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Comparing the functional components, SOD-like activities, antimutagenicity, and nutrient compositions of *Phellinus igniarius* and *Phellinus linteus* mushrooms

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ABSTRACT

Many species of the genus *Phellinus* possess beneficial properties, including antioxidant, immune-enhancing, and antimutagenic effects. Phenolic compounds and polysaccharides are two kinds of bioactive compounds; however, few studies have compared the differences between *Phellinus igniarius* and *Phellinus linteus* in their functional components, functional activities, and nutrient compositions. Herein, the proximate compositions and microelements of the fruiting body of *P. igniarius* and *P. linteus* were determined. The fruiting body of *P. igniarius* and *P. linteus* were extracted by boiling water [water extract of *P. igniarius* (WEPI) and *P. linteus* (WEPL)]. The contents of total phenolics and polysaccharides, as well as superoxide dismutase (SOD)-like and antimutagenic activities of WEPI and WEPL, were compared. We found that WEPI was rich in phenolics and polysaccharides and had higher SOD-like activity than WEPL. Nutrient compositions were mainly different in minerals, whereas anitmutagenicity was similar. All of these results suggested that *P. igniarius* has greater potential for the development of antioxidant and immunomodulating food products than *P. linteus*.

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1. Introduction

The genus *Phellinus* is a group of medicinal mushrooms belonging to the family *Hymenochaetaceae* (*Basidiomycetes*) [1]. In folk medicine, several species of *Phellinus* (*Phellinus* spp.) improve health and prevent or treat various diseases, such as alleviating oral ulcers, some gastroenteric disorders, and some lymphatic diseases [2,3]. In the last decade, some *Phellinus* spp. have demonstrated additional medicinal properties, including anti-inflammatory, antidiabetic, antioxidant, immune-enhancing, antimutagenic, anticarcinogenic, and antimetastastic effects [1–3]. Within the genus of *Phellinus* mushrooms, *P. igniarius* and *P. linteus* are two of the most well-known mushrooms [1]; however, few studies have compared the differences in functional components, functional activities, and nutrient compositions between *P. linteus* and *P. igniarius* mushrooms.

Phenolic compounds and polysaccharides are two main kinds of bioactive compounds responsible for the medicinal properties of *Phellinus* spp. [1]. Phenolic compounds have been associated with inhibition of atherosclerosis and cancer [4]. Potential mechanisms of action include the scavenging of reactive oxygen species, metal chelation, and lipooxygenase inhibition [4]. Additionally, superoxide dismutase (SOD) is an important enzyme that defends against the harmful effects of superoxide, which is one of the most common free radicals in the body [5]. Phenolics are specific nonenzymatic scavengers of superoxide, and can prevent biological damage by trapping radical oxidants, such as SOD (SOD-like activity) [5]. However, polysaccharides isolated from *Phellinus* spp. confer immunomodulating activities, including enhancement of nonspecific immunity and humoral- and cell-mediated immunity [2,6–8]. The immunostimulating activity of polysaccharides isolated from *Phellinus* spp. also play important roles in tumor suppression [9,10]. Additionally, 1,3-β-glucan is the most active polysaccharide isolated from *Phellinus* spp. and other medicinal mushrooms [1,2,11,12]. However, the phenolic compounds, polysaccharides, 1,3-β-glucan contents, and SOD-like activity of *P. linteus* and *P. igniarius* mushrooms remain unclear.

One study compared the antimutagenic activity of extracts of cultured mycelium from *P. igniarius* and *P. linteus* against several direct- and indirect-acting mutagens [13], finding no difference in the antimutagenicity of the extracts. However, it is still unknown whether the aqueous extracts of the fruiting bodies rather than the mycelium have different antimutagenic properties. Moreover, to the best of our knowledge, the nutrient compositions of the fruiting body of *P. linteus* and *P. igniarius* mushrooms also remain unknown.

