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Comparison of Biological Activities using Several Solvent Extracts from Ramariabotrytis

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Abstract

Background/Objectives: To investigate the antibacterial as well as antioxidant activity of *Ramariabotrytis.* **Methods/Statistical analysis:** The antibacterial activities of the solvent extractions (acetone, ethyl acetate, ethanol, and methanol) of *R. botrytis* were determined against Gram-(+)/Gram-(-) bacteria by disc diffusion method. The antioxidant activities were also checked by measuring free radical scavenging activities (DPPH and ABTS). **Findings:** All solvent extracts showed an inhibitary effect against *E. coli,* and especially the ethyl acetate extracts showed higher antibacterial activities against *Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. In addition, methanol extracts were effective in DPPH and ABTS activities. The negative correlations were observed between radical scavenging activities of extracts with TPC and TFC. The TPC and TFC were the highest at acetone and ethyl acetate extracts. **Improvements/Applications:** *Ramaria botrytis* showed antioxidant and antibacterial activities and so they can be widely used in naturally health food combinations.

Keywords: ABTS, Antibacterial and Antioxidant, DPPH, *Ramaria* botrytis, TFC, TPC

1. Introduction

At present, depending on the raise of aging population and diversification of dietary life, the frequency of adult disease and senile disorder has increased. Therefore, an interest in food and health has also increased, and a concept of food is changed into a direction laying stress on nutrition, preference, hygiene, and function. With this, studies on functional component of bioactive substance and food natural products are actively conducted. One of them, because natural products of mushroom has unique taste, outstanding aroma, low lipid, and rich fiber, the consumption has increased as diet food.

Generally, mushroom contains natural bioactive of various secondary metabolites that are functional components[1], and they has reinforcement of immunity, antibacterial and antiviral action, and cholesterol lowering for prevention of adult diseases such as arteriosclerosis, cardiac disorder, and diabetes[2]. In addition, β-glucan of mushroom enhances immune function so shows the inhibitory effect on cancer. Therefore, people are interested in functional food or drug using mushroom lately[3]. In Korea, about 1,150 species of mushrooms are self-begotten, and about 330 species of them are used as edible and medicinal mushrooms. Thus, more than about 20 species of mushrooms are artificially cultivated and they are used for food[4].

Especially, *Ramaria botrytis* is widely distributed in fields and mountains in Korea, East Asia, Europe, and North America, and they lives in herds or alone within a broadleaf forest from the middle of August to late October. More than about 10 species including *Ramariaaurea*, *Ramariaeumorpha*, *Clavulinacoralloides*, and *Clavicoronapyxidata* are self-begotten in this country. Appearance of a fruiting body is similar to lespedeza, so it is called *Ramaria botrytis*. And because a flavor is richer than other mushrooms, they are widely used as health food with high preference[5]. *Ramaria botrytis* contains

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aspartic acid, crysteine, histidine, glutamic acid, organic acid, and free sugar, and minerals content is high, but calcium is less contained[6]. *Ramariaformosa* was reported to have the effect on cancer of sarcoma 180 in mouse[7], and many studies on anti-mutagenic effect, cancer cell growth inhibitory effect, immunocyte invigoration, and anti-tumor have conducted[8,9]. However, studies on its antibacterial and antioxidant activities are insufficient. Therefore, this study is to examine antibacterial activities against Gram-(-)/Gram-(+) bacteria by using the extracts of *Ramaria botrytis* with various solvents. Also, we want to compare and analyze antioxidant activities such as radical scavenging (DPPH and ABTS) including TPC and TFC.

2. Materials and Methods

2.1 Extraction of *Ramaria botrytis* and Determination of Yield

Frozen *Ramaria botrytis* used in this experiment was purchased from Yangyang-gun, Gangwon-do, Korea. 400 mL of each organic solvent (acetone, ethyl acetate, ethanol, and methanol) was added to 50 g of *Ramaria botrytis* that is naturally dried and pulverized, and then, it was extracted and evaporated. All extracts were dissolved in 5 mL of DMSO. The experiment was conducted by diluting these extracts as needed. On measuring antioxidant activity, all extracts were diluted by DMSO for 100mg/mL concentration.

