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Cytotoxicity and antimicrobial effects of the methanolic extract of *Sophora flavescens* Ait. (IV)

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SUMMARY

This study was carried out to evaluate cytotoxicity of the methanol extract from *Sophora flavescens* Ait. against L1210 (lymphocytic leukemia) and $P388D_1$ (lymphoid neoplasma) Cells *in vitro*. We have determined cytotoxicity by the MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H- tetrazolium bromide) assay. The order of cytotoxicity of *Sophora flavescens* Ait. extracts against L1210 and $P388D_1$ cells *in vitro* is as follows : Fr. 4 > Fr. 3 > Fr. 5 > Fr. 2 > Fr. 1. These results suggest that the fraction 4 of the methanol extracts from *Sophora flavescens* Ait. may be a valuable choice for the development of antitumor agents. In order to develop an antimicrobial agent, dried *Sophora flavescens* Ait. was extracted with hot methanol, and then antimicrobial activity (MIC test) was investigated. In this study, the fraction 3 of the methanol extracts from the roots of *S. flavescens* showed strong growth inhibition activity against gram-positive and gram-negative bacteria (MIC, 3.125 μg/ml) such as *S. mutans*, *S. epidermidis* and *P. putida*. These results indicate that fractions 3 and 4 inhibit tumor cells and bacteria.

INTRODUCTION

Sophora Radix extraction, the roots of *Sophora flavescens* Ait. *(Leguminosae)* has been widely used in traditional medicine as a diuretic, stomachic, antipyretic and anthelmintic uses in Korea (Lee, 1996). The plant, *S. flavescens* Ait. grows as a perennial herb and is widely distributed in Korea (Lee, 1989). Phytochemical studies of this plant has so far yielded such alkaloids as flavonoids, chromones, and saponins (Tang and Ecsenbrand., 1992; Ryu *et al.*, 1996; Ding *et al.*, 1992). In the constant effort to improve the efficacy and

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ethics of modern medical practice, researchers are increasingly turning their attention to folk medicine as a source of new drugs (Haslam, *et al.*, 1989).

We recently reported that the methanol extract of the roots of S. *flavescens* showed strong growth inhibition activity against gram-positive bacteria (MIC, 25-50 ㎍/㎖) such as B. *subtilis* and S. *aureus*. Among gram-positive bacteria tested, B. *subtilis* was the most susceptible to the extracted substance. The antimictobial activity of the methanol extract from the sample had also s strong growth inhibition activity against gram-negative bacteria such as P. *aeruginosa* (MIC, 25 ㎍/㎖) (Cho *et al.*, 1999a). A previous study was carried out to evaluate the cytotoxicity of the extracts from *Sophora flavescens* Ait. against L1210 (lymphocytic leukemia) and P388D_1 (lymphoid neophasma) cells *in vitro*. We have determined cytotoxicity by MTT assay. The order of cytotoxicity of S. *flavenscens* Ait. extracts against L1210 and P388D_1 cells *in vitro* is as follows: adriamycin > ethyl acetate extract of S. *flavenscens* Ait. > chloroform extract of S. *flavenscens* Ait. > methanol extract of S. *flavenscens* Ait. > water extract of S. *flavenscens* Ait. > hexane extract of S. *flavenscens* Ait. > and adriamycin > ethyl acetate extract of S. *flavenscens* Ait. > chloroform extract of S. *flavenscens* Ait. > methanol extract of S. *flavenscens* Ait. > hexane extract of S. *flavenscens* Ait. > water extract of S. *flavenscens* Ait. (Cho *et al.*, 1999b).

Nowadays, the development of resistance by pathogens to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents. Hence this *in vitro* study was aimed at screening selected Korean medicinal plants for their antimicrobial activity, evaluating their potential use in treating dermatomucosal infections caused by bacteria and fungal species, and determining whether their use in folkloric medicine is justified.

MATERIALS AND METHODS

Plant collection

Mature plants were collected at Kochang Chonbuk, South Korea, in July 1998. The roots of *Sophora flavescens* Ait. were collected and dried in the shade. Voucher specimens (980705-10) of the plants were deposited at the Department of Natural Products, Professional Graduate School of Oriental Medicine, Wonkwang University, South Korea.

