on a column for further purification.

2.2.1 (+)-catechin

Fraction T1 and T2 were retreated with 100% water to get a pure crystal compound, (+)-catechin(3.8g), through recrystallization. These crystals gave a strong red spot on a cellulose sheet with the spray reagent. R_f values were 0.52(solvent A) and 0.44(solvent B).

^{1}H-NMR(200MHz, δ, MeOH-d_4) : 2.50(1H, dd, J=8.2, 16.1Hz, H-4_{ax}), 2.85(1H, dd, J=5.4, 16.2Hz, H-4_{eq}), 3.95(1H, m, H-3), 4.55(H-1, d, J=7.6Hz, H-2), 5.82(1H, d, J=2.3Hz, H-6), 5.89(1H, d, J=2.3Hz, H-8), 6.8(3H, m, H-2', 5', 6').

^{13}C-NMR(200MHz, ppm, Acetone-d_6) : 82.3(C-2), 68(C-3), 28.5(C-4), 157.1(C-5), 96.1(C-6), 156.6(C-7), 95.2(C-8), 157.5(C-9), 100.5(C-10), 131.7(C-1'), 115.4(C-2'), 145.6(C-3'), 145.7(C-4'), 115.9(C-5'), 119.9(C-6').

2.2.2 (-)-epicatechin

This compound was obtained by repeated col-

umn chromatography using ethanol-hexane(4:1 and 2:1, v/v) from fraction TE2. R_f was 0.39 (solvent A) and 0.31(solvent B).

^{1}H-NMR(200MHz, δ, MeOH-d_4): 2.67~2.94 (2H, m, H-4_{eq}, H-4_{ax}), 4.20(1H, br s, H-3), 4.82 (H, br s, H-2)), 5.95(2H, m, H-6,8), 6.76~7.0 (3H, m, H-2',5',6').

^{13}C-NMR(200MHz, ppm, MeOH-d_4): 79.74(C-2), 67.33(C-3), 29.08(C-4), 157.97(C-5), 96.31(C-6), 157.62(C-7), 95.81(C-8), 157.35(C-9), 99.98 (C-10), 132.24(C-1'), 115.25(C-2'), 145.73(C-3'), 145.89(C-4'), 115.85(C-5'), 119.36(C-6').

2.2.3 Quercetin-3-O-α-L-arabinopyranosyl-(1'''→6'')-β-D-glucopyranoside

Fraction TW2 was retreated on a column chromatography using ethanol-hexane(4 : 1 and 2 : 1, v/v) and recrystallized to isolate pale yellow crystals, quercetin-3-O-α-L-arabinopyrasyl -(1'''→6'')-β-D-glucopyranoside(250mg). R_f values were 0.50(solvent A) and 0.26(solvent B).

^{1}H-NMR(400MHz, δ, MeOH-d_4): 3.3~4.1(11H, br m, sugar protons), 5.17(1H, d, J=7.24Hz, H-1''(glc)), 6.20(1H, d, J=2.07Hz, H-6), 6.41(1H, d, J=1.93Hz, H-8), 6.89(1H, d, J=8.27Hz, H-5'), 7.68(2H, m, H-2', 6').

^{13}C-NMR(400MHz, ppm, MeOH-d_4): 158.74(C-2), 135.4(C-3), 179.25(C-4), 162.41(C-5), 100.39(C-6), 166.74(C-7), 95.37(C-8), 158.41(C-9), 105.37(C-10), 122.97(C-1'), 116.35(C-2'), 145.76(C-3'), 149.27(C-4'), 117.43(C-5'), 123.65(C-6'), 103.58(C-1''), 75.36 (C-2''), 77.94(C-3''), 71.05(C-4''), 77.52(C-5''), 69.40 (C-6''), 104.68(C-1'''), 73.75(C-2'''), 72.11(C-3'''), 68.97(C-4'''), 66.74(C-5''').

2.2.4 Quercetin-3-O-α-L-rhamnopyranosyl-(1'''→6'')-β-D-glucopyranoside(rutin)

Pure yellow crystals, quercetin-3-O-α-L-rhamnopyranosyl-(1'''→6'')-β-D-glucopyranoside(30mg) was also obtained from fraction TW2 with ethanol-hexane(4:1 and 2:1, v/v) followed by recrystallization. R_f values were 0.53(solvent A) and 0.28(solvent B).

