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A Study on the Transformation of Baicalin or Antibacterial, Antitumor Effect of the Active Ingredients in Scutellariae Radix

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ABSTRACT

A Study on the Transformation of Baicalin or Antibacterial, Antitumor Effect of the Active Ingredients in Scutellariae Radix

Scutellariae Radix has been widely used as oriental herbal medicine for the treatment of bacterial infections in the respiratory or the gastrointestinal tract. In partition experiment for better understanding of herbal medicine with various solvents, baicalein or wogonin have more hydrophobic characteristics than baicalin or wogonoside. Unexpectedly, methylene chloride could extract more for baicalin or wogonoside over other active ingredients.

New compound from baicalin is discovered casting frointier area on herbal medicine in the future. Application study with new molecule hydrolyzed from baicalin is on the way for better treatment of the patient against specific disease.

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The baicalin modified with reaction has been shown weak antibacterial activity against *Streptococcus pyogenes* 308A and *Pseudomonas aeruginosa* 1771. The minimum inhibitory concentration (MIC) of the baicalin modified compound against those strains were about 600 μg/mL, respectively. In vitro antitumor experiment, EC_{50} of baicalin modified with reaction was more than 300 μg/mL and EC_{50} of baicalein was 100 μg/mL. Among these compounds, baicalin exhibited high level of antitumor activity. EC_{50} of baicalin was less than 33.3 μg/mL.

Keywords : Scutellariae Radix, baicalin, wogonoside, baicalein, wogonin

I. Introduction

Since Scutellariae radix is described at the first phytology text, <神農本草經>[15] 中品 as "味苦平, 主諸熱黃疸, 腸澼洩利, 逐水, 下血閉, 惡創疽蝕, 火瘡", it has been used for "瀉實火, 除濕熱, 止血, 安胎 效能" as one of important oriental herbal medicine[1,2,3,5,8,19,23,28,36,37,39,42]. It has originated from Labiatae in Scutellaria as perennaltion species. As herbal medicine, the root of *Scutellaria baicalensis* Georgi is peeled off and dried for treatment. [1,2,5,8,23,28,36,37,39,42-45] *Scutellaria baicalensis* Georgi is one of the most widely used oriental herbal medicine against bacterial infections of the respiratory and the gastrointestinal tract. The study on the clinical effect in the area of "抗炎症作用[2,52], 抗 allergy作用[2,28,39], 膽汁分泌促進作用[2,8,28,36,39], 肝障碍 豫防作用[28], 抗菌作用[8,14,36,39], 利尿作用[2,8,28,36,39], 鎭痙作用[2,28], 消化管粘膜 內因性 PG增加作用, 血壓降下作用[2,8,28,36,39], 高脂血症 改善作用[2,14], 脂質過敏化反應 抑制作用, 緩下作用, 腸管運動抑制作用[2,8,36,39], 解熱作用[2,8,28,36,39], 皮膚 and 毛髮 保護 作用, 血栓症, 抗癌, 抗皮膚腫瘍 作用[8,12,29,36,42,43,53]".

For the qualitative analysis of *Scutellaria baicalensis* Georgi, since 高橋[47] had reported wogonin in 1891, lots of study have been progressed on baicalin, baicalein, wogonin as major components[1,2,5,8,14,36,37,39,44,45]. Except that, around forty species have been reported[42,48,49,51]. Especially for the quantitative study reported that it contain baicalin 10-20%, baicalein 0.2-1.4%, wogonin 0.1-0.4% respectively[46]. The reason why Scutellariae radix is showing yellow color would be due to yellow flavon derivatives on baicalin, baicalein, wogonin.[7,12,14] The most yellow crystal, baicalin rich in root part could be hydrolyzed into baicalein and glucuronic acid[12,39,54] at certain cases.

In the present study, the extract from *Scutellaria baicalensis* Georgi was partitionated for better understanding of its character. The baicalin hydrolyzed was identified followed by screening test including antibacterial effect and antitumor effect as initial characterization of Scutellariae radix for better usage of herbal medicine.

II. Experimental

1. Material or Instruments

1) Samples

Each Scutellariae radix collected from

Bulgyo(Junnam) were dried without sunlight for few weeks followed by cutting into samall pieces, drying in vacuum desiccator for two days and crushing into about 40 mesh for next experiments.

2) Reagents and Instruments

(1) Standards or Reagents

The standard compounds-baicalin, baical ein, wogonin- over 98.0% in purity were purchased from Wako Pure Chemical Co. (Tokyo, Japan). The solvents used without further purification during the experiments were HPLC grade acetonitrile, methanol, chloroform, methylene chloride, hexane, buta nol, benzene, ethanol. It was provided by J. T. Baker Co.(Phillipsburg, U.S.A.). Phosphoric acid(over 85.4%) and other analytical reagent grade acids including acetic acid were obtained from Sigma Chemical Co.(St. Louis, MO, U.S.A.).

(2) Instruments

For the separation of Scutellariae radix, HP 1050 High performance liquid chroma tography made by Hewlett Packard Company with Diode Array Detector at the range of 200-650nm UV spectrum for the identification from structual information. Fourier-Transform Infrared Spectrometer(IFS 120HR) was insta lled by Bruker High Tech. Co.(Karlsruhe, Germany) and DEP/MS for vital structual analysis was provided by Finnigan Co.(San Jose, U.S.A.) as quadrupole SSQ7000 or ion trap Magnum. Distilled water for all through the experiments was made of Milli-Q water system from Millipore Co.(Tokyo, Japan). The herbal medicine was crushed with Grinder (Thomas Co., Philadelphia, U.S.A.) to proper size. The HPLC Column was ODS HYPERSIL(5 μm, 4.6×250mm, Shandon, Great Britain) or C18 Sheseido from Japan.

