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The Efficacy of B-Glucans Enzyme and Secondary Metabolites of the White Fungus (Agaricus Bisporus) as Inhibitory Agents for Lung Cancer Cells

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Abstract: β-glucans are long- or short-chain polymers made up of glucose subunits, with β-1,3 and β-1,6 bonds, responsible for the linear and branched structures respectively, found in the cell walls of the fungus Agaricus bisporus, which have an effective role in immune modulation and as an anti-inflammatory using β-glucans extracted from the white fungus.

Qualitative inferential studies were conducted on the primary and secondary metabolites of A. bisporus extract. The results indicated that A. bisporus contains phenolic compounds, flavonoids, tannins, alkaloids, and carbohydrates, but the proportions of alkaloids, saponins, tannins, and reducing sugars were the highest compared to phenols and flavonoids. The active compounds in the extract of A. bisporus fungus were also determined, namely ash, moisture, oil, fiber, and protein. The results showed that the concentrations of fiber, moisture, protein, and oil were 14.34%, 12.18%, 9.96%, and 7.99%, respectively.

The rate of cancer cell death was determined by the effect of β-glucans enzyme on lung cancer cell line A549 pass 21 after 24 hours of treatment. Seven different concentrations of the enzyme were used, and the inhibition rates of the seven concentrations were compared for MB 231 lung cancer cells. All concentrations of β-glucans enzyme caused a significant inhibition rate of lung cells. The results showed that a concentration of 1 mg/ml achieved a significant inhibition ratio (IR) of 70.54% in the lung cancer cell line, while the same concentration showed an inhibition ratio of 98.14% in the normal cell line.

Keywords: β-glucans enzyme, Agaricus bisporus, lung cancer, active compounds.

Fungi are eukaryotic, heterotrophic organisms that play a vital role in the environment as decomposers, contributing to the recycling of elements in nature (1,2). Some are symbiotic, such as endophytes. In addition, some types have significant nutritional and medicinal value, while others contain toxic components (3).The white edible mushroom Agaricus bisporus is one of the most important types of edible mushrooms worldwide (4). In addition to being a desirable food source in terms of flavor and taste, it is distinguished by its health importance due to its content of important proteins, minerals, and vitamins, and its low fat content in general, except for saturated fats (5). It has environmental and economic importance, as it contributes greatly to the recycling of agricultural and animal waste, which constitutes a burden on humans and their environment, into an important food and economic source that provides profits for its producers. It is even called white gold, so interest in it has increased in a number of countries to produce different types of edible mushrooms (6). The white edible mushroom A. bisporus has high nutritional qualities as it contains all the essential amino acids, as well as unsaturated fatty acids with a special flavor and taste. The white cultivated mushroom is distinguished by its distinctive flavor, the composition of which is 1-octen-3-ol, the amount of which in the fruiting bodies reaches 19.3-37.2 mg L-1 (7). The white edible mushroom A. bisporus is characterized by its high protein content, reaching 37.1% of the dry weight. The amount of some vitamins per 100 grams reaches 1.1 mg Thiamin, 5 mg Riboflavin, and 55.7 mg Niacin (8). Many studies have confirmed that edible mushrooms contain compounds with high health benefits for humans, as they are anti-cancer and antiviral, and antioxidant (9). Fungi are characterized by their anti-cancer properties, due to their ability to produce many compounds and enzymes that effectively combat the growth of cancer cells (10).The edible mushroom contains compounds such as Iso-flavones and Flavones that inhibit the aromatase enzyme, which works with the hormone estrogen to stimulate the proliferation of cancer cells in women, and the inhibition rate has reached 50% (11).

β-glucans are long- or short-chain polymers made up of glucose subunits with β-1,3 and β-1,6 bonds, responsible for the linear and branched structures, respectively, found in the cell walls of the fungus Agaricus bisporus, which have an effective role in modulating immunity and as an anti-inflammatory agent. The use of β-glucans extracted from the fungus has been clinically tested in various cases. For example, respiratory infections and in the treatment of some types of cancer, as an adjunct to traditional chemotherapy (12). The immunomodulatory function of beta-glucan depends on the type of bonds, branching, and solubility of beta-glucan, with highly soluble beta-glucan showing a higher degree of performance in modulating inflammatory cytokines than low-soluble beta-glucan (13). Studies have shown that soluble, mushroom-based fiber containing beta-glucan can accelerate intestinal transit and increase stool contents, ultimately protecting the body from inflammatory bowel disease and colon cancer in humans (14).

