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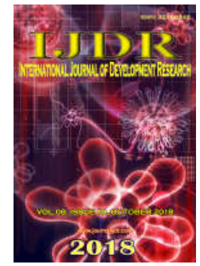

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTITUMOR ACTIVITIES OF AQUEOUS ETHANOLIC EXTRACT OF THE GOLDEN OYSTER MUSHROOM *PLEUROTUS CITRINOPILEATUS* (FR.) SINGER

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ABSTRACT

Pleurotus citrinopileatus, an excellently edible mushroom, was examined for its antioxidant, anti-inflammatory and antitumor activities. The aqueous ethanolic extract of the mushroom was used for the study. The mushroom possessed profound antioxidant activity as evidenced from its ability to scavenge $DPPH^{\cdot}$, $ABTS^{\cdot+}$, hydroxyl and nitric oxide radicals. The extract also reduced ferric ions into ferrous ions and prevented ROS induced lipid peroxidation to a significant level. Anti-inflammatory and antitumor properties of this mushroom was tested on Swiss albino mice model. The extract, at a dose of 1000 mg/Kg body weight, reduced formalin induced chronic inflammation by 40 % which was near to the anti-inflammatory effect of diclofenac (10mg/Kg body weight). The extract also possessed antitumor property against DLA induced tumor, reducing tumor mass by 33 % on the administration of the extract at 1000 mg/Kg dose for ten days. Preliminary chemical examination revealed that major chemical constituents of the extract were steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates.

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INTRODUCTION

The role and significance of oxidative stress in the initiation and progression of many diseases have become increasingly evident in recent years. Oxidative stress and inflammation operate mutually in a large number of diseases such as neurodegenerative, cardiovascular and autoimmune diseases and also in diabetes (Khansari *et al*., 2009). Both these conditions play significant role in the formation of neoplastic tissue and its progression into malignant condition. A large number of studies have shown that many of the compounds having antioxidant activity also possessed significant anti-inflammatory and antitumor properties. These results on one hand indicate the etiologic effect of oxidative stress on inflammation and neoplastic growth and on the other encourage the search for novel compounds with strong antioxidant activity for their anti-inflammatory and antineoplastic properties. Several natural products are potent sources of strong antioxidants. Though a large number of such compounds have been isolated and identified, many of them remain inadequately explored. In this respect, mushrooms are largely unexplored for such bioactive compounds. Among the mushrooms, the genus *Pleurotus* with about 40 species is noted for its cosmopolitan distribution (Deepalakshmi and Mirunalini, 2014). Most of them have high edible status. *Pleurotus citrinopileatus*, native to eastern part of Asia, is an excellently edible mushroom. We examined the antioxidant, anti-inflammatory and antitumor activities of this edible mushroom and the findings are reported in this communication.

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MATERIALS AND METHODS

Animals: Male Swiss albino mice were purchased from Small Animal Breeding Station, Agricultural University, Mannuthy, Thrissur, Kerala. They were kept under standard conditions with free access to standard food and water. Animal

experiments were carried out according to guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and with approval of Institutional Animal Ethics Committee (IAEC).

Preparation of the extract: Fruiting bodies of the mushroom *P. citrinopileatus* were obtained from Indian Institute of Horticultural Research, Bangalore. The fruiting bodies were dried under the sun and then in a hot air oven at 50°C. Hundred gram samples of the dried material were powdered and extracted using 70% aqueous ethanol on a boiling water bath for 9-10 hours. The extract was separated from suspended particles by filtering through a Whatman No.1 filter paper. The filtrate was concentrated and evaporated at low temperature under vacuum. The residue thus obtained was used for experiments as aqueous ethanol extract (ETH). Yield was found to be 22.46%.

