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Studies on Biological Activity of Wood Extractives(XIV)*1
- Antifungal activity of isoflavonoids -

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ABSTRACT

Five isoflavonoids, biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-gluco- pyranoside (1), (-)-maackiain (2), calycosin (3), trifolirhizin (4) and genistein (5), were tested for antifungal activity against nine fungi. These compounds were isolated from the wood (compound 1 and 2) and from the bark (compound 3, 4 and 5) of *S. japonica*. According to the results of antifungal activity test, (-)-maackiain was evaluated as the best antifungal compound among the isolated compounds. In this regard, it could be mentioned that high antifungal activity of *S. japonica* wood extracts was originated from (-)-maackiain.

Keywords: antifungal activity, *Sophora japonica*, isoflavonoid, biochanin A glycoside, (-)-maackiain, calycosin, trifolirhizin, genistein

1. INTRODUCTION

From wood, many compounds can be extracted by solvents or water. Some compounds isolated from wood extracts have the toxic or deterrent activities to bacteria or fungi (Mori *et al.*, 1994). For example, the woods of *Castanea sativa* and *Quercus sesseliflora* are resistant to wood-decaying fungi because of ellagitannins, such as castalin and castaligin (David & Shiraishi, 1990). Recently, environmental pollution and the toxicity of synthetic antiseptics have posed serious problem. For this reason, great concern is focused on natural products including wood extractives as natural antifungal agent.

Among many extractives from wood, isoflavonoids are almost restricted to the subfamily Papilionoidae of Leguminosae such as *Sophora japonica* and *Pueraria lobata*. In contrast to the flavonoids, the isoflavonoids are based on a 3-phenylchroman skeleton, instead of a 2-phenylchroman skeleton. There are several new classes of isoflavonoids not directly represented in the flavonoids series, such as pterocarpans.

In the previous study (Park *et al.*, 2000), we have already reported the isolation and structural determination of four isoflavonoids, irisolidone, biochanin A, formononetin and dihydroxyformononetin, from the woods of *Sophora japonica* and tested their antifungal activity because the ethanolic extracts have high antifungal activity.

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In this paper, we report the antifungal activities of five isoflavonoids in which two compounds were isolated from the wood and three compounds from the bark which were isolated previous work (Park *et al.*, 2001).

2. MATERIALS AND METHODS

2.1. Extractives

2.1.1. Materials

The woods of *S. japonica* were collected from Jiri mountain, Kyungnam, the southern part of Korea on June, 1996. The voucher specimens are deposited at the Korea Forest Research Institute, Seoul, Korea.

2.1.2. Extraction and Fractionation

Dried and ground wood of *S. japonica* were extracted twice with ethanol (EtOH) and then evaporated to give the crude extracts. The crude extracts was successively partitioned with organic solvents, such as *n*-hexane, methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc) and butanol (*n*-BuOH).

2.1.3. Isolation of Compounds

The CH_2Cl_2 soluble fraction (51.55 g) was subjected to column chromatography on Sehpadex LH-20 eluted with MeOH-EtOH (1:1, v/v) to yield 9 sets of fraction (SJD1～SJD9). Fraction SJD5 (29.17 g) was rechromatographed on a silica gel column with CH_2Cl_2-MeOH (150:1～50:1, v/v) to give 17 subfractions (SJD5-1～SJD5-17). Fraction SJD5-17 (4.65 g) was subjected to column chromatography on silica gel with benzene-MeOH (5:1, v/v) to give 8 fractions (SJD5-17-1～SJD5-17-8). Among 8 fractions of SJD5-17, the last fraction (SJD5-17-8, 183 mg) was purified by column chromatography on silica gel and eluted with EtOAc-MeOH-H_2O (10:2:1, v/v) to yield compound 1 (25 mg). Fraction SJD6 was further subjected to column chromatography on silica gel with benzene-EtOAc-MeOH (20:1:1, v/v) to give compound 2 (13 mg) from second fraction among 5 fractions of SJD6.

