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Anticancer Potential of *Pleurotus ostreatus* (Jacq.ex.fr) P. Kumm. ethanol extract: *In vitro* cytotoxicity and apoptotic effects on HT29 colorectal cancer cells

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Abstract

Pleurotus ostreatus (Jacq.ex.fr) P. Kumm. (oyster mushroom) is a medicinal fungus known for its bioactive compounds, which exhibit potential anticancer properties. This study aimed to evaluate the cytotoxic effects of *P. ostreatus* extract on HT29 human colorectal adenocarcinoma cells. Fresh mushrooms were collected, authenticated, and subjected to ethanol extraction. The extract was analyzed for phytochemical constituents, followed by *in vitro* cytotoxicity assessment using MTT and lactate dehydrogenase (LDH) assays. Apoptosis induction was determined using acridine orange/ethidium bromide (AO/EB) staining. The results demonstrated significant dose-dependent cytotoxicity and apoptosis in HT29 cells, suggesting that P. ostreatus could be a promising source of natural anticancer agents.

Keywords: Mushroom; HT29 Cell Line; MTT Assay; LDH Assay; Secondary Metabolites

1. Introduction

Cancer remains one of the most formidable health challenges of the 21st century, with colorectal cancer ranking as the third most commonly diagnosed malignancy worldwide [1]. Despite advances in conventional therapies including surgery, chemotherapy, and radiation, the search for novel anticancer agents continues due to issues of drug resistance, severe side effects, and high treatment costs [2]. This has led to growing interest in natural products as potential sources of safer and more effective anticancer compounds [3]. Among these, medicinal mushrooms have emerged as particularly promising candidates, with a long history of use in traditional medicine and increasing scientific validation of their therapeutic properties [4].

Mushrooms of the genus *Pleurotus*, commonly known as oyster mushrooms, have attracted significant attention from researchers due to their rich content of bioactive compounds. These fungi contain various pharmacologically active components including polysaccharides (particularly β-glucans), phenolic compounds, terpenoids, and sterols, which have demonstrated immunomodulatory, antioxidant, and antitumor activities in numerous studies [5]. *Pleurotus ostreatus* specifically has shown potential in preliminary research, with reports indicating its ability to inhibit cancer cell proliferation and induce apoptosis through multiple pathways [6]. The mushroom's widespread availability, nutritional value, and safety profile make it an attractive subject for anticancer drug discovery research.

The need for the present study arises from several critical gaps in existing research. While several studies have examined the anticancer potential of various mushroom species, systematic evaluation of *P. ostreatus* against colorectal cancer remains limited [7]. Most existing studies have focused on crude extracts without detailed investigation of the underlying mechanisms of action. Furthermore, there is a paucity of research examining the dose-dependent cytotoxic effects of *P. ostreatus* extracts on specific cancer cell lines using standardized in vitro assays. The HT29 colorectal

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adenocarcinoma cell line, being one of the most studied models for colorectal cancer research, provides an excellent system for such investigations but has been underutilized in mushroom research.

This study aims to address these gaps by conducting a comprehensive *in vitro* evaluation of *P. ostreatus* ethanol extract against HT29 cells. The research employs multiple complementary approaches including cell viability assessment (MTT assay), membrane integrity evaluation (LDH assay), and apoptosis detection (AO/EB staining) to provide a robust analysis of the extract's anticancer potential. By establishing dose-response relationships and characterizing the mode of cell death induced by the extract, this work contributes valuable data to the growing body of research on mushroom-based anticancer therapies. The findings may provide a foundation for future studies involving compound isolation, mechanism elucidation, and in vivo evaluation, potentially leading to the development of novel adjunctive or alternative therapies for colorectal cancer management.

The significance of this research extends beyond academic interest, as it explores the therapeutic potential of a readily available natural resource that could be developed into affordable treatment options. In an era of increasing cancer burden and healthcare costs, such investigations into natural anticancer agents assume particular importance, offering the possibility of complementary approaches that may enhance existing treatment regimens while minimizing adverse effects. The study also contributes to the broader scientific understanding of fungal metabolites and their potential applications in modern medicine.

2. Materials and Methods

2.1. Collection and Authentication of Mushroom

Fresh *P. ostreatus* specimens were collected from a local forest and authenticated by a mycologist. The fruiting bodies were cleaned with distilled water to remove debris, shade-dried at room temperature for 72 hours, and ground into a fine powder using a sterile blender [8].

2.2. Extraction Process

The powdered mushroom material (100 g) was subjected to Soxhlet extraction using 70% ethanol as the solvent (water: ethanol ratio 30:70) following the method described by Patel et al. [9]. The extraction process was carried out for 6 hours at 60°C. The resulting extract was filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator (Buchi R-210, Switzerland) at 40°C under reduced pressure. The concentrated extract was stored at 4°C until further use [10].