Determination of in vitro antioxidant activity

DPPH˙ Radical Scavenging Assay: In this method, a stable free radical DPPH˙ (2,2-diphenyl-1-picryl hydrazyl) was used. The extract at different concentrations was added to freshly prepared DPPH solution prepared in methanol (100 µM) and kept under dim light for 20 minutes. Then absorbance of the test samples was measured at 517 nm against methanol. The ability of the extracts to scavenge DPPH˙ radicals was calculated by comparing the absorbance values of the control with that of treated. Reaction mixture without extract was used as control (Aquino *et al*, 2001).

Assay of Hydroxyl Radical Scavenging Activity: Hydroxyl radicals were produced by inducing Fenton's reaction . The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), K_2HPO_4-KOH buffer (20 mM, pH 7.4), EDTA (0.1 mM), H_2O_2 (1.0 mM), ascorbic acid (0.1 mM) and various concentrations of the extract in a final volume of 1 mL. The hydroxyl radicals formed would react with deoxyribose and produce TBARS. Scavenging activity of the extracts was determined by measuring TBARS by the method of Ohkawa *et al*,1974**.** Briefly, the reaction mixture was incubated at 37°C for 60 min. After incubation, 0.4mL of the reaction mixture was treated with 0.2 mL of SDS (8.1%), 1.5 mL of TBA (0.8%) and 1.5 mL of acetic acid (20% V/V, pH 3.5). The total volume was made up to 4mL using distilled water and kept in a boiling water bath for 60 minutes. The reaction mixture was cooled by dipping the tubes in tap water. 1mL of distilled water and 5mL of butanol : pyridine mixture (15:1, V/V) were added to the reaction mixture. The tubes were vortexed and centrifuged at 2750 g for 10 minutes. The OD of the upper layer was measured at 532 nm in a double beam spectrophotometer. The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated (Elizabeth and Rao, 1990).

Inhibition of Lipid Peroxidation: Lipid peroxidation was induced by Fe^{2+}-ascorbate system in the rat liver homogenate in the presence and absence of the extract to form TBARS. The reaction mixture contained 0.1 mL of rat liver homogenate (25%, w/v) in Tris-HCl buffer (20 mM, pH 7), KCl (30 mM), $FeSO_4(NH_4)_2SO_4.6H_2O$ (0.16 mM), ascorbate (0.06 mM) and various concentrations of the extract in a final volume of 0.5 mL (Bakshi *et al*, 2009). The TBARS formed is measured according to the method of Ohkawa *et al*, 1979.

$ABTS^{\cdot+}$ Radical Scavenging Assay: In this assay the extracts were allowed to react with $ABTS^{\cdot+}$, a model stable- free radical derived from 2,2'-azinobis (3-ethylbenzothiazolin-6-sulphonic acid)**.** Ammonium persulphate (2.45 mM, final concentration) was added to a solution of ABTS (7 mM) and allowed to react for more than 16 hrs in dark at room temperature. ABTS and persulphate react with each other leading to the incomplete oxidation of ABTS to generate $ABTS^{\cdot+}$ radical. The $ABTS^{\cdot+}$ radical solution was diluted to an absorbance of 0.75 at 734 nm using ethanol. Different concentrations of the extract was added to 2 mL of $ABTS^{\cdot+}$ radical solution. The decrease in the absorbance was measured against ethanol by a spectrophotometer after 6 minutes of initial mixing (Pino *et al*, 2005).

Ferric Reducing Antioxidant Power (FRAP) Assay: The ferric reducing ability of the extracts was measured at low pH**.** The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl and ferric chloride (20 mM) in a ratio of 10:1:1. Different concentrations of the extract was added into the reagent solution and incubated in dark for 30 minutes. The intense blue color developed was measured at 593 nm. The reducing power of the extract was calculated from a standard graph drawn by using different concentrations of $FeSO_4$. $7H_2O$ (10-100 µM in distilled water) (Benzie and Strain, 1996).

