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Anticancer And Antibacterial Potential Of A Protein Isolated From The Mushroom, *Bondarzewia Berkeleyi*

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ABSTRACT

The main goal of this study is to examine *in vitro* antibacterial and anticancer activity of the protein isolated from the mushrooms *Bondarzewia berkeleyi*. The protein were isolated from the mushroom by precipitation method. The studied mushroom proteins underwent SDS-PAGE analysis, which revealed significant heterogeneity in the divided protein fractions. However, there were some commonalities amongst the mushroom samples (protein and crude). By calculating the lowest inhibitory concentration using the microdilution plate technique on *Proteus vulgaris*, the antibacterial activity was calculated. The crude extracts and protein from mushroom had relatively strong antibacterial activity against the *Proteus vulgaris*. The anticancer activity was investigated by MTT assay and the results showed, the protein has the good anticancerous activity against A549 with an IC_{50} value of 19.37 µg/ml and significantly increased ($p < 0.05$) than the standard drug (Doxorubicin). Mushroom protein treated with IC_{50} concentration representing the changes in nuclear morphology of cells. In G_0/G_1 phase (Growth Phase), 51.08%, 31.41% and 41.88% cells get arrested more than other phases in Untreated, Standard and Mushroom protein with IC_{50} concentration respectively. Accordingly, the current study demonstrates that examined mushroom protein showed a great potential for both antibacterial and anticancer properties. It implies that mushrooms might be effective natural resources to employ in pharmaceuticals for the treatment of microbial and cancer diseases.

Keywords: anticancer activity, MTT assay, mushrooms, *Proteus vulgaris*, SDS-PAGE.

Abbreviations: DMSO, Dimethyl Sulfoxide; ELISA, Enzyme linked Immunoassay; A549, Lung cancer cell line; HepG2, uman liver cancer cell line; IC_{50}, Inhibitory Concentration 50; L929, Normal fibroblast cell line; MCF-7, Human breast cancer cell line; MIC, Minimum Inhibitory Concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

1. INTRODUCTION

Mushrooms are rich in high-quality protein, unprocessed fibre, minerals, and vitamins. In addition to their nutritional value, mushrooms include bioactive compounds that are physiologically advantageous and improve health. They generate a variety of secondary metabolites with significant medicinal potential (Kosanic et al., 2013). Some types of

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mushrooms have been shown to have health-promoting qualities, such as antioxidant, antimicrobial, cholesterol-lowering, anticancer, and immunostimulatory activities. The mycelium and fruiting bodies both include substances with a variety of antioxidant and antibacterial properties (Oyetayo et al., 2009).

To survive in their natural surroundings, mushrooms require substances that are antibacterial and antifungal (Alves et al., 2013). In fact, a large number of compounds produced by mushrooms are known to be bioactive. These natural compounds include polysaccharides, proteins, minerals, fats, glycosides, volatile oils, alkaloids, terpenoids, tocopherols, flavonoids, phenolics, carotenoids, lectins, folates, enzymes, ascorbic acid, and organic acids in general. They can also be discovered in fruiting bodies, cultured mycelium, and Modern medicine places the most emphasis on polysaccharides, and β-glucan is the most well-known and adaptable metabolite with a broad range of biological activities, including antibacterial activity. As a result, antimicrobial substances may be extracted from a variety of mushroom species, eliminating the negative effects of synthetic chemicals on the body (Finimundy et al., 2013).