2. Experimental Procedure

1) Preparation of Standards

The standard compounds, baicalin, baicalein, wogonin were weighed 3.02, 3.07, 2.99mg eactly and transferred to 10mL volumetric flask with 100% MeOH for further dilution whenever it is necessary.

2) Experiment for Partition Constant on Ex tractant

For efficient separation of active ingredients in Scutellariae radix, extraction coefficient(D) was measured at following conditions. In sepa ratory funnel, the solvent system composed of ether(Et_2O)/H_2O, methylethyl ketone(MEK)/ H_2O, ethylacetate(EA)/H_2O, methylene chloride (MC)/H_2O, butanol(BuOH)/H_2O, chloroform ($CHCl_3$)/H_2O were added 7mL respectively followed by partition of extractant 200 μL from Scutellariae radix ingredient with pure methanol. After vigorous shaking for few minutes, stand by the funnel until two liquid layer separated completely as clear solution. Injection of each solution with same volume into HPLC will yield the important information of extraction coefficient(D).

3) Structual Transformation of Baicalin with Acetic Acid

In order to investigate structual change of Scutellariae radix, sample 10g was extracted with MeOH 100mL in conjunction with concentrated CH_3COOH 0, 10, 30, 40mL for 2

hours at elevated reflux temperature 60℃ followed by quantitation of each components with HPLC. For the effect of time passage, concentrated CH_3COOH 30mL was added as same procedure maintaining reflux duration for 0, 2, 4, 6 hours at over 60℃ followed by quantitation with HPLC on baicalin, wogonosi de, baicalein, wogonin.

4) Hydrolysis of Active Ingredient in Scute llariae Radix

After baicalin standard or extract from Scutellariae radix 1g was placed in 200mL rouund bottomed flask with 2mL concentrated hydro chloric acid in 100mL methanol at 60℃ for 2 hours reflux, this solution was filtered through 0.45 micrometer membrane filter and concentrated to 10mL followed by semipre parative purification with HPLC. The compound hydrolyzed and purified with this process was used for structual identification and screening test.

5) Biological Activity Assay of the Compo nents of Scutellariae Radix

(1) Antibacterial Effect

A. Strain

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

Table I. 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* DC2
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

B. Medium

Fleisch Extract Broth which contains 1% beef extract, 1% Bacto peptone, 0.3% NaCl, 0.2% $Na2HPO_4 \cdot 12H_2O$ at pH 7.4~75 and agar were used for culture medium. Some cases, sheep blood or horse serum or fildes extract from Oxoid Co. were used additionally. Müeller Hinton broth and agar were brought from Difco Co,.

C. Maintenance of strain

① Stock Preparation

Strains were incubated at 37℃ for 16~18 hours after inoculation on Fleisch extract broth. To this, equal volume of 40% glycerol solution was added, shook well and stored at -70℃ deep freezer or under liquid nitrogen as 0.5mL aliquot in Cryo vial.

② Slent Preparation

Agarose slent medium was used for short time storage of strains. Pseudomonas was inoculated in Mueller Hinton Medium medium and Mueller Hinton Medium with 10% sheep

blood was used for Streptococcus and Fleisch extract agar medium for all other strains.

D. Activity Test

① Strain Solution Prepation

Fleisch extract broth, 10mL was placed in 20 tesι tubes in each, then sterilized in autoclave under 1.2 atm at 121℃ for 15min utes. After cooling to room temperature, horse serum was added to make to 10% and *Strep tococcus* was inoculated using platinum line, incubated at 37℃ for 16~18 hours and dil uted to 100 times for desirable concentration.

② Preparation of Agarous Mediun with antibiotics and Inoculation of Strain

ⓐ In each test tube, 0.5mL of sterilized distilled water was placed and gave number.

ⓑ Solution of antibiotics, 3mL(1mg/mL) was added to number 1 test tube and shook well, then took 1.5mL and trasfered to number 2 test tube. Repeated this dilutions 15 times to number 17 test tube.

ⓒ Mueller Hinton argar medium 13.5mL and antibiotic solution 1.5mL were mixed well and placed in petri dish to make final concention of antiboitics to 100~0.002μg/mL respectively.

ⓓ Test strain solutions which were incubated in Fleisch extract broth or same broth with 10% horse serum, were diluted for inoculation.

ⓔ Each test strains was placed ino culation tray and inoculated by using Auto matic Inoculator(Dynatech, U.S.A.) on prepared petri dish plate with antibiotics(10^4CFU/spot).

ⓕ After incubation at 37℃ for 18 hours, determined minimum concentration for inhibition of strain growth(MIC).

(2) Antitumor Effect

A. Tumor cell lines

K-562 (Human chronic myelogenous leukemia)
SNU-1 (Human stomach)
SKMEL-2 (Human melanoma)
A549 (Human lung)
SKOV-3 (Human ovarian)

B. Test Materials

Antitumor effect was tested for Bai calin, baicalein reference standard and baicalin derivative(baicalin*)

C. Assay Method[55,56,57]

ⓐ Adequate number of cells were suspended in 180 μL of medium and inoculated on 96 well plate.

