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Effects of storage and extraction methods on antioxidant activities and total phenolic compounds of *Termitomyces clypeatus*

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Abstract

The effects of storage and extraction methods on the total phenolic content and antioxidant activity for the preservative of *Termitomyces clypeatus* (wild edible mushroom) were investigated. The mushrooms were prepared fresh, dried (with extraction periods of 3, 5, and 10 min), boiled, and then froze in a refrigerator for 1-3 months after boiling. The crude extracts were evaluated for total phenolic content and antioxidant activity. The antioxidant properties of samples were evaluated by several biochemical assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay. The boiled and frozen mushrooms in a refrigerator for 1-3 months as folk wisdom for mushroom preservation, results show a slight decrease in the antioxidant activities and total phenolic content after storage. The dried mushroom has higher antioxidant activities and total phenolic content than fresh mushrooms; extracting dried mushrooms with boiling water for 3 min is the best condition for extraction. The mushroom boiled water exhibits phenolic content and antioxidant activity, indicating its nutritional value and suitability for consumption. Interestingly, the magic quadrant analyzed the antioxidant properties of all samples, revealing that dried mushrooms extracted with boiling water, akin to tea, for 3 min exhibited the highest antioxidant activity. This information was interesting for developing this mushroom into a tea product. This study provides valuable information concerning the storage and extraction of *T. clypeatus* for eating off-season and developing into another product.

Keywords: *Termitomyces clypeatus,* Storage mushroom, Folk wisdom preservative of mushroom, Antioxidant, Phenolic compound

1. Introduction

Oxidants are the compounds that initiate oxidation, referred to as free radicals (or sometimes just radicals). A free radical is a molecule containing one or more unpaired electrons [1]. Radicals are one of the causes of various diseases such as cancer, vascular disease, pathological processes of the nervous system including Alzheimer's disease, and growing older [2]. Antioxidants are bioactive compounds that scavenge and neutralize free radicals, and oxidants, inhibiting lipid peroxidation reactions and preventing other oxidative damage [3].

Phenolic compounds are important and interesting owing to their antioxidant properties. The structure of phenolic compounds consists of the hydroxy group being attached directly to the aromatic ring [4]. They are secondary metabolites produced by plants, fungi, marine organisms, and bacteria [5]. Mushrooms are a type of fungi that are an interesting natural food source but mushrooms rot quickly because they lack a protective layer, have a high respiration rate, and contain high moisture. So, numerous conventional techniques are employed to

uphold their quality and prolong their storage duration such as cooling, drying, coating, and packaging after harvest [6].

More than 200 varieties of mushrooms are edible [7], an important food source in many countries in Asia [7-8]. Mushrooms are popular as food and medicine, which is a good taste and favor [9]. They contain a rich source of primary and secondary metabolites such as high protein, low fat, and cholesterol contents, rich in minerals and vitamins, phenolic compounds, polysaccharides, polyketides, terpenes and steroids [10-11]. Owing to a rich source of biologically active compounds responsible for antioxidants, antibacterial, hepatoprotective, antiradical, antihyperglycemic, antiangiogenic, and even anti-inflammatory, antitumor, antiallergic, antiatherogenic, and hematological properties [11]. So, mushrooms are interesting food as a good source of nutraceuticals.

Termitomyces clypeatus is an edible mushroom that belongs to the genus Termitomyces, family Lyophyllaceae [12]. Sporocarps of *T. clypeatus* appear during the rainy season (June-September) and are abundant in the natural forests of Thailand [13]. They are popular as food because of their flavor, texture, and delicacy. Recently pyrogallol (0.223 μg/mg), cinnamic acid (0.095 μg/mg), and polysaccharide were observed in this mushroom [12,14]. *T. clypeatus* was found to be cytotoxic against cancer cells, the human leukemic monocyte lymphoma cell line (IC_{50} 25 ± 1.02 μg/mL), with antiproliferative, and antioxidative properties [12, 14-16]. Due to the *T. clypeatus* having ethnomedicinal uses, a food source and only had in the rainy season. Therefore, they are famous for preserving food for eating out of season. Based on folk wisdom, this mushroom is boiled and then stored in the freezer in the refrigerator.

