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Analysis of Chemical Constituents of Saccharides and Triterpenoids in the Korean Native Mistletoes[*1]

– Ⅱ. Screening the Extractives of Korean Camellia Mistletoe (*Pseudixus japonicus*) for Cytotoxicity –

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韓國産 겨우살이類의 糖類와 triterpenoids의 化學的 組成 分析[*1]

– Ⅱ. 동백나무겨우살이 추출물의 항암활성 성분 검색 –

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要　約

한국산 동백나무겨우살이(*Pseudixus japonicus*) 추출물의 암치료를 위한 생약으로서 활성 유효성을 검증하기 위하여 메탄올, 석유에테르, 클로로포름, 초산 에틸 용매로 순차적으로 추출하여 겨우살이 추출물의 다섯가지 분획을 얻어, 이에 대하여 *in vitro*로 1차와 2차 검색 시스템을 사용해 항암활성 성분을 체계적으로 검색하였다.

다섯 가지 분획중 클로로포름 가용성 분획이 1차 검색 세포인 $P388D_1$에 대해 가장 높은 항암활성을 나타내어 MSB1, NIH/3T3, SNU-1, SNU-C2A 등 2차 검색 시스템에 대해 클로로포름 가용성 분획의 항암활성을 다양한 농도하에서 비교 검색하였다.

혈액암 세포중 특히 $P388D_1$의 생장이 클로로포름 추출물에 의해 강하게 저해되었으며, 형질전환된 생쥐의 태아 섬유아세포와 사람의 대장암, 위암세포들도 어느 정도의 생육저해를 나타내었다.

이 클로로포름 가용성 분획의 주성분은 원소분석, 발색시약과의 반응, IR, GC-MS, ^{13}C-NMR의 스펙트럼의 결과로 세 종류의 알칼로이드 화합물로 확인되었고, 부성분으로는 지방산 메틸 에스테르와 프탈라이드 화합물이 MS 스펙트럼을 통해 동정되었다.

Keywords : Camellia mistletoe, cytotoxicity, lymphoma, cancer cell lines, alkaloids

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1. INTRODUCTION

The mistletoe is perenial evergreen parasitic plant which occurs on branches and stems of either trees or shrubs. There are some 1,300 species of mistletoe and what is called the 'common mistletoe' varies from country to country(Anderson & Phillipson, 1982). The word mistletoe is generally applied to plants with a similar lifestyle and a certain degree of taxonomical relationship in three families(Loranthaceae, Viscaceae and Ermolepidaceae)(Becker, 1986). Mistletoes are hemiparasitic, i.e. they depend for water and mineral nutrition on their respective host but are able to produce carbohydrates by photosynthesis(Beecher *et al*, 1989).

While a prodigious amount of papers on European mistletoe(***Viscum album*** L.) has been published, attracting special interest in folklore and medicine, there has been scarcity of publication on camellia mistletoe(***Pseudixus japonicus*** Hayata)(Franz, 1986).

Camellia mistletoe inhabits the subtropical forest, ranging from Australia to China and is parasitic on ***Camellia japonica*** var. ***hortensis, Camellia sasanqua, Cinnamomum japonicum, Ligustrum ovalifolium, Ligustrum japonicum, Masakia japonica, Ilex integra, Ilex crenata*** var. ***microphylla, Eurya japonica*** and so forth. Camellia mistletoe has been called as 'Pseudicis Herba' in the field of pharmacognosy and prescribed for the treatment of paralysis and high blood pressure in herb medicine. But there has been lack of scientific investigation on the pharmacological activities of components in camellia mistletoe(Song, 1985).

In this study, extractives of Korean camellia mistletoe(***Pseudixus japonicus*** Hayata) were fractionated into 5 fractions and screened for cytotoxicity ***in vitro***, using primary and secondary assay systems. And the fundamental structures of the constituents in the fraction showing the highest cytotoxicity among 5 fractions were elucidated.