ⓑ After addition of series of diluted test samples, incubated at 37℃ under 5% CO_2 for 4 days.

ⓒ Known concentration of MTT sol ution, 50μL was added to well and incubated at 37℃ under 5% CO_2 for 4-6 hours.

ⓓ After centrifugation at 450 x g for 5 minutes, supernatant was removed by making the plate up side down immediately.

ⓔ To each well, 150μL of DMSO was added and shook until formazon crystall was desolved to dark red solution.

ⓕ Absorbance at 540nm was masured and made growth-curve, from that IC_{50} value was calculated.

D. Calculations

ⓐ When test sample was added and started incubation, some aliquot was taken and masured for SRB protein and made SRB protein Time zero(Tz).

ⓑ Absorbance of well without test sample was defined as control value(C). Absorbance of well treated with test sample

was defined as drug-treated test value(T).

ⓒ From Tz, C and T, effect of test sample, such as, growth stimulation, net grow th inhibition and net killing was determined.

ⓓ If T≥Tz, cellular response func tion is 100×(T-Tz)/(C-Tz), in case of T<Tz, cellular response function is calculated as 100 ×(T-Tz)/Tz.

Ⅲ. Results

1. Partition constant on Extractant of Scutellariac Radix

In the solubility test of Scutellariac Radix, baicalin and wogonoside were more soluble in water than organic solvents shown in Table Ⅱ. On the other hand baicalein and wogonin were more soluble in organic solvents than that in water. But in case of methylene chloride, oposite result was observed. In case of methyl ethyl ketone or butanol, baicalein and wogonoside were very soluble both in organic solvents or in water. Therefore baicalin and wogonoside are hydrophylic and regarded as sweet, in other hand baicalein and wogonin are lipophylic and regared as somewhat bitter taste.

2. Veriation of Major Components from scutellariae Radix by Ace tic acid

Variation of major components from Scutellariac Radix by acetic acid is shown in Table Ⅲ and Ⅳ. When acetic acid was added to extracting solvent, initially baicalin was extracted 0.1-0.18wt% more than without acetic extraction. But initial concentration was decreased from 2.77 to 2.65, 2.38 and 2.22wt% after 2, 4 and 6 hours respectly. This means that solvent extraction with acetic acid of herbal medicine give more baicalin than solvent extraction without acetic acid. But prolonged heatring could make baicalin to other component. When Scutellariac Radix was extraced, using acetic acid can make alternation of content of component and can give activity difference.

Table Ⅱ. Extraction Coefficient(D) at Organic Solvent to Aqueous layer

solvent / acrive ingradient	Et_2O/H_2O	MEK/H_2O	EA/H_2O	MC/H_2O	BuOH/H_2O	$CHCl_3/H_2O$
baicalin	0	0.59	0	∞	0.19	0
wogonoside	0	1.48	0.97	22.4	0.42	0
baicalein	35.9	20.6	64.3	0.03	∞	39.7
wogonin	26.3	41.8	35.7	0.01	∞	29.4

3. Acid Hydolysis of Baicalin and Structure Identification

Baicalin reference standard was treated with HCl instead of weak acetic acid for fast conversion toward intermediate state and its

Table IV. Effect of the Reflux Time on the Content of the Major Components from Scutellariae Radix (Unit : Wt%)

	Passage of Time after Addition of AcOH (hrs.)			
	0	2	4	6
baicalin	2.77	2.65	2.38	2.22
wogonoside	1.30	1.41	1.51	1.58
baicalein	1.35	1.34	1.34	1.34
wogonin	1	1	1	1

Table III. Effect of CH_3COOH Concentration on the Content of the Major Components from Scutellariae Radix (Unit : Wt%)

(AcOH added)	0mL	10mL	30mL	40mL
baicalin	1.68	1.78	1.85	1.86
wogonoside	1.20	1.40	1.41	1.57
baicalein	1.38	1.40	1.40	1.40
wogonin	1	1	1	1

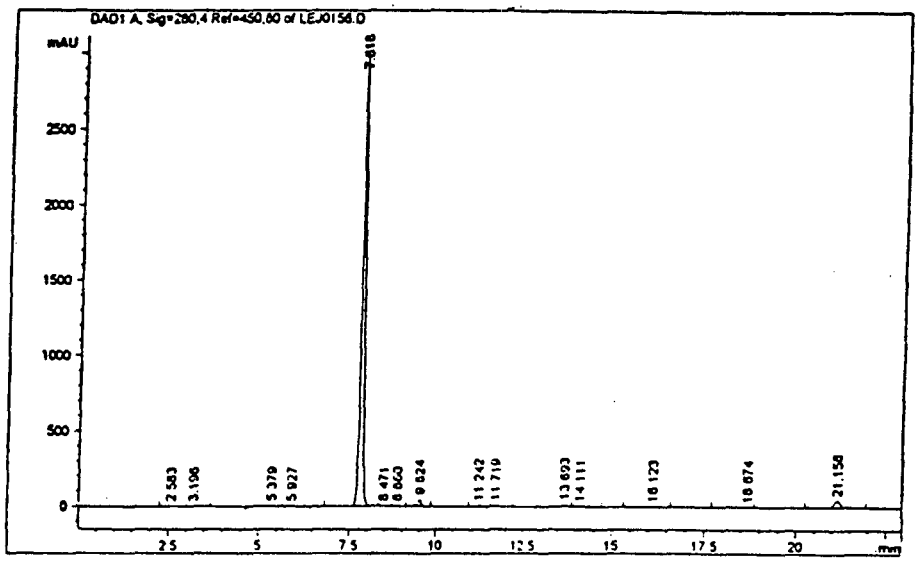
product was analyzed by HPLC, UV/VIS, IR, DEP/MS and NMR.