Assay of Nitric Oxide Scavenging Activity: Nitric oxide radical inhibition can be estimated by employing Griess Ilosvay reaction**.** Sodium nitroprusside (10 mM) was prepared in phosphate buffered saline (pH 7.4) and 3 mL of the solution was incubated with various concentrations of the extract at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of Sulphanilamide solution (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min at 25°C for completing diazotization. Then 1 mL of naphthyl ethylene diamine dihydrochloride (0.1%) was added, mixed, and allowed to stand for 30 min at 25°C. A pink colored chromophore is formed. The absorbance of the solutions was measured at 540 nm against the PBS (Sesti, 2012).

Determination of anti-inflammatory activity: Male Swiss albino mice weighing 30±4 g were used for the study. The animals were divided into 5 groups having 6 animals in each group. Inflammation was induced by a single subcutaneous injection of 0.02 mL of freshly prepared 2% formalin to the paw region of all the mice. Diclofenac was used as standard reference drug. The extract/ diclofenac was orally administered for six consecutive days starting on the day after formalin injection. One group, which received neither extract nor diclofenac, was kept as untreated control. Three groups received the extract in three different doses (1000, 500 and 250 mg/ Kg body weight). The remaining one group was treated with diclofenac (10 mg/ Kg body weight) and kept as standard reference. After six days, the paw thickness was measured using a Vernier caliper and the net increase in paw thickness was calculated by deducting the initial paw thickness. Percentage of reduction in paw thickness in the extract/ diclofenac treated group of animals was calculated by comparing paw thickness of the treated groups with that of untreated control (Ajith and Janardhanan, 2001).

Determination of antitumor activity: The antitumor activity of the extract was determined by solid tumor model on Swiss

albino mice. Animals were divided in to 4 groups with 6 animals in each group. Viable DLA cells ($1x10^6$) in 0.1ml PBS was transplanted subcutaneously in to the thigh of right hind limb of all the mice. One group acted as untreated control and one group received single dose of cyclophosphamide orally (25 mg/Kg body weighjt) as standard drug. ETH extract was administered orally to two groups in two different doses (1000 and 500 mg/kg body weight). The extract was administered for ten consecutive days starting from the day of DLA cell implantation. Development of tumor on animals in each group was measured once every week for the first 5 weeks. The tumor volume was calculated using the formula $V=4/3\pi (r_1)^2 (r_2)$, where r_1 is the minor radius and r_2 is the major radius of the tumor. At the end of the 5th week, animals were sacrificed, tumor was extracted and weighed. The inhibition of tumor growth was calculated by comparing the tumor volume and weight of drug/extract administered animals with that of the animals of control group and the value was expressed as percentage using the relation ((A – B) /A) X 100, where A is average tumor volume/weight of the control group and B is that of the treated group (Chihara *et al*, 1970).

Preliminary phytochemical analysis: Preliminary photochemical analysis of ETH was carried out using standard methods.

Statistical analysis: The values were reported as mean ± sd. Statistical significance of the results were analyzed by One-way ANOVA. A P value <0.05 was considered significant.

RESULTS

In vitro antioxidant activities

DPPH· radical scavenging activity: The extract showed marked DPPH· radical scavenging activity. The scavenging activity was found concentration dependent. The three different concentrations of the extract, 500, 250 and 50 µg/mL, showed 86.13, 46.6 and 9.47 % radical scavenging activity respectively (Fig .1).

