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Antioxidant/anti-inflammatory activities and chemical composition of extracts from the mushroom *Trametes versicolor*

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Abstract: The mushroom, Trametes versicolor is commonly used as a traditional Chinese medicine and is known to exhibit various biological activities. However, its antioxidant activity has remained unknown. In the present study, various extracts obtained from the Trametes versicolor mushroom were examined for antioxidant and anti-inflammatory activities. Among the extracts obtained with Soxhlet extraction, the extract from acetone exhibited the highest antioxidant activity (50.9%), followed by the extracts from methanol (33.9%), n-hexane (29.5%), and chloroform (15.2%) at the level of 500 μg/mL. This acetone fraction displayed a dose-dependent anti-inflammatory activity or lipoxygenase inhibitory activity by 76.4% at 500 μg/mL, 55.6% at 200 μg/mL, and 37.0% at 100 μg/mL. Among a total of 76 compounds identified in this acetone extract, hexadecanoic acid was the largest component (18.11 mg/kg), followed by 5-hydroxy-2-pentanone (17.33 mg/kg), lactic acid (3.25 mg/kg), and acetic acid (3.21 mg/kg). The following possible principles of antioxidant activity were identified: furfural (1.48 mg/kg), γ-butyrolactone (0.51 mg/kg), furfuryl alcohol (0.49 mg/kg), 2-methoxy-4-vinylphenol (0.49 mg/kg), and 2,6-dimetoxy-4-vinylphenol (0.33 mg/kg), and benzaldehyde (0.15 mg/kg).

Keywords: Trametes Versicolor Mushroom, Medicinal Plants, Antioxidant, Anti-Inflammatory, Lipoxygenase Inhibitor

1. Introduction

Edible mushrooms have been consumed since ancient times and about 200 species are consumed in the world today [1]. In 2008, the world production total of edible mushroom was over one million tons, wherein China produced approximately 50% of global total productions [2]. Currently, edible mushrooms are either commercially available in grocery stores or found in the wild. Mushrooms are rich in proteins and compounds with various biological activities including antioxidant [3], anti-tumor [4], antimicrobial [5], anti-hyperglycemic [6], anticholinesterase [7], immunostimulant [8], and anti-mutagenic [9] properties. Therefore, mushrooms are ideal health foods [10]. Also, many mushrooms have been used as folk medicine since the eras of Ancient China, Rome, and Greece [11].

Trametes versicolor is a Chinese medicinal mushroom known to possess a wide range of biological activities including immune-enhancing activity [12], antitumor [13], and antiviral effects [14]. A prophylactic bioactive of the mushroom extract, known as PSK, has been demonstrated to be effective against carcinogenesis [15]. Derived from the extract of T. versicolor mycelia, PSK (Krestin; PSK) is a protein-bound polysaccharide approved for use in cancer treatment by the Japanese Ministry of Health and Welfare in 1977 [16]. This was the first polysaccharide antitumor drug approved by the regulatory agency. Although T. versicolor has been known to possess various biological effects, its antioxidant activity is not well established. It is important to know about antioxidant activity in order to elucidate the medicinal mechanisms of biological effects because most diseases are associated with oxidative damage [17]. Free radical and oxygen reactive species affect the mechanisms of many diseases including diabetes, atherosclerosis,

aging, neurodegenerative disorders, as well as cancer [18]. Therefore, some antioxidants found in natural plants may be effective in combating these diseases [19].

In the present study, the antioxidant/anti-inflammatory activities and chemical components of extracts from mushroom, T. versicolor was investigated to learn more about their healthful effects on humans.

2. Materials and Methods

2.1. Chemicals and Reagents

n-Hexane, chloroform, acetone, methanol, water (HPLC-grade), sodium dodecyl sulfate (SDS), hydrogen peroxide, and butylated hydroxytoluene (BHT) were purchased from Fisher Scientific Co. (Rochester, NY, USA). Dichloromethane, cod liver oil, ferrous chloride tetrahydrate ($FeCl_2$•$4H_2O$), N-methylhydrazine, 1-methylpyrazole (1-MP), 2-methylpyrazine, tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane hydrochloride, nordihydroguaretic acid (NDGA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A Lipoxygenase Inhibitor Screening Assay (LISA) kit® was purchased from Cayman Chemical Co. (Portland, OR, USA). Standard volatile chemicals for analysis of the acetone extract were a gift from TAKATA KORYO Co., Ltd., (Hyogo, Japan).

