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Antioxidant properties of solid-state fermented adlay and rice by *Phellinus linteus*

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ABSTRACT

Phellinus linteus (Berkeley & Curtis) Teng (Hymenochaetaceae) was inoculated into cooked grains, and new products were formed after the colonisation of fungal mycelia. Our objective was to evaluate the antioxidant properties of ethanolic and hot water extracts from fermented products [*Phellinus*-fermented adlay (PFA) and rice (PFR)] as compared to uninoculated controls [polished adlay (PA) and rice (PR)]. PFA and PFR were more effective than were PA and PR in antioxidant activity by the conjugated diene method, reducing power, scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals and chelating ability on ferrous ions. Total phenols were the major antioxidant components found in both extracts (1.31–9.10 mg/g). Flavonoid contents were in the range of 0.07–1.26 mg/g. Total phenols and flavonoids of two extracts were associated with antioxidant properties. Based on the results obtained, *Phellinus*-fermented products possessed effective antioxidant properties.

1. Introduction

Phellinus linteus (Berkeley & Curtis) Teng (Hymenochaetaceae), a tan-yellow-coloured mushroom, usually grows on mulberry trees. It has been used as a traditional medicine in China, Korea, Japan and other Asian countries for the treatment of various diseases, including oral ulcer, gastroenteric disorder, lymphatic disease and other cancers. It has been reported that *P. linteus* showed anti-inflammation, and anti-angiogenesis effects, stimulating humoral and cell-mediated immunity, as well as inhibiting tumor growth and metastasis (Cho et al., 2002; Han et al., 1999; Kim et al., 1996; Kim et al., 2004; Kim et al., 2007; Shon & Nam, 2001; Song et al., 2003).

The technique of solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solid substrates in the absence of free flowing water (Babu & Satyanarayana, 1995). Recently, many studies have shown that SSF of dietary fungus could induce enzyme activity which could also lead to the release of phenolic antioxidants, especially those found in bound forms within the food matrix. SSF of *Lentinula edodes* could enhance the total content of phenolic antioxidants (McCue & Shetty, 2005) and increase the production of laccase (McCue, Horii, & Shetty, 2004).

Fruit bodies of *P. linteus* are precious due to their rareness and difficulty in cultivation. Currently, *P. linteus* is mainly obtained in the form of mycelia from submerged culture and used in the formulation of nutraceuticals and functional foods. Using SSF, the Japanese food "natto (*Bacillus*-fermented soybean)" and the Chinese traditional food "anka (*Monascus*-fermented rice)" were produced. Similarly, *P. linteus* can be inoculated into cooked grains, such as rice, adlay, soybean and wheat, and a fermented grain product is formed after mycelial colonisation. This *Phellinus*-fermented product will be a novel functional food, providing beneficial effects. Therefore, the objective of this study was to examine the antioxidant properties of ethanolic and hot water extracts from fermented products [*Phellinus*-fermented adlay (PFA) and rice (PFR)] as compared to uninoculated controls [polished adlay (PA) and rice (PR)]. Antioxidant properties assayed included antioxidant activity by the conjugated diene method, reducing power, scavenging ability on radicals and chelating ability on ions. Contents of potential antioxidant components in extracts were also evaluated.

2. Materials and methods

2.1. Materials and preparation of extracts

Adlay and rice were purchased at a local market in Taichung City, Taiwan. *P. linteus* was obtained from Laiyang Agricultural College, Shandong, China. The fungus was inoculated onto potato dextrose agar (PDA) plates and incubated at 25 °C for 14 days. After pure culture was obtained, the mycelium was reinoculated into basal medium and incubated at 25 °C for 14 days. The basal medium contained the following (g/l): glucose, 20; yeast extract, 5; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.875; K_2HPO_4, 0.125; $CaCl_2 \cdot 2H_2O$, 0.1; and NaCl, 0.1. The culture was then homogenised in a Waring blender and inoculated into autoclaved adlay or rice supplemented with 1% glucose and yeast extract at rates of 8 and 12%, respectively.