2.2 Antibacterial Susceptibility Testing

Gram-(+) bacteria containing *Bacillus subtilis* (KCTC1918), *Micrococcus luteus* (KCTC1915), and *Staphylococcus aureus* (KCTC1928), and Gram-(-) bacteria containing *Enterobacter cloacae* (KCTC1685), *Escherichia coli* (KCTC2441), and *Pseudomonas aeruginosa* (KCTC1637) are purchased from KCTC (Daejeon, Korea). Antibacterial activity of *Ramaria botrytis* extracts against above all bacteria was measured by disc diffusion method[10]. All bacteria were grown in LB liquid culture at 37□ for 24 hours. A paper discs (ø 6 mm, Whatman AA disc) soaked with 20 μL *Ramaria botrytis* extracts were placed on the preloaded plates. The plates were incubated at 37□ during 24 hours. After that, antibacterial activity was measured by comparing the size of inhibition zone around disc.

2.3 Determination of TPC

TPC of *Ramaria botrytis* extract were measured by the modified Folin-Ciocalteureagent method[11,12]. 45 μL of *Ramaria botrytis* extracts and 45 μL of 1N Folin-Ciocalteureagents were mixed. After 3 minutes, 910 μL of 2% Na_2CO_3 was added, and then the mixture was reacted (RT, 30 minutes). Absorbance was checked at 760 nm, and their calibration curve was obtained by the gallic acid standard. All data was expressed as mg of Gallic Acid Equivalent (GAE)/g extract.

2.4 Determination of TFC

Using a method to form aluminium-flavonoid complexes, flavonoid contents of *Ramaria botrytis* extract were measured by two methods as follows[12,13]. First, 500 μL of *Ramaria botrytis* extracts and 500 μL of 2% $AlCl_3$ were mixed and reacted (RT, an hour). Absorbance was measured at 420 nm, and their calibration curve was obtained by the quercetin standard. All data were expressed as mg Quercetin Equivalent (QE)/g extract. Second, 250 μL of *Ramaria botrytis* extract and 1,000 μL of distilled water were mixed and 75 mL of 5% $NaNO_2$ was added. And then 150 μL of 10% $AlCl_3$ and 500 μL of 1M NaOH were added. After 15 minutes, absorbance was confirmed at 510 nm. Calibration curve was obtained by the catechin standard. All data was expressed as mg of Catechin Equivalents (CE)/g extract.

2.5 DPPH Activity

DPPH activity of *Ramaria botrytis* extract was determined by using Blois method with a little modification[14,15]. 30 μL of *Ramaria botrytis* extracts was added to 970 μL of 0.1 mM DPPH solution. The absorbance (MECASYS, Daejeon, Korea) was measured at 517 nm as a blank control. 1 mM ascorbic acid was used as a positive control. The DPPH radical scavenging activity (%) = $(1 - A_{sample}/A_{control}) \times 100$.

2.6 ABTS Activity

ABTS activity of *Ramaria botrytis* was measured by using a method of Re with some modifications[15,16]. 7.4 mM ABTS and 2.6 mM potassium per sulfate were mixed and reacted in dark place during 24 hours. And then the mixture was diluted until to 0.7 at 734 nm. 970 μL of above solution was mixed with 30 μL of extracts and it was kept in the dark (RT, 30 minutes). Then, the absorbance was

measured against a blank control at 734 nm. The mixture with addition of ascorbic acid served as a positive control. The ABTS radical scavenging activity (%) = (1 - A_{sample}/ $A_{control}$) × 100.

2.7 Statistical Analysis

All experiments are repeated three times and showed as average±standard deviation. Statistical analysis was performed ANOVA followed by PASW Statistics 23.0 (SPSS Inc.). $P<0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Extraction and Yield

The extraction yields obtained from *Ramaria botrytis* powder were shown in Table 1. The extraction with methanol extract resulted in the highest yield of 11.28%, and the extraction yield of ethanol, acetone, and ethyl acetate were 6.86%, 2.94%, and 2.08%, respectively.