The development of nanovectors for drug delivery and diagnostics (Ferrari, 2005), the plan of prodrugs that could be transformed into active form when targeting tumour cells (Huennekens, 1994), gene therapy (Hunt and Vorburger, 2002), and other techniques that have an influence on malignant cells but don't affect normal cells are some of the most recent approaches to treating cancer. When paired with traditional chemotherapy and radiation, these technologies produce the best results in the early stages of the disease (Hunt and Vorburger, 2002). As a result, chemotherapy includes more than 100 anticancer medications with various modes of action. In order to maintain and enhance the immunological state of patients, several adverse effects of chemotherapy and radiation necessitate the use of additional treatments. For instance, herbal medicines with cytotoxic qualities and little adverse effects may be used as part of anticancer therapy. More people are becoming interested in using mushrooms as a raw material to make these medications. Since ancient times, folk medicine practises across the world have taken use of the curative characteristics of medicinal mushrooms (Wasser and Weis, 1999). Approximately 14,000 different types of mushrooms are recognised today (Wasser, 2012). The number of mushroom species with anticancer action varies, ranging from 200 to 331 (out of the 540 mushroom species utilised in Chinese medicine) (Dai et al., 2009). One may estimate that 150 of these mushroom species are native to Ukraine after examining these species. To identify effective herbal treatments for cancer treatment, several researchers turned to Macromycetes. There are few research that demonstrate the antibacterial and anti-cancer properties of fresh mushrooms, but there are none that address the separated protein from mushrooms. Because of this, the goal of this study is to investigate the *Bondarzewia berkeleyi* protein's *in vitro* antibacterial and anticancer properties.

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2. Materials and methods

2.1. Preparation of crude extract

The crude extract of the mushroom *Bondarzewia berkeleyi* was prepared using ethyl acetate. The dried powdered mushroom was subjected to the soxhlet extraction using the solvent ethyl acetate (10 g of dried mushroom powder for 10 hours in 100 ml solvent).

2.2. Protein extraction

To extract protein from mushroom, the fruiting bodies were first washed using distilled water and then they are crushed using lysis buffer and the homogenate was kept at 20℃ overnight. Then, for 10 minutes, the cold homogenate was centrifuged at 10,000 rpm. To the supernatant obtained ammonium sulphate was added to precipitate the protein. The protein precipitated was collected using centrifugation and then dissolved in a buffer and kept at -20℃. They are subjected to SDS-PAGE.

2.3. SDS-polyacrylamide gel electrophoresis

To run gel electrophoresis two types of gels were prepared they are stacking and separating with 4% and 10% respectively using tris-SDS, acrylamide and ammonium persulphate. The gels were cast in the casting tray. The sample to be loaded was prepared using bromophenol blue to track them while they are in the run, which was preheated at 90℃ for about 5 minutes. The casted gel was then loaded with the sample and allowed to run using tank buffer at 100V for about 3-4 hours until the sample reaches the bottom of the gel. After running the gel was removed and stained using bromophenol blue to stain the bands kept rocker for more than one hour, then the gel was removed and destained using acetone for 30 minutes. The bands are visible in the gel (Domas et al., 1981).

2.4. Antibacterial activity

The Mueller-Hinton Agar Medium was prepared in which the test organism was swabbed throughout the plate. The disc that is loaded with the sample was kept on the plate, and then they are incubated at 37℃ overnight. The zone of inhibition was measured and noted. Positive and negative controls are also used.

2.5. MIC Determination

Antibacterial activity of the mushroom protein and the crude extract was evaluated by the visible growth of bacteria in the broth present in the 96 well-plate. Serial two-fold dilution of both the samples was made in the wells containing the broth which gives the different concentration of samples present in each well which was then adjusted with the bacterial concentration from which the MIC of the broth can be measured. The control wells have only the inoculum broth without the sample in it. The plates were then incubated for 24 hours at 37°C. After which the plates are read in the ELISA plate reader at 470nm (Sarkar et al., 2007).

2.6. Anticancer activity (MTT assay)

MTT was performed in triplicate. In a 96 well plate, 5000 cells per well were seeded. Once, the cell got adhered which will take a minimum of 24 hours. After 24 hours the cells were treated with different dilution of mushroom protein (20 µg to 1 mg), except in the control well. After 24 hours of treatment, the cells were processed for MTT analysis, where

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the media of the cells were replaced by the media containing MTT (5 mg/ml) and incubated for 3 hours at 37°C in the incubator. After 3 hours remove all previous media from each well and add 100 µl of DMSO per well to keep it in the dark at room temperature for 30 mins. Take the absorbance reading at 517 nm on an ELISA plate reader.