Therefore, our study was to evaluate the modifications induced by different conservation treatments (drying, boiling, and freezing) and extract in the total phenolic content and antioxidant properties of *T. clypeatus*. In this research, we studied different storage periods (1-3 months) after boiling mushrooms and freezing them in the refrigerator, as well as various extraction periods (3, 5, and 10 m.) for dried mushrooms. This is the first report on the total phenolic content and antioxidant properties of *T. clypeatus* for various preservation treatments and extracts based on traditional folk wisdom for preserving mushrooms. It aims to provide information on preserving and storing this mushroom for consumption.

2. Materials and methods

2.1 Chemicals and reagents

DPPH, ABTS, and Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich Chemical Co. USA. All the other chemicals and solvents were analytical grade.

2.2 Plant materials

T. clypeatus as an edible mushroom, was purchased from a local market in Sakon Nakhon District, Sakon Nakhon Province, Thailand. The *T. clypeatus* after boiled samples were subjected to freezing storage for different periods (1, 2, and 3 months), and dried samples were extracted with boiled water (60 ºC) for various durations (3, 5, and 10 m.), while extracts with distilled water at room temperature were also prepared. Finally, all sample extracts were evaporated to yield eleven crude extracts, as presented in Figure 1, dissolved in a known volume of the respective solvent, and used for the Folin-Ciocalteu method and antioxidant assays.

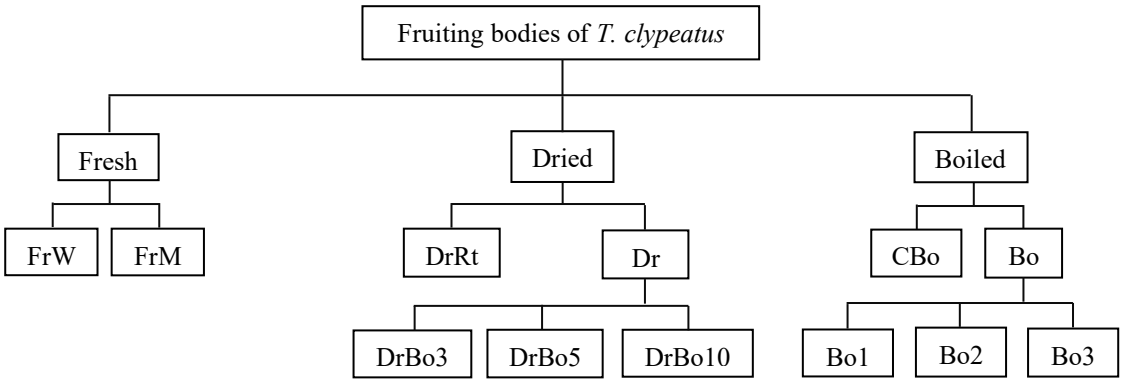

Figure 1 The scheme for preparation and extraction of *T. clypeatus*: friedmann robertson walker (FrW) fresh mushrooms extracted with water, (FrM) fresh mushrooms extracted with methanol (MeOH), demand-responsive transport (DrRt) dried mushrooms extracted with water, (DrBo3-DrBo10) dried mushrooms extracted with water for 3, 5, and 10 m, chief business officer (CBo) mushroom boiled water, backorder (Bo) boiled mushrooms, (Bo1-Bo3) mushrooms boiled and refrigerated for 1, 2, and 3 months before being extracted with water.

2.3 Preparation of crude fresh fruiting bodies extract (Fr)

Fresh fruiting bodies of *T. clypeatus* 250 g were ground in an electric grinder and extracted three times (24 hours each) with 1L distilled water or MeOH (250 g for each sample), respectively. The extraction was carried out using the maceration method at room temperature. The aqueous and MeOH crude extracts were then evaporated under reduced pressure to obtain the crude aqueous (FrW) and MeOH (FrM) extracts.

2.4 Preparation of crude dried fruiting bodies extract (Dr)

Fresh fruiting bodies of *T. clypeatus*, weighing 250 g, were dried at 70 ºC for 24 hours in a hot air oven and then ground in an electric grinder. The dried samples were then divided into two equal groups.

The first group was extracted three times (24 hours each) with 1L of distilled water using the maceration method at room temperature. Subsequently, the aqueous crude extract was evaporated under reduced pressure to obtain the crude aqueous extract (DrRt).

The second group of samples was divided into three samples in equal amounts for extraction with 300 mL boiling distilled water at 60 ºC for different durations (3, 5, and 10 m.). All crude extracts obtained from maceration were then evaporated under reduced pressure to obtain three crude extracts: DrBo3, DrBo5, and DrBo10.

