

Evaluation of Antioxidant Activity of Selected Indian Mushrooms

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Abstract

Cellular damage caused by reactive oxygen species (ROS) has been implicated in several diseases, and hence antioxidants have significant importance in human health. In this study, five methods were used to test and compare the antioxidant activity at different levels involving formation and scavenging of free radicals by the extracts of four mushrooms that have medicinal values. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) and ascorbic acid equivalent antioxidant capacity (AEAC). In the DPPH (1,1-diphenyl 2-picryl hydrazyl) assay, the ethyl acetate extract of *Phellinus rimosus* showed more potent activity than the methanolic extracts of *Pleurotus florida*, *Pleurotus sajou-caju*, and *Ganoderma lucidum*. The ethyl acetate extract of *P. rimosus* at a concentration of 0.1% showed a high TEAC value (12.488). In the ABTS (2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid) spectrophotometric assay, it possessed the most effective antioxidant activity (TEAC 4.84) compared to methanolic extracts of *P. florida*, *P. sajou-caju*, and *G. lucidum*. The extract of *P. rimosus* also possessed higher activity compared to other extracts in the ferric-reducing antioxidant power (FRAP) assay. In the pulse radiolysis studies and the ORAC (oxygen radical absorbance capacity) assay also, these mushrooms showed significant activities. Results of the DPPH, ABTS, FRAP, and ORAC assays indicate that all the four mushrooms examined showed significant antioxidant activities. Among these, *P. rimosus* extract seems to be the more effective antioxidant.

Keywords: ABTS assay, antioxidant activity, DPPH assay, FRAP assay, medicinal mushrooms, ORAC assay, *Phellinus rimosus*, pulse radiolysis.

Introduction

Potentially harmful reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism. The reactive species is usually inactivated *in vivo* by a variety of antioxidants. Antioxidants are deployed to prevent generation of ROS or to scavenge those formed. Thus, oxidatively induced tissue damage is minimized. However, deficiency of antioxidant defenses may lead to oxidative stress, which might be associated with a variety of disorders including coronary heart disease, neural disorders, diabetes, arthritis, and cancer (Yoshikawa et al., 2000; Spiteller et al., 2001). When natural defenses are overwhelmed by excessive generation of pro-oxidants, a situation of oxidative stress evolves, and cellular macromolecules might suffer oxidative damage (Sies, 1996; Weisman & Halliwell, 1996). A number of methods have been developed to measure the efficiency of antioxidants. These methods focus on different mechanisms of the antioxidant defense system such as scavenging or inhibiting free radicals or chelation of metal ions that otherwise may lead to free-radical formation. These free radicals are capable of inducing damage to all cellular molecules, which can lead to diseased states (Yoshikawa et al., 2000; Spiteller et al., 2001). Synthetic antioxidants also have been developed to retard lipid peroxidation, but their use as food supplements and therapeutic agents have been hindered due to their possible toxicity.

A number of natural antioxidants have already been demonstrated from different kinds of plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Ramarathnam et al., 1995; Packer & Ong, 1997; Cullen et al., 1997; Kamat et al., 2000; Devasagayam et al., 2001; Nigris et al., 2003; Negi & Jayaprakasha, 2003).

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Chinese herbs have been used for diet therapy for several millennia. Some of them are alleged to exhibit significant antioxidant activity (Su et al., 1992; Kim et al., 1994; Weng & Chen, 1996; Yun, 1999). Mushrooms are traditionally used in Chinese medicine and are commonly used for pharmaceutical purposes and health foods. Increasing experimental evidence indicates that mushrooms contain a large number of biologically active components that offer health benefits and protection against degenerative diseases. A number of medicinal mushrooms have recently been reported to possess significant antioxidant activity. (Jose et al., 2002; Jones & Janardhanan, 2000; Ajith & Janardhanan, 2001; Mau et al., 2001; Ekanem & Ubengama, 2002).