2.6.1. Apoptosis

2 x 105 cells/2ml of cells are cultured in a 6-well plate, which is then incubated in a CO_2 incubator at 37°C overnight for 24 hours. In 2 ml of culture media, aspirate the used medium, treat the cells with the necessary concentrations of the experimental substances, and then incubate the cells for 24 hours. Remove the medium from each well and then give it a PBS wash at the conclusion of the treatment. Harvest the cells into 2ml eppendorf tubes by trypsinization. Stain cells with 200µL staining solution for 10min. Remove staining solution and wash with PBS to remove excess of dye. Carefully load the 50ul of cell suspension on glass slide and mount it under the cover slip with a drop of mounting medium before imaging Use a filter cube and a fluorescence microscope to observe under conditions where EtBr is excited at 560/40 nm and emitted at 645/75 nm, and acridine orange is excited at 470/40 nm and emitted at 525/50 nm. Image J Software version 1.48 was used to overlay the images.

2.6.2. Cell cycle analysis by Flow cytometry

The cells were treated as per the above said procedure. After that, discard the PBS, add 300 µl of trypsin-EDTA solution, and incubate the mixture for 3–4 minutes at 37 °C. Harvest the cells immediately into 12 x 75 mm polystyrene tubes, then add 2 ml of culture medium. Centrifuge the tubes at 300 x g for five minutes at 25 °C. Decant the supernatant with caution. Use PBS to wash. Decant all of the PBS. Add to 1 ml of cold, 70% ethanol. While the cell pellet is vortexed, add drops at a time. This should prevent clumping and guarantee that all cells are fixed. Incubate for 30 minutes in -20˚C freezer. For five minutes, pellet cells more quickly than live cells while aspirating the supernatant with caution so as not to lose the pellet. Be aware that as they become more buoyant after being treated with ethanol, cells that have been fixed need greater centrifugal speeds to pellet than cells that have not been fixed. Use PBS to wash twice. Use 400 µL of Propidium Iodide/RNase staining buffer on the cell pellet to make sure that only DNA is stained (PI stains all nucleic acids). Mix thoroughly. Cells should be incubated at room temperature in the dark for 15 to 20 minutes. Utilize flow cytometry to analyse the samples in PI/RNase solution (no need to wash cells).

2.7. Statistical analyses

The SPSS software programmes were used to carry out statistical analysis. To determine the statistical significance of antimicrobial and anticancerous activity activity, one-way ANOVA was used. All data are shown as the average of three parallel measurements, or mean ± SE.

3. Results and Discussion

Medicinal mushrooms exhibit an optimal food nature, due to their low sugar and fat content, nutritional value, and especially since they are healthy diet items. New therapies against secondary metabolites generated from fungus and for the identification of precursor chemicals are now of increasing interest. These bioactive substances are in demand as natural

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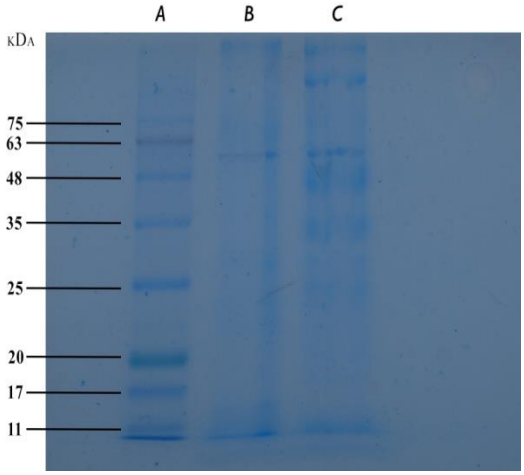
sources of immunostimulatory, antiviral, anticancer, antioxidant, and antiviral compounds. The antibacterial and anticancer properties of the Bondarzewia berkeleyi mushroom protein were examined in this study.