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New corresponding products, PFA and PFR, were then produced after the colonisation of fungal mycelia for 22 and 14 days, respectively, at 30 °C. Two *Phellinus*-fermented products (PFA and PFR) as well as two uninoculated products (PA and PR) that were also autoclaved and used as controls were air-dried in an oven at 50 °C. For each product, three dried samples (~50 g of each) were randomly selected and ground using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany) to obtain a coarse powder (40 mesh). For the hot water extract, a subsample (10 g) was extracted by boiling with 100 ml of deioinised water at 100 °C for 10 min and filtering. For the ethanolic extract, a subsample (10 g) was extracted by shaking with 100 ml of 95% ethanol at 25 °C at 100*g* for 24 h and filtering through Whatman No. 1 filter paper. Both residues were then extracted with two additional 100 ml portions of deioinised water and ethanol, respectively, as described above. The combined hot water extract was freeze–dried and the combined ethanolic extract was rotary evaporated at 40 °C to dryness. Both dried extracts obtained were used directly for analyses of antioxidant components or redissolved in deionised water or ethanol, respectively to a concentration of 50 mg/ml and were then diluted to 0.01, 0.1, 1, 5, 10 and 20 mg/ml for further uses.

2.2. Antioxidant activity

Antioxidant activity was determined by the conjugated diene method (Lingnert, Vallentin, & Eriksson, 1979). The antioxidant activity assayed is the ability of the extracts, from solid-state fermented products, to inhibit the peroxidation of linoleic acid in which the double bond is converted into conjugated diene. Each extract (0.01–20 mg/ml) in deionized water or ethanol (100 ml) was mixed with 2 ml of 10 mM linoleic acid emulsion in 0.2 M sodium phosphate buffer (pH 6.6) in test tubes and placed in the dark at 37 °C to accelerate oxidation. After incubation for 15 h, 6 ml of 60% methanol in deionized water were added, and the absorbance of the mixture was measured at 234 nm against a blank in a Hitachi U-2001 spectrophotometer. The antioxidant activity was calculated as follows: $\text{Antioxidant activity}(\%) = [(\Delta A234 \text{ of control} - \Delta A234 \text{ of sample})/\Delta A234 \text{ of control}] \times 100$. A value of 100% indicates the strongest inhibitory ability. EC50 value (mg extract/ml) is the effective concentration at which the antioxidant activity was 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid, butylated hydroxyanisole (BHA), and α-tocopherol were used for comparison.

2.3. Reducing power

Reducing power was determined according to the method of Oyaizu (1986). The reducing power assayed is the ability of the extracts to form a coloured complex with ferricyanide which is an electron acceptor. Each extract (0.5–20 mg/ml) in deionized water or ethanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid were added, the mixture was centrifuged at 200*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. EC50 value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA, and α-tocopherol were used for comparison.

2.4. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). The scavenging ability assayed is the ability of the extracts to react rapidly with DPPH radicals and reduce most of them. Each extract (0.5–20 mg/ml) in deionized water or ethanol (4 ml) was mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.2 mM DPPH·. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. The scavenging ability was calculated as follows: $\text{Scavenging ability}(\%) = [(\Delta A517 \text{ of control} - \Delta A517 \text{ of sample})/\Delta A517 \text{ of control}] \times 100$. EC50 value (mg extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.5. Chelating ability against ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Ferrous ions play an important role as catalysts in the oxidative process, leading to the formation of hydroxyl radicals and hydroperoxide decomposition by the Fenton reaction. The chelating ability assayed is the ability of the extracts to inhibit the complex formation of ferrozine with ferrous ions. Each extract (0.01–20 mg/ml) in water or ethanol (1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicates a higher chelating power. EC50 value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50% and was obtained by interpolation from linear regression analysis. Citric acid and ethylenediaminetetraacetic acid (EDTA) were used for comparison.

2.6. Determination of antioxidant components

Ascorbic acid was determined according to the method of Klein and Perry (1982). β-Carotene and tocopherols were extracted and analysed as described by Rundhaug, Pung, Read, and Bertram (1988) and Carpenter (1979), respectively. Total phenols and flavonoids were determined according to the method of Taga, Miller, and Pratt (1984) and Meda, Lamien, Romito, Millogo, and Nacoulma (2005), respectively. Content of antioxidant components

Table 1
Extraction yield of hot water and ethanolic extracts from *Phellinus*-fermented adlay, polished adlay, *Phellinus*-fermented rice and polished rice.

