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Biochemical Analysis and Therapeutic Potential of Extract from Mushroom (*Pleurotus ostreatus*)

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Abstract: *Pleurotus ostreatus* is a popularly consumed species due to its taste, flavor, large values of nutrition, and pharmacological products. This research aims to investigate the nutritional, medicinal, and toxicological properties of locally cultivated oyster mushrooms in Khyber Pakhtunkhwa, Pakistan. The study consists of the evaluation of the presence of toxins using HPLC, proximate composition, nutritional profile, hemolytic activity (against human erythrocytes), cytotoxic activity (against brine shrimp), antibacterial potential, and anticancer properties of crude methanolic extract of *P. ostreatus*. Toxicological evaluation through HPLC revealed the presence of ochratoxin A (3.4 ppb) and Penicillic acid (2.7 ppb). Proximate analysis showed that the moisture content in 5 g of *P. ostreatus* was approximately 3.00±01%, 3.6±0.5% of proteins, 10.5±0.4% of crude fiber, 1.5±01% of lipids, and 89.3% of carbohydrates. Similarly, the mineral profile showed the presence of Fe, Mg, Na, Zn, Cr, Mn, and K. During hemolytic activity, the biocompatibility of extract towards human RBCs was free from the risk. The cytotoxic effects on shrimps were minimal and were found to be safe for living cells with a 64.57 LC^{50} value. The extract showed maximum antibacterial activity against *P. aeruginosa, E. amnigenus, E. coli*, *E. cloacae*, Citrobacter, and *S. Serratia*. The extract also showed noticeable cytotoxic activity on human Rhabdomyosarcoma cell lines. Overall, our results indicated that locally cultivated mushroom (*P. ostreatus*) is a rich source of nutrients and therapeutic agents with obscure toxicity.

Keywords: Edible mushroom; hypocholesterolemia activities; polysaccharide content; Nutritional benefits; Food production

Introduction

Mushrooms are fruiting bodies of higher fungi, belonging to Ascomycetes and Basidiomycetes. They play an important role in maintaining the equilibrium of Earth's ecosystem. Their deep mycorrhizal root networks help feed most of the forests and help in recycling dead leaves into the soil [1]. There are two major groups of mushrooms: non-edible mushrooms and edible mushrooms. Non-edible mushrooms contain toxic secondary metabolites and are antiviral, antibiotic, mind-altering, bioluminescent, and some possess psychoactive properties [4-6]. Non-edible mushrooms with psychoactive

characteristics have played a native role as traditional medicines in many cultures to treat mental instability. They have been used as drugs to heal mental and physical illnesses and to facilitate visionary statuses. They are also known as psychedelic mushrooms that produce psychedelic bioactive compounds i-e, Psilocin and Psilocybin. Magic mushrooms are commonly used for their recreational effects. Additionally, some types are known to help prevent migraines, *Psilocybe semilanceata* is an example [2]. Edible mushrooms are approximately 2000 species and only 25 species are generally recognized as truly favorable to eat. Among them, few are recommended to cultivate commercially. For the last fifteen years, they have been growing continuously, particularly due to their nutritional and attractive culinary benefits, and they were considered as an important food item with many sensory characteristics. Since they are low in calories with low content of fats, carbohydrates, and sodium, hence, they are good for lowering cholesterol levels in the body. Besides this, they are enriched with important nutrients including vitamin D, proteins, selenium, potassium, niacin, riboflavin, and fibers[3]. Apart from being a nutrient supplement, mushrooms have also played a major role in therapeutic activities. [4][5]. Some species enhance immunity when consumed in the form of tablets. The polysaccharide content is used in the treatment of cancer, as they have been used to treat HIV effectively [6][7]. Some members of this group possess antiviral, antibacterial, antitumor, antibiotic, hematological, immunomodulatory, and hypocholesterolemia activities [8]. The most popularly known species of Pleurotus genera are P. ostreatus (oyster mushroom), *P. eryngii* (king oyster), *P. djamor* (pink oyster), *P. tuber regium* (king tuber oyster), *P. citrinopileatus* (golden oyster), *P. pulmonarius* (phoenix oyster), P. cystidiosus (abalone mushroom), *P. nebrodensis* (white ferula mushroom), *P. cornucopiae* (branched oyster mushroom) and *P. sajor-caju* (grey abalone oyster) [9]. This is why *P. ostreatus* is one of the most grown mushrooms relative to other edible mushrooms and is an excellent choice for food production that requires critical environmental conditions [10]. In recent years, compounds with medicinal properties have been isolated from fructifying species of basidiomycetes, including Pleurotus spp. These compounds have been recognized for their clinical benefits, including anticancer, immunomodulatory, anti-viral, antibiotic anti-inflammatory, and cholesterol reduction effects. P. ostreatus, the third-largest cultivated mushroom for food purposes, is nutritionally rich in protein, fiber, carbohydrates, vitamins, and minerals, with a special aromatic property. Its high protein and vitamin content and low-fat content make it a popular substitute for refined foods like bread and dairy products. The moisture percentage of fresh fruiting bodies depends on the mushroom species [11][12]. In this study, we evaluated the nutritional properties of P. ostreatus [Figure 1] and evaluated the presence of mycotoxins (ochratoxin A and Penicillic acid).

2. Methodology

2.1 Study Design

The research was carried out to determine the nutritive value of locally grown P. ostreatus as well as to evaluate the beneficial biological properties of its crude methanolic extract with acceptable toxicity. The study was performed in the Microbiological Research laboratory at the "Centre of Biotechnology and Microbiology (COBAM), University of Peshawar (UOP), Peshawar" with the collaboration of "Post Graduate Laboratory Plant Science Building, Department of Chemistry from Agricultural University Peshawar", "PCSIR Laboratories Complex Peshawar, Ministry of Science and Technology, Government of Pakistan" and "Pakistan Institute of Engineering and Applied Sciences (PIEAS) Islamabad".