In HPLC analysis, baicalin reference stand ard had retention time of 7.8 minutes, in other hand acid treated baicalin has been detected the retention time at 9.5 minute as shown in Figure 1. Its transition could be opposite result from other reported fact that baicalin would be hydrolyze into baicalein and glucronic acid. This may be confirmed with UV/VIS at 280nm showing sound flavonoid skeleton without breakage as in Figure 2. According to IR spectra in of both species as in Figure 3, the intensity of -OH group is increased showing hypsochromic shift around 3273~3233cm^{-1} due to hydrogen bonding. Whereas the carboxylic group on glucronide could rotate more random way showing weaker hydrogen bonding at 1657~1670cm^{-1} with blue shift. The aromatic double bond streching 1576cm^{-1} from 1560cm^{-1} represents for the ring opening on glucronide acompany with interference by it. As another -OH group is generated, the intensity of absorbance at 1082cm^{-1} is increased accordingly.

According to mass spectrum (A) of standard baicalin as in Figure 4, the fragment through McLafferty rearrangement m/e=270 without glucronide is appeared followed by Retro-Diels-Alder fragmentation at m/e=168. When baicalin was hydrolyzed with HCl as shown (B) in Figure 4, the flavone ring without glucronic acid was substituted with $-OCH_3$ at m/e=284 whereas CD3OD and DCl have used, m/e=288 with -OCD3 is seen as (C) in Figure 4. In three cases, m/e=270 could be deduced as baicalein ion at extrme condition showing the possibility of conversion to baicalein from baicalin as described on the references.

On NMR study of standard baicalin as in Figure 5(A), it can be intrepreted as δ 3.7(2", 3", 4" on glucronic acid, multiplet), δ 4.21 and δ 4.24(5",doublet), δ 5.27 and δ 5.25(1",

(A)



(B)

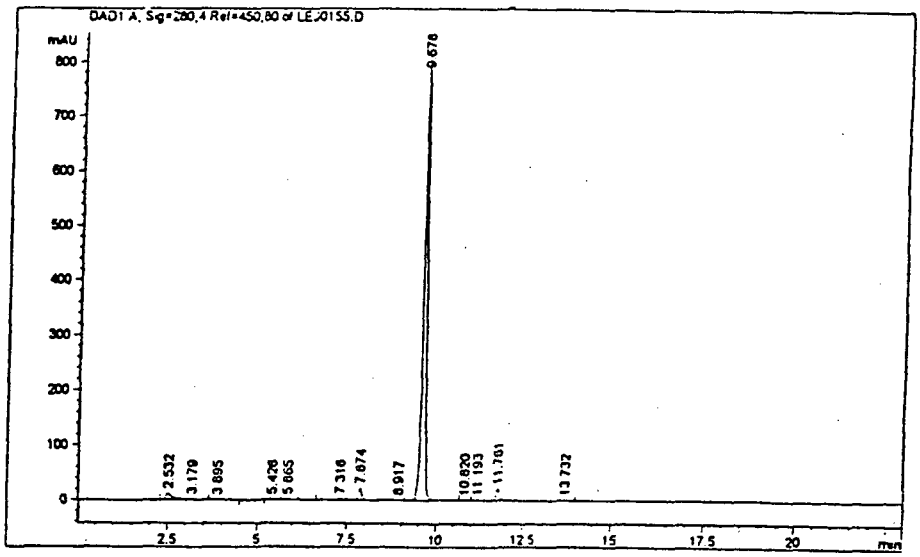


Figure 1) HPLC chromatogram of baicalin(A) and new compound(B, baicalin*) after hydrolysis with hydrochloric acid.
Transform toward new compound is almost completed.