2. MATERIALS & METHODS

2.1 Experimental Material

Aerial shoots of Korean camellia mistletoe (***Pseudixus japonicus*** Hayata) were collected from heavily infected camellia(***Camellia japonica*** L.) growing in the vicinity of Arboretum of Chonnam National University, situated on Bogil Island, Wando-gun, Chollanam-do on Jan. 4. 1994. Both twigs and leaves were used as vouched sample.

2.2 Tumor cell lines

To assay cytotoxic activity ***in vitro***, 5 cell lines were used, as follows :

$P388D_1$(mouse T cell lymphoma, non-adherent : ATCC HB 8065), MSB1(chicken T cell lymphoma induced by Marek's disease virus (MDV), non-adherent : Veterinary Research Institute, Anyang, Korea), NIH/3T3(transformed mouse embryo fibroblast, adherent : ATCC CRL 1658), SNU-1(human gastric cancer cell line, non-adherent : Cancer Research Institute, College of Medicine, Seoul National University, Seoul, Korea) and SNU-C2A(hu-man colon cancer cell line, adherent : ATCC CCL 250.1). All the cell lines were maintained in suspension in complete RPMI 1640 tissue culture medium(Gibco) and cultivated in a humidified CO_2(5%) incubator at 37℃.

2.3 Preparation of mistletoe extracts

The pulverized mistletoe sample was extracted with 40 *l* of aqueous methanol(80%), 3 *l* of diethyl ether and 5 *l* of aqueous acetic acid(2%) sequentially by continuous agitation and filtered through Whatman No. 2 filter paper. The extract with aqueous methanol was concentrated to 1 *l* ***in vacuo***. Water insoluble precipitate was separated from the extract by filtration and dissolved in 1 *l* of methanol(fraction M). The

diethyl ether extract was condensed to syrup, combined with the extract solutions of aqueous methanol and acetic acid, and left to stand for 24hr. The combined solution was concentrated to 1.6 l (Kjwaja *et al*, 1980 · 1986).

2.4 Fractionation of the extract solution

The aqueous extract solution was extracted with 19 l of petroleum ether(fraction P) and brought to pH 9 with saturated aqueous $NaHCO_3$. And then the aqueous layer was extracted with 13 l of chloroform(fraction C). pH was adjusted to 7.5 with acetic acid and extracted repeatedly with 8 l of ethyl acetate (fraction E).

Five solvent fractions were filtered through Whatman No. 2 filter paper, respectively. The filtrates were concentrated and the concentration of each solvent fraction was measured.

Fraction P was dissolved in aqueous dimethyl sulfoxide(DMSO), fractions of C and M in aqueous ethanol, and fractions of A and E in distilled water, respectively.

The sample for cytotoxicity assay was prepared by filtration through sterilized disposable membrane filter(0.2μm : Korea green cross corp.) and twofold serial dilution.

2.5 Cytotoxicity assay on tumor cell lines

$P388D_1$ was adopted as primary screening tumor cell line to select the most efficacious fraction among 5 fractions of mistletoe extractives.

MSB1, NIH/3T3, SNU-1 and SNU-C2A were used as secondary screening tumor cell lines to test the cytotoxic activity of the fraction selected by primary screening system.

All experiments were performed in triplicate, using 96-well micro titer plate.

The percentage of cytotoxicity on non-adherent tumor cell was determined with the following formula, by means of trypan blue exclusion method.

$$\% \text{ Cytotoxicity} = \left(1 - \frac{\text{viable cells in test}}{\text{viable cells in control}}\right) \times 100$$

Adherent cell was stained with crystal violet and the absorbance in each well was read at 540nm with a microplate spectrometer(ELISA reader: Titer Multiskan MCC/340).