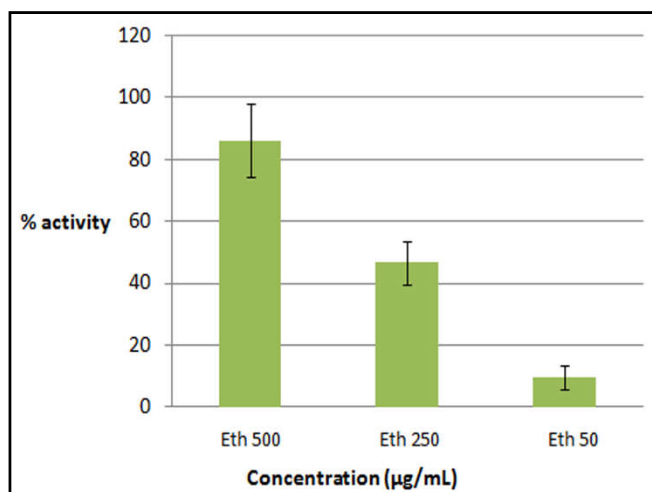

Figure 1. DPPH· radical scavenging activity : The amount of DPPH· radicals scavenged by the extract is expressed in percentage

ABTS·+ radical scavenging activity: The extract was able to reduce the ABTS·+ radicals in a concentration dependent manner.100µg/mL concentration of the extract reduced 77% of the radicals and 50 and 10 µg/ml concentrations of the extract showed 50 and 22% reduction respectively (Fig. 2).

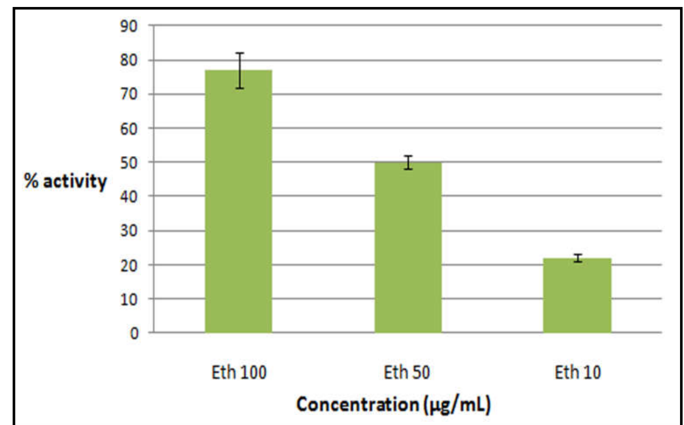

Figure 2. ABTS·+ radical scavenging activity : The ability of the extract to reduce ABTS·+ radicals is shown in percentage

FRAP radical scavenging assay: The extract showed ferric ion reducing activity. The activity was found dose dependent. The extract at concentrations of 250, 125 and 50 µg/mL of the extract reduced 65.44, 35.65 and 15.17 nanomoles of Fe^{3+} ions respectively (Fig. 3).

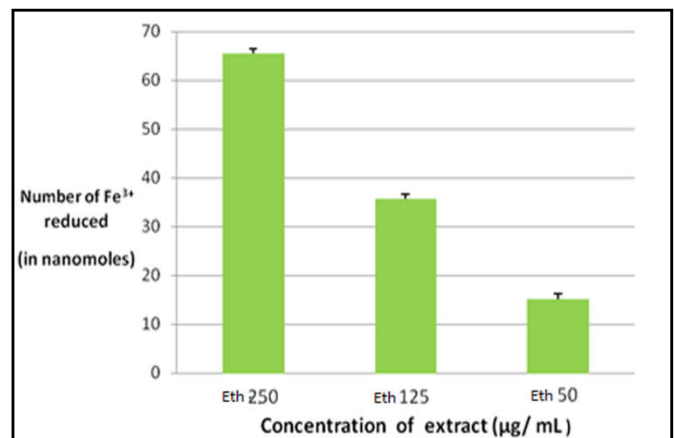

Figure 3. FRAP assay: The extract reduced ferric ions in a dose dependent manner

Nitric oxide scavenging activity: Nitric oxide scavenging activity of the extract was found to be weak. A concentration of 1000 µg/ml was required for 24.03% scavenging activity and 500 and 250 µg/mL concentrations showed 21.5 and 7.6 % activities respectively (Fig. 4).