In addition, mushroom beta-glucans may improve Crohn's disease, depression, gut health, physical fitness, skin infections, dental problems, hepatitis, herpes, HIV, and urinary tract infections in humans. (15). Compared to soluble, amplified fungal beta-glucan derivatives, water-insoluble beta-glucans exhibit minimal biological activity in terms of antitumor or antiviral activities (16). It has been reported that enlarged fungal beta-glucan derivatives have shown better anti-tumor activity in the human cancer cell lineage HepG2 compared to water-soluble branched beta-glucans (17). Beta-glucan depends on the sugar chain, chemical modification, molecular weight, and type of glycosidic bond, as it has a direct effect on immunity and acts as an anti-cancer agent due to its stimulatory effects on the immune system (18, 19).Through the role of beta-glucan in inhibiting immunity, the current research aimed to investigate the effectiveness of the β-glucanase enzyme and the secondary metabolites of the white fungus (Agaricus bisporus) as inhibitory agents for lung cancer cells.

Materials and Methods

Preliminary qualitative Screening

Preliminary investigations of secondary metabolites were carried out using tests on the alcoholic extract to identify the active compounds present, as follows:

Detection of tannins and phenols: Tannins and phenols were detected in the alcoholic extract of the white mushroom (Sofowara) using the method (20).

Detection of flavonoids: The following tests were used to detect the presence of flavonoids in the alcoholic extract of the white mushroom (Sofowara) (20).

Alkaloid Detection: Several tests were performed on the alcoholic extract to detect the presence of alkaloids in the white mushroom, using the Gibbs method (21).

Protein Detection: Biuret solution was used to detect the presence of proteins in the alcoholic extract of the white mushroom, using the Kokate method (22).

Carbohydrate detection: The method of (Kokate et al.)(22) was used to detect the presence of proteins in the alcoholic extract of the white mushroom.

Detection of saponins: The (Gibbs21) method was used to detect the presence of proteins in the alcoholic extract of the white fungus.

Assessing certain qualitative characteristics

Estimation of the ash content in the fungus A. Bisporus

Weigh 2 grams of the dried and ground fruiting body and place it in a ceramic dish, then burn it in an incineration furnace at a temperature of 600 degrees for two hours until the protein turns white. Then leave the samples to cool. After that, the protein and the amount of protein content of the fruiting body were weighed using the following equation:

% Ash = Weight of ash in the sample (g) × 100 / Weight of sample (g)

Estimating the protein content in the fungus A. bisporus

The amount of protein depends on the percentage of total nitrogen in the fruiting body and is calculated using the method described in (Sawhney and Singh) (23) using the Kjeldahl apparatus, which includes three steps: digestion, distillation, and titration, and the percentage of nitrogen is calculated according to the following equation:

$$N\% = (\text{ml of HCl} \times 0.00014 \times 50) \times 100 \text{ gm} / 10$$

The percentage of protein was estimated according to the following equation:

% of protein = % of nitrogen × 6.25

Estimation of moisture content and dry weight in the fungus A. bisporus

It was calculated as follows:

Moisture % = (Weight of fresh fruit – Dry weight / Wet weight) × 100

Dry matter % = 100 – Moisture content

It was calculated as follows:

Moisture % = (Weight of fresh fruit – Dry weight / Wet weight) × 100

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Cancerous and normal lines

The cancer cell line and normal cells were prepared from the laboratories located at the Biotechnology Research Center / Al-Nahrain University / Department of Molecular Medical Biotechnology - Baghdad. The cells for the lines were grown in RPMI culture medium and supplied with 10% bovine fetal serum. Upon formation of a confluent monolayer, the cells were treated with 2-5 ml of trypsin-ferricin solution for 5-10 minutes to prepare the subculture. The cells were then prepared for treatment with the substances under investigation, as will be described later. The study included the following lines:

Lung cancer line 549A:

The 549A cell line was first developed in 1973 by Giard et al. (24). It is a human alveolar glandular basal epithelial cell carcinoma that forms a cell line. The cancerous lung tissue was removed and cultured into the tumor of a 58-year-old Caucasian man. These cells are used in lung cancer research and the development of drug therapies against it. The 16th pass was prepared by the research center.

Rat Embryo Fibroblast cell line

Rat embryo natural culture is an important and essential source that provides a culture of normal undifferentiated embryonic fibroblast cells. REF cells were used at pass number 12.

