대한한의학회지 : 제18권 제1호
K.O.M.S. Vol. 18. No. 1. 1997

A Study for the isolation of the Berberine-type Alkaloid from Coptidis Rhizoma and for their Antitumor Activities

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ABSTRACT

A Study for the isolation of the Berberine-type Alkaloid from Coptidis Rhizoma and for their Antitumor Activities

The purpose of this study is the separation of biologically active ingredients from Coptidis Rhizoma which has been widely used as one of oriental herbal medicine for body fever. In this study, berberine-type alkaloids were tested on their biological activities in the aspect of antibacterial, antitumor, anti-herpetic and anti-HIV activity.

Contents of five major alkaloids for the various origin of Coptidis Rhizoma were assayed by HPLC. As the results, the content of berberine from Coptis chinensis and Coptis japonica were 6.78% and 7.09%, respectively. The contents of coptisine, jatrorrhizine, berberastine from Coptis chinensis were higher than those of Coptis japonica. The amount of palmatine from both species were almost the same.

Surprisingly for antitumor experiment, all compounds have been shown remarkable activity, es

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pecially against SNU-1(human stomach cancer) cell line. Among the compounds purified through column chromatography, palmatine, coptisine, and jatrorrhizine inhibited the growth of K-562 (human chronic myelogenous leukemia) cell line whereas jatrorrhizine has been shown the effective inhibition of A-549 (human lung) cell line at the same time.

Ⅰ. Introduction

Since Coptidis Rhizoma is described at the first phytology text, <神農本草經> 上品 as '味苦寒 主熱氣目痛眥傷泣出明目腸澼腹痛下痢婦人陰中腫痛 久服令人不忘 一名玉連 生川谷'[8], another text in 傷寒論 it is explained as "心, 胃經處 歸經"[13,14,16], "入心, 肝, 胃, 大腸-淸熱燥濕 淸心除煩 瀉火解毒"[3,4,16] as well as othe herbologic text book[7,9,10,11,12] and regarded as one of important oriental herbal medicine.

This medeicine belongs to "毛茛科"[2,16,17] and its family will be 川黃連 Coptis chinensis FRANCH[3,5,16], 日黃連 Coptis japonica MAKINO var. dissecta NAKAI[4,17,38] or 雅連(三角葉黃連) C. deltoidea C.Y. CHENG et HESIAO[15,16], 雲連(雲南黃連) C. teetoides C.Y. CHENG, 野連(野黃連, 峨眉野連) C. omeiensis C.Y. CHENG.[15,16,17]

Untill present study relating Coptidis Rhizoma, study on alkaloid, organic salts, inorganic salts, and coumarin[19,274] are under progress. It was found that the components of alkaloid listed on Fig. 1, berberine[1,4,16,17] and nine other alkaloid[18,19,21,22,26], magnoflorine[20,24,25]. Except for magnoflorine, others are berberine type alkaloid[34]. And berberrubine[27] is reported as modified berberine type.

There was comparison between Chinese C. chinensis, C. deltoides and Japanese C. japonica having quantitative analysis on berberine, coptisine, palmatine, epiberberine, berberastine, jatrorrhizine.[19,21,23]

Concerning screening test, there were reports on antibacterial test[16,17], antiviral test[41], antiphlogistic test[20], anticancer activity[28], antifungal activity[36,37] as well as the leather bottle gastritis[30], prewarning diarrhea treatment[39], treatment on mouth[31], treatment on ophthalmology[41] and in vitro test[32,33,35].

In the area of screening test among alkaloids in Coptidis Rhizoma, only the result with berberine[20,24], berberrubine[28], coptisine[40] were reported. On the other hand, the difference on screening effect between single pure compound and mixed compound is not reported yet. Therefore isolated pure compound is need to be tested on antibacterial effect, antiviral effect, antitumor effect for comparison and understanding the efficiency of mixed herbal medicine as the initial stage investigating oriental herbal medicine.

In present study, antibacterial effect, antiviral effect, antitumor effect with Coptis chinensis and Coptis japonica were carried out. In addition, the quantitative difference between two species was compared for next experiment.