SDS-PAGE revealed significant protein heterogeneity in the samples of mushrooms under investigation. However, the protein and crude electrophoretic profiles of the mushroom samples with an estimated molecular mass of 56 kDa were found to be identical (Fig. 1).



A-Marker; B-Protein from Mushroom; C- Crude Mushroom

Fig. 1. SDS –PAGE analysis of protein isolated from mushroom

3.1. Antibacterial activity

The test organism chosen for the analysis of antibacterial activity was *Proteus vulgaris*, the crude protein extract gave high activity of about 21 mm, then the crude solvent extract gave the zone of inhibition was 19 mm. The positive control disc gave a 22 mm diameter of zone of inhibition. Between protein and control, there was a statistically significant difference ($P < 0.05$). From the results obtained from the antibacterial activity that is well known that Berkeley's polypore mushroom protein has high activity against the *Proteus vulgaris* bacterial species, so protein present in the mushroom can act against the bacterial species (Fig. 2).

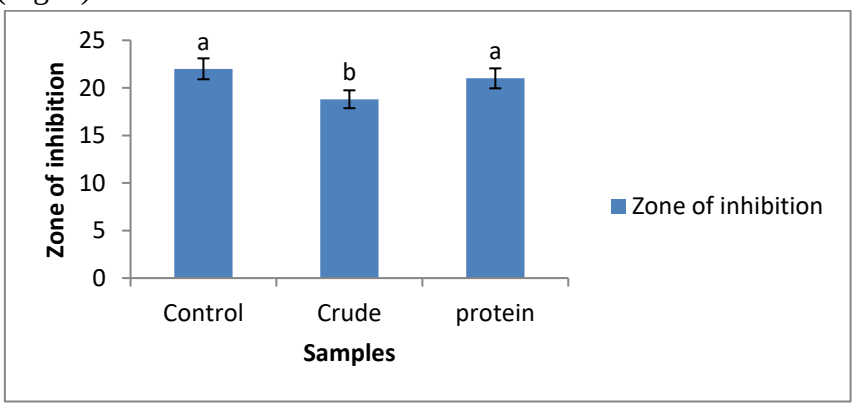


Fig. 2. Antibacterial activity of the crude solvent and protein extract against *Proteus vulgaris*

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In quest of the novel antimicrobial agents, a variety of mushrooms were tested for antibacterial activity (Ramesh and Manohar, 2010; Kosanic et al., 2013). It was discovered that the antibacterial activity shown by various mushroom species varies. The existence of various components with antibacterial activity is likely the cause of these variations in antimicrobial activity between different species of mushrooms.

Antibacterial substances are necessary for mushrooms to thrive in their natural habitats. Various molecules produced by mushrooms are in fact known to be bioactive, and these molecules include polysaccharides, fats, proteins, minerals, alkaloids, glycosides, volatile oils, tocopherols, terpenoids, phenolics, carotenoids, flavonoids, folates, enzymes, lectins, ascorbic acid, and organic acids in general. These bioactive molecules are also present in cultured mycelium, fruiting bodies, and culture broth. According to our earlier research (Sreedevi and Hespizbah Beulah, 2021), the presence of terpenoids, steroids, carbohydrate, and protein is responsible for the antibacterial action. Therefore, it is possible to extract antimicrobial substances from a variety of mushroom species, which would reduce the negative effects of synthetic chemicals on the body (Alves et al., 2013).

Minimum inhibitory concentration was measured by ELISA plate reader at 470 nm (Table 1). The result revealed that, the maazimum antimicrobial activity found in the protein against *P. vulgaris* (MIC- 0.4 µg/ ml).

