

# Hepatoprotective efficacy of medicinal mushroom *Pleurotus tuber-regium*

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## Abstract

*Pleurotus tuber-regium* (Rumph. ex Fr.) Singer is a medicinal mushroom traditionally used for curing various diseases. The aim of the present study was to perform chemical screening and analyze hepatoprotective efficacy of *P. tuber-regium* extracts in CCl<sub>4</sub> hepatotoxic rat. The highest concentration in fungal extract was found for alkaloids (28.41 ± 0.5 mg 100 g<sup>-1</sup>) and the lowest for tannins (2.74 ± 0.28 mg 100 g<sup>-1</sup>) with flavonoids and phenolics in moderate concentrations. *P. tuber-regium* extract showed high total antioxidant activity (12.7 and 21.5% at 10 and 50 to 100 µg mL<sup>-1</sup>, respectively). The activity of aspartate aminotransferase, alanine transaminase, alkaline phosphatase and concentration of bilirubin significantly increased in *P. tuber-regium* extract-treated hepatotoxic rats while concentration of total protein and serum albumin significantly decreased in comparison to control animals, averting toxic effects of CCl<sub>4</sub>. As the *P. tuber-regium* extract possessed no acute oral toxicity, it can be used as hepato protective tonic.

**Key words:** antioxidant activity, hepatoprotective efficacy, oxidative stress, *Pleurotus tuber-regium*, mushrooms.

**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; TAC, total antioxidant capacity.

## Introduction

The liver is a vital organ of the human body, maintaining homeostasis of the body by regulating various physiological functions such as carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamins (Subramoniam, Pushpangadan 1999; Pushpalatha, Ananthi 2012). The liver is also involved in most of the biochemical pathways related to growth, fight against diseases, nutrient supply, energy provision and reproduction (Sharma et al. 1991; Pandit et al. 2012).

Reactive oxygen species and reactive nitrogen species are the major initiators of oxidative stress and they appear due to various pathogenic diseases, exposure to radiation, tissue injury etc. (Atawodi 2005). Oxidative stress has been implicated in the pathogenesis of acute and chronic liver injury in a variety of pathophysiological conditions such as hepatotoxin exposure, intrahepatic cholestasis, alcoholic liver injury, liver ischemia and viral hepatitis, fibrosis, cirrhosis, steatohepatitis and biliary disease, inflammation and necrosis of liver, etc. (Stephens 2003; Parvathi et al. 2013; Adeyemi et al., 2014). Thus, maintaining a healthy liver is a crucial for overall health and human well-being.

Carbon tetrachloride (CCl<sub>4</sub>), also known as tetrachloromethane or carbon tet, is widely used in fire

extinguishers, as a precursor to refrigerants, as a cleaning agent and for other purposes. CCl<sub>4</sub> is a potent hepatotoxin that causes hepatic damage by oxidative stress (Seifert et al. 1994).

Mushrooms have been used in folk medicine and healthy food since ancient times (King 1993). Edible macrofungi or mushrooms belong to the two major taxonomic groups Ascomycota and Basidiomycota, which include 80 families, 550 genera and 10 000 species. Of these, approximately 700 species have been reported for their significant pharmacological activity (Wasser et al. 1999; Karaman et al. 2012). Medicinal mushrooms contain various bioactive secondary metabolites such as tannins, alkaloids, flavonoids, phenolics etc., which possess therapeutic efficacy and can act as antioxidants. *Pleurotus tuber-regium* (Rumph. ex Fr.) Singer, commonly called king tuber mushroom, is an edible gilled fungus belonging to family Pleurotaceae. It has been traditionally used as an antioxidant, antiinflammatory, hepatoprotective, antipathogenic, and antimutagenic agent, and as nutraceutical food supplement (Patel et al. 2012).

The aim of the present study was to evaluate chemical constituents associated with antioxidant activity and to assess hepatoprotective efficacy of *P. tuber-regium* as hepatoprotective agent using a mammalian model.

## Materials and methods

### Collection of fungi

Fresh fruiting bodies of *P. tuber-regium* were collected by the corresponding author from different sites of three National Parks (Orang National Park, Kaziranga National Park and Manas National Park) of Assam and were identified in the Department of Botany, Gauhati University, Guwahati, Assam, and brought to the Department of Zoology, Ranchi University, Ranchi to evaluate their pharmacological efficacy.