Ⅱ. Reagents and Instruments

1. Material and Reagents

1) Oriental Herbal Medicine

The material for present study was purchased at "同仁堂集用南城批發大樓" Beijing, China for Coptis

chinensis and Tochimoto Tenkaido Co. LTD product for Coptis japonica through Seoul Guangmeong pharmateutical Co.. Two species of Coptis rhizoma was dried without sunlight for several weeks and was grinded into 40 mesh for next experiments.

2) Standards and Reagents

The berberine standard over 98.0% in purity was bought at Wako Co.(Tokyo, Japan) without further purification. Other solvents, such as acetonitrile, methanol, chloroform, methylene chloride, hexane, butanol, benzene, ethanol were high quality HPLC grade and were provided by J. T. Baker Co.(Phillipsburg, U.S.A.). Potassium phosphate or SDS were reagent grade and were suplied by Sigma Chemical Co.(St. Louise, MO, U.S.A.). All solvents prepared for HPLC separation were filtered through membrane filter or treated in ultrasonicator degassing process with water vacuum.

2. Instrumentation

For qualitative and quantitative analysis on Coptis rhizoma, High Performance Liquid Chromatograph was used with HP-HPLC 1050 model by Hewlett Packard Co. equipped with Diode Array Detector at the range of 200~650nm UV full spectrum. Distilled water was prepared through Milli-Q water system by Millipore Co., The herbal sample was crushed with Grinder(Thomas사, Philadelphia, U.S.A.). HPLC Column was C18(Capcell pak, Type UG 120Å, 5㎛, 4.6×250mm, Shisheido, Japan).

Ⅲ. Experimental

1. Preparation of Standard Soluti on

Berberine reference standard 2.09 mg was weighed exactly, transferred to 10mL volume tric flask and filled with absolute methanol to the mark. This stock solution was dilluted and used for each experiment.

2. Relative Composition of Coptis Cheinesis and Coptis Japonica

For quantitative analysis on alkaloids of Coptis rhizoma originated from China or Japan, 40 mesh grinded sample were weighed at 1.00g and placed in round bottomed flask followed by reflux with 50mL methanol for 2 hours at 60oC. The extract was filtered and measured with HPLC at the condition listed on Table I. By comparison of peak area with standard solution as following equation will yield the content of berberine in the tested sample.

content of berberine(%)=C. of std soln (㎎/mL)× $\frac{A_T}{A_S} \times \frac{S_{mL}}{S_g} \times \frac{100}{100}$

A_T : area under curve of test sample (mAU)
A_S : area under curve of berberine (mAU)
S_g : weight of Coptidis Rhizoma (g)
S_{mL} : volume of extract (mL)

Table I. Analytical Conditions of HPLC for Copti dis Rhizoma from China or Japan

Column : Shisheido, Capcell Pak UG120Å 4.6×250mm 5㎛
Detector : UV detector 254nm
Mobile phase : 1/15M KH_2PO_4/CH_3CN/SDS(300 : 270 : 3.3)
Flow rate : 1mL/min
Oven temp : 40℃