Phellinus rimosus is a wood-inhabiting polypore-macrofungus often found growing on Jackfruit tree trunks in Kerala, India. The basidiocarps of this fungus have been used by some local tribes for the treatment of ailments such as mumps (Ajith & Janardhanan, 2001). Our earlier investigations showed that ethyl acetate extracts of *P. rimosus* possessed antioxidant, antitumor, anti-inflammatory, hepatoprotective, and nephroprotective activities (Ajith & Janardhanan, 2002). Oyster mushrooms (*Pleurotus* spp.) are widespread throughout the hardwood forests of the world; they are edible, nutritious, and rank second among the cultivated mushrooms of the world. They are known as efficient blood pressure lowering agents, diuretics, cholesterol reducers, adjuvants, and aphrodisiacs. They have also been found to modulate the immune system. *Ganoderma lucidum*, commonly known as Reishi, is considered a panacea in Chinese medicine because of its effectiveness in the treatment of large number of diseases. It is known to possess DNA-protective properties (Kim & Kim, 1999). Recent investigations in our laboratory have shown that fruit bodies of *G. lucidum* occurring in South India possessed significant antitumor, anti-inflammatory, and antinociceptive properties (Jones & Janardhanan, 2000; Sheena et al., 2003). Because the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of the mushrooms is of significant importance in exploiting their therapeutic potential. The proof of their antioxidant activity can also explain their mechanism of action. Hence, it was considered desirable to evaluate the antioxidant activity of *Pleurotus* spp., *G. lucidum*, and *P. rimosus* acting at different levels of free radical scavenging, by various methods using standard equivalent references.

Materials and Methods

Materials

Ascorbic acid, 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid diammonium salt (ABTS), butylated hydroxytoluene (BHT), 1,1-diphenyl 2-picryl hydrazyl (DPPH), ethylene diamine tetraacetic acid (EDTA), ferric chloride, hydrogen peroxide, methanol (HPLC grade), myoglobin, potassium ferricyanide, sodium acetate, Trolox (6-hydroxy-2,5,7,8-

trtramethyl chroman-2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ), 2-thiobarbituric acid, triphenyl phosphene (TPP), trichloroacetic acid, and xylenol orange were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used in the studies were of analytical grade.

Fruiting bodies of *Pleurotus florida* and *Pleurotus sajor-caju* were obtained from the small-scale cultivation unit of Integrated Rural Technology Centre (Palakkad, Kerala, India). *Ganoderma lucidum* and *Phellinus rimosus* were collected locally from the outskirts of Thrissur, India. The identifications of the specimens were authenticated by Prof. K.M. Leelavathy, Department of Botany, Calicut University (Calicut, India). Voucher specimens were deposited in the herbarium of the Centre of Advanced Studies in Botany, University of Madras (Chennai, India) (*G. lucidum*: HERB.MUBL-3172; *P. rimosus*: HERB.MUBL-3171).

Methods

Preparation of mushroom extracts

Fruiting bodies of *P. florida*, *P. sajor-caju*, *G. lucidum*, and *P. rimosus* were dried at 45–50°C for 48 h and were powdered. The powdered material was defatted by extraction with petroleum ether in a Soxhlet apparatus for 8–10 h. The defatted material of *P. rimosus* was then extracted with ethyl acetate and that of *P. florida*, *P. sajor-caju*, and *G. lucidum* with 70% methanol. The extracts were concentrated at low temperature under vacuum and solvents evaporated completely. The residues thus obtained were used for experiments.

For *in vitro* antioxidant activity studies, the ethyl acetate extract of *P. rimosus* was dissolved in methanol, and the extracts of *P. florida*, *P. sajor-caju*, and *G. lucidum* were dissolved in distilled water. Aqueous extracts of *P. florida*, *P. sajor-caju*, and *G. lucidum* were prepared by dissolving 0.1 g extract in 10 ml distilled water and stirring for 1 h. Methanolic extracts of *P. rimosus* were prepared by dissolving 0.01 g ethyl acetate extract in 10 ml of methanol and stirring for 30 min. All the extracts were centrifuged for 15 min, and supernatants were stored at 20°C.

Determination of antioxidant activity

Antioxidant activity of the extracts were assayed by the ferric-reducing antioxidant power (FRAP), DPPH, and ABTS methods. For the antioxidant activity assays of *P. florida*, *P. sajor-caju*, and *G. lucidum*, 0.1, 0.5, and 1% concentrations of the extracts were used, and for *P. rimosus* the concentrations used were 0.01, 0.05, and 0.1%. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) and ascorbic acid equivalent antioxidant capacity (AEAC). TEAC is the concentration of Trolox (%) required to give the same antioxidant capacity as test substance and AEAC is that of ascorbic acid (Gil et al., 2000).