2.2. Plant Samples

T. versicolor mushrooms were purchased from Marue pharmacy (Tokyo, Japan). The T. versicolor mushroom used in this study was harvested in Nagano prefecture in Japan in 2010.

2.3. Sample Preparations

Samples were prepared for analysis of components and antioxidant tests [20].

2.3.1. Steam Distillation

The fruit body of T. versicolor was ground into a fine powder using a blender. Samples of the resultant powder (50 g) were placed in 2 L two-necked flasks with 1 L water. Each sample was steam distilled at 55°C and 95 mmHg for 7 h. The steam distillate (800 mL) was extracted with 100 mL dichloromethane for 6 h using a liquid-liquid continuous extractor. The extract was dried over anhydrous sodium sulfate for 12 h. After removal of the sodium sulfate, the extract was condensed to approximately 2 mL using a rotary evaporator. The extract was further condensed under a purified nitrogen stream to 575 mg. This sample was tested for antioxidant activity by an MA/GC assay, DPPH assay, and an anti-inflammatory lipoxygenase assay.

2.3.2. Column Chromatography of Residual Aqueous Solution

The residual aqueous solution (800 mL) after dichloromethane extraction was condensed to 5 mL and placed in a glass column (30 cm x 2.5 cm i.d.) packed with Amberlite XAD-2 resin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The sample was eluted sequentially with 100 mL each of water and acetone. The water and acetone fractions were then condensed to 2 mL and subsequently reduced to 14.7 mg and 10.3 mg, respectively, using a purified nitrogen stream. These samples were tested for antioxidant activity with the MA/GC assay and the anti-inflammatory assay.

2.3.3. Soxhlet Extraction of Mushroom Powder Sample

A powder sample (50 g) was extracted sequentially with 250 mL each of n-hexane, chloroform, acetone, and methanol using a Soxhlet extractor. The extracts were condensed using a rotary evaporator to 2 mL and then condensed further with a purified nitrogen stream to 46.9 mg for the n-hexane extract, 242.4 mg for the chloroform extract, 200.9 mg for the acetone extract, and 468.0 mg for the methanol extract. These samples were tested for antioxidant activity with MA/GC and DPPH assays and anti-inflammatory (lipoxygenase) assay.

2.4. Antioxidant Activity Tests

The antioxidant activities of samples were tested according to previously reported methods [17]. The levels of antioxidant activity were calculated by a previously reported method [21]. All experiments were repeated three times.

2.4.1. Malonaldehyde/Gas Chromatography (MA/GC) Assay

MA/GC assays were performed according to a previously reported method [17]. Briefly, a test sample was added to an assay solution (5 mL) containing 10 μL cod liver oil in a 20 mL test tube. After a sample was incubated at 37 °C for 18 h, the MA formed in the sample was analyzed as 1-methylpyrazole derivative by an Agilent Technologies model 6890N GC equipped with a 30 m x 0.25 mm i.d. (df = 0.25 μm) DB-wax fused-silica capillary column (Agilent Technologies Inc., Santa Clara, CA, USA) and a nitrogen-phosphorus detector (NPD). The GC oven temperature was programmed to rise from 70°C to 120 °C at 4°C/min. The injector and detector temperatures were 260 °C and 280 °C, respectively. The helium carrier gas flow rate was 1.4 mL/min. The entire assay was performed in duplicate.

2.4.2. DPPH Radical Scavenging Assay

The DPPH radical scavenging assay on samples from Soxhlet extraction was conducted according to a previously reported method [17]. Briefly, a mixture containing a test sample and DPPH was incubated at room temperature for 30 min and then the absorbance at 517 nm was measured with a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA, USA).

2.5. Anti-inflammatory Test

An anti-inflammatory test was performed on samples prepared by steam distillation and Soxhlet extraction using

the LISA kit (Cayman Chemical Co., Ann Arbor, MI, USA) as previously described [21]. Briefly, A 15-LOX lipoxygenase enzyme solution (90 µL) and a testing sample (10 µL) were placed in the testing well. The reaction was initiated by adding a 10 µL of arachidonic acid substrate solution to a testing sample well. All testing wells were then covered and placed on a shaker (Bellco Biotechnology, Vineland, NJ, USA) for 5 min. Chromogen (100 µL) was added to the reaction wells to stop the enzyme catalysis and prevent further development of the reaction. The level of hydroperoxides (HP) produced by 15-LOX from arachidonic acid were measured by the UV absorbance at 490 nm.