Table 1. Yield of extracts from *Ramaria botrytis*

	Methanol	Ethanol	Acetone	Ethyl acetate
Yield (%)	11.28	6.86	2.94	2.08

3.2 Antibacterial Activity of *Ramaria botrytis* Extracts

The antibacterial activity of *Ramaria botrytis* extracts were investigate against several bacteria with paper disc diffusion (Table 2). An inhibitory effect on the growth by *Ramaria botrytis* extracts was confirmed against four cultures. Ethyl acetate extracts showed antibacterial activity against four cultures, and also showed the highest antibacterial activity against *P. aeruginosa*. Ethanol extracts showed antibacterial activity against three cultures, and also showed a somewhat larger inhibition zone of *P. aeruginosa*. In addition, acetone and methanol extracts showed antibacterial activity only against *E. coli*. All *Ramaria botrytis* extracts showed antibacterial activity against *E. coli*, Gram-(-) bacteria, but did not show any clear zone against *B. subtilis* and *M. luteus,* Gram-(+) bacteria. As a result, *Ramaria botrytis* extracts showed high antibacterial activity on Gram-(-) bacteria in comparison with Gram-(+) bacteria. In addition, ethyl acetate extracts showed high activity against *E. coli*, *P. aeruginosa*, *Ent. cloacae,* and *S. aureus*in comparison with other extracts. Like this, antibacterial activity of mushroom was affected by species of mushroom, extraction solvent, and culture used in an experiment[17]. Barros confirmed that methanol extracts of *Agaricusbisporus* and *Agaricussilvicola* showed antibacterial activity against *B. subtilis*, *B. cereus*, and *S. aureus*[18]. In addition, ethyl acetate extract of *Lentinusedodes* showed an inhibition effect against *B. subtilis*, *Ent. cloacae, E. coli, M. luteus, P. aeruginosa,* and *S. aureus*[19]. Similarly, our results with *Ramaria botrytis*, showed that ethyl acetate was the best solvent to extract antibacterial substance rather than acetone, ethanol, and methanol.

Table 2. Antibacterialactivities of various solvent extracts from *Ramaria botrytis* against gram-positive and gram-negative bacteria

Bacterial strains	Extracts	Inhibition zone diameter (mm)
Enterobacter cloacae	Acetone	-
	Ethanol	-
	Ethyl acetate	7.7±0.95
	Methanol	-
Escherichia coli	Acetone	8.0±0.95
	Ethanol	7.7±0.65
	Ethyl acetate	8.0±0.89
	Methanol	7.7±0.60
Pseudomonas aeruginosa	Acetone	-
	Ethanol	8.6±1.07
	Ethyl acetate	9.0±0.89
	Methanol	-
Bacillus subtilis	Acetone	-
	Ethanol	-
	Ethyl acetate	-
	Methanol	-
Micrococcus luteus	Acetone	-
	Ethanol	-
	Ethyl acetate	-
	Methanol	-
Staphylococcus aureus	Acetone	-
	Ethanol	6.5±0.75
	Ethyl acetate	6.4±0.95
	Methanol	-

Values are mean±SD (n=3); -, not detected (6 mm)

3.3 TPC of *Ramaria botrytis* Extracts

Various bioactive substances have an impact on anticancer and antibacterial activities. As secondary metabolite in the vegetable kingdom, polyphenol (phenolic hydroxyl, -OH) so provides electron, and they suppresses oxidation by active oxygen. And they has various biological activities such as not only outstanding anti-oxidation but also antibacterial and anticancer effects[20,21]. A result of TPC extracted from *Ramaria botrytis* by using various solvents was shown as Table 3. As acetone extracts was 3.28±0.13 mg GAE/g extract and ethyl acetate was 3.34±0.04 mg GAE/g extract, they showed a significant difference and high contents in comparison to methanol and ethanol extracts. In addition, as extract by methanol was 0.43±0.01 mg GAE/g extract and ethanol was 0.58±0.01 mg GAE/g extract, phenolic contents were looked like a little. A correlation between phenolic compound and other compounds was not revealed, so the selections of solvent and extraction condition are so important. Turkmen investigated an effect of various solvents about phenolic contents extracted from black tea, and 80% acetone showed the highest contents[22]. In addition, Yu reported that when *Auriculariaauricular-judae* is extracted by several solvents, there was no significant difference in phenolic contents[23]. As a result, in case of *Ramaria botrytis*, a content difference of phenolic compounds can be accurately confirmed depending on solvents used in extraction, and when acetone and ethyl acetate are used as solvent rather than methanol and ethanol extracts, phenolic compound contents are much higher.