2.5 Preparation of crude boiled fruiting bodies extract (Bo)

Fresh fruiting bodies of *T. clypeatus*, weighing 250 g, were boiled with 1L distilled water at 80 ºC for 30 min, and the resulting solutions were collected and filtered using Whatman No. 1 filter paper. The aqueous solution was evaporated under reduced pressure to obtain crude CBo extract. Thereafter, the boiled fruiting bodies of *T. clypeatus* were ground in an electric grinder and extracted three times (24 hours each time) with 1L of distilled water. The resulting aqueous solution was evaporated under reduced pressure to obtain the crude Bo extract.

2.6 Preparation of crude boiled and frozen fruiting bodies extract (Bo)

The extraction procedure is the same as described above in section 2.5. The boiled fruiting bodies of *T. clypeatus* were divided into three parts and frozen (-18 ºC) in the refrigerator for 1, 2, and 3 months, respectively. Each group was extracted three times (24 hours each) with 1L of distilled water. The resulting aqueous solutions were evaporated using a rotary evaporator to obtain crude extracts Bo1, Bo2, and Bo3, respectively.

Finally, the extraction yield of all crude extracts was calculated as a percentage (%yield) using the following equation:

$$\%yield = (Weight\ of\ crude\ extract\ (g) / Weight\ of\ raw\ mushroom\ (g)) \times 100 \quad (1)$$

2.7 DPPH free radical scavenging assay

The scavenging activity of all crude extracts from mushrooms on DPPH radicals was measured according to the method of Cheung et al. (2003) [17] with some modifications. An aliquot of 150 µL of 0.1 mM DPPH radical (Sigma) in methanol was added to a 96-well plate with 50 µL of mushroom crude extract of different concentrations (50 to 1000 ppm). Methanol was used instead of the mushroom sample as a control. The mixture was mixed and then discarded in the dark at room temperature for 30 min. The absorbance (Abs) was determined immediately after mixing by measuring at 520 nm with a microplate reader. The analysis was done in triplicate. Gallic acid was used as the standard. The scavenging activity on DPPH radicals was calculated by the equation below. The results were expressed as percent inhibition.

$$\%DPPH\ inhibition = ((A_{control} - A_{sample}) / (A_{control})) \times 100 \quad (2)$$

where $A_{control}$ is the mixture of methanol and DPPH solution; and A_{sample} is the mixture of sample extract and DPPH solution.

2.8 ABTS radical scavenging assay

The ABTS assay was done by following the methods of Thaipong et al. (2006) [18] and Tachalerdmanee et al. (2016) [19] with some modifications. The stock solutions included 7.4 mM $ABTS^{\bullet+}$ solution and 2.4 mM potassium persulfate. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing the mixture to stand in the dark at room temperature for 16 hours before use. Before use,

the $ABTS^{\bullet+}$ cation radical solution was diluted with distilled water to provide an absorbance of 0.70±0.05 at 734 nm. Fifty microliters of the sample solution were reacted with 150 µl of ABTS reagent in a 96-well plate, then incubated in darkness at room temperature for 7 min. The absorbance was measured at 734 nm with a UV-Vis microplate reader. The positive control was gallic acid. Each reaction was performed in triplicate. The percentage of $ABTS^{\bullet+}$ was determined using the equation below. The results were expressed as percent inhibition.

$$\%ABTS\ inhibition = ((A_{control} - A_{sample}) / (A_{control})) \times 100 \quad (3)$$

where $A_{control}$ is the mixture of distilled water and $ABTS^{\bullet+}$ solution; and A_{sample} is the mixture of sample extract and $ABTS^{\bullet+}$ solution.

2.9 Determination of total phenolic content

The content of total phenolic compounds in the crude extracts of mushroom was determined using Folin-Ciocalteu method by UV-Vis microplate reader according to the method of Cheung et al. (2003) [17] and Tachalerdmanee et al. (2016) [19] with some modifications. Twenty microliters of each mushroom extract solution were reacted with 100 µl of 1:10 Folin-Ciocalteu's regent in a 96-well plate. Then, 80 µl of 7.5% of sodium carbonate solution was added. The mixture was kept in darkness at room temperature for 30 min after which its absorbance was read at 750 nm. A calibration curve was constructed with different concentrations of gallic acid (4–20 ppm) as standard. Each reaction was performed in triplicate. Gallic acid was used as a positive control to provide a calibration curve. The total phenolic content of each crude extract was calculated using the calibration curve and expressed as mg gallic acid equivalents per gram (mg GAE/g) of fresh weight [20].