2.2 Sample Collection

A locally cultivated oyster mushroom (P. ostreatus) that is commercially available was purchased in spring of 2019, from the Mushroom farm of Agriculture University, Peshawar. A total of 5 kg sporocarp (fruiting body) was collected for extract preparation. The collected samples were deposited in the collection of the Microbiology lab, COBAM, University of Peshawar to evaluate their different biological activities. The collected samples were stored and maintained in 4℃ freezers.

Figure 2. Sample collection from Mushroom farm of Agriculture University Peshawar (a) Oyster mushroom in saw dust bags. (b) Newly arise mushroom fruiting body from the substrate. (c) Collected fruiting bodies of P ostreatus.

2.3 Cutting and Drying of sample

The fresh fruiting bodies of mushrooms were cut into sizable pieces (about 2 cm) with the help of knife and were shaded-dried for about three days at room temperature. Dried mushroom fruiting bodies (10 g) were ground to powder of particle size less than 0.5 mm in a high-speed blender. A sample of finely dried out 20 mesh mushroom powder was prepared.

2.4 Sample Preparation and evaluation of toxins using HPLC

After grinding, the powdered material was weighed and then divided into two parts: part A (500 g), for proximate analysis; part B (1.5 kg) for preparing liquid extract. Part B (1.5 kg) was added to 2000 mL round bottom flask containing 1000 mL of HPLC methanol for extract preparation. The suspension was stirred manually for 3 hours and then was transferred to 250 mL flasks for continuous shaking in methanol at 25 ℃ at 150 rpm for three days (72 hrs.) in a shaking incubator [13]. After three days of continuous stirring the flasks containing the extract suspensions were taken and then the extract was filtered in a sterile 1000 mL flask [14]. The contents were passed through gauze fabric to get maximum quantity of extract, and Whatman filter paper No. 41 was used to filter it for 5 hrs. The filtrate was evaporated using a rotary evaporator to remove the solvent. The pressure was kept below 700 mmHg and temperature was 45 °C in order to avoid degradation of phenolic compounds. The remaining content (extract) was re-dissolved in methanol for preparing its stock solution and then moved to a falcon tube (50 mL) and weighed. The solution was stored at 4 °C until being used for further research [15]. A part of extract was refined for the evaluation of toxins by using HPLC. For this 5 mL of total 25 mL extract was taken into a falcon tube and 5 mL of H-hexane was added to remove unnecessary fatty acids. Centrifugation of this solution (10 mL) was performed for 10 minutes at 3000 rpm. Fractions were separated from each other by solvent-solvent distillation. After fractionation with H-hexane, the remaining portion was evaporated by rotary evaporator and 5 mL of extract was obtained as residual fraction which was used for HPLC. The residual 5 mL extract obtained after fractionation was again filtered using syringe filters (0.22-micron filter). The solution will be pushed down via the filter into the vials drop wise. This results in the purification and filtration of samples directly into the 2 ml HPLC vials. After that these vials will be carefully labelled and preserved. Standards of Ochratoxin A and Penicillic acid were obtained from Department of Chemistry, Agricultural University Peshawar with >98 percent purity. 5 µL of the OTA and Penicillic acid each as reference standard were injected onto the HPLC column under specified chromatographic conditions to detect the minimum detectable level. The chromatograms of standards were recorded. The peaks showed the appearance of many toxins, particularly Penicillic acid and Ochratoxin.

2.5 Proximate analysis and mineral analysis

To determine the composition of the fruiting bodies of the mushroom, the part A (500 g) of dried mushroom powder was utilized which was already prepared and stored in air-tight bottle [66-67]. For Moisture contents in the fruiting bodies of oyster mushroom, 2 g of the powdered fruiting bodies were placed on clean and dry flat-bottom dishes whose weight was taken in electrical balance. These dishes were then kept in oven at 102 ℃ for 6-8 hrs to remove complete moisture. After oven drying the dishes containing the sample were again weighed to measure constant weight. The activity was done in triplets and average weight was taken [16].The percentage of moisture of dry sample was calculated as follows:

$$Moisture\ (\%) = \frac{Initial\ weight\ of\ sample - final\ weight\ of\ sample}{weight\ of\ sample} X\ 100 \quad (Equation.1)$$

For ash content, two grams of the sample were weighed into a clean silica-crucible. Over a low flame, the crucible was charred completely by using a Bunsen burner while being placed on a clay pipe triangle, then the crucible was heated in a muffle furnace at 600 ℃ for about 5~6 hours. After heating the crucible was cooled in a desiccator for 30 minutes and weighed. The ash was almost grey or whitish. The process was carried out in triplets to obtain the average weight of ash. The following formula was used to calculate the total ash content:

$$Ash\ content\% = \frac{wt.\ of\ ash}{wt.\ of\ sample\ taken\ [57]} \times 100 \quad (Equation.2)$$

Total fat content was evaluated by the slightly adjusted strategy of Folch et al.,(1957).[17] Two gram crushed mushroom was suspended in 50 mL of chloroform: methanol (2:1 v/v) mixture in a flask which was already measured, then with continuous stirring, it was kept in a shaking incubator for 3 days. After three days, a table centrifuge system was used to filter the suspension and centrifuged at 1000 rpm. By using a Pasteur pipette, the upper layer of meweighed, was expelled and chloroform was evaporated using a rotary evaporator. The remaining was the unrefined lipid that was weighed, and the weight of the flask was subtracted to get the precise weight of fat content only.

