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The Cytotoxic Effects of Phenolic Compounds Extracted from Caps of White Beech Mushroom (*Hypsizygus tessulatus)* on HepG2 (Hepatocellular Cancer Cell Line)

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Abstract: Cancer remains the world's leading cause of death, despite advancements in detection and treatment techniques. The current direction in scientific study is the development of novel anticancer medications derived from plants. Unlike other cancer therapies, phytochemicals are thought to work selectively and specifically, not to harm healthy cells. Liver cancer is a global health concern that has a high fatality rate. Because certain therapeutic mushroom extracts have anticancer qualities, they are becoming more and more popular. Using varying concentrations of white beech mushroom caps (15.60 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, and 500 µg/ml) for 48 hours at 37 °C, this study examined the function of extracts from the caps of the mushroom (*Hypsizygus tessulatus*) as a cytotoxic agent against two cell lines: the colon cell line cancer (HepG2) and the normal cell line HFF-1. The viability findings showed that the sensitivity of HepG2 was 532.88µg/ml. The study found that because white beech mushroom caps have little cytotoxicity on HFF-1, there is no danger involved in employing them in pharmaceuticals. The extract of white beech mushroom caps had a viability percentage of 55.84% on HepG2 cell lines at a 500 mg/ml concentration. Following 48 hours of incubation, there was no sensitivity and 100% viability in HFF-1 cells at control (IC50>100 µg/ml). In summary, the extract had an inhibitory impact on cancer cell lines, suggesting that it may be used as an anticancer medication.

Keywords: antiproliferative; cytotoxicity, liver cancer; *Hypsizygus tessulatus*.

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Introduction

One of the most important medical problems of the modern period and a major source of mortality for communities is cancer. (1). Important information concerning the biological features of cancer has been provided by research, and this information is updated daily. Several characteristics define cancer as a disease, including an unregulated proliferation of aberrant cells and dynamic alterations in the genetic material. Malignant cells proliferate faster than healthy cells divisions. The infiltration of neighboring normal tissues and subsequent metastases to distant regions are what cause this overpowering (2).

According to some estimates, the third most frequent cause of death associated with cancer is liver cancer (LC). Hepatocellular cell carcinoma (HCC) and intrahepatic cholangiocarcinoma are the two most common forms of liver cancer. It is currently the most frequent cause of

death for those with cirrhosis and the most prevalent form of primary carcinoma of the liver in adults (3).

Edible mushrooms have been demonstrated to have restricted effectiveness despite having important pharmacological properties. Plants have been attributed to the bulk of phytochemicals with demonstrated bioactive potential. It is well known that edible mushrooms are an abundant supply of nutrients and bioactive compounds (4). Edible mushrooms are often seen in diets due to their distinctive flavor, texture, and aroma. They are also easily available from grocery store shelves. They can be served as a side dish or as a main ingredient. Edible mushrooms can be rich in nutrients (5). Mushrooms are known to be an essential food source required for food security (6).

East Asia is inhabited by *Hypsizygus tessulatus.* Locally grown in the temperate regions of North America, Australia, and Europe, fresh vegetables can be found in stores. In the wild, these gill-bearing mushrooms grow on wood. The mushroom is often found on beech trees, hence its common name (7).

Hypsizygus tessulatus, often known as the Shimeji mushroom, is a type of fungus that contains glucans, niacin, and vitamins B and D. Vitamin B aids in weight loss and immunity, while vitamin D is thought to be beneficial against osteoarthritis and anti-aging (8). Polysaccharides, l-ergothioneine, sterols, and ergosterol, a type of provitamin D2, are a number of physiologically active compounds found in this fungus, which was initially identified as *Hypsizygus tessulatus*, and have a range of medicinal applications (9). The *H. tessellatus* samples contained the phenolic compounds vanillic acid, cinnamic acid, gallic acid, protocatechuic acid, and p-hydroxybenzoic acid (10). These compounds are thought to be accountable for their hepatoprotective, antioxidant, radical scavenging, antiviral, antibacterial, anticancer, immunomodulatory, and antidiabetic properties (11).

