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Biological Activity of Different Solvent Extracts from *Hypsizygus marmoreus*

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Abstract: It is to analyze antioxidant and antibacterial activities of several solvent extracts from *Hypsizygus marmoreus*. Antioxidant activity was evaluated by determining DPPH/ABTS activities, TPC and TFC. In addition, the antibacterial activity was determined against six bacterial pathogensby disc diffusion method. DPPH radical scavenging activity (93.3±0.10%) and ABTS radical scavenging activity (97.2±1.22) were significantly high in methanol extracts which also showed higher value of TPC and TFC as compared other extracts. In addition, all extracts showed an inhibition effect against *P. aeruginosa* and one of them, the ethyl acetate extractsespecially showed a very high inhibition effect against Gram (-) bacteria (*Ent. cloacae*, *E. coli* and *P. aeruginosa*). *Hypsizygus marmoreus* has a high biological active effect related to antioxidant and antibacterial activity and so their utility value is so high in various health foods or functional cosmetic products.

Key words: Antibacterial activity, antioxidant activity, flavonoid, *Hypsizygus marmoreus*, polyphenol

INTRODUCTION

As attack rate of chronic diseases such as diabetes, high blood pressure, cardiac disorder and cardiovascular disorder is increase, consumption tendency of health-oriented foods related to low-calorie natural food also increases to prevent above diseases. In addition to overcome many difficulties on usage by antimicrobial resistance, many researchers have tried to develop new natural compounds with an antimicrobial effect and without a side effect. At present, many studies are confirming various biological activities of natural product and establishing their structure and function in the world.

Mushroom is flavor and not only contains all sorts of nutrients such as carbohydrate, protein, lipid and minerals but also has various biologically active functions so has been widely used for food and medicine. Major biological activities of mushroom revealed until now are cholesterol lowering, dropping in blood sugar level, antimicrobial and anticancer effects (Fukushima *et al*., 2001; Smith *et al*., 2002), anti-high-pressure vitality of *Pleurotus cornucopiae* (Jang *et al*., 2011), platelet aggregation inhibitory activity of *Inonotus obliquus* mycelial extract (Hyun *et al*., 2006), anti-dementia activity of *Umbilicaria esculenta* (Lee *et al*., 2009) and hyperlipidemia prevention of *Pholiota adipose* (Yu *et al*., 2007). Especially, many antibacterial activities are reported as below; *Agaricus bisporus* methanol extract against *Bacillus subtilis* (Barros *et al*., 2008), *Hericium erinaceum* ethyl acetate extract against *Micrococcus luteus* (Han *et al*., 2015a), *Lentinula edodes* ethyl acetate extract against *B. subtilis*, *Enterobacter cloacae*, *Escherichia coli*, *M. luteus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Han *et al*., 2015c) and *Pleurotus ostreatus* methanol extract against *B. megaterium*, *E. coli*, *Kiebsiella pneumonia* and *S. aureus* (Wolff *et al*., 2008).

Hypsizygus marmoreus belongs to genus of Tricholomataceae of basidiomycetes and has a boast of unique texture and rich taste. It can be found in East Asia such as Korea, Japan and China and is grown in a bundle in stump of beech, maple and dead tree. Some studies about *Hypsizygus marmoreus* are also conducted as other mushrooms. Matsuzawa *et al*. (1997, 1998) confirmed antitumor and antioxidant effects of *Hypsizygus marmoreus*, Akavia *et al*. (2009) reported that *Hypsizygus marmoreus* contains antitumor polysaccharide β-(1, 3) D-glucan (Akavia *et al*., 2009). In addition, Kim confirmed antioxidant and tyrosinase inhibition effects by using methanol extract (Kim *et al*., 2013). However, there was no data of antimicrobial and antioxidant activities by various solvents targeting *Hypsizygus marmoreus* therefore this study makes each extracts by using various solvents and investigates their antioxidant and antimicrobial activities to examine usefulness of *Hypsizygus marmoreus* as a naturally functional compound in detail.