Table 1. Minimum inhibitory concentration (MIC) of protein from mushroom *Bondarzewia berkeleyi*

Sample Conc. (µg/ ml)	**Optical Densityof *Proteus vulgaris***			
200	0.0047	0.0050	0.0048	0.0050
100	0.0135	0.0135	0.0134	0.0136
50	0.0590	0.0598	0.0598	0.0597
25	0.0625	0.0623	0.0624	0.0623
12.5	0.0965	0.0953	0.0964	0.0964
6.25	0.1086	0.1087	0.1085	0.1087
3.12	0.1890	0.1898	0.1897	0.1898
1.6	0.2180	0.2179	0.2173	0.2178
0.8	0.2835	0.2863	0.2838	0.2830
0.4	0.3097	0.3099	0.3096	0.3099
PvC	0.3067	0.3098	0.3108	0.3098
NC	0.0001	0.0004	0.0006	0.0004

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between different species of mushrooms. The tested mushroom protein has a comparatively robust antibacterial action in our studies. Bacterial sensitivity can be attributed to variable levels of cell wall transparency (Yang and Anderson, 2001). The gram-positive bacteria's cell wall is made up of peptidoglycans (mureins) and teichoic acids (Jean Van, 2001; Farkas, 2003). Additionally, our prior study (Sreedevi and Hespizbah Beulah, 2021) revealed that the mushroom had the potential to exhibit antibacterial action and that it included terpenoids, carbohydrates, proteins, and steroids. Based on these findings, mushroom protein appears to be a strong source of natural antibiotics and may be important for treating human diseases as well as ailments in both animals and plants.

3.2. Cytotoxic/Anticancer activity

The cytotoxicities of protein isolated from musheoom against L929 (Normal fibroblast cell line) was shown in Fiure 3. The results revealed that the protein have the potency to target cancer cells than the normal cells. Table 3 shows the cytotoxicities of protein extracted from musheoom against A549 (Lung cancer cell line). The protein were strongly inhibited the viability of A549 cells with an IC_{50} of 19.37 µg/ml. Phase contrast images of A549 and L929 cell linetreated with different concentration of protein from *Bondarzewia berkeleyi*was. There was statistically significant difference between protein and standard drug, doxorubicin ($P < 0.05$). Of all the bioactive components of mushrooms, mushroom polysaccharides have been studied the most. However, bioactive proteins make up another significant portion of the functional components in mushrooms, which are also gaining popularity because of their potential as drugs and the ability to create protein engineering with certain capabilities.

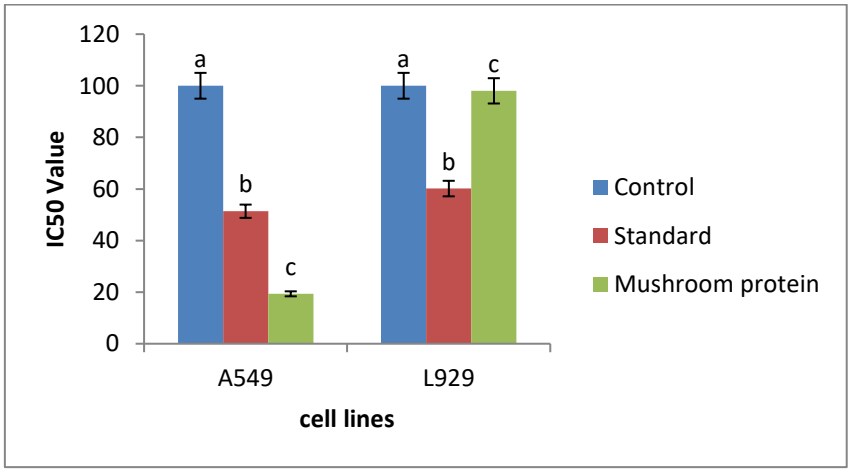

Fig. 3. Cytotoxic/Anticancer activity of protein isolated from *Bondarzewia berkeleyi*

Mushroom proteins with anticancer properties may be split into two categories: immunomodulating proteins and proteins with direct antiproliferative effect on cancer cells. Both of these methods of action may be found in the class of lectins. The most popular cancer cell lines utilised to test the antiproliferative activity of mushroom proteins *in vitro* were HepG2 and MCF-7 (Ivanova et al., 2014), but in this investigation A549 was significantly suppressed by mushroom protein.

