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Procyanidins from *Lindera obtusiloba* Bark[*1]

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생강나무 수피의 Procyanidins[*1]

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요 약

생강나무의 수피를 아세톤-물(7:3)의 혼합용액으로 추출하여 에틸아세테이트용성과 수용성 화합물로 분리하였고 이 중에서 에틸아세테이트용성 부분을 Sephadex LH-20과 TSK 40F로 충진한 칼럼크로마토그래피를 사용하여 5개의 화합물, 즉 (+)-catechin, (-)-epicatechin, quercetin 그리고 epicatechin-(4β → 6)-catechin 이량체와 epicatechin-(2β → 7, 4β → 8)-epicatechin 이량체를 단리하였다. 단일물의 확인을 위하여 박층크로마토그래피를 실시하였으며, 화합물의 정확한 구조는 ^{1}H-NMR과 ^{13}C-NMR 스펙트럼을 이용하여 규명하였다.

Keywords : *Lindera obtusiloba*, bark, procyanidins, ethylacetate soluble fraction, column chromatography

1. INTRODUCTION

Lindera obtusiloba, which is called a camellia plant and is one of hardwood tree species, widely distributes in most area of Korean peninsula, China and Japan. The tree is a small arbor or shrub growing up to 7 meter high. It has black ash bark, yellow green little branches, eggshape leaves and small globular fruits(Kim, 1994; Krussmann, 1977; Kubitzki *et al.*, 1993). It's trunks, branches or leaves emit very similar odor to ginger fragrance when is cut. This tree has been used for a medicinal woody plant to formulate a chinese herbal medicine(Kim, 1994). Recently, there is a great concern on the search of new functional materials with any bioactive effect from the extractives of medicinal plants or woody plants. Generally, hardwood tree species are known to contain many useful medicinal materials compared to softwoods. The purpose of this research is to isolate any functional materials from the bark extractives of *Lindera obtusiloba* which is one of useful medicinal woody plants and to get basic chemical information by structure determination on the isolated compounds for further utilization in the future.

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2. METERIALS & METHODS

2.1 Equipments

Chromatographic glass columns were packed with Sephadex LH-20 or TSK 40F and eluents used methanol, ethanol, aqueous alcohols and ethanol-hexane mixture. Eluting solvents were collected using a Gilson FC 204 fraction collector. Analytical one and two dimensional thin layer chromatography(TLC) were performed on precoated cellulose plates(Merk DC-plastikfolien cellulose F) and developed with *t*-butanol-acetic acid-water(TBA, 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) solution followed by heating with hot air. ^{13}C-NMR and ^{1}H-NMR spectra were obtained from a Varian Gemini 200 spectrometer with samples dissolved in methanol-d_4 and acetone-d_6 and chemical shifts are given in δ values.

2.2 Extraction and purification

Lindera obtusiloba collected at Experimental Forests of Kangwon National University in October 1996 was debarked at once and then its wood and bark were air-dried in laboratory for about three weeks. After grinding, 1.3kg of the bark was extracted with acetone-water(7:3, v/v) for three days at room temperature, then the extract was concentrated under vaccum evaporation and this process was repeated three times. The crude samples were extracted with chloroform to remove high polar materials and then hexane was used to eliminate waxy materials and fatty acid. The residue was separated into ethylacetate and water soluble fraction and each fraction was freeze dried after vaccum concentration to give 41.4g of ethylacetate soluble powder and 103.8g of water soluble materials.

A protion of the freeze dried ethylacetate soluble powder(30g) was applied to a Sephadex LH-20 or a TSK 40F columns(3×40cm). The columns were washed with methanol, ethanol, aqueous alcohols and ethanol-hexane solutions until the eluent was almost colorless. Four major fractions were collected and labeled E1, E2, E3 and E4. Fraction E1 first eluted and E4 was the last. Then each fraction was rechromatographed for further purification.

2.2.1 (+)-catechin

Fraction E2 was retreated on a Sephadex LH-20 column using ethanol-hexane(4 : 1 and 2 : 1, v/v) and ethanol-water(1 : 2, v/v) to give 620mg of a brown freeze dried powder. R_f 0.47(solvent A) and 0.35(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.4~2.6(1H, dd, J = 8.2Hz, J = 16.1Hz, H-4(axial)), 2.77~2.92 (1H, dd, J = 5.4Hz, J = 16.2Hz, H-4(equatorial)), 3.95-4.0(1H, m, H-3), 4.55~4.6(1H, d, J = 7.42Hz, H-2), 5.85~5.86(1H, d, J = 2.3Hz, H-6), 5.93~5.94(1H, d, J = 1.3Hz, H-8), 6.69~6.88(3H, m, H-2', 5', 6').

