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Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus of Hymenochaetaceae*) extracts

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Abstract

This study was carried out to expand the utilization (for the pharmaceutical and food industries) of extracts of *Phellinus baumii*. Fractions rich in phenolics were extracted from *P. baumii* using various solvents. Antioxidant and free radical scavenging activity were studied using a β-carotene-linoleate model system and reactive oxygen species generation system. Total phenolic compounds in methanol and hot water extracts were 33.3 and 20.7 mg/100 ml, respectively; these values are higher than other solvent extracts. Therefore, methanol and hot water extracts exhibited good inhibition rates, of about 80–90%, by hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity, effect of reducing power on metallic compound formation and antioxidant activity.

Keywords: Antioxidants; Free radicals; *Phellinus baumii*; Phenolics; Scavenging effect

1. Introduction

Reactive oxygen species play a critical role in many diseases, such as cancer (Muramatsu et al., 1995), atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989), gastric ulcer (Das, Bandyopadhyay, Bhattacharjee, & Banerjee, 1997), and other conditions (Babizhayev & Costa, 1994; Oliver, Ahn, Moerman, Goldstein, & Stadtmaan, 1987; Smith et al., 1996). The intake of antioxidants such as polyphenols has been effective in the prevention of these diseases (Cao, Sofic, & Prior, 1997; Cohly, Taylor, Angel, & Salhudeen, 1998; Lotito & Fraga, 1998; Vinson, Dabbagh, Serry, & Jang, 1995). Polyphenols belong to a heterogeneous class of compounds with a great variety of effects. Recently, physiological functions of polyphenols have received much attention (Frankel, 1995). Many natural antioxidants have already been isolated from different kinds of plant materials, such as oilseeds, cereal crops, vegetables, leaves, roots, spices, and herbs (Ramarathnam, Osawa, Ochi, & Kawakishi, 1995). Among natural antioxidants, phenolic compounds are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical (Wanasundara & Shahidi, 1996: Yuting, Rongliang, Zhongjian, & Young, 1990). Also, phenolic compounds of plant materials have been shown to neutralize free radicals in various model systems (Ruch, Cheng, & Klaunig, 1989; Silva, Darman, Fernand, & Mitiavila, 1991; Zhang et al., 1996). Polyphenols, including vitamins, pigments and flavonoids, possess antimutagenic properties as well as blood glucose decreasing activity (Thompson, Yoon, Jenkins, & Wolever, 1984). The antioxidant effect of polyphenols has been demonstrated in many systems from in vitro studies (human low density lipoprotein, liposomes) (Leanderson, Feresjo & Tagesson, 1997; Teissedre, Frankel, Waterhouse, Peleg & German, 1996; Wiseman, Balentine & Frei, 1997) to investigations in normal human subjects, although their effects remain controversial (Goldbohm, Hertog, Brants, van Poppel, & van den Brandt, 1996; Graham, 1992; Hayatsu et al., 1992).

Phellinus baumi is a mushroom used as folk medicine for a variety of human diseases, such as diabetes, cancer and toxification in Korea. However, the beneficial effects of this *P. baumii* extract have not been investigated and are largely overlooked at the biological and biochemical levels. The aim of the present study was to evaluate the antioxidant activity, reducing power and free radical scavenging activity of the extracts of

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P. baumii and to find out which properties contribute to this effect.

2. Material and methods

2.1. Materials

2,2-Diphenyl-picrylhydrazyl (DPPH), linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween40), sodium potassium tartarate, 2-thiobarbituric acid (TBA), 2-deoxyribose, ethylenediaminetetraacetic acid (EDTA), potassium phosphate, potassium ferricyanide and L-ascorbic acid were all purchased from Sigma Chemical Co. (St Louis, MO). H_2O_2 (30%, v/v) was obtained from Aldrich Chemical. Co (Milwaukee, WI). All other solvents/chemicals used were of analytical grade and purchased from Sigma Aldrich.

