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Isolation and Characterization of Antitumor Agents from *Dictamnus albus*

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Abstract – This study was carried out to find new antitumor agents from plant resource. Three cytotoxic agents were isolated from the root of *Dictamnus albus* by hexane extraction, silica gel column chromatography and HPLC. They were identified to be dictamnine ($C_{12}H_9NO_2$), preskimmianine ($C_{17}H_{21}NO_4$) and fraxinellone ($C_{14}H_{16}O_3$) on the basis of spectroscopic evidences. In this study, it was newly found that these compounds possess a cytotoxic activity against lung lymphoma L1210 cell line. Among them, preskimmianine was the most potent against the lymphoma L1210 with a IC_{50} of 3.125 μg/ml (10.3 μM). Toxicity of preskimmianine against normal lymphocyte was observed at the concentration of 50 μg/ml (165 μM). These results support the pharmacological role of *D. albus*, a herb known as Paeksun in Korea and used as an anticancer agent in folk medicine.

Key words – *Dictamnus albus*; cytotoxicity; dictamnine; preskimmianine; fraxinellone.

Plant resources have been widely used as a folk (herbal) medicine throughout the world.[1-3] Among the numerous numbers of herbal plants, *Dictamnus albus* (*D. dasycarpus* Turcz, 白鮮) has been used for the treatment of jaundice, leprosy, headache, colds, rheumatism,[4] dermatitis, psoriasis, and itching of the skin[1](Huang, 1993) in folk medicine. The herb was also noted for its antifertility,[5] antifungal and antipyretic activities.[6] In some Korean district like Choongchong Province, it has been used for the treatment of cancer (personal communication). They decoct roots and drink the liquid after filtration. It was also reported to be effective against icteric hepatitis at the clinical dosage of 3~10 g/day.[1] Chemical studies have been performed to isolate psoralen, xanthotoxin, isomaculosindine[1] and furoquinoline alkaloids[6] from *D. albus*. However, chemical studies have been carried out independently of biological activities with the exception of antifertility activity.[5] In the course of our screening programe to find new antitumor agents from the plant origin, the root of *Dictamnus albus* demonstrated a potent antitumor activity against lymphoma L1210 cell line. Thus, isolation and characterization of the active compounds from *D. albus* were attempted. The active compounds were identified to be dictamnine,[1] preskimmianine[7] and fraxinellone.[5] Among them preskimmianine showed the most potent activity against lung lymphoma L1210 cell line with a IC_{50} of 10.3 μM. All of the 3 compounds showed relatively mild cytotoxic activity compared with taxol,[8] helenolides, cucurbi-

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tacins, taxane diterpenes,[9] or benzyl isothiocyanate.[10] However, it was newly found in our study that they possess antitumor activity and thus support the pharmacological role of *Dictamnus albus* having been used in folk medicine for the treatment of cancer.

MATERIALS AND METHODS

Isolation and purification of active compounds – As shown in Fig. 1, dried root of *Dictamnus albus* was pulverized and extracted with hexane for 48 hr. Hexane extract was filtered and concentrated under the reduced pressure to obtain an oily residue. The residue was then subjected to silica gel (70-230 mesh) column chromatography with a solvent system of hexane-ethylacetate (9 : 1 → 1 : 1), stepwisely. Final purification of active compounds was achieved by HPLC (Waters 991, Nova-pak ODS, 20×250 mm, UV 220 nm, flow rate: 5 ml/min) with a mobile phase of 72% aqueous MeOH (Fig. 2). Isolation of the active compounds was carried out by monitering cytotoxicity against lung lymphoma L1210 cell line.

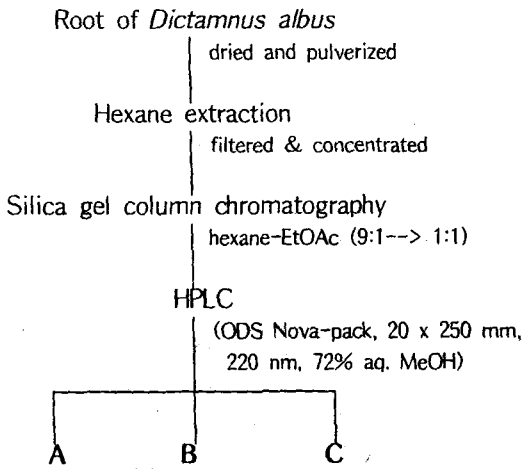

Fig. 1. Procedure for the isolation and purification of antitumor agents from *Dictamnus albus*.