The percentage of cytotoxicity was calculated by the following equation(Woo, 1988).

$$\% \text{ Cytotoxicity} = \left(1 - \frac{\text{Abs. at 540nm with sample}}{\text{Abs. at 540nm with control}}\right) \times 100$$

2.6 Structural analysis of components in C fraction

2.6.1 Elementary analysis

Elementary composition of fraction C, dried up under reduced pressure was analysed by Perkin Elmer Model 240C.

2.6.2 IR spectrum

Fraction C of mistletoe extract was evaporated to dryness and acetylated with pyridine/acetic anhydride(1 : 1) at 40℃ for 24hr. The IR spectra of fraction C with and without acetylation were recorded by Perkin Elmer System 2000 FT-IR.

2.6.3 Color reaction with spray reagents

Fraction C was subjected to TLC(silica gel $^{60}F_{254}$, Merck), developed with methanol/chloroform(2:15, v/v) system and sprayed with a variety of spray reagents. These reagents include Dragendorff's, Mayer's and Marquis' for alkaloids and ninhydrin for amino acids (Browning, 1967).

2.6.4 GC-MS analysis

Fraction C was analysed by gas chromatography-mass spectrometry(GC-MS). And GC-MS conditions were as follows: Hewlett-Packard 5980 II GC equipped with 30m * 0.25mm DB-1 capillary column and Hewlett-Packard 5971 A MSD with an ionizing energy of 70eV. Column temperature was programmed from 50℃ to 290℃.

2.6.5 ^{13}C-NMR spectrum

Fraction C was dried up under reduced pres-

sure and dissolved in $CDCl_3$. To investigate the carbon structure of major components in fraction C, ^{13}C-NMR spectrum was taken using FT-NMR Bruker AMX500(125.7MHz).

3. RESULTS & DISCUSSION

3.1 Primary cytotoxicity screening

Five solvent fractions of extractives, obtained from Korean camellia mistletoe(*Pseudixus japonicus* Hayata) were primarily screened for cytotoxicity against $P388D_1$ to select the fraction showing the highest cytotoxicity among 5 fractions. Fraction C was selected as the most efficacious fraction. Some of the $P388D_1$ cells were necrotized by treating with fraction C and cell fragments were observed by microscopy. Cytotoxicity was increased in proportion to the sample concentrations, as shown in Fig. 1.

3.2 Secondary cytotoxicity screening

Comparison of the effect of fraction C on 5 cell lines, $P388D_1$, MSB1, NIH/3T3, SNU-1 and SNU-C2A is shown in Fig. 2. Fraction C had wide spectrum of cytotoxicity on various tumor cell lines, but possessed specific cytotoxicity against lymphoma cell lines, $P388D_1$ and MSB1.

Cytotoxicity was expressed dose-dependently. It is interesting that in low concentration, fraction C was more active on human colon cancer cell line than on human gastric cancer cell line but in high concentration, *vice versa*.

3.3 Structural analysis of components in fraction C

3.3.1 Elementary analysis

Elementary composition of fraction C was C : 59.74%, H : 7.82% and N : 1.37%. From the result, it is conjectured that fraction C contains nitrogeneous compounds.

3.3.2 IR spectrum

Hydrogen bonded OH stretching vibration was detected around 3427cm^{-1}(Fig. 3(A)). To discriminate amine peak from broadened hydroxyl peak, fraction C was acetylated with pyridine and acetic anhydride. IR spectrum of acetylated fraction C(Fig. 3(B)) showed N-H stretching vibration around 3451cm^{-1}. Amine group presumably belongs to secondary amine, based on the pattern of N-H stretching vibration.