2.6. Identify the Chemical Components of Acetone Extract Using GC/MS

The acetone extract prepared by Soxhlet extraction was analyzed for its components using GC/MS because antioxidant and anti-inflammatory activities of this extract exhibited the highest levels of antioxidants and anti-inflammatories. The chemical components in the acetone extract were identified by comparison with the Kovat's gas chromatographic retention index and mass spectrometric pattern of each component and those of authentic compounds. An Agilent model 6890 GC interfaced to an Agilent 5973 Network Mass Selective Detector (Foster City, CA, USA) was used for mass spectral identification of the GC components at MS ionization voltage of 70 eV. A 60 m x 0.25 mm i.d. (df = 0.25 µm) DB-WAX bonded phase fused silica capillary column (J & W Scientific, Folsom, CA, USA) was used for GC. The linear velocity of the helium carrier gas was 30 cm/sec. The injector and the detector temperatures were 250°C. The oven temperature was programmed to rise from 40 to 200°C at 2°C/min. The identification of chemicals was also confirmed with the MS library of the NIST AMDIS version 2.1 software.

2.7. Statistical Processing

Results are expressed as the mean ± SD (n = 3). Data were analyzed by one-way ANOVA, followed by Dunnett's test for separate comparisons with the control group. Differences were considered significant at a P value of < 0.05.

3. Results and Discussion

3.1. Antioxidant Activities of Samples

Figure 1 shows the results of MA/GC assay on the samples prepared by steam distillation. The extracts were tested at the level of 500 µg/mL. A standard antioxidant, BHT was examined at 100 µg/mL and exhibited high inhibitory effect to MA production (99.1 ± 0.2%), indicating that the method used in this study was valid. Among the samples tested, the acetone fraction showed the highest antioxidant activity (52.0 ± 2.2%), followed by the dichloromethane extract (7.0 ± 11.0%) and the water fraction (5.3 ± 8.5%).

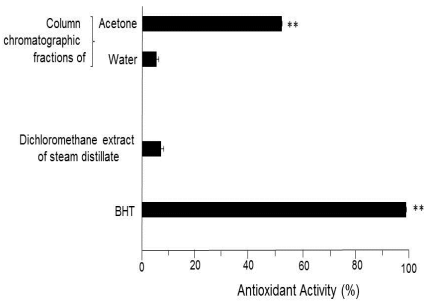

Figure 1. *The results of the MA/GC assay on samples prepared with steam distillation. Values are means ± SD (n = 3). *: p < 0.05、**: p < 0.01.*

Figure 2 shows the results of the MA/GC assays of the samples prepared by Soxhlet extraction. The amounts of the extracts collected from each solvent fraction after removal of the solvent were 46.9 mg from n-hexane, 242.4 mg from chloroform, 200.9 mg from acetone, and 468.0 mg from methanol. Three concentrations of each extract, 100, 200, and 500 µg/mL were tested. BHT exhibited 96.6 ± 0.19% antioxidant activity at the level of 100 µg/mL, indicating that this assay is valid. The acetone extract showed moderate antioxidant activity with a dose response ranging from 50.9 ± 5.2% (500 µg/mL) to 32.5 ± 0.5% (100 µg/mL). Also, the methanol and hexane extracts exhibited moderate activities of 33.9 ± 0.4% and 29.5 ± 6.0% at the levels of 500 µg/mL, respectively. Both extracts also showed dose responses. The chloroform extract exhibited slight antioxidant activity without dose response. These results suggest that antioxidant principles are polar compounds.

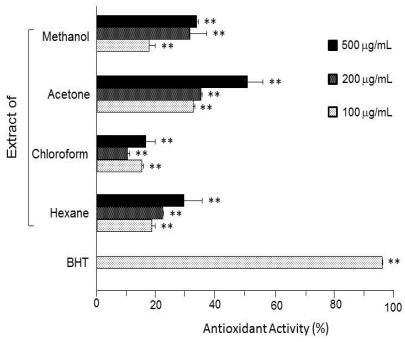

Figure 2. *The results of the MA/GC assay on samples prepared by Soxhlet extraction. Values are means ± SD (n = 3). *: p < 0.05、**: p < 0.01.*

The antioxidant activities of the samples from Soxhlet extraction were also tested by DPPH assays. Figure 3 shows the results of the DPPH assay on the samples from Soxhlet extraction. BHT exhibited 89.2 ± 5.7% antioxidant activity. All extracts showed dose response antioxidant activity. Acetone extract exhibited the highest antioxidant activity at 54.9 ± 0.1%, followed by the methanol extract at 40.0 ± 0.2% at the level of 500 µg/mL. The values obtained from the chloroform and hexane extracts were relatively low but they also showed appreciable antioxidant activities.