For crude fiber determination, two grams of fat-free powdered mushroom sample were taken, and 200 mL of boiling 0.255 NH_2SO_4 to it. The mixture was boiled at regular intervals for 30 minutes while keeping the volume steady by adding water. The mixture was then filtered through a muslin cloth and cleaned the excess with hot water until it was free of acid. The product was then moved to the same beaker and 0.313 NaOH was applied to 200 mL of boiling. The mixture was filtered through a muslin cloth after boiling for 30 minutes (keeping the amount steady as before) and the residue was washed with hot water until free of alkali, then washed with ether and alcohol. It was then moved to a crucible, dried at 80~100 ℃ overnight, and weighed (We) in an electric balance. The crucible was heated for 5~6 hours in a muffle furnace, cooled, and measured (Wa) again. The weight difference (We-Wa) is the weight of the raw fiber.

$$Crude\ fiber = \frac{[100 - (moisture + fat)] \times (We - Wa)}{Wt.\ of\ the\ sample\ [57].} \quad (Equation.\ 3)$$

For total protein content, the micro-kejldahl technique was carried out to analyze the amount of raw protein in the specimen. This technique first calculated the maximum nitrogen content and multiplied the sum by 6.25 coefficients [18]. Total protein content was calculated as such; raw protein = the total Nitrogen multiplied by 6.25.

The percentage of carbohydrate content was calculated by adding the total percentages of moisture, ash, fats, and proteins given by:

100-(% of moisture + % of ash + % of fats + % of proteins) [19]. *(Equation. 4)*

For mineral analysis, the powdered material (5 g) was placed in a silica crucible and heated at 400 °C in a muffle furnace until no smoke formed. The crucible was left to cool

in a desiccator at room temperature and concentrated H2SO4 (0.5 mL) moistened the ash. The crucible was put on a hot plate and heated until H2SO4 fumes stopped evolving. The sulfated ash crucible was heated at 600 °C in a muffle furnace until the material weight was constant. The ash obtained was cooled, dissolved in 6NHCl 5 mL, and let to stand for 30 minutes. It was purified then and deionized water made up the volume up to 50 mL. Using a Hitachi Zeeman Japan Z-2000, Atomic Absorption Spectrophotometer, the resulting solution was used to measure minerals, while Na and K concentrations were measured on the Jenway PFP7 Flame Photometer.

2.6 Haemolytic activity of extract

The activity was carried out to determine the hemolytic properties of various concentrations of mushroom extract using human RBCs. Methanolic extracts of oyster mushrooms were applied on human Red blood cells for hemolytic activity as described previously [20]. A blood sample was collected from a healthy human volunteer and centrifuged at 5000 rpm for 5-10 minutes. The supernatant was discarded, leaving a pellet containing red blood cells. The pellet was washed with saline solution to remove contaminants. A 2% erythrocyte stock solution was prepared by adding 1.5 mL of RBCs in 48.5 mL sterile phosphate buffer saline. The crude mushroom extract was tested at different concentrations. After incubation at 25 ℃, cells were centrifuged again at 1500 rpm for 15 minutes. The absorbance value of the liberated hemoglobin was measured at 540 nm. The activity was conducted in triplicates to calculate the average value. The percentage of hemolysis was calculated by dividing the sample's absorbance value by positive control absorbance (complete hemolysis) multiplied by 100 [21].

$$\% \text{ of hemolysis} = \frac{\text{Sample's absorbance OD} \quad x\ 100}{\text{positive control absorbance (complete hemolysis)}} \qquad \textit{(Equation. 5)}$$

2.7 Cytotoxic activity of extract

The cytotoxic effects of the mushroom extract were investigated on Artemia salina leach (brine shrimp eggs) using a protocol described by Meyer et.al. (1982). Artificial seawater (ASW) was prepared using 1000 mL distilled water in a beaker and 38 g of sea salt was mixed with a magnetic stirrer for 5 minutes. The eggs were incubated for 48 hours at 27 ℃. After two days, active nauplii were collected and placed in Petri dishes containing fresh brine solution for five different concentrations [15, 30, 60, 120, 240 µl] of mushroom extract. In each dish, five different concentrations, i-e. 15, 30, 60, 120, and 240 µl of the crude extract were added and maintained at room temperature for 24 hrs under light along with a negative control. The positive control was methanol 95% in this experiment. The next day, surviving larvae were counted. This activity was repeated three times to get average results [64-65].

2.8 Lethality concentration determination

The percentage of Lethality concentration of larvae was calculated by comparing the mean surviving larvae of the test and control petri dishes. LC50 values were calculated from the accurate line concentration plotted against lethality percentage.

$$\left(\text{Mortality \%} = \frac{100 - \text{dead shrimps in sample} \times 100}{\text{Total shrimps}} \right) \qquad \textit{(Equation. 6)}$$

The lethal concentration was measured using a program (Probit analysis) after the percentage estimate.

2.9 *Antibacterial activity*

Antibacterial activity of the crude methanolic mushroom extracts was evaluated against 7 bacterial species: *Shigella* (12), *Enterobacter cloacae* (13), Enterobacter amnigenus (15), *Serratia odoriface* (24), *Citrobacter* (25), *Escherichia coli* (57), and *Pseudomonas aeruginosa* P(N1). All the strains were maintained in sterile Eppendorf tubes containing Muller-Hinton Agar at 4 ℃ in a refrigerator. For antibacterial activity corn borer diffusion method was used [13]. Fresh test microorganism culture was spread on agar plates with the help of sterile swabs. Small plastic cork wells were placed carefully in each plate and various extract concentrations (15, 25, 30, 50 µL) using a micro-pipette. Ciprofloxacin antibiotics were taken as a positive control while DMSO was used as a negative control. Cultures were incubated at 37℃ for 24 hours, and growth inhibition was checked. Results were matched with ciprofloxacin antimicrobial agent, and inhibition zones were measured in millimeters.