Ferric-reducing antioxidant power assay

The ferric-reducing ability was measured at low pH (Benzie & Strain, 1996; Pulido et al., 2000). The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl₃·6H₂O, and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37 °C. Then, 900 µl FRAP reagent was mixed with 90 µl water and 30 µl test sample/methanol/distilled water standard antioxidant solution. The reaction mixture was then incubated at 37 °C for 30 min, and absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyl triazine (Fe³⁺ TPTZ) complex was reduced to the ferrous (Fe²⁺) form, and the absorption at 595 nm was recorded. The calibration curve was plotted with absorbance at 595 nm *versus* concentration of FeSO₄ in the range 0–1 mM (both aqueous and methanol solutions). The concentrations of FeSO₄ were in turn plotted against concentrations of standard antioxidants (L-ascorbic acid or Trolox).

DPPH radical scavenging assay

In this method, a commercially available and stable free-radical (DPPH⁺, 2,2-diphenyl-1-picrylhydrazil), which was soluble in methanol, was used (Aquino et al., 2001). DPPH in its radical form has an absorption peak at 515 nm, which disappeared on reduction by an antioxidant compound. An aliquot (37.5 µl) of the extract was added to 1.5 ml of freshly prepared DPPH solution (0.25 g/l in methanol). Absorbance was measured at 515 nm 20 min after the reaction was started. The DPPH concentration in the reaction medium was calculated from the calibration curve of %DPPH scavenged *versus* concentration of the standard antioxidant (L-ascorbic acid or Trolox).

ABTS radical scavenging assay by spectrophotometry

In this assay, the radical scavenging activities of extracts were determined by using ferrylmyoglobin/ABTS protocol (Alzoreky & Nakahara, 2001). The stock solutions of 500 µM ABTS diammonium salt, 400 µM myoglobin, 740 µM potassium ferriyocyanide, and 450 µM H₂O₂ were prepared in phosphate-buffered saline (PBS) (pH 7.4). Metmyoglobin (MbIII) was prepared by mixing equal amounts of myoglobin and potassium ferriyocyanide solutions. The reaction mixture (total volume 2 ml) contained the following substances (final concentrations in the reaction mixture): ABTS (150 µM), MbIII (2.5 µM), 16.8 µl of the sample, and 978 µl PBS. The reaction was initiated by adding 75 µM H₂O₂ (330 µl) and the lag time in seconds, before absorbance of ABTS⁺ at 734 nm began to increase was recorded. The calibration curve was plotted with lag time in seconds *versus* concentration of the standard antioxidants (L-ascorbic acid or Trolox).

ABTS radical scavenging by pulse radiolysis

Antioxidant activity of the extracts was also assayed by ABTS radical scavenging by pulse radiolysis technique using a linear accelerator. For pulse radiolysis measurements, the absorbed dose was kept to a minimum to avoid decomposition of the test compound. Typical maximum doses with 50 pulses were 15 Gy. The standard pattern of decay with ascorbic acid having four different concentrations of 2.5, 5, 7.5, and 10 µg per ml showed typical concentration-dependent curves (Scotl et al., 1993). The ascorbic acid equivalent was computed by extrapolating the results with the standard graph.

Oxygen radical absorbance capacity assay

In the oxygen radical absorbance capacity (ORAC) assay, oxygen radical absorbance capacity was measured on detection of chemical damage to β-phycoerythrin through the decrease in the fluorescence emission. The fluorescence was recorded every 5 min until the last reading was less than 5% of the 0 min reading. ORAC values were calculated in terms of µmol/g of fresh weight (Lussingoli et al., 1999).

