Composition of Methanol Extract from Hwangryeon (Coptidis rhizoma) and Antimicrobial Activity against Food Spoilage and Foodborne Disease Microorganisms

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Abstract : Hwangryeon (*Coptidis rhizoma*) was extracted by methanol and its antimicrobial activities against food spoilage and foodborne disease microorganisms were investigated by the paper-disc method. The microorganisms used in this experiment included 5 species of bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Klebsiella pneumoniae, and Staphylococcus aureus*) and 3 species of fungi (*Fusarium solani, Aspergillus flavus, and Penicillium citreonigrum*). The Hwangryeon extract showed antimicrobial effect against *P. aeruginosa, S. aureus, S. typhimurium*, and *K. pneumonia*. The minimum inhibitory concentration on *S. aureus, S. typhimurium*, and *K. pneumoniae* was 300 mg/mL, but on *P. aeruginosa* it was 200 mg/mL. In the methanol extracts from Hwangryeon, 190 compounds were separated by GC/MS. The extraction yields of phenols, furans, alcohols, acids and esters, ketones, and miscellaneous compounds were 28.10%, 2.67%, 1.79%, 12.89%, and 2.35%, respectively. The phenolic compounds, generally understood to be an antimicrobial active substance, was measured at 28.10%, a relatively remarkable amount.

Key Words : Hwangryeon (*Coptidis rhizoma*), antimicrobial activity, minimum inhibitory concentration

I. Introduction

Currently the use of chemical preservatives is widespread. Preservatives are used not only to prevent putrefaction and deterioration but also to extend shelf-life of foods. However, the increasing number of chemical preservatives raises the question about their safety. There are 14 kinds of food preservatives permitted in Korea that include such as sorbic acid, benzoic acid, butyl ρ-hydroxybenzoate and so on. However, in many cases their use does not follow prescribed protocols. The accumulation of preservatives in the human body can cause reactions such as stomach disability, chronic poisoning, and mutation (Lee & Lim, 1998). Owing to growing awareness about the safety problems of chemical preservatives, consumers want natural substances to be used as preservatives (Ahn *et al*., 1998). Therefore, it is urgent to develop alternatives which are harmless to humans by using natural substances such as medicinal herbs when the food industry must use chemical preservatives (Bullerman *et al*., 1977; Sharma *et al*., 1979; Yamamoto *et al*., 1984; Zaika & Kissinger, 1981; Park *et al*., 1992; Kim *et al*., 1998). Following this trend, researchers have concentrated on the antimicrobial activity of herbal medicinal agents. The major antimicrobial active substances existing in plants are secondary metabolic products or derivatives such as phenol, terpene, and volatile aroma ingredients

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(Song *et al*., 1998). Natural substances exhibiting antimicrobial activity are contained in protein materials such as polylysine, lysozyme, protamin, avidin, and conalbumin and the volatile components of spices that have esters of short-chain fatty acids. Garlic, pepper, onion, medical herbs, and extracts of edible plants contain various antimicrobial components. Especially, studies on the antimicrobial activity of extracts of medicinal plants and herbs such as *Lycium chinense, Glycyrrhiza uralensis, Gardenia jasminoides, Plantago asiatica, Chaenomeles sinensis, Salvia miltiorrhiza*, and *Chrysanthemum sibiricum* have been performed (Park *et al*., 1995; Lim, 1999).

Hwangryeon (*Coptidis rhizoma*) belongs to *Ranunculaceae* and is a perennial herb which is often called a dried root of *Coptis Chinensis*. It has hollows in the roots which look like a cock's claws. When winter begins, it is picked and dried in the sun after its stems, leaves, and soil are removed. The cork layer is then peeled and used as a medicinal herb. The main components of Hwangryeon are alkaloid, berberine, jateorrhizine, palmitine, magnoflorine, and ferulic acid. Berberine and coptisine are reported to have antimicrobial activity, hypotensive action, and anti-inflammatory action. The current study was carried out as part of a base study to develop natural food preservatives using antimicrobial substances obtained from nature. We examined the antimicrobial effect of methanol extract from Hwangryeon against food spoilage and foodborne disease microorganisms and analyzed the components of the methanol extracts.