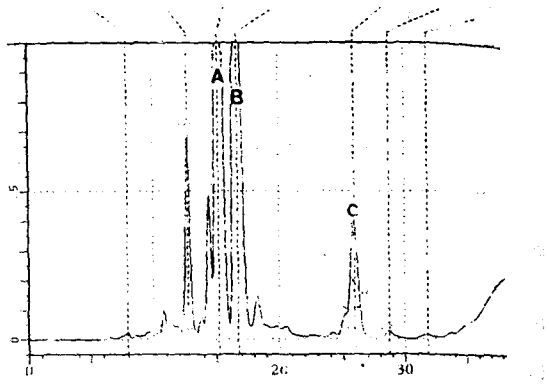

Fig. 2. HPLC profile of antitumor agents from *Dictamnus albus*.

Measurement of physico-chemical properties – ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra of the active compounds were determined by Brucker ARX-400 NMR spectrophotometer. Tetramethylsilane (TMS) was employed as an internal reference. Molecular weights were determined with Jeol JMX DX-303 FAB/mass spectrophotometer. Melting points of the active compounds were measured by the Fisher Johns melting point apparatus. Thin-layer chromatographic analysis was performed on precoated silica gel TLC glass plate (Merck, 60 F_{254}) and visualized under the UV lamp or iodine vapor. Color reactions of Dragendorff, vanillin, anisaldehyde-sulfuric acid, bromocresol green, and ninhydrin were investigated by the methods described by Otake *et al.*[11]

Bioassay for cytotoxicity against cancer cell lines – Cytotoxic activity of the test materials against lung lymphoma L1210 cell line was investigated by the trypan blue exclusion method.[12] The cell was cultured in RPMI 1640-HEPES medium supplemented with 10% fetal bovine serum (GIBCO), and L-glutamate in the presence of penicillin G (100 units/ml) and streptomycin (100 μg/ml) under a humidified atmosphere of 5% CO_2 and 95% air. Two ml/well of the suspended

cells (density: 5.0×10^4/ml) were dispensed into a 24 multi-well plate. Plant extract was diluted with 75% methanol to a desired concentration and added 5 μl/well. The cell was then incubated for 48 hr and treated with trypan blue. Cell death was calculated to compare with negative control without chemical agent. Antitumor activity of the sample was expressed as a percentage to the death rate of the negative control. Five replicate wells were employed for the determination of IC_{50} of the active compounds. Two-fold dilution was made from 100 μg/ml as a final concentration for the test materials.

Antitumor activity against carcinoma cell line A549 was determined by the MTT (2-4, 5,-dimethyl thiazol-2,5-diphenyl tetrazolium bromide) method described by Mosmann *et al.*[13] Cell was cultured under the same condition as the lymphoma L1210 and 250 μl of cell suspension (1.0×10^4/ml) was dispensed into a 96 multi-well plate. After 24 hr of preincubation at 37℃, plant extract and MTT (20 μl) dissolved in Dubelcco's phosphate buffered saline (DPBS-A) were added and cultured for an additional 48 hr. The plate was wrapped with aluminium foil before incubation. The plate was then spinned at 1,000 rpm for 5 min to eliminate medium. One hundred μl/well of hydrochloric acid (0.04 N)-isopropanol mixture was supplemented, centrifuged again for 20 min, and shaked in a titer plate shaker (Lab-Line) for 5 min. Optical density was measured at 570 nm using ELISA-reader (EMax). Death rate of test group was calculated by comparing with that of the control. Chemical reagents for cell culture were purchased from Sigma unless otherwise mentioned and were the highest purity among commercially available products.

Cytotoxicity in normal cell–Peripheral blood from male healthy adults was diluted two folds with Mg^+- and Ca^{2+}- free HBSS and loaded on Ficoll-Hypaque (density: 1.119, Pharmacia, Uppsala, Sweden) and centrifuged at the speed of 700×g, room temperature, for 30 min to obtain monocyte and neutrophils. Addition of sample, incubation conditions, and calculation of the death rate were performed by the same method as for the lung lymphoma L1210 cell line.

RESULTS AND DISCUSSION

Isolation and physico-chemical properties of the active compounds–As shown in Fig. 2, five active compounds were observed on HPLC. Among them, 3 major compounds (A, B, C) were isolated and their physico-chemical properties characterized. They were amorphous white powder. Rf values for A, B, and C were 0.24, 0.72, and 0.40, respectively on silica gel TLC with a solvent system of hexane-EtOAc (6 : 4). Compounds A and C showed positive color reaction to Dragendorff reagent, and B to vanillin and anisaldehyde-sulfuric acid reagents and weakly to Dragondorff. However, they showed negative color reactions to ninhydrin and bromocresol green reagents. Melting points of A, B, and C were 123-125℃, 93-95℃ and 140-144℃ (dec), respectively. Molecular weight of A was $(M+H)^+$ 200 (FAB-MS), B (M^+) 303 (EI-MS), and C $(M+H)^+$ 233 (FAB-MS).