2.1.4. Instrumental Analysis

For the determination of molecular weights of the isolated compounds, EI-MS was performed at 70 eV ionization energy by direct inlet probe method, using JEOL JMS-600W mass spectrometer. About 10 mg of each sample was dissolved in 0.75 mL of methanol-d_4 (CD_3OD) with TMS (tetramethylsilane) as an internal standard and NMR spectra were obtained using a Varian UI 500 spectrometer at the operating frequency of 500 MHz (^1H) and 150 MHz (^{13}C) at Korea Basic Science Institute in Seoul.

2.1.5. Spectral data of Compounds

2.1.5.1. Compound 1

EI-MS *m/z* : 284 (M^+-Glu, Xyl), 269, 255, 241, 213, 173, 152, 132, 117, 89.

^1H-NMR (500 MHz, CD_3OD) : δ 3.17 (1H, *m*, H-5'''), 3.30 (2H, *m*, H-2''',3'''), 3.35 (1H, *m*, H-3''), 3.49 (1H, *m*, H-2''), 3.56 (1H, *m*, H-4'''), 3.80 (3H, *m*, H-4'',5'',6''), 3.83 (3H, *s*, 4'-OCH_3), 3.89 (1H, *m*, H-5'''), 4.12 (1H, *d*, *J* = 10.5 Hz, H-6''), 4.33 (1H, *d*, *J* = 7.5 Hz, H-1'''), 4.99 (1H, *d*, *J* = 7.5 Hz, H-1''), 6.56 (1H, *d*, *J* = 2.0 Hz, H-6), 6.81 (1H, *d*, *J* = 2.0 Hz, H-8), 6.99 (2H, *d*, *J* = 8.5 Hz, H-3',5'), 7.50 (2H, *d*, *J* = 8.5 Hz, H-2',6'), 8.20 (1H, *s*, H-2). ^{13}C-NMR (125 MHz, CD_3OD) : δ 55.7 (*q*, 4'-OCH_3), 66.9 (*t*, C-5'''), 70.5 (*t*, C-6''), 71.1 (*d*, C-4'''), 71.6 (*d*, C-5''), 74.7 (*d*, C-2''), 75.0(*d*, C-2'''), 77.3 (*d*, C-4''), 77.7(*d*, C-3'''), 77.8 (*d*, C-3''), 95.9 (*d*, C-8), 101.3 (*d*, C-6), 101.6 (*d*, C-1''), 105.8 (*d*, C-1'''), 108.2 (*s*, C-10), 114.9

Table 1. List of microorganisms used for antifungal activities test

Microorganisms	Characteristics
Glomerella cingulata	plant pathogenic fungus
Fusarium oxysporum	plant pathogenic fungus
Fusarium subglutinans f. sp. *pini*	tree pathogenic fungus
Cryphonectria parasitica	tree pathogenic fungus
Trametes versicolor	white-rot fungus
Tyromyces palustris	brown-rot fungus
Libertella betulina	mushroom pathogenic fungus
Trichoderma viride	soft-rot fungus
Trichoderma harzianum	sapstain fungus

(*d*, C-3',5'), 124.4 (*s*, C-1'), 124.8 (*s*, C-3), 131.4 (*d*, C-2',6'), 155.5 (*d*, C-2), 159.3 (*s*, C-9), 161.3 (*s*, C-4'), 164.7 (*s*, C-5, 7), 182.4 (*s*, C-4).

HMBC correlations : H-2→C-1'/C-3/C-4, H-6→C-5/C-6/C-7, H-8→C-5 /C-6/C-7/C-10, H-1"→C-7, H-1'"→H-6", H-2'/6'→C-1'/ C-2'/C-3/C-4', H-3'5'→C-1'/C-3'/C-4'/C-5'. NOESY correlations : H-1"↔H-6/H-8.

2.1.5.2. Compound 2

EI-MS *m/z* : 284 (M^+, molecular ion), 69, 134, 162, 197, 267.