2.10 Activity on cancer cell lines

To evaluate the bioactivity of methanolic extract of P. ostreatus, human Rhabdomyosarcoma cell lines were used. These cells were cultured with 10 percent fatal bovine serum and L-glutamine of 2 mM in combination with a few non-essential amino acids in a Minimum Essential Medium (MEM). The cells were then separated and washed with phosphate buffer to remove trypsin inhibitors. The cells were then mixed with trypsin and placed in an incubator. The cells were then isolated and transferred to a new cultured flask. The cells were incubated at 37°C for 24 hours, with sufficient MEM containing a 10% FBS by volume to achieve 80% confluency. The inverted microscope was used to monitor cell growth, viability, division, and morphology.

2.11 Treatment with P. ostreatus extracts

The 96-microwell plate configuration was used for sample reactions. A microplate reader was used to identify biochemical, chemical, and physical events happening in the 96-microwell. The light in the microplate reader travels to microwell and a detector quantifies the transmission of light. The microplate analyzer used was AMP PLATOS R-496. An optical fiber device of wavelengths 405nm, 450nm, 492nm, and 630 nm was.

2.12 Density of Viable Cells

To determine the quantity of cells in a suspension hemacytometer was used. 1 mL cell suspension was moved by a sterile serological pipette into cryovials for the count of cells using a haemocytometer. A cover slip over the counting surface was mounted and a micro pipettor was used to transmit ten microns of cell suspension into compartments of the hemacytometer to perform capillary operation. This chamber was placed under the microscope. In the following formula, the density of viable cells of the mixture is established:

$$Cells/ml = T/4 \times d \times 104 \qquad (Equation.\ 7)$$

Where "T" indicates the sum of four square cell numbers and "d" the dilution factor is 1 here [24]. The experiments have been conducted three times. Averages of data from three results were used as ± standard deviations (SD) using Microsoft Excel.

3. Results

The current research was carried out to determine nutritive value of locally grown *Pleurotus ostreatus* as well as to evaluate the beneficial biological properties of its crude methanolic extract with acceptable toxicity. Total 5 kg sporocarp (fruiting body) of fresh oyster were collected for extract preparation. Figure 3 shows the freshly cut mushroom

fruiting bodies of one day dried fruiting bodies [a], and completely dried fruiting bodies after three days of shade drying [b].

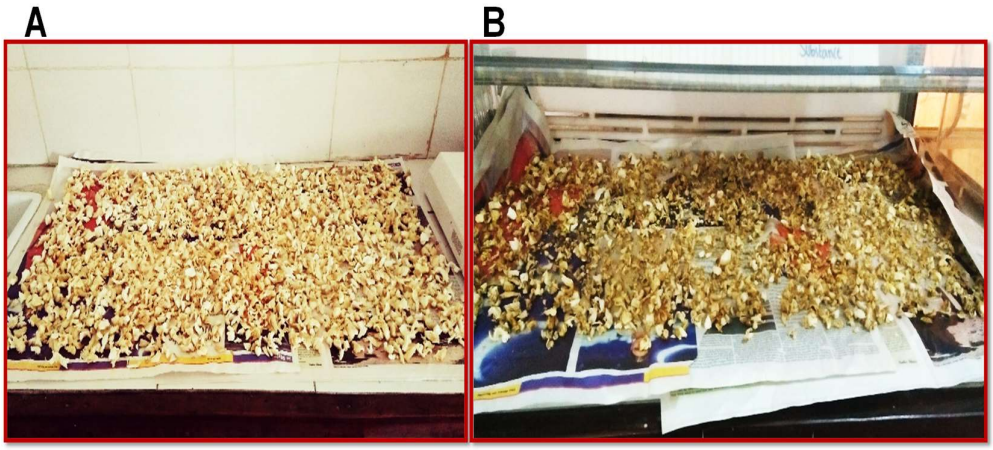

Figure 3. (a) Freshly cut mushroom fruiting bodies (b) Completely dried fruiting bodies after three days of shade drying.

Thus, the extract was run through analytical HPLC along with the standard and then by comparing the retention times of sample with the standard having same experimental condition, like solvent system, UV and column. Thus, Ochratoxin A and penecillic acid were slightly detected in the extract as shown in [Fig 4]. The concentration of ochratoxin A in extract was 3.4 ppb while the penecillic acid was 2.7 ppb. The reported data confirmed the presence of toxins in oyster mushrooms with acceptable concentration that is below the permissible limit 20 ppb as described by FDA.