### Preparation of extract

Fresh mushrooms were washed and disinfected by treating with  $\text{HgCl}_2$  and washed again. The mushrooms were dried in shade under room temperature for six to seven days, powdered and sieved. An aliquot of the fine powder (50 g) was subjected to Soxhlet extraction using distilled water for aqueous extraction. The obtained extract was filtered, concentrated and dried in a rotary flash evaporator maintained at 45 °C for proper dehydration and the dried extract was stored in air-tight containers at room temperature for further studies (Dandapat et al. 2014).

### Chemical screening

Estimation of total phenolics was performed by phenolic-catechol method according to the procedure described by Malick and Singh (1980). Diluted aqueous extract (1:10, 0.5 mL) was pipetted in a series of test tubes and volume was made up to 3 mL with distilled water. Folin-Ciocalteu reagent (0.5 mL) was added to each tube and incubated for 3 min at room temperature and then sodium carbonate (20%, 2 mL) was added, mixed thoroughly and the tubes were incubated for 1 min in boiling water bath. Total phenolics was determined by colorimetry at 650 nm against a reagent blank. The standard curve was made using 10, 50, 100  $\mu\text{g mL}^{-1}$  solution of catechol. From the standard curve, concentration of phenolics in the test samples was determined and expressed as mg of catechol equivalent.

For estimation of total flavonoids, 10, 50 and 100  $\mu\text{g mL}^{-1}$  of samples were pipetted in a series of test tubes and volume was made up to 0.5 mL with distilled water. Sodium nitrite (5%, 0.03 mL) was added to each tube and incubated for 5 min at room temperature. Aluminium chloride solution (10%, 0.06 mL) solution was added and incubated for 5 min at room temperature. Sodium hydroxide solution (1 M, 0.2 mL) was added and total volume was made up to 1 mL with distilled water. Absorbance was measured at 510 nm against a reagent blank. A standard curve using 10, 50 and 100  $\mu\text{g mL}^{-1}$  concentrations of rutin was prepared, concentration of flavonoids in the test samples was determined and expressed as mg of rutin equivalent (Helmja et al. 2007).

For estimation of total alkaloids, 1 g of the dry extract was added to a 250 mL beaker together with 40 mL 10%

acetic acid in 60 mL ethanol. Tubes were covered and allowed to stand for 4 h. Then the solution was filtered and the extract was concentrated in a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop-wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with diluted ammonium hydroxide and then filtered. The residue represents alkaloid, which was dried and weighed (Harborne 1973).

For estimation of tannins, 50 mL of the aqueous extract evaporated to dryness in an oven at 105 °C for 4 h and the dried residue was weighed ( $T_1$ ). A fresh amount of extract (80 mL) was mixed with 6.0 g of hide powder and shaken for 60 min, and filtered, and 50.0 mL of the clear filtrate was evaporated to dryness in an oven at 105 °C. The residue was weighed ( $T_2$ ). Hide powder (6 g) was shaken with 80 mL of water, filtered, evaporated and residue was dried at 105 °C and weighed ( $T_0$ ). The quantity of tannins was calculated as a percentage using the formula suggested in the Quality control methods (WHO, 1998):

$$\text{Tannin (\%)} = (T_1 - T_2 - T_0) \times 500W,$$

where W is the weight of the extract in grams.

### Total antioxidant capacity

Total antioxidant capacity (TAC) of aqueous extracts of *P. tuber-regium* was evaluated by phospho-molybdenum method following Prieto et al. (1999). Amounts of 10, 50 and 100  $\mu\text{g mL}^{-1}$  of extract were added in a series of test tubes and 1.9 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The tubes were incubated at 95 °C for 90 min and allowed to cool, then absorbance of samples was measured at 695 nm against a blank. Antioxidant capacity was expressed as equivalents of ascorbic acid. Ascorbic acid equivalents were calculated using a standard graph of ascorbic acid. A synthetic antioxidant butylated hydroxy anisole was used to compare the TAC of aqueous extract of *P. tuber-regium*.

