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Original Article

Partially Purified Polysaccharides from *Lentinus edodes* (Mushroom) Scavenge Free Radicals and Induce Apoptosis in MCF-7 Cancer Cells by Regulating Apoptotic Genes

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ABSTRACT

Background: Therapeutic products obtained from nature have been gaining attention for health improvement, disease treatment, and drug development. Traditional therapeutic compounds from plants, animals, and microorganisms and their bioactive constituents have led to the development of therapeutics for various diseases, including cancer. Mushrooms are widely consumed products because of their nutritional and therapeutic potential. **Materials and Methods:** This study aimed to extract partially purified polysaccharides from *Lentinus edodes* (PLE) to a study to identify bioactive compounds by GC-MS and HPLC. Furthermore, we examined PLE to identify its antioxidant potential and effect on breast cancer cells (MCF-7 cell lines). **Results:** GC-MS revealed the presence of 45 bioactive components from PLE, and HPLC analysis showed the presence of β-glucan. Furthermore, PLE showed potential antioxidant activity, which was confirmed by significant free radical (DPPH, FRAP, and ABTS) scavenging and total antioxidant activity analysis. In addition, PLE showed noteworthy anticancer activity, substantiated by decreased cell viability in a dose-dependent manner (100, 200, 300, and 400µg/mL) manner. On the other hand, RT-PCR analysis revealed that PLE potentially up-regulated caspase-3, toll-like receptor 4, and Bcl-2-associated X protein and concurrently down-regulated B-cell lymphoma 2 (Bcl-2), which confirmed that PLE can induce apoptosis in MCF-7 cancer cells. **Conclusion:** Hence, this study revealed that PLE can induce apoptosis in cancer cells and fight against breast cancer through its antioxidant potential. These results recommend that PLE might be considered a nutraceutical for managing breast cancer.

Keywords: Mushrooms, Nutraceuticals, Breast Cancer, Apoptosis, Antioxidants.

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INTRODUCTION

Cancer is a global problem associated with uncontrolled cell proliferation and is often considered fatal because of a lack of rapid diagnostic techniques and effective therapeutics.[1,2] Although unprecedented advances have been made in medicine in terms of new diagnostic techniques and therapeutic modalities, cancer remains the leading cause of mortality and morbidity around the world.[3-5] Recent estimates from the International Agency for Research on Cancer indicate that cancer will remain the leading cause of death worldwide in the twenty-first century.[6] The Indian National Cancer Registry Programme report shows that there will be approximately 1.4 million cancer cases in India by the end of 2020. According to the report, at this rate, there will be approximately 1.6 million cancer cases in India alone by 2025.[7] According to another study; 2.3 million women worldwide will develop breast cancer by 2020, including 685,000 with fatal outcomes. Another study found that 7.8 million women will develop breast cancer from 2015 to 2020, making it the cancer with the highest incidence.[8] To date, chemotherapy is the most effective cancer treatment. Several naturally occurring compounds are used as chemotherapeutic agents, such as vincristine, bleomycin, vinblastine, camptothecin, and paclitaxel. It is noteworthy that currently available chemotherapeutic agents are often associated with several drawbacks, such as a low success rate, low specificity and particularity, and high risk of relapse.[9] This warrants the need for new, safer, and more effective chemotherapeutic agents.[10]

Naturally occurring bioactive compounds from medicinal mushrooms and herbs exhibit pronounced pharmacological and anticancer activities.[11] Several epidemiological and pharmacological studies have shown that the daily consumption

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of certain phytochemicals can reduce the risk of various cancers.[12] *L. edodes* is an edible mushroom that has been extensively studied for its anticancer, antihypertensive, hypocholesterolemic, hypoglycemic, antifungal, antibacterial, and antioxidant compounds.[13-15] Among the bioactive compounds extracted from *L. edodes*, lentinan, a pure β-(1,3)-D-glucan, is the best-studied compound. Several *in vivo* and *in vitro* studies have shown that extracts of *L. edodes* exhibit anticancer activities.[16] We hypothesized that the polysaccharides purified from *L. edodes* (PLE) may cause MCF-7 cells to undergo apoptosis, mainly by controlling apoptotic genes, to achieve their anticancer effects. Therefore, in the current study we attempted to reveal the processes by which these polysaccharides induce apoptosis and scavenge free radicals, with the goal of exposing their possible therapeutic possibilities for preventing cancer.

MATERIALS AND METHODS

Sample collection and preparation

The edible mushroom *L. edodes* was collected from the Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. The fruiting bodies of *L. edodes* were washed and freeze-dried at -40 to -50°C. The dried mushroom was then ground, sieved, and stored in an airtight container in the freezer until further analysis.

Isolation of polysaccharides from *L. edodes* fruiting bodies

To find out the bioactive of polysaccharides, their extraction process plays a pivotal role in ensuring their effectiveness. For this study, 50 g of air-dried, powdered cap parts were treated with a chloroform: methanol: water (200:200:60) solution. The residue was autoclaved with 500 mL of distilled water at 121°C for 1 hr and then filtered. The extract was then concentrated at 40°C under reduced pressure and gradually precipitated with cold ethanol. The solution was then centrifuged at 6026x g for 20 min. The precipitate was collected.