Methanolic extract

The dried and chopped roots (45.0 g) of S. *flavescens* were extracted with hot MeOH (3 × 950 ㎖) thrice at 62℃ for 4 hours. Methanol was removed by rotary evaporatior from the combined, filtered to give a brown gum (84.7 g). This gum (4.0 g) was coated onto C18 (8.0 g) and packed onto a C18 column (40.0 g). This column was developed in a stepwise manner with H_2O, CH_3CN : H_2O mixtures, CH_3CN : $CHCl_3$ mixtures, and 95% EtOH. Details of eluents and fraction volumes are given in Table I (Blunt *et al.*, 1987). Each fractions were assayed for antimicrobial activity.

Biological activity test

Microorganisms

The microorganisms used included : *Staphylococcus aureus* (ATCC 6538P), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 10539), *Pseuomonas aeruginosa* (ATCC 1636), *Micrococcus luteus* (ATCC 9341), *Aspergillus niger* (ATCC 9029) and *Candida albicans* (ATCC 10231).

Screening for antimicrobial activities

The dried plant extracts were dissolved in 10% aqueous dimethyl sulfoxide (DMSO)

to a final concentration of 2000 ㎍/㎖ and sterilized by filtration through a 0.45 ㎛ membrane filter. Antimicrobial tests were then carried out by the Agar serial Dilution method (You *et al*,. 1994; Min *et al.*, 1996). Each of several concentrations of the tested extracts in molten agar was poured into a petri plate, and allowed to solidify. The organisms containing 10^6 bacterial cells/㎖ or 10^8 yeast cells/㎖ were inoculated into the petri plates. After the plates have been incubated for 24h at 37℃ for bacteria and for five-seven days at 22℃ for fungi, the lowest concentration of methanol extracts that inhibits growth of the organisms, was determined as the MIC of the antimicrobial agents. Amoxicillic acid sodium served as positive controls for *S. aureus, B. subtilis, E. coli* and P. *aeruginosa,* consequently, whereas, ketoconazole served as a positive control for C. *albicans and A.niger.* Each test was carried out in triplicates.

Screening for cytotoxocity (MTT assay)

Cell lines (L1210, P388D1, BSC) were suspended at 2×10^5 cells/㎖ in complete medium (10% fetal bovine serum) containing each concentration of two-fold dilution series of the sample, vigorously vortexed and then 100 ㎕ aliquots were dispensed into 96-well flat-bottomed microtiter plates using a multichannel pipet. Plates were then incubated at 37℃ for 72 h in 5% CO_2 incubator. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at 5 mg/㎖ and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. An aliquot of 10 ㎕ of MTT stock solution was added to each well using a multichannel pipette and the plate was incubated at 37℃ for 4 h. To each well 150 ㎕ of 0.01N HCl solution containing 10% sodium dodecyl sulfate was added to solubilize the MTT formazan. Plates were gently shaken until all formazan crystals were dissolved, and the absorbance at 540 nm was determined with a Microplate Reader (SPECTRA MAX 340). All results were corrected for background absorbance detected in wells without added MTT. Preliminary experiments established a linear relationship between the cell numbers and the absorbance at 540 nm, when cells in the range of 4×10^2 to 4×10^5 per well examined. Results were expressed as an IC_{50} represented as a dilution factor ($* 10^{-6}$) of the concentration of the extract. Extract concentrations were not determined, but normally fall in the range 5 to 10 ㎎/㎖. An extract with an activity of 1000 and a concentration of 10 ㎎/㎖ would have an IC_{50} of 10 ㎍/㎖.

Statistical analysis

All values, expressed as mean ± standard deviation, were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

The dried and chopped roots of *S. flavescens* were extracted three times with MeOH for 4 hours at 62℃. In order to fractionate the resultant MeOH extracts, it was applied over reverse-flash chromatography using the solvents of H_2O - CH_3CN mixture, CH_3CN, $CHCl_3$, EtOH and CH_3CN - $CHCl_3$ mixture All fractions studied in this work showed antimicrobial activity against test microorganisms. This result showed that some of the studied fractions are potentially a rich source of antimicrobial agents. However, the fractions differ significantly in their activity against test microorganisms. The most active fraction was 1 : 9 H_2O : CH_3CN, whereas, 9 : 1, 3 : 1 and 1 : 1 H_2O : CH_3CN, 100% $CHCl_3$ and EtOH (Table 1).