### Animals

Wistar albino rats (*Ratus norvegicus*) with mass of 175 to 200 g were obtained from the National Institute of Nutrition, Hyderabad, India. They were maintained under standard laboratory conditions at ambient temperature of  $25 \pm 2$  °C and relative humidity at  $50 \pm 15$  %, with dark-light cycle of 12 h. Animals were fed with a commercial pallet diet (Sadguru Shri Shri Industries Pvt. Ltd. Pune, India) and water. The experiment was performed after prior approval of the Ethics committee of Ranchi University, Ranchi (Proceeding no. 46, page no. 137).

### Acute toxicity studies

Various doses of *P. tuber-regium* extract (50 to 2000 mg  $\text{kg}^{-1}$  body weight) were administered orally by oral feeding tube

to Wistar albino rats. The animals were observed for gross behaviour, neural and autonomic toxicity as described on OECD guidelines (OECD 2004). No mortality was observed at doses up to 2000 mg kg<sup>-1</sup> body weight of dose within 24 h.

#### Hepatoprotective efficacy studies

Animals were equally distributed among four treatment groups (group 1, 2, 3 and 4) containing five animals in each group as follows. Liver damage was induced in rats of group 2, 3 and 4 by intraperitoneal administration of a mixture containing carbon tetrachloride (CCl<sub>4</sub>) and liquid paraffin (1:2 v/v; 1 mL kg<sup>-1</sup>) (Shahjahan et al. 2004; Parmar et al. 2009).

Group 1 rats served as a control and were neither treated with mushroom extract nor CCl<sub>4</sub> and received 1 mL of distilled water orally throughout the entire period of the experiment. Rats of the group 2 were considered as hepatotoxic, and received 30% CCl<sub>4</sub> suspended in liquid paraffin (1.0 mL kg<sup>-1</sup> body weight i.p.) every 72 h for 14 days. The hepatotoxic rats treated as above (group 3) received 250 mg kg<sup>-1</sup> body weight (low dose, LD) of *P. tuber-regium* extract orally for 14 days. Hepatotoxic rats treated as above (group 4) received orally 500 mg kg<sup>-1</sup> body weight (high dose, HD) of *P. tuber-regium* extract orally for 14 days.

#### Sample collection and assessment of hepatoprotective efficacy

All of animals were sacrificed on day 14 under light ether anaesthesia. A blood sample (5 mL) was collected from each animal by cardiac puncture using a sterile needle and syringe. Three blood samples were taken from each rat group. Blood samples were placed into test tubes and allowed to clot for 30 min at 37 °C. The clear serum was separated by centrifuge (Wisperfuge 1384 Samson, Holland) at 2500 rpm for 10 min and biochemical investigations were carried out. Total bilirubin was estimated following the standard method of Mallory and Evelyn (1956), total protein and albumin was measured following the standard method of Kingsley and Frankel (1939), serum alanine transaminases

(ALT) and aspartate amino transferase (AST) activity was estimated following the standard method of Reitman and Frankel (1957) and serum alkaline phosphatase (ALP) activity was estimated following the standard method of Bessey et al. (1964).

#### Statistical analysis

Data were taken in triplicate and results were expressed as a mean ± standard error of mean. Statistical analysis was performed using one-way ANOVA followed by Student's *t*-test, *p* < 0.05 was considered as statistically significant.

## Results

#### Chemical analysis and total antioxidant capacity

Preliminary mycochemical analysis indicated the presence of alkaloids, tannins, flavonoids and phenolics in the extract of *P. tuber-regium*. Quantitative analysis of these chemicals performed showed alkaloids present in higher concentration (28.41 ± 0.5 mg 100 g<sup>-1</sup>), followed by phenolics and flavonoids, while tannins were in the lowest concentration (2.74 ± 0.28 mg 100 g<sup>-1</sup>; Fig. 1).

Aqueous extract of *P. tuber-regium* showed comparably high total antioxidant capacity (TAC). However, TAC of crude extract was less than 50% of the antioxidant capacity of butylated hydroxy anisole (EC<sub>50</sub> = 62.5 µg mL<sup>-1</sup>; Fig. 2).