Results**Antioxidant capacity of extract: FRAP assay**

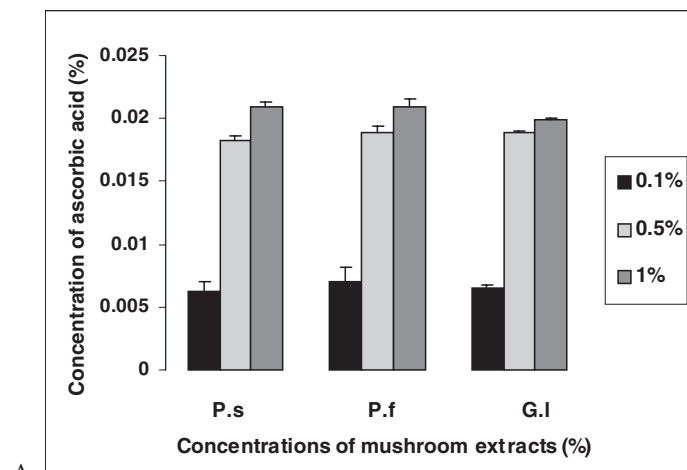
The ferric-reducing antioxidant power of the extracts was found to increase in a concentration-dependent manner. The extracts of *P. rimosus* possess the highest activity compared to other extracts. The ethyl acetate extracts of *P. rimosus* at a concentration of 0.1% showed 0.31 TEAC, whereas *P. florida*, *P. sajor-caju*, and *G. lucidum* at a concentration of 1% showed AEAC values of 0.021, 0.021, and 0.020, respectively (Fig. 1).

Radical scavenging activity of the extracts: DPPH assay

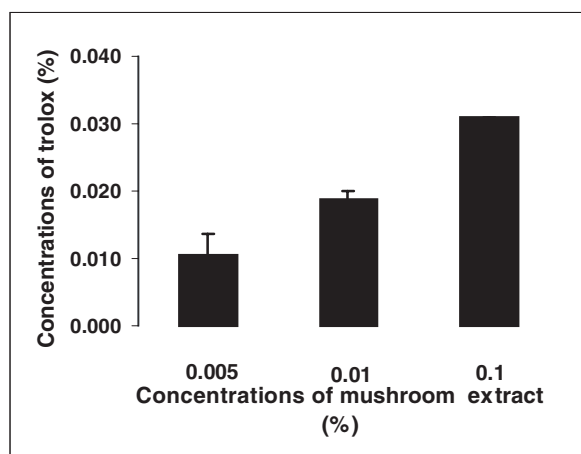
The ethyl acetate extracts of *P. rimosus* at a concentration of 0.1% showed 12.49 TEAC, whereas *P. florida*, *P. sajor-caju*, and *G. lucidum* at a concentration of 1% showed AEAC values of 0.66, 0.40, and 0.47, respectively. In the DPPH assay, the results indicate that the ethyl acetate extracts of *P. rimosus* have more potent activity than methanol extracts of *P. florida*, *P. sajor-caju*, and *G. lucidum*. *P. florida* showed higher free-radical scavenging activity than *P. sajor-caju* (Fig. 2).

Effect of the extract on radical scavenging: Ferrylmyoglobin/ABTS assay

Ethyl acetate extracts of *P. rimosus* possessed the most effective antioxidant abilities compared to methanol extracts of *P. florida*, *P. sajor-caju*, and *G. lucidum*. There was a concentration-dependent increase in radical scavenging activities (Fig. 3). *P. rimosus* had a TEAC value of 4.84. *P. sajor-caju*



A



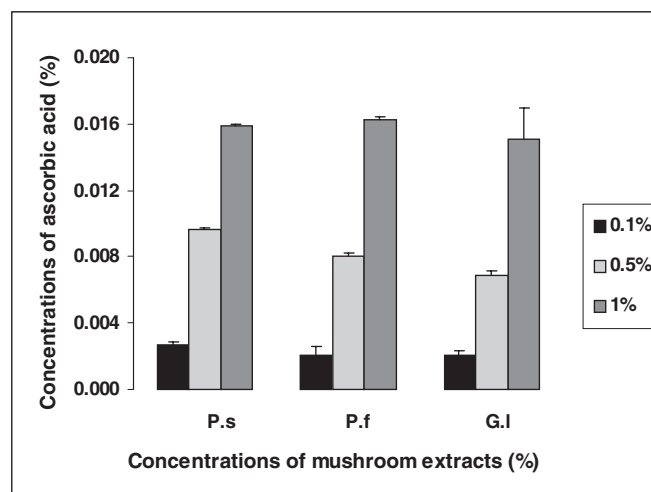
B

Figure 1. Antioxidant capacity of different mushroom extracts as estimated by the FRAP assay: (A) represents the results for aqueous extracts (*P. florida*, *P. sajor-caju*, *G. lucidum*), whereas (B) shows the results of the methanolic extracts (*P. rimosus*). The antioxidant capacities of the aqueous extracts are equivalent to indicated concentrations of ascorbic acid (%). The antioxidant activities of the methanolic extracts are equivalent to Trolox (%). Values are mean $\pm$ SE from four estimations each.