2.3. Phytochemical Analysis

Qualitative phytochemical screening was performed using standard protocols [11]

- Alkaloids: Wagner's reagent test
- Flavonoids: Aluminum chloride test
- Phenols: Folin-Coatue method
- Terpenoids: Salkowski test
- Polysaccharides: Molisch's test

Quantitative analysis was performed to determine

- Total phenolic content using the Folin-Coatue method [12], with results expressed as mg gallic acid equivalents (GAE) per gram of extract
- Total flavonoid content using the aluminum chloride colorimetric method [13], with results expressed as mg quercetin equivalents (QE) per gram of extract

2.4. Cell Culture Maintenance

HT29 human colorectal adenocarcinoma cells (ATCC® HTB-38™) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 go/mL amphotericin B) [14]. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in a HERA cell 150i CO_2 incubator (Thermon Scientific, USA).

2.5. MTT Assay for Cell Viability

The MTT assay was performed as described by Mossman [15] with modifications. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere for 24 hours. Various concentrations (50-500 µg/mL) of *P. ostreatus* extract were added and incubated for 24 and 48 hours. After treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours. The formazan crystals formed were dissolved in 100 µL DMSO, and absorbance was measured at 570 nm using a microplate reader (BioTek Synergy HT, USA).

2.6. LDH Release Assay

LDH activity was measured using a commercial LDH cytotoxicity assay kit (Takara Bio, Cat. No. MK401) according to the manufacturer's instructions [16]. After treatment with different concentrations of the extract, 100 µL of cell culture supernatant was collected from each well. The reaction mixture was prepared by adding 50 µL of catalyst solution to 50 µL of dye solution, incubated for 30 minutes at room temperature protected from light, and absorbance was measured at 490 nm.

2.7. Apoptosis Detection by AO/EB Staining

Apoptosis was assessed using the dual staining technique with acridine orange (AO) and ethidium bromide (EB) as described by Ribble et al. [17]. HT29 cells (1×10^5 cells/well) were seeded in 6-well plates and treated with IC_{50} concentration of the extract for 24 hours. Cells were then washed with PBS and stained with AO/EB solution (100 µg/mL each in PBS) for 5 minutes. Stained cells were immediately observed under a fluorescence microscope (Nikon Eclipse Ti, Japan) at 40× magnification.

3. Results

3.1. Phytochemical Analysis

The ethanol extract of *P. ostreatus* tested positive for phenols, flavonoids, and polysaccharides. The total phenolic content was 45.2 mg gallic acid equivalents (GAE) per gram, while the flavonoid content was 28.6 mg quercetin equivalents (QE) per gram (Table 1).

Table 1 Phytochemical Composition of P. ostreatus Extract

Phytochemical	Result
Total Phenolic Content	45.2 mg GAE/g
Total Flavonoid Content	28.6 mg QE/g
Alkaloids	Present
Terpenoids	Present
Polysaccharides	Present

3.2. MTT Assay for Cell Viability

The MTT assay revealed a dose-dependent reduction in HT29 cell viability after treatment with *P. ostreatus* extract. The IC_{50} value at 48 hours was 220 µg/mL (Table 2).

Table 2 Cell Viability of HT29 Cells Treated with P. ostreatus Extract

Concentration (µg/mL)	Viability (%) at 24 h	Viability (%) at 48 h
50	85.2 ± 3.1	72.4 ± 2.8
100	70.5 ± 2.6	58.3 ± 2.1
200	55.8 ± 1.9	42.7 ± 1.5
300	40.1 ± 1.4	30.6 ± 1.2
500	25.3 ± 0.9	18.9 ± 0.7

3.3. LDH Release Assay

The LDH assay indicated increased enzyme release at higher extract concentrations, suggesting membrane damage and cell death (Table 3).

Table 3 LDH Release in HT29 Cells After Treatment

Concentration (µg/mL)	LDH Release (U/L)
Control	120 ± 10
50	180 ± 15
100	250 ± 20
200	380 ± 25
300	520 ± 30
500	680 ± 35

3.4. Apoptosis Detection by AO/EB Staining

Fluorescence microscopy revealed that untreated cells remained green (viable), while treated cells exhibited orange (early apoptosis) and red (late apoptosis/necrosis) staining. At 200 µg/mL, approximately 40% of cells showed apoptotic features (Table 4).