Partial purification of polysaccharide compounds using Ion Exchange Chromatography (IEC) and Size-Exclusion Chromatography (SEC)

IEC was performed using a Diethylaminoethyl (DEAE) cellulose column (530 cm; Sigma Chemical, St. Louis, MO) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Bound peptides were eluted at a flow rate of 0.2 mL/min using a stepwise salt concentration gradient (0, 0.1, 0.5, and 1 M NaCl). Fractions eluted at 280 nm were pooled and lyophilized. These fractions were further purified by SEC using a Sephadex G25 column (5×30 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Again, the peptides were eluted as before at IEC. The purification steps were performed at 4°C. Fractions eluted at 280 nm were pooled and lyophilized.

Gas Chromatography-Mass Spectrophotometry (GC-MS)

PLEs were subjected to GC-MS as described by Uddandrao *et al.*[17] Bioactive compounds were identified based on their mass spectra and the National Institute of Standards and Technology database (NIST).

High-Performance Liquid Chromatography (HPLC)

β-Glucan was identified from PLE using an established HPLC method, as previously described.[18] HPLC was performed using a C18 column (5 mm, 250×4.6 mm, temperature: 35 °C). Acetonitrile (A) and aqueous solutions (B) were used for gradient elution (flow rate: 0.5 mL/ min). The gradient was changed (v/v) from A to B as follows: 5-25% (0-5 min), 25-55% (5-10 min), and 55-100% (10-15 min). The UV detector was set at 254 nm.

Determination of Antioxidant Capacity

The antioxidant capacity of PLE was determined using Trolox as a reference. The EnSpire multimode reader (PerkinElmer, MA, USA) was used to determine absorbance at the desired wavelength. Results are expressed as μmol TE/g polysaccharides.

DPPH assay

The DPPH scavenging activity of PLE was determined using the method proposed by Valko *et al.*[19] Briefly, 200 μL DPPH solutions were mixed with 10 μL standard, blank, or sample. The solution was incubated in the dark for 60 min, and the absorbance at 517 nm was determined. Then, the reduction in absorbance, which indicated the proton-donating activity of the test sample, was evaluated.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was performed as proposed by Benzie *et al.*[20] However, with some modifications. Briefly, 200 μL FRAP working solution was mixed with 10 μL standard, blank, or sample. The solution was incubated in the dark for 60 min, and absorbance was determined at 593 nm.

2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The ABTS assay was performed as described previously proposed by Re *et al.*[21] Briefly, 200 μL FRAP working solution was mixed with 10 μL standard, blank, or sample. The solution was incubated in the dark for 60 min, and the absorbance at 734 nm was determined. The decrease in the color intensity of the solutions reflected the radical scavenging activity.

Anticancer Assays of Breast Cancer Cell Lines

Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin-G, streptomycin, L-glutamine, 3-(4,5 dimethylthiozol

-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate-buffered saline (PBS), 2'7'diacetyl-dichlorofluorescein, sodium dodecyl sulphate, trypan blue, trypsin EDTA, ethylenediaminetetraacetic acid, acridine orange, ethidium bromide, rhodamine-123, Triton X-100, Dimethyl Sulfoxide (DMSO), ethanol, and bovine serum albumin were purchased from Sigma Aldrich Chemicals Pvt. Ltd., India. The remaining analytical grade reagents were purchased from Hi Media Laboratories Pvt. Ltd., India.

Cell culture

The breast cancer cell line MCF-7 was obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and cultured in DMEM containing 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), and penicillin (100 U/mL). Cell culture was maintained at 37°C and 5% CO_2.

MTT assay

The cytotoxicity of PLE was evaluated using a previously proposed method.[22] In this assay, yellow-stained MTT is reduced to a purple-stained compound, formazan, after a reaction with mitochondrial dehydrogenase produced by viable cells. Formazan is then dissolved in the dissolved solution. The absorbance of the solution was determined at 500-600 nm.

Cell viability assay

DMEM culture was prepared using viable MCF-7 cells (density: 1×10^4 cells/mL). The cells were then seeded in 96-well plates and incubated for 24 hr. The cells were then treated with different concentrations of PLE (100 to 400 µg/mL) and control and then incubated in a 5% CO_2 incubator for 24 hr. The incubated cells were then washed with fresh culture medium and treated with MTT. The plate was then incubated again at 37°C for 4 hr. The resulting formazan was precipitated and dissolved in 100 µL DMSO. The viability of the cells was determined by determining the absorbance at 540 nm. Results are expressed as percentages of stable cells. Inhibition of cell proliferation (%)=[(Mean absorbance of control-Mean absorbance of the sample)/(mean absorbance of control)]×10. All analyses were performed in triplicate.

RT-PCR analysis

Frozen MCF-7 cells were thawed and homogenized, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse transcribed using a DNA synthesis kit (Applied Biosystems, Foster City, USA) to produce cDNA. For semiquantitative PCR, 20 ng of cDNA was extracted. PCR amplification was performed for 38 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 1 min. The sequences of the primers for caspase-3, Toll-like receptor 4 (TLR-4), Bcl-2 associated X protein (BaX), and B-cell lymphoma 2 (Bcl-2) are listed in Table 1. The housekeeping gene β-actin was used for normalization.

Statistical Analysis

The experimental results are expressed as the mean±Standard Deviation (SD) of three replicates. Where relevant, the results were treated as a one-way analysis of variance (ANOVA), and the significant difference ($p<0.05$, 0.01) between means was determined by the Least Significant Difference (LSD) using Statistical Package for Social Sciences (SPSS) version 15.0 for Windows.