Structure determination of the active compounds–^1H NMR spectrum of compound A revealed four aromatic protons at 8.29 (dd), 7.88 (dd), 7.72 (t), 7.46 (t) ppm and two furan ring protons at 7.83 (d) and 7.38 (d) ppm (Fig. 3). Sharp singlet methyl signal at 4.52 ppm indicated the presence of methoxy moiety. Presence of nitrogen could

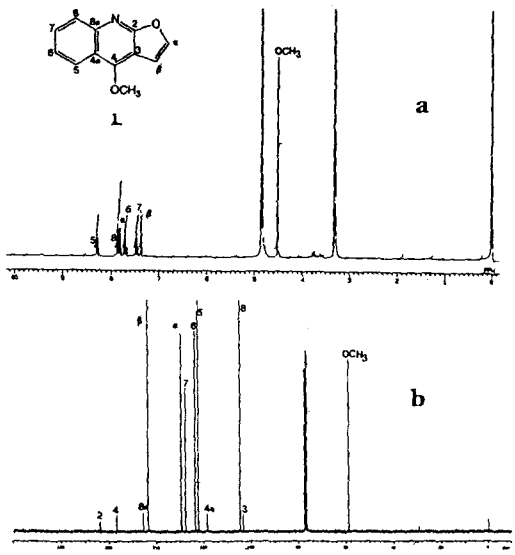

Fig. 3. ^{13}H (a; in CD_3OD) and ^{13}C (b; in $CDCl_3$) NMR spectra of compound A (400 MHz).

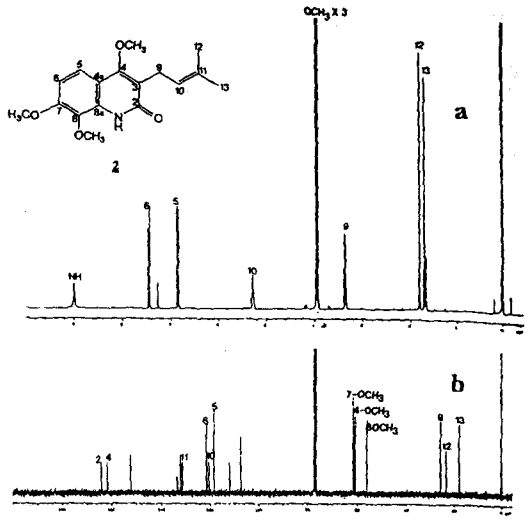

Fig. 4. ^{1}H (a) and ^{13}C (b) NMR spectra of compound B ($CDCl_3$, 400 MHz).

be considered from the molecular weight (199) of the compound. Total of 12 carbon atoms were observed on ^{13}C NMR spectrum. Presence of methoxy moiety was further supported by the signal at 59 ppm. Six sp^2 methine carbons were found at 104 and 144~122 ppm, and five sp^2 quaternary carbons were observed at 103.5, 109.5, 143.0, 148.5, and 162 ppm. Molecular weight of compound A could reasonably be accounted by the molecular formula ($C_{12}H_9NO_2$). From these spectroscopic evidences, we could deduce the chemical structure of compound A as 1. Consequently, compound A was identified to be dictamnine,[5] a furoquinoline alkaloid commonly occurring in Rutaceae.

Broad singlet at 9.0 ppm and three sharp singlets at 3.9~4.0 ppm could be assigned to NH and methoxy protons, respectively in compound B (Fig. 4). Presence of nitrogen was further supported by the molecular weight (303) of compound B. Ortho-coupled aromatic protons were observed at 7.4 and 6.8 ppm. ^{1}H-^{1}H COSY spectrum (data not shown here) showed that methine proton at 5.2 (t) and methylene proton at 3.4 ppm (d) were found to be coupled each other. Three oxymethyl protons were observed at 4.0~3.8 ppm, and two terminal methyl protons at 1.82 and 1.72 ppm. Seventeen carbon atoms were observed on the ^{13}C NMR spectrum of compound B. The presence of three methoxy was further supported by the carbon signals at 62.2, 61.7 and 56.3 ppm. Signal at 164.2 ppm was ascribed to carbonyl carbon. From these NMR data, chemical structure of compound B was established as 2 and molecular formula was determined to be $C_{17}H_{21}NO_4$. Storer *et al.* identified this compound from *Dictamnus albus* L. and named it as preskimmianine because of its probable implication in the biosynthesis of skimmianine.[7] However, cytotoxic activity of preskimmianine has not been reported. In this paper, we suggest the pharmacological role of preskimmianine as a potential antitumor agent.