^{1}H-NMR(400MHz, δ, MeOH-d_4): 1.12(3H, d, J=5.96, H-6''' (rha), 3.26~3.81(10H, br m, sugar protons), 4.52(1H, br s, H-1'' (rha)), 5.11(1H, d, J=7.42Hz, H-1''(glc)), 6.19(1H, br s, H-6), 6.38 (1H, br s, H-8), 6.87(1H, d, J=8.36Hz, H-5'), 7.66(2H, br m, H-2', 6').

^{13}C-NMR(400MHz, ppm, MeOH-d_4): 159.33(C-2), 135.66(C-3), 179.39(C-4), 162.97(C-5), 99.96 (C-6), 166.05(C-7), 94.89(C-8), 158.49(C-9), 105.61(C-10), 123.10(C-1'), 116.06(C-2'), 145.84(C-3'), 149.83 (C-4'), 117.71(C-5'), 123.59(C-6'), 102.44(C-1''), 75.74(C-2''), 78.17(C-3''), 71.38(C-4''), 77.19(C-5''), 68.56(C-6''), 104.77(C-1'''), 72.23(C-2'''), 71.38(C-3'''), 73.94(C-4'''), 69.73(C-5'''), 17.92(C-6''').

3. RESULTS & DISCUSSION

Two flavonoid glycosides, quercetin-3-O-α-L-arabinopyranosyl-(1'''→6'')-β-D-glucopyranoside and quercetin-3-O-α-L-rhamnopyranosyl-(1'''→6'')-β-D-glucopyranoside(rutin) including two flavans, (+)-catechin and (-)-epicatechin, were isolated by repeated column chromatography packed with Sephadex LH-20 from the extractives of *Taxus cuspidata* needles in this study. Eluents were methanol, ethanol, queous alcohols and ethanol-hexane.

Ethylacetate soluble fraction contained a large amount of flavan-3-ols in addition to a trace amount of the other phenolic materials. Flavonoid glycosides from water soluble portion could be easily crystallized using 100% water after preliminary isolation of the mixture with organic solvents. Preliminary structure elucidation of the isolated compounds was done by one and two dimensional thin layer chromatography using vanillin-hydrochloric acid-ethanol solution as a spraying reagent.

NMR spectroscopy was also a very useful tool to determine the structures of the isolated compounds.

(+)-catechin(1) and (-)-epicatechin(2) gave strong red spots on a cellulose plate when visualized with the spraying reagent and their Rf values were very similar to authentic samples reported by other researchers(Agrawal, 1989; Bae *et al.*, 1994; Foo *et al.*, 1983; Foo & Karchesy

1989: Ham & Bae, 1995).

^{1}H and ^{13}C-NMR spectra also showed typical resonances corresponded well to phloroglucinol A-ring, catechol B-ring and heterocyclic C-ring of flavan-3-ols.

3.1 Quercetin-3-O-α-L-arabinopyranosyl-(1‴ → 6″)-β-D-glucopyranoside

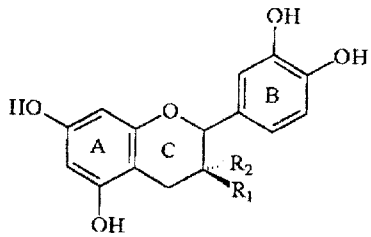
Compound 3 gave a pale yellow spot on a cellulose sheet when visualized with the detecting reagent and R_f values were 0.50(solvent A) and 0.26(solvent B).

^{1}H-NMR spectrum of the aglycon gave typical meta-coupled phloroglucinolic A-ring signals at δ6.20(J = 1.93) and δ6.41(J = 2.07Hz) for H-6 and H-8, respectively. Catechol B-ring also showed a ortho-coupled signal at δ6.89(J = 8.27Hz) for H-5′ and one signal appeared at δ 7.68 for H-2′ and H-6′.