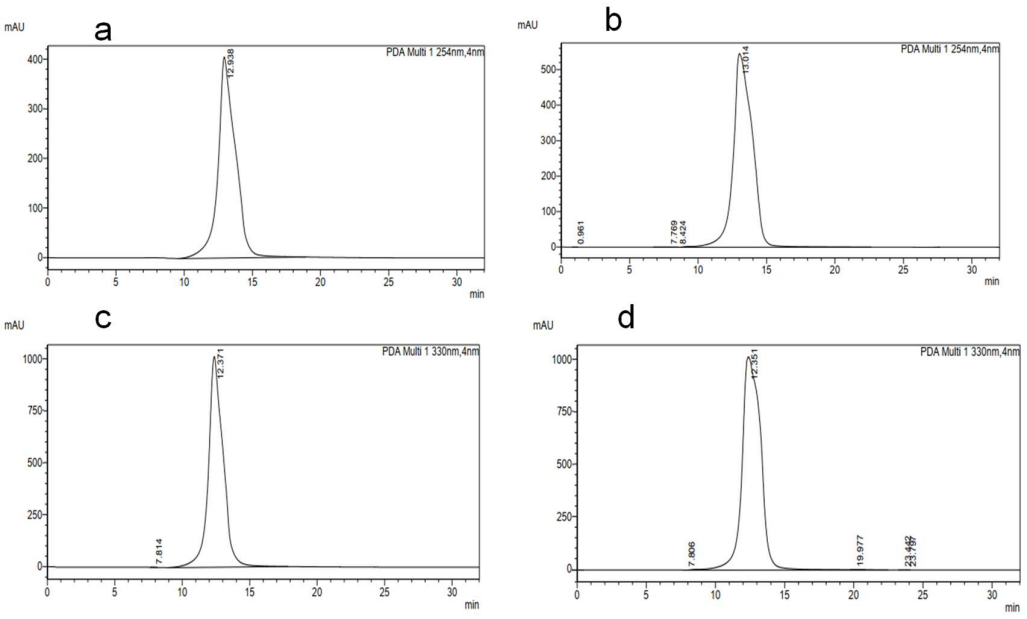

Figure 4. HPLC profile of *Pleurotus ostreatus* extract and standards. (a) HPLC chromatogram of Penicillic acid standard (10 µL). (b) HPLC chromatogram of Penicillic acid detected in sample extract of (20 µL). (c) HPLC chromatogram of Ochratoxin A standard (10 µL). (d) HPLC chromatogram of OTA detected in sample extract (20 µL).

3.1 Nutritional analysis of Oyster mushroom

Besides previous knowledge of the therapeutic potential of bioactive compounds, very few details on the nutritional composition of *Pleurotus ostreatus* are available in Pakistan. For dry mushrooms, several nutritional parameters were measured. The moisture content of *P. ostreatus* was approximately 3.00±01 percent, 3.6±0.5 percent of proteins, 10.5±0.4 percent of crude fiber, 1.5±01 percent of lipids, and 89.3 percent of carbohydrates were identified. The mushroom species were examined for their proximate composition before extraction. Table 1 assembles data obtained from the results.

Table 1. Proximate Analysis of dried sample of *Pleurotus ostreatus*

S. No	Parameters	Result
1	Moisture%	3.00±01
2	Ash%	2.6±01
3	Crude Fat %	1.5±01
4	Crude Fibre %	10.5±0.4
5	Crude Protein %	3.6±0.5
6	Carbohydrate %	89.3

Each value is a mean of 3± SD.

The data demonstrated a raw protein content of 3.6±0.5 percent per 5 g on dry weight basis in selected commercial exotic and local mushrooms. Hung and Nhi *et al.,* 2012, suggested that the *P. ostreatus* protein content was (28.6%) [9], Whilst the protein content calculated by Sumaira Sharif *et al.,* 2016, was 21.14±0.4 per 100 g of dry mushrooms [25]. Nuhu Alum *et al.,*2008, indicated that 100 g of dehydrated *P. ostreatus* accommodated a lower amount of lipid 4.6±0.26 g and were rich with protein 23.9±2.0 g while the fibre content was 24.34±1.8 g in the specimens. Carbohydrate levels calculated to be 37.8±2.5 g. The mineral content was rich as well. The total ash content was 9.36±0.5 g. The pileus and gills were rich in protein and lipids, and the stripe was rich in carbohydrates and fibre. Mushroom moisture content ranged from 86 to 86.9 percent [26].

3.2 Minerals Analysis

Mushrooms are capable of assimilating and storing large quantities of both macro and micro-elements in their fruiting bodies which are important for fungi as well as their consumers [78,79,80]. Mg, K, Na, and Ca are the primary macro-nutrients, and the most essential micro-nutrients are Fe, Zn, Se, Mn, Cu, Co, Cr, and Mo [30][31]. Macro-nutrient minerals act to maintain acid-base equilibrium, fluid, and oxygen osmotic control in the body [32][33]. In the enzyme system's catalytic procedures which comprise a broad variety of metabolic, endocrine, and immune systems enzymes, the micro-nutrient minerals (Se, Zn, Mo, Fe, V, Cu, Co, and Cr) are crucial components. Table 2 describes the conclusions gained from an analytical detection of minerals in a dry mushroom sample.

Table 2: Minerals Analysis

	Minerals	Symbols	Results (ppm)
1	Sodium	Na	20±01
2	Potassium	K	243±01
3	Iron	Fe	70±02
4	Zinc	Zn	80±01
5	Magnesium	Mg	150±02
6	Manganese	Mn	40±02
7	Copper	Cu	ND
8	Chromium	Cr	3.0±00
9	Lead	Pb	ND

10	Nickel	Ni	ND

ND= Not detected, each value is a mean of 3± SD

Detection limit Ni= 0.018 mg/l, Pb= 0.1 mg/l, Cu=0.01 mg/l

It is important to note that the composition of Fe, Mg, Na, Zn, Cr, Mn, and K is rich in oyster mushrooms. Such values have been accepted by some previous mineral content studies in mushrooms. The table above gives an overview of values in mineral composition [34,35].

3.3 Haemolytic Activity

The effectiveness of natural extracts of mushrooms as an antibacterial agent was tested for hemolytic activity [Figure 5] to analyze the use of extract for therapeutic purposes. Methanol was used as a positive control in this study. This activity was performed to check the biocompatibility of crude extract against human RBCs, and it was concluded that *P. ostreatus* extract is safe for them. Table 3 and Figure 5 outline the results obtained.