RESULTS

GC-MS analysis

GC-MS analysis of PLE (Figure 1) confirmed the presence of numerous phytochemicals, which are listed in Table 2. The identification of phytoconstituents through GC-MS analysis relied on data sourced from the National Institute of Standards and Technology (NIST) database, which houses an extensive collection of more than 62,000 samples. GC-MS was used for quantitative determination by linking the respective peak areas to the areas from TIC. Test materials were identified by their name, retention time, and peak area fraction. The presence of 45 peaks in the chloroform: methanol: water extract of *L. edodes* indicates forty-five compounds (Table 2).

HPLC Analysis

HPLC analysis confirmed the presence of β-glucan which is a key polysaccharide component in *L. edodes*, as shown by the HPLC chromatogram peak at a retention time of 2.434 (Figure 2). This alignment highlights the extracted β-glucan's purity and indicates a reliable and efficient extraction process used in this investigation.

Table 1: Sequences of the primers used for RT-PCR analysis.

Gene	Primer Sequence
Caspase-3	Forward 5'-AGCAAACCTCAGGGAAACATT-3′ Reverse 5'-CTCAGAAGCACACAAACAAAACT-3′
TLR-4	Forward 5'-TGGCATCATCTTCATTGTCC-3′ Reverse 5'-CAGAGCATTGTCCTCCCACT-3′
BaX	Forward 5'-TCAGGATGCGTCCACCAAGAAG-3′ Reverse 5'-TGTGTCCACGGCGGCAATCATC-3′
Bcl-2	Forward 5'-ATGTGTGTGGAGACCGTCAA-3' Reverse 5'-GCCGTACAGTTCCACAAAGG-3'
β-actin	Forward 5'-GGCACCACACTTTCTACAAT-3′ Reverse 5'-AGGTCTCAAACATGATCTGG-3′

Table 2: Tentative compounds identified in PLE through GC-MS analysis.

Peak	Name	Retention Time	Area	Area %	Height	Height %	A/H
1	1-Dodecanamine, N,N-Dimethyl-	18.314	1162542	1.17	318128	1.36	3.65
2	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	18.402	309266	0.31	104620	0.45	2.96
3	Cyclooctasiloxane, Hexadecamethyl-	20.711	1243073	1.25	594809	2.55	2.09
4	1-Tetradecanol	21.715	336503	0.34	107719	0.46	3.12
5	Ethanol, 2-(Dodecyloxy)-	22.432	247242	0.25	81406	0.35	3.04
6	Cyclononasiloxane, Octadecamethyl-	23.51	2203757	2.22	1012485	4.34	2.18
7	Octadecane	23.903	426254	0.43	193983	0.83	2.2
8	Neophytadiene	24.533	555858	0.56	245241	1.05	2.27
9	Z-25-Tetratriaconten-2-One	24.635	273665	0.28	96487	0.41	2.84
10	Dibutyl Phthalate	24.942	243212	0.24	102298	0.44	2.38
11	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16, 16,18,18,20,20-Icosamethylcyclodecasiloxane	25.999	2363402	2.38	990131	4.25	2.39
12	1,2-Benzenedicarboxylic Acid, Bis(2-Methoxyethyl) Ester	26.541	237153	0.24	90403	0.39	2.62
13	Diethylene Glycol Monododecyl Ether	27.162	658093	0.66	198742	0.85	3.31
14	Eicosane	27.268	442501	0.45	179966	0.77	2.46
15	Cyclooctasiloxane, Hexadecamethyl-	28.274	2536473	2.55	1101183	4.72	2.3
16	Phytol	28.982	2267210	2.28	498316	2.14	4.55
17	Gamma.-Sitosterol	29.181	2033649	2.05	225941	0.97	9
18	Octadecanoic Acid, Methyl Ester	29.23	750766	0.76	242339	1.04	3.1
19	Gamma.-Sitosterol	29.372	5586774	5.63	679831	2.92	8.22
20	Gamma.-Sitosterol	29.522	3339482	3.36	421879	1.81	7.92
21	Gamma.-Sitosterol	29.905	1581356	1.59	282001	1.21	5.61
22	Cyclononasiloxane, Octadecamethyl-	30.336	3467370	3.49	1404565	6.03	2.47
23	Phytol, Acetate	30.514	394708	0.4	168240	0.72	2.35
24	Cyclononasiloxane, Octadecamethyl-	32.244	3467585	3.49	1405909	6.03	2.47
25	Cyclononasiloxane, Octadecamethyl-	34.039	3569894	3.6	1458183	6.26	2.45
26	Bis(2-Ethylhexyl) Phthalate	34.884	1240104	1.25	448870	1.93	2.76
27	Methyl 7-Ethyl-10-Hydroxy-11-Hydroxy(18o)-3,11-Dimethyl-2,6-Tridecadienoate	35.12	252684	0.25	97353	0.42	2.6
28	Methyl 2-[1-(2-Methoxy-2-Oxoethyl)-2,4b,6a,9,9,10b,12a-Heptamethyloctadecahydro-2-Chrysenyl]Propanoate #	35.189	775291	0.78	260989	1.12	2.97
29	D:A-Friedo-2,3-Secooleanane-2,3-Dioic Acid, Dimethyl Ester, (4R)-	35.21	573197	0.58	240688	1.03	2.38
30	3,17di(Heptafluorobutyryl)-Trideuterio-17. Beta.-Nortestosterone	35.275	355360	0.36	114656	0.49	3.1
31	Cyclononasiloxane, Octadecamethyl-	35.733	4633964	4.67	1556313	6.68	2.98
32	Dl-.Alpha.-Tocopherol	36.129	12900915	12.99	2294941	9.85	5.62
33	9,19-Cyclolanost-24-En-3-Ol, (3.Beta.)-	36.332	4586348	4.62	507172	2.18	9.04
34	9a-(Acetyloxy)-5,7b-Dihydroxy-3-(Hydroxymethyl)-1,1,6,8-Tetramethyl-1a,1b,4,4a,5,7a,7b,8,9,9a-Decahydro-1h-Cyclopropa[3,4]Benzo	36.5	841178	0.85	149657	0.64	5.62