In this study, the nutrient compositions of the fruiting bodies of *P. igniarius* and *P. linteus* were determined by analysis of the proximate and mineral compositions. The fruiting body of *P. igniarius* and *P. linteus* were then extracted by boiling water [water extract of *P. igniarius* (WEPI) and *P. linteus* (WEPL)], and the dried extracts were used to determine the contents of functional components, including total phenolic compounds, flavonoids (a subgroup of phenolics) [14], polysccahrides, and β-1,3-glucan. Additionally, SOD-like activity was determined by a commercialized SOD kit using an automatic biochemical analyzer. Antimutagenic activity was detected by Ames test [15] using *Samonella typhimurim* TA98 and TA 100 with direct- and indirect-acting mutagens of 4-nitro-quinoline-N-oxide (4-NQO) and aflatoxin B_1 (AFB), respectively.

2. Methods

2.1. Chemicals

All chemicals used were of analytical grade. Aluminum nitrate, sodium dihydrogen phosphate, sodium chloride, gallic acid, quercetin, aniline blue, laminarin, histidine, 4NQO, AFB, and Folin-Ciocalteu's reagent were obtained from Sigma-Aldrich(St. Louis, MO, USA). Sodium hydroxide, sodium carbonate, sodium bicarbonate, and Na, Mg, K, Ca, Mn, Fe, Cu, and Zn standard solutions were purchased from Merck (Darmstadt, Germany). Potassium acetate was obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Investigative mushrooms

The fruiting bodies of *P. igniarius* and *P. linteus* were obtained from Bao-Jia Biotech Co., Ltd. (Taiwan, Nantou). *P. igniarius* fruiting bodies were harvested from the trunk of broadleaf trees in Taiwan, which are perennial and hard mushrooms with a large body size and belong to a white-rot fungus. *P. linteus* fruiting bodies were harvested from the trunk of mulberry trees in Taiwan, which are also perennial and hard mushrooms with a yellow color inside, and also belong to a white-rot fungus, but have a body size less than the fruiting body of *P. igniarius*. The morphology of the fruiting bodies of *P. igniarius* and *P. linteus* is shown in Fig. 1A and 1B, respectively. After cutting, *P. igniarius* (PI) and *P. linteus* (PL) fruiting bodies were crushed and strained through a 40-mesh sieve. The powders were stored at 4°C before analysis of the water extractions.

2.3. Determination of proximate compositions and microelements of P. igniarius and P. linteus fruiting bodies

The proximate composition of *P. igniarius* and *P. linteus* mushrooms were assessed, including moisture and crude protein, fat, ash, and fiber contents. These parameters were analyzed by the methods referred to as the Association of Official Agricultural Chemists (AOAC) 15.950.02, AOAC 15.976.05, AOAC 15.920.39, AOAC 15.955.03, and AOAC 15.962.09, respectively [16]. Microelements, including Na, Mg, K, Ca, Mn, Fe, Cu, and Zn, were analyzed by a flame atomic absorption analyzer (Varian AA-1275, Varian Techtron, Springvale, Australia) [16]. Nitrogen-free solubles were calculated by 100-(moisture + crude protein + crude fat + ash + crude fiber).

2.4. Preparation of WEPI and WEPL

The powders of the fruiting bodies of *P. igniarius* and *P. linteus* were extracted in triplicate using 15-fold distilled water at 100°C for 3 h. After filtration, the solutions were dehydrated in a vacuum (VirTis 12ES, VirTis Co. Inc., Gardiner, NY, USA), and