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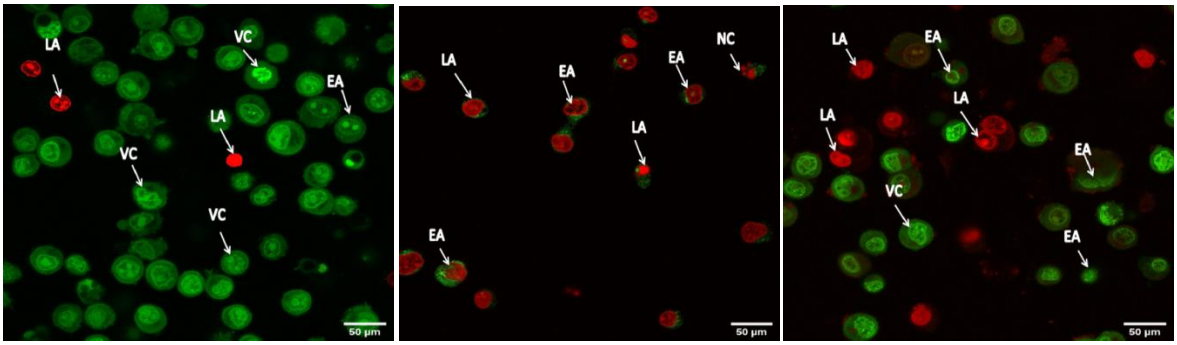
Acridine orange (AO) and Ethidium bromide (EB) dual staining study of A549 cells in Untreated, Std control (Cisplatin-8uM/ml) and Mushroom protein treated with IC_{50} concentration demonstrating the changes in nuclear morphology of cells. AO represents viable cells (green) and EtBr express dead cells (red colour). The images were captured at 40x magnification (Fig. 4).

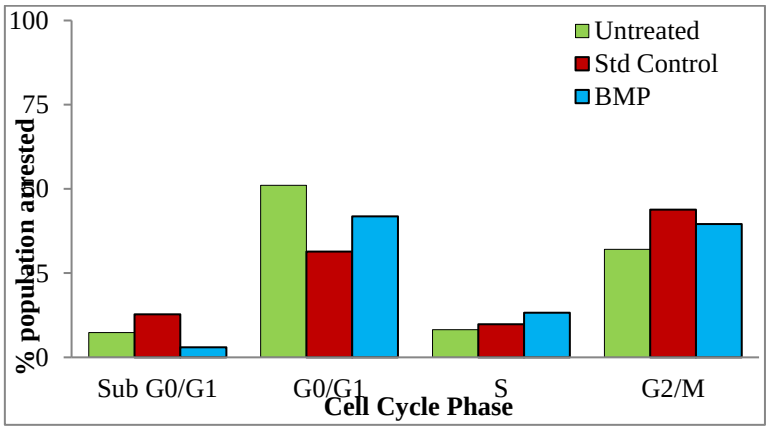


Untreated **Standard** **Mushroom protein**

Fig. 4. Changes in Morphology of the cell by treating Standard and Mushroom protein

VC-Viable cells, EA-Early apoptotic cells, LA-Late apoptotic cells and NC-Necrotic cells

Fig. 5. showed the % cells arrested in the different phases of A549 cell cycle. In Sub G_0/G_1 phase (Apoptotic phase), 7.39%, 12.81% and 2.97% cells get arrested in Untreated, Standard and Mushroom protein with IC_{50} concentration respectively. In G_0/G_1 phase (Growth Phase), 51.08%, 31.41% and 41.88% cells get arrested in Untreated, Standard and Mushroom protein with IC_{50} concentration respectively. In S phase (synthetic phase), 8.23%, 9.79% and 13.29% cells get arrested in Untreated, Standard and Mushroom protein with IC_{50} concentration respectively. On the other hand, in G_2/M phase, 32.07%, 43.84% and 39.62% cells get arrested in Untreated, Standard and Mushroom protein with IC_{50} concentration respectively.