^{13}C-NMR(ppm, MeOH-d_4) : 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'), 146.4(C-4'), 116.2(C-5'), 120.2(C-6').

2.2.2 (-)-epicatechin

This compound was obtained from fraction E2 using ethanol-hexane(4 : 1 and 2 : 1, v/v). R_f 0.43(solvent A) and 0.30(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.65~2.98(2H, m, H-4), 4.2(1H, s, H-3), 4.8(H, s, H-2), 5.92~5.96(2H, dd, H-6, 8), 6.74~6.99(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 Quercetin

40mg of a yellow freeze dried powder was obtained from fraction E3 using ethanol-hexane(4 : 1 and 2 : 1, v/v). R_f 0.41(solvent A) and

0.02(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 6.17(1H, d, J=2.0Hz, H-6), 6.38(1H, d, J=2.0Hz, H-8), 6.88(1H, d, J=8.48Hz, H-5'), 7.64(1H, dd, J=2.0Hz, and 8.49Hz: H-6'), 7.73(1H, d, J=2.0Hz, H-2').

^{13}C-NMR(ppm, MeOH-d_4) : 148.1(C-2), 137.4 (C-3), 177.6(C-4), 162.7(C-5), 99.3(C-6), 165.8 (C-7), 94.5(C-8), 158.4(C-9), 104.6(C-10), 123.5 (C-1'), 116.3(C-2'), 146.4(C-3'), 149.0(C-4'), 116.1(C-5'), 121.8(C-6').

2.2.4 Epicatechin-(4β→6)-catechin

Fraction E3 gave 20mg of a brown freeze dried compound by column chromatography using ethanol-hexane(4 : 1 and 3 : 2, v/v) and ethanol-water(1 : 2, v/v) eluents. R_f 0.47(solvent A) and 0.20(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.46(1H, dd, J=8.8, 15.3Hz, H-4ax), 2.86(1H, dd, J = 5.5, 16.6Hz, H-4eq), 4.0~4.2(2H, m, H-3(B), H-4(T)), 4.52(1H, d, J = 8.06Hz, H-2(B)), 4.62(1H, br, s, H-3(T)), 4.96(1H, br, s, H-2(T)), 6.0~6.12(3H, m, H-6, 8(T, B)), 6.7 ~7.1(6H, m, H-2', 5', 6'(T, B)).

^{13}C-NMR(ppm, Acetone-d_6)

Top unit : 77.0(C-2), 72.0(C-3), 37.2(C-4), 155.3(C-5), 96.6(C-6), 157.8(C-7), 95.8 (C-8), 159.4(C-9), 101.3(C-10), 131.8(C-1'), 115.3(C-2'), 145.9(C-3'), 145.5(C-4'), 115.6(C-5'), 119.2(C-6').

Bottom unit : 82.5(C-2), 68.2(C-3), 29.5(C-4), 159.4(C-5), 107.7(C-6), 157.8(C-7), 96.0 (C-8), 154.8(C-9), 100.4(C-10), 132.0(C-1'), 115.5(C-2'), 145.7(C-3'), 145.4(C-4'), 115.8 (C-5'), 120.2(C-6').

2.2.5 Epicatechin-(2β→7, 4β→8)-epicatechin

Fraction E3 was rechromatographed on a column using ethanol-hexane(4 : 1 and 3 : 2, v/v) and ethanol-water(1 : 2, v/v) eluents to give 24mg of a brown freeze dried powder. R_f 0.43 (solvent A) and 0.26(solvent B).

^{1}H-NMR(δ, MeOH-d_4) : 2.7-3.1(2H, m, H-4(B)), 4.15(1H, d, J=3.24, H-4(T)), 4.21(1H, m, H-3(B)), 4.35(1H, d, J = 3.11, H-3(T)), 4.97(1H, s, H-2(B)), 6.0(1H, d, J=2.34, H-6(T)), 6.06(1H, s, H-8(T)), 6.15(1H, s, H-6(B)), 6.8-7.4(6H, m, H-2', 5', 6'(T, B)).

