Journal of Phytology 2010, 2(1): 022–028
ISSN: 2075-6240

Available Online: www.journal-phytology.com

REGULAR ARTICLE

ANTIOXIDANT AND ANTIPROLIFERATIVE EFFECT OF *PLEUROTUS OSTREATUS*

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SUMMARY

In this study, ethanol extract of an edible mushroom *Pleurotus ostreatus* cultivated under the laboratory condition was investigated for its antioxidant and anticancer property in vitro. To confirm the total antioxidant activity, ABTS, DPPH free radical-scavenging assay was carried, along with total phenolic and flavonoid concentration. The ethanolic extract showed a potent antioxidant activity against both DPPH and ABTS radicals, with the EC50 value of 0.202±0.55 mg/mL and 6.42±0.261 mg/mL. Antioxidant components like total flavonoids were 1.82±0.15 µg/mg (Quercetin equivalent) and the total phenols were 8.52±0.6 mg/g (Catechin equivalent). Against the cancer cell (HL-60) in vitro *P. ostreatus* extracts exhibited the cytotoxic effect. The HL-60 cells treated with ethanol extract was further stained with propidium iodide and analyzed through flow cytometry, to identify whether the cytotoxicity induction was due to apoptosis or necrocis. The results of the flow cytometry confirm the cytotoxic effect of the mushroom extract was found to be mediated by the induction of apoptosis. In conclusion, our results supported the consumption of edible mushroom that act as a good dietary supplement and functional food.

Keywords: *Pleurotus ostreatus*; Anticancer; Antimicrobial; ABTS; DPPH; HL-60 cells.

V. Venkatakrishnan et al. Antioxidant and Antiproliferative Effect of *Pleurotus ostreatus*. J Phytol 2 (2010) 022-028
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1. Introduction

The awareness of functional food and their positive effect in human system has evolved the concept of functional food science [1]. These functional foods are in different name forms, e.g. dietery supplements, nutria-ceuticals, medicinal foods, vita foods, pharma foods, phytochemicals and mycochemicals [2]. In search of these functional foods scientific community has studied in different natural habitats like plants, algae, fungi and bacteria. In addition to that wild edible mushrooms were also used for many years in oriental cultures as tea and nutritional food [3]. In which many mushrooms have been reported of having variable therapeutic properties such as anti-inflammatory, immuno-stimulator and immunomodulater[4] especially against certain cancer types [5].

Energy production by oxidation is needed for many living organisms to fuel biological process. The increased oxidation rate leads to uncontrolled production of oxygen derived

free radicals involved in the onset of many diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes associated with aging [6]. Against these radicals organisms are well protected or balanced by the enzymes present in the system such as superoxide dismutase, catalase and glutathione peroxidase. Compounds such as polyphenols, flavonoids, ascorbic acid, tocopherols and glutathione, are an integral part of human diet which are available in fruits vegetables, seeds, tea, wines and juices [7]. These external factors had received much attention based on the, many epidemiological studies which suggests that consumption of poly phenol-rich foods and beverages is associated with a reduced risk of cardiovascular diseases, stroke and certain types of cancer [8].

There is no doubt that edible mushrooms are nutritionally sound tasteful food source for most people and can be a significant dietary component for vegetarians. Nevertheless, the edible mushrooms consumed and cultivated in most of the countries, among that *Pleurotus ostreatus* is the third most important cultivated mushroom for human consumption. This genus comprises of group of edible ligninolytic mushrooms with medicinal properties and important biotechnological and environmental applications. Nutritionally, it has unique flavor and aromatic properties that are considered to be rich in proteins, fibers, carbohydrates, vitamins and minerals [9]. To the best of our knowledge, no work has been available on antiproliferative property of *P.ostreatus* extract against HL-60 Cells (Human prohaemolytic leukemia cells). Therefore, the aim of the present work is to evaluate the antioxidant and antiproliferative effect of *Pleurotus ostreatus* ethanol extract in *in vitro.*