Maintaining and preparing cancerous and normal cell lines

The cancer cell lines used in the study, MDA, A549, and the normal cell line REF were maintained according to the method of Freshney (25) (2012). When a complete, fluent monolayer was formed, a subculture was performed by discarding the old growth medium, and then the cells were washed with sterile PBS phosphate buffer. Then add 2-3 ml of trypsin-ferricin TV solution to Falcon 25 cm tissue culture vessels containing the cell lines, so that the surface of the cells is covered when the bottle is placed horizontally, for 5-10 seconds, while gently moving the culture bottle to loosen the adhesion of the cells to the wall of the bottle from each other. After that, add the culture medium to get rid of TV and redistribute it in the special culture bottles, then incubate at a temperature of 37°C. The complete information is then written on each vial (cell type, New passage number, and date of secondary transplantation for each cell line).

Estimation of beta-glucanase enzyme

The reaction mixture for the determination of beta-glucanase enzyme consists of adding (1) ml of β-glucan solution to (1) ml of enzyme extract. Then the mixture was incubated at a temperature of 35 in a water bath for 40 minutes. Then 1 ml of the mixture was taken after incubation and 1 ml of DNS solution was added to it, followed by heating in a water bath at a temperature of 100°C for 5 minutes. After that, rapid cooling of the tubes was carried out and the absorbance was measured with an optical spectrometer at a wavelength of 540 nanometers (26).

β-glucans Toxicity test

The following tissue culture steps were performed under sterile conditions according to the Freshney (2012) protocol to investigate the toxicity of the enzyme under study towards cell lines: Five concentrations of β-glucans enzyme extracted from A. bisporus were prepared for each purification stage, which included crude extract, ion-exchange purified enzyme, and gel filtration purified enzyme, using sterile serum-free medium (SFM).The concentrations were (100, 50, 25, 12.5, and 6.25 mg/ml). Then it was sterilized using a 0.22 micrometer perforated filter under fully sterile conditions, and all prepared concentrations were used immediately after the preparation process was completed.

B The cell suspension was prepared by treating the contents of a 25 cm2 tissue culture vial prepared according to paragraph (3-7-2-1) with Trypsin-Ferrin TV solution after emptying the old culture medium and gently stirring the vial to break up the cells. Then 20 ml of culture medium containing 5% serum and 5% growth media was added, and the cell suspension was mixed well. One hundred microliters of the suspension containing the loosened cells is transferred into the wells of a 96-well tissue culture plate using a micropipette. The plates are then incubated at 37°C for twenty-four hours until the cells adhere to the wells and form a confluent monolayer.

The old culture medium is disposed of in the pits, and 100 microliters of new serum-free culture medium are added, along with 100 microliters of the previously prepared concentrations, with three replicates for each concentration. In addition, 12 control replicates were prepared containing only cancerous lines and culture medium as a control model. The plates were incubated after treatment in a special culture incubator for living cells, at a temperature of 37°C and in the presence of 5% CO2 gas for 24 hours.

After the 20-hour exposure period, the plates were removed and their contents were poured out. The cells were then washed with a phosphate buffer solution. After that, 0.1 ml of MTT stain was added to each hole in the plate. The samples were then incubated again at 37°C for an additional four hours. After the incubation period, 50 microliters of DMSO solution were added to each well, and the color results were read using a microplate reader (ELISA) at a wavelength of 620 nm.

E- The inhibition values of the enzyme solutions (crude extract, ion exchange, gel filtration) in the cell lines were converted to percentages as follows:

Percentage of cell viability = Absorption reading of treated cells per concentration / Absorption reading of control cells × 100

Percentage of cell inhibition = Absorption reading of control cells - Absorption reading of treated cells per concentration / Absorption reading of control cells × 100

Statistics Analysis

The Statistical Analysis System program was used to detect the impact of the factors of difference in the study criteria. To analyze the data of results obtained from the steps of extracting and purifying the enzyme and its effect on cancer cells and normal lymphocytes, the significant differences between the means were compared with the Least LSD Significant Difference test for the least significant difference at a probability level of ($P<0.05$).

Results and Discussion

Qualitative determination of secondary metabolism

The results showed a clear variation in the components of the mushroom extract, as shown in Table (1).

Table (1): Results of qualitative detection of secondary metabolites present in the fungus A. bisporus.

Sample		Flavonoids	Saponins	Polyphenol	Alkaloids	Tannins	Reducing sugar
10 %		+	+ + +	+	+ + +	++	+ + +
20%		+	+ + +	+	+ + +	++	+ + +
30%		+	+ + +	+	+ + +	++	+ + +
40%		+	+ + +	+	+ + +	++	+ + +

Preliminary test results indicated that the fungus A. bisporus had high proportions of alkaloids, saponins, tannins, and reducing sugars compared to phenols and flavonoids.