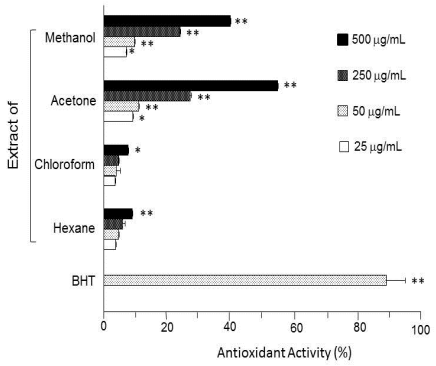

Figure 3. *The results of the DPPH assay on samples from Soxhlet extraction. Values are means ± SD (n = 3). *: p < 0.05、**: p < 0.01*

A previous study demonstrated that methanol extracts of black and red ear mushrooms exhibited 100% antioxidant activity at the level of 1.0 mg/mL by DPPH assay [22]. Also, ethanol extracts from mushroom species Phellinus Quél and their isolated fractions exhibited antioxidant activity and protected PC 2 cells against oxidative damage [23]. Another study reported that ethanolic extracts from the mushroom Coprinus comatus possessed higher antioxidant activity (47.0% at 20 μg/mL) than hot water extract [24]. A peptide obtained from another fermented mushroom (Ganoderma lucidum) showed inhibitory activities toward lipid peroxidation and lipoxigenase activity, which has been used to examine anti-inflammatory activity [25].

3.2. Anti-inflammatory Activity of Samples

This anti-inflammatory assay involves the measurement of inhibitory activity of samples toward lipoxygenase formation in the test solution.

Figure 4 shows the results of the anti-inflammatory assay on the samples prepared by steam distillation. Values are mean ± SD (n = 3). A standard lipoxygenase inhibitory chemical, NDGA, inhibited 15-lipoxygenase activity by 66.7 ± 3.5% at 100 μg/mL. The dichloromethane extract exhibited the greatest lipoxygenase inhibitory effect by 81.3 ± 9.4%, followed by the acetone extract (50.0 ± 10.0%), and the water extract (16.4 ± 1.6%) at a 500 g/mL.

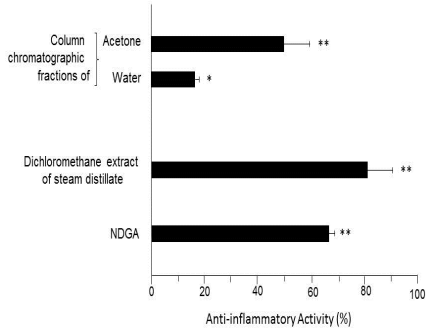

Figure 4. *The results of the anti-inflammatory assay on samples prepared by steam distillation. Values are means ± SD (n = 3). *: p < 0.05、**: p < 0.01.*

Figure 5 shows the results of the anti-inflammatory test on the samples prepared by Soxhlet extraction. Values are mean ± SD (n = 3). A standard antioxidant, NDGA exhibited clear anti-inflammatory activity (67.5 ± 4.0%), indicating the method used in this study was valid. All samples tested showed dose response activity. The acetone extract showed strong anti-inflammatory activity (70.6 ± 18.2%) at the level of 500 μg/mL. Extracts of hexane, chloroform, and methanol also showed appreciable anti-inflammatory activities. These results indicate that acetone extract contains strong anti-inflammatory chemicals.