2. Material and methods

Mushroom

The wild edible mushroom *Pleurotus ostreatus* collected and identified from southern part of India, was cultivated in our laboratory condition to raise the fruitbody by following the standard method, using paddy straw as a substrate (Fig 1). The mushroom cultivated was harvested and shade dried at room temperature. Dried mushroom samples (50g) was extracted by stirring with 500 mL of ethanol at 30 ^{o}C at 150 rpm for 24 h and filtered through Whatman No. 4 filter paper. The residue was then extracted with two additional 500 mL of ethanol as described above. The combined ethanol extracts were then subjected rotar evaporator at 40^{o}C to dryness, and re-dissolved in ethanol to a concentration of 10 mg/mL and stored at 4^{o}C for further use.

Fig. 1. *Pleurotus ostreatus* Cultivated, using Paddy straw

Chemicals

2,2-Azino-bis (3-ethyl benzo thiazoline-6-sulfonic acid) 1,1-Diphenly-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and Vitamin E were purchased from Sigma (Sigma, Aldrich GmbH, Sternheim, GERMANY). Catechin, Quercetin, Folin-ciocalteu's phenol reagent (FCR), sodium carbonate, ethanol, RPMI 1640 medium supplemented with 10% fetal bovine serum, Penicillin (100 IU/ml), and Streptomycin (100µg/ml), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, propidium iodide. and the other chemicals and reagents were purchased from Merck (Darmstat, GERMANY). All other unlabelled chemicals and reagents were of analytical grade.

Antioxidant activity

ABTS radical scavenging activity

The Trolox equivalent antioxidant capacity (TEAC) was estimated using the Feryl Myoglobulin/ABTS method for total antioxidant activity [10]. In this assay, ABTS is

oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\cdot+}$, which is intensely colored, and AOC (Antioxidant capacity) is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\cdot+}$ radical. Results of test compounds are expressed relative to Trolox or Vitamin E. The stock solution included 7.4 mM ABTS solution and 140 mM Potassium per-sulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with ethanol to obtain an absorbance of 0.7 ± 0.02 units at 734 nm using UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The antioxidant property was determined by reduction in the O.D compared with the standard Vitamin E. Inhibition of free radical by $ABTS^{\cdot+}$ in percent (I%) was calculated in following way:

I% = $(A_{blank} - A_{sample}/A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of ethanol extracts. Tests were carried out in triplicates.

DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2, 2 diphenylpicrylhydrazyl (DPPH). DPPH the stable free radical was used in this spectrophotometric assay [11]. A stock solution of 1.3 mg/mL in methanol was prepared from that 75 µl of it was added in 3 mL methanol and gave an initial absorbance of 0.9. Decrease in absorbance in the presence of sample extract at different concentration was noted after 15 min. Inhibition of free radical by DPPH in percent (I%) was calculated in following way:

I% = $(A_{blank} - A_{sample}/A_{blank}) \times 100$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph, plotted in percentage against extract concentration. Tests were carried out in triplicate.

Determination of total phenolic compounds

Total phenolic compounds in the ethanol extracts were determined using Folin–Ciocalteu method [12]. One mL of the extract was added to 10.0 mL distilled water mixed with 2.0 mL of Folin-Ciocalteu phenol reagent. The mixture was allowed to stand at room temperature for 5 min and then 2.0 mL of 20% sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The phenol compound was calibrated using the linear equation based on the calibration curve. The contents of the phenolic compound were expressed as mg catechin equivalent/g dry weight.

Determination of total flavonoid concentration

The $AlCl_3$ method [13] was used for the determination of total flavonoid content in the mushroom extract. Aliquot of 1.5ml of extracts was added to equal volume of 2% $AlCl_3$ (2 g in 100 mL methanol) solution. The mixture was vigorously shaken, and absorbance at 367 nm was read after 10 min of incubation. Quercetin was used as a standard for the calibration curve. The contents of the flavonoid compound were expressed as mg Quercetin equivalent/g dry weight.