Table 3. Total phenolic and flavonoid contents of extracts from *Ramaria botrytis*

solvent	Total phenolic content (mg GAE/g extract) 1)	Total flavonoid contents	
		mg QE/g extract 2)	mg CE/g extract 3)
Acetone	3.28±0.13 [a 4)]	0.71±0.03 [a]	11.75±0.17 [a]
Ethyl acetate	3.34±0.04 [a]	0.61±0.01 [b]	11.57±0.25 [a]
Methanol	0.43±0.01 [c]	0.27±0.00 [d]	4.59±0.10 [c]
Ethanol	0.58±0.01 [b]	0.52±0.00 [c]	7.15±0.60 [b]

The results represent the mean±SD of values obtained from three independent experiments.

1) Values are expressed as mg of Gallic Acid Equivalent (GAE) per g extract (mg GAE/g extract).

2) Values are expressed as mg of Quercetin Equivalent (QE) per g extract (mg QE/g extract).

3) Values are expressed as mg of Catechin Equivalent (CE) per g extract (mg CE/g extract).

4)[abcd]Means with the different letters within a column are significantly different by Duncan's multiple range test ($P<0.05$).

3.4 TFC of *Ramaria botrytis* Extracts

As one of polyphenol compounds, flavonoids is variously existed in plant, and they suppressed pathogenic bacterium, block the ultraviolet rays, and are good for antivirus and anti-inflammatory[24]. Flavonoids contents of *Ramaria botrytis* extracts were found by standard substances such as quercetin and catechin, and the result was as Table 3. When quercetin is standard substance, acetone extracts was 0.71mg QE/g extract, so is significantly higher than other extracts. And ethyl acetate extracts showed 0.61 mg QE/g extract of TPC, and ethanol extracts showed 0.52 mg QE/g extract of TPC. As 0.27 mg QE/g extract, methanol extracts showed the lowest content. TFC are in the order of acetone > ethyl acetate > ethanol > methanol, and they showed a similar tendency with TPC. About TFC measuring by making catechin as standard substance, acetone extract was 11.75 mg CE/g extract, and other extracts showed different results such as ethyl acetate extracts (11.57mg CE/g extract), ethanol extracts (7.15 mg CE/g extract), and methanol extracts(4.59 mg CE/g extract). Value of flavonoid contents measured by making catechin as standard substance is much higher than value of flavonoid contents measured by making quercetin. Generally, it was known that a method using quercetin as standard substance is used in flavonoid and flavones (luteolin) and a method using catechin is also used in rutins, luteolins, and catechins[13]. As a result of this study, *Ramaria botrytis* extracts contained flavonoid affiliation such as rutins, luteolins, and catechins more.

3.5 DPPH Activity of *Ramaria botrytis* Extracts

DPPH method was used to measure the standard to bleach purple by getting electron with reacting DPPH radical, and it was one of method to measure antioxidant activity of single compound/extract. A result to measure DPPH activity using ascorbic acid, a positive control, and *Ramaria botrytis* extracts was as Figure 1. As methanol extract was 92.1±0.19 % and ethanol extract was 91.4±0.62 %, these extracts showed considerably high DPPH activity. Ethyl acetate extracts showed 55.9±2.34 % as relatively low scavenging activity, but this activity was similar to 59.4±6.98 % of scavenging activity of ascorbic acid, a positive control. Generally, Cheung reported that DPPH activity of *Lentinusedodes* was 29.4±0.59 % in methanol extract (9mg/mL) and 40.4±4.41 % in hot water extract (9mg/mL)[25]. And Yoon reported that DPPH activity was

76.94% in hot water extract of *Sarcodonaspratus* (1,000 ppm) and was 73.06% in ethanol extract (1,000 ppm)[26]. As a result, comparing with above studies, radical scavenging activity of *Ramariabotrytis* was more excellent than radical scavenging activity of other mushrooms. That is, DPPH activity of *Ramaria botrytis* extracts differs depending on polarity of solvent that is used to extract, and is mostly excellent in polar solvent. In addition, this activity showed a negative correlation with polyphenol and flavonoid contents (r = -0.70). Because antioxidant activity was affected by various natural bioactivity substances such as phenol, flavonoid, peptides, and organic acids, it was difficult to find a consistent correlation with polyphenol and flavonoid contents[27].