A study by Amin (27) details the qualitative tests performed on the methanolic extract of the fungus. These tests confirmed the presence of various compounds, including phenols, flavonoids, proteins, amino acids, alkaloids, tannins, and carbohydrates. Both fungi were free of saponins. Alkaloids were detected only in Pleurotus ostreatus.

Approximate chemical estimation or crude chemical analysis of the fungus A. bisporus

The active compounds in the extract of A. bisporus fungus, represented by (ash, moisture, oil, fiber, protein), were estimated. The results showed that the highest concentration was for fiber at 13.04%, followed by moisture at 10.18%, then protein at 8.96%, and oil at 6.99%. The lowest percentage was for moisture at 0.72%, according to Table (2).

Table (2): Shows the proportions of components of the fungus A. Bisporus

Protein %	% Fiber	% Oile	Moisture %	%Ash
9.86	14.34	7.99	12.18	0.92

A. bisporus mushroom is widely consumed due to its nutritional value. A study by (Zakari) (28) indicated the determination of the approximate composition of types of mushrooms, including Macrocybe lobayensis and Agaricus subsaharianus, which are two types of edible wild mushrooms. The approximate composition was determined based on dry weight using the standard method. The results of the approximate analysis showed that the moisture content ranged from 9.08% to 10.03%, while the ash content ranged from 10.46% to 8.48%. The mushrooms are characterized by high protein content (20.66% and 28.09%) and carbohydrate content (57.55% and 54.42%), and low fat content (2.45% and 3.28%). These nutrients varied considerably among the mushroom species. According to the findings, A. subsaharianus and M. lobayensis could be good sources of protein for rural populations in Niger suffering from nutritional deficiencies.

Other studies of fungi have also indicated similar findings. For example, preliminary analysis of two edible wild mushroom species showed crude protein content ranging from 18.71% to 28.09%. The highest protein content was found in A. subsaharianus (28.09%). The lowest protein content

was found in the fungus *M. lobayensis* (20.66%). The protein content of most mushrooms studied in scientific literature ranged from 19% to 39% on a dry matter basis (29). It has been reported that the protein content of mushrooms is influenced by several factors, including the species, genetic makeup, physical and chemical properties, and the environment (30)

Increased mushroom consumption is beneficial for preventing malnutrition, although it can be an alternative source of protein instead of meat (31).A. subsaharianus fungi also showed the lowest moisture content (9.08 and 10.03%). Low moisture content indicates that the fungus will be less susceptible to microbial contamination, since high water content encourages microbial growth (32).

A study by Abu-Riya et al. (33) indicated the approximate composition and nutritional quality of two mushroom species cultivated in Egypt (Agaricus bisporus and Pleurotus ostreatus). The mushroom samples contained 26.05% and 33.85% crude protein in the dried species (P. ostreatus and A. bisporus), respectively.

The crude fat content in the two mushroom cultivars (P. ostreatus and A. bisporus) was almost identical ($P > 0.05$), at 2.79% and 2.41%, respectively. Other approximate composition values ranged from 9.65% to 11.81% for moisture, 5.86% to 7.97% for ash, 8.25% to 13.21% for crude fiber, and 57.05% to 42.56% for carbohydrates in P. ostreatus and A. bisporus, respectively. The oils of the dried mushrooms Agaricus bisporus and Pleurotus ostreatus also contained higher amounts of unsaturated fatty acids.

Determining the killing rate in lung cancer cell lines and normal cells

Effect of β-glucans enzyme on lung cancer cell lines compared to normal cell lines

Table (1) shows the anticancer effect of the β-glucans extract on A549 Pass 21 lung cancer cells after 24 hours of treatment. Seven concentrations of the enzyme were used in the treatment, and the inhibition rates of the seven concentrations for MB 231 lung cancer cells were then compared. All concentrations of the β-glucans enzyme caused a significant inhibition rate in lung cells. The results showed that a concentration of 1 mg/mL reached an inhibition ratio (IR) of 70.54% in the lung cancer cell line, while the same concentration showed an inhibition ratio of 98.14% in the normal cell line.

Table (3) Effect of β-glucans enzyme on lung cancer compared to the normal cell line after 24 hours

Concentration (mg/dl)	Mean ± SE IR%	
	Iung Cancer	Normal Cells
1	70.54±1.15	98.14±1.15
0.5	55.26±1.73	62.28±1.73b
0.25	42.43±1.15	59.49±1.15
0.125	22.51±3.24	40.5±0.58
0.063	15.41±2.72	28.08±1.15
0.031	8.48±2.00	20.16±0.58
0 (control)	0.00 ±0.00 f	
Means having with the different letters in same column differed significantly, * (P≤0.05).		