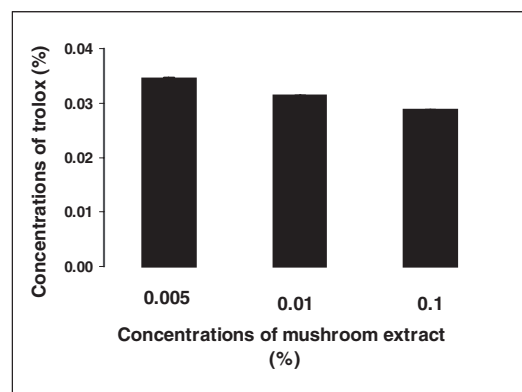
(0.12 AEAC) showed more radical scavenging activity than *P. florida* (0.11 AEAC). This method involves scavenging of secondary radicals.

Radical scavenging activity of the extract: Pulse radiolysis assay of ABTS

Pulse radiolysis study of ABTS involves scavenging of primary radicals. In this assay, extract of *P. florida* showed highest activity (4.8 μ g ascorbic acid equivalent per ml) followed by *G. lucidum* (4.2 μ g ascorbic acid equivalent per ml) and *P. sajor-caju* (3.1 μ g ascorbic acid equivalent per ml) (Figure 4). The ABTS radical scavenging activity of *P. rimosus* could not be estimated due to constraints in solubility.



A



B

Figure 2. Antioxidant capacity of different mushroom extracts as estimated by the DPPH assay: (A) represents the results for aqueous extracts (*P. florida*, *P. sajor-caju*, *G. lucidum*), whereas (B) shows the results of the methanolic extracts (*P. rimosus*). The antioxidant capacities of the aqueous extracts are equivalent to indicated concentrations of water-soluble standard antioxidant ascorbic acid (%). The antioxidant activities of the methanolic extracts are equivalent to ethanol-soluble standard antioxidant Trolox (%). Values are mean $\pm$ SE from six estimations each.

Oxygen radical absorbance capacity assay

The ORAC values for the mushroom extracts determined by spectrofluorimetric assay were Pf (45.65 $\pm$ 5.85), Ps (43.94 $\pm$ 8.66), Gl (38.94 $\pm$ 9.0), and Pr (30.59 $\pm$ 1.59) as micromoles of Trolox equivalent per gram of fresh weight. Hence, *P. florida* was the most potent against scavenging peroxy radicals in the ORAC assay.

Discussion

The results of the investigation reveal that extracts of *P. rimosus*, *P. florida*, *P. sajor-caju*, and *G. lucidum* possess a property to inhibit free-radical formation and scavenging activity. The extracts act at two different levels as primary antioxidants. Antioxidants show activities at different levels