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MATERIALS AND METHODS

Materials: *Hypsizygus marmoreus*, Baekmansong-I [trade name, a product mixing *Hypsizygus marmoreus* (brown cultivar) and *Hypsizygus marmoreus* (white cultivar)] was purchased from Co. Pureuni Food in Yeoju-gun, Gyeonggi-do. All chemical compounds containing DPPH, ABTS, gallic acid, quercetin, catechin and ascorbic acid were obtained from Sigma-aldrich Co. and other all reagents were used as special grade chemical for analysis. In order to measure antibacterial activity of *Hypsizygus marmoreus* extracts, 6 kinds of bacteria containing *Bacillus subtilis* (KCTC1918), *Staphylococcus aureus* (KCTC1928), *Micrococcus luteus* (KCTC1915), *Escherichia coli* (KCTC2441), *Pseudomonas aeruginosa* (KCTC1637) and *Enterobacter cloacae* (KCTC1685) werepurchased from KCTC (Microorganism Resource Center).

Extraction of *Hypsizygus marmoreus*: The 400 mL of each solvent including acetone, ethyl acetate, ethanol and methanol was added to 50 g of powder and then, the agitated extract was conducted during 3 days. After 2 h, three vacuum filtrations were conducted by using separating funnel and then it was concentrated by using rotary evaporator (EYELA A1000S) and dissolved in DMSO to be 100 mg/mL and then it was used to an experiment by keeping at 4°C.

DPPH activity: DPPH activities of *Hypsizygus marmoreus* extracts were measured after changing some of method by Blois (1958) and Han *et al*. (2015b). After mixed 30 μL of *Hypsizygus marmoreus* extracts and 970 μL of 0.1 mM DPPH solution in dark place during 30 min, absorbance was measured at 517 nm. The 1 mM ascorbic acid was used as a control and DPPH activity (%) showed a difference of absorbance between sample addition and control in percentage:

$$\text{Activity percentage} = (1-A_{sample}/A_{blank})\times 100$$

ABTS activity: ABTS activities of *Hypsizygus marmoreus* extracts were measured using the modified method by Re *et al*. (1999) and Han *et al*. (2015b). About 7.4 mM ABTS and 2.6 mM potassium persulfate were mixed on a one for one basis (1:1) and it was reacted in dark place during >12 h. After controlling ABTS solution to be 0.7 of absorbance at 734 nm, 970 μL of this solution and 30 μL of *Hypsizygus marmoreus* extracts were mixed.After reacted in dark place during 30 min, absorbance was measured at 734 nm. The 1 mM ascorbic acid is used as a control and ABTS activity (%) showed a difference of absorbance between sample addition and control in percentage:

$$\text{Activity percentage} = (1-A_{sample}/A_{blank})\times 100$$

Determination of TPC: Phenolic content of *Hypsizygus marmoreus* extractswas measured using the modified method by Folin and Denis (1915), Han *et al*. (2015a). After mixed with *Hypsizygus marmoreus* extracts (45 μL) and 45 μL of 1 N Folin-Ciocalteu reagent, 910 μL of 2% Na_2CO_3 was added and then absorbance was measured at 760 nm. TPCwas calculated by calibration curve with gallic acid as standard material: mg of Gallic Acid Equivalents (GAE)/100 g DW.

Determination of TFC: Flavonoid content of *Hypsizygus marmoreus* extractswas measured by using the modified $AlCl_3$ method as following two methods (Quettier-Deleu *et al*., 2000; Pekal and Pyrzynska, 2014). After mixed 500 μL of *Hypsizygus marmoreus* extracts and 0.5 L of 2% $AlCl_3$, absorbance was checked out at 420 nm. TFC was calculated using quercetin as a standard material: mg of Quercetin Equivalents (QE)/100 g DW. The 250 μL of *Hypsizygus marmoreus* extracts and 1 L of distilled water were mixed and 75 mL of 5% $NaNO_2$ was added. About 150 μL of 10% $AlCl_3$ and 500 μL of 1 M NaOH were added and then absorbance was confirmed at 510 nm. TFC was calculated by making catechin as standard material: mg of Catechin Equivalents (CE)/100 g DW.

Antimicrobial susceptibility testing: Antimicrobial activities of *Hypsizygus marmoreus* extractsagainst several bacteria were confirmed by disc diffusion method (Piddock, 1990). After each culture was inoculated in LB liquid culture medium, it was cultivated at 37°C during >12 h and then absorbance was changed into 0.1. Eventually it was painted out by the sterilized cotton swab on LB plate. After 20 μL of *Hypsizygus marmoreus* extracts (2 mg/disc) were put on paper disc (6 mm diameter, ADVANTEC, Japan), it was incubated at 37°C during 24 h. Antimicrobial activity was confirmed by measuring diameter including clear zone made around disc.