Cell culture

Human promyelotic leukemia HL-60 cells were purchased from NCCS (Pune, INDIA) were grown in a humidifier atmosphere containing 5% CO_2 in RPMI-1640 medium supplemented with 10% FBS and penicillin (100 IU/ml), and streptomycin (100µg/ml), HL-60 cells were seeded at a density of 1×10^5 cells/ml and treated with the extract after 24h.

MTT assays

Cell suspensions were prepared at a concentration of 2 X 10^4 cells/ml were seeded in 96-wells micro-culture plates with or without extract treatment in a volume of 100µl. After 72h incubation at 37°C, 20µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide was added to each well, and the samples were incubated for further 2h at 37 °C. Plates were analyzed on a micro-titer plate reader at 492 nm. The inhibition ratio was calculated according to the following equation: Inhibition ratio (%) = ($A_{control\ group}$ – $A_{AB\ treated\ group}$) / $A_{control\ group}$ X 100

Assessment of apoptosis

Apoptotic cell death was measured as the percent of cells with hypodiploid DNA. HL-60 Cells cultured with or without the samples (100 µg/ml) at 37°C for 48 h were harvested, washed with PBS, and fixed with 75% ethanol at 4°C for 2h. cells were then treated with RNase A (0.25 mg/ml) at 37°C for 1 h. after washing, the cells were stained with 50 mg/ml propidium iodide at room temperature for 10 min. cell cycle analysis was performed on FACS calibur flow cytometer (Becton-Dickinson, San Jose, CA)

Results and Discussion

Antioxidant activity of ethanolic extract Pleurotus ostreatus

To find out the antioxidant activity of the ethanolic extracts of *P.ostreatus* four complementary test systems, namely ABTS free radical-scavenging, DPPH free radical scavenging systems, total phenolic and total flavonoid concentration were used for the analysis.

ABTS, a stable free radical with a characteristic absorption at 734 nm, generally it was used to study the radical-scavenging effects of different organic extracts. The advantageous of using ABTS radical is for their simplicity in operation, solubility and adaptability for wide range of pH and used in multiple media to determine both hydrophilic and liphophilic antioxidant sources [14].

It was found that inhibition values of ethanol extracts of *Pleurotus ostreatus* and the standards increased with increase in concentration. Inhibition values were found to be 13.23%, 65.21%, 87.74% and 90.42% at a concentration of 100, 200, 400 and 800µg/ml respectively. And the inhibitory concentration (EC_{50}) (minimum concentration scavenging 50% of free radicals) was found to be from 200µg/ml (Table 1). The EC_{50} value of ethanolic extract of *Pleurotus* sps, reported earlier was 250µg/mL and similar results were also inferred in *Agaricus bisporus* extract having the scavenging ability of $ABTS^{\cdot+}$ [15,16].

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts. As antioxidants donate protons to these radicals, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging activity of the samples. The ethanolic extract of *Pleurotus ostreatus* was tested against this radical at different concentrations ranging from (100 to 6400 µg/mL). Inhibition values of 12.57% 18.37% 24.62% 34.60% 40.87% 46.65% and 61.74% at a concentration of 100, 200, 400, 800, 1600, 3200 and 6400 µg/ml respectively. The EC_{50} value *P.ostreatus* against DPPH scavenging effect found to be in 6.4 mg/mL only (Table 1), when compared with other mushroom *Lactarius deliciosus* (8.52 mg/mL) and *Tricholoma protentosum* (22.9 mg/mL) which was reported earlier [17]. Generally the compounds involved in biological relevance were found to reduce DPPH less when compared to the synthetic antioxidant compounds like BHT, BHA [18].

Amount of Total Phenol and Flavonoids

The role of phenolic compounds as scavengers of free radicals is widely reported and accepted that phenolic compound plays a major role in antioxidant activity of many vegetables [19,20]. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in

humans, when ingested daily from a diet rich in fruits and vegetables [21]. Amounts of total flavonoid components of ethanolic extract of *Pleurotus ostreatus* was found to be 1.82±0.5mg/g (quercetin equivalent) while the phenolics was 8.52±0.14 mg/g (catechin equivalent) respectively (Table 1). Polyphenolic compounds seem to have important role in stabilizing lipid oxidation and to be associated with antioxidant activity [22,23].