2.2. Extraction and fractionation of extracts

P. baumii was obtained from local mushroom-cultivating industries. Dried *P. baumii* was powdered and extracted with methanol (75%, v/v) for 12 h, three times, at 70 °C and the methanol extract was concentrated in a vacuum evaporator (EYELA, Japan). The extracts obtained were fractionated in a separatory funnel with 1000 ml of extractants, such as *n*-hexane, chloroform, ethyl acetate and butanol. The fractions were concentrated in a vacuum evaporator. Also 30 g of the sample were extracted with 1000 ml of distilled water in a boiling water bath. The extract in water was concentrated in a vacuum evaporator. The extract was lyophilized and stored at −20 °C.

Total phenol contents of each fraction were determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965), reading samples on a UV/Vis spectrophotometer at 765 nm. Each fraction was mixed with 0.5 ml of 2% Na_2CO_3 for 3 min at room temperature and treated with 1 ml of Folin & Ciocalteu's phenol reagent and diluted by a factor 10 with distilled water. The total phenol content of each fraction was estimated by comparison with a standard curve generated from analysis of caffeic acid.

2.3. DPPH radical scavenging

The DPPH test was carried out as described by Cuendet et al. (1997) with some modification; 5.5 ml of various dilutions of the extracts or BHT/ascorbic acid were mixed with 1 ml of a 0.001% ethanolic solution of DPPH. The samples were incubated for 15 min in the dark at 30 °C and the decrease in the absorbance at 517 nm was measured using a (CECIL, CE2021, England) spectrophotometer. Ethanol was used to zero the spectrophotometer. The DPPH solution was freshly prepared daily, stored in a flask, covered and kept in the dark at 4 °C between the measurements. All determinations were performed in triplicate.

2.4. Non-site-specific hydroxyl radical scavenging activity

Non-site-specific $OH^{\bullet}$ radical acavenging activity of extracts was measured according to the method of Halliwell, Gutteridge, and Aruoma (1987). One millilitre of the final reaction solution consisted of aliquots (500 μl) of various concentrations of the extract, 1 mM $FeCl_3$, 1mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37 °C, and further heated in a boiling water-bath for 15 min after addition of 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/w) 2-thiobarbituric acid. The colour development was measured of 532 nm against a blank containing phosphate buffer.

2.5. Site-specific hydroxyl radical scavenging activity

This procedure is similar to that used to measure the non-site-specific hydroxyl radical scavenging activity. For the EDTA assay, the only exception made was the replacement of EDTA with a similar volume of buffer (Aruoma, Grootveld, & Halliwell, 1987).

2.6. Hydrogen peroxide-scavenging activity

The extract was dissolved in 3, 4 ml of 0.1 M phosphate buffer (pH7.4) and mixed with 600 μl of a 43 mM solution of hydrogen peroxide. The absorbance value (at 230 nm) of the reaction mixture was recorded from 0 to 40 min and then at every 10 min. For each concentration, a separate blank sample was used for background subtraction (Ruch, Chung, & Klaunig, 1984).

2.7. Reducing power activity

The reducing power of extracts was determined by the method of Yen and Duh (1993). Different concentrations of extracts were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50 °C. After incubation, 2.5 ml of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation at 650 × *g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride and the absorbance of the resultant solution was measured at 700 nm.

2.8. Antioxidant assay using a β-carotene linoleate model system

The antioxidant activity of extracts was evaluated by the β-carotene linoleate model system (Miller, 1971). A

solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two mililitres of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations of the extract. BHA was used for comparative purposes. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470nm using a spectrophotometer (CE2021, CECIL, England). The tubes were placed at 50 °C in a water bath. Measurement of absorbance was continued until the colour of β-carotene disappeared; a blank, devoid of β-carotene, was prepared for background subtraction. Antioxidant activity (AA) was calculated using the following equation;

$$\mathrm{AA} = \left(\frac{\beta\text{-carotene content after 2 h of assay}}{\text{initial } \beta\text{-carotene content}}\right) \times 100$$

3. Results and discussion

The yields obtained by using various extractants and their contents of total phenols are shown in Table 1. Of the various solvents used for the extraction of total phenols, extraction with MeOH and hot water gave high yields of the extracts. Chloroform extracts contained significantly less phenols than methanol and hot water extracts.

In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. Methanol and hot water extracts showed radical scavenging activity with 85% of 500 μg (Fig. 1).