Statistical analysis: All experiments are repeated three times and showed by average±standard deviation and SPSS Statistics 23.0 (SPSS Inc.) was used. In addition, after variance analysis (ANOVA), significance in $p<0.05$ level was verified by Duncan test.

RESULTS AND DISCUSSION

DPPH activity of hypsizygus marmoreus extracts: Free-radical glut causes cancer, diabetes, cardiovascular disease and liver ailment promotes body aging. Antioxidant material erases this free-radical and it can

Table 1: Yield, total phenolic and flavonoid contents of extracts from *Hypsizygus marmoreus*

Solvent	Yield (g/100 g DW)	Total phenolic content (mg GAE/100 g DW)[1]	Total flavonoid contents (mg QE/100 g DW)[2]	(mg CE/100 g DW)[3]
Acetone	3.46	3.49±0.33[b, 4]	0.66±0.05[c]	20.36±0.69[d]
Ethyl acetate	2.08	3.04±0.20[c]	0.61±0.06[c]	13.52±0.11[b]
Methanol	13.44	4.40±0.09[a]	3.18±0.06[a]	36.57±0.24[a]
Ethanol	6.86	1.10±0.14[d]	2.26±0.16[b]	17.48±0.35[c]

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 100 g dry weight (mg GAE/100 g DW); [2]Values are expressed as mg of Quercetin Equivalent (QE) per 100 g dry weight (mg QE/100 g DW); [3]Values are expressed as mg of Catechin Equivalent (CE) per 100 g dry weight (mg CE/100 g DW) 4); [a-d]Means with the different letters within a column are significantly different by Duncan's multiple range test ($p<0.05$)

prevent various chronic diseases. DPPH, stable free radical is returned by antioxidant material. At this moment while dark purple turns pale, absorbance decreases so antioxidant activity of extracts can be measured (Kedare and Singh, 2011). DPPH radical scavenging result of *Hypsizygus marmoreus* extracts from organic solventswas showed in Fig. 1. As methanol extract was 93.3±0.10% and acetone extract was 77±3.72%, these extracts showed scavenging activity much higher than 59.4±6.98% of ascorbic acid used as a control. In addition, ethanol extract was 53.6±1.61% and ethyl acetate extract was 57.0±1.60% and they showed no significant difference with ascorbic acid. Hong *et al*. (2012) reported that the whole part of *Hypsizygus marmoreus* was 80.8%, stem of them was 79.4% and surface of them was 85.8% when they checked DPPH activity of methanol extract from *Hypsizygus marmoreus*. And Zanabaatar also reported that DPPH activity in ethanol extract of *Hypsizygus marmoreus* (brown cultivar) was 5.6% and ethanol extract of *Hypsizygus marmoreus* (white cultivar) was 5.8% (Bolormaa *et al*., 2011, 2012). As above, our studies confirmed that radical scavenging activities in methanol extracts were higher than ethanol extract because *Hypsizygus marmoreus* (brown cultivar) and *Hypsizygus marmoreus* (white cultivar) are also mixed and used.

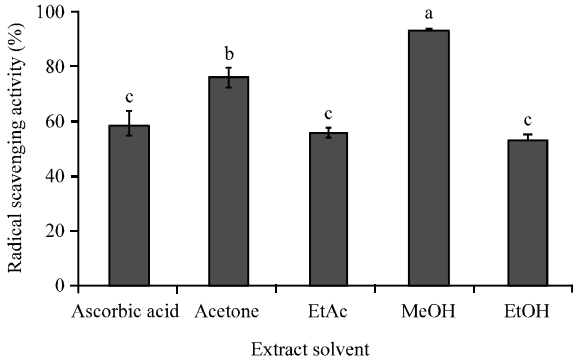

Fig. 1: DPPH radical scavenging activity of extracts from *Hypsizygus marmoreus*. The results represent the mean±SD of values obtained from three independent experiments. 1 mM ascorbic acid was used as positive control. EtAc, ethyl acetate; MeOH, methanol; EtOH, ethanol; [a-c]Means with different letter on the bars are significantly different by Duncan's multiple range ($p<0.05$)