^{1}H-NMR (500 MHz, acetone-d_6) : δ 3.55 (1H, *m*, H-6a), 3.62 (1H, *dd*, *J* = 10.8, 22.4 Hz, H-6_{ax}), 4.26 (1H, *dd*, *J* = 4.5, 10.8 Hz, H-6_{eq}), 5.48 (1H, *d*, *J* = 7.0 Hz, H-11a), 5.91 (2H, *dd*, *J* = 1.0, 13.2 Hz, OCH_2O), 6.35 (1H, *d*, *J* = 2.5 Hz H-4), 6.38 (1H, *s*, H-10), 6.55 (1H, *dd*, *J* = 2.5, 8.4 Hz, H-2), 6.88 (1H, *s*, H-7), 7.29 (1H, *d*, *J* = 8.4 Hz, H-1). ^{13}C-NMR (125 MHz, acetone-d_6) : δ 41.0 (C-6a), 66.9 (C-6), 79.4 (C-11a), 93.9 (C-10), 102.1 (OCH_2O), 103.9 (C-4), 105.9 (C-7), 110.5 (C-2), 112.8 (C-11b), 119.35 (C-6b), 133.0 (C-1), 142.4 (C-8), 148.9 (C-9), 155.3 (C-10a), 157.7 (C-4a), 159.7 (C-3).

HMBC correlations : H-6→C-11a, H-6a→C-11a, H-1→C-11a/C-3/C-4a, H-2→C-11b, H-7→C-10a/C-9/C-8. NOESY correlations : H-6a↔H-11a, H-11a↔H-1, H-1↔H-2.

2.2. Antifungal Activity

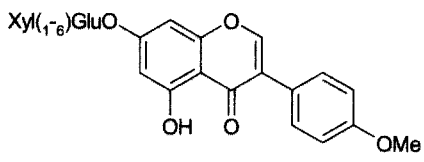
2.2.1. Fungi

Fungi strains used in antifungal test experiment were showed in Table 1.

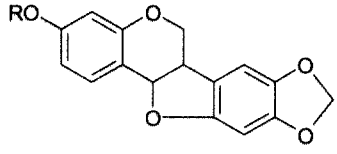
2.2.2. Antifungal Activity Test

Five isoflavonoids were used in antifungal activity test. Among theses compounds, biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside (1) and (-)-maackiain (2) were isolated from the wood and calycosin (3), trifolirhizin (4) and genistein (5) were from the bark of *S. japonica* in previous work (Park *et al.*, 2002) (Fig. 1).

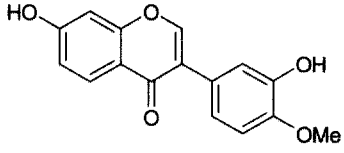
Test compounds were dissolved in EtOH which did not affect the growth of any fungi employed to a final concentration of 0.1 μg/μL and added into sterile PDA medium. PDA medium without any additive was used as a control. 8 mm diameter agar discs of pre-incubated fungi were inoculated at the center of petri dish (Hostettmann, 1991). The cultivation was carried out at 28°C for specified incubation periods. The hyphal growth inhibition rate (HGIR) was calculated as follows;



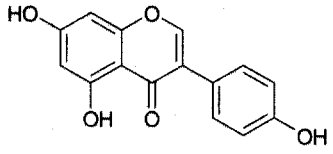
biochanin A 7-O-B-D-xylo-(1-6) glucoside (1)



R-H :(-)maackiain (2)
R-Glc : trifolirhizin(4)



calycosin (3)



genistein (5)

Fig. 1. Structures of isoflavonoids used in antifungal activities test.

$$HGIR(\%) = \left[\frac{(Gc)-(Gt)}{(Gc)}\right] \times 100$$

Gc : diameter of hyphal growth on control medium (mm)

Gt : diameter of hyphal growth on treated medium (mm)