	Extraction (% dry weight)[a]			
	PFA[b]	PA[b]	PFR[b]	PR[b]
Ethanol	26.61 ± 0.71A	1.01 ± 0.03C	6.39 ± 0.29B	0.56 ± 0.03C
Hot water	31.38 ± 0.46B	40.44 ± 0.32A	27.77 ± 0.55C	20.08 ± 1.12D

[a] Each value is expressed as mean ± SE ($n = 3$). Means with different letters within a row are significantly different ($P < 0.05$).
[b] PFA, *Phellinus*-fermented adlay; PA, polished adlay; PFR, *Phellinus*-fermented rice; PR, polished rice.

was calculated on the basis of the calibration curve of the corresponding authentic compounds and gallic acid and quercetin were used for total phenols and flavonoids, respectively.

2.7. Statistical analysis

For each of the extracts from PFA, PA, PFR and PR, three samples were prepared for assays of every antioxidant attribute and component. The experimental data were subjected to an analysis of variance for a completely random design, to determine the least significant difference at the level of 0.05. Linear regression analysis was completed to obtain a correlation coefficient (r) between EC_{50} value of each antioxidant attribute and content of total phenols and flavonoids.

3. Results and discussion

3.1. Extraction yields

The extracts were obtained from air-dried samples using hot water or ethanol and the extraction yields were expressed as the percentage of samples on the basis of dry weight. The yields of hot water extracts from four products (20.08–40.44%) were higher than those of ethanolic extracts (0.56–26.61%) (Table 1). The yields of hot water extracts from the four products might be due to the fact that the four products contained more water-soluble substances. Regardless of extractants, the extracted yields from *Phellinus*-fermented grains were much higher than those from grains, except for the hot water extract from PFA. The higher yields from PFA and PFR were the consequence of mycelial growth. During the metabolic process of fermentation on the grains, mycelia excreted enzymes, such as cellulose and amylase, leading to more extractable solids (Mau & Li, 2002).

3.2. EC_{50} values in antioxidant properties

The antioxidant properties assayed herein are summarised in Table 2 and the EC_{50} values (mg various extracts per ml) were calculated for comparison. Effectiveness of antioxidant properties correlated inversely with their EC_{50} values. With regard to the EC_{50} values in antioxidant activities by the conjugated diene method, the effectiveness of ethanolic extracts was in a descending order: PR ~ PFA > PA > PFR, whereas that of hot water extracts was in a descending order: PFR > PR > PA > PFA. It is noteworthy that EC_{50} values of ethanolic extracts from PFA and PR and the hot water extract from PFR were less than 1 mg/ml. However, EC_{50} values of BHA and α-tocopherol were both <0.05 mg/ml, whereas that of ascorbic acid was 8.86 ± 0.20 mg/ml.

The effectiveness of ethanolic extracts in their reducing powers was in the descending order: PFA ~ PFR > PR > PA whereas that of hot water extracts was in a descending order: PFA > PFR > PR > PA. For both extracts, PFA and PFR were more effective than were PA and PR. It is obvious that the reducing power of fermented products was greatly enhanced as mycelia grew. However, the EC_{50} value of BHA was <0.05 mg/ml whereas those of ascorbic acid and α-tocopherol were 0.15 ± 0.02 and 0.10 ± 0.01 mg/ml, respectively.

With regard to the scavenging ability on DPPH radicals, ethanolic extracts were more effective than were hot water extracts as evidenced by lower EC_{50} values (2.10–4.49 mg/ml versus 9.28–25.5 mg/ml). In addition, the fermented products (PFA and PFR) were more effective than the uninoculated controls (PA and PR). However, EC_{50} values of BHA and α-tocopherol were both <0.05 mg/ml, whereas that of ascorbic acid was 18.9 ± 1.17 mg/ml.

With regard to chelating ability on ferrous ions, hot water extracts were more effective than were ethanolic extracts, whereas fermented products (PFA and PFR) were more effective than were uninoculated controls (PA and PR). In addition, both extracts from PR showed no effect on ferrous ion-chelating ability. However, the fermented products (PFA and PFR) were more effective than were the uninoculated controls (PA and PR). However, the EC_{50} value of EDTA was <0.05 mg/ml, whereas citric acid had no effect.