The antioxidant activity of extracts using a water-soluble deoxyribose on site-specific and non-site-specific assay is presented in Figs. 2 and 3. A concentration-dependent inhibition against hydroxyl radical-induced

Table 1
Total phenols contents of *Phellinus baumii* extracts

Solvents used for extraction	Total phenols (μg/ml of caffeic acid equivalents)
Methanol	338±8
Chloroform	2±11
Ethyl acetate	32±10
Butanol	28±15
Water	42±3
Hot water	307±18

Data are presented as means±SE (n=5–6).

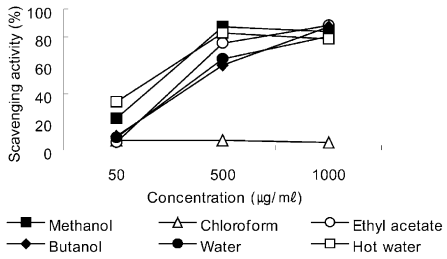

Fig. 1. DPPH scavenging activity of *Phellinus baumi* extracts. Data are presented as means±SE (n=6–8).

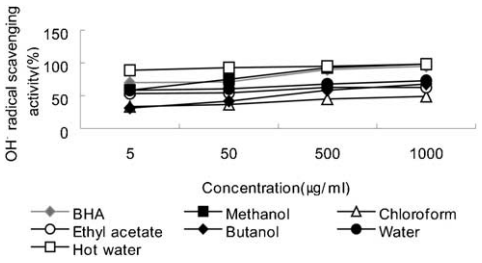

Fig. 2. The scavenging activity of hydroxyl radical in *Phellinus baumi* extracts. Data are presented as mean > ±SE (n=9–10).

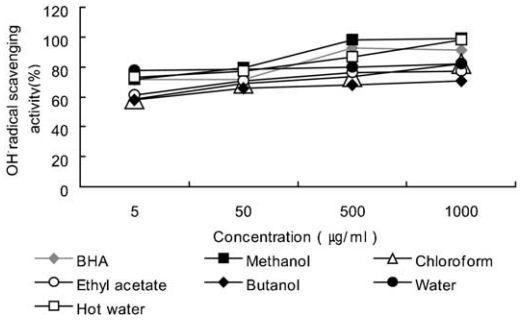

Fig. 3. The scavenging activity of hydroxyl radical in *Phellinus baumi* extracts (-EDTA). Data are presented as means±SE (n=7–12).

deoxyribose degradation was observed in both the site-specific and non-site-specific assays. Extracts of methanol and hot water were the most effective inhibitors of free radical in both assays.

In the site-specific reactions, extract of hot water, at concentrations from 5 to 1000 μg, inhibited the production of hydroxyl radicals by 88.8 – 98%. Thiourea reference compound and extracts of methanol at 500 μg showed complete inhibition of the production of hydroxyl radicals. In the non-site-specific reactions, extract of methanol at concentration 500 μg inhibited the production of hydroxyl radicals by 98%. Furthermore, extracts of methanol and hot water were high in their total phenol contents, phenols act as antioxidants and scavenge the hydroxyl radical generated from the Fenton reagent. However, even if the concentration of extracts was changed, the inhibition curve was not largely shifted, i.e. extracts inhibited the hydroxyl radical generating system, rather than scavenging hydroxyl

radical. Therefore, hydroxyl radicals scavenging activity was not due to direct scavenging but inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{+}.

Phenolic compounds tested in this study were able to form complexes with Fe^{3+} according to the EDTA assay of deoxyribose degradation. This general chelating ability of phenolics is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule (Aust & Koppenol, 1991; Gelvan & Saltman, 1991; Becana & Klucas, 1992; Gutteridge, Rowley, &n Halliwell, 1981). And also, in the absence of EDTA, damage to deoxyribose was diminished, due to chelation of Fe^{2+} by phenolics.

Hydrogen peroxide scavenging activity of the extract is presented in Fig. 4; the extract exerted a concentration-dependent scavenging. Scavenging activities of methanol extract (10 μg) and tocopherol (10 μg), reference compounds, were 80 and 83% of the initial concentrations, respectively. The concentration of hydrogen peroxide in water may vary according to the phenolic compounds.

Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent $OH^{\bullet}$ mediated oxidative DNA damage (Spencer et al., 1996). Levels of H_2O_2 at or below about 20–50 mg seem to have limited cytotoxicity to many cell types (Abe & Berk, 1999; Dalton, Shertzer, & Puga, 1999; Schreck, Ricber, & Puga, 1991 Wang, Martindale, Liu, & Holbrook, 1998). Since phenolic compounds present in the extract are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O (Ruch et al., 1984).

Table 2 shows the reducing power of the extracts using the potassium ferricyanide reduction method. At 1 mg/ml concentration, the extracts obtained using methanol, ethyl acetate, butanol, water and chloroform showed absorbances of 1.94, 3.00, 2.86, 1.99 and −3.00, respectively. Thus, all extracts exhibited high reducing activity, except for chloroform. The reducing power might be due to hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992), and is generally associated with the presence of reductones (Pin-Der Duh, 1998).

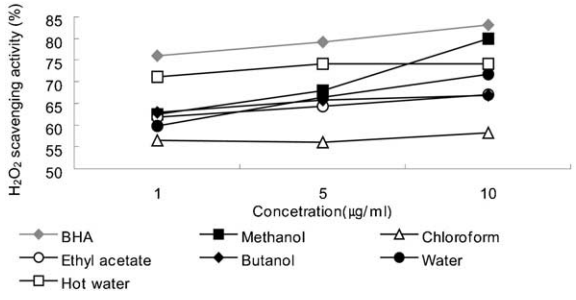

Fig. 4. The scavenging activity of hydrogen peroxide in *Phellinus baumi* extracts, Data are presented as means±SE (n=4–5).

Table 2
Reducing power of *Phellinus baumi* extracts[a]

Extracts	5 μg/ml	50 μg/ml	500 μg/ml	1000 μg/ml
Methanol	−0.019±0.00[b]	0.239±0.01	0.706±0.05	1.94±0.03
Chloroform	−0.185±0.00	−3±0.00	−3±0.00	−3±0.00
Ethylacetate	0.038±0.20	0.589±0.02	2.74±0.21	3±0.27
Butanol	0.004±0.00	0.312±0.01	2.375±0.22	2.86±0.21
Water	0.009±0.00	0.497±0.03	1.875±0.04	2.53±0.20
Hot water	0.001±0.00	0.128±0.00	1.53±0.03	1.99±0.07
BHA[c]	0.038±0.31	0.599±0.04	2.91±0.23	3.2±0.30

[a] Data are presented as mean±SE (n=7–8).
[b] Absorbance at 700 nm.
[c] Reference compound.

Table 3
Antioxidant activity (%) of *Phellinus baumi* extracts in the β-carotene-linoleate system[a]

Extracts	50 μg	100 μg	200 μg
MeOH	80.8±1.21	90.8±1.30	100±1.04
Chloroform	15.4±1.32	13.6±1.42	5±1.33
EtOAc	61.9±1.41	64.3±1.51	66.8±1.53
Butanol	62.9±1.04	65.7±1.01	53.8±1.53
Water	60.3±1.51	69.2±1.76	88.5±1.55
Hot water	53.8±1.21	83.3±1.33	91.0±1.45
BHA[b]	64.1±1.72	89.4±1.51	95.2±1.82

[a] Data are presented as means±SE (n=6–7).
[b] Reference compound.

Table 3 shows the antioxidant activity of the extracts as measured by the bleaching of β-carotene. Extracts in methanol and hot water, as well as BHA itself, were found to give the maximum antioxidant activity of 100, 91 and 95%, respectively. The antioxidant activity of carotenoids is based on the radical adducts of carotenoid with free radicals from linoleic acid. The linoleic acid free radical attacks the highly unsaturated β-carotene models. The presence of carotenoid shows, not only a decrease of the free radical concentration, but the reduction of Fe^{3+} to Fe^{2+} by carotenoids. The presence of different antioxidants can hinder the extent of β-carotene- bleaching by neutralising the linoleate-free radical and other free radicals formed in the system (Jayaprakasha, Singh, & Sakariah, 2001).

4. Conclusion

The results of the present work indicate that the extracts possess high antioxidant activity and free radical scavenging activity. These assays are useful for establishing the ability of phenolics to chelate and reduce Fe^{3+} and have important applications for the pharmaceutical and food industries. However, further investigation of individual phenolic compounds, their in vivo antioxidant activity and the different antioxidant mechanisms is warranted.

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