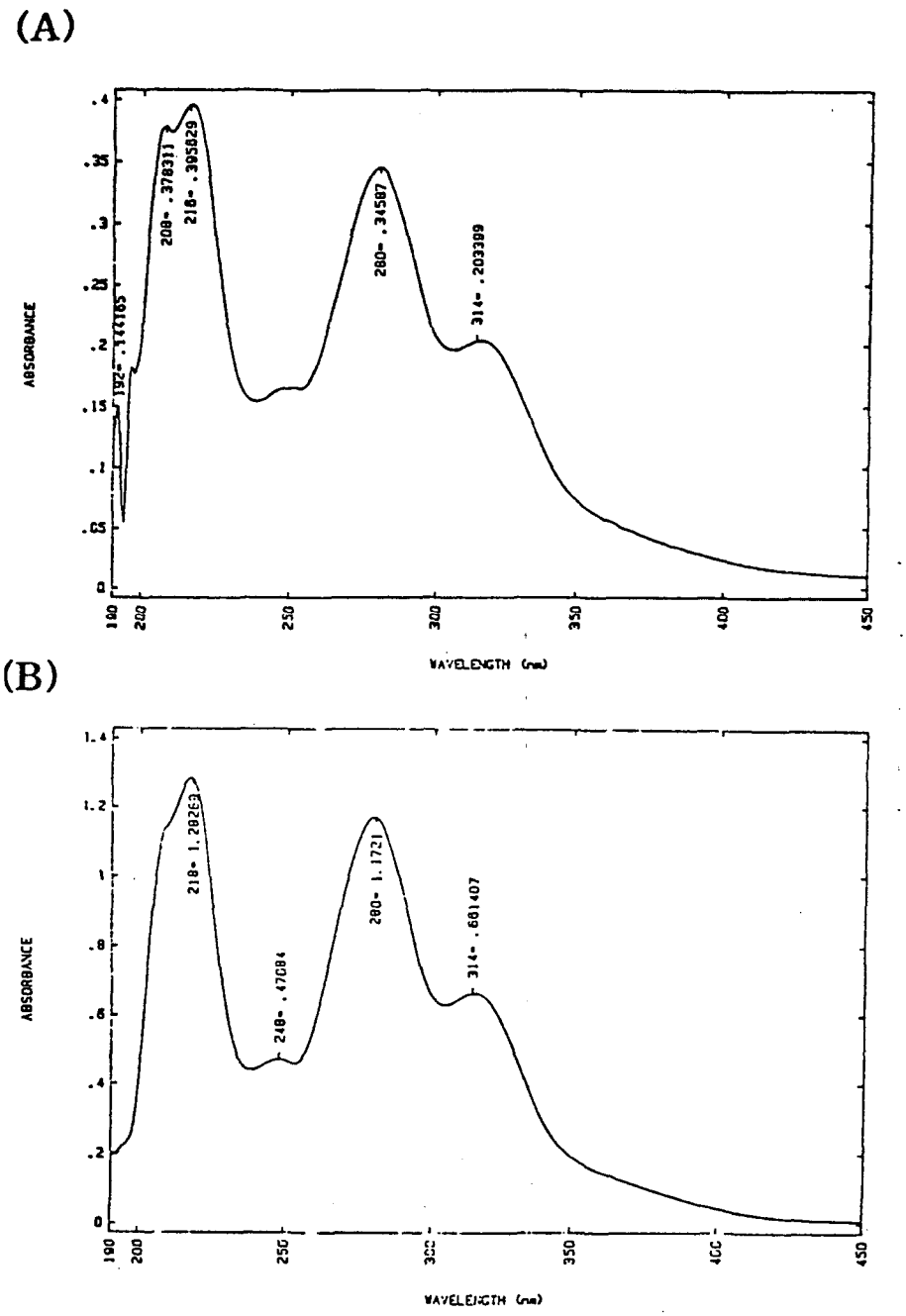


Figure 2) The UV/VIS spectra of baicalin standard(A) and baicalin changed(B) after bydro lysis with hydrochloric acid.
The chromophore from flavone skeleton remain intact inspite of modification of baicalin.

