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FIRST REPORT OF THE ANTICANCER EFFICIENCY OF AGARICUS BRASILIENSIS MUSHROOM ON HUMAN EMBRYONIC LIVER WRL68 AND HUMAN PANCREATIC ASPC-1 CANCER CELLS THERAPY

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Abstract

Mushrooms are food traditionally consumed in Asia, Europe and America. They are being studied for medicinal benefits. Extensive studies have shown that *Agaricus brasiliensis* mushroom used as a medical product to combat cancers.

Our data reveal that the determined inhibitory concentration fifty (IC₅₀) values were observed maximum dose responses (IC₅₀) of WRL68 and AsPC-1 cancer cells reported of 172.6 µg/ml and 158.2 µg/ml respectively at 2.23 and 2.1 µg/ml ethanolic mushroom concentrations. The highly cytotoxic activity of the extract on growth inhibition AsPC-1 and WRL68 were generally observed 97.9% and 95% at extract concentrations of 25 µg/ml and 50 µg/ml respectively.

Finally, Phytochemical profile of *Agaricus brasiliensis* mushroom extract found to be flavonoids, glycosides, saponins, phenols, alkaloids, tannins. The extracts of *Agaricus brasiliensis* were tested through Gas Chromatography-Mass (GC-MS). There were five different compounds analyzed from the extracts of *Agaricus brasiliensis*. The compounds in the ethanolic extract of *A. brasiliensis* mushroom were comprised mostly of Acetic acid ethyl ester (38.39%), followed by (3-Methyloxiran-2-yl)-methanol (34.71%), Chlorbromuron (22.86%), n-Hexadecanoic acid (3.85%) and Heptane, 1-(1-butenyloxy) (0.19%).

However, no studies were done using *Agaricus brasiliensis* mushroom extract against WRL68 and AsPC-1 human cell lines. Therefore, the anticancer efficiency of *Agaricus brasiliensis* mushroom on human embryonic liver WRL68 and human pancreatic AsPC-1 cancer cell therapy is being reported for the first time in the current study.

Keywords: *Agaricus brasiliensis*, Phytochemical profile, MTT assay, GC-Mass, WRL68, AsPC-1.

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1. Introduction

According to the American Cancer Society, WRL68 and AsPC-1 are a particular name for a cancer that starts with human's liver and pancreas respectively. The latest estimates of the liver and pancreatic cancers deaths in the world are accounting for more than 700000 and 330000 deaths respectively (American Cancer Society, 2021).

Current therapy involves a mixture of radiation, surgery and chemotherapy, although the level of relapse seems to be extremely high. Therefore, the lack of clinical strategies and the desire to support the patient's health demands the assessment of an additional or supplementary therapeutic approach (Buchanan et al., 2005; Nakamura et al., 2019). In the recent decades, the scientific experiment and technological advances of novel drugs have expanded and tumoricidal substances have become increasingly important (Patridge et al., 2016).

Currently, Mushrooms were used as humans' nutrition for thousands of years and are remarkably poor in calories but rich in minerals, vitamins, fibers, and essential amino acids (Lima et al., 2011; Tepsongkroh et al., 2020). Among of these mushrooms, *Agaricus brasiliensis* mushroom, an edible fungus in the agaricaceae family. It was historically used as a medical food to combat cancer, diabetes, hyperlipidemia, coronary artery disease, and chronic liver disease and is widely believed to activate the immune response (Misgiati et al., 2021). They considered as nutritional value due to their diverse chemical properties and large ratio of phytochemical components, providing numerous health benefits to those who eat them, in addition to their lack of toxicity (Orsine et al., 2012). The majority of mushroom content constitutes water (90%), protein (2-40%), carbohydrate (1-55%), fiber (3-32%), and ash (8-10%) (Zivkovic et al., 2017).

To our knowledge, this is the first discovery in the world on the cytotoxic activities of *Agaricus brasiliensis* extract against WRL68 and AsPC-1 human cell lines. Based on the above findings, the goal of this work was to discover the anticancer efficiency of *Agaricus brasiliensis* mushroom on human embryonic liver WRL68 and human pancreatic AsPC-1 cancer cells therapy.

Materials and Methods

Preparation of *Agaricus brasiliensis* mushroom extracts

The *Agaricus brasiliensis* mushroom was collected from a local producer in Al-Anbar Governorate in Iraq, cleaned, dried at 40 °C, grinded and then weighed. The mushroom dried powder 20g was used for 200ml of solvent 70% ethanol, and then extracted by using soxhlet. After 24h, The solution was centrifuged for 15min at 1000 rpm /min, and then collected liquid phase was used for further process. The liquid portion was dried at 50 °C in a rotary evaporator and then preserved at -20 °C for further studies.

In-vitro Anticancer activity

The anticancer efficiency of ethanolic extract from *Agaricus brasiliensis* mushroom against WRL68 and AsPC1 human cell lines that obtained from the Iraqi Center For Cancer Research in Al-Mustansiriyah university. Cells have been grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin at 37 °C in a humid condition of 5% CO₂ for 24h.

Cell viability MTT assay was included by the standard instructions (Promega Corporation, Madison, WI, USA). At first, the 96-well tissue culture plate was filled with 100 µl/well of cells (106 cell/ml). Various concentrations of *Agaricus brasiliensis* mushroom extract test solution were prepared to evaluate cytotoxic effect against two examined cell line (400, 200, 100, 50, 25, 12.5, 6.25 µg/ml) in water.

After that, 100 µl of different concentrations was applied to each well in an incubator at 37 °C with 5% CO₂ humid conditions for 24h. During the incubation, 10 µl of 5mg/ml MTT solution was transferred to each well and incubation at 37 °C for 2h.

An extraction buffer (20% sodium dodecyl sulphate (SDS) and 50% dimethylformamide) introduced to each well and incubated at 37 °C overnight. Then negative control wells included cells with the medium in 0.4% DMSO.

The cell suspension absorbance was calculated using ELISA Microplate Reader (Bio Tek, USA) at 570nm.

Cell viability was described as the ratio of the mean absorbance of the treated cells to that of control cells. The tumors cells' sensitivity to the extract was represented as IC₅₀ values.

This experiment was replicated three times, and the statistical data was analysed to give the final results.

Phytochemical profile analysis

phytochemical profile was performed by standard instructions (Trease and Evans, 1989; Harborne, 1998).

Gas Chromatography-Mass Spectrometry

By using a high-temperature column, Agilent Technologies (SHIMADZU-Japan) bought a high-temperature column (Inert cap IMS; 30 m x 0.25 mm id x 0.25 µm film thickness) for GC-MS research. Each sample's derivatization was withdrawn. The injector and detector were calibrated to 280°C, whereas the original column heated at 100°C. The column was loaded with a 5 µl sample volume and operated in split (1:10) system. The sample was heated to 225°C at a level of 12.5°C/min after 1 minute. After that, this sample was gradually increased to 300°C at a level of 7.5°C/min. The helium carrier gas optimized to preserve a stable flow rate of 17.5 mL/min. The data reported and analyzed through Agilent GC-Mass Solution (SHIMADZU-Japan) and postrun software. The compounds were detected by matching their mass and NIST database.

Data analysis

For statistical research, the program SPSS 20.0 has been used. Three different values have been represented as mean + SD values. In all instances, the adopted levels was 5% ($p < 0.05$).

Results and Discussion

Cell line growth and cytotoxicity assay

Figure1: Dose responses (IC₅₀) of anti-tumor mushroom on WRL68 