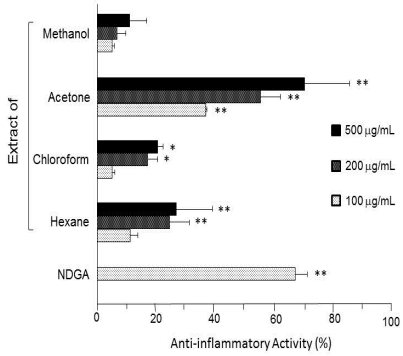

Figure 5. *The results of the anti-inflammatory test on samples prepared by Soxhlet extraction. Values are means ± SD (n = 3). *: p < 0.05、**: p <.*

A previous study demonstrated that polysaccharides possessed potent anti-inflammatory activity in rats with burn injuries [26]. As mentioned above, an extract of T. versicolor, containing a polysaccharide known as PSK [16] displays some potent biological activities, including immune activity [13], antiviral activity [14], and antimetastatic activity [27]. However the precise chemical source of this mushroom's antioxidant activity is not known. Because the chemical composition of the extract is complex, choice of the extraction solvent is an important factor in obtaining target chemicals. However, the present study and previous studies mentioned above indicate that polar solvents, such as methanol, ethanol, and acetone as well as water, can isolate antioxidants from mushrooms. On the other hand, in the case of anti-inflammatory activity, a dichloromethane extract obtained from the steam distillate exhibited potent anti-inflammatory activity (81.3 ± 9.4%). A previous study, also found that the chloroform extract of the mushroom Agaricus blazel contained potent anti-inflammatory chemicals [28]. These results indicate that some volatile anti-inflammatory chemicals are present in mushrooms.

3.3. Chemicals Identified and Their Relationships to Antioxidant Activity

In the present study, among all samples examined by antioxidant assays and anti-inflammatory assay, the acetone extract exhibited the highest antioxidant activity. In the case of anti-inflammatory activity, the dichloromethane extract of the steam distillate showed the highest activity, whereas the column chromatographic acetone fraction of the residual steam distillation exhibited the second highest

activity. These results suggest that acetone extract contains potent antioxidants as well as chemicals with anti-inflammatory activity. Therefore, the acetone extract prepared by Soxhlet was analyzed by GC/MS and the results are shown in Table 1.

Table 1. *Compounds identified in the acetone extract prepared by Soxhlet extraction.*

Compounds	KI[a]	Amount (mg/Kg)
Alkyl Alcohols		
Isoamyl alcohol	1248	0.02
5-Hydroxy-2-pentanone	1357	17.33
2-Butoxyethanol	1398	0.94
Threo-2,3-butanediol	1530	0.23
Erythro-2,3-butanediol	1579	0.13
1,4-Pentaediol	1892	0.15
1,4-Hexanediol	1971	0.10
1,5-Hexanediol	2000	0.05
Phenyl propyl alcohol	2053	0.25
-Eudesmol	2216	0.32
1,4-Nonanediol	2269	0.15
Alkyl Aldehydes and Ketones		
2-Methylbutanal	910	0.30
Isovaleraldehyde	913	0.47
Hexanal	1073	0.45
3-Penten-2-one	1119	0.20
4-Methyl-3-penten-one	1124	0.44
3-Hexen-2-one	1208	0.03
3,3-Octanedione	1318	0.04
6-metthyl-(E)-3-hepten-2-one	1329	0.06
1,4-Cyclohexanedione	1945	0.11
Alkyl Esters and Lactones		
-buryrolactone	1612	0.51
-Hexalactone	1687	0.05
-Hexalactone	1778	0.20
-Valerolactone	1791	0.34
5-Hydroxy-2-hexenoic acid lactone	1818	0.22
3-Hydroxy-2,4,4-trimethylpentyl isobutyrate	1863	0.19
2,2,4-Trimetyl-1,3-pentanediol 3-monobutyrate	1873	0.25
3-Hydroxy-2-pyranone	1980	0.49
-Nonalactone	2016	0.03
-Hydroxy- -butyrolactone	2160	0.62
-Eudesmol	2216	0.32
(S)-(-)- -Hydroxy- -butyrolactone	2580	1.73
Alkyl Acids		
Acetic acid	1440	3.21
Formic acid	1498	0.11
Isobutyric acid	1560	0.12
2-Propenoic acid	1618	0.09
Valeric acid	1712	0.07
(E)-2-Butenoic acid	1760	0.11
Hexanoic acid	1838	0.75
3-Methyl-2-ovovaleric acid	1934	0.09
Succinic anhydride	2104	0.13
Nonanoic acid	2154	0.33
Lactic acid	2172	3.25
3-Hydroxylbutyric acid	2233	1.16
Tetradecanoic acid	2688	1.74
Pentadecanoic acid	2797	2.51
Hexadecanoic acid	2811	18.11
Alkyl Esters and Acetals		
Acetone propylene glycol acetal	933	0.26
Acetone 2,3-bytyleneglycol acetal	971	0.08
Acetone glycerine acetal	1604	2.33
Diethyleneglycol monobutyl ester	1789	0.67
Hydrocarbons		
-Bissabolene	1695	0.22
Nonacosan	1898	0.15
Heneicosane	2116	0.21
Heterocyclic Compounds		
2-Pentylfuran	1222	0.21
5-Methyl-2(H)-furanone	1419	0.12
Furfural	1452	1.48
Furfuryl alcohol	1655	0.49
2(5H)-Furanone	1737	0.21
Acetamide	1768	0.12
Dihydro-3-hydroxy-4,4-diethyl-2(3H)-furanone	2021	0.38
-Butyrolactam	2069	0.65
5-Pentyl-2(5H)-furanone	2098	0.06
2-Furoic acid	2409	0.34
3-Hydroxypyrdine	2417	0.15
Succinimide	2461	0.09
(S)-(+)-Dihydro-5-(hdroxyethyl)-2(3H)-furanone	2468	0.22
Diisobutyl phtalate	2530	0.18
Aromatic Compounds		
1,2,3-Trimethylbenzene	1267	0.03
Benzaldehhyde	1507	0.15
o-Cresol	1994	0.15
2-Hydroxylacetophenone	2134	0.11
2-Methoxy-4-vinylphenol	2183	0.49
Benzoic acid	2412	1.31
Phenyl acetic acid	2540	0.17
2,6-dimethoxy-4-vinylphenol	2545	0.33