In both in vitro and in vivo settings, *Hypsizygus tessulatus* extracts have shown beneficial properties in suppressing tumor growth. Methanol extracts from *H. tessulatus* demonstrated concentration-dependent inhibitory effects on cancer cell lines, particularly human hepatocarcinoma and colon carcinoma cells, without exhibiting mutagenicity (12).

Materials and Methods

Preparation of Mushroom Extracts

After being recognized based on an archive of existing literature, the white beech mushroom, also referred to as Bunapi shimeji, was purchased from a local supermarket in Baghdad, Iraq. The mushroom caps have been removed from the stems and dried at 40°C until their weight stabilized. The dried mushroom tops were subsequently ground into a powder and placed into conical flasks. Before the extraction process utilizing 100% pure methanol, the dried caps were stored in plastic containers after being ground into a powder. 400 mL of 100% methanol and 40g of ground-up mushroom tops were extracted in a conical flask. Following the incorporation of solvents, the flasks were filled with aluminum foil, plugged, and shaken at 150 rpm overnight at 25°C for 24 hours (13).

Total Phenolic Content (TPC) examination

A standard Folin-Ciocalteu reagent was used to measure the total amount of phenolic components in the methanolic extract. The reaction mixture included

1.5 ml of 20% sodium carbonate, 0.5 ml of the Folin-Ciocalteu reagent, and 100 µl of the extract. The sample was diluted with distilled water until it reached a final volume of 10 milliliters after being mixed in a vortex mixer. Using a calibration curve engendered with gallic acid, the absorbance at 765 nm was measured following a two-hour reaction and used to determine the phenolic content. Milligrams of gallic acid equivalent (GAE) per gram of dry weight were used to prompt the overall amount of phenolic compounds (14).

Total Flavonoid Content (TFC) examination

The total quantity of flavonoids in the crude extract was estimated using the aluminum chloride colorimetric method. The procedures were as follows: After reducing 50 µL of crude extract (1 mg/mL ethanol) to 1 mL with methanol, 4 mL of distilled water, and 0.3 mL of 5% $NaNO_2$ solution, the mixture was incubated for 5 minutes after placing 0.3 mL of 10% $AlCl_3$ solution and being allowed to stand for 6 minutes. The total volume of this mixture was then minimized to 10 mL via the addition of double-distilled water after 2 mL of a 1 mol/L NaOH solution. The absorbance of the mixture at 510 nm was determined after it had remained for 15 minutes. A calibration curve, which contained the total flavonoid content indicated as milligrams of rutin equivalent per gram of dry weight, was utilized to calculate the total flavonoid content (15).

Polyphenol compound isolation, identification, and quantification in mushroom extract:

Reversed-phase HPLC analysis was used to determine the quantity of individual phenolic components. A SYKAM HPLC chromatographic system with a UV detector was the equipment utilized, and the -C18-OSD (25 cm, 4.6 mm) column was utilized. The temperature of the column was 30ºC. The following processes were taken when performing the gradient elution method: 40% B for the first 0–4 minutes; 50% B for the next 4–10 minutes; and a flow rate of 0.7 mL/min. The solvent A was methanol, while eluent B was 1% formic acid in water (v/v). The autosampler was used to automatically inject 100 µL of samples and 100 µL of standards. The spectra were obtained at 280 nanometers (16).

$$Concentration(ppm) = \frac{Area\ of\ the\ sample}{Area\ of\ the\ standard} \times concentration\ of\ the\ standard \times Dilution\ factor$$

Cell Culture:

Two cell lines were identified, namely HepG2 (Liver Hepatocellular Cancer Cell Line) and HFF-1(Foreskin fibroblast cell). These cells were established in RPMI1640 culture media and were then treated with (10%) heated inactivated fetal bovine serum at a humidified 37°C temperature and (5%) CO_2 (17).

