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Biological Activity Analysis of Different Solvent Extracts from *Pleurotus Ostreatus*

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Abstract

We have evaluated antioxidant and antimicrobial activities of various solvents extracts from *Pleurotus ostreatus*. Organic solvents such as acetone, ethyl acetate, and ethanol were used to produce *P. ostreatus* extracts. Antioxidant activities were determined by measuring total polyphenol and flavonoid contents, DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging activity, and ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity. The acetone extract showed the highest antioxidant activities except total flavonoid contents. Antimicrobial activities of *P. ostreatus* extracts were determined against four strains of Gram-negative bacteria and eleven strains of Gram-positive bacteria including nine oral bacteria by disc diffusion method. The acetone extracts had antimicrobial activities against only *B. subtilis* and *E.* coli, whereas the other extracts inhibited the growth of most oral bacteria. These extracts showed a significant growth inhibition against *S. sanguinis*.

Keywords: ABTS Radical Scavenging Activity, Antioxdant Activity, Antimicrobial Activity, DPPH Radical Scavenging Activity, *Pleurotus Ostreatus* (Oyster Mushroom)

1. Introduction

Recently, as it moves into an aging time, modern people came to be more interested in health. So, as interest in functional food originated from natural substances for treatment of disease and suppression of aging is growing, studies on functionality of natural substances like antibiosis, antioxidant, and anticancer and effects of biological activity of secondary metabolites are being processed briskly[1]. Among them, since mushrooms have various nutrients necessary for human bodies such as saccharinity, protein, minerals, vitamin, and so on and are low in fat content, they have been used as low calorie food and medical purposes. Also, because it was revealed that they contain diverse biological activity matters with the efficacy of anticancer, immune reinforcement, protection of lung function, anti-diabetes, anti-inflammation, and antibiosis, they are being used materials of functional food and medicines[2-5]. *Pleurotus ostreatus* is the most consumed representative mushroom.

Especially, it is known that it is effective in preventing cardiovascular disorders from containing lovastatin which lowers cholesterol[6-8]. It was reported that water extract of the oyster mushroom has antioxidant and antitumor activity[9] and effect of treatment and prevention of breast cancer and colon cancer[10]. Including the antioxidant effect[11] of hypha extract, diverse results such as comparison analysis[12] of polyphenol content according to kinds of strains, immunity-boosting effect[13] and so forth were reported.

Also, as problems of antibiotic resistance reach serious levels, because development of new antibiotics to control resistant bacteria is in an urgent situation, especially, along with the effect of existing antibiotics, for the development of antimicrobial substances originated from natural products harmless to human bodies, it is in a state that studies on antibacterial activity of mushrooms are being realized diversely. Methanol extract of *Agaricus bisporus* shows higher antibacterial activity against *Bacillus subtilis* than *ampicillin*. It was reported that methanol extract of boletus showed antibacterial activity against *Staphylococcus aureus*[14]. Plus, it was reported that high antibacterial activity of *Ganoderma lucidum* against *Micrococcus luteus*[15], high antibacterial activity

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of shiitake against Gram-positive and Gram-negative bacteria[16] and antibacterial activity of ethyl acetate extract of shiitake against *Streptococcus mutans* of oral bacteria[17,18]. In addition, inhibition effect[19] of methanol extract of the oyster mushroom against *Escherichia coli*, *Bacillus megaterium*, *Staphylococcus aureus* and *Klebsiella pneumonia* and activity of anti-caries against mushroom extract are reported as well[17,18].

Therefore, targeting *P. ostreatus* with the most consumption, after extracting it from using different solvents, by investigating antioxidant activity such as total content of polyphenol, total content of flavonoid, DPPH radical scavenging ability and ABTS radical scavenging ability and antibacterial activity against Gram-positive bacteria, Gram-negative bacteria, and oral bacteria, various biological activities of oyster mushrooms are to be researched.

2. Materials and Methods

2.1 Materials

P. ostreatus in this experiment was used from being dried and pulverized after purchasing live mushrooms which can be obtained at a regular retail market. Clearly prepared oyster mushrooms had been kept in a cool place for a week and dried. Then, after compounding them in powder, they were extracted from using several solvents. Reagents except extractants were purchased at Sigma-Aldrich (St. Louis, MO, USA).