Table 1. C_{18} Reverse-phase flash chromatography of the methanol extract from *Sophora flavescens* Ait

Fraction	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5	Fr. 6	Fr. 7	Fr. 8	Fr. 9	Fr. 10
Mobile phase	H_2O	H_2O : CH_3CN (9 : 1)	H_2O : CH_3CN (3 : 1)	H_2O : CH_3CN (1 : 1)	H_2O : CH_3CN (1 : 3)	H_2O : CH_3CN (1 : 9)	CH_3CN	CH_3CN : $CHCl_3$ (3 : 1)	$CHCl_3$	EtOH
Mass (mg)	520	208	276	2,247	289	32	34	63	220	57

For A. niger and C. *albicans*, all fractions against these yeasts were inactive. Differences in antimicrobial activity of the test plants are obviously related to differences in their contents of active compounds. The most active fractions studied in this work seem to possess antimicrobially active compounds of including essential oils (especially thymol), flavanoids and triterpenoids and other components of phenolic nature or with free hydroxyl group, which are classified as active antimicrobial components (Rojas *et al.*, 1992). However, some of the moderately active and least active plants were also reported to have similar and other active compounds but probably in smaller amounts. 1 : 9 H_2O : CH_3CN fraction (MIC = 6.25 $\mu g/m\ell$) showed the most antimicrobial activity against gram-positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacterium (*P. aeruginosa*). This fractions (MIC = 12.5 $\mu g/m\ell$) showed antimicrobial activity only against gram-positive bacterium (*M. luteus*). 1 : 3 H_2O : CH_3CN fraction (MIC = 12.5 $\mu g/m\ell$) showed antimicrobial activity against gram-positive bacteria (*S. aureus*, *B. subtilis* and *P. aeruginosa*) and gram-negative bacterium (*P. aeruginosa*). H_2O fraction (MIC = 25 $\mu g/m\ell$) showed antimicrobial activity against gram-positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacterium (*P. aeruginosa*). 3 : 1 CH_3CN : $CHCl_3$ (MIC = 50 $\mu g/m\ell$) showed antimicrobial activity against gram-positive bacteria (*S. aureus*, *B. subtilis* and *M. luteus*) and gram-negative bacterium (*P. aeruginosa*). The hot methanol extract of the plant, 9 : 1, 3 : 1 and 1 : 1, H_2O : CH_3CN, 100% $CHCl_3$ and EtOH (MIC = 50 $\mu g/m\ell$) showed antimicrobial activity against three gram-positive and negative and fungi (Table 2).

The cytotoxic activities of fractions of the methanol extract of *Sophora flavescens* Ait. evaluated against L1210 and $P388D_1$ cell lines by the MTT assay. Reverse-flash chromatography concentrated the cytotoxic activity in fractions eluted with H_2O : CH_3CN 1 : 3. As shown in Table 3, they exhibited strong inhibitory effects on the growth of these cell lines with IC_{50} ($\mu g/m\ell$) values of 4.98 and 3.98, respectively. The cytotoxicity of fraction 4 appears to be weaker than that of fraction 5. However, the other fractions showed no significant activities (Table 3).

Table 2. Minimum inhibitory concentrations (MIC) of fractions from *Sophora flavescens Ait.* extracted with methanol against various microorganisms

Microorganism	MIC (㎍/㎖)[a]												
	Amocla	ketoco-nazole	Fr.1	Fr.2	Fr.3	Fr.4	Fr.5	Fr.6	Fr.7	Fr.8	Fr.9	Fr.10	HMTSF
S. aureus	<6.25	>200	25	>200	>200	>200	12.5	6.25	-	50	>200	>200	>200
B. subtilis	<6.25	>200	25	>200	>200	>200	12.5	6.25	-	50	>200	>200	>200
M. luteus	<6.25	>200	>200	>200	>200	>200	12.5	12.5	-	50	>200	>200	>200
E. coli	<6.25	>200	>200	>200	>200	>200	>200	>200	-	>200	>200	>200	>200
P. aeruginosa	<6.25	>200	25	>200	>200	>200	12.5	6.25	-	500	>200	>200	>200
A. niger	>200	<6.25	>200	>200	>200	>200	>200	>200	-	>200	>200	>200	>200
C. albicans	>200	<6.25	>200	>200	>200	>200	>200	>200	-	>200	>200	>200	>200

Plants extracts : HMTSF; Hot methanol of *Sophora flavescens* Ait.
[a] Extract and fractions were examined in triplicate experiments. Not tested: -