Cytotoxicity Assay:

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay was utilized to investigate the cytotoxic impact of white beech mushroom caps extract on cell viability and growth. MTT powder was dissolved in sterile PBS to produce an MTT solution that worked (5 mg/ml). In advance of being treated to the extract, 7000 cells were put into each well. For optimal cell adhesion, cells were then incubated in 96-well plates at 37°C for 24 hours. After that, cells were co-cultured for 48 hours with increasing concentrations of mushroom extract (15.60 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, and 500

µg/ml) in contrast to the concentration (control). For each treatment, three replicate wells were utilized. Following a 48-hour incubation period, the wells were cleaned with PBS after the old solution was removed. Later, furthermore, to a comparable volume of MTT working solution (5 mg/ml), 20 µl of serum-free medium was added. After three hours of dark incubation at 37°C, 50 µl of MTT formazan dissolver (DMSO) was added to the plate. A microplate reader set at 570 nm was utilized to measure absorbance (18). This formula, which was employed by (19), was used to calculate the viability percentage:

viability %: = (A test \ A control) X 100

A: Absorbance the viability curve was used to determine the growth inhibitory concentration (IC50), at which viability is reduced to 50%.

Analysis of the cell cycle arrest

Cell cycle analysis is the most straightforward technique for identifying DNA fragmentation. Apoptotic cells are indicated by a sub-G0/G1 population, and the fragmented 182bp DNA multimers leave the cell when it is permeabilized. The cells were cultivated in 6-well plates and given medicine for a specific amount of time. Following treatment, the suspended and attached cells were collected by trypsinization, vortexed, and fixed in 70% ethanol at 4°C. After two hours, the cells were rinsed with a cold PBS solution containing 100 µg/ml RNase A and then incubated for an additional hour at 37°C. The cells were then stained with propidium iodide (20 µg/ml) for 30 minutes at room temperature without light. With a flow cytometer and flow software, the DNA concentration was analyzed (20).

Analysis of statistics

The data was shown utilizing the mean ± standard deviation (SD). For statistical comparisons between groups, one-way analysis of variance (ANOVA) was employed. All statistical analyses were conducted utilizing GraphPad Prism (version 9.5.1; Institute Inc., Cary, NC, USA) and Microsoft Excel 2016. The P-value was regarded as statistically significant if it was less than 0.05 (21).

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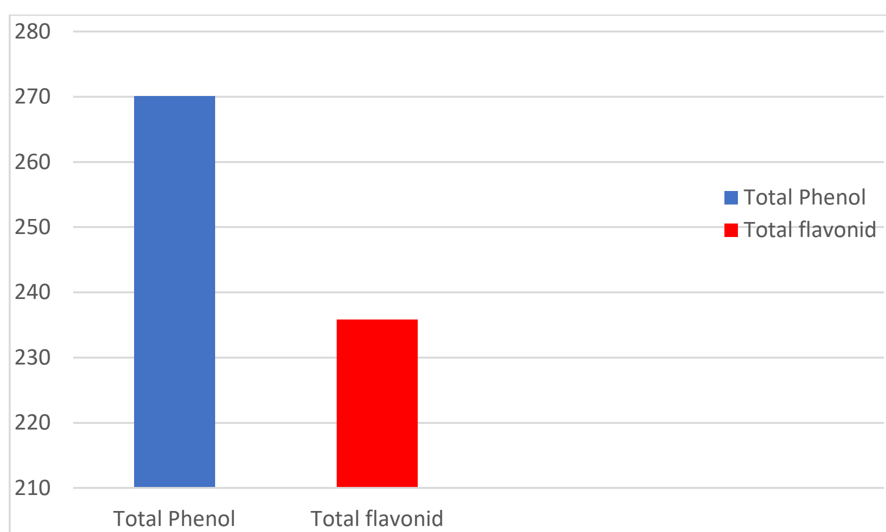
Total Phenolic Content and Total Flavonoid Content Determination