2.2 Extraction of Pleurotus Ostreatus

To the 50 gm powder of the pulverized oyster mushroom, 400 ml of acetone, Ethyl Acetate (EtAc) and Ethanol (EtOH) is put. After shaking them for three days from using an agitator, they were shaken once again for two hours. For each solution, after filtering the extraction by gauze, vacuum filtration was conducted and decompression concentration was implemented with a rotary evaporator. Each extraction was used in the test from melting it in 5 ml Dimetyl Sulfoxide (DMSO). An anti-bacteria test against oral bacteria was implemented from making the concentration diluted into 50 mg/ml. The extraction was used while keeping it at 4°C.

2.3 Bacterial Strains and Culture Conditions

Antibacterial activity of the oyster mushroom extracts was investigated targeting three kinds of Gram-positive bacteria like *Bacillus subtillis*, *Staphlyococcus aureus* and *Micrococcus luteus* and three kinds of Gram-negative bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. Strains used in the antibacterial activity investigation against oral bacteria of the oyster mushroom were *Streptococcus sobrinus* (KCTC3308), *Staphylococcus aureus* (KCTC1927) *Streptococcus mutans* (KCTC3065), *Streptococcus ratti* (KCTC3655), *Streptococcus sanguinis* (KCTC3284), *Actinomyces viscosus* (KCTC5531), *Actinomyces naeslundii* (KCTC5525), *Streptococcus anginosus* (KCTC3983) and *Aggregatibacter actinomycetemcomitans* (KCTC3698). Strains for the test were used from being distributed by Korean Collection for Type Cultures (KCTC). The used medium and culture conditions are like (Table 1). The rest bacteria except oral bacteria were cultured in the incubator of 37°C and the oral bacteria was cultured in the anaerobic incubator of 37°C for 24 hours. After adjusting the turbidity of bacterial culture media into the 0.5 McFarland standard (about 1.0×10^6 CFU/ml), they were used at the test.

Table 1. List of Stains used for antibacterial experiments

	Strains	Media	Temp(°C)
Gram-positive bacteria	*Bacillus subtilis*	LB	37
	Staphylococcus aureus	LB	37
	Micrococcus luteus	LB	37
Gram-negative bacteria	*Escherichia coli*	LB	37
	Pseudomonas aeruginosa	LB	37
	Enterobacter cloacae	LB	37
Oral bacteria	*Streptococcus sobrinus*	BHI	37
	Staphylococcus aureus	BHI	37
	Streptococcus mutans	BHI	37
	Streptococcus ratti	BHI	37
	Streptococcus sanguinis	BHI	37
	Actinomyces viscosus	TSB	37
	Actinomyces naeslundii	TSB	37
	Streptococcus anginosus	TSB	37
	Aggregatibacter actinomycetemcomitans	TSB	37

BHI: Brain-Heart Infusion, TSB: Trypticase Soy Broth.

2.4 Antimicrobial Susceptibility Testing

The antibacterial effect of the *P. ostreatus* extracts was measured by the disk diffusion method from changing

some of the Bauer method[20]. After the cultivated strains were smeared on the agar plate which was prepared already with cotton swabs, placing paper discs (ϕ6 mm, Whatman AA discs, Whatman International) that each extract of 30 µl was absorbed and dried on the smeared plate, Gram-positive strains and Gram-negative strains were cultivated in the 37°C incubator and the oral bacteria were cultivated in the 37°C CO_2 incubator for 24 hours. Whether clear zones around discs were created or not were checked, and the sizes were measured. As negative control, DMSO was used. Each experiment was repeatedly measured three times.

2.5 Determination of the Total Phenolic Content

To check the polyphenol content of the oyster mushroom extracts, it was measured from changing some of the Folin-Denis method[21] that blue is shown from responding with phosphomolybdic acid as a phenolic substance. From adopting 45 µl extract, mixed with 45 µl of 1 N Folin-Ciocalteu (Folin-Ciocalteu: extract, 1:1, v/v), after fixing it at room temperature for three minutes, 910 µl of 2% Na_2CO_3 was added. From letting the mixed liquid responded at room temperature for 30 minutes, the absorbance was measured at 760 nm (MECASYS, Daejeon, Korea). By writing down standard curves with gallic acid, total phenolic content was decided and expressed by mg (GAE) per g extract. Experiments of each extract were implemented three times.