Formula: Haemolysis percentage: $\frac{\textit{Optical Density at 576 nm in sample x 100}}{\textit{Optical Density at 576 nm in 0.1\% Triton-X}}$

Table 3. Haemolytic activities of selected mushroom extracts at varying concentrations (mg/mL)

S. No	Sample	conc. (µg)	%Absorbance	%Transmittance	% Hemolysis
1	Sample 1	100	0.578	26. 4	51%
2	Sample 2	150	0.603	24.9	54%
3	Sample 3	200	0.764	17.2	68%
4	+ive control	100	1.114	7.7	98%

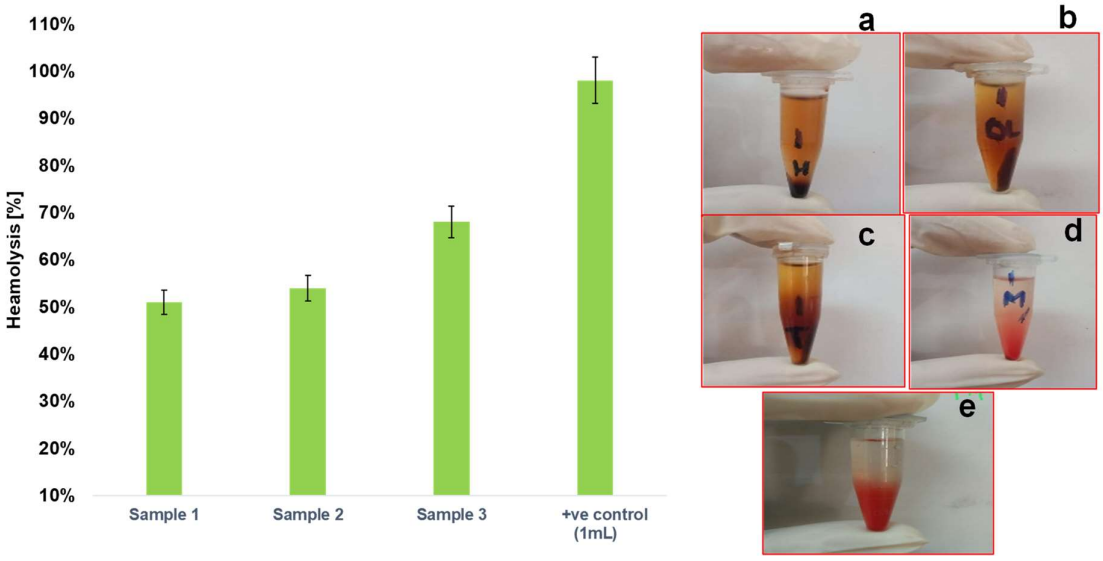

Figure 5. Percentage of haemolytic activity of mushroom crude extract. a) sample-1 with 100 µl of mushroom extract. (b) sample-2 with 150 µl of mushroom extract. (c) sample-3 with 200 µl of mushroom extract. (d) sample-4 with 100 µl of methanol. (e) sample-5 with 100 µl of Triton-X.

3.4 Cytotoxic activity of extract

Present study shows that crude methanolic extract of oyster mushroom has lesser cytotoxicity towards brine shrimps, which means that methanol alone is a good solvent for extraction of metabolites from *P. ostreatus*. The results from brine shrimps assay indicated that the percentage of death rate is directly proportional to the concentration of extract. We used minimal concentrations of crude *P. ostreatus* extract and after 24 hours, the shrimps remained alive. Even though maximum mortality rate was observed at 240 µg/ml. The data obtained from the lethality of methanolic extract of *P. ostreatus* on brine shrimps is with LC50 value was 64.57mg/mL as shown in Table **Error! No text of specified style in document.**.

Table 4: Cytotoxic activity at concentrations (µg/mL) measured for mushroom extract against Brine shrimps

S.no	Conc. (µg/ml)	No. of surviving nauplii (after 24 hrs.)	Total No. of nauplii	% Mortality	LC50 (µg/ml)
1	15	29	30	3%	
2	30	17	30	46%	
3	60	16	30	46%	
4	120	16	30	50%	64.57
5	240	1	30	96%	

3.5 Antibacterial activity

Preliminary testing of mushroom extract with antimicrobial properties was conducted against seven bacterial species, i-e, *Shigella* (12), *Enterobacter cloacae* (13), *Enterobacter amnigenus* (15), *Seretia odoriface* (24), *Citrobactor* (25), *Escherichia coli* (57), and *Pseudomonas auroginosa* N1(P). Quantitatively, our results indicated that mushroom action opposing these specified microorganisms was average. The extracts produced a zone of inhibition of selected bacteria between 4 to 14 mm. A notable action was observed by the fractions against *Pseudomonas aeruginosa* N1(P) followed by *Enterobacter amnigenus* (15), *E. coli* (57), *Enterobacter cloacae* (13), *Citrobactor* (25), *Shigella* (12) and *Seretia odoriface* (24) as shown in Figure 6. According to Sumera Sharif *et al*., *P. ostreatus* ethanolic extract demonstrated a high inhibition zone (30±0.2) against Gram positive bacteria *B. Subtilis* [25]. Throughout this study, it was observed that *Pseudomonas auroginosa* N1(P) and *Enterobacter amnigenus* (15) were very prone to mushroom extracts. The outcomes generated can be related to the study of Hearst et al., (2009)[36], who tested the aqueous extracts of two exotic mushrooms *L. edodes* and *P. ostreatus,* against 29 bacterial species and The extracts displayed substantial antimicrobial action opposing 85 percent of the species in the experiment. The previous studies proved that Gram-negative bacteria have more resistance towards mushroom extract as compared to Gram-positive strains due to more peptidoglycan in their cell layer [92,93,94,95].