BMP- Mushroom protein

Fig. 5. Overlaid bar graph showed the % cells get arrested in the different phases of A549cell cycle

Flow cytometric histograms depicting the different phases of cell cycle distribution in the A549cell line treated with Test compound, BMP with IC_{50} value and standard drug, Cisplatin with 8uM/ml concentration compared to the control.

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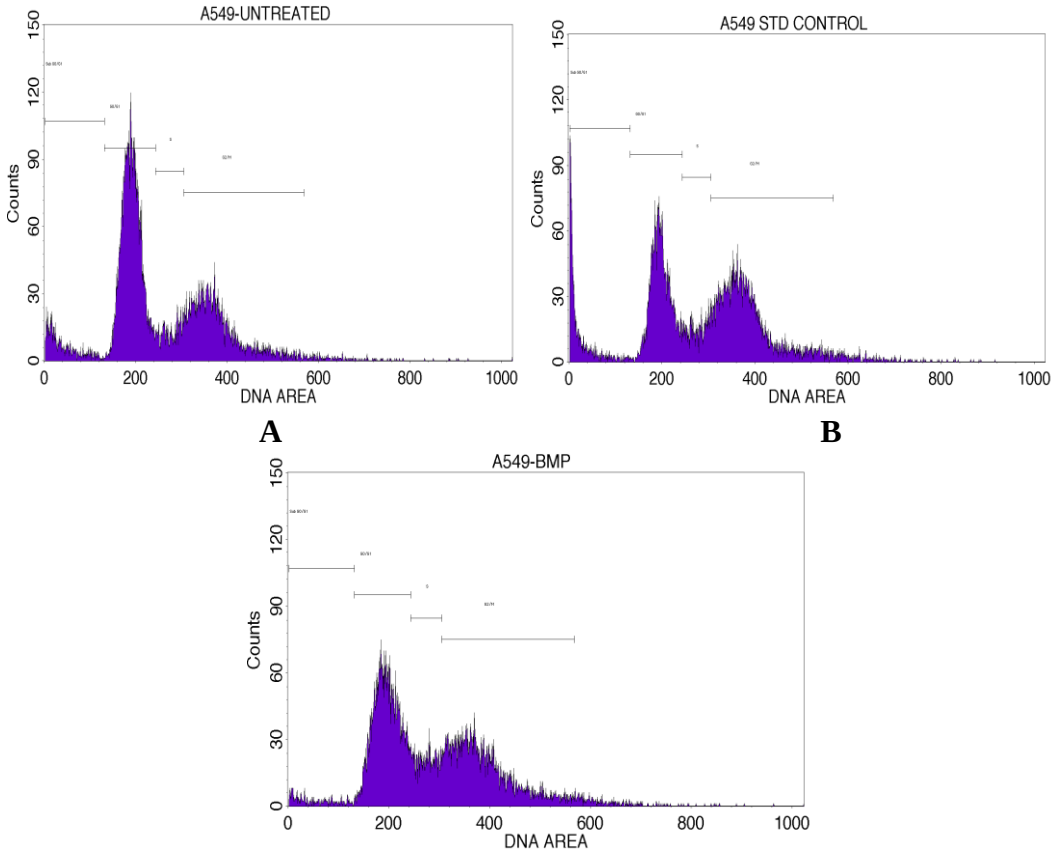

A-Cell Control, B –Std Control, C-BMP

Fig. 6. Flow cytometric histograms showed the different phases of cell cycle distribution in the A549cell line treated with mushroom protein

Medicinal mushrooms that have an anticancer effect contain polysaccharides, dietary fibre, polysaccharide-protein complexes, certain types of proteins, steroids, phenols, terpenoids, and other biologically active substances. Another significant component of mushrooms' functional components is their bioactive proteins, which are gaining interest owing to their potential for use in pharmaceuticals and the potential for protein engineering with specific features (Ayeka, 2018). When tested on MCF-7 cells, a 43 KDa antitumor protein from *Pholiota nameko* altered the distribution of cells in different cell cycle stages, disturbed the mitochondrial transmembrane potential, and caused apoptosis (Qian et al., 2016). By attaching to pathogen recognition receptors, chemicals produced from mushrooms stimulate immune cells to cause either cell-mediated or direct cytotoxicity in cancer cells. The proliferation of cytotoxic T cells and macrophages is increased by substances like lentinan, which also causes non-specific immunological reactions (Wasser and Weis, 1999). The activation of lymphocytes and natural killer (NK) cells, as well as the number of T helper cells, the CD4/CD8 ratio, and the population of macrophages, were all demonstrated to be stimulated by pleurotus tuber and pleurotus rhinoceros extracts and to have anticancer effects (Jeitler et al., 2020).