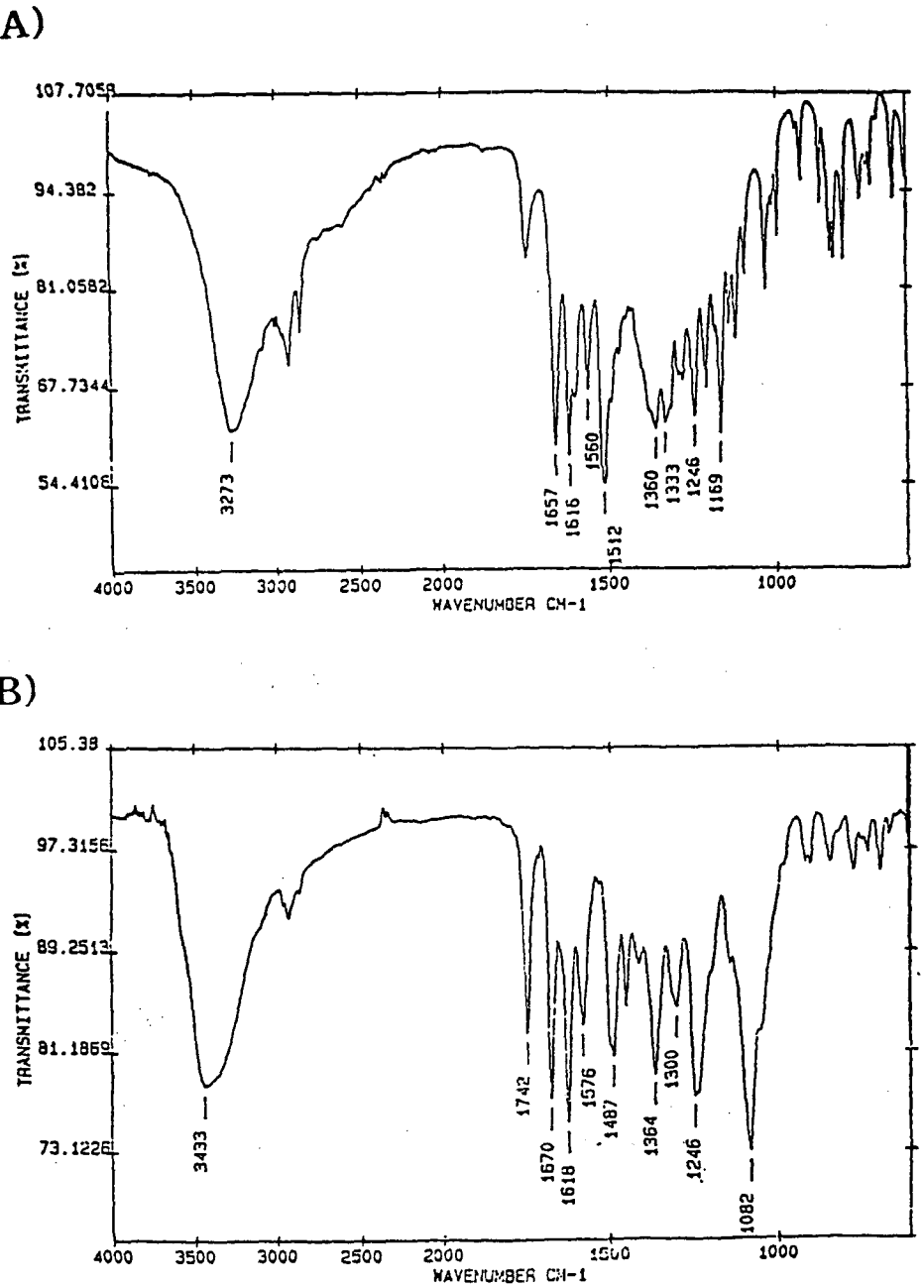


Figure 3) The FT/IR spectra of baicalin standard(A) and baicalin modified(B, baicalin*) after hydrolysis.

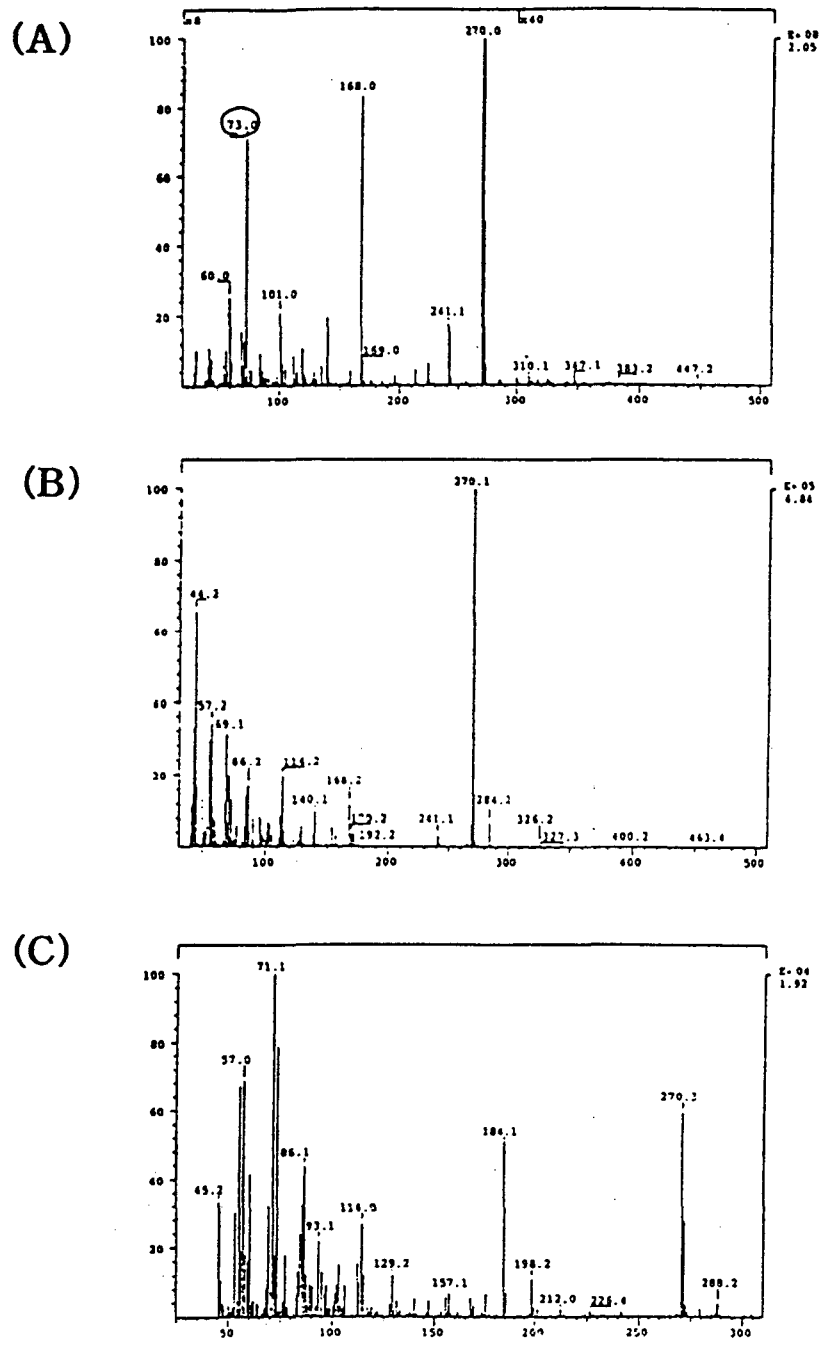


Figure 4) The mass spectra of baicalin standard(A), baicalin modified(B) with HCl in methanol solution and baicalin modified(B) with HCl in methanol solution and baicalin modified(C) with DCl.