II. Materials and Methods

1. Materials

Hwangryeon was bought at Yakryng-si (Herb Drug Market) in Daegu. In order to compare with antimicrobial activity, benzoic acid (Duksan Co., Korea), which is permitted as a chemical food preservative was used.

2. Strains and culture media

Strains for the experiments, *Escherichia coli* KCTC 1682, *Pseudomonas aeruginosa* KCTC 1750, *Salmonella typhimurium* KCTC 2515, *Klebsiella pneumoniae* KCTC 2001, *Staphylococcus aureus* KCTC 1916, *Fusarium solani* KCTC 6636, *Aspergillus flavus* KCTC 6633, *Penicillium citreonigrum* KCTC 6927, were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The experiment on the bacteria was performed using nutrient agar (Difco Co., USA) and the fungi using potato dextrose agar (Difco Co., USA).

3. Extraction method

Dried Hwangryeon was extracted with methanol 20 times of Hwangryeon's weight for 1 week at 25°C with occasional agitating and the extract was filtrated by filter paper (Toyo No. 2). Then, it was concentrated with rotary vacuum evaporator (Heidolph, VV 2000, Germany) at 50°C. It was filtered through a syringe filter (cellulose acetate membrane, pore size 20 μm, Advantec Co., Japan) to eliminate microorganisms and conserved at 4°C in a refrigerator.

4. Antimicrobial activity assay

Antimicrobial assay of methanol extract of Hwangryeon was performed by a modified paper-disc method (Cho *et al*., 1994). After pouring the media to be applied to each strain into the sterilized Petri dishes and making them stiff, strains were streaked and cultivated. A colony from the strain was inoculated into liquid media (Nutrient broth, Difco Co., USA). It was cultivated until the optical density was 0.3 at 600 nm by using a spectrophotometer (Beckman DU-650, USA). This cultivated liquid media (150 μl) was added and spread into a plate with solid media, the sterilized paper disc (10 mm diameter, Advantec Co., Japan) was placed on, and 60 μl of the extract was injected onto the paper. After keeping it at 20°C for an hour, the plates with bacteria were incubated at 37˚C for 24 hours and the plates with fungi were incubated at 24˚C for 48 hours. The clear zone around the disc created by the extract against microorganisms was measured after incubation. As a control group, benzoic acid was also tested.

5. Minimum inhibitory concentration (MIC)

The MIC of Hwangryeon methanol extract was measured by the modified Kang *et al*.' method (Kang *et al*, 1994). After extraction with methanol, concentrating the extract, and sterilizing it with a syringe filter, 80, 100, 150, 200, 250, and 300 mg/mL of the extract was poured into the plate. After inoculating strains of 150 μl cultivating media into the plate, the plate was incubated for bacteria at 37˚C for 24 hours and for fungi at 24˚C for 48 hours. The MIC for each microorganism was measured by confirming the growth possibility of the microbes.

6. Component analysis of extract by gas chromatography/mass spectrometer (GC/MS)

Methanol from the extract was removed by rotary vacuum evaporator (Heidolph, VV 2000, Germany). Ethyl ether was added to the concentrate and it was shaken. It was transferred to a separatory funnel and the ether layer was collected. After adding anhydrous Na_2SO_4 into the ether layer and keeping it for a day, it was then filtered with filter paper. The components of the extract were analyzed using GC (GC 8000 series, CE Instruments Co., Italy) and MS (Micromass Quattro II, Micromass Quattro Co., UK) under conditions shown in <Table 1>.

<Table 1> Operation Conditions of Gas Chromatography/mass Spectrometry

Condition	<Gas chromatography>
Model	CE Instruments GC 8000 series (CE Instruments CO., Italy)
Column	DB-17 (50% phenyl/50% methyl silicon) 30m length × 0.25mm i.d. × 0.25 μm (Film Thickness)
Carrier gas	He: 1 mL/mm
Programmed	40˚C held for 5 min
Temperature	5˚C/min to 120˚C (1 min held) 10˚C/min to 280˚C (10 min held)
Injector temperature	220˚C
Condition	**<Mass spectrometer>**
Model	Micromass Quattro II (Micromass Quattro Co., UK)
Column	DB-17 (50% phenyl/50% methyl silicon) 30m length × 0.25mm i.d. × 0.25μm (Film Thickness)
Carrier gas	He: 1 mL/mm
Programmed	40˚C held for 5 min
Temperature	5˚C/min to 120˚C (1 min held) 10˚C/min to 280˚C (10 min held)
Injector temperature	220˚C
Ionization	El
Ionization voltage	70 ev
Ion source temperature	230˚C
Mass scan range	40-550 a.m.u
Injection mode	splitless