Peak	Name	Retention Time	Area	Area %	Height	Height %	A/H
35	Fenretinide	36.75	1044541	1.05	215546	0.92	4.85
36	26-Dehydroxy-Dihydropseudoprogenin-25-Ene	36.828	1182501	1.19	270387	1.16	4.37
37	3-N-Pentylthiolane, S,S-Dioxide	36.965	1278052	1.29	202131	0.87	6.32
38	Cyclononasiloxane, Octadecamethyl-	37.315	3229850	3.25	1231600	5.28	2.62
39	Dichloroacetic Acid, 2-Methyloct-5-Yn-4-Yl Ester	38.3	513589	0.52	117440	0.5	4.37
40	Stigmast-4-En-3-One	38.524	7155542	7.21	808308	3.47	8.85
41	Tetracosamethyl-Cyclododecasiloxane	38.817	5506566	5.55	1105843	4.74	4.98
42	Cyclohexanecarboxylic Acid, 2,2,3,3,4,4,5,5-Octafluoropentyl Ester	38.98	2176994	2.19	225380	0.97	9.66
43	Stigmasta-5,23-Dien-3-Ol, (3.Beta.)-	39.194	9481969	9.55	1017381	4.36	9.32
44	Spiro[7h-Cyclohepta[B]Furan-7,2'(5'h)-Furan]-2,5'(3h)-Dione, Octahydro-8-Hydroxy-6,8-Dimethyl-3-Methylene-, [3as-(3a.Alpha.),6.B	39.42	802725	0.81	143897	0.62	5.58
45	Z-2-Octadecen-1-Ol	39.66	1058805	1.07	95921	0.41	11.04

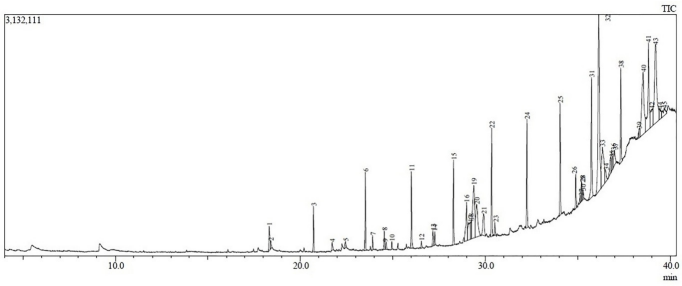

Figure 1: GC-MS chromatogram of PLE.

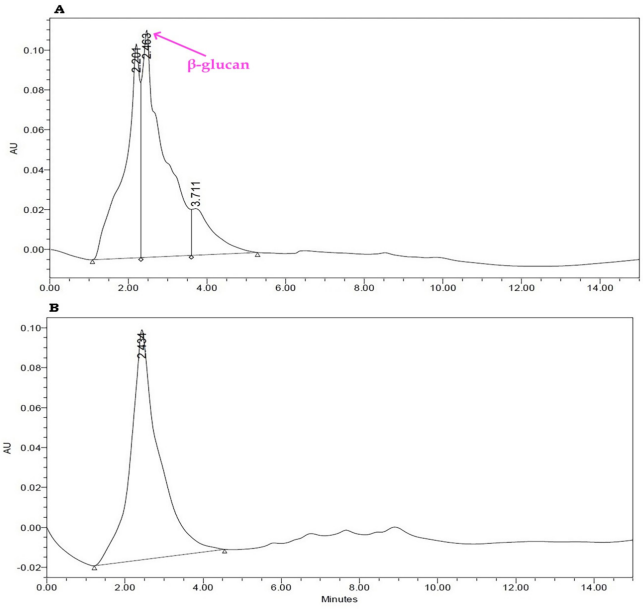

Figure 2: HPLC chromatogram of (A) PLE and (B) standard β-glucan.

Antioxidant Activity

DPPH assay

In this study, the antioxidant capacity of PLE was compared with that of ascorbic acid as the standard. Compared with the standard, PLE showed higher antioxidant activity at a concentration of 50 mg (Figure 3A).

ABTS scavenging activity

Figure 3B shows the ABTS radical scavenging capacity of PLE. A concentration-dependent increase in radical scavenging activity was observed. ABTS scavenging activities were effective at 50 mg mL^{-1}.

Determination of the antioxidant capacity

Our results demonstrate the antioxidant capabilities of PLE compounds at different doses, and show dose-dependent antioxidant activity (Figure 3C).

FRAP assay

According to the principle of this assay, all antioxidants present in PLE would reduce ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}). PLE showed higher antioxidant activity than ascorbic acid, which was used as a standard (Figure 3D).

Assessment of the anticancer activity

We investigated the anticancer activity of PLE against MCF-7 cell lines to determine its anticancer properties. Our results show that the anticancer effect of PLE is dose-dependent (Figure 4). Of all the concentrations studied, the highest anticancer effect of PLE was observed at a concentration of 400 µg/mL (Figure 4).