2.6 Determination of Total Flavonoid Content

The total flavonoid content of the oyster mushroom extract was measured from transforming some of the method of Lee et al[22]. By mixing 500 µl extract and 500 µl of 2% $AlCl_3$, after acting it while leaving it at room temperature for one hour, the absorbance was measured at 420 nm. From the standard curve written by using quercetin, the total flavonoid content was calculated. It was expressed by mg (QE) per g extract. Tests for each extract were conducted three times.

2.7 DPPH Radical Scavenging Activity

When DPPH reacts with a substance of antioxidant activity, cobalt violet DPPH is bleached. By measuring the decreasing absorbance, radical scavenging activity can be measured easily. So, by using 1,1-diphenyl-2-picrylhydrazyl (Sigma-Aldrich), from using the Cheung method measuring the effect of radical scavenging, the test result was gained[21]. By adding 970 µl of 0.1 mM DPPH solution to the extract of 30 µl, it was reacted in the dark for 30 minutes. All tests were processed in a state of blocking light. By measuring the absorbance at 517 nm, the absorbance decrease by reduction of DPPH was investigated. For the positive control, 1 mM ascorbic acid was used. The average value was found from implementing tests three times.

Radical scavenging activity (%) = $\{1 - A_{sample}/A_{blank}\} \times 100$

2.8 ABTS Radical Scavenging Activity

ABTS assay is an antioxidant measurement method using the principle that blue-green ABTS free cation is eliminated and bleached by an antioxidant substance of the extract which was formed from responding with potassium persulfate. Since the bleach response of ABTS anion ends within one minute, it can be measured in a short time and be applied to hydrophobic and hydrophilic properties all. The antioxidant power using ABTS radical scavenging activity was measured according to the method of Re et al[23]. After mixing 7.4 mM ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and 2.6 mM potassium persulphate, ABTS cations were formed from placing it in the dark for 24 hours, by adding 30 µl extract to 970 µl ABTS solution, the absorbance was measured at 734 nm. By using 1 mM ascorbic acid for the positive control, the ABTS radical scavenging activity was found. The average value was found from implementing three time experiments.

Radical scavenging activity (%) = $\{1 - A_{sample}/A_{blank}\} \times 100$

2.9 Statistical Analysis

All tests were implemented three times repeatedly and the results were represented by average ± standard deviation. For the statistical analysis of data, the significance was verified in the level of $P<0.05$ with the ANOVA test and Tukey using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1 Antimicrobial Susceptibility Testing

Antibacterial activity about six kinds of multi-resistant strains of the oyster mushroom extracts which were

extracted by organic solvents like acetone, EtOH, and EtAc was measured by the disc diffusion assay. The result was presented in (Table 2). Among extracts of the oyster mushroom, acetone and EtAc extracts showed antibacterial activity only against *B. subtilis* as Gram-positive bacteria and *E. coli* as Gram-negative bacteria. In the EtOH extract, antibacterial activity was not shown against all bacteria. Acetone extract showed clear zones of 7.33 mm and 8.33 mm, respectively about *B. subtilis* and *E. coli*. EtAc extract showed clear zones of 8.33 mm and 7.67 mm, respectively about *B. subtilis* and *E. coli*. Therefore, in case of *E. coli*, activity was somewhat high in acetone extract. In *B. subtilis*, activity was somewhat high in EtAc extract. In the other four strains of *S. aureus*, *M. luteus*, *P. aeruginosa*, and *E. cloacae*, activity was not presented. Chowdhury et al[24] reported that methanol extract of the oyster mushroom had activity against *B. subtilis*, *S. aureus*, and *E. coli*. Kalyoncu et al[25] reported that ethanol extract of the oyster mushroom represented high growth inhibition effect (>= 16 mm) about *S. lutea*, *S. aureus*, *C. cloacae*, *E. coli* and *B. cereus*. However, Iwalokun et al[26] reported that extracts of petroleum ether and acetone of the oyster mushroom showed 7.0~8.2 mm clear zones about *S. aureus*, *B. subtilis* and *E. coli*. The test results could be checked to be similar to the experiment result of this study. Like this, antibacterial activity against resistant bacteria using extracts of the oyster mushroom has been processed diversely. On the other hand, it is in a situation that antibacterial activity against oral bacteria related with the oyster mushroom has not been realized at all.