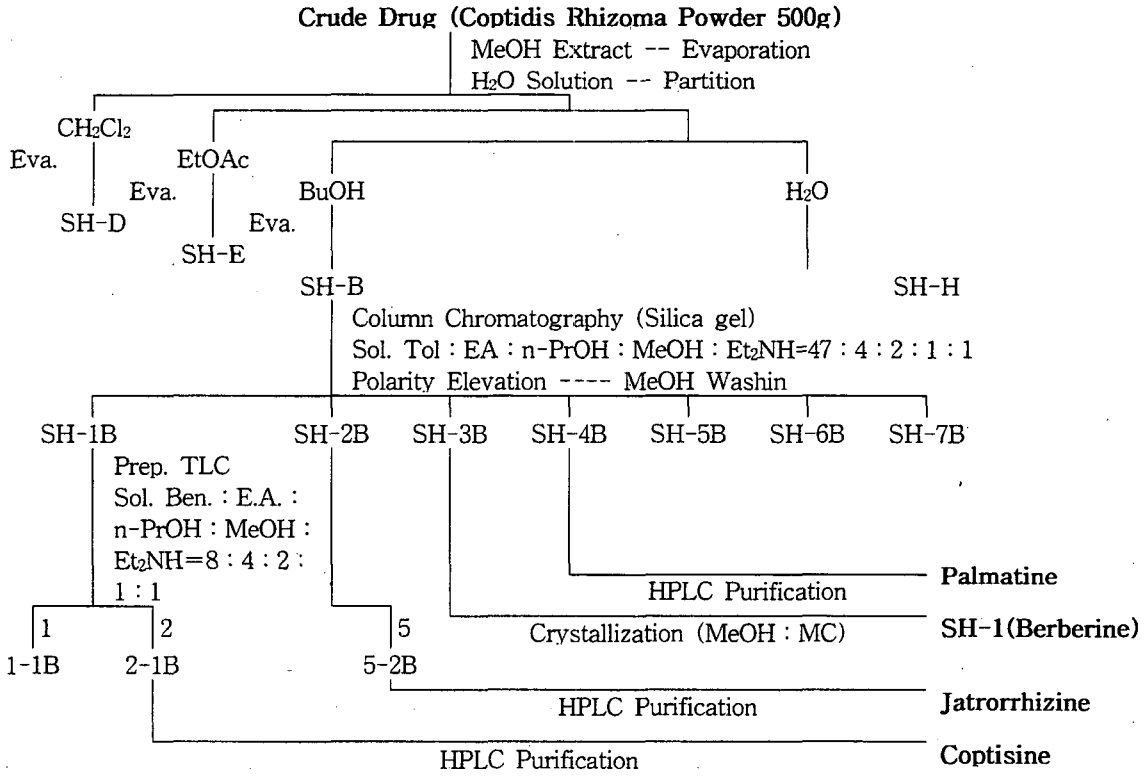

Figure 1. Flow Diagram of the Isolation and Purification of Components from Coptidis Rhizoma

(1) Strain

The microorganisms which were used for MIC (Minimum Inhibitory Concentration) determination, were transfered from KRICT, (Table II) and MIC values were compared with reference anribiotics each time.

(2) Medium

Fleisch Extract Broth which contains 1% beef extract, 1% Bacto peptone, 0.3% NaCl, 0.2% $Na_2HPO_4 \cdot 12H_2O$ at pH 7.4~75 was used for culture medium for MIC test. For streptococcus, 10% horse serum was used additionally.

For the slent medium, Fleisch Extract Broth with 2% Bacto agar was used, Müeller Hinton Medium for pseudomonas, Müeller Hinton Medium with 10% Sheep blood for streptococcus, were used.

(3) Reference Antibiotics and Test Samples

Cefpirome was used as reference abtibiotics and SH-1 was assayed for antibacterial activity.

3. Isolation and Purification of Coptidis Rhizoma by Column Chromatography

The flow diagram of the isolation and purification method for the alkaloids from Coptidis Rhizoma is shown in Figure 1.

4. Biological Activity Assay of the Alkaloids from the Coptidis Rhizoma

1) Antibacterial Effect

Antibacterial activity was assayed using Agar Dilution Method[43] at KIST screening la boratory.

Table III. Virus, Host Cell and Assay Information

Virus Information			Host Cell Information		Assay Information	
Virus	HSV-1	HSV-2			Assay	Cep/MTT
Strain	F	MS	Cell Passage	Vero(CCL81) 10	Day	3ed
Batch No.	A1	A5				
Inoc. size	56	66			Patee No.	95H024A
Dilution	44.00	73.33	Remark			

2) Anti HIV Activity Assay

Anti HIV activity was assayed at National Hygenic Institute Immune Deficiency Lab. by MTT assay method[6]. The components which were extracted with organic solvents or water, such as SH-B(BuOH), SH-D (CH_2Cl_2), SH-E(EtOAc), SH-H(H_2O) were tested for antivial activity.