RT-PCR analysis

Figure 5 shows the effect of PLE on the mRNA expression of apoptotic and anti-apoptotic genes in control and treated

MCF-7 cancer cells. Analysis of RT-PCR revealed significant downregulation of mRNA of caspase-3, TLR-4, and BaX and concomitant upregulation of the mRNAs expression of Bcl-2 in the cancer cells of MCF-7. On the other hand, the study revealed that treatment of these cells with PLE (100 to 400µg) resulted in significant upregulation of mRNA expressions of apoptotic genes (caspase-3, TLR-4, and Bax) and concomitant downregulation of mRNA expressions of anti-apoptotic genes (Bcl-2) in a dose-dependent manner.

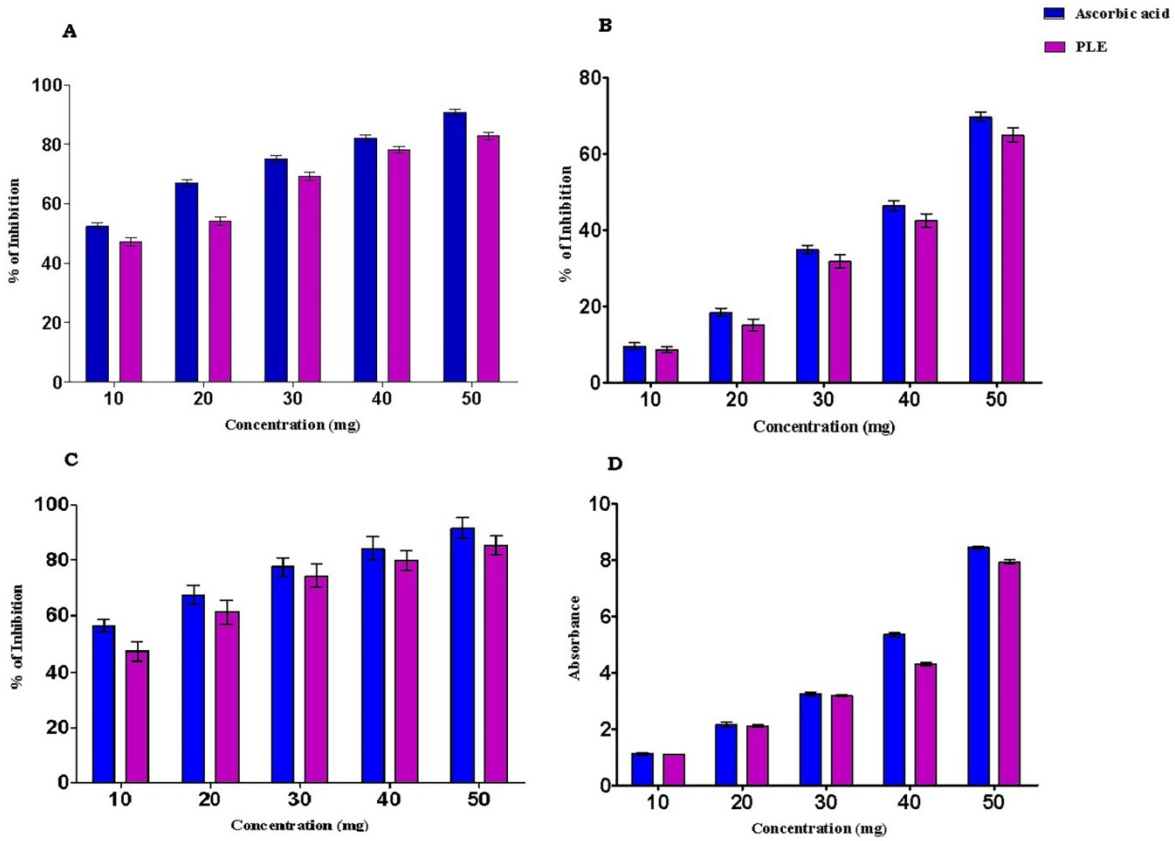

Figure 3: Antioxidant potential of PLE (A) DPPH radical scavenging, (B) ABTS radical scavenging, (C) total antioxidant and (D) FRAP. All the values are expressed as ±SD, *n*=3.