III. Results and Discussion

1. Antimicrobial activity assay

The result of antimicrobial activity of the methanol extract from Hwangryeon (*Coptidis rhizoma*) is shown in <Table 2>. In the methanol extract, antimicrobial effect appeared against *S. aureus, P. aeruginosa, S. typhimurium*, and *K. pneumoniae*. The Hwangryeon extract showed a distinctive clear zone of 24.5 mm in *S. aureus*, 22.5 mm in *S. typhimurium*, and 16.5 mm in *P. aeruginosa*. However, antimicrobial activity against fungi did not appear. Therefore, it was concluded that methanol extract from Hwangryeon had stronger antimicrobial activity against bacteria than to fungi. Hwang *et al*. (2000) reported that the ethylacetate fraction of Jakyak (*Paeonia japonica* var. *pilosa* NAKAI) showed a clear zone at 500 μg/disc against *S, aureus, B. subtilis*, and *V. parahaemolyticus* and at 2,000 μg/disc against *L. monocytogenes* and *E. coli.* Therefore, it was determined that a methanol extract of Hwangryeon has comparatively strong antimicrobial activity when compared with this study using 60 μg/disc.

The antimicrobial effect of benzoic acid against food spoilage and foodborne disease microorganisms is shown in <Table 2>. Benzoic acid is used as a preservative agent in foods like soy sauce in Korea.

<Table 2> Antimicrobial Activities of Methanol Extracts from Hwangryeon Against Microorganisms

Microorganisms	Inhibition zone (mm)[1]	
	Hwangryeon	Benzoic acid
Staphylococcus aureus KCTC 1916	24.5	17
Escherichia coli KCTC 1682	10	15
Psudomonas aeruginosa KCTC 1750	16.5	22
Slmonella typhimurium KCTC 2515	22.5	15
Klebsiella pneumoniae KCTC 2001	21.5	16
Fusarium solani KCTC 6636	10	19
Penicillium citreonigrum KCTC 6927	10	17
Aspergillus flavus KCTC 6633	10	18

[1] diameter of paper disc: 10 mm

Benzoic acid showed a clear zone of 22 mm about *P. aeruginosa* and 15 mm, 17 mm, 16 mm, and 15 mm about *S. typhimurium*, *S. aureus*, *K. pneumonia*e, and *E. coli* bacteria, respectively. In the fungi, each clear zone was 18 mm, 19 mm, and 17 mm about *A. flavus, F. solani*, and *P. citreonigrum*. Benzoic acid was reported to have antimicrobial activity against *A. niger*, *P. roqueforti*, *M. pusillus*, *S. cerevisae*, *S. rouxii, P. membranaefaciens, B. sutilis, L. mesenteroides*, and *B. coagulans* strains (Song & Park, 1998). In this experiment, a high antimicrobial effect appeared against *S. typhimurium*, *S. aureus*, *K. pneumonia*e, and *P. aeruginosa*. When compared with methanol extract and benzoic acid, benzoic acid exerted distinctive antimicrobial activity against both bacteria and fungi, but methanol extract from Hwangryeon exerted strong antimicrobial activity against only bacteria in this experiment. Therefore, Hwangryeon extract would be useful to foods that would be easily contaminated by these bacteria.

2. Minimum inhibitory concentration (MIC)

The MIC of methanol extract of Hwangryeon is shown in <Table 3>. The methanol extract of Hwangryeon inhibited the growth of *S. aureus*, *S. typhimurium*, and *K. pneumonia*e at a concentration of 300 mg/mL, but inhibited the growth of *P. aeruginosa* at a concentration of 200 mg/mL. However, the methanol extract of Hwangryeon did not inhibit the growth of *A. flavus*, *F. solani*, and *P. citreonigrum* at 300 mg/mL concentration. Compared with the paper-disc experiment, the case not showing antimicrobial activity on the paper-disc experiment did not inhibit the microbial growth in 300 mg/mL concentration in the MIC experiment. Kim and Han (1997) reported that the methanol extract of Sancho (*Zanthoxylum Schinifolium*) showed antimicrobial activity at 1,000 μg/mL against *B. subtilis* and *S. aureus*. Lee and Shin reported that 1,000 ppm of amur cork extract inhibited *B. cereus* and 2,000 ppm of elm root and plantain showed fairly good inhibition against *L. mesenteroides*