2.10 Statistical analysis

The data was represented as the mean value ± standard deviation (SD). Statistical comparisons were conducted using Tukey's further test, with statistical significance set at $p \leq 0.05$.

3. Results and discussion

3.1 Extraction yield in various extracts and various storage

In this study, various storage and extraction methods of *T. clypeatus* were investigated, including fresh mushrooms (extracted with distilled water and MeOH), dried mushrooms (extracted with distilled water at room temperature and boiling distilled water for 3, 5, and 10 min), and boiled mushrooms (stored frozen in the refrigerator for 1, 2, and 3 months before extraction). Eleven crude extracts were obtained from this extraction, and the result of the %yield of crude extracts is shown in Table 1.

The %yield of crude extracts using water as a solvent (FrW), 10.93%) from fresh fruiting bodies of *T. clypeatus* is higher than that of MeOH (FrM, 3.93%). This suggests that the majority of soluble components in these mushrooms are highly polar, likely due to the presence of heteroglycans as the major component [12]. The boiled fruiting bodies of *T. clypeatus* chief business officer (CBo) exhibited a lower %yield of crude extract (4.09 %) compared to extracts from fresh fruiting bodies of *T. clypeatus* (FrW) (10.93 %), likely due to the release of components such as soluble sugars and proteins during boiling [21]. To confirm the result, the %yield of crude extract for mushroom boiled water backorder (Bo) was determined to be 3.63%.

3.2 Dried fruiting bodies of T. clypeatus

The dried fruiting bodies of *T. clypeatus* were extracted using distilled water at room temperature and boiled distilled water at 60 ºC for various durations (3, 5, and 10 m.) to simulate tea preparation. The method for scavenging DPPH free radicals in the extracts of DrBo3, DrBo5, and DrBo10 showed the percent of inhibition value of 14.34±0.03, 9.18±0.06, and 7.72±0.15 at 400 ppm, respectively (Table2). In addition, the ABTS radical scavenging for DrBo3, DrBo5, and DrBo10 extract showed inhibition percentages of 50.39±0.22, 33.94±0.07, and 22.71±0.16, respectively (Table 2). Additionally, the total phenolic content of the DrBo3, DrBo5, and DrBo10 extract was found to be 13.06±0.11, 10.03±0.10, and 9.73±0.15 mg GAE/g fresh weight, respectively (Table 2). The antioxidant levels in dried mushroom extracts were found to be associated with the total phenolic content, showing a slight decrease over extended extraction periods.

Table 1 The yield of *T. clypeatus* extracts by various storage and extraction.

Crude extract	% yield*
Fresh (Fr)	
FrW	10.93
FrM	3.93
Dried (Dr)	
DrRt	9.93
DrBo3	10.93
DrBo5	11.22
DrBo10	11.36
Boiled (Bo)	
Bo	3.63
CBo	4.09
Bo1	8.59
Bo2	6.29
Bo3	4.84

*% yield is the ratio of the crude extracted yield to the raw sample yield.

These results indicate that the choice of extraction methods influences the antioxidant activities and the presence of phytochemical compositions in the extracts of dried fruiting bodies of *T. clypeatus*. Table 2 demonstrates that the antioxidant activities of DrRt are similar to those of DrBo3 in DPPH and ABTS assays. Conversely, prolonged extraction times result in reduced antioxidant activities and phenolic content. The decrease in antioxidants and phenolic contents of dried fruiting bodies of *T. clypeatus* during boiled extract could be due to either the decomposition of phenolic compounds or other chemical changes of phenolic compounds such as the formation of new insoluble substances with other organic components [21]. The dried *T. clypeatus* (DrRt) exhibits more potent antioxidant activity and higher phenolic content compared to fresh *T. clypeatus* (FrW). This trend is similar to that observed in dried *Boletus* species, which typically contain substantially more total polyphenols than fresh or frozen varieties [22]. During the drying process of mushrooms, heat reactions occur, including the Maillard reaction and non-enzymatic glycosylation. These reactions can result in the formation of new chemical compounds that enhance the antioxidant activities and phenolic content of dried *T. clypeatus* mushrooms [23].