The total phenolic content and total flavonoid content of the phenolic compound extracted from *Hypsizygus tessulatus* (white beech mushroom caps). In general, TFC (as a subclass of polyphenols) was much lower than TPC, as expected. Analysis of the results showed that the methanol extracts of Bunapi shimeji exposed the results of TPC and TFC values (TPC µgGAE/mg and TFC = 235.8µg Rutin/m as shown in Figure. Total phenolics are recognized as the highest typical antioxidant components and contributors to the biological activities of white beech mushroom caps (22).



Figure(1): Total Phenolic and Flavonoid Content of *Hypsizygus tessulatus* (white beech mushroom caps) extract.

Separation and identification of phytochemicals in the caps of white beech mushroom extract

High-performance liquid chromatography (HPLC): In the existing pharmaceutical industry , HPLC is the utmost important and fundamental analytical tool in all stages of drug discovery, development, and production. The chemical study results by HPLC directed that numerous phenolic phytochemicals were naturally present in the extract of white beech mushroom caps such as gallic, chlorogenic acid, rutin, ferulic, quercetin, and caffeic acid (Table 2, Fig. 2). The concentration of isolated phenolic phytochemicals was found to be the highest for gallic acid but the lowest for caffeic acid. White beech mushrooms would be a significant natural anti-proliferative food resource. To explore the strong contribution of the furthermost important compounds to the antioxidant and anti-proliferative effects and their further mechanisms, tests on individual compounds should be shown as an effective resource for enormous antioxidants.

Table (1): Isolation and quantification of polyphenol compounds in caps of white beech mushroom extract:

Active compound	N0. 0f compound	Retention time (minutes)	Area (%)	Concentration (ppm)
Ferulic acid	1	3.18	14.00	65.0
Chlorogenic acid	2	3.90	23.00	80.9
gallic acid	3	4.20	16.00	112.6
Quercetin	4	5.82	14.00	88.1
Caffeic acid	5	6.30	19.00	55.8
Rutin	6	7.92	14.00	70.9

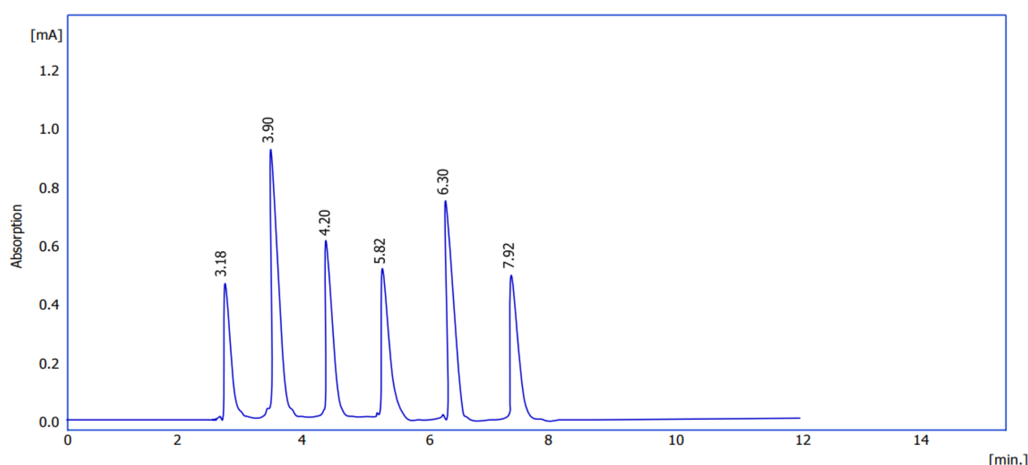


Figure 2: HPLC chromatogram *Hypsizygus tessulatus* (white beech mushroom caps)