ABTS activity of *Hypsizygus marmoreus* extracts: ABTS radical is made by oxidizing agents such as potassium persulfate.While it is changed into colorlessness by reacting with antioxidant material, antioxidant activity of extracts can be measured with difference of absorbance (Jun *et al*., 2013). Not only it can be applied to all antioxidant materials related to water solubility and fat solubility but also an experimental method is relatively simple and sensitivity is good. And it is possible to measure in a variety of pH (Re *et al*., 1999; Awika *et al*., 2003). A result to measure ABTS activity of *Hypsizygus marmoreus* extracts was as Fig. 2. ABTS activity of methanol extract was 97.2% with acetone extract (31.0%), ethanol extract (27.6%) and ethyl acetate extract (16.3%). Especially, scavenging activity of methanol extract was 3 times higher than other extracts and there was no significant difference with activity of ascorbic acid as a control. Hong *et al*. (2012) reported that ABTS activity in 80% methanol extract of *Hypsizygus marmoreus* (brown cultivar) was 88.5%, however this study we found that ABTS activity was 97.2% that is much higher. As these results, activities are differently showed according to extractant because elution degrees of antioxidant substances are different according to polarity of solvents and various bioactive substances of *Hypsizygus marmoreus* which are applied to different solvent according to drying methods (Kim *et al*., 2012).

Yield, TPC and TFC of *Hypsizygus marmoreus* extracts: Table 1 showed yield of *Hypsizygus marmoreus* extracts obtained by several solvents. *Hypsizygus marmoreus* extracts showed 2.60~13.44% of extraction yield and are investigated in the order of methanol>ethanol>acetone >ethyl acetate. Especially, yield of methanol extract was about five times higher than yield of ethyl acetate extract.

Table 2: Antimicrobial activities of various solvent extracts from *Hypsizygus marmoreus* against gram-positive and gram-negative bacteria

	Inhibition zone diameter (mm)					
	Gram (+)			Gram (-)		
Solvent	*B. subtilis*	*M. luteus*	*S. aureus*	*Ent. cloacae*	*E. coli*	*P. aeruginosa*
Acetone	-	-	-	-	-	6.6±1.1
Ethanol	-	-	-	-	8.3±0.3	7.4±0.2
Methanol	-	-	-	-	6.8±1.4	7.6±0.8
Ethyl acetate	-	-	-	7.7±0.4	8.9±1.1	8.0±0.2

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

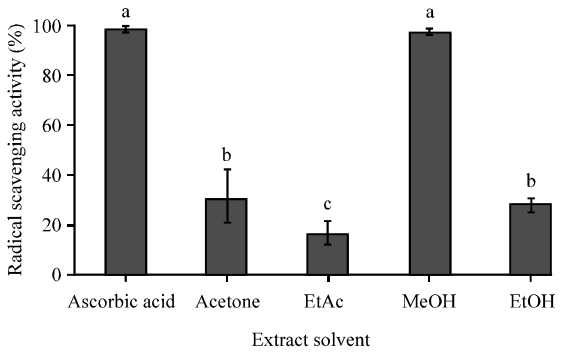

Fig. 2: ABTS radical scavenging activity of extracts from *Hypsizygus marmoreus*. The results represent the mean±SD of values obtained from three independent experiments. The 1 mM ascorbic acid was used as positive control. EtAc, ethyl acetate; MeOH, methanol; EtOH, ethanol; [a-c]Means with different letter on the bars are significantly different by Duncan's multiple range ($p<0.05$)

As second metabolite that is widely distributed in the vegetable kingdom, phenolic compounds include not only simple structure such as phenolic acid and anthocyanin but also substance with high molecular weight such as tannin which showed various biological activities (Chen and Yen, 2007). This study showed TPC of *Hypsizygus marmoreus* extracts and the measured results were as Table 1. Methanol extract was 4.40 mg GAE/100 g DW with acetone extract (3.49 mg GAE/100 g DW), ethyl acetate extract (3.04 mg GAE/100 g DW) and ethanol extract (1.10 mg GAE/100 g DW). And there was a meaningful difference according to solvents ($p<0.05$). Generally as there was a positive correlation between phenolic content and antioxidant activity in mushroom (Choi *et al*., 2006) this study confirmed a positive correlation as r = 0.86 in a relation between phenolic content and DPPH activity. In the future, if TPC is raised by optimizing extracting conditions of *Hypsizygus marmoreus* more, antioxidant activity will be considerably raised.