3.3 Boiled fruiting bodies of T. clypeatus

Folk wisdom suggests boiling the fruiting bodies of mushrooms before storing them in the freezer for consumption out of season. This practice is based on the fact that mushrooms contain enzymes that can cause rapid deterioration after harvesting. Boiling can help denature these enzymes, slowing down enzymatic browning and degradation, thereby prolonging the freshness of the mushrooms [24]. The results show that antioxidants and phenolic compounds can dissolve in mushroom boiled water (CBo), as presented by percent inhibition (6.75±0.09 for DPPH assay and 30.54±0.04 for ABTS assay) and total phenolic content (7.79±0.12 mg GAE/g fresh weight) in Table 2. When comparing the periods of storing boiled mushrooms in the refrigerator for 1 (Bo1), 2 (Bo2), and 3 (Bo3) months, it was observed that the antioxidant activity and phenolic compound content of the mushrooms decreased with longer storage durations, as indicated in Table 2. The storage of mushroom products resulted in a substantial decline in their antioxidant activity due to losses of vitamin C or tocopherol, polyphenols, and total flavonoids [25].

Table 2 Antioxidant activities and total phenolics of *T. clypeatus* extracts by various storage and extraction.

Crude extract	%inhibition at 400 ppm DPPH assay	%inhibition at 400 ppm ABTS assay	Total phenolic (mg GAE/g fresh weight)
Fresh (Fr)			
FrW	7.55^{f}±0.04	29.13^{i}±0.12	12.78^{b}±0.10
FrM	8.38^{e}±0.04	49.20^{e}±0.06	12.06^{c}±0.15
Dried (Dr)			
DrRt	13.50^{c}±0.04	54.87^{b}±0.04	15.60^{a}±0.06
DrBo3	14.34^{b}±0.03	50.39^{c}±0.22	13.06^{b}±0.11
DrBo5	9.18^{d}±0.06	33.94^{f}±0.07	10.03^{d}±0.10
DrBo10	7.72^{f}±0.15	22.71^{k}±0.16	9.73^{d}±0.15
Boiled (Bo)			
Bo	8.29^{e}±0.04	49.82^{d}±0.05	12.95^{b}±0.02
CBo	6.75^{g}±0.09	30.54^{h}±0.04	7.79^{f}±0.12

Bo1	8.46^{e}±0.17	31.12^{g}±0.05	9.08^{e}±0.07
Bo2	8.37^{e}±0.10	23.92^{j}±0.18	8.94^{e}±0.10
Bo3	8.24^{e}±0.11	17.82^{l}±0.16	8.88^{e}±0.10
Gallic acid	85.78^{a}±0.05	82.51^{a} (5 ppm)	-

Values are presented as Mean ± SD. Values with the different letters in superscript along the same column are significantly different by Tukey's test ($p \leq 0.05$).

The antioxidant levels in boiled mushroom samples stored in the refrigerator were found to be associated with the total phenolic content, showing a slight decrease over extended storage periods. All the crude extracts were tested for both antioxidant methods using DPPH and ABTS assays. Due to differences in principle, reaction kinetics, sensitivity, and conditions, the ABTS radical cation reacts with both lipophilic and hydrophilic antioxidants in various matrices, while the DPPH assay primarily relies on the electron transfer reaction. Therefore, evaluating the mushroom containing the unknown chemical composition through multiple antioxidant assays offers a more comprehensive and nuanced assessment of its antioxidant properties, considering different mechanisms, radicals, sensitivities, and types of information obtained [26].

3.4 Comparison of various storage of T. clypeatus

In this research, Gartner's Magic Quadrant for analysis of antioxidant activity for all samples is necessary to consider certain parameters, including %inhibition of DPPH assay and ABTS assay were applied from Friedman and Bitterer (2011) [27]. Crude extracts are positioned on a graph with two axes: the %inhibition " DPPH assay " and " ABTS assay ". The quadrants are labelled as follows: Leaders (the crude extracts with high %inhibition of both assays), Challengers (the crude extracts with high %inhibition of only one assay), Visionaries (the crude extracts with low %inhibition of both assays), and Niche Players (the crude extracts with moderate %inhibition which one or both assay). This makes it easier to understand the comparative landscape without needing to delve into complex reports.