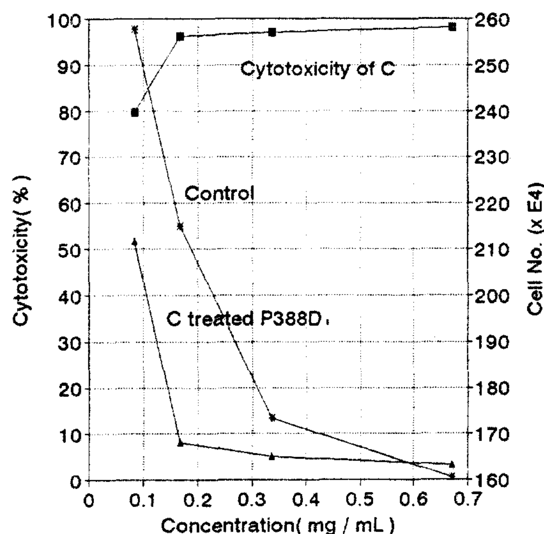

Fig. 1. Cytotoxicity of fraction C on $P388D_1$, mouse.

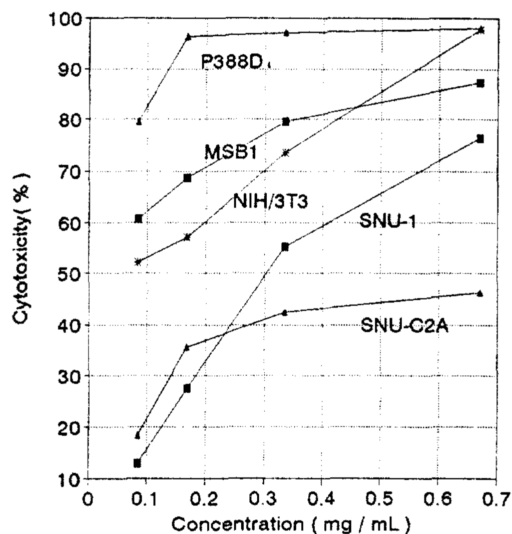

Fig. 2. Cytotoxicity of fraction C on $P388D_1$, MSB1, NIH.