Table 2. Antimicrobial activities of various solvent against gram-positive bacteria and gram-negative bacteria from Pleurotus ostreatus

	Acetone	EtAc	EtOH
P. aeruginosa	-	-	-
E. coli	++	+	-
M. Luteus	-	-	-
E. cloacae	-	-	-
S. aureus	-	-	-
B. subtilis	+	++	-

-; no inhibition(<6 mm), +; slight inhibition(6-8 mm), ++; moderate inhibition(9-10 mm). EtOH: ethanol, EtAc: ethyl acetate.

So, antibacterial effect of the extract of the oyster mushroom against 9 kinds of oral bacterial was investigated through the disc diffusion assay. The measured result was represented in (Table 3). In all of *S. sobrinus*, *S. aureus*, *S. mutans*, *S. ratti*, *S. sanguinis*, *A. viscosus*, *A. naeslundii*, *S. anginosus*, and *A. actinomycetemcomitans* except *A. viscosus*, antibacterial activity could be checked. Especially, in acetone extract, somewhat high activity was shown in *S. sobrinus*, *S. mutans*, *S. ratti*, and *S. sanguinis*. In *A. naeslundii* and *S. anginosus*, there were clear zones. But, in *S. aureus* and *A. viscosus*, activity could not be checked at all. In case of EtAc extract, somewhat good activity was shown in *S. mutans*, *S. ratti*, *S. sanguinis*, and *S. anginosus*. In *S. aureus* and *A. naeslundii*, weak activity was shown. In particular, acetone and EtAc extracts of the oyster mushroom showed the greatest growth inhibition effect (all 9.7 mm) in the investigation of antibacterial activity about *S. sanguinis*. As seen in the result, it can be concluded that the oyster mushroom extracts present better activity in oral bacteria than resistant bacteria.

Table 3. Antimicrobial activities of various solvent against oral bacteria from Pleurotus ostreatus

	Acetone	EtAc	EtOH
Streptococcus sobrinus	++	-	-
Staphylococcus aureus	-	+	-
Streptococcus mutans	++	++	-
Streptococcus ratti	++	++	-
Streptococcus sanguinis	++	++	-
Actinomyces viscosus	-	-	-
Actinomyces naeslundii	+	+	-
Streptococcus anginosus	+	++	-
Aggregatibacter actinomycetemcomitans	++	-	-

-; no inhibition(<6 mm), +; slight inhibition(6-8 mm), ++; moderate inhibition(9-10 mm), +++; heavy inhibition(>10 mm). EtOH: ethanol, EtAc: ethyl acetate.

3.2 Determination of Total Phenolic and Flavonoid Contents

Phenolic compounds are secondary metabolites of plants. Because they contain a dihydric group (-OH), they are biological substances representing anti-oxidative activity from eliminating radicals[27]. The total polyphenol content of *P. ostreatus* were measured from using gallic acid as a standard substance. The result is like (Table 4).

The polyphenol content of acetone and EtAc extracts were investigated to be 3.88 mg GAE/g extract and 3.10 mg GAE/g extract, respectively. In EtOH extract, polyphenol content of 1.37 mg GAE/g extract was shown. These study results were investigated similarly in other reports. There are cases that Sala et al[28] reported that the total polyphenol content of EtAc extract was 3.69 mg GAE/g extract and Um et al[29] reported that polyphenol

content of acetone extract was 20-40 mg% and Choi et al[9] reported that polyphenol content of water extract of the oyster mushroom was 30.2 mg%. Overall, if it is calculated from being converted into other unit, almost similar polyphenol content could be known.

Also, the result that the total flavonoid content of *P. ostreatus* is presented into quercetin content per gram of extract is like (Table 4). The flavonoid content of *P. ostreatus* according to solvents was the highest with 1.04 mg QE/g extract in the ethanol extract. In EtAc and acetone extracts, 0.77 mg QE/g extract and 0.61 mg QE/g extract were measured each. The reason why polyphenol content is higher than the flavonoid content is thought that other kinds of polyphenolic compounds except flavonoids are extracted more. Plus, there is a report that as polarity of extraction solvents increases, the content of polyphenol components increases[21], but it was investigated different from the result of this study. In general, the contents of polyphenols and flavonoids showed negative correlation (r = -0.9977) in this study. It was investigated that they were not related with polarity of extraction solvents.