3. RESULTS AND DISCUSSION

3.1. Identification of the Compounds

3.1.1. Compound 1 (biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside)

The compound 1 was isolated as a yellow powder. The ^{1}H NMR spectrum showed signals for six aromatic protons, one olefinic proton and one methoxy methyl proton. A total of 27 carbons appeared in the ^{13}C NMR spectrum which included one methyl, seven methine, eight quaternary carbons and eleven carbons of sugar moiety. A singlet at δ 7.83 (H-2) was coupled with C-2 (δ 154.3) in the HMQC spectrum and HMBC showed cross peaks between H-2 and C-3, C-4 and C-9, indicating the isoflavone nature of this compound (Iqbal *et al.*, 2001). The ^{1}H NMR spectrum of compound 1 indicated the presence of a methoxyl group at δ 3.83 (3H, s, OMe) and determined the position of the methoxy group to be 4' by using HMBC. These results strongly support the presence of biochanin A as aglycone. The connectivities of the monosaccharide units were established on the basis of HMBC cross peak indicating long-range ^{13}C-^{1}H couplings. The 1" anomeric proton (δ 4.99) correlates with C-7 (δ 164.7) and the 1'" anomeric proton (δ 4.33) gives a strong cross-peak with C-6" signal (δ 70.5) of glucose. Therefore, the position of glucose was suggested to be at C-7 of ring A and the position of xylose to be at C-6" of glucose. Consequently the structure of 1 was

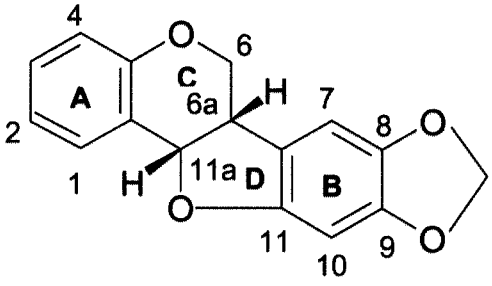


Fig. 2. The numbering systems of pterocarpans.

concluded to be biochanin A-7-O-β-D-xylopyranosyl(1-6)-β-D-glucopyranoside (Takeda *et al.*, 1977) (Fig. 1).

3.1.2. Compound 2 ((-)-maackiain)

The compound 2, a yellow powder, exhibited [M+] at *m/z* 284 in the EI-Mass spectrum with molecular formula $C_{16}H_{11}O_5$. This compound is a kind of pterocarpans which acts as phytoalexins. The numbering systems of pterocarpans are different from that of flavonoid such as Fig 2.

Characteristic features of this compound is the chemical shift values for 6b and 10a, which form a part of the five membered heterocyclic ring D. Both of these carbon resonances are of quaternary nature resonating at 117.3～119.4 (C-6b) and 153.6～160.6 ppm (C-10a) in the compounds (Agrawal, 1989). The ^{1}H-NMR signal at δ 5.91 (2H, *dd*) and ^{13}C-NMR signal at δ 102.1 revealed the presence of a methylendioxy group in compound 2 (Eliane *et al.*, 2000). The position of this group on ring B was suggested to be between C-8 and C-9 owing to HMBC spectrum. Consequently the structure of compound 2 was concluded to be (-)-maackiain (Soby *et al.*, 1996) (Fig. 1).

3.2. Antifungal Activity

Searching for antifungal agents, nine fungi were used in this study. *G. cingulata* is a plant pathogenic fungus (Mitsuo *et al.*, 1995). *F. oxysporum* is a fungus to cause wilt disease and attacks crops such as tomatoes, bananas, sweet potatoes and pears (Alexopoulos *et al.*, 1996). *C. parasitica* is a fungus that causes devastating chestnut blight disease. *T. versicolor* is a common white-rot fungus and grows on hardwood logs. The decay by this fungus is a uniform simultaneous type of attack (Otjen & Blanchett, 1986). *T. palustris* is a brown-rot fungus of genus *Tyromyces*, family polyporaceae, division basidiomycetes, which accumulates oxalic acid in culture media (Tokimatu *et al.*, 1998). *T. viride* is one of those soft-rot fungi that produce a complete cellulase complex of endo-and exocellulase which is able to attack crystalline cellulose (Eaton & Hale, 1993). *T. harzianum* is a sapstain fungus that has smooth conidial walls.