Table II. List of Bacterial Strains

No.	strains
1	*Streptococcus pyogenes* 308A
2	*Streptococcus pyogenes* 77A
3	*Streptococcus faecium* MD8b
4	*Staphylococcus aureus* SG511
5	*Staphylococcus aureus* 285
6	*Staphylococcus aureus* 503
7	*Escherichia coli* 055
8	*Escherichia coli* DCO
9	*Escherichia coli* DC_2
10	*Escherichia coli* TEM
11	*Escherichia coli* 1507E
12	*Pseudomonas aeruginosa* 9027
13	*Pseudomonas aeruginosa* 1592E
14	*Pseudomonas aeruginosa* 1771
15	*Pseudomonas aeruginosa* 1771M
16	*Salmonella typhimurium*
17	*Klebsiella oxytoca* 1082E
18	*Klebsiella aerogenes* 1522E
19	*Enterobacter cloacae* P99
20	*Enterobacter cloacae* 1321E

HIV-1 virus was obtained from H9/HTLV-III cells (ATCC No. CRL-8543) and stored at -70oC deep freezer (REVCO, ULT-1685) in small aliquots (0.5mL). Antiviral activity was determimed by observation of Cytopathic effect(CPE) at MT-4 cells.

3) Anti Herpes Simplex Virus (HSV) Activity Assay

Activity was assayed at KRICT Screening Lab. by CPE/MTT assay method[6]. In Table III, informations on Virus and host cell is listed.

4) Antitumor Effect

Primary test was carried out at KRICT Screening Lab. by SRB assay method and, second test was carried at KIST Screening Lab. by SRB assay method.

(1) Tumor cell lines

A549 (Human lung)
SKOV-3 (Human ovarian)
SKMEL-2 (Human melanoma)
XF-498 (Human CNS)
HCT-15 (Human colon)
K-562 (Human chronic myelogenous leukemia)

SNU-1 (Human stomach)

(2) Test Samples

1st Experiment : SH-D, SH-E, SH-B, SH-H

2nd Experiment : 1-1B, 2-1B, 5-2B, SH-1, SH-4B, SH-6B, SH-7B

3ed Experiment : palmatine, coptisine, jatro rrhizine which were puri fied once again

(3) Assay Method

In vitro screeningSRB assay method[42] which was used for fast In vitro screening in general, was adaped.

Ⅳ. RESULT

1. Relative Composition of Coptis cheinesis and Coptis japonica

Contents of berberine-type ingredients were analyzed by HPLC and the result are shown in the Table Ⅳ.

Table Ⅳ. Quantitative analysis of Rhizoma of Cop tis Chinensis and Coptis japonica.

Compound	*Coptis chinensis*	*Coptis japonica*
Berberine	55.64%	56.71%
Palmatine	12.13%	12.13%
Coptisine	16.24%	15.93%
Jatrorrhizine	8.34%	7.87%
Berberastine	7.64%	7.31%

2. Isolation and Purification of Co pidis Rhizoma by Column Chro matography

After column chromatography, SH-4B, 5-2B and 2-1B fractions were purified again by prerative HPLC. The operating conditions are shown in Table I. Excess MeOH was added into each fractions to remove SDS and phosphoric acid salt and stood by for one day. After filtration, filtrate was concentrated and dissolved in water and re-extracted with E.A. and CH_2Cl_2. Solvent was evaporated and recrystalized from MeOH to give palmatine 18.0mg, coptisine 19.3mg, jatrorrhizine 11.2mg respectively.

3. Biological Activity of the Alkalo ids from the Coptidis Rhizoma

1) Antibacterial Effect 0f Alkaloids from Coptidis Rhizoma

Test sample SH-1 contains 97% berberine which is major alkaloid of Coptidis Rhizoma. Antibaterial activity of SH-1 was very weak. Only the MIC for Streptococcus pyogenes 77A strain was 25μg/mL (Table V) and others were more than 100μg/mL. Therefore, berberine, major ingradient of Coptidis Rhizoma, has practically no antibacterial activity.

2) Antiviral(HIV) Effect of Alkaloids from Coptidis Rhizoma

CD50 values, which indicate cytotoxic activity to host organism, of SH-1, SH-D, SH-E, SH-B and SH-H were too low to observe the antival effect. Therefore SH-1, SH-D, SH-E, SH-B and SH-H could not give reseanable result for antivial effect. (Table VI)