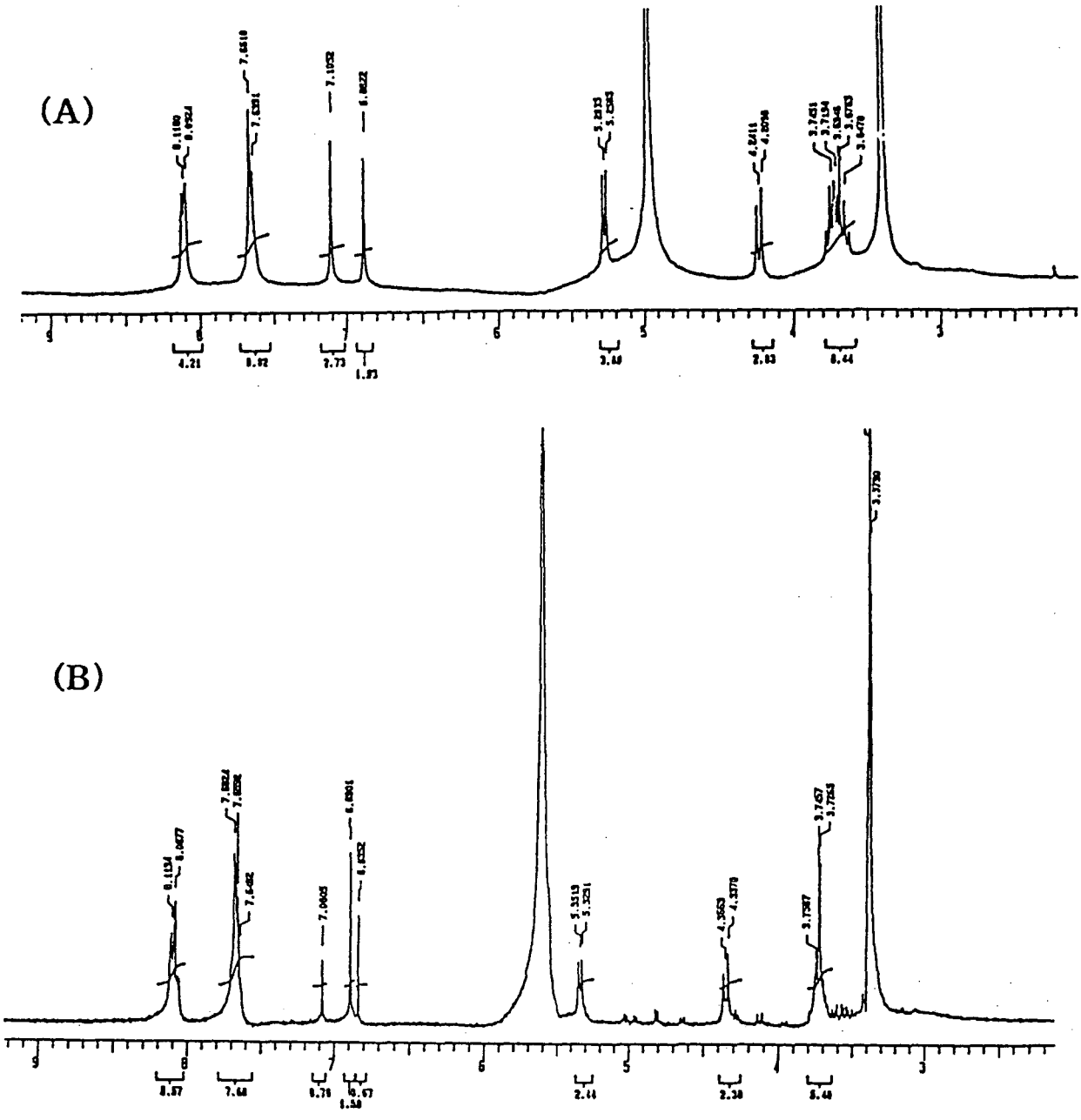
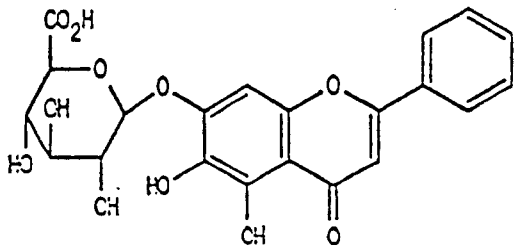


Figure 5) The 300MHz nmr spectra of baicalin standard(A) and baicalin hydrolyzed(B) in acidic solution. The proton at 8 position of flavone ring has been shown blue shift due to bond breakage at 5″ C.

(A)



(B)

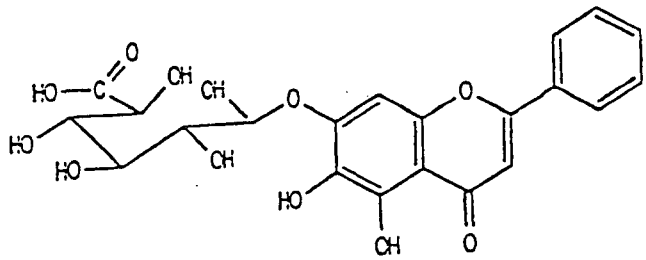


Figure 6) The molecular structure of baicalin(A) which is a major ingredient in Scutellariae Radix and baicalin hydrolyzed(B) from baicalin at elevated temperature.