Overall, PFA and PFR were much more effective in the antioxidant properties assayed than were PA and PR. It seems that the increased effectiveness of antioxidant properties in the *Phellinus*-fermented products was the result of mycelial growth. More extractable solids produced by metabolic enzymes during the fermentation of mycelia on the grains might be responsible for the more effective antioxidant properties. For fermented products (PFA and PFR), most EC_{50} values were less than 15 mg/ml, except for ethanolic extracts from PFA and PFR, in chelating ability on ferrous ions, and the hot water extract from PFR, in scavenging ability on DPPH radicals, indicating that both extracts of PFA and PFR were effective in these antioxidant properties.

Among the antioxidant properties assayed, ethanolic extracts were more effective in antioxidant activity and scavenging ability on DPPH radicals except for that from PFR in antioxidant activity, whereas hot water extracts were more effective in reducing power and chelating ability on ferrous ions, except for those from PFR and PR in reducing power. For ethanolic extracts, PFA was more effective in antioxidant activity, reducing power and chelating ability on ferrous ions, whereas PFR was more effective in reducing power, scavenging ability on DPPH radicals and chelating ability on fer-

Table 2
EC_{50} values of ethanolic and hot water extracts from *Phellinus*-fermented adlay, polished adlay, *Phellinus*-fermented rice and polished rice in antioxidant properties.

		EC_{50} value[a] (mg extract/ml)			
		PFA[b]	PA[b]	PFR[b]	PR[b]
Ethanolic	Antioxidant activity	0.84 ± 0.01 bC[c]	2.24 ± 0.02 bB	6.84 ± 0.22 aA	0.71 ± 0.02 bC
	Reducing power	9.77 ± 0.15aC	54.0 ± 3.88 aA[d]	10.5 ± 0.20 bC	21.7 ± 0.22 bB[d]
	Scavenging ability	2.88 ± 0.06 bC	3.79 ± 0.14 bB	2.10 ± 0.08 bD	4.49 ± 0.13 bA
	Chelating ability	27.7 ± 0.65 aB[d]	83.6 ± 2.99 aA[d]	25.8 ± 0.32 aB[d]	–[e]
Hot water	Antioxidant activity	11.3 ± 0.01 aA	8.89 ± 0.02 aB	0.39 ± 0.01 bD	6.75 ± 0.14 aC
	Reducing power	3.22 ± 0.01 bD	47.2 ± 1.18 bA[d]	14.1 ± 0.19 aC	31.3 ± 0.02 aB[d]
	Scavenging ability	9.28 ± 0.05 aC	23.5 ± 0.48 aAB[d]	22.4 ± 1.96 aB[d]	25.5 ± 0.29 aA[d]
	Chelating ability	4.78 ± 0.01 bC	8.70 ± 0.01 bB	11.7 ± 0.18 bA	–

[a] EC_{50} value: the effective concentration at which lipid oxidation was inhibited by 50%; the absorbance was 0.5 for reducing power; 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.
[b] PFA, *Phellinus*-fermented adlay; PA, polished adlay; PFR, *Phellinus*-fermented rice; PR, polished rice.
[c] Each value is expressed as mean ± SD (n = 3). Means with different capital letters within a row are significantly different (P < 0.05). Means with different small letters within a column at a specific antioxidant attribute are significantly different (P < 0.05).
[d] EC_{50} value was obtained by extrapolation from linear regression analysis.
[e] No effect.

Table 3
Contents of ascorbic acid, β-carotene, tocopherols and total phenols of ethanolic and hot water extracts from *Phellinus*-fermented adlay, polished adlay, *Phellinus*-fermented rice and polished rice.

	Content[a] (mg/g)			
	PFA[a]	PA[b]	PFR[b]	PR[b]
Ethanolic				
Ascorbic acid	0.51 ± 0.01 bA	0.40 ± 0.02 aB	0.19 ± 0.01 aC	nd[c]
β-Carotene	0.03 ± < 0.01 A	nd	0.02 ± < 0.01 A	nd
α-Tocopherol	0.08 ± < 0.01A	nd	nd	0.09 ± < 0.01 A
γ-Tocopherol	nd	0.01 ± < 0.01 A	nd	0.01 ± < 0.01 A
Total phenols	2.67 ± 0.03 bC	5.88 ± 0.03 aA	4.54 ± 0.02 aB	2.34 ± 0.03 aD
Flavonoid	0.59 ± 0.01 aB	0.49 ± < 0.01 aC	1.26 ± 0.01 aA	0.09 ± 0.01 aD
Hot water				
Ascorbic acid	0.66 ± < 0.01 aA	0.09 ± < 0.01 bC	0.12 ± 0.01 bB	0.06 ± < 0.01 D
Total phenols	9.10 ± 0.22 aA	2.23 ± 0.02 bC	4.38 ± 0.03 bB	1.31 ± 0.01 bD
Flavonoid	0.52 ± < 0.01 bA	0.15 ± < 0.01 bC	0.20 ± < 0.01 bB	0.07 ± < 0.01 aD