In the previous work (Park *et al.*, 2000), we isolated four isoflavonids from *Sophora japonica* and examined the antifungal activity of isolated compounds. Among four isoflavonids, 7-hydroxy-4'-methoxyisoflavanone exhibited the highest antifungal activity against *C. parasitica* with antifungal activity values 70.2%.

In this work, compounds isolated from the woods (biochain A glycoside and (-)-maackiain) and barks (calycosin, trifolirhizin and genistein) of *S. japonica*, especially from CH_2Cl_2 fraction, were tested for their antifungal activity. Fig. 3 showed the antifungal activity (%) of isoflavonoids isolated.

The order of antifungal activity against *L. betulina* was in (-)-maackiain 〉 biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranosid e 〉 calycosin 〉 genistein 〉 trifolirhizin. The order of the antifungal activity against *T. harzianum* was as follows. (-)-maackiain 〉 calycosin 〉 genistein 〉 biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside =

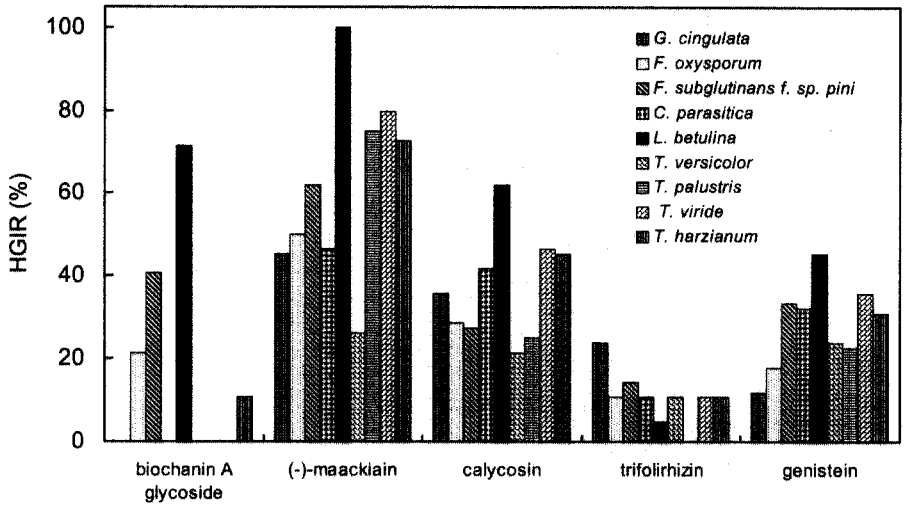


Fig. 3. Antifungal activities of biochanin A-7-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside, (-)-maackiain, calycosin, trifolirhizin and genistein.

trifolirhizin. Regarding to antifungal activity, (-)-maackiain exhibited significant antifungal activity against all fungi except *T. versicolor* with antifungal activity values from 45.2 to 100%, which meant that this compound was regarded as the main compound of CH_2Cl_2 fraction which showed high antifungal activity (Park *et al.*, 2000). It can be also considered that the position of hydroxyl group on aromatic ring may influence the biological activity. (-)-maackiain have no double bond at the C-2 position in ring C. It is suggested that this structural feature could be related to the antifungal activity (Osawa *et al.*, 1992). The antifungal activity of (-)-maackiain was much higher than that of trifolirhizin (a glycoside of (-)-maackiain). The above results means that the glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Rice-Evans *et al.*, 1996).

4. CONCLUSIONS

In order to find out the biological activity of compounds isolated from *S. japonica* wood and bark, the test of antifungal activities against nine fungi was subjected to isoflavonoids obtained from this study. From woods of *S. japonica*, biochanin A-7-O-β-D-xylopyranosyl- (1→6)-β-D-glucopyranoside (1) and (-)- maackiain (2) were isolated and calycosin (3), trifolirhizin (4) and genistein (5) were from barks of *S. japonica* in previous work.