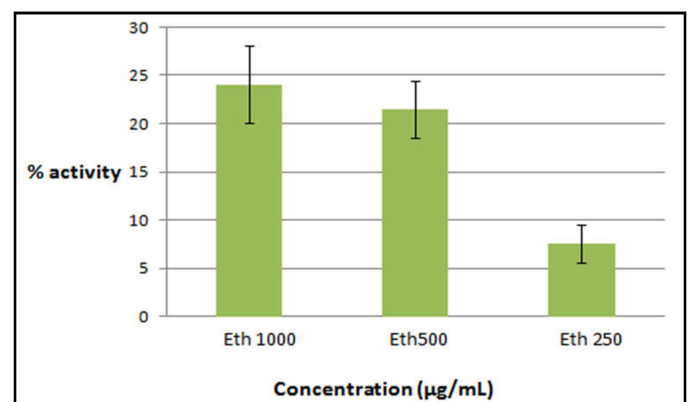

Figure 4. Nitric oxide radical scavenging assay: The extract scavenged nitric oxide radicals generated in the reaction mixture

Hydroxyl radical scavenging activity: Hydroxyl radicals were efficiently scavenged by the extract. The extract at a concentration of 500 µg/mL scavenged about 69.86% of hydroxyl radicals and 250 and 50 µg/mL concentrations of the extract showed 50.72 and 13.63 % activity respectively (Fig. 5).

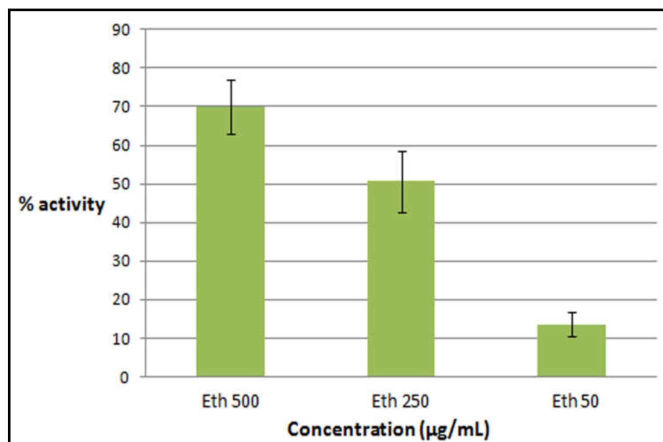

Figure 5. Hydroxyl radical scavenging assay : OH· Radicals scavenged by the extract is expressed in percentage

Lipid peroxidation inhibition activity: Two concentrations of the extract, 500 and 250 µg/mL, inhibited lipid peroxidation by 26 and 8% respectively. The lowest concentration, 50 µg/ml, was found to be insufficient to bring about detectable inhibitory activity (Fig. 6).

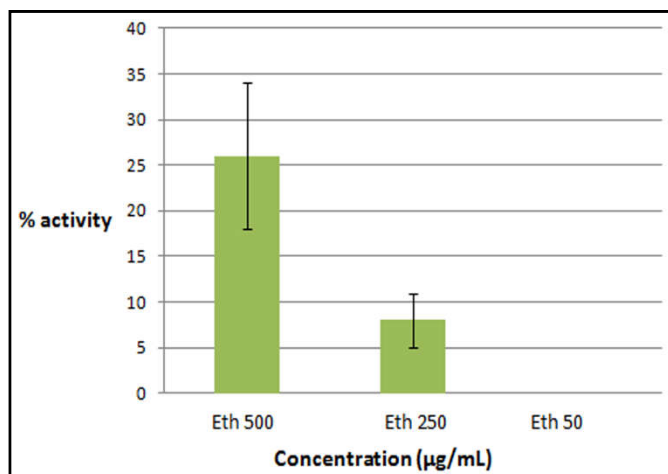

Figure 6. Lipid peroxidation inhibition assay: The extent to which lipid peroxidation was inhibited by the presence of the extract is expressed in percentage

Anti-inflammatory activity: The extract possessed significant activity against chronic inflammation. Treatment with 1000 mg/Kg dose of the extract resulted in about 40 % reduction in the edema which was almost equal to the effect of standard drug diclofenac at a dose of 10 mg/Kg (Fig.7).