Table V. Result of Antimicrobial Susceptibility Test with Sample SH-1

	strains	Minimal Inhibitory Concentration (μg/mL)	
		Cefpirome	SH-1(Berberine)
1	*Streptococcus pyogenes* 308A	0.007	>100
2	*Streptococcus pyogenes* 77A	0.004	25
3	*Streptococcus faecium* MD8b	6.25	>100
4	*Staphylococcus aureus* SG511	0.391	100
5	*Staphylococcus aureus* 285	0.781	>100
6	*Staphylococcus aureus* 503	0.098	>100
7	*Escherichia coli* 055	0.013	>100
8	*Escherichia coli* DCO	0.013	>100
9	*Escherichia coli* DC2	0.049	>100
10	*Escherichia coli* TEM	0.049	>100
11	*Escherichia coli* 1507E	0.025	>100
12	*Pseudomonas aeruginosa* 9027	3.125	>100
13	*Pseudomonas aeruginosa* 1592E	1.563	>100
14	*Pseudomonas aeruginosa* 1771	0.391	>100
15	*Pseudomonas aeruginosa* 1771M	0.391	>100
16	*Salmonella typhimurium*	0.013	>100
17	*Klebsiella oxytoca* 1082E	0.781	>100
18	*Klebsiella aerogenes* 1522E	0.013	>100
19	*Enterobacter cloacae* P99	3.125	>100
20	*Enterobacter cloacae* 1321E	0.013	>100

Method : Agar Dilution Method

Table VI. Antiviral(HIV) Effect of SH-1 Separated and Ingredients Partitioned from Coptidis Rhizoma

Sample Name	CD50 (μg/mL)
SH-D(CH_2Cl_2 Layer)	1.163
SH-E(EA Layer)	7.040
SH-B(BuOH Layer)	≤0.781
SH-H(H_2O Layer)	≤0.781
SH-1(Berberine)	≤0.391

CC : 0.945 VC : 0.112 (11.9%)

3) Antiviral(HSV) Effect 0f Alkaloids from Coptidis Rhizoma

For test sample SH-D, SH-E, SH-B and SH-H, CC_{50} which indicate cytotoxicity activity to host organism, was higher than EC_{50} for infected cells by HSV-1 or HSV-2. SI value was smaller than 1 or uncalculable. Therefore test sample SH-D, SH-E, SH-B and SH-H have no significant antiviral effect for HSV-1and HSV-2. (Table VII).

Table VII. Antiviral(HSV) Effect of SH-1 Separa ted and Ingredients Partitioned from Coptidis Rhizoma

Sample Name	Cytotoxicity Activity CC_{50}	HSV-1(Strain : F)		HSV-2(Strain : MS)	
		EC_{50}	SI	EC_{50}	SI
SH-1	56.8	>56.8	<1	>56.8	<1
SH-B	>300	>300	NC	>300	NC
SH-E	62.4	>62.4	<1	>62.4	<1
SH-H	>300	>300	NC	>300	NC

SH-1 : Berberine
SH-B : BuOH Layer
SH-E : E.A. Layer
SH-H : H2O Layer

4) Antitumor Effect 0f Alkaloids from Coptidis Rhizoma

(1) Result of First Experiment

Test sample SH-D, SH-E, SH-B and SH-H gave EC50 value of 90.3 - 117.9 μg/mL to HCT 15 cell lines, that means test samples have no significant antitumor effect. But to other tumor cell lines, considerable antitumor effect was obserbed. Espescially to XF 498 cell lines, Test samples have been shown ED50 of 0.615 - 9.54μg/mL. But there has been no evidence for selective antiviral effect to specific tumor cell line. (Table VIII).

(2) Result of Second Experiment

Samples of 1-1B, 2-1B, 5-2B, SH-1, SH-4B, SH-6B and SH-7B were tested for antitumpr effect. Samples of 1-1B, SH-6B and SH-7B have no considerable result toward all cell lines. Significant antitumor effect of test sample 2-1B, 5-2B, SH-1 and SH-4B to SNU-1 (human stomach) cell line, test sample 5-2B to SKMEL-2 (human melanoma) cell line, test sample SH-4B to K-562 (human chronic myelogenous leukemia) cell line were observed. 5-2B and SH-4B have been shown meaningful antitumor effect to all cell lines. (Table IX.)