Table 4. Total polyphenol and flavonoid contents of extracts from Pleurotus ostreatus

	Total polyphenol (mg GAE/g extract)1)	Total flavonoid (mg QE/g extract)2)
Acetone	1.81±0.49[a]	0.61±0.03[a]
Ethyl acetate	1.53±0.30[b]	0.77±0.18[a]
Ethanol	2.12±0.79[c]	1.04±0.15[b]

The results represent the mean±SD of values obtained from three independent experiments
1) Values are expressed as mg gallic acid equivalent(GAE) per g of extract.
2) Values are expressed as mg quercetin equivalent(QE) per g of extract
[a,b,c] Mean with different letter within a column are significantly different by Tukey (P<0.05).

3.3 DPPH Radical Scavenging Ability

The anti-oxidative substances of natural substances have properties to provide electrons to activity radicals and control fat oxidation of food and play a role in controlling aging by activity radicals. So, the radical scavenging activity comes to perform so important a role in preventing disease and aging of human bodies. In this study, radical scavenging ability of the oyster mushroom was measured by using DPPH. The DPPH radical elimination ability is measured from using the principle that DPPH radicals are removed by antioxidants and deep purple changes into bright yellow. With a small amount, antioxidant activity can be observed easily. It is comparatively simple and useful to explore antioxidant activity. The measured result of the DPPH radical scavenging ability is like (Figure 1).

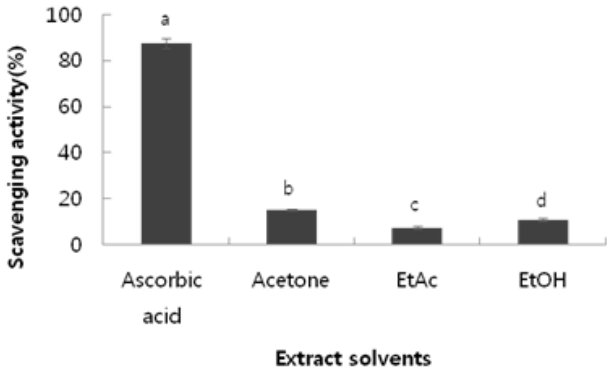

Figure 1. DPPH free radical scavenging activity of extracts from *Pleurotus Ostreatus*.

The results represent the mean±SD of values obtained from three independent experiments. Ascorbic acid was used as positive control. EtOH: ethanol, EtAc: ethyl acetate. a, b, c, d Mean with different letter within a column are significantly different by Tukey (P<0.05).

Generally, the DPPH radical scavenging ability showed low result compared with 87.5% elimination ability of ascorbic acid. Acetone extract, EtOH extract, and EtAc extract of the oyster mushrooms were 15.1%, 10.9%, and 7.5%. According to solvents, different results can be checked. Kalyoncu et al[25] showed 6.11% DPPH radical elimination ability in EtOH extract of *P. ostreatus*. Choi et al[9] reported that there was 44.1% DPPH radical elimination ability in water extract (5.00 mg/ml) of *P. ostreatus*. And, according to Kang et al[30], as the content of polyphenol is more, the DPPH radical elimination ability increases. Differently, in this study result, the DPPH radical elimination ability and polyphenol content did not show correlation (r = 0.3588). Generally, as can be known in the report that antioxidant activity is complex activity of phenolics, peptides, organic acids, and other antioxidant substances[31-33], in the case of antioxidant activity investigation of the oyster mushroom, it is self-judged that other various bioactive substances including polyphenol are contained.

3.4 ABTS Radical Scavenging Ability

The ABTS elimination method is an activity measurement method using a principle that ABTS cation radicals which were formed responded with potassium persulfate are scavenged and bleached by antioxidant substances of extracts. Also, the ABTS scavenging ability has characteristics that it acts with hydrophilic and hydrophobic antioxidant substances all and the measuring

is possible in diverse pH[34]. At the result of measuring the ABTS elimination ability, like (Figure 2.), 46.2% of acetone extract, 38.2% of EtOH extract, and 21.2% of EtAc extract were checked as lower results compared with ascorbic acid as a comparison group. The correlation of the ABTS scavenging ability and polyphenol content was $r = 0.0915$. The content did not provide significant influence on the ABTS radical elimination ability. From showing $r = 0.9623$ of the ABTS radical elimination ability and the DPPH radical elimination ability, it was checked that high correlation was shown. In general, like the report[35] that because pigment components like anthocyanine and carotenoid hinder the measurement of the DPPH radical elimination ability, comparatively low measured values are presented, in this study, it was checked that measured values related to the ABTS scavenging ability were substantially higher in activity than the DPPH radical scavenging ability.