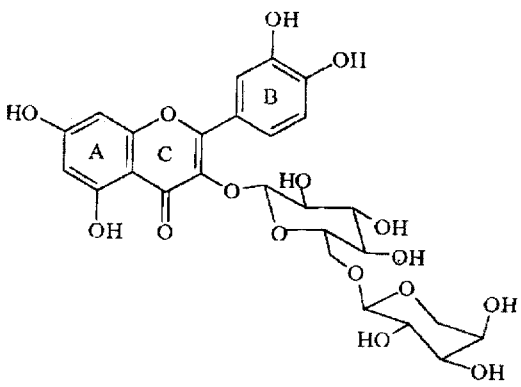
Most of sugar protons gave complex signals at δ3.3~4.1. Glucose showed a signal at δ5.17(J= 7.24Hz) for H-1″ as a doublet and H-1‴ of arabinose was eclipsed at about δ 4.8~5.0 with the hydroxyl group of NMR solvent.

However, this ^{1}H-NMR spectrum might not be useful to identify the correct structure of the sugar moiety.

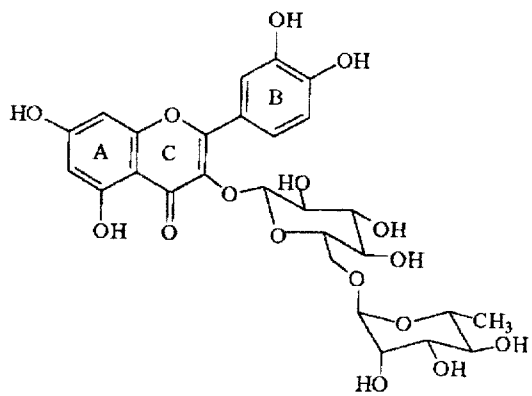
^{13}C-NMR spectra also showed characteristic aglycon and sugar carbon signals of quercetin-3-O-α-L-arabinopyranosyl-(1‴ →6″)-β-D-glucopyranoside. In A-ring of the aglycon, three oxygen-bearing carbons gave signals at 162.41, 166.74



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



(3) Quercetin-3-O-α-L-arabinopyranosyl (1‴ →6″)-β-D-glucopyranoside



(4) Quercetin-3-O-α-L-rhamnopyranosyl-(1‴ →6″)-β-D-glucopyranoside(rutin)

and 158.41ppm for C-5, C-7 and C-9, respectively. The chemical shifts of C-6 and C-8 absorbed at 100.39 and 95.37ppm.

B-ring also showed typical catechol resonances of flavonol. Four peaks appeared at 122.97, 116.35, 117.43 and 123.65ppm for C-1', C-2', C-5' and C-6', respectively. The hydroxyl-containing C-3' and C-4' gave two signals at 145.76 and 149.29ppm.

In etherocyclic C-ring, C-2 and C-3 observed at 158.51ppm and 135.4ppm, respectively and carbonyl C-4 gave a signal at 179.25ppm. Generally, C-ring carbons of quercetin give three signals at 146.9, 135.6 and 175.7ppm for C-2, C-3 and C-4, respectively, in DMSO-d_6(Agrawal, 1989; Harbone & Mabry, 1982). However, substitution at C-3 causes a slight upfield shift by 0.5ppm at C-3 as well as a large downfield shift at C-2 and C-4 due to the result of the electronegativity from a substituent(Agrawal, 1989; Harbone & Mabry, 1982; Sen *et al.*, 1992). In this spectrum, C-3 was shifted upfield by about 0.2ppm, and C-2 and C-4 were shifted downfield by 11.61 and 3.55ppm, respectively. Therefore, this compound should have a substituent at C-3.

The sugar moieties also corresponded well to the carbon resonances of α-L-arabinopyranosyl-(1‴→6″)-β-D-glucopyranoside. ^{13}C-NMR spectrum of β-D-glucopyranosyl gave six signals at 69.4~103.58ppm. C-3″ and C-5″ were very similarly corresponded to β-D type glucosyl signals(Harbone & Mabry, 1982; Markham *et al.*, 1992). The signal for C-1″ shifted more downfield ca. 6ppm than that of a free glucose C-1 resonance(96.8ppm) due to the electronegativity by the ether linkage with the aglycon(Nilsson *et al*, 1973; Sen, 1992). Glucosyl C-6″ signal also shifted downfield ca. 7ppm due to the linkage with arabinopyranose compared to a free glucose(61.8ppm). In this glucosyl ^{13}C-NMR spectrum, these two signals, C-1″ and C-6″, were very important keys to determin the structure of the sugar moities.