#### Hepatoprotective efficacy

The results of hepatoprotective efficacy of *P. tuber-regium* extract are presented in Table 1. The activity of AST, ALT, ALP and bilirubin concentration significantly (*p* < 0.001) increased and total protein and serum albumin significantly (*p* < 0.001) decreased in the CCl<sub>4</sub>-treated hepatotoxic group, compared to levels in the control group. When *P. tuber-regium* extract was administered to hepatotoxic rats, activity of AST, ALT and ALP significantly (*p* < 0.001) decreased and concentration of serum albumin and protein significantly increased, compared to the hepatotoxic group (Table 1).

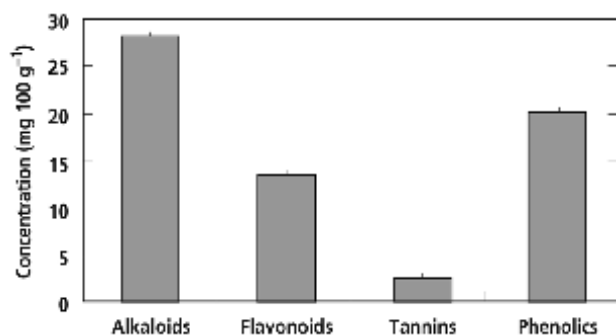


Fig. 1. Quantitative estimation of different groups of chemical constituents in aqueous extract of *Pleurotus tuber-regium*. Data are means ± SE, n = 3

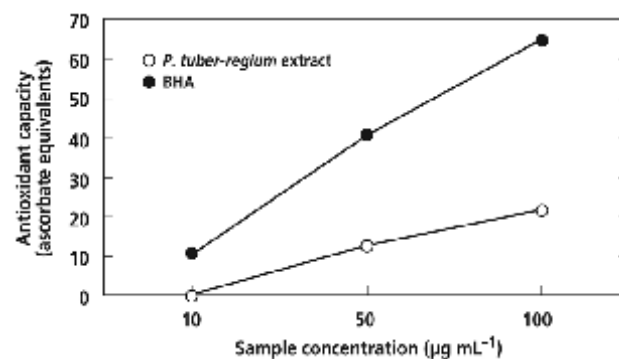


Fig. 2. Total antioxidant capacity of aqueous extract of *Pleurotus tuber-regium* in comparison to that of butylated hydroxy anisole. Data are means from three measurements.

**Table 1.** Hepatoprotective efficacy of *Pleurotus tuber-regium* extract against CCl<sub>4</sub>-induced hepatotoxicity in rats. Data are means ± SE, n = 3. <sup>a</sup>Statistically significant when compared to control group (p < 0.05). <sup>b</sup>Statistically significant when compared to hepatotoxic group (p < 0.05)

Animal groups (treatments)	Total protein (g dL <sup>-1</sup> )	Serum albumin (g dL <sup>-1</sup> )	Bilirubin (mg dL <sup>-1</sup> )	AST (U L <sup>-1</sup> )	ALT (U L <sup>-1</sup> )	ALP (U L <sup>-1</sup> )
Group 1 (control)	8.57 ± 0.17	4.23 ± 0.13	0.62 ± 0.09	52.30 ± 1.15	146.63 ± 3.79	176.24 ± 2.80
Group 2 (hepatotoxic)	5.20 ± 0.08 <sup>a</sup>	2.30 ± 0.52 <sup>a</sup>	2.86 ± 0.27 <sup>a</sup>	107.20 ± 3.17 <sup>a</sup>	206.20 ± 4.82 <sup>a</sup>	508.26 ± 5.22 <sup>a</sup>
Group 3 (hepatotoxic plus low dose of extract)	6.46 ± 0.21 <sup>ab</sup>	3.35 ± 0.23 <sup>ab</sup>	1.25 ± 0.13 <sup>ab</sup>	98.25 ± 2.65 <sup>ab</sup>	192.61 ± 2.31 <sup>ab</sup>	412.43 ± 3.82 <sup>ab</sup>
Group 4 (hepatotoxic plus high dose of extract)	8.20 ± 0.10 <sup>b</sup>	4.01 ± 0.41 <sup>b</sup>	0.68 ± 0.03 <sup>b</sup>	61.38 ± 1.72 <sup>ab</sup>	162.31 ± 1.82 <sup>ab</sup>	197.23 ± 2.64 <sup>ab</sup>