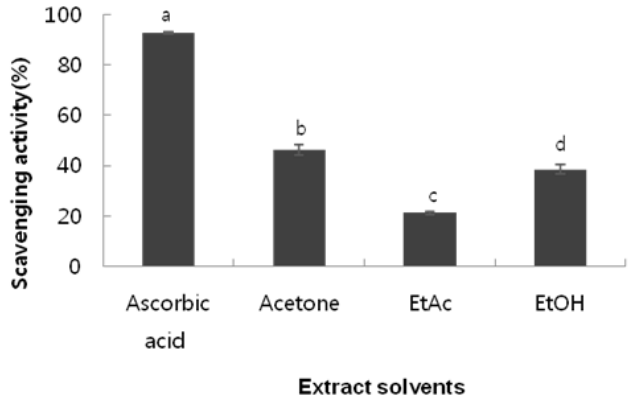

Figure 2. ABTS activity of extracts from Pleurotus Ostreatus.

The results represent the mean±SD of values obtained from three independent experiments. Ascorbic acid was used as positive control. EtOH: ethanol, EtAc: ethyl acetate. a, b, c, d Mean with different letter within a column are significantly different by Tukey ($P<0.05$).

4. Conclusions

In order to check antioxidant activity and antibacterial effect of multi-resistant bacteria and oral bacteria of *P. ostreatus* which is eaten commonly, in this study, acetone, EtOH, and EtAc were extracted. About each extract, total polyphenol, total flavonoid content, DPPH radical scavenging ability, ABTS radical scavenging ability, antibacterial activity through disc diffusion assay were measured. At the result of measuring activity against multi-resistant bacteria, in acetone and EtAc extracts, clear zones against *B. subtilis* and *E. coli* could be checked. And, in acetone extract, better activity about *E. coli* could be checked. In EtAc extract, a bigger clear zone about *B. subtilis* could be checked. At the result of oral antibacterial activity, in acetone extract, antibacterial activity of the other *S. sobrinus*, *S. mutans*, *S. ratti*, *S. sanguinis*, *A. naeslundii*, and *S. anginosus* except *S. aureus* and *A. viscosus* could be checked. In case of EtAc extract, clean zones could be checked in *S. mutans*, *S. ratti*, *S. sanguinis*, *S. anginosus*, *S. aureus*, and *A. naeslundii*.

Conclusively, it could be known that the oyster mushroom shows better activity in oral bacteria than resistant bacteria. The total polyphenol contents of *P. ostreatus* were investigated to be 3.88 mg GAE/g, 3.10 mg GAE/g, and 1.37 mg GAE/g in acetone, EtAc and EtOH extracts respectively. The total flavonoid contents were investigated to be 1.04 mg QE/g, 0.77 mg QE/g, and 0.61 mg QE/g in acetone, EtAc and EtOH extracts respectively. In the DPPH radical elimination ability, acetone, EtOH, and EtAc extracts were high in order. There was no correlation with polyphenol content. At the result of measuring the ABTS radical scavenging ability, in acetone, EtOH, and EtAc extracts, activity of 46.2%, 38.2%, and 21.2% was shown.

Although there was no significant correlation with polyphenol content and the radical elimination ability in extracts of the oyster mushroom, polyphenol content, DPPH radical scavenging ability, and ABTS radical scavenging ability were relatively higher in acetone extract than other solvent extracts. As seen in the study results above, in conclusion, since higher measured values of acetone extract of the oyster mushroom were checked than other solvent extracts in most cases such as antibacterial activity and antioxidant activity against oral bacteria as well as antibacterial activity against multi-resistant bacteria, it is thought to include diverse relevant bioactive substances and it was seen that acetone extract is the most proper extract solvent to check activity. Hence, all contents of this study is thought to be available for basic data related to diverse antibacterial activities and anti-oxidation of *P. ostreatus* and also to be precious data and research fields in finding functional materials originated from natural substances.

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