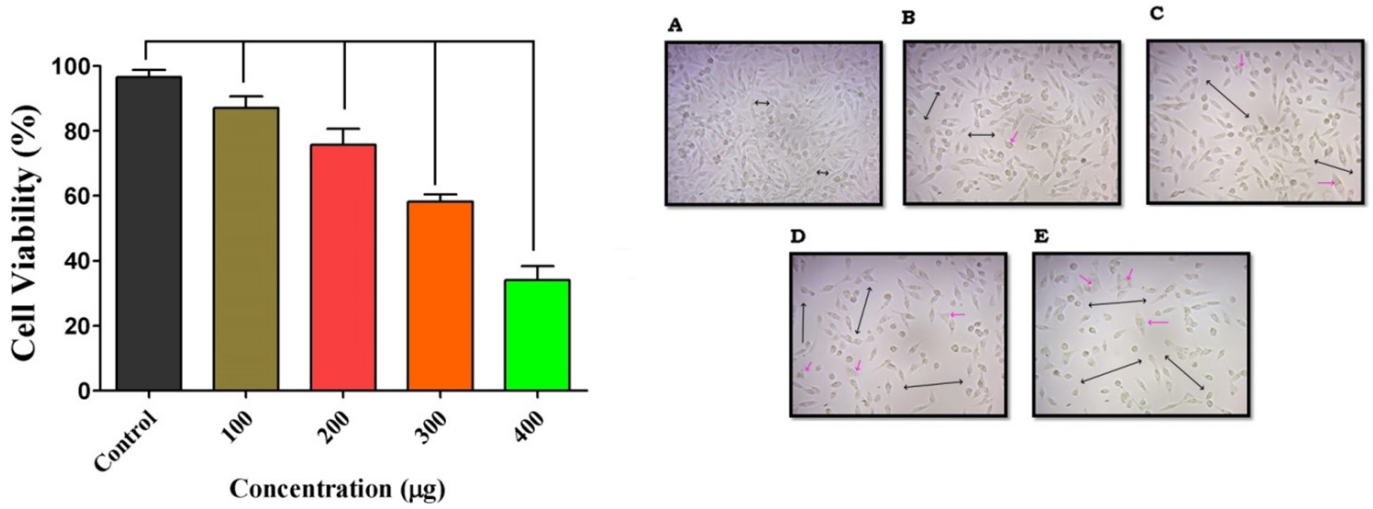

Figure 4: PLE demonstrated anticancer activity in a dose-dependent manner and significantly caused cell death in MCF-7 cell lines, (A) Normal control, (B) PLE (100 µg), (C) PLE (200 µg), (D) PLE (300 µg) and (E) PLE (400 µg). The black arrow indicate significant inhibition of proliferation, and Pink arrow indicate apoptosis or necrosis. All the values are expressed as mean±SD, *n*=3.

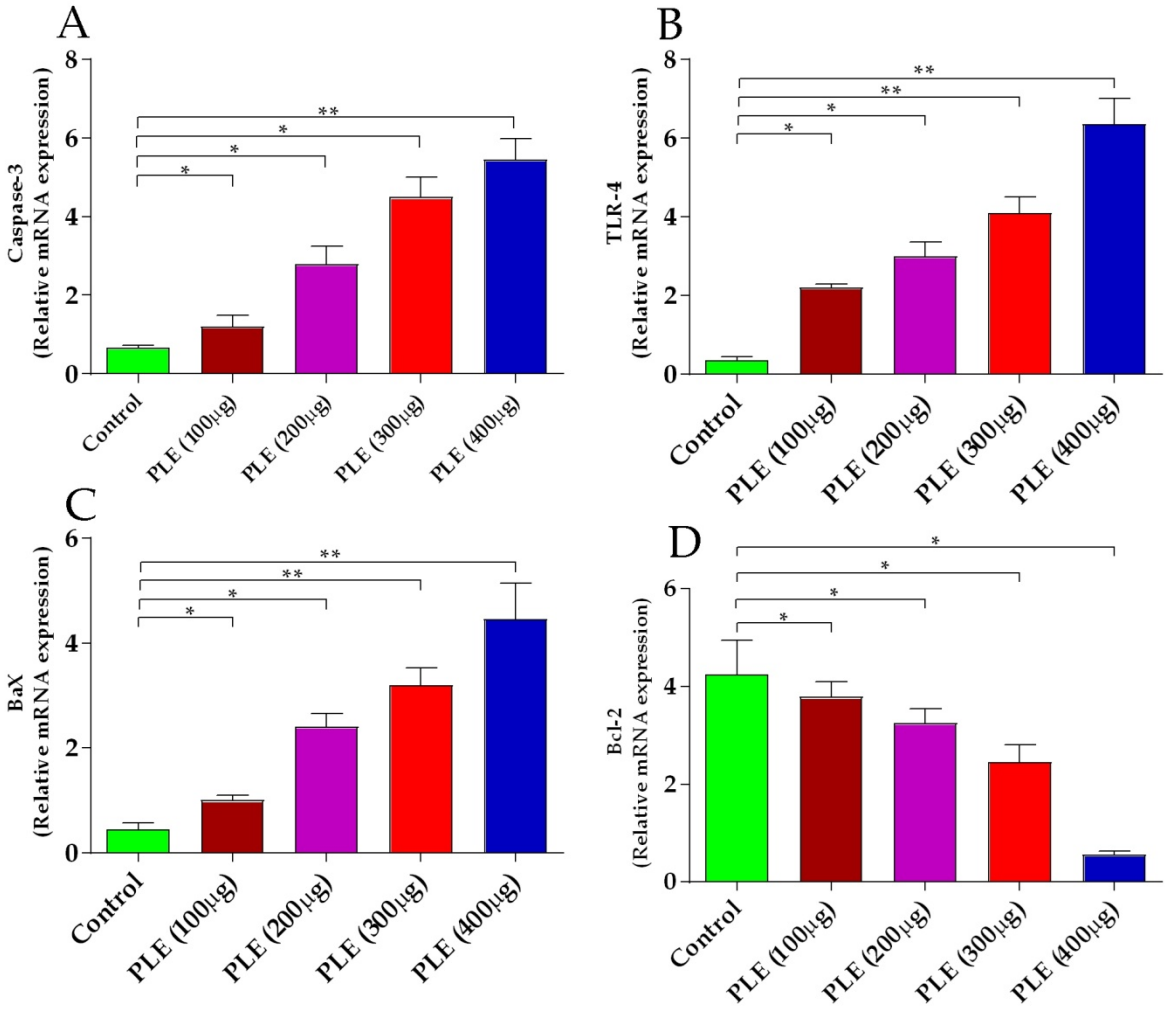

Figure 5: PLE induced apoptosis in MCF-7 cell lines via up-regulation of A) Caspase-3, (B) TLR-4 (C) BaX and concomitant down-regulation of (D) Bcl-2. Values are expressed as mean±SD, n=3, *p<0.05 and **p<0.01 vs Cancer control.