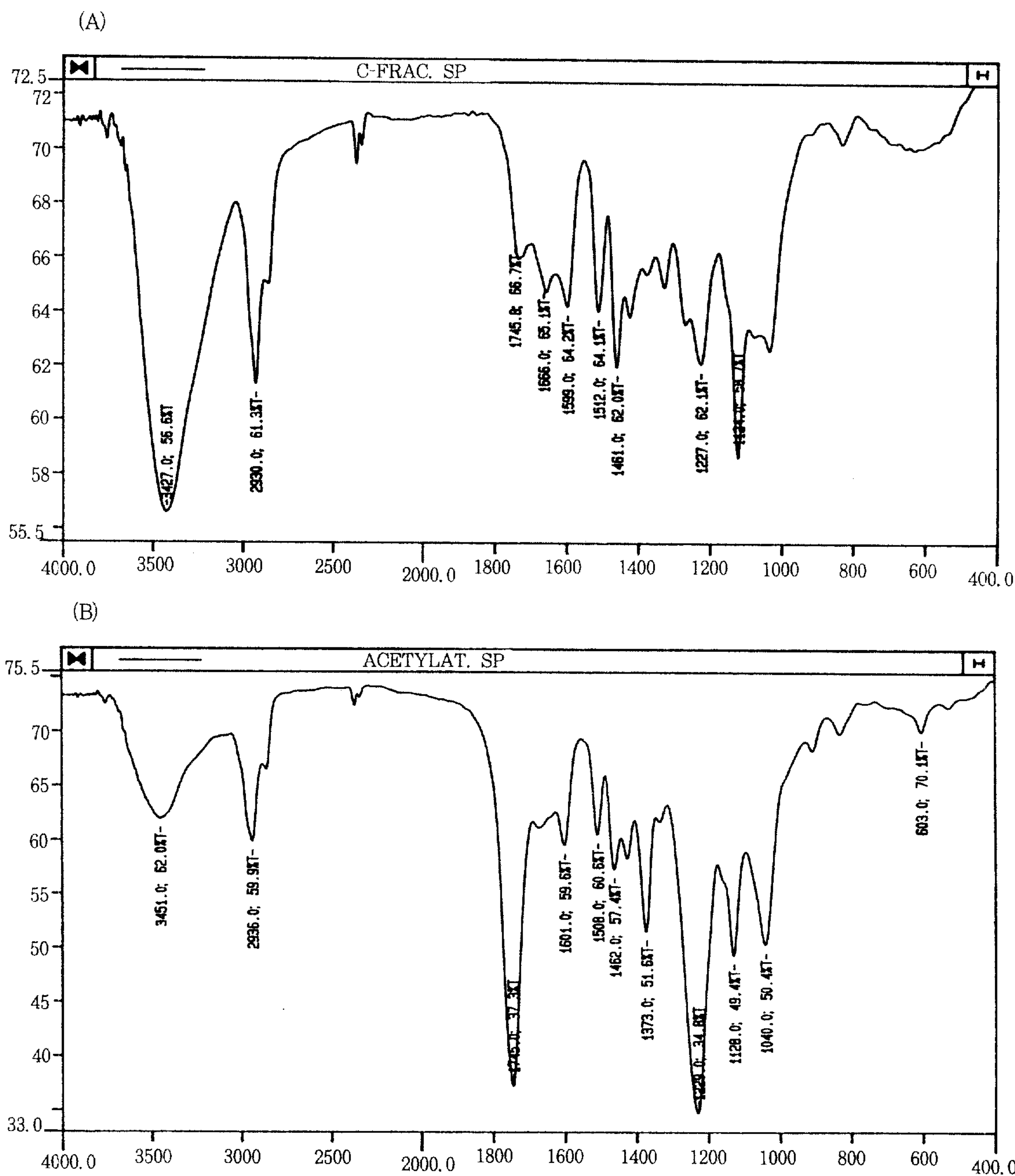

Fig. 3. IR Spectra of Fraction C without acetylation(A) and with acetylation(B).

3.3.3 Color reaction with spray reagents

When TLC chromatogram was sprayed with Dragendorff's, Mayer's and Marquis' reagents, alkaloidal components visualized as red, white and brown bands respectively. But ninhydrin spray reagent did not induce color reaction with TLC chromatogram.

From the result of color reaction, it is concluded that the nitrogeneous compounds in fraction C belong to not proteineous compound but

(A)

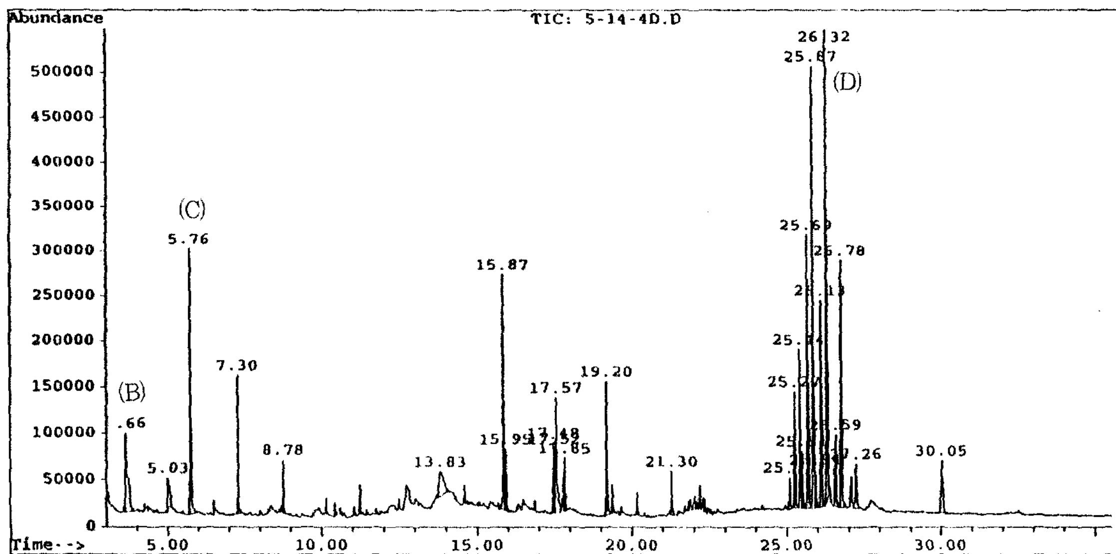

Fig. 4. GC-MS Spectra of Fraction C. (A) FID Chromatogram of Fraction C. (B) MS Spectrum of type Ⅲ alkaloid compound. (C) MS spectrum of type Ⅱ alkaloid compound. (D) MS spectrum of type Ⅲ alkaloid compound.