The position in the upper right corner of the magic quadrant (Figure 2) indicates the effectiveness of the associated procedure. Due to its superior capability in comparison to the other approaches in obtaining high %inhibition at 400 ppm DPPH assay and high %inhibition at 400 ppm ABTS assay, the method found in Q1 is categorized as the leader. The DrBo3 sample exhibited a higher percentage of inhibition at 400 ppm DPPH assay than DrRt, DrBo5, Bo1, and FrM, according to the figure. These samples are located in the Q1 area. In Q2, the Bo sample is a rank contender, followed by the Bo2 in Q3, the Bo3 in Q4, and the DrBo10, FrW, and CBo in Q4. In summary, the DrBo3 delivers the best %inhibition at 400 ppm DPPH assay, causing the procedure to generate the best %inhibition at 400 ppm DPPH assay when predicting the antioxidant activities. DrRt also fared better than other approaches from a different angle. The outcomes were comparable to DrBo3 as well. DrBo3 is one of the competing samples as a result.

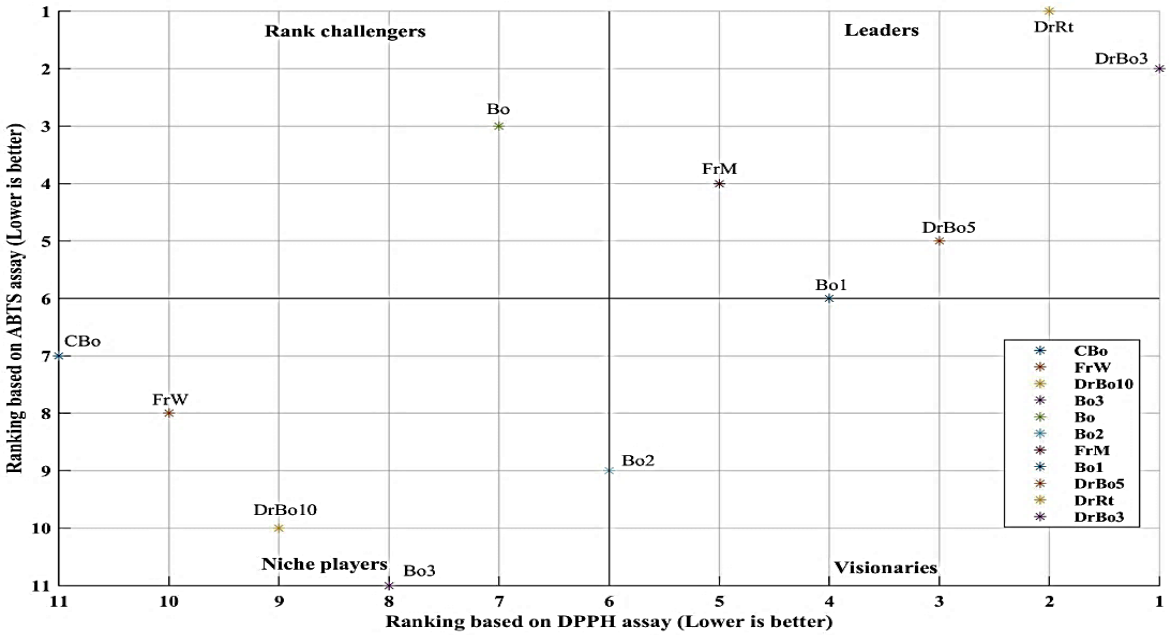

Figure 2 The magic quadrant for analysis of antioxidant activity from *T. clypeatus*.

4. Conclusions

In conclusion, this study compares the antioxidant activities and total phenolic content of mushrooms preserved according to folk wisdom for consumption out of season. Fresh mushrooms were boiled and stored in the freezer for 1 – 3 months, resulting in a slight decrease in antioxidant activities and total phenolic content. Additionally, we investigated the antioxidants in dried mushrooms and extracts with boiled water, to process them into other products such as tea. Our findings indicate that the boiled water extract processed for 3 min exhibited higher antioxidant activity compared to those processed for 5 and 10 min. While boiling mushrooms in water, the phenolic compounds dissolved into the boiling water, and the antioxidants were observed in the resulting mushroom broth. Furthermore, the water solvent extract exhibited a higher total phenolic content compared to the organic solvent (MeOH) extract. This evidence is helpful for the preservation and extraction of mushrooms (*T. clypeatus*). Interestingly, our study demonstrates that DrBo3 exhibits the highest antioxidant activity. Therefore, this mushroom could be further developed into a tea product.

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