DISCUSSION

The chromatographic techniques used in this study showed that the yield of purified polysaccharides was 15.06% (w/w) on a dry weight basis. GC-MS analysis of PLE confirmed the presence of octadecane,[23] neophytadiene,[24] dibutyl phthalate,[25] phytol,[26] and fenretinide.[27] Previous studies on PLE revealed a glucan consisting of a β-(1-3)-D-glucan backbone and a single -(1-6)-D-glucosyl side branch after every three residues.[16] A previous study reported that most people in developing countries use medicinal herbs and mushrooms as primary therapeutic agents.[28,29] Although modern medicines are very effective in treating various diseases, traditional and natural remedies have attracted much attention because of their better toxicity profile and fewer adverse effects.[30] This study used GC-MS to screen PLE for possible health-promoting effects, such as antioxidant and anticancer properties. The DPPH free radical assay is commonly used to determine free radical scavenging abilities.[31] Our results showed that the free radical scavenging activity of PLE was directly proportional to polysaccharide concentration, with the highest activity observed at 50 mg/mL. The radical scavenging activity of PLE can be attributed to the hydrogen-donating ability of the polysaccharides.[32] The ABTS radical scavenging assay is widely used to evaluate the antioxidant activity of compounds.[33] The antioxidant activity of PLE is primarily mediated by the promotion of antioxidant enzymes and radical scavenging activity. We observed that PLE at a concentration of 50 mg/mL reduced ABTS. Our results are consistent with those of Chikari *et al.*,[34] who also demonstrated the antioxidant activity of PLE against ABTS. The antioxidant activity of PLE can be attributed to its polyphenolic components.[35]

Cancer cells are characterized by drastically altered signaling pathways leading to uncontrolled proliferation, induced metastatic activity, and accelerated angiogenesis.[36] Chemotherapeutic agents primarily disrupt the signaling pathways that induce proliferation and metastasis, leading abnormal cells to apoptosis.[37] Our results suggest that PLE and its parent fungus have great potential as natural antioxidants. One of the significant challenges in current cancer research is the formulation of a fungal-based

therapeutic modality with minimal side effects.[38] Our results also demonstrated the high cytotoxic potential of PLE against breast cancer cell lines. The compounds identified by GC-MS in this study, including Stigmast-4-En-3-One (Zhiyun *et al.*, 2023),[39] Fenretinide (Mody *et al.*, 2014),[40] Bis(2-ethylhexyl) phthalate (Habib *et al.*, 2012),[41] Phytol (Pejin *et al.*, 2014),[42] γ-Sitosterol (Sundarraj *et al.*, 2012),[43] and dl-alpha-tocopherol (Vitamin E) (Sigounas *et al.*, 1997),[44] have been previously reported for their anticancer properties. Furthermore, the anticancer effect of any drug is due to the host's immune system. Therefore, we hypothesized that the observed anticancer activity in this study might be due to the presence of these compounds and the β-D-glucan contained in PLE binds to the surface of lymphocytes or the serum proteins that activate TH cells, macrophages, natural killer cells, etc. This interaction leads to the upregulation of the synthesis of antibodies and other interferons and interleukins.

Caspases are proteolytic enzymes that mediate the apoptotic process leading to cell death. Because of its association with the 'death cascade' and its promotion and enhancement, caspase-3 is thought to play an essential role in cell entry into the apoptotic pathway.[45] Apoptosis, often called "programmed cell death," is an essential gene-regulated process in pathological and healthy conditions. Death receptors, caspase activation, mitochondrial responses, BaX, and modulation of Bcl-2 gene expression are the primary regulatory mechanisms of apoptosis.[46] The relative abundance of available dimerization partners alters the balance of cell fate and favors either viability or cell death. The products of the Bcl-2 and BaX genes can hetero- or homodimerize.[47] After treatment, we showed that apoptosis and Bcl-2 have an antagonistic association in the MCF-7 cell line.

The expression of Bcl-2 and BaX genes was also inversely correlated. Apoptosis-promoting or -inhibiting proteins are produced by the Bcl-2 gene family. BaX, Bak, Bad, and Bcl-xs are proapoptotic proteins, whereas Bcl-2 and Bcl-xL are anti-apoptotic. According to Krajewski *et al.*[48], Bcl-2 is expressed in approximately 80% of breast tumors in humans and is associated with the presence of estrogen and progesterone receptors, which are favorable prognostic indicators of disease. This unexpected correlation between an apoptosis inhibitor and favorable prognostic indicators is supported by the higher survival rates of patients with Bcl-2-positive tumors than those with Bcl-2-negative tumors. According to previous studies, a decrease in BaX protein has been associated with a weaker response to chemotherapy in metastatic breast cancer,[49] however no significant associations were found between BaX protein and prognosis. Caspase-3 is essential for apoptosis induced by radiation and chemotherapy in breast cancer. According to Yang *et al.*,[50] it may contribute to radiation and chemoresistance in breast cancer patients. The Toll-like family of proteins, localized to the cell membrane and cytoplasm, is primarily studied in immune cells. TLR-4 is a member of this family. Numerous ligands, including DNA, RNA, viral particles, chemotherapeutic agents, and lipopolysaccharides, activate TLR-4.