ppm 162.69 153.26 153.02 152.72 147.18 147.01 146.64 144.79 134.28 131.80 130.06 128.16 128.07 118.73 114.27 102.73 86.72 85.60 77.26 77.20 77.00 76.75 72.40 71.64 70.38 60.37 56.24 56.08 55.82 54.26 38.58 36.44 33.82 31.76 31.38 29.53 29.19 28.98 27.95 27.06 25.74 25.47 25.38 24.72 23.45 22.52 15.48 13.96

Fig. 5. ^{13}C-NMR Spectrum of Fraction C.

alkaloid.

3.3.5 GC-MS analysis

A variety of compounds were isolated from fraction C by gas chromatography(Fig. 4) and mass fragmentation pattern of each compound was recorded by mass spectrometer.

Several methyl ester of fatty acids and phthalides were identified by Wiley138 library of MS spectra. These compounds includes methyl hexadecanoate, methyl 9, 12, 15-octadecatrienoate, methyl 8, 11-octadecadienoate, methyl 15-octadecenoate, methyl octadecanoate, dibutyl phthalate, bis(2-ethylhexyl)phthalate and butyl phthalate, ester with butylglycolate.

The unknown compounds, having odd number of molecular ion fragment, amounted to ca 78% of fraction C on the basis of integrated GC peak areas. These major components in fraction C are categorized into alkaloid in view of the result of elementary analysis, color reaction with spray reagents and IR spectra.

According to the mass fragmentation pattern, they can be divided into 3 types of alkaloids and postulated to comprise of odd number of nitrogen in accordance with 'nitrogen rule'. In the MS spectra of these compounds, M+2 peak does not allow for the presence of sulfur, chlorone or bromine.

Among 3 types of alkaloids, type Ⅲ alkaloids were main components of fraction C(about 62%, based on integrated GC peak areas). In the MS spectrum of type Ⅲ alkaloid, methyl fragment cluster proves the presence of long aliphatic side chain(Fig. 4).

3.3.6 ^{13}C-NMR spectrum

Judging from the intense peaks around 30ppm in the spectrum, the carbon skeleton of major components in fraction C is supposed to be made up of mainly secondary carbon. Several peaks around 60ppm may result from the carbons bonded with amine or hydroxyl group. Triplet around 76ppm is the peak of CDC_{l3} solvent(Fig. 5).

4. CONCLUSION

Chloroform soluble fraction, obtained from Korean camellia mistletoe(***Pseudixus japonicus*** Hayata) showed the highest cytotoxicity on primary screening tumor cell line, $P388D_1$ among 5 fractions. By treatment of chloroform extract, lymphoma cells were necrotized strongly and the growth of transformed mouse embryo fibroblast and human cancer cell lines were inhibited to some extent.

Through elementary analysis, color reaction with spray reagent and spectra of IR, GC-MS and ^{13}C-NMR, major components of chloroform extract were ascertained to be 3 types of alkaloids, and a minority of methyl ester of fatty acids and phthalides were identified.

In the sequel, it can be carefully surmised that synergistic effect of alkaloids, methyl ester of fatty acids and phthalides might be responsible for the cytotoxicity of chloroform extract, isolated from Korean camellia mistletoe.

It is highly recommended that purification, cytotoxicity test and *in vivo* assay of individual compounds in chloroform soluble fraction should be followed to elucidate the component showing anticancer activity on molecular level.

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