(3) Result of Third Experiment

EC_{50} value of coptisine, jatrorrhizine and palmatine to SNU-1 (human stomach) and K-562 (human chronic myelogenous leukemia) cell line were 59-75μg/mL, which means those comporits have excellent antitumor effect. Especially jatrorrhizine has been shown EC_{50} value 64μg/mL to A-549 (human lung) cell line and all three other components had EC_{50} value lower than 300μg /mL toward all cell line indicating significant antitumor effect (Table X).

Table VIII. Antitumor Effect (EC50) of Ingredients Partitione from Coptidis Rhizoma (unit : μg/mL)

Cell Line / Sample Name	A-549	SKOV-3	SKMEL-2	XF-498	HCT-15
SH-D(CH_2Cl_2 Layer)	28.10	14.70	12.90	9.54	117.90
SH-B(BuOH Layer)	44.70	26.20	10.40	2.58	90.30
SH-E(EtOAc Layer)	15.00	7.59	3.96	0.615	96.40
SH-H(H_2O Layer)	32.20	24.30	8.98	2.12	

A-549 : Human Lung SKOV-3 : Human Ovarian
SKMEL-2 : Human Melanoma XF-498 : Human CNS
HCT-15 : Human Colon

Table IX. Antitumor Effect (EC50) of Ingredients Separated by Column Chromatography and Prep-TLC(unit : μg/mL)

Cell Line / Sample Name	SNU-1	SKOV-3	SKMEL-2	K-562	A-549
1-1B	523	BAD	BAD	BAD	BAD
2-1B(Coptisine Layer)	GOOD	BAD	224	97	124
5-2B(Jatrorrhizine Layer)	GOOD	159	GOOD	40	150
SH-1(Berberine Layer)	GOOD	BAD	211	108	158
SH-4B(Palmatine Layer)	GOOD	207	98	GOOD	300
SH-6B	BAD	BAD	BAD	BAD	BAD
SH-7B	108	BAD	BAD	BAD	BAD

** GOOD : EC_{50}<33.3 ** BAD : EC_{50}>300
SNU-1 : Human Stomach A-549 : Human Lung
SKOV-3 : Human Ovarian SKMEL-2 : Human Melanoma
K-562 : Human Chronic Myelogenous Leukemia

Table X. Antitumor Effect (EC_{50}) of Coptisine, Palmatine and Jatrorrhizine Separated from Coptidis Rhizoma(unit : μg/mL)

Cell Line / Sample Name	SNU-1	SKOV-3	SKMEL-2	K-562	A-549
Coptisine	61	217	185	70	180
Jatrorrhizine	59	182	160	75	64
Palmatine	71	181	107	75	157

SNU-1 : Human Stomach A-549 : Human Lung
SKOV-3 : Human Ovarian SKMEL-2 : Human Melanoma
K-562 : Human Chronic Myelogenous Leukemia

V. Conclusion

The quantitative comparison of Coptis rhizoma as well as antibacterial effect, antiviral effect, antitumor effect were performed reaching following results :

1. On the comparison between Coptis chinen sis and Coptis japonica, the content of berberine were 6.78%, 7.09% respectively. Though the palmatine level was similar each other, the concentration of coptisine, jatrorr hizine, berberastine were higher in C. chinen sis.

2. Unfortunately not only berberine but also the fraction SH-D, SH-E, SH-B, SH-H could not be found significance on antibacterial effect or HIV, HSV-1, HSV-2 antiviral effect. Similar trend was found on SH-1 fraction against HIV antiviral effect.

3. Surprisingly, purified berberine, palmatine, coptisine, jatrorrhizine have been shown signifi cant antitumor effect against SNU-1(human stomach cancer) cell line.

4. Meaningful results could be observed at purified palmatine, coptisine, jatrorrhizine aga inst K-562(human chronic myelogenous leuke mia) cell line showing significant antitumor effect. Purified jatrorrhizine also very effective against A-549(human lung) cell line in the aspect of antitumor effect.

5. Compared to highly purified single alkalo ids, less purified complex alkaloids have been shown relatively stronger antitumor effect against variety of cancer cell line. These mechanism or synergism may be worth to study in the future for better treatment on the patient.

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Acknowledgement

Present study could be possible only by meaningful discussion with Professor Ho Koon Park at KIST. Especially in the course of screening test, his interpretation was helpful on this research.