Discovering and identifying new drugs that enhance immune function has become an important goal of research in pharmacology, especially immunology, particularly if the source of this treatment is natural and has few side effects. Testing the inhibitory effect in normal cells is one of the essential things that must be investigated when studying the effect of any extract for the purpose of using it as a treatment, and these immune cells are among these. Especially lymphocytes, which can divide outside the body (34). Therefore, the effect of the β-glucans enzyme of the local species A. bisporus fungus on normal human lymphocytes was studied in vitro. The cytotoxic effect of the enzyme as an anticancer agent was also investigated by treating cancer cells. In this study, the A549 21-pass lung cancer cell line was used, and the effects were observed in terms of inhibition percentages according to different enzyme concentrations. Based on the calculations shown in the tables above, the effect ratios varied at certain concentrations of the enzyme purification stages when treated with these concentrations, with three replicates for each concentration.

Likewise, normal human cell lines were treated to determine the immunological effect of these cells so that they could later be used on living organisms or on humans as an active ingredient to treat some types of cancer and to strengthen the immune system (35). New research in various fields continues to highlight the importance of this enzyme and its effective results in the possibility of extracting and purifying it and completing the requirements for its manufacture and use as an effective drug in treating various types of tumors and cancers after investigating all its toxic effects and avoiding them if they exist (36).

There are multiple approaches, including combining 5-FU with other chemotherapy drugs, antitumor proteins, or radiation therapy, to enhance the sensitivity of cancer cells to 5-FU. The initial 5-FU drug was generated by a compact enzyme disc, and therefore, incorporating the compact enzyme into the gene encoding the 5-FU into cancer cells may make them more sensitive to the 5-FU drug. Despite the paradoxical feature of killing target cells, the enzyme tablets extracted from bacteria suffered from a major flaw: the enzyme was less efficient at converting 5-FC to 5-FU, i.e., 20 times less than the natural substrate of the cytosine deaminase enzyme in some cases, to achieve the desired response. Increasing doses of 5-FC led to several dose-dependent side effects due to the enzyme's toxic effect (37).

The association of proteolytic enzymes with cancer is of great importance, as serine proteases, plasmin, plasminogen activators, human tissue kallikreins, and multiple myeloma proteins (MMPs) all play a role in tumor growth, invasion, angiogenesis, and metastasis. In addition, other trypsin-like enzymes have been identified in various types of cancer known as tumor-associated trypsinogen, which promotes the metastatic phenotype of cancer cells (38, 39).

In recent years, the number of tumors and cancer cases has increased. This research highlights the historical progress in cancer treatments, showcasing significant achievements in medical and surgical oncology. The medical field comprehensively addresses targeted therapies, while also emphasizing new adjuvant therapies, also known as alternative therapies. Among these natural sources are the byproducts of secondary metabolism or enzymes produced by microorganisms such as bacteria and yeasts, which have important biological activities that are useful and economical for the manufacture and production of many medicines and treatments for many of these diseases and disorders. It also addresses innovations, challenges, and the pivotal role of patient-centered care. It provides insight into future trends and expectations in the field of integrated care for constantly evolving tumors (40,41).

From the above, we discover that the β-glucans enzyme increases mutations responsible for cancer genomes, as it has contributed to genetic heterogeneity within and between tumors and resistance to treatment in lung cancer. Understanding the available methods for clinical detection of these enzymes is of utmost importance. This could serve as a useful diagnostic predictive biomarker in many standard lung cancer treatment plans of care and may be a new target for therapy. This study noted that the effect of the β-glucans enzyme as an anti-lung cancer agent is very small. The

current study may be unique in clarifying the toxic effect of the enzyme, which is extracted from local mushrooms and produced through several local purification stages and steps, on some types of cancerous and normal cells, which is unprecedented in the field of scientific research at the local level.

Conclusions:

Qualitative inferential analyses of the primary and secondary metabolites of the mesquite extract revealed a clear diversity in its components. It contains phenolic compounds, flavonoids, tannins, alkaloids, and carbohydrates. However, the proportions of alkaloids, saponins, tannins, and reducing sugars were highest compared to phenols and flavonoids. The highest percentage of active compounds in the Bosphorus extract was fiber (14.34%), while the lowest was moisture (0.92%). Additionally, the cancer cell death rate was determined by the effect of beta-glucan enzyme on lung cancer (line A549, 21) after 24 hours of treatment.

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