Arabinosyl four signals, 73.75(C-2‴), 72.11(C-3‴), 68.97(C-4‴) and 66.74ppm(C-5‴), were very similar to α-L-arabinopyranose that was shifted more downfield by 3-4ppm for C-2‴, C-3‴ and C-5‴ than those of arabinofuranose. C-1‴ gave a peak at 104.68ppm shifted downfield by about 7ppm as a result of the ether linkage with glucosyl C-6″.

Therefore, the sugar moieties should have the structure of α-L-arabinopyranosyl-(1‴→6″)-β-D-glucopyranoside. Finally, this compound was identified as quercetin-3-O-α-L-arabinopyranosyl-(1‴→6″)-β-D-glucopyranoside and identical to an authentic sample studied by other scientists(Agrawal, 1989; Harbone & Mabry, 1982).

3.2. Quercetin-3-O-α-L-rhamnopyranosyl-(1‴→6″)-β-D-glucopyranoside(rutin)

Compound 4 also showed a yellow spot on a cellulose sheet with the spraying solution and R_f was 0.53(solvent A) and 0.28(solvent B).

^{1}H and ^{13}C-NMR spectra on the aglycon, quercetin, gave the same chemical resonances corresponding to phloroglucinolic A-ring and catechol B-ring as already mentioned the above.

In ^{1}H-NMR spectrum of the substituent, most of sugar protons exhibited very complex signals at δ 3.26~3.81. But glucosyl H-1″ gave a doublet(J = 7.42Hz) at δ5.11 and rhamnosyl H-1‴ and H-6‴ showed several signals at δ4.52 and δ1.12(Markham, 1992; Nilsson *et al.*, 1973).

However, these proton signals might not be useful to identify the correct structure of the sugar moiety.

^{13}C-NMR spectrum of the substituent showed typical α-L-rhamnopyranosyl-(1‴→6″)-β-D-glucopyranoside carbon resonances.

Glucosyl portion gave very similar carbon signals to β-D type. Six glucosyl signals appeared at 68.56~102.44ppm. A signal at 102.44ppm corresponded to C-1″ attached to oxygen of the aglycon C-3 and C-6″ at 68.56ppm shifted downfield by 7.5ppm compared to a free glucose due

to the electronegativity by the ether linkage with rhamnopyranose(Agrawal, 1992; Harbone *et al.*, 1975; Markham, 1992; Nilsson *et al.*, 1973).

Rhamnosyl carbons gave six signals at 17.92 ~104.77ppm. A signal at 17.92ppm was a characteristic methyl group of rhamnosyl C-6‴ and C-1‴ linked to glucosyl C-6″ showed a signal at 104.77ppm shifted downfield by 10ppm compared to a free rhanmose(94ppm)(Agrawal, 1992; Markham *et al.*, 1992).

From these NMR data, the sugar moiety was characterized as α-L-rhamnosyl-(1‴ →6″)-β-D-glucopyranoside(rutin) substituted at C-3 of quercetin by ether linkage and the chemical shifts of this compound were also identical to an authentic sample reported by other people (Agrawal, 1989; Harbone & Mabry, 1982).

4. CONCLUSIONS

Needles of *Taxus cuspidata* were extracted with acetone-water(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. Four compounds were isolated and characterized by two dimensional thin layer chromatography and NMR spectroscopy. The ethylacetate soluble fraction contained a lot amount of flavan compounds, (+)-catechin and (-)-epicatechin, in addition to a trace amount of procyanidins and these flavans could be easily and successfully isolated using ethanol-hexane solvents as eluents.

Quercetin-3-O-α-L-arabinopyranosyl-(1‴→6″)-β-D-glucopyranoside and quercetin-3-O-α-L-rhamnopyranosyl-(1‴→6″)-β-D-glucopyranoside (rutin) were also isolated on repeated column chromatography followed by recrystallization from the water soluble portion. The aglycon of the isolated glycosides was quercetin with phloroglucinolic A-ring and phenolic B-ring and these flavonoid glycosides have never been reported in Taxus cuspidata species although they are already well-known compounds in the other tree species(Dictionary of natural products, 1994).

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