Antitumor activity: The extract showed profound inhibitory effect on the developing tumor in a dose dependent manner. Treatment with 1000 and 500 mg/Kg of the extract for ten days reduced tumor mass by 33 and 28.3% respectively while the standard drug cyclophosphamide (25 mg/Kg b. wt) reduced tumor mass by 92% at the end of five weeks (Fig .9).

Preliminary phytochemical analysis: The extract responded to various phytochemical tests indicating the presence of steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates.

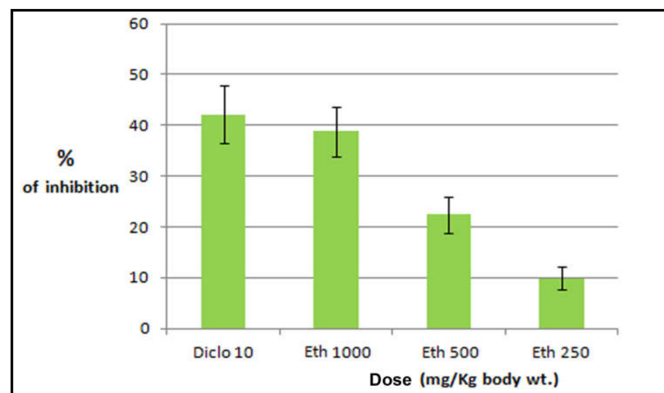

Figure 7. Anti-inflammatory assay: Paw edema developed on mice due to formalin injection was reduced by the administration of the extract. The values are calculated by comparing the paw edema in control group with that of the test group

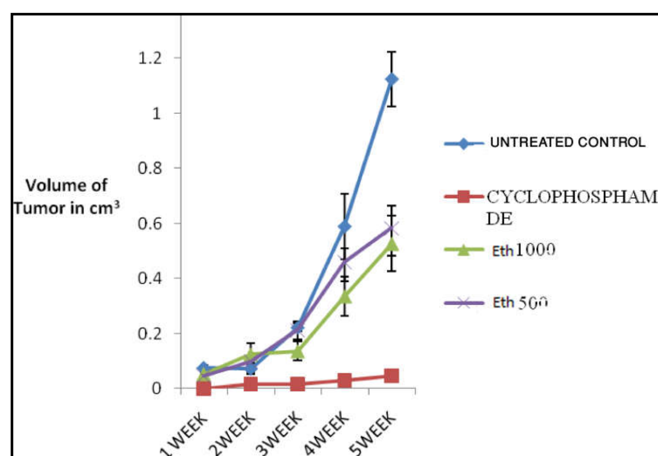

Figure 8. Antitumor activity: Volume of the tumor developed on mice was measured for the first five weeks after DLA implantation is shown in the graph. Cyclophosphamide was used as the standard drug

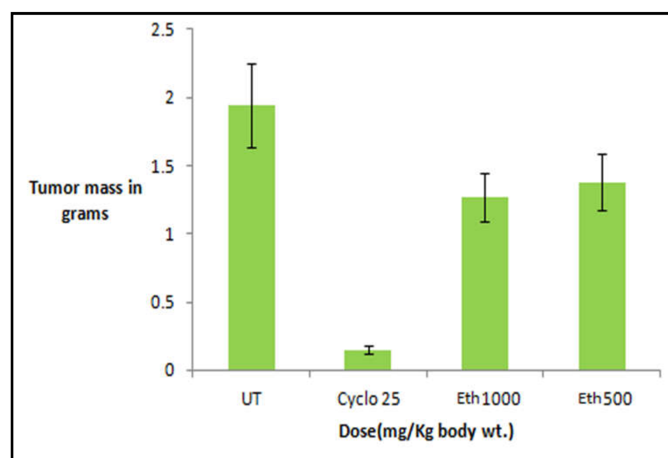

Figure 9. Anitumor activity: After five weeks of DLA implantation, tumor weight on different groups of mice was determined and compared with that of untreated control (UT)