<Table 3> Minimum Inhibitory Concentration (MIC) of the Methanol Extracts from Hwangryeon Against Microorganisms

Microorganisms	Concentration (mg/mL)						MIC
	80	100	150	200	250	300	(mg/mL)
Staphylococcus aureus KCTC 1916	++	++	++	++	+	-	300
Escherichia coli KCTC 1682	++	++	++	++	++	+	>300
Psudomonas aeruginosa KCTC 1750	++	++	+	-	-	-	200
Salmonella typhimurium KCTC 2515	++	++	++	++	+	-	300
Klebsiella pneumoniae KCTC 2001	++	++	++	++	++	-	300
Fusarium solani KCTC 6636	++	++	++	++	++	+	>300
Penicillium citreonigrum KCTC 6927	++	++	++	++	++	+	>300
Aspergillus flavus KCTC 6633	++	++	++	++	++	+	>300

++ : moderate growth
+ : slight growth
- : no growth

(Lee & Shin, 1991). Additionally, it has been reported that the MICs of sodium benzoate and *p*-hydroxybenzoate that had been used as typical preservatives were 100,000 μg/mL and 125-4,000 ppm, respectively (Mok *et al*., 1994). Therefore, it is possible that Hwangryeon extract could be used as a natural preservative.

3. Component analysis of methanol extract

The results obtained from GC/MS analysis of the components of Hwangryeon methanol extracts are showed in <Tables 4-6> and <Figure 1>. In the methanol extracts from Hwangryeon, 190 kinds of compounds were separated by GC/MS. The extraction yields of phenolics, furans, alcohols, acids and esters, ketones, and miscellaneous compounds were 28.10%, 2.67%, 1.79%, 12.89%, and 2.35%, respectively. Phenolic compounds, generally reported as antimicrobial active substances, were major components and existed 28.10%. In the type of phenols, 4-vinyl-2-methoxy-phenol existed 7.39% and 4-hydroxy-3-methoxy-benzaldehyde, 3-(4-hydroxy-3-methoxyphenyl)-methyl-2-propen-noate, and methyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate existed 4.11%, 4.78% and 4.47% in it, respectively. It was reported that magnolol, honokiol, and 3,5'-diallyl-2-hydroxy-4-methoxy biphenyl extracted from *Magnolia grandiflora* exerts antimicrobial activity against Gram positive bacteria and that 8 types of phenolic compounds extracted from *Acer ginnala* and 4-hydroxycinnamic acid extracted from *Aralia elata* showed antimicrobial activity, too (Song & Park, 1998; Clark *et al*., 1981). In the Hwangryeon methanol extracts, 4,5-dimethoxy-isobenzofuran-1,3-dione among furan compounds amounted to 1.61% and alcoholic compounds contained 2,6-dimethyl-1,5-heptadien-3-ol, 2-methyl-1,3-oxatholane-2-propanol, 2,3-epoxyhexanol, and benzylalcohol. Song *et al*. (1998) reported that the extract form Smilax china root contained 5-(hydroxy-methyl)-2-furancarboxaldehyde as a major furan and this aldehyde was also identified in 0.39% in our experiment.

In acid and ester compounds, ethyl acetate was the major component whose amount was 8.40%, and 2-hydroxy-1-(hydroxymethyl) ethyl-octadecanoate and tetra-hydrofurylacetate each existed 4.55% and 2.39%. Ketone compounds existed in 6.66% of all compounds. Among them, 4-hydroxy-4-methyl-2-pentanone and α-methylene-γ-butyrolactone contained 4.16% and 0.36%, respectively. Generally, phenol compounds are known to possess strong antimicrobial activity. In this study, the methanol extract from Hwangryeon contained many kinds of phenols and the ratio of phenolic compounds was the highest.