Cytotoxic activity:

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT assay) tests were performed to assess the cytotoxic effects of different concentrations of the white beech mushroom methanol extract on human Foreskin fibroblast cells (HFF-1) and liver cancer cell lines (HepG2) when compared to all of the concentrations of methanol extract that were observed (15.60μg/ml, 31.25μg/ml, 62.5μg/ml, 125μg/ml, 250μg/ml, and 500μg/ml). The viability percentage in HFF-1 at control was 100%, while the viability percentage in HepG2 at 500μg/ml concentration was 55.840%, but it was 73.357% in HFF-1 at 500μg/ml concentration. It was shown that the viability decreased with increasing extract concentration. Figure 3 also displays the growth inhibition (IC) of cancer cell lines, HepG2 (IC50 = 532.88 μg/ml concentration) and HFF-1 (IC50 > 100 μg/ml concentration), after a 48-hour co-cultivation with crude methanol extract. white beech mushroom extract cytotoxicity effect on liver cancer cell line (HepG2) shown in (Table 2).

Table2. Cell Death Percentage (Cytotoxicity Assay) Measured on HepG2 exposed white beech mushroom caps extract for 48 hr.

White beech mushroom caps	0	15.60%	31.25%	62.5%	125%	250%	500%
Mean	419.5	414.5	409	392.5	333.75	294	234.25
Viability	100	98.808	97.497	93.563	79.558	70.083	55.840
SD	13.304	7.937	8.406	5.196	13.375	5.887	18.997
SEM	6.652	3.968	4.203	2.598	6.687	2.943	9.498

The inverted cell lines utilized in Figure 4 further highlight the white beech mushroom extract's ability to suppress the cellular development of HepG2 after 48 hours of treatment. White beech mushroom cap methanol extracts showed anti-proliferative action against the cells under consideration ($p < 0.05$). The presence of certain phytochemicals (such as gallic, ferulic, rutin, quercetin, chlorogenic acid, and caffeine) may be crucial for the extracts' anti-proliferative activity. These phytochemicals' synergistic interactions may enhance the extracts' cytotoxic effect on cells. Their anti-proliferative characteristics may also be related to other quercetin-derived extracts. For instance, normal cells probably do not produce all of the proteins that cancer cells do, which minimizes the binding potential of the phytochemicals found in mushrooms to the cells (23).

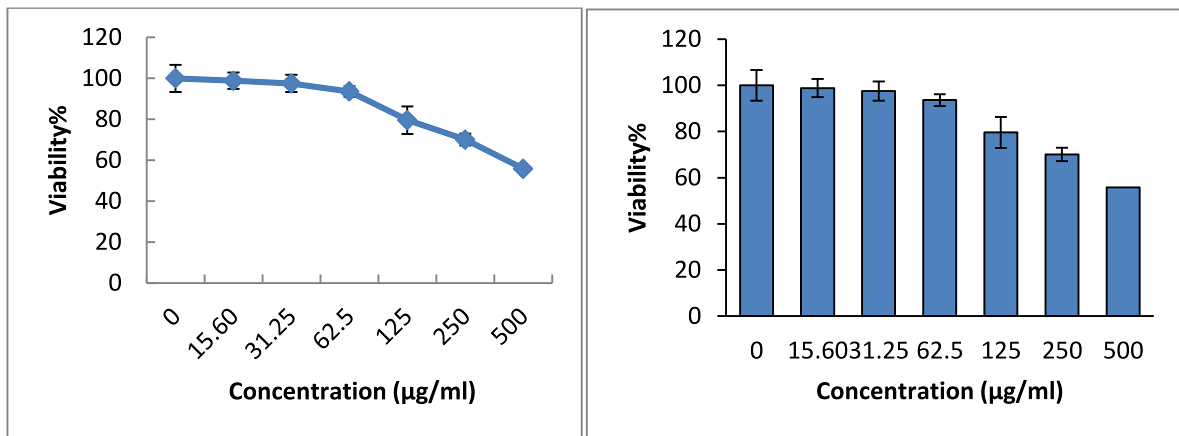


Figure (3): Cytotoxicity effect caps of white beech mushroom extracts on HepG2 cells using MTT assay