Table 4 Apoptosis Induction in HT29 Cells

Concentration (µg/mL)	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis/Necrosis (%)
Control	95 ± 0.01	3 ± 1.00	2 ± 0.01
50	80 ± 1.13	15 ± 0.21	5 ± 0.03
100	65 ± 0.02	25 ± 0.32	10 ± 1.2
200	45 ± 0.03	35 ± 0.04	20 ± 1.03
300	30 ± 1.2	45 ± 0.52	25 ± 1.07
500	15 ± 1.34	55 ± 0.61	30 ± 0.05

4. Discussion

The present study demonstrates significant anticancer potential of *Pleurotus ostreatus* ethanol extract against HT29 human colorectal adenocarcinoma cells, as evidenced by dose-dependent cytotoxicity and induction of apoptosis. Our findings align with and expand upon current understanding of mushroom-derived anticancer compounds while providing novel insights specific to colorectal cancer treatment.

The observed phytochemical profile of *P. ostreatus* extract, particularly its high phenolic (45.2 mg GAE/g) and flavonoid (28.6 mg QE/g) content, provides a plausible explanation for its biological activity. These results corroborate previous findings by Gazeka et al. [18] who reported similar phenolic content (38.7-52.4 mg GAE/g) in *P. ostreatus* extracts from different geographical regions. The presence of these bioactive compounds is particularly significant as phenolics and flavonoids are known to modulate multiple cancer-related pathways, including NF-ƙb. signaling and caspase activation [19].

Our MTT assay results showing dose-dependent reduction in cell viability (IC50 = 220 go/mL at 48 h) compare favorably with recent studies on mushroom extracts. Zhang et al. [20] reported IC50 values of 195-310 go/mL for various *Pleurotus* species against colon cancer cells, while Patel et al. [21] found slightly higher IC50 (280 egg/mL) for *P. ostreatus* water extract in the same cell line. The superior efficacy of our ethanol extract may be attributed to better extraction of non-polar bioactive compounds, consistent with findings by Taufiq et al. [22] regarding solvent polarity effects on mushroom bioactivity.

The LDH release data indicating membrane damage at higher concentrations (680 U/L at 500 egg/mL) provides compelling evidence of the extract's cytotoxic mechanism. These results parallel observations by Nowacka et al. [23] who demonstrated similar LDH release patterns (550-720 U/L) in breast cancer cells treated with *P. ostreatus* polysaccharides. Our findings extend this knowledge to colorectal cancer and suggest that membrane disruption may be a common mechanism across different cancer types for *Pleurotus* compounds.

The AO/EB staining results revealing significant apoptosis induction (55% early apoptotic cells at 500 egg/mL) offer important mechanistic insights. These findings are particularly noteworthy when compared to recent work by Wang et al. [24] who reported 40-60% apoptosis in lung cancer cells using *P. ostreatus* β-glucans. Our study not only confirms these effects in colorectal cancer but also demonstrates that whole extracts may be more effective than isolated compounds, possibly due to synergistic interactions between multiple bioactive components [25].

Several aspects of our results warrant special discussion in the context of current literature. First, the relatively low IC50 value compared to many synthetic drugs suggests potential for clinical development, especially considering the extract's likely lower toxicity [26]. Second, the dual mechanism involving both direct cytotoxicity (LDH release) and programmed cell death (apoptosis) indicates multifaceted activity that could help overcome drug resistance - a major challenge in colorectal cancer treatment [27].

However, certain limitations must be acknowledged. While our *in vitro* results are promising, they require validation in animal models to assess bioavailability and systemic effects. Recent work by Chen et al. [28] has shown that some mushroom compounds may have limited oral bioavailability, suggesting potential need for formulation optimization. Additionally, the specific bioactive compounds responsible for the observed effects remain to be identified, though current evidence points to polysaccharide-protein complexes and phenolic derivatives as likely candidates [29].

5. Conclusion

The findings of this study provide compelling evidence supporting the anticancer potential of *Pleurotus ostreatus* ethanol extract against HT29 human colorectal adenocarcinoma cells. Mechanistic studies revealed that the anticancer activity was mediated through dual pathways—membrane damage, as evidenced by increased LDH release, and induction of apoptosis, confirmed by AO/EB staining. These results align with recent research on mushroom-derived bioactive compounds while providing novel insights specific to colorectal cancer treatment. The high phenolic and flavonoid content of the extract likely contributes to its bioactivity, suggesting that *P. ostreatus* could serve as a valuable source of natural anticancer agents. Moving forward, further research should focus on isolating and characterizing the specific bioactive compounds responsible for the observed effects, as well as evaluating their efficacy and safety in in vivo models. The results not only contribute to the scientific understanding of mushroom-based anticancer agents but also highlight their potential for clinical translation in cancer management. Future studies should prioritize preclinical validation to assess bioavailability, pharmacokinetics, and long-term safety, paving the way for potential therapeutic applications.

Compliance with ethical standards

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