Table 3. Results of the *in vitro* cytotoxicity of various fractions of *S. flavenscens* Ait. extracts by the MTT assay[a]

Cells	IC_{50} (㎍/㎖)[b]											
	HMTST	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5	Fr. 6	Fr. 7	Fr. 8	Fr. 9	Fr. 10	AM
L1210	29.67	30.34	28.51	26.68	14.87	4.98	25.86	26.15	26.45	32.56	26.4	0.06
P388	29.18	28.48	24.23	28.32	8.73	3.98	20.79	24.68	22.72	25.43	18.72	0.5

Plants extracts : HMTSF; Hot methanol of *Sophora flavescens* Ait. AM : Adriamycin.
[a]Each fraction was examined in triplicate experiments.
[b]IC_{50} represents the concentration of a fraction required for 50% inhibition of cell growth.

Si gel column chromatography concentrated the cytotoxic activity in fractions eluted with ethyl acetate - hexane (50-90%). It spreads this activity across several fractions. Fraction 4 was the major cytotoxic component of *Sophora flavescens* Ait., active against L1210 and $P388D_1$ cells at about 8.1 ㎍/㎖, showed cytotoxic effects against monkey kidney (BSC) cells at about 29.5 ㎍/㎖. However, it has less active than adriamycin as a reference compound (Table 4).

Table 4. The antitumor activities of fraction 5 of the methamol extract of *Sophora flavescens* Ait. Comparison of IC_{50} for fraction 5 of the methamol extract of *Sophora flavescens* Ait. by the MTT assay[a]

Fraction	IC_{50} (μg/mℓ)[b]						
	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5		
Mobile phase / Cell	2-10 % EA/Hx	10-20 % EA/Hx	20-50% EA/Hx	50-90% EA/Hx	90-100% EA/Hx, 100% EtOH, CH_3CN	AM	HMTSF
L1210	>20	23.03	8.40	8.07	17.33	0.02	20.67
$P388D_1$	60.01	18.23	8.38	8.18	15.10	0.03	29.18
BSC	80.28	26.92	12.80	29.48	27.77	-	-

Plants extracts; HMTSF; Hot methanol extract of *Sophora flavescens* Ait.; AM; Adriamycin. Not tested: -

[a]Each fraction was examined in triplicate experiments (mean ± standard deviation (n=3)).
[b]IC_{50} represents the concentration of a fraction required for 50% inhibition of cell growth.

The antimicrobial effects of fraction 4 appears to be weaker than that of fraction 3. Ethyl acetate : hexane (20 - 50%) fraction (MIC = 3.125 μg/mℓ) showed the most antimicrobial activity against gram-positive bacteria (*S. aureus* and *S. epidermidis)* and gram-negative bacterium (*P. putida*). Fraction 3 exhibited the same antimicrobial activity against gram-positive bacteria (*S. mutans;* MIC = 3.125 μg/mℓ) with ampicillin as a reference. However, Ethyl acetate : hexane (50 - 90%) fraction showed the antimicrobial activity against gram- positive bacteria (*S. mutans;* MIC = 12.5 μg/mℓ and *S. epidermidis;* MIC = 6.25 μg/mℓ) and gram-negative bacterium (*P. putida;* MIC = 25 μg/mℓ). Ethyl acetate : hexane (10 - 20%) fraction showed the antimicrobial activity against gram-positive bacteria (*S. aureus* and *S. epidermidis;* MIC = 50 μg/mℓ) and gram-negative bacterium (*P. putida;* MIC = 25 μg/mℓ). However, the other fractions showed no significant activities (Table 5).

In conclusion, the results obtained proved that the fraction 4 of the methanol extracts from *Sophora flavescens* Ait. may be a valuable choice for the development of antitumor agents. The fraction 3 will be interesting to test inhibition activity against bacteria and fungi.

Table 5. Minimum inhibitory concentrations (MIC) of fraction 5 of the methanol extract of *Sophora flavescens* Ait. against microorganisms[a]

Microorganisms	MIC (μg/mℓ)					
	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5	AP
S. mutans	>200	50	3.125	12.5	>200	3.125
S. epidermidis	>200	50	3.125	6.25	>200	50
S. aureus	>200	50	3.125	3.125	>200	3.125

[a]Each fraction was examined in triplicate experiments (mean ± standard deviation, n=3). AP:ampicillin

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