Signals at 7.44 (d), 7.42 (s) and 6.37 (d) ppm indicated the presence of furan moiety

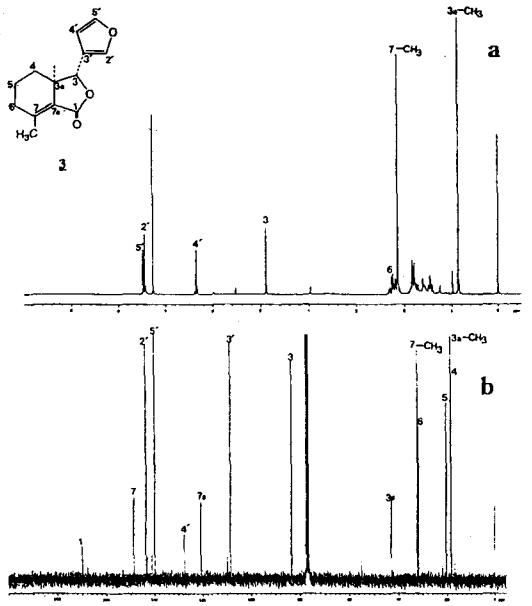

Fig. 5. ^{1}H (a) and ^{13}C (b) NMR spectra of compound C ($CDCl_3$, 400 MHz).

in compound C (Fig. 5). Singlet methine proton at 4.91 ppm was assigned to oxymethine from its low-field shift. Another singlet methyl proton (δ_H 2.18) indicated CH_3-C=. The presence of carbonyl carbon was indicated by the signal at 170 ppm in ^{13}C NMR spectrum. Interpretation of NMR data allowed us to deduce the chemical structure of compound C as 3. Finally, compound C was identified to be fraxinellone ($C_{14}H_{16}O_3$). Fraxinellone is a natural product of unique structure with a β-substituted furan ring.[14]

Cytotoxic activity of the active compounds– Antitumor activity against lymphoma L1210 cell line indicated preskimmianine was the most potent among the three active compounds (Table 1). IC_{50} values for dictamnine, preskimmianine and fraxinellone were 12.5, 3.125, and 25.0 μg/ml against lymphoma L 1210 cell line, respectively. However, they were not active against lung carcinoma A 549 cell line, indicating rather selective cytotoxicity. Toxicity against normal lymphocyte was observed at almost 10 times higher concentration for dictamnine and preskimmianine than the cytotoxic activity against lymphoma L1210. Fraxinellone showed the weakest activity among the 3 compounds and toxicity was observed at the 2-fold concentration of the IC_{50} value.

All of the three antitumor agents were identified to be known natural compounds. However, it was newly found in this experiment that they possessed antitumor activity. Preskimmianine showed the most potent antitumor activity with a IC_{50} of 3.125 μg/ml (10.3 μM) against lung lymphoma L 1210. In case of preskimmianine, toxic concentration against normal lymphocyte was almost 17 times higher than the IC_{50} against the lymphoma L1210. Cytotoxicity of fraxinellone (IC_{50}: 25 μg/ml) against lymphoma L1210 was weaker than dictamnine (IC_{50}: 12.5 μg/ml) or preskimmianine. In addition, it showed toxic effect at the 2-fold concentration (50 μg/ml) of IC_{50}. Although fraxinellone was known to have antifertility activity,[5] its clinical use should, therefore, be carefully reviewed due to its toxicity against normal cell. Klier *et al.* said that dictamnine acted as a promutagen in *Salmonella typhimurium*.[15] An unstable epoxide

Table 1. IC_{50} values of the active compounds against cancer cell lines

Cell line \ Compound	Dictamnine	Preskimmianine	Fraxinellone
Lymphoma L1210	12.50 μg/ml (62.5 μM)	3.13 μg/ml (10.3 μM)	25.00 μg/ml (107.3 μM)
Lung carcinoma A549	>100 μg/ml	>100 μg/ml	>100 μg/ml
Normal lymphocyte	100 μg/ml	50 μg/ml	50 μg/ml

is postulated as an intermediate which acts as the ultimate mutagen in the Ames assay. However, dictamnine is known to be easily metabolized by the cell into non-mutagenic dictamnic acid.[16] Due to the short-lived unstable character of the epoxide it could hardly be supposed that the epoxide intermediate can reach the target molecules without converted to dictamnic acid. In addition, *Dictamnus albus* has been used in some korean districts as an anticancer agent without any severe side effect. And our results support the pharmacological role of *D. albus* which has long been used in folk medicine, especially in the treatment of cancer patients. From these results, we propose *D. albus* as a potential anticancer material.

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