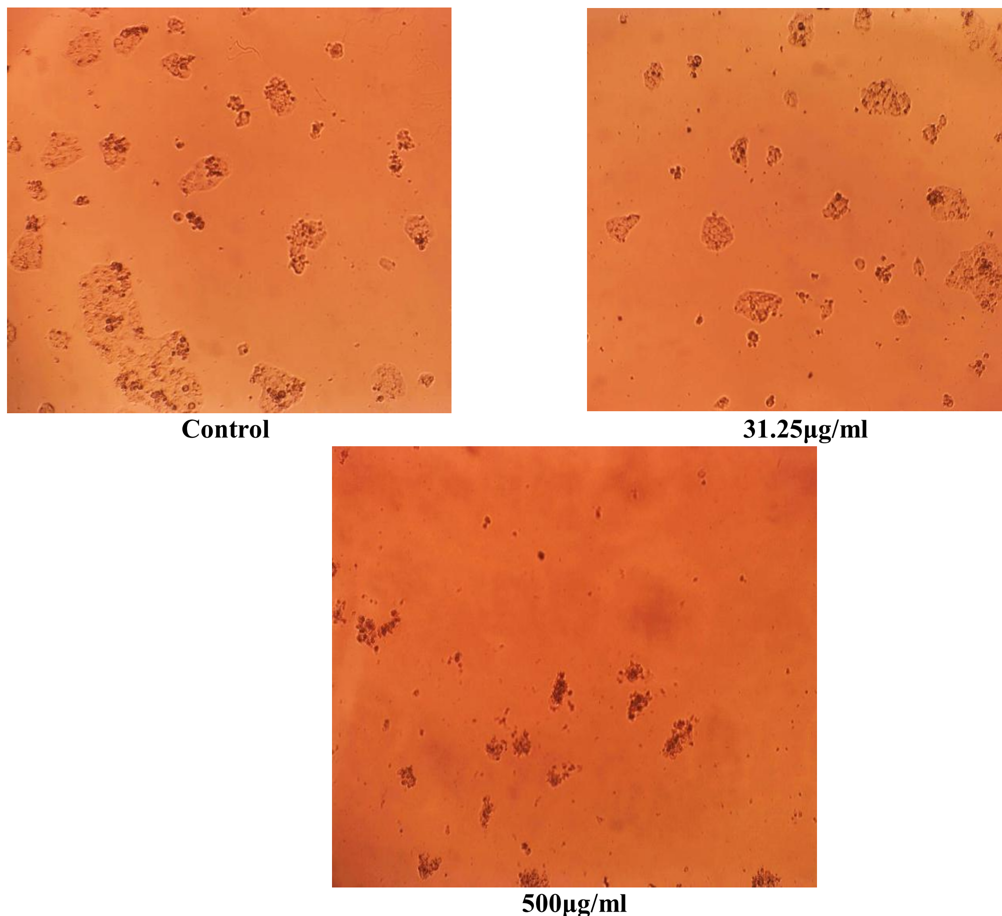
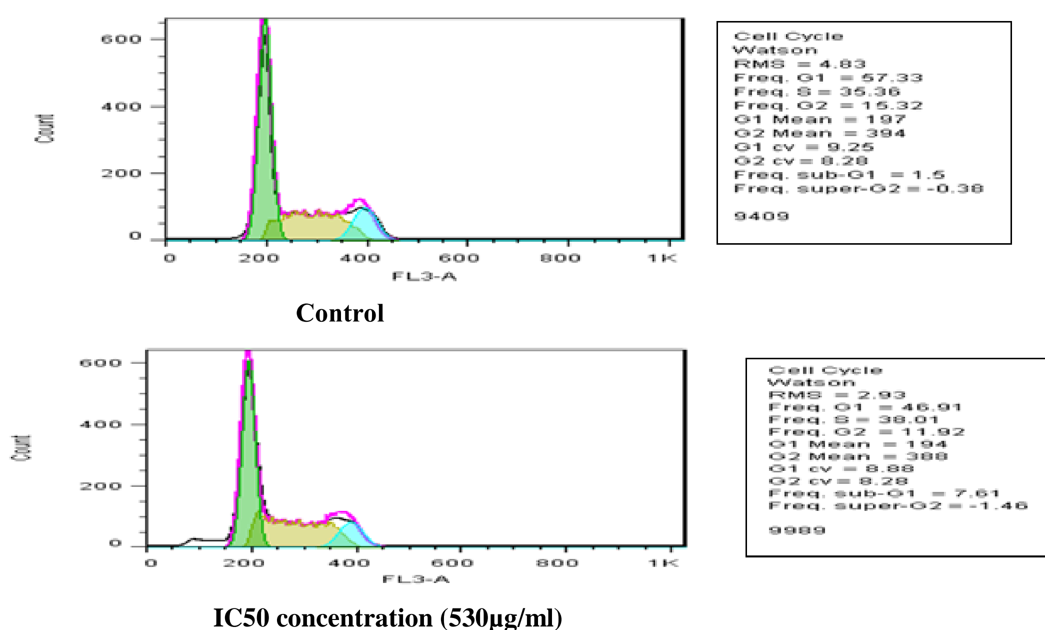