^{13}C-NMR(ppm, Acetone-d_6)

Top unit : 100.0(C-2), 66.0(C-3), 29.3(C-4), 152(C-5), 98.1(C-6), 155.8(C-7), 96.2 (C-8), 157.1(C-9), 104.1(C-10), 132.5(C-1'), 116.4(C-2'), 145.2(C-3'), 146.2(C-4'), 115.7(C-5'), 119.8(C-6').

Bottom unit : 81.7(C-2), 67.6(C-3), 28.9(C-4), 154.1(C-5), 96.3(C-6), 156.3(C-7), 107.0 (C-8), 158.1(C-9), 102.4(C-10), 131.0(C-1'), 115.4(C-2'), 145.8(C-3'), 146.4(C-4'), 115.8(C-5'), 120.7(C-6').

3. RESULTS & DISCUSSION

Five compounds, (+)-catechin, (-)-epicatechin, quercetin, epicatechin-(4β→6)-catechin and epicatechin-(2β→7, 4β→8)-epicatechin, were obtained by repeated column chromatography packed with Sephadex LH-20 and TSK 40F from the ethylacetate soluble fraction of Lindera obtusiloba bark. Eluents were methanol, ethanol, aqueous methanol and ethanol-hexane mixture. The mixture of flavan compounds such as (+)-catechin and (-)-epicatechin could be easily isolated on a TSK 40F column using ethanol-hexane(4 : 1 and 2 : 1, v/v) as eluents and dimeric procyanidins were purified on a

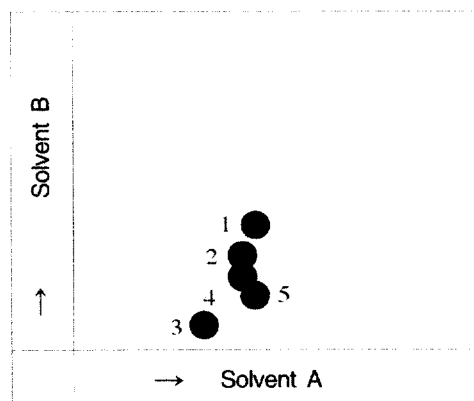


Fig. 1. Two dimensional thin layer chromatogram of the isolated compounds.

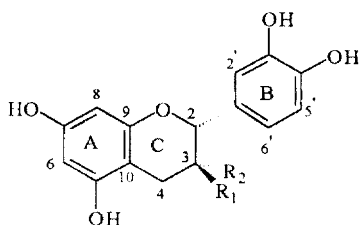
Sephadex column. Identification of the isolated compounds was carried out by two dimensional thin layer chromatography(Fig. 1) and NMR spectroscopy.

3.1 (+)-catechin

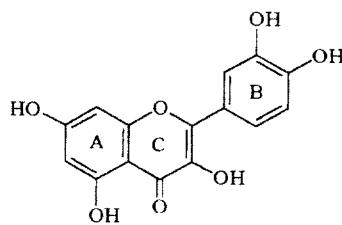
Compound 1 gave a dark red spot on a cellulose plate when visualized with the spray reasent. Its R_f value, 0.47(solvent A) and 0.35 (solvent B), were very similar to an authentic sample charicterized by several researchers (Agrawal, 1989; Ham & Bae, 1995; Harbone & Mabry, 1982; Markham, 1982; Newman *et al.*, 1987). Its ^{1}H-NMR and ^{13}C-NMR spectra also gave typical signals corresponded to phloroglucinol A-ring, catechol B-ring and heterocyclic C-ring of (+)-catechin.