Table 1. EC_{50} values (mean±SD) obtained in the antioxidant activity assays ethanolic extract *P. ostreatus* and the total amount of phenolic and flavonoid content in the extract.

Organisms	Antioxidant properties (EC values: mg/ml)		Total phenolic (Gallic acid equivalent mg/g)	Total flavonoid (quercetin equivalents (mg /g)
	ABTS	DPPH		
P. ostreatus	0.202±0.55[a]	6.42±0.261	8.52±0.14	1.82 ±0.14

[a]Means ± SD from triplicate determination.

MTT Assay

To examine the effect of *Pleurotus ostreatus* extracts on HL-60 leukemia cell proliferations. The cells were treated with increasing concentration of (0 – 0.8mg/ml) mushroom extract and the cell growth was determined, given in the (Table 2) the extract of *Pleurotus ostreatus* inhibited the cell proliferation in dose dependent manner, because after 72h of treatment with 0.1, 0.2 and 0.4 and 0.8 mg/ml of mushroom extract inhibited the proliferation of HL-60 cells by 32.42, 47.2, 52.66 and 78.27 respectively.

Table 2. The inhibitory effect of *Pleurotus ostreatus* extracts on HL-60 leukemia cells using cell proliferation or MTT assay

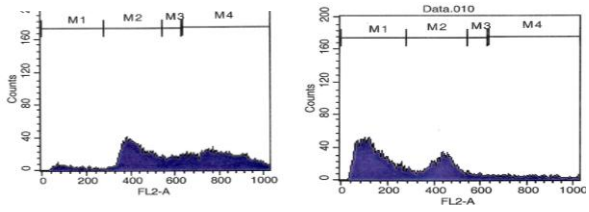
Cell proliferation or MTT Assay	*Pleurotus ostreatus* (PO) *Raw*			
	0.1mg	0.2mg	0.4mg	0.8mg
Control	-	-	-	-
% Inhibition	32.42 ± 0.24 [a]	47.21±0.54	52.66±0.48	75.66±0.66

[a]Means ± SD from triplicate determination.

Assessment of apoptosis

To evaluate whether the cytotoxic effect observed upon treatment of HL-60 cells was due to the induction of apoptosis or necrosis; HL-60 cells were treated with the extract at the concentration of 0.8mg/ml were maximum cytotoxicity was observed and incubated for 48h and the cell cycle distribution was determined by flow-cytometry (Fig. 3). Thus, the treatment of *P. ostreatus* extract against HL-60 leukemia cells significantly changes the amount of cells at M1 phase of around 58.41%. These data suggests that *Pleurotus ostreatus* extract inhibited the growth of HL-60 cells by cell cycle arrest that is by the induction of apoptosis. Our results were in agreement with the earlier report in *Agaricus bisporus* extract against HL-60cells [16]. Though many reports indicated that most of the polysaccharide or polysaccharide protein complexes from mushroom or natural sources cannot exert direct cytotoxicity on tumor cells, but predominantly by host mediated immune response, this type of inhibitory action may be interwoven and the mechanism of action is varied from person to person and the type of cancer, so the mechanism of action is urgently needed [24]. The current approach was undertaken to maximize the extraction of small molecule existing direct cytotoxicity in relation with antioxidant compounds like phenol and flavonoids. Ergosterol a phenolic compound extracted from white button mushroom showed inhibitory effect on breast cancer cell line by aromatase inhibition without side effects [25].

Fig. 2. Induction of apoptosis by *Pleurotus ostreatus* extracts against HL-60 cells. Cells were treated with extract (0.8mg/ml) for 48h (a) Control (b) cells treated with ethanol extract. The percentage of apoptotic cells was assessed by flow cytometry.



In conclusion, that edible mushrooms consumed has essential medicinal properties. And it is imperative to identify the biological and pharmacological potential of mushrooms especially of wild edible one, collected indigenously and should be cultivated. The

research on mushrooms in identifying its nutraceutical and medicinal properties will help to commercialize the mushrooms as functional food.

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