[a] Each value is expressed as mean SD ($n = 3$). Means with different capital letters within a row are significantly different ($P < 0.05$). Means with different small letters within a column at a specific antioxidant component are significantly different ($P < 0.05$).
[b] Not detected.

rous ions. For hot water extracts, PFA was more effective in reducing power, scavenging ability on DPPH radicals and chelating ability on ferrous ions, whereas PFR was more effective in antioxidant activity and reducing power. Overall, both extracts from fermented products (PFA and PFR) were more effective, in most antioxidant properties, than were those from uninoculated controls (PA and PR).

Although BHA, ascorbic acid and α-tocopherol were more effective in antioxidant activity, reducing power and scavenging ability on DPPH radicals, and EDTA was a good chelator for ferrous ions, they are additives and are used or present at milligram levels in foods. However, both extracts from PFA and PFR could be used at gramme levels as food or as a food ingredient. Therefore, these two extracts from PFA and PFR might serve as possible protective agents in human diets to help human reduce oxidative damage.

3.3. Antioxidant components

Naturally occurring antioxidant components, including ascorbic acid, tocopherols, total phenols and flavonoids, were found in both extracts from PFA and PFR (Table 3). For four products, β-carotene and tocopherols were not detected in hot water extracts and were at 0–0.09 mg/g in ethanolic extracts. In addition, ascorbic acid was detected in a small amount (0–0.66 mg/g) in ethanolic and hot water extracts from four products. Total phenols were the major naturally occurring antioxidant components found in both extracts (1.31–9.10 mg/g). Flavonoid contents were in the range 0.07–1.26 mg/g. Contents of flavonoids were higher in PFA and PFR than in PA and PR for both extracts. However, high amounts of total phenols and flavonoids in PFA and PFR might explain their increased effectiveness in antioxidant properties. The higher total phenol and flavonoid contents in the fermented products might be due to metabolic activities during the mycelial growth, which degraded large molecules into small phenolic compounds and synthesised these compounds.

Due to wide variations found, a correlation between total phenols or flavonoids and antioxidant activity was not established for the two extracts from the four products. For hot water extracts, total phenols and flavonoids correlated well with reducing power, with correlation coefficients of 0.845 and 0.764, respectively. Flavonoid contents of ethanolic and hot water extracts were extremely associated with scavenging ability on DPPH radicals ($r = 0.952$ and 0.995, respectively) whereas total phenol content of the hot water extracts was also associated with scavenging ability ($r = 0.974$). Similarly, correlations were established between flavonoids and chelating ability on ferrous ions for ethanolic and hot water extracts ($r = 0.797$ and 0.573, respectively), whereas some correlation was established between total phenols and chelating ability for the hot water extracts ($r = 0.578$). It seems that total phenols and flavonoids of two extracts were associated with antioxidant properties.

Phenols, such as BHT and gallate, are known to be effective antioxidants (Madhavi, Singhal, & Kulkarni, 1996). Due to their scavenging abilities on free radicals and chelating abilities on ferrous ions, phenols might possess good antioxidant, antimutagenic and anticancer properties (Ahmad & Mukhtar, 1999). Tsai, Tsai, and Mau (2007) found that contents of total antioxidant components were moderately to highly associated ($r = 0.636–0.907$) with antioxidant properties. In this research, both total phenol and flavonoid contents of the hot water extracts were responsible for their effective reducing power, scavenging ability and chelating ability. In addition, the flavonoid content of the ethanolic extracts might play an important role in the effective scavenging ability and chelating ability. Overall, total phenols and flavonoids in extracts from PFA and PFR were responsible for their effective antioxidant properties. Since *Phellinus*-fermented products possessed effective antioxidant properties, PFA and PFR might be potential antioxidants for use in food products and could be developed as a new dietary supplement and functional foods.

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