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Cui et al. (2007) examined the biological role of the new polysaccharide-peptide GFPPS1b, which was isolated from *Gtifola frondosa* GF9801 cultivated mycelia. The growth of L-02 (human normal liver cell line) was very little impacted by GFPS1b's anti-tumor action, however considerably reduced the growth of SGC-7901 (human gastric adenocarcinoma). SGC-7901 cells were subjected to apoptosis after being exposed to GFPS1b, as shown by the death of the villus, the emergence of apoptotic bodies on the cell surface, volume decrease, and chromatin condensation. The SGC-7901 cell cycle was halted in the G2/M phase, according to the findings of flow cytometry analysis and the annexin V-PI test. Reduced mitochondrial transmembrane potential, increased Bax, decreased Bcl-2, and activated caspase-3 were all signs of the apoptotic machinery. Purified recombinant fungal immunomodulatory protein from *G. tsugae* (reFIP-gts) suppresses telomerase in human lung adenocarcinoma (A549) cells. According to Liao et al. (2008), lung cancer cells treated with reFIP-gts experience early cellular senescence and are arrested at the G1 phase.

Researcher interest in mushroom products has grown as a result of their anticancer properties, according to analysis of published data. A class of proteins known as mushroom lectins can have both direct cytotoxic and immunomodulating effects on cancer cells. Additionally, it is feasible to analyse changes in membrane glucoconjugates and cancer formation using the specificity of lectins to glucoconjugate. Other applications include the classification of mutant and tumour cells, diagnostics, and the development of prodrugs. Hemolysin proteins, ribosome inactivating proteins, enzyme laccase, and ubiquitin-conjugated proteins are under-researched anticancer mushroom proteins that exhibit direct cytotoxicity *in vitro*. Because genetic engineering and large-scale amplification are possibilities, proteins can be a useful tool for medical therapy (Kosanic et al., 2013).

According to Kumar et al. (2011), antroquinonol therapy dramatically decreased the growth of A549 as shown by cell shrinkage, hole formation, apoptotic vacuoles, TUNEL positive cells, and an increase in the population of Sub-G1 cells in a dose- and time-dependent manner. In A549 cells, antroquinonol-induced apoptosis was linked to mitochondrial membrane potential disruption, caspase 3 activation, and PARP cleavage. Additionally, antroquinonol therapy lowered PI3K and mTOR protein levels without affecting pro- and anti-apoptotic proteins, while downregulating the expression of the apoptosis regulatory protein Bcl2. In A549 cells, antroquinonol changed the level of miRNA expression in comparison to the untreated control. The information taken in its entirety indicates that antroquinonol's anti-proliferative action may be a potential chemotherapeutic treatment for lung cancer.

4. CONCLUSION AND RECOMMENDATION

In conclusion, the protein isolated from mushroom *Bondarzewia berkeleyi* is a 56 kDa protein that shown as a good antimicrobial and anticancer activities. The isolation and characterisation of novel mushroom compounds with antibacterial and anticancer action merits further investigation.

A novel approach to the treatment and prevention of cancer is vaccineotherapy. In this regard, adjuvants that immunise against mushrooms can be effective parts of

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conventional and DNA vaccines. Another innovative method for treating cancer is the development of nanovectors for medication delivery and diagnostic purposes. An environmentally friendly and effective reductant in the conversion of ions to nanoparticles with targeted cytotoxicity against cancer cells is mushroom mycelia.

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