3.2 (-)-epicatechin

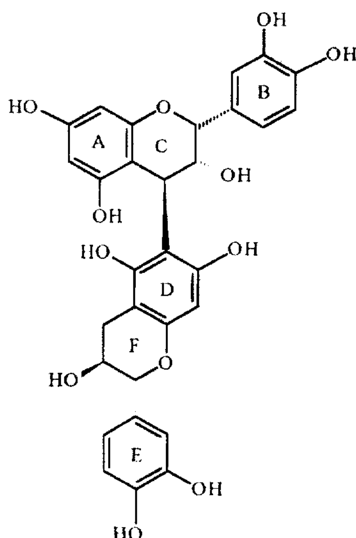
Compound 2 also showed a dark red spot on a cellulose sheet with vanillin solution and R_f was the same as an authentic compound isolated by several scientists(Agrawal, 1989; Harbone & Mabry, 1982; Markham, 1982; Newman *et al.*,



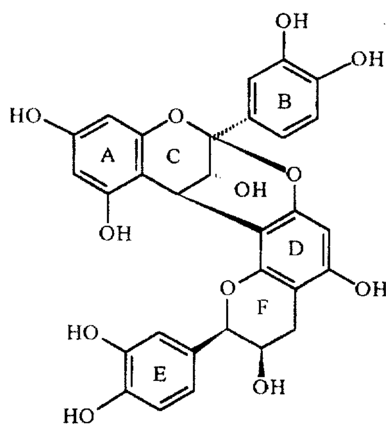
(1) R_1=OH, R_2=H, (+)-catechin
(2) R_1=H, R_2=OH, (-)-epicatechin



(3) quercetin



(4) Epicatechin-(4β→6)-catechin



(5) Epicatechin-(2β→7, 4β→8)-epicatechin

1987). Its NMR pectra also were very much similar to the standard signals of (-)-epicatechin.

3.3 Quercetin

A yellow freeze dried powder, compound 3, was isolated from fraction E3 and its R_f values were 0.41(solvent A) and 0.30(solvent B). ^{1}H-NMR and ^{13}C-NMR spectra were also identical to the chemical shifts of an authentic quercetin reported by several researchers(Agrawal, 1989; Harbone & Mabry, 1982; Markham, 1982).

3.4 Epicatechin-(4$\beta\rightarrow$6)-catechin

Brown compound 4 gave a light pink color on a cellulose sheet with the detecting solution and R_f values were 0.47(solvent A) and 0.20 (solvent B).

In the ^{1}H-NMR spectrum, H-6 and H-8 of A-ring and H-8 of D-ring appeared at δ6.0~6.12. However, H-6 of D-ring did not give a signal because C-6 of D-ring was linked with C-4 of C-ring. H-4 of C-ring was shifted downfield to δ4.0~4.2 due to the electronegativity from the bottom unit. The multiplet at δ2.4~2.9 were similar to two H-4 protons of (+)-catechin.

^{13}C-NMR spectrum also gave characteristic epicatechin-(4$\beta\rightarrow$6)-catechin carbon signals with typical hydroxylation patterns of phloroglucinol A-ring and catechol B-ring. C-5, C-7 and C-9 of A- and D-ring, six oxygen-containing carbons, appeared at 154.8~159.4ppm. C-6 and C-8 of the same rings gave four signals at 95.8, 96.0, 96.6 and 107.7ppm. The chemical shifts of C-6 or C-8 of the bottom unit was shifted downfield by about 11ppm due to the electronegativity of the top unit(Agrawal, 1989; Bergmann *et al.,* 1987; Hemingway *et al.*, 1982; Newman *et al.*, 1987; Song & Oh, 1996).

There is a characteristic difference between 4$\beta\rightarrow$6 and 4$\beta\rightarrow$8 linkages at C-10 of the top unit of dimeric procyanidins. 4$\beta\rightarrow$8 linkage gives a signal at about 102ppm and 4$\beta\rightarrow$6 linkage at about 101ppm(Agrawal, 1989; Bergmann *et al.*, 1987; Song & Oh, 1996). This compound corresponded to 4$\beta\rightarrow$6 linkage because C-10 gave a signal at 101.3ppm.

The signals of catechol B- and E-ring gave typical carbon shifts. Two C-1' signals observed at 131.8 and 132ppm, four C-2' and C-5' resonances appeared at 115.3~115.8ppm and four hydroxyl-containing carbons at C-3' and C-4' resonated at 145.4~145.9ppm.

Three resonances of heterocyclic C-ring observed at 77, 72 and 37.2ppm corresponding to C-2, C-3 and C-4 of (-)-epicatechin, respectively. The signal at 37.2ppm was shifted downfield by about 8ppm due to the electron donating effect of the bottom unit. F-ring also gave three signals at 82.5, 68.2 and 29.5ppm corresponding to (+)-catechin, the bottom unit.