Table V. Result of Antimicrobial Susceptibility Test for baicalin*

	strains	Minimal Inhibitory Concentration (μg/mL)
		baicalin*
1	*Streptococcus pyogenes* 308A	600
2	*Streptococcus pyogenes* 77A	>600
3	*Streptococcus faecium* MD8b	>600
4	*Staphylococcus aureus* SG511	>600
5	*Staphylococcus aureus* 285	>600
6	*Staphylococcus aureus* 503	>600
7	*Escherichia coli* 055	>600
8	*Escherichia coli* DCO	>600
9	*Escherichia coli* DC2	>600
10	*Escherichia coli* TEM	>600
11	*Escherichia coli* 1507E	>600
12	*Pseudomonas aeruginosa* 9027	>600
13	*Pseudomonas aeruginosa* 1592E	>600
14	*Pseudomonas aeruginosa* 1771	>600
15	*Pseudomonas aeruginosa* 1771M	>600
16	*Salmonella typhimurium*	>600
17	*Klebsiella oxytoca* 1082E	>600
18	*Klebsiella aerogenes* 1522E	>600
19	*Enterobacter cloacae* P99	>600
20	*Enterobacter cloacae* 1321E	>600

Method : Agar Dilution Method
Baicalin* : Baicalin derivative with acid treatment

doublet), δ7.1 and δ6.9(8, 3, singlet), δ7.66 and δ7.64(3', 4', 5',multiplet), δ8.12 and δ 8.09(2', 6', multiplet). In order to get Figure 5(B), the baicalin standard is heated for 30hours with CD_3OD and DCl in NMR tubes. Due to acidity as well as ionic strength, the water peak shifts to δ5.0 from δ5.7. The protons at 2", 3", 4" on glucronic acid become sharper providing important evidence of ring opening. As the time of heating is progressed the bond breakage at 5" in glucronic acid affecting the chemical shift at proton 8 in flavonone ring showing new peak at δ6.83 from δ7.10.

According to interpretation from instrumental method, the baicalin hydrolyzed(B) has the structure as shown in Figure 6. It is converted from breakage of 5"C bond in glucronic acid.

4. Biological Activity of Components from Scutellariae Radix

1) Antibacterial Effect

Baicalin derivative wirh acid treatment was tested for antibacterial activity uasing 20 strains which were generally used for antibacterial test. Only against *Streptococcus pyogenes* 308A and *Pseudomonas aeruginosa* 1771, test sample showed groth inhibition at

600μg/mL concentration. This result might be due to the low solubility of test sample (baicalin*) in water. (Table V)

2) Antitumor Effect

IC_{50} value can give us the information of antitumor activity. Baicalin derivative with acid treatment((baicalin*) showed IC_{50} over 300μg/mL against Human chronic myelogenous leukemia(K-562), Human stomach(SNU-1), Human melanoma(SKMEL-2), Human lung(A-549) and Human ovarian(SKOV-3) Tumor cell line. Baicalein reference standard inhibited tumor groth to give IC_{50} 200μg/mL and Baical in reference standard has significant antitumor effect to gave IC50 33.3μg/mL. (Table VI)

Table VI. Anticancer Drug Screening Result

Sample	IC50(μg/mL)				
	K-562	SNU-1	SKMEL-2	A-549	SKOV-3
BAICALIN*	>300	>300	>300	>300	>300
BAICALEIN	223	256	221	228	252
BAICALIN	33.3>	33.3>	33.3>	33.3>	33.3>

Method : MTT Assay
Wavelength : 540nm
Concentration (μg/mL) : 300, 100, 33.3
Cell line : K-562(Human chronic myelogenous leukemia)
SNU-1 (Human stomach)
SKMEL-2 (Human melanoma)
A-549 (Human lung)
SKOV-3 (Human ovarian)

Ⅳ. Results

After identification of the compound hydro lyzed from the major component baicalin in Scutellariae radix, antiviral and anticancer effect were tested.

1. In the experiment on the extract from Scutellariae radix, more hydrophylic baicalin and wogonoside could be extracted more in aqueous layer whereas opposite result was observed at baicalein and wogonin. Unexpect edly methylene chloride could extract baicalin, wogonoside more than that in water implying some interaction with analytes.

2. Even with weak acid such as acetic acid during extraction, the content of baicalin was decreased providing the possibility on the conversion toward new compound in oriental herbal medicine. Therefore special care is needed be taken into account when acidic herbal medicine is treated as prescription.

3. A new compound from baicalin in Scutell ariae radix was converted through hydrolysis process with heating. Its sturucture was identified by NMR, DEP/GC/MS, FT/IR and UV/VIS. The new compound was hydrolyzed baicalin derived from breakage of bond at C5" glucronide ring. Modification study toward

new compound from traditional herbal medi cine will be beneficial for better treatment of the patient against specific disease.

4. The new compound has been shown weak antibacterial activity around 600μg/mL due to low solubility in water. In spite of relatively weak anticancer effect by hydroly zed baicalin(Baicalin*, IC50 300μg/mL), Baical ein(IC50 200μg/mL) and Baicalin(IC50 33.3μg/mL), the scope of application may be extended on further study.

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