Figure (4): HepG2 cells were exposed to extracts from white mushroom caps at concentrations of (0, 31.25, and 500 µg/ml) for 48 hours.

Flow cytometry analysis of the Cell cycle

Researchers have shown a growing interest in using the cell cycle as a new method for cancer therapy in the last decade. It is well documented, for example, that there is a link between cell cycle advancement and prevention of cell proliferation and apoptosis in cancer cells. The apoptotic induction was detected by flow cytometry, and by analyzing cell distribution in different

stages of the cell cycle, we used flow cytometric studies in conjunction with DNA staining with PI (24). Our findings show that treating HepG2 with the IC50 value of phenolic extract of white beech mushroom caps results in cells at G2 (11.95%) and S (38.01%) phases in treated cells compared to control (G2 (15.32%), and S (35.32%), while G1 (46.51%) phase in treated cells was lower than control (G1 (57.33%)), Figure 5(A and B). As a result, HepG2 cells were arrested in the G1 phase. The cell cycle is the most important regulator of the proliferation of eukaryotic cells (25). G1 arrest is a stage of the cell cycle that gives cells the chance to either go through repair processes or follow the apoptotic pathway (26). DNA damage generally happens during the G1 or G2 phases (25). After DNA fragments break to generate apoptosis, cells disintegrate to form apoptotic bodies (27).



Control

IC50 concentration (530µg/ml)

Figure (5): White beech mushroom effects on HepG2 cell-cycle phase distributions. Flow cytometry is used to measure cycle dispersion.

Conclusion

The impact of several substrates on the metabolomics profile and biological characteristics of extracts from the caps of the medicinal fungus *H. tessulatus* was examined in this work. The presence of phenolic compounds was measured by the HPLC instrument after phenol extract was prepared using a variety of extraction techniques. HepG2 was cytotoxically affected by higher concentrations of phenolic substances, including gallic, ferulic, rutin, quercetin, chlorogenic acid, and caffeine, found in caps of white beech mushrooms. The results of *H. tessulatus* on malignant lines are encouraging. In the worldwide fight against liver cancer cell lines, medicinal mushrooms have to be taken into consideration as an alternative natural chemopreventive agent.

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