Activation of TLR-4 in immune cells can activate Mitogen-Activated Protein Kinase (MAPK) and NF-kB signaling pathways, among others.[51,52] By altering secreted cytokines in the tumor microenvironment, TLR-4 activity in tumor-recruited immune cells controls antitumor immunity and T-cell maturation. TLR-4 has also been detected in breast epithelial carcinoma cells at the protein level.[53] Proliferation, growth arrest, and apoptosis are some mechanisms that control cell homeostasis. An imbalance in the ratio between cell development and cell death often causes carcinogenesis.[54] The cell cycle, a complex cellular growth and replication sequence, controls how fast cells divide.[55] It is now generally accepted that cancer can most readily be defined due to problems with cell cycle control, in which damaged or altered cells that should be eliminated can instead progress through the cell cycle and accumulate mutations. Cells tend to suffer genetic damage during this process. However, evidence suggests that organized control mechanisms can detect damage. According to Dixon *et al.*,[56] this can lead to malignancy or the beginning of carcinogenesis. The cell cycle has several regulatory points. According to Vermuelan *et al.*[57] and Singh *et al.*[58] restriction points lead to necrosis, senescence (permanent arrest), or apoptosis, all pathways by which cells can heal or eliminate damage. It is still unknown how to solve the problem of selective direct selection of cells in the apoptotic pathway. The p53, pRb, and Bcl-2 gene families and the proteins they produce have dual functions in cell division and death. When further stimulated, these chemicals can cause cell elimination, cell cycle arrest, or cell growth. The outcomes depend on several elements that promote or prevent apoptotic cell death. Mutations in the gene responsible for checkpoints are observed in different cancers.[59] Apoptosis plays a vital role in the development of human diseases, particularly malignancies. At the same time, the factors that regulate the apoptotic process (mutation, phosphorylation, and acetylation) are either inhibited, overexpressed, or altered in function.[60] Defects in this signaling pathway can potentially prolong cancer cell survival and induce resistance to antineoplastic treatments. Research on apoptosis is progressing rapidly, and new therapeutic strategies for some human diseases may be possible.[57] The upregulation of caspase-3, BaX, TLR-4, and Bcl-2 downregulation gene expression in response to PLE treatment indicates its potential as an inducer of apoptosis in breast cancer cells. Apoptosis induction is a desirable therapeutic strategy in cancer treatment because it promotes selective elimination of cancer cells while sparing normal cells. Further investigations are warranted to elucidate the molecular mechanisms underlying PLE-mediated apoptosis and to explore its clinical applications in breast cancer therapy.

When free radical and antioxidant levels are not balanced, oxidative stress occurs. Cellular elements such as DNA, proteins, and lipids can be damaged by excessive oxidative stress, which can

promote cancer development. By neutralizing free radicals that can indirectly protect cells from damage, including healthy cells, antioxidants help minimize oxidative stress. Some antioxidants contribute to DNA repair processes. This can prevent mutations in normal cells and prevent further DNA damage in cancer cells, thus delaying their growth. Antioxidants are essential for preventing oxidative damage to healthy, normal cells. Antioxidants support the integrity of cellular structures and activities by preventing oxidative stress. This is critical because some cancer therapies, such as radiation therapy and certain chemotherapy drugs, can kill both healthy and cancer cells simultaneously. Overall, these results indicate the potential of PLE in cancer therapy.

CONCLUSION

In conclusion, our study demonstrated the potent antioxidant and apoptosis-inducing potential of compounds derived from *Lentinula edodes* (PLE) against MCF-7 breast cancer cells. While acknowledging the limitations of using extracts rather than whole mushrooms, these findings suggest promising therapeutic implications. Future investigations to isolate and characterize specific bioactive compounds from mushrooms could pave the way for the development of novel anti-cancer agents with minimal side effects.

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CONFLICT OF INTEREST

The authors declare tht there is no conflict of interests.

ABBREVIATIONS

ABTS: 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid; **BaX:** Bcl-2-associated X protein; **Bcl-2:** B-cell lymphoma 2; **cDNA:** Complementary Deoxyribonucleic acid; **CO_2:** Carbon dioxide; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl sulfoxide; **DNA:** Deoxyribonucleic acid; **DPPH:** 2,2-diphenylpicrylhydrazyl; **FBS:** Foetal bovine serum; **FRAP:** Ferric ion reducing antioxidant power; **GC-MS:** Gas chromatography-mass spectrometry; **HCl:** Hydrochloric acid; **HPLC:** High-performance liquid chromatography; **MAPK:** Mitogen-activated protein kinase; **MCF-7:** Michigan Cancer Foundation-7; **mRNA:** Messenger ribonucleic acid; **MTT:** 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; **NaCl:** Sodium chloride; **NF-kB:** Nuclear factor kappa B; **p53:** Tumor protein p53; **PCR:** Polymerase chain reaction; **PLE:** Partially purified polysaccharides from *Lentinus edodes;* **pRb:** Retinoblastoma protein; **RNA:** Ribonucleic acid; **RT:** Retention time; **RT-PCR:** Real-time reverse transcription-polymerase chain reaction; **TIC:** Total Ion Chromatogram; **TLR-4:** Toll-like receptor 4; **Tris-HCl:** Tris (hydroxymethyl) aminomethane (THAM) hydrochloride; **UV:** Ultraviolet.

SUMMARY

In the present study, we attempted to isolate the polysaccharides from the edible mushroom and evaluate their anti-cancer potential against breast cancer using MCF-7 cell lines. The study revealed that the polysaccharides extracted from the mushrooms showed significant antioxidant activity and potential antiproliferative activity against MCF-7 cell lines *in vitro*. Furthermore, these polysaccharides induced apoptosis in the MCF-7 cell lines by activating caspase-3, BaX, and TLR-4 while suppressing Bcl-2. Therefore, this study revealed that the polysaccharides isolated from mushrooms could be a potential nutraceutical against breast cancer.

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