DISCUSSION

Chronic oxidative stress and inflammation are found to be the leading causes of a large number of diseases which include

cancer. Mushrooms contain a great variety of compounds which can ameliorate oxidative stress and inflammation thereby offer protection from many diseases. The results of current investigation indicate that aqueous ethanol extract of *P. citrinopileatus* possesses significant antioxidant, anti-inflammatory and antitumor activities. The four assays, namely, DPPH$^{\cdot}$, ABTS$^{\cdot+}$, hydroxyl and nitric oxide radical scavenging assays, revealed the ability of the extract to scavenge free radicals. Similarly the extract was found to reduce ferric ions in the FRAP assay indicating that it contained compounds with loosely bound electrons, a property of immense value for an antioxidant. The phytochemical analysis of the extract showed that it contained steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates. All of these classes of compounds can act as reducing agents. Phenols often present various functional groups and a delocalized electron system to the oxidants thereby acting as strong antioxidants. A previous study has estimated the amount of phenolic compounds in aqueous ethanolic extract of fruiting bodies of *P. citrinopileatus* is in the range of 8.62–12.38 mg/g of material (Lee *et al*, 2007). Ascorbic acid is a strong antioxidant and its quantity in ethanolic extract of *P. citrinopileatus* has been quantified to 31 ng/mg (Adhiraj *et al*, 2013).

The presence of ergosterol and nicotinic acid in this mushroom has also been reported. These compounds are also known to possess antioxidant activity (Shu Hui Hu *et al*, 2006). These results support our observation on the antioxidant activity of the ETH extract. Lipids are easy targets for various oxidative reactions because of the presence of multiple bonds. Hence overproduction of hydroxyl radicals in the cells results in lipid peroxidation. The extract was effective in reducing the extend of peroxidation of lipids. Since phenols, coumarins, tannins, alkaloids, steroids etc. often possess conjugated ring structure, these compounds can act as alternative targets for the prooxidants thereby mask lipids from overexposure to the oxidative agents. The anti-inflammatory activity of the extract is evident from the significant reduction in the edema in mice after the treatment with the extract. The phytochemicals that can cause a reduction in the inflammation, include flavanoids and terpenoids (John and Shobana, 2012). Flavonoids like quercetin have been reported to possess anti-inflammatory reaction by inhibiting histamine release in mast cells (Park *et al*, 2008). Since aqueous alcohol can extract both these types of compounds, it is possible that similar bioactives might have caused the observed anti-inflammatory activity. Chen *et al*, 2012 has isolated a nonlectin glycoprotein from fruiting body of *P. citrinopileatus* that can down-regulate the pro-inflammatory mediators, like iNOS and NF-kB in RAW 264.7 cells (Yashvant *et al*, 2012). Though the extract could not prevent drastic development of tumor, it was able to reduce the average tumor size by 33%. Antitumor activity of this mushroom has also been shown by another study using a different model, against Sarcoma induced tumor in mice (Li *et al*, 2008). It is also reported that it can increase the number of T cells (both CD4+ and CD8+) in mice; (Humberto *et al*, 2011) probably contributing to its antitumor activity.

Conclusion

Aqueous ethanol extract of the fruiting bodies of *P. citrinopileatus* was examined for its antioxidant, anti-inflammatory and antitumor properties. The mushroom possessed significant antioxidant, anti-inflammatory and antitumor activities. Priliminary phytochemical analysis of the extract showed that it comprised steroids, alkaloids, coumarins, phenols, tannins, saponins and carbohydrates. Since this mushroom is highly edible, studies on the medicinal properties of this mushroom has considerable social dietary importance.

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Conflict of Interest: The authors of this article declare no conflict of interest in this study.

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