<Table 4> Phenols in the Methanol Extract from Hwangryeon

Peak No	RT[1)] (min)	Area (%)	Compound Name
			Phenolic compounds (28.10%)
9	15.2	0.18	Phenol
19	19.4	0.16	2-methoxyphenol
23	23.0	0.09	1,2-benzenediol
27	25.8	7.39	4-hydroxy-methoxyl benzoate
33	27.3	0.33	2,6-dimethoxy-phenol
34	27.4	0.52	4-hydroxy-methyl benzoate
37	27.9	0.60	butyl hydroxy toluene
38	28.2	0.14	3-hydroxy-methyl benzoate
39	28.3	4.11	4-hydroxy-3-methoxy-benzaldehyde
40	28.7	0.60	4-hydroxy-benzeneethanol
45	29.7	0.40	4-hydroxy-3-methoxy-methyl benzoate
47	30.1	0.74	3,4-dimethoxy-5-hydroxybenzaldehyde
49	30.4	0.60	methyl-(3-methoxy-4-hydroxy-benzyl)-ether
57	31.9	0.25	3-(3-hydroxyphenyl)-methyl 2-propenoate
58	32.0	0.17	3,4-dihydroxy-methyl benzoate
59	32.1	0.16	1-(4-hydroxy-3-methoxyphenyl)-ethanone
60	32.2	0.50	4-hydroxy-3,5-dimethoxy-benzaldehyde
61	32.3	4.78	3-(4-hydroxy-3-methoxyphenyl)-methy-2-propenoate
64	32.8	0.80	3-(p-hydroxy-m-methoxyphenyl)-2-propenal
65	32.9	0.49	4-(3-hydroxy-1-propenyl)-2-methoxy-phenol
70	33.7	4.47	methyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate
78	35.9	0.44	(2-hydroxy-4-methoxyphenyl)-phenylmethanone
96	42.1	0.18	1-(2,4-dihydroxy-5-methoxyphenyl)-3-phenyl-2-propen-1-one
			Furans (2.67%)
24	23.4	0.14	2,3-dihydro-benzofuran
26	25.3	0.39	5-(hydroxymethyl)-2-furancarboxaldehyde
69	33.6	1.61	4,5-dimethoxy-isobenzofuran-1,3-dione
73	34.2	0.27	4,5-methylenedioxy-1,3-isobenzofurandione
87	37.9	0.26	tetrahydro-2-isopentyl-5-propyl-furan