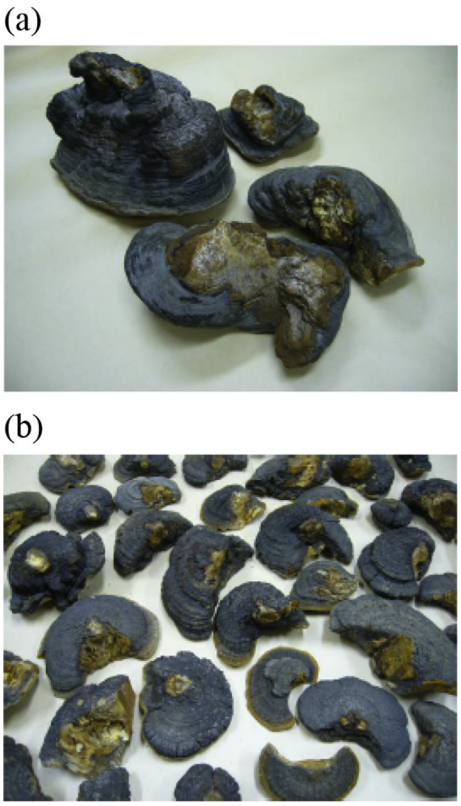

Fig. 1 – The morphology of fruiting bodies of *Phellinus igniarius* and *Phellinus linteus*. Photographs show the fruiting bodies of (a) *P. igniarius* and (b) *P. linteus*.

these dried extracts were used as WEPI and WEPL. The extraction rates (%) were calculated by the weight of vacuum-dried powder of water extract (g) per weight of crushed powder of fruiting body (g) × 100.

2.5. *Determination of total phenolic compounds*

Total phenolic compounds were measured using the method of Singleton et al. with some modifications [17,18]. Samples (0.2 mL) with adequate concentration were mixed with 1 mL Folin-Ciocalteu's reagent and 0.8 mL sodium carbonate (75 mg/mL). After standing for 30 min, the absorbance was determined by a spectrophotometer (U-2000 Spectrophotometer, Hitachi, Ltd., Tokyo, Japan) at 760 nm. The calibration curve was achieved by using gallic acid as the standard. The total phenolic compounds were reported as the gallic acid equivalent in mg/g of dried sample.

2.6. *Determination of flavonoids*

Flavonoid content was determined by the method proposed by Jia et al. with slight modifications [19,20]. Briefly, samples (0.5 mL) with proper concentration were mixed with 1.5 mL distilled water, 0.1 mL 0.1 g/mL aluminum nitrite, 0.1 mL 1 M potassium acetate, and 2.8 mL H_2O. After standing for 40 min, the absorbance was determined by a spectrophotometer (Hitachi, Ltd) at 415 nm. The calibration curve was achieved by using quercetin as the standard. The flavonoid content is reported as the quercetin equivalent in mg/g of drying sample.

2.7. *Determination of polysaccharides*

Polysaccharide levels were determined by the method of Chaplin and Kennedy by ethanol precipitation with slight modifications [21]. Briefly, proper sample concentration was precipitated by ~4–5-fold volumes of 95% ethanol. After standing overnight at 4°C, the sample was centrifuged at 1500*g* for 10 min at 4°C. After completely eliminating the ethanol by aspiration, followed by oven- and freeze-drying, the dried powder was weighed and the polysaccharide content expressed as crude polysaccharide (mg)/dried extract (g).

2.8. *Determination of β-1,3-D-glucan*

β-1,3-D-glucan was measured using the aniline blue assay [22]. Samples were dissolved in 3 mL of 3 mM NaOH solution for 30 min, and the pH of the solution adjusted to 11.5. The solution was then mixed with 50 mM Na_2HPO_4 buffer (pH 11.5) containing 0.5 mM NaCl. The mixed solution (2 mL) was then mixed with 0.2 mL aniline blue (1.0 mg/mL) for 2 h, and the fluorescence determined by a fluorimeter (F-2500, Hitachi, Japan), with excitation and emission wavelength of 395 nm and 495 nm, respectively. The calibration curve was obtained using laminarin as the standard. The β-1,3-D-glucan content was reported as the laminarin equivalent in mg/g of dried sample.