Among pure compounds, (-)-maackiain, which has no double bond at the C-2 position in ring C, showed high antifungal activity, which meant that this compound was regarded as the main compound of CH_2Cl_2 fraction which showed high antifungal activity while other compounds showed low or moderate antifungal activity. It is suggested that this structural feature could be related to the antifungal activity.

REFERENCES

1. Agrawal, P. K. 1989. Carbon-13 NMR of Flavonoids. Elsevier, New York. pp. 192~196.
2. Alexopoulos, C. J., C. W. Mims and Blackwell.

1996. Introductory Mycology Fourth edition. John wiley & sons, INC., New York. p.244.
3. David, N. S. Hon, and N. Shiraishi. 1990. Wood and Cellulosic Chemistry. Marcel Dekker INC., New York. pp. 241~242.
4. Eaton, R. A. and M. D. C. Hale. 1993. Wood - decay, pests and protection -. Chapman & Hall, London. pp. 183.
5. Eliane, O. F. and D. A. Dias. 2000. A methylenedioxyflavonol from aerial parts of *Blutaparon portulacodides*. Phytochemistry. 53: 145~147.
6. Hostettmann, K. 1991. Methods in plant biochemistry volume 6 -Assays for bioactivity-. Academic press, London. pp. 33~46.
7. Iqbal, M., M. Nur-e-Alam, I. Baig, F. Akhtar, A. M. Khan, P. O. Ndognii, T. Badarchiin, G. Purevsuren, N. Nahar, and A. Rahman. 2001. Four new flavones and a new isoflavone from *Iris bungei*. Journal of natural product. 64: 857~860.
8. Mitsuo M., N. Hirokazu, and K. Hiromu. 1995. Biotransformation of (-)-α -bisabolol by plant pathogenic fungus, *Glomella cingulata*. Phytochemistry. 39(5): 1077~1080.
9. Mori, M., S. Doi, M. Aoyama, A. Kanetosh, and T. Hayashi. 1994. Antifungal activity of bark extracts of trees collected in Hokkaido. Hokkaido For. Prod. Res. Inst. 8(6): 12~17.
10. Osawa, K., H. Yasuda, T. Maruyama, H. Morita, K. Takeya, and H. Itokawa. 1992. Isoflavones from the heartwood of *Swartzia polyphylla* and their bacterial activity against cariogenic bacteria. Chem. Pharm. Bull. 40(11): 2970~2974.
11. Otjen, L. and R. A. Blanchett. 1986. A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. Can. J. Bot. 64: 905~911.
12. Park, Y. K., H. J. Lee, S. S. Lee, D. H. Choi, W. H. Yeo, and J. S. Oh. 2001. Studies on biological activity of wood extractives (Ⅷ)-Antifungal activity of isoflavonoids from *Sophora japonica*-. Mokchae Konghak. 29(4): 89~96.
13. Park, Y. K., H. J. Lee, D. H. Choi, Y. H. Kwon, and J. S. Oh. 2002. Extractives from the bark of *Sophora japonica* L. Mokchae Konghak. 30(3): 42~47.
14. Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure -antioxidant activity relationships of flavonoids and phenolic acids. Free radical Biology & Medicine. 20(7): 933~956.
15. Soby, S., Caldera, S., Bates, R., and Van Etten, H. D. 1996. Detoxification of the phytoalexins maackiain and medicarpin by fungal pathogens of alfalfa. Phytochmistry. 41(3): 759~765.
16. Takeda, T., I. Ishiguro, M. Masegi, and Y. Ogihara. 1977. New isoflavone glycosides from the woods of *Sophora japonica*. Phytochemistry. 16(5): 619~ 620.
17. Tokimatu, T., Y. Nagi, T. Hattori, and M. Shimada. 1998. Purification and Characteristics of a novel cytochrome C dependent glyoxylate dehydro- genase from a wood-destroying fungus *Tyromyces palustris*. FEBS Lett. 437: 117~121.