[1)] Retention Time

<Table 5> Alcohols, Acids and Ester in the Methanol Extract from Hwangryeon

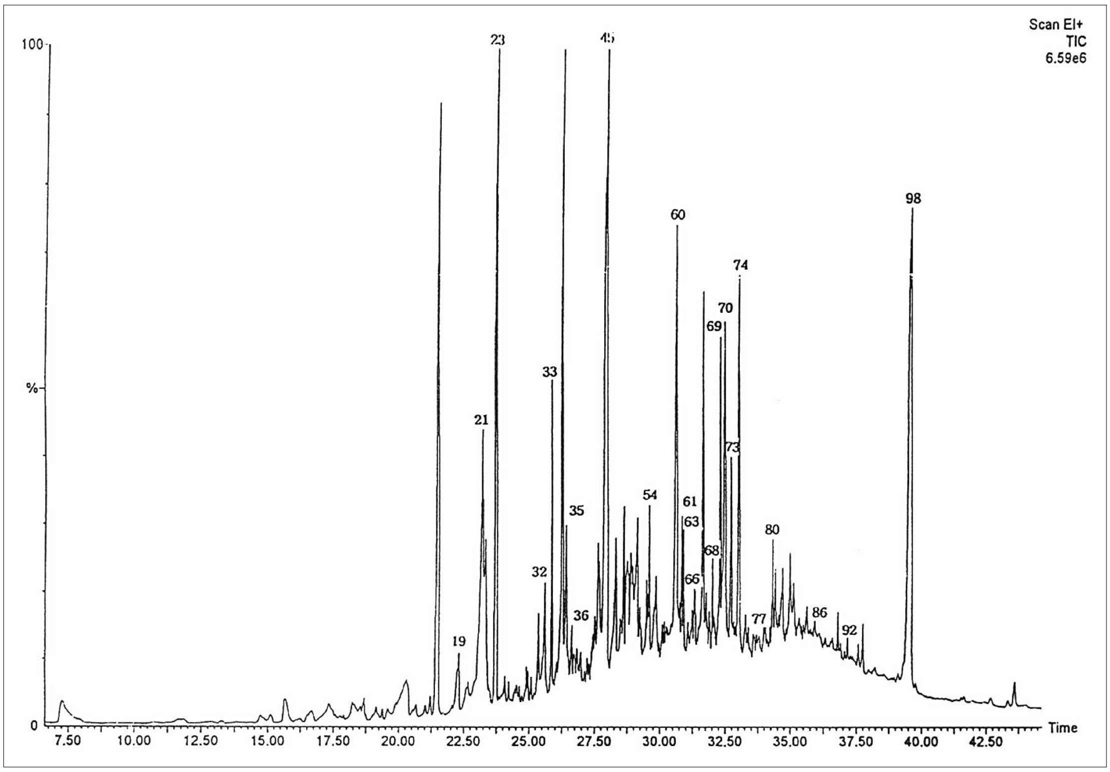
Peak No	RT[1)] (min)	Area (%)	Compound Name
			Alcohols (1.79%)
1	6.2	0.19	2,6-dimethyl-1,5-heptadien-3-ol
4	9.8	0.40	2-methyl-1,3-oxathiolane-2-propanol
5	12.0	0.71	2,3-epoxyhexanol
15	18.3	0.20	benzyl alcohol
20	20.3	0.17	phenylethyl alcohol
23	21.3	0.12	2-(2-butoxyethoxy)-ethanol
			Acids & Ester (12.89%)
2	7.6	8.40	ethyl acetate
6	12.6	2.39	tetrahydrofurfurylacetate
7	13.0	0.24	hexanoic acid
13	17.6	0.20	phenyl dodecanoate
17	19.0	0.08	octyl acetate
22	21.1	0.10	2-hydroxy-dimethylbutanoate
71	33.9	0.72	1,1’-(oxydiethylidene)bis-benzene
72	34.0	0.63	methyl-9,12-octadecadienoate
88	38.3	1.64	2-hydroxy-1-(hydroxymethyl)ethyl-hexadecanoate
93	93	4.55	2-hydroxy-1-(hydroxymethyl)ethyl-octadecanoate

[1)] Retention Time

<Table 6> Ketones and Miscellneous Compounds in the Methanol Extract from Hwangryeon

Peak No	RT[1)](min)	Area (%)	Compound Name
			Ketones (6.66%)
3	9.2	4.16	4-hydroxy-4-methyl-2-pentanone
8	14.5	0.02	2-cyclohexen-1-one
10	15.9	0.23	butyrolactone
11	16.3	0.23	3-methylbutyrolactone
12	17.0	0.15	4,4-dimethyl butyrolactone
14	17.9	0.36	α-methylene-γ-butyrolactone
16	18.5	0.18	pantoic lactone
18	19.2	0.12	1-(pyrrol-2-yl)-ethanone
31	27.0	1.21	2,6-di-butyl-2,5-cyclohexadiene-1,4-dione
			Miscellaneous compounds (2.35%)
22	22.4	0.20	tetradecane
25	24.1	2.04	N-ethyl-N-[(1-methylethoxy)methyl]-ethanamine
81	36.6	0.11	3,4-dihydro-7-hydroxy-6-methoxy-1-isquinolinone

[1)] Retention Time



<Figure 1> GC-MS Spectrum of Methanol Extracts from Hwangryeon (Coptidis rhizoma)

Therefore, it is concluded that phenolic compounds of Hwangryeon contributed to the antimicrobial activity against the microorganisms studied.

IV. Conclusions

In the antimicrobial activities of Hwangryeon methanol extract, the extract showed antimicrobial effect against *P. aeruginosa*, *S. aureus*, *S. typhimurium*, and *K. pneumonia*. The MIC of the extract on *P. aeruginosa* was 200 mg/mL while the MIC on *S. aureus*, *S. typhimurium*, and *K. pneumonia*e was 300 mg/mL. This means that Hwangryeon methanol extract possesses antimicrobial activity against some bacteria and that it could be used as a natural preservative. In the component of methanol extract of Hwangryeon, the yield of phenols that are generally known as an antimicrobial active substance, was highest. Therefore, it was concluded that phenol compounds of the methanol extract would play an important role in the inhibition of microorganisms.

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