## Discussion

Activity of liver markers such as AST, ALT, ALP and concentration of bilirubin increase in the blood due to liver biliary obstruction, and degradation of hepatic cell membrane (Huo et al. 2011), while serum albumin and protein level decreases due to damage of intracellular structures such as endoplasmic reticulum, mitochondria, DNA etc. (Huang et al. 2012; Uru et al. 2013). CCl<sub>4</sub> causes hepatotoxicity by changing to the form of trichloromethyl radical (CCl<sub>3</sub>·) and trichloromethylperoxy radical (CCl<sub>3</sub>OO·), which initiates activation of cytochrome P450 2E1, lipid peroxidation, release of pro-inflammatory mediators such as TNF-α results in necrosis and apoptosis of hepatocytes and enhances oxidative stress-mediated hepatic damage by release of reactive oxygen species and reactive nitrogen species (Edwards et al. 1993; Huang et al. 2012; Sanghai et al. 2013). In the present study the hepatic biomarkers AST, ALT, ALP and bilirubin level increased and albumin and protein level decreased significantly in hepatotoxic rats due to hepatic damage mediated by oxidative stress.

In the present study, *P. tuber-regium* extract possessed antioxidant activity in a dose-dependent manner, possibly due to the presence of relatively high concentration of bioactive chemicals like flavonoids, tannins, alkaloids and phenolics. Phenolics and flavonoids have antioxidant activity due to their redox properties, which allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators (Kanimozhi et al. 2011). Alkaloids cause antioxidizing effects by reduction of nitrate generation, which involves protein synthesis (Isaac, Chinwe 2001; Dandapat et al. 2014). Tannins inhibit free radical-mediated lipid peroxidation by blocking the propagation of free radicals and inhibiting 3-hydroxy-3-methyl CoA reductase (Chang et al. 2001; Auger et al. 2002; Dandapat et al. 2015). In the present study chemicals of the extract contains bioactive components possessing antioxidant activity and preventing hepatic damage from free radicals.

Chatterjee et al. (2011) studied the hepato protective efficacy of wild edible mushroom *Calocybe indica* on

CCl<sub>4</sub>-induced hepatotoxic mice and reported that elevated ALT, AST, ALP, and total bilirubin level of hepatotoxic mice significantly decreases in *C. indica* extract-treated animals due to stabilization of plasma membrane, repair of hepatic injury by healing of hepatic parenchyma and regeneration of hepatocytes through the antioxidant defense mechanism of bioactive components such as flavonoids, phenolics, polysaccharides etc. of *C. indica* extract. Huo et al. (2011) studied hepatoprotective efficacy of *Glycyrrhiza glabra* root extract and found that ALT, AST and ALP level significantly decreases and total protein and total albumin level significantly increases in CCl<sub>4</sub>-treated hepatotoxic rat group when treated with *G. glabra* extract.

In present study *P. tuber-regium* extract at both low and high doses lowered ALT, AST, ALP activity, while total bilirubin level significantly decreased but total protein and total albumin level significantly increased in CCl<sub>4</sub> hepatotoxic rats. Similar effects have been reported for licorice extract (Huo et al. 2011) and extract of *Calocybe indica* (Chatterjee et al. 2011).

It has been reported that for animals, treated with the plant or fungal extract, lowered serum AST, ALT and ALP activity indicates stabilization of plasma membrane as well as repair of hepatic injury and regeneration of hepatocytes with healing of hepatic parenchyma (Thawbrew et al. 1987; Acharya et al. 2012). Healing agents are the major bioactive components such as flavonoids, tannins, phenolics, polysaccharides etc. Membrane-stabilizing and antioxidant properties has been described for a drug Silymarin, which is a mixture of flavonoids and polyphenols, through promotion of hepatocyte regeneration, reduction of inflammatory reactions, and inhibition of fibrogenesis (Feher, Lengyel 2012).

In conclusion, *P. tuber-regium* possessed antioxidant activity and exhibited hepatoprotective efficacy against CCl<sub>4</sub>-induced hepatotoxicity in rats. Therefore, *P. tuber-regium* can be used as liver tonic against the diseases such as hepatotoxin exposure, intrahepatic cholestasis, alcoholic liver injury, liver ischemia and viral hepatitis, fibrosis, cirrhosis, steatohepatitis and biliary disease, inflammation and necrosis of liver etc.



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