Flavonoid is dominated by phenolic compound which is known as very effective and strong antioxidant (Yu and Oh, 2016). Flavonoid content of *Hypsizygus marmoreus* according to extractant was investigated by making quercetin and catechin as standard substance and the measured result was showed in Table 1. According to standard substances, a difference was confirmed in flavonoid content of *Hypsizygus marmoreus* and especially, when catechin was used as standard substance, flavonoid content was generally higher. When quercetin was used as standard substance, the highest flavonoid content was showed as 3.18±0.06 mg QE/100 g DW in methanol extracts and as 2.26±2.26 mg QE/100 g DW in ethanol extracts. In extract whose standard substance was catechin, methanol extract contained the most as 36.57 mg QE/100 g DW and they showed a meaningful difference in comparison with other solvent extracts. Pekal and Pyrzynska (2014) reported that rutin, luteolin and catechin are suitable in flavonoid measuring method whose standard substance was catechin. And flavonol and luteolin can be used to decide in flavonoid measuring method whose standard substance was quercetin. Therefore, a difference of flavonoid content confirmed by this study was a result by a difference of absorbance between different standard substances and consequentially, flavonoid of catechin was contained more than flavonoid of flavonol in *Hypsizygus marmoreus* extracts.

Antibacterial activity of *Hypsizygus marmoreus* extracts: A result to measure antibacterial activity of *Hypsizygus marmoreus* extracts with paper disc diffusion against several bacteria was as Table 2. *Hypsizygus marmoreus* extracts showed antibacterial activity of Gram (-) bacteria but do not showed an inhibition effect of Gram (+) bacteria. In case of ethyl acetate, clear zone of *E. coli* was 8.9 mm with *Ent. cloacae* (7.7 mm), *P. aeruginosa* (8.0 mm) and in case of ethanol extract, clear zone of *E. coli* was 8.3 mm with *P. aeruginosa* (7.4 mm). As a result, this study confirmed that antibacterial activity of ethyl acetate extract was the highest activity. This result was partly reported in other mushrooms. It was reported that acetone and ethyl acetate extracts of *Hericium erinaceum* showed antibacterial activity against *E. coli* and *B. subtilis*

(Han *et al*., 2015a). And it was reported that ethyl acetate extract of *Coriolus versicolor* mycelial culture medium showed high activity against *P. aeruginosa* and ethyl acetate extract of *Pleurotus eryngii* shows high activity against *E. coli* and *P. aeruginosa* (Lee *et al*., 2006; Kim *et al*., 2006). In addition, ethyl acetate extract of *Lentinus edodes* showed high activity in comparison with ethanol and acetone extracts (Han *et al*., 2015c). Clark *et al*. (1981) reported that phenolic compound of plants showed antibacterial function. However, in this study showed the highest suppressive activity in ethyl acetate extracts with low phenolic content and not only polyphenol and flavonoid but also various substances with antibacterial activity which can be existed depending on natural materials therefore it is considerably important to select extractant.

CONCLUSION

This study examined a generally biological active effect of *Hypsizygus marmoreus* by investigating yield, TPC, TFC, antioxidant activities such as DPPH/ABTS activities and disc diffusion antimicrobial activity against 6 bacteria. Extraction yield of methanol extract was 2~5 times higher than other solvents. And as a result of DPPH activity, acetone and methanol extracts showed higher scavenging activity than ascorbic acid as a control. Especially, this study confirmed that there was stronger antioxidant scavenging activity when solvents are mixed and extracted with brown and white *Hypsizygus marmoreus*. In ABTS activity, methanol extracts showed activity similar to ascorbic acid, a control and methanol extract was relatively high in TPC and TFC according to dry weight of *Hypsizygus marmoreus*. Antibacterial activity of *Hypsizygus marmoreus* was confirmed in all solvents against *P. aeruginosa* and ethyl acetate extracts showed the biggest growth inhibition effect. As a result, this study can prove that *Hypsizygus marmoreus* was natural ingredient with a high biological active effect related to antioxidant and antibacterial activity therefore, the utility value of *Hypsizygus marmoreus* is so high in various health functional foods or functional cosmetic development in the future.

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