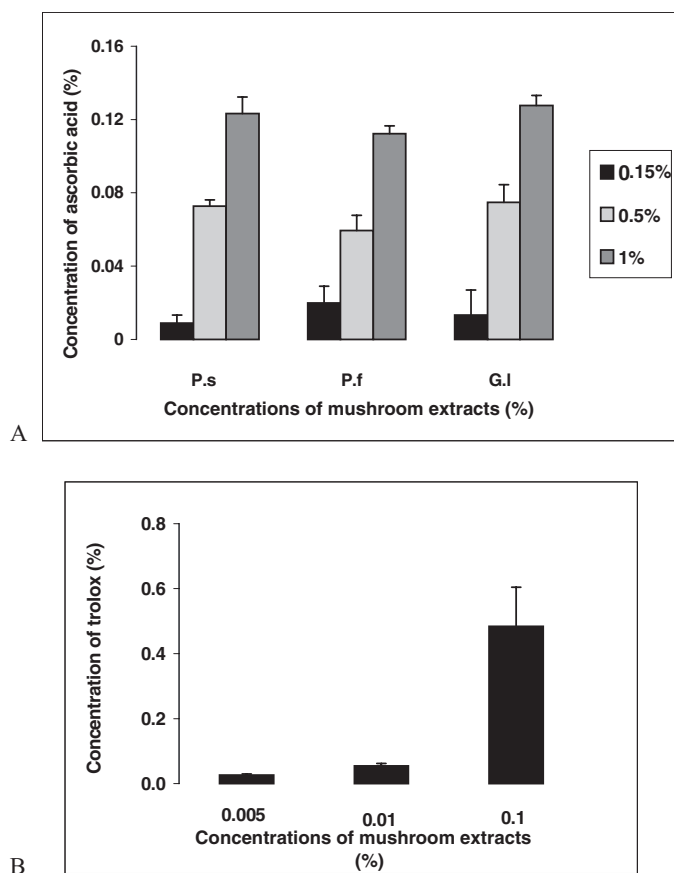


Figure 3. Antioxidant capacity of different mushroom extracts as estimated by using spectrophotometric ferrylmyoglobin/ABTS⁺ method: (A) represents the results for aqueous extracts of *P. florida*, *P. sajor-caju*, and *G. lucidum* equivalent to indicated concentrations of water-soluble standard antioxidant ascorbic acid (%). In (B) is shown the results of the methanolic extract of *P. rimosus* equivalent to ethanol-soluble standard antioxidant Trolox, (%). Values are mean $\pm$ SE from four estimations each.

of protection (Cadenas & Packer, 1996). Although organisms are bestowed with antioxidant and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage totally (Hemnani & Parihar, 1998). Hence, antioxidants in diet are important as possible protective agents to help the human body to reduce oxidative damage. Recently, a large number of natural antioxidants have been isolated from different plant materials (Packer & Ong, 1997; Jovanovic & Simic, 2000; John et al., 2002). Mushrooms are functional foods and are traditionally used in folk medicine of several systems of medicine. The presence in the human diet of would medicinal mushrooms that possess antioxidant properties would potentially be useful to help the human body to reduce oxidative damage.

In Chinese medicine, use of fruiting bodies of *G. lucidum* is considered a panacea because of its proven ability to treat a number of diseases. The broad-spectrum medicinal property of *G. lucidum* might be the result of its significant

antioxidant activity. Our earlier reports also confirm the significant antioxidant activity of this medicinal mushroom (Jones & Janardhanan, 2000). *Pleurotus* species are excellent, edible mushrooms used as delicacies. Antioxidant activity of the edible mushrooms has significant importance because this activity greatly contributes to their nutraceutical properties, thus enhancing their nutritive value.

Nonenzymatic antioxidants react with pro-oxidants and inactivate them. In this redox reaction, antioxidant acts as a reductant. In this context, the antioxidant power can be referred to as reducing ability. In the FRAP assay, an easily reducible oxidant Fe III is used in excess. Thus, on reduction of Fe III–TPTZ complex by antioxidant, Fe II–TPTZ is formed, which can be measured spectrophotometrically at 595 nm (Benzie & Strain, 1996). Our results indicate that all the extracts have hydrogen-donating capacity. The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. In ferrylmyoglobin/ABTS assay, on addition of antioxidant, formation of ABTS radical by reaction of ferrylmyoglobin and ABTS is delayed, and inhibition of formation of the radical is measured as the lag time in seconds (Alzoreky & Nakahara, 2001). All the extracts had the ability to prevent radical formation, and the ability was more in the case of *P. rimosus*. The second line of defense of antioxidants to scavenge free radicals is to suppress chain initiation and/or break the chain propagation reactions. In the DPPH assay, the ability of antioxidant to scavenge stable purple-colored primary radical DPPH is tested by its depolarization spectrophotometrically at 515 nm (Aquino et al., 2001). In the pulse radiolysis study, ABTS radicals are generated as “primary radicals” due to electron bombardment in a linear accelerator. In this system, too, the mushrooms show significant antioxidant activities. *P. florida* shows the highest scavenging of ABTS radicals in terms of ascorbic acid equivalent (4.8 μ g/ml). In the ORAC assay, inhibition of peroxy radical-induced β -phycoerythrin oxidation and the protection by the mushroom extracts is measured. The ORAC values are used as “standard” measures for comparing antioxidant activity of food materials. Our results show that the values of mushroom extract ranging from 30 to 45 are higher than the values observed for some fruits and vegetables including those for garlic (19.4), spinach (12.6), and onion (4.5) (micromoles of Trolox equivalent per gram of fresh weight).

In conclusion, our studies reveal that all the four mushrooms show high radical scavenging activities and potent reducing power using five standard assays. Our results indicate that among the samples examined, the extract of *P. rimosus* is the most effective in rendering antioxidant protection, followed by *P. florida* among the aqueous extracts.