As a result of the above data, this compound was characterized to epicatechin-(4$\beta\rightarrow$6)-catechin.

3.5 Epicatechin-(2$\beta\rightarrow$7, 4$\beta\rightarrow$8)-epicatechin

A brown freeze dried compound 5 was isolated on a Sephadex LH-20 column and gave a red spot with the vanillin solution. R_f values were 0.43(solvent A) and 0.26(solvent B).

NMR spectra on this compound were very similar to an authentic sample reported by several scientists(Agrawal, 1989; Balde *et al.*, 1991; Botha *et al.*, 1981; Barrett *et al.*, 1979; Karchesy & Hemingway, 1986; Nonaka *et al.*, 1983).

In the ^{1}H-NMR spectrum, H-6, H-8 of A-ring and H-6 of D-ring appeared at δ6.0, δ6.06 and δ6.15, respectively. However, H-8 of D-ring did not give a peak because C-4 of C-ring is linked with C-8 of D-ring. H-4 of C-ring was shifted downfield to δ4.15 due to the electronegativity from the bottom unit. H-2 of C-ring and H-7 of D-ring also did not show any peak because C-2 of C-ring was linked with C-7 of D-ring. Four signals at δ2.7~3.1 corresponded to (-)-epicatechin, the bottom unit.

^{13}C-NMR spectrum showed typical signals at 152~158.1ppm corresponding to C-5, C-7 and C-9 of phloroglucinolic A- and D-ring, respective-

ly. C-6 and C-8 of the same rings also gave characteristic four signals at 96.2, 96.3, 98.2 and 107ppm and C-6 or C-8 of the bottom unit was shifted downfield by about 11ppm due to the electronegativity of the upper unit. C-10 also showed two signals at 104.1 and 102.4ppm. There is a typical difference between 4β→6 and 4β→8 linkages at C-6 and C-8 of the lower unit. C-6 of the 4β→6 linkage gives a signal at 108.8ppm and C-8 of the 4β→8 linkage at 106.8ppm(Agrawal, 1989; Balde *et al.*, 1991; Karchesy & Hemingway, 1986). Consequently, this dimeric compound was similar to the 4β→8 linkage.

B- and E-ring also showed typical catechol resonances corresponding to two C-1' at 131 and 132.5ppm and to six C-2', C-5' and C-6' at 115.4~120.7ppm. The hydroxyl-containing carbons at C-3' and C-4' also gave four signals at 145.2~146.4ppm. C-2, C-3 and C-4 of the heterocyclic C-ring showed three signals at 100, 66 and 29.3ppm, respectively and the C-2 signal was shifted downfield by 18ppm due to the linkage with C-7 of the bottom epicatechin unit. C-2, C-3 and C-4 of the heterocyclic F-ring also gave three signals at 81.7, 67.6 and 28.9ppm, respectively.

All of these carbon signals were very similar to an authentic epicatechin-(2β→7, 4β→8)-epicatechin sample(Agrawal, 1989; Balde *et al.*, 1991; Botha *et al.*, 1981; Barrett *et al.*, 1979; Karchesy & Hemingway, 1986; Nonaka *et al.*, 1983).

4. CONCLUSIONS

Five compounds, (+)-catechin, (-)-epicatechin, quercetin, epicatechin-(4β→6)-catechin and epicatechin-(2β→7, 4β→8)-epicatechin, were isolated by repeated column chromatography using Sephdex LH-20 and TSK 40F from the ethylacetate soluble extractives of *Lindera obtusiloba* bark and characterized by NMR spectroscopy.

The ethylacetate soluble fraction of the bark contained a large amount of (+)-catechin and (-)-epicatechin and these flavan compounds were easily isolated by column chromatography packed with TSK 40F using ethanol-hexane which has not been tried to separate these procyanidin compounds yet.

Quercetin, a flavonol, was also purified in this work although it was a trace amount.

One branched dimeric procyanidin, epicatechin-(4β→6)-catechin and one A-type dimeric procyanidin, epicatechin-(2β→7, 4β→8)-epicatechin, were isolated. All of the isolated compounds were composed of the hydroxylation patterns of phloroglucinol A-ring and catechol B-ring.

It was an interesting finding that the bark of *Lindera obtusiloba* contained a large amount of flavans and procyanidins because most of hardwood tree species does not contain significant amount of procyanidins unlike softwoods.

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