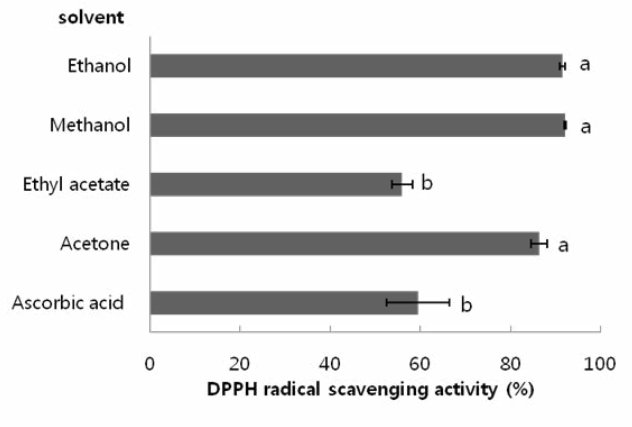

Figure 1. DPPH radical scavenging activity of extracts from *Ramaria botrytis*. The results represent the mean±SD of values obtained from three independent experiments. 1 mM ascorbic acid was used as positive control. [a,b,c]Means with different letter on the bars are significantly different by Duncan's multiple range ($P<0.05$).

3.6 ABTS Activity of *Ramaria botrytis* Extracts

ABTS activity is a method using ABTS radical that are made by reacting with potassium persulfate[28]. A result to measure ABTS radical scavenging activity was as Figure 2. *Ramaria botrytis* extracts showed 87.6±1.81% of ABTS activity in methanol extract, 60.8±1.92 % in acetone extract, 60.6±7.23 % in ethanol extract, and 2.8±3.59 % in ethyl acetate extract that was considerably lower than other extracts and similar to DPPH activity. Hong reported that methanol extract of *Lentinusedodes* showed excellent scavenging activity[29], and Kang reported that ethanol extract of *Flammulinavelutipes* showed excellent scavenging activity[30]. In case of *Ramaria botrytis*, an object of this study, it was suitable the most to use methanol as solvent to extract bioactive substance from *Ramaria botrytis*. There was a positive correlation (r = 0.96) between DPPH and ABTS activities, but there was a somewhat difference because kinds of radical and reaction strength of antioxidant and radical are different.

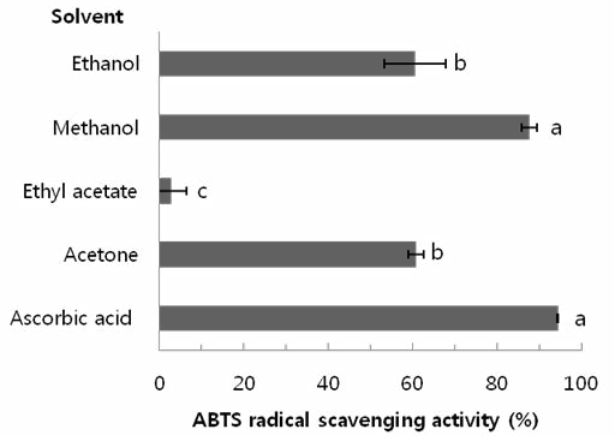

Figure 2. ABTS radical scavenging activity of extracts from *Ramaria botrytis*. The results represent the mean±SD of values obtained from three independent experiments. 1 mM ascorbic acid was used as positive control. [a,b,c]Means with different letter on the bars are significantly different by Duncan's multiple range ($P<0.05$).

4. Conclusion

All extracts obtained from *Ramaria botrytis* by using different solvents were investigated for their antibacterial and antioxidant activities. Extraction yield was in the order of methanol extract (11.28%) > ethanol extract (6.86%) > acetone extract (2.94%) > ethyl extract (2.08%), and ethanol and methanol with a strong polarity showed somewhat high extraction yield. Ethyl acetate extracts showed high antibacterial activity against *Ent. cloacae, E. coli*, *P. aeruginosa*, and *S. aureus* in comparison with other solvent extracts. Generally, *Ramaria botrytis* extracts have an inhibition effect against Gram-(-) bacteria rather than Gram-(+) bacteria. TPC/TFC in acetone and ethyl acetate extracts were much higher than TPC/TFC in ethanol and methanol extracts with contrasting to extraction yield. DPPH activity was considerably high in methanol and ethanol extracts, and ABTS activity was similar to DPPH activity. *Ramaria botrytis* has antioxidant and antibacterial activities, and so it can be used as naturally functional material. Furthermore, it will be

necessary to confirm the structure by separating substance related to biological activity from *Ramaria botrytis*.

5. References

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