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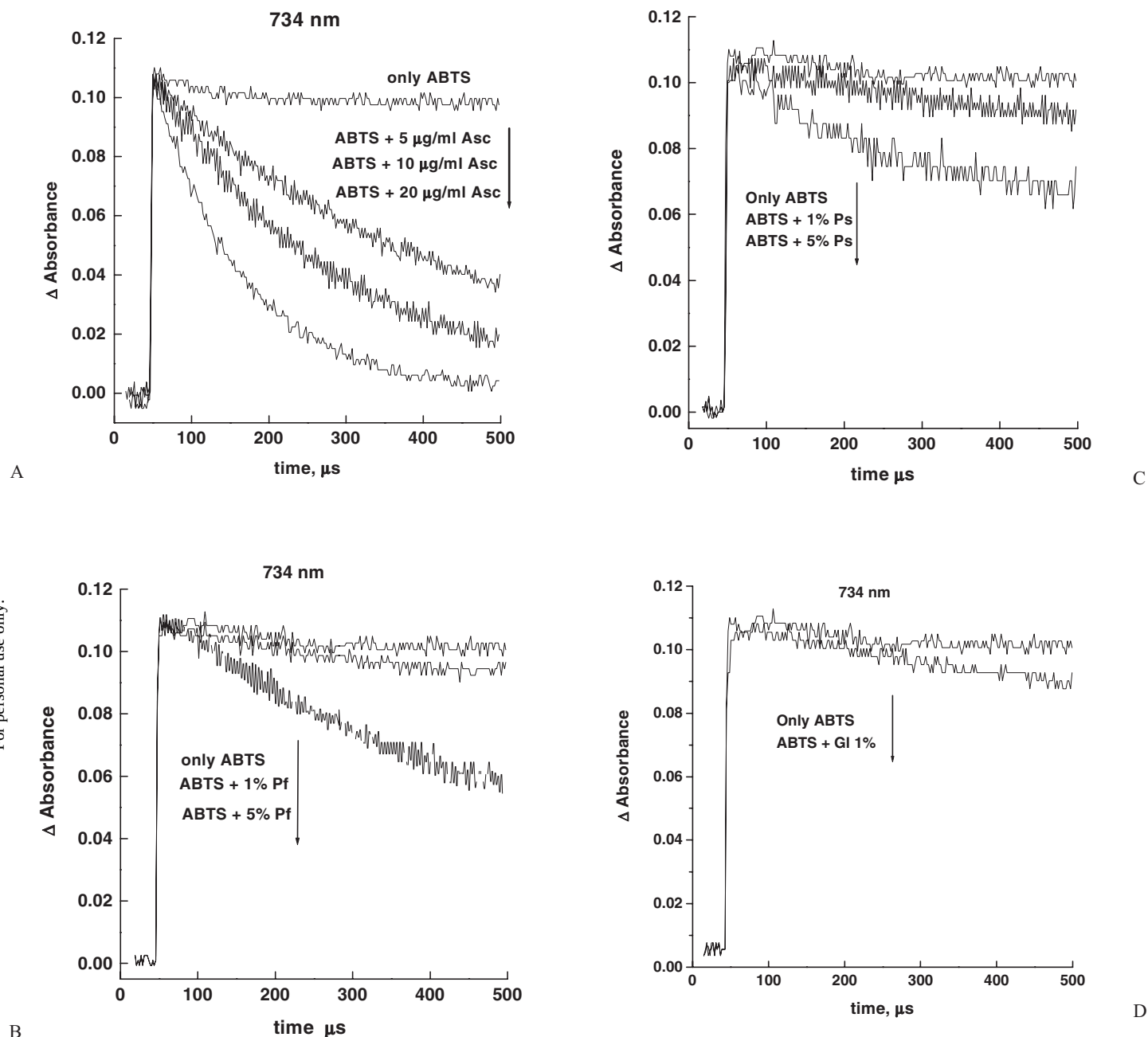


Figure 4. The antioxidant capacities of the mushroom extracts as determined by pulse radiolysis by measuring the decay of ABTS^{•-}. (A) represents the decay in absence and in presence of standard antioxidant, ascorbic acid (Asc) 5, 10, and 20. (B) shows ABTS radical scavenging patterns with aqueous extracts of *P. florida*, whereas (C) and (D) show the ABTS radical scavenging by extracts of *P. sajor-caju* and *G. lucidum*, respectively.

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