[a]*Kovats Index on DBWAX.*

A total of 76 compounds were positively identified. They are 16 alkyl esters, acetals, and lactones (8.29 mg/kg), 15 alkyl acids (total 31.78 mg/kg), 14 heterocyclic compounds (4.7 mg/kg), 11 alkyl alcohols (19.67 mg/kg), 9 alkyl aldehydes and ketones (2.10 mg/kg), and 8 aromatic compounds (2.74 mg/kg). Hexadecanoic acid was the largest component (18.11 mg/kg), followed by 5-hydroxy-2-pentanone (17.33 mg/kg), lactic acid (3.25 mg/kg), and acetic acid (3.21 mg/kg).

The antioxidant activity of many of the constituents identified in T. versicolor in the present study has been previously reported. The compounds with the antioxidant activity are mainly heterocyclic and aromatic compounds. Furfural (1.48 mg/kg) and furfuryl alcohol (0.49 mg/kg), which are sugar degradation products [29], inhibited aldehyde oxidation for over 40 days at the level of 500 μg/mL [30]. Furanones, which are formed from the Maillard reactions between carbohydrates and proteins [31] and lipids [32], are also known to have antioxidant activity [33]. Mushrooms are rich in the reactants of the Maillard reaction such as carbohydrates, proteins, and lipids [6]. Moreover, some furanones are found in fruits and their antioxidant and anti-inflammatory activities are also reported [34]. 2-Methoxy-4-vinylphenol (0.49 mg/kg) and 2,6-dimetoxy-4-vinylphenol (0.33 mg/kg), which are found in coffee [35], possess antioxidant activity as well as anti-inflammatory activity [36]. Phenols are well-known, naturally occurring antioxidants and are believed to be beneficial to human health [37]. A recent study has demonstrated that an ethanolic extract of white button mushroom (Agaricus bisporus) possessed potent antioxidant activity associated with its phenolic components [38]. The antioxidant and anti-inflammatory activities of benzaldehyde (0.15 mg/kg) are reported and is expected to be approved for use in anti-cancer therapy [39]. Various γ-lactones have been known to possess antioxidant activity [40]. γ-Butyrolactone (0.51 mg/kg) showed prophylactic effects against hepatotoxicity by its anti-oxidative effect toward hepatocytes [41].

4. Conclusion

Traditional medicinal extract of mushroom T. versicolor is usually prepared by simply boiling the mushroom in water. Bioactive PSK was isolated only with water because it is highly water-soluble. The antioxidant activity of water extract of this mushroom was considerably lower than that of acetone extract in the present study. These results suggest that the components of the acetone extract possessing antioxidant and anti-inflammatory activities are different from PSK found in an extract of a cultured mushroom, mycelia of T. versicolor, previously. Therefore, in addition to PSK, T. versicolor contains various antioxidants and anti-inflammatory compounds, which contribute to the beneficial medicinal activities of this mushroom.

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