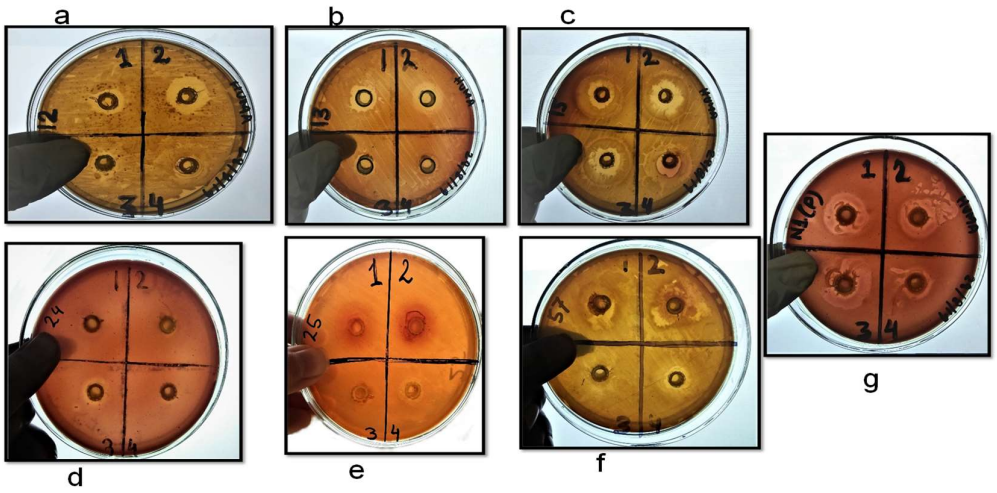

Figure 6. Antibacterial activities of *P. ostreatus* extracts by disc diffusion method. (a) (12) *Shigella* (b) (13) *Enterobacter cloacae* (c) (15) *Enterobacter amnigenus* (d) (24) *Seretia odoriface* (e) (25) *Citrobactor* (f) (57) *Escherichia coli* (g) N1(P) *Pseudomonas aeruginosa*.

3.6 Activity on cancer cell lines

In the present research a critical analysis revealed that methanolic extract of *Pleurotus ostreatus* showed significant cytotoxic activity on human Rhabdomyosarcoma cell lines compared with control. While having potential cytotoxic activity against cancerous cells, the mushroom extract has mild to lesser effect on normal cells with the correspondence of control which clearly indicates that our potential extract has 100% biocompatibility with human normal cells as shown in Figure 7.

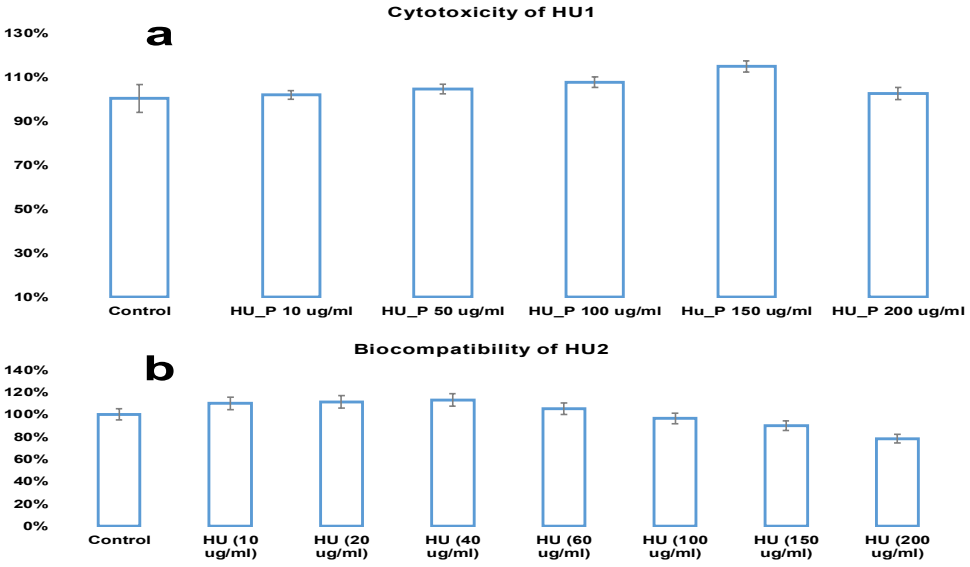

Figure 7. (a) HU1 Sample: Cytotoxicity of HU1, (b) HU2 Sample: Biocompatibility

4. Discussion

Plant-based natural products and other materials have been used as a leading source of prophylactic agents for avoiding and curing diseases. Mushrooms have managed to create a great deal of interest in their use as fruit, and they have found great promise for