2.9. *Determination of SOD-like activity*

SOD-like activity was determined using the commercialized SOD kit (Randox Laboratories Ltd., Crumlin, UK) following manufacturer instructions and using an automatic biochemical analyzer (Chiron/Diagnostics Express Plus, East Walpole, MA, USA). This method uses xanthine/xanthine oxidase system to generate superoxide radicals, which react with electron acceptor 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan. The absorbance of red formazan was detected at 505 nm, and SOD activity was measured by the degree of inhibition of this reaction. The SOD activity (U/mL) was calculated by using the SOD standards provided by the kit to create a calibration curve for quantification.

2.10. *WEPI and WEPL antimutagenicity assay*

Antimutagenicity was determined using the Ames test on two *Salmonella typhimurium* strains (TA98 and TA100) [15]. TA98 has a frame-shift mutation, whereas TA100 carries a base-substitution mutation. Briefly, bacterial cultures (0.1 mL) and mutagens (0.1 mL) with or without S9 (0.5 mL) were mixed with soft agar and poured onto plates. After a 48-h incubation, the numbers of revertant His^+ bacterial colonies were calculated. Percent inhibition (%) was also assessed, which was calculated by [(number of revertants in control – number of revertants in treatments) / (number of revertants in control – number of spontaneous revertants) *100%]. The

doses of 4NQO and AFB were both 0.5 μg/plate. The S9 mixture was prepared from the livers of male Sprague-Dawley rats and induced with phenobarbital. WEPI and WEPL at concentrations of 0, 0.5, 1, and 2 mg per plate were used for the antimutagenicity tests, having previously determined that <2.5 mg/plate of the extracts induced no toxic or mutagenic effects on the *S. typhimurim* TA98 or TA100 strains.

2.11. *Statistical analysis*

Data are expressed as means ± standard deviation (SD) and were analyzed using a Student t test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Nutrient compositions of* P. igniarius *and* P. linteus *fruiting bodies*

The proximate composition and microelement contents of *P. igniarius* and *P. linteus* fruiting bodies were analyzed. As shown in Table 1, the two mushrooms contained similar levels of nitrogen-free solubles (>67%) and moisture (~15%; Table 1). The *P. igniarius* fruiting body contained higher levels of fiber and fat than *P. linteus* ($p < 0.05$), whereas *P. linteus* had higher ash content than *P. igniarius* ($p < 0.05$). Furthermore, the calories of *P. igniarius* and *P. linteus* were estimated, respectively, as 302 Kcal/100 g and 295 Kcal/100 g, which were calculated by [(4 × protein weight (g)) + (4 × nitrogen-free soluble (g)) + (9 × fat weight (g))] per 100g of sample. Regarding the microelements, the *P. linteus* fruiting body contained higher levels of K, Ca, and Na than *P. igniarius*. In particular, the levels of K and Ca in *P. linteus* were much higher than *P. igniarius*. By contrast, the Mg content in *P. igniarius* was ~5-fold higher than that of *P. linteus* (Table 1).

Table 1 – Nutrient compositions of *Phellinus igniarius* and *Phellinus linteus* fruiting bodies.

Component	*P. igniarius*	*P. linteus*
Proximate composition (%)		
Moisture	16.0 ± 1.28	15.2 ± 1.20
Crude protein	5.6 ± 0.11	5.4 ± 0.10
Crude fat	1.3 ± 0.07	**0.3 ± 0.07**
Crude fiber	7.9 ± 0.52	**6.8 ± 0.39**
Ash	2.2 ± 0.04	**4.6 ± 0.01**
Nitrogen-free solubles	67.1 ± 0.59	67.7 ± 0.22
Microelements (μg/g)		
Na	0.8 ± 0.3	**2.6 ± 0.1**
Mg	5.1 ± 0.1	**1.0 ± 0.1**
K	44.0 ± 2.8	**130.6 ± 5.0**
Ca	14.8 ± 2.8	**118.1 ± 15.1**
Mn	0.6 ± 0.0	0.6 ± 0.0
Fe	1.0 ± 0.0	1.0 ± 0.4
Cu	0.3 ± 0.0	**0.1 ± 0.0**
Zn	0.2 ± 0.0	**0.1 ± 0.0**

Bold highlighted values (mean ± SD; n=3) indicate statistically significant differences ($p < 0.05$).
SD = standard deviation.

Table 2 – Yield of the water extract from crushed powders of *Phellinus igniarius* and *Phellinus linteus* fruiting bodies.

	Yield (%)
P. igniarius	7.1 ± 1.6
P. linteus	**13.9 ± 1.6**

The bold highlighted value (mean ± SD; n=3) indicates a statistically significant difference ($p < 0.05$).
SD = standard deviation.

3.2. *The extraction rate*

The yield of water extracts from crushed powders of the fruiting bodies were analyzed. The results showed that the *P. igniarius* and *P. linteus* extraction rates were 7.1% and 13.9%, respectively (Table 2). These results indicated that the *P. linteus* extraction rate was ~2-fold higher than that of *P. igniarius*.

3.3. *Contents of total phenolics, flavonoids, polysaccharide, and β-1,3-D-glucan in WEPI and WEPL*

As shown in Table 3, the levels of total WEPI phenolic compounds and flavonoids were higher than those of WEPL ($p < 0.05$). Additionally, the contents of total WEPI phenolics and flavonoids were both ~1.5-fold higher than those of WEPL, and polysaccharide content was much higher in WEPI, which contained ~2-fold more than that observed in WEPL. In contrast, WEPL contained about 1.2-fold higher β-1,3-glucan than WEPI ($p < 0.05$).

3.4. *WEPI and WEPL SOD-like activity*

WEPI and WEPL SOD-like activity were determined, revealing that WEPI possessed a 1.7-fold higher SOD-like activity ($p < 0.05$) than WEPL (Fig. 2).

3.5. *Antimutagenicity of WEPI and WEPL*

As shown in Table 4, WEPI and WEPL inhibited colony formation of histidine revertants (His^+) in a dose-dependent manner in both strains of *Salmonella typhimurium* TA98 and TA100, regardless of the presence of S9 and use of 4NQO or AFB mutagens. Notably, the antimutagenic activities against the different mutagens in both strains were almost equal.

Table 3 – WEPI and WEPL functional compositions.

Components	WEPI	WEPL
Total phenolics (mg/g dry weight)	12.0 ± 0.02	**8.3 ± 0.06**
Flavonoids (mg/g dry weight)	12.0 ± 0.07	**7.9 ± 0.06**
Polysaccharide (mg/g dry weight of extract)	49.7 ± 0.4	**25.4 ± 0.2**
β-1,3-Glucan (mg/g dry weight)	4.3 ± 0.02	**5.3 ± 0.01**

Bold highlighted values (mean ± SD; n=3) indicate statistically significant differences ($p < 0.05$).
SD = standard deviation; WEPI = water-extracted *Phellinus igniarius*; WEPL = water-extracted *Phellinus linteus*.

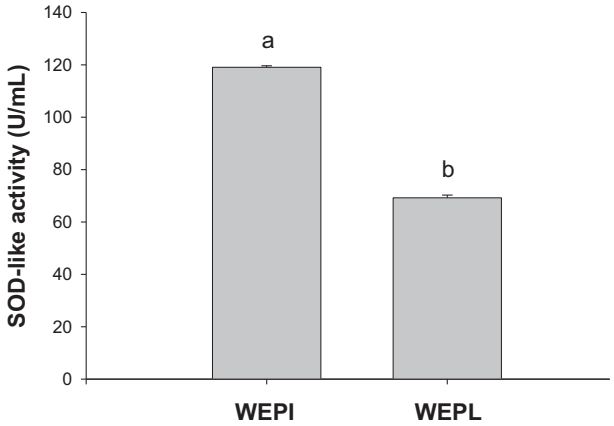

Fig. 2 – SOD-like activity in aqueous extracts from WEPI and WEPL. Equal quantities of WEPI and WEPL powders were dissolved in distal water at a proper concentration, and the SOD-like activity was determined as described in Section 2. Values (mean± SD; n=3) not sharing a common letter are significantly different ($p < 0.05$). SOD = superoxide dismutase; WEPI = water-extracted Phellinus igniarius; WEPL = water-extracted Phellinus linteus.

4. Discussion

P. igniarius and *P. linteus* are two of the most well-known *Phellinus* mushrooms. However, limited data exist regarding the differences between these two mushrooms in their functional components, functional activities and nutrient compositions. Upon analyzing the compositions of both mushrooms, we found that WEPI (the hot water extract of *P. igniarius*) possessed higher SOD-like activity coupled with higher phenolic and flavonoid contents than WEPL (the hot water extract of *P. linteus*). Additionally, the WEPI polysaccharide content was ~2-fold higher than WEPL, suggesting that the immunomodulating and antitumor potential of WEPI may be higher than WEPL. Both mushrooms showed similar antimutagenic activities, regardless of which strains (TA98/TA100) or mutagens (4NQO/AFB) were used. Taken together, our results suggested that *P. igniarius* may be a more suitable choice for the development of antioxidant and immunomodulating functionality in food products than *P. linteus*.

SOD defends against the harmful effects of superoxide, one of the most common free radicals in the body [5]. Nonenzymatic superoxide scavengers have become the subject of much attention, because, unlike SOD, they are found in natural foods and are not degraded by the digestive system. Phenolic compounds are nonenzymatic scavengers of free radicals that can prevent biological damage by trapping radical oxidants, such as superoxide [5]. Here, we found that WEPI contained ~1.5-fold higher levels of phenolic compounds than WEPL. We also demonstrated that WEPI has ~1.7-fold higher SOD-like activity than WEPL. To the best of our knowledge, this paper is the first study to compare the SOD-like activity of *Phellinus* spp. mushrooms and report that *P. igniarius* water extract exhibits greater SOD-like activity than that of *P. linteus*.

Polysaccharides isolated from *Phellinus* spp. confer immunomodulating [2,6,7] and tumor-suppressing activities [9,10]. Here, we found that WEPI contained ~2-fold more polysaccharides than WEPL, suggesting that WEPI may have greater immunomodulating and tumor-suppressing potential than WEPL. Although 1,3-β-glucan is the most active polysaccharide isolated from several medicinal mushrooms [1,2,11,12], we found that the difference in β-1,3-glucan content between WEPI and WEPL was minimal. As shown in Table 4, the difference was only ~1 mg/g. By comparing the immunomodulating activities of *P. linteus* and *P. gilvus*, Chang et al. [6] found that an equal concentration of polysaccharides from *P. gilvus* had a greater immunostimulating activity than that from *P. linteus*. Such results suggest that the types of polysaccharide can significantly affect the immunostimulating activity of these mushrooms. Additionally, different physiochemical parameters, such as solubility, primary structure, molecular weight, breaching, and polymer charge influence the biological activities of β-1,3-glucan [23]. Therefore, the types of polysaccharide and the physiochemical properties of β-1,3-glucan in WEPI and WEPL may influence the functional activities of mushrooms. Further investigation is needed to confirm such immunomodulating and tumor-suppressing activities *in vivo*.

AFB is a naturally occurring mycotoxin that is the most carcinogenic substance capable of inducing hepatic tumors.

Table 4 – WEPI and WEPL antimutagenicity.

Extracts of mushrooms (dose)	His$^+$ revertants/plate (% inhibition)			
	TA98		TA100	
	4NQO (without S9)	AFB (with S9)	4NQO (without S9)	AFB (with S9)
Spontaneous revertants	55 ± 6	79 ± 9	155 ± 12	154 ± 10
Control	615 ± 14 ^{a}	633 ± 14 ^{a}	1133 ± 22^a	745 ± 10 ^{a}
WEPI (0.5 mg/plate)	522 ± 14 ^{b} (16.6)	567 ± 11 ^{b} (11.9)	957 ± 4 ^{b} (18.0)	699 ± 15 ^{b} (7.7)
WEPL (0.5 mg/plate)	529 ± 8 ^{b} (15.3)	573 ± 8 ^{b} (10.7)	964 ± 7 ^{b} (17.3)	702 ± 15 ^{b} (7.3)
WEPI (1 mg/plate)	470 ± 8 ^{c} (25.9)	514 ± 6 ^{c} (21.5)	879 ± 8 ^{c} (26.0)	664 ± 8 ^{c} (13.7)
WEPL (1 mg/plate)	492 ± 5 ^{d} (21.9)	522 ± 9 ^{c} (20.0)	887 ± 8 ^{c} (25.2)	649 ± 8 ^{c} (16.2)
WEPI (2 mg/plate)	396 ± 7 ^{e} (39.2)	462 ± 12 ^{e} (30.9)	843 ± 8 ^{d} (29.7)	612 ± 5 ^{d} (22.5)
WEPL (2 mg/plate)	397 ± 8 ^{e} (38.9)	465 ± 13 ^{e} (30.3)	846 ± 6 ^{d} (29.4)	627 ± 5 ^{d} (19.9)

Values (mean ± SD; n=3) not sharing a common letter in the same column are significantly different ($p < 0.05$).
SD = standard deviation; WEPI = water-extracted *Phellinus igniarius*; WEPL = water-extracted *Phellinus linteus*.

4NQO is a synthetic water-soluble carcinogen used in the assessment of the efficacy of diets and drugs in the prevention and treatment of cancer [24]. Both WEPI and WEPL protected against mutagenesis induced by either AFB or 4NQO in a dose-dependent manner, suggesting that WEPI and WEPL may contain potent antigenotoxic compounds that might contribute to the anticarcinogenic or chemopreventive actions of WEPI and WEPL. Although we did not address the antimutagenic mechanisms of WEPI and WEPL against AFB and 4NQO, extracts from a species of genus *Phellinus* (*P. rimosus*) exert antimutagenic effects by mechanisms, including direct inactivation of mutagens, inhibition of the metabolic activation of promutagens, and antioxidant activities [25,26]. It is possible that the antimutagenic mechanisms of *P. igniarius* and *P. linteus* may be similar to those of *P. rimosus*.

It is known that the moisture and crude-fiber content of edible mushrooms are ~90% and ~1%, respectively [27]. Since both *P. igniarius* and *P. linteus* are hard mushrooms with much higher extents of lignification, the two mushrooms contained less moisture (~15%) and higher fiber (~6% to 8%) content than other edible mushrooms [27]. Interestingly, we also found that *P. igniarius* and *P. linteus* fruiting bodies contained high levels of nitrogen-free solubles. In general, nitrogen-free solubles in edible mushroom are below 10% [27], while the nitrogen-free solubles in *P. igniarius* and *P. linteus* mushrooms were >67%. This observation suggested that *Phellinus* mushrooms have a significantly different nutritional profile in their carbohydrate composition than other commonly eaten mushrooms. Although *P. igniarius* has higher contents in fiber and fat than that of *P. linteus* mushrooms, we believe that these differences were limited from the viewpoint of the nutrient compositions. The most different item in the proximate compositions was the ash between these two mushrooms. The ash content of *P. linteus* was 2.2-fold higher than *P. igniarius*, and the K and Ca contents of *P. linteus* were higher than *P. igniarius*, suggesting that the mineral composition of *P. linteus* were higher than *P. igniarius*. However, the Mg content was still higher in *P. igniarius*.

In summary, we found that WEPI had higher contents of phenolic compounds, flavonoids, and polysaccharides, as well as higher SOD-like activity. These results suggested that *P. igniarius* has greater potential for the development of antioxidant and immunomodulating functionality in food products than *P. linteus*. In contrast, the mineral composition, especially of K and Ca, in *P. linteus* was higher than that of *P. igniarius*. Furthermore, the antimutagenic activities of these two mushrooms were similar.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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