both medicinal and therapeutic applications. The main objective of this analysis was to draw attention to the current outlook, developments, facts, challenges and further growth in 21st century mushroom science [25]. Mycotoxins are low molecular weight compounds produced by chemically diversified fungal generation as secondary metabolites. They are the products of *Penicillium*, *Aspergillus*, *Fusarium*, *Claviceps* and *Alternaria* which have a deleterious effect on human beings and animals [41]. In addition, the crops as fresh or in storage, contaminated with mycotoxin may also have an impact on foodstuffs for human utilization. Inadequate level of humidity and temperatures are the primary sources of mycotoxin growth [42]. As a result of poor processing and storage, many of the foods are contaminated by the proliferation of toxin producing fungi. [43]. Thirty-four dried market samples were examined for the purpose of establishing that dried mushrooms are foodstuffs less prone to toxin infection and therefore mycotoxins contamination. A number of studies have shown the primary contaminants in foodstuffs processed by *Aspergillus*, *Penicillium* and *Fusarium* species and their toxic secondary metabolites [14]. The first study on toxicity of microflora and mycotoxins in dried morels from Jammu and Kashmir was published in 2017. They classified different mycotoxins in dry morels for the toxigenic moulds. Aflatoxins, citrinin, ochratoxin and zearalenol were the most frequently isolated essential mycotoxins. Amongst all other mycotoxins, the mean level of aflatoxin B1 (125.44± 78.14) was higher[44]. Mushrooms are capable of assimilating and storing large quantities of both macro and micro-elements in their fruiting bodies which are important for fungi as well as their consumers [78,79,80]. Mg, K, Na and Ca are the primary macro-nutrients, and the most essential micro-nutrients are Fe, Zn, Se, Mn, Cu, Co, Cr and Mo [30][31]. Macro-nutrient minerals act to maintain acid-base equilibrium, fluid and oxygen osmotic control in the body [32][33].

In the present study, the HPLC results showed minimal concentration of ochratoxin A and penecillic acid in the crude mushroom extract but there is no distinctive indication that *P. ostreatus* produced these toxins by itself. There is possibility that the toxigenic moulds growing in media are responsible for the production of toxins. The culture medium for mushrooms includes waste animal manure heaps, sugarcane bagasse, plants dead waste material, corn cobs, wheat straw and sawdust so chances are there that during the cultivation of oyster mushrooms locally, thus the fungus naturally growing in the vicinity of oyster mushroom may be the reason of toxin production. Additionally, it is also concluded from the present work that varieties of other fungus may contaminate the cultivated mushroom because of poor cultivation processes and sterilization practices in Pakistan. The mycotoxicological precautions of *Pleurotus* species sold in local markets must therefore be assessed with regular interval, and the possible threats if any may be communicated to the consumers [45]. Fibrinolytics dysfunction or myocardial infarction induces a major blood thrombus, blocking the blood vessels with fibrin clots leading to artery complications such as thrombosis, heart attack, myocardial infarction and embolism of the lungs [46]. Natural extracts are being used in disease's treatment and are an inexpensive healthy substitute to cardiovascular and contagious ailments [47].

A few cytotoxic compounds are bio-assayed with the brine shrimp nauplii (*Artemia salina*). According to Rahman M. Faridur *et al*., 2010, the petroleum ether (PE) extract, crude hot water (HW) extract, methanol-chloroform (MC) extract, and residual (R) fractions showed positive values for the extracts and fractions to be cytotoxic in the bioassay of brine shrimp lethality. MC, PE and R extracts of oyster mushrooms were found to contain compounds such as steroids and terpenoids. The PE fraction showed to be extremely cytotoxic compound verified by this analysis [48]. According to Sumera Sharif *et al*., *P. ostreatus* ethanolic extract demonstrated a high inhibition zone (30±0.2) against Gram positive bacteria *B. Subtilis* [25]. Throughout this study, it was observed that *Pseudomonas*

auroginosa N1(P) and *Enterobacter amnigenus* (15) were very prone to mushroom extracts. The outcomes generated can be related to the study of Hearst et al., (2009)[36], who tested the aqueous extracts of two exotic mushrooms *L. edodes* and *P. ostreatus,* against 29 bacterial species and The extracts displayed substantial antimicrobial action opposing 85 percent of the species in the experiment. The previous studies proved that Gram-negative bacteria have more resistance towards mushroom extract as compared to Gram-positive strains due to more peptidoglycan in their cell layer [92,93,94,95].

This study clearly indicates that edible mushrooms of family basidiomycetes can act as potent anticancer products. Similarly, oyster mushrooms are composed of many bioactive compounds that have demonstrated profound in vitro and in vivo anticancer activity against different cancer cell lines animal models. Cancer disorders are one of the major causes of death globally [49]. The development of new biological compounds is a worldwide tendency to make natural substances less harmful at present [50]. Several bioactive compounds from the environment, classified and separated as an suppressor of different cancer cell lines, have been investigated [51]. Thetsrimuang *et al.* (2011), studied the anticarcinogenic action of selected mushrooms in some cell lines, including HT-29 colon and H-1299 lungs carcinoma cell lines. The greater the concentration, the lesser the percentage of cell sustainability, was found [52]. In year 2004, analysis of crude methanol extracts of 58 mushroom species from Basidiomycetes was conducted by Tomasi *et al*., in which mushroom extracts were inspected for their cytotoxic activity towards two murine cell lines, L1210 and 3LL. The findings showed that the majority of extracts (74 per cent) had IC50 > 100 mg / ml relative to both cell lines [53].

5. Conclusions

The findings of the current investigation have shown that the MeOH obtained wild mushroom extracts, have high contents of micro and macronutrients. The biological evaluations indicated that *Pleurotus ostreatus* not only have large levels of cytotoxic and antimicrobial activities, but even human erythrocytes were found to be safe when subjected to mushroom extract. Also, the levels of reported toxins by HPLC are minimal and negligible. Therefore, methanol extracts of *Pleurotus ostreatus* may be further investigated as dietary supplements and chemo-preventive medicinal products. This can open the door for grass-root people to start growing oyster mushrooms for commercial purposes that can boost the quality of life of Pakistan's poor populations.

Data availability

All data generated during this study is present within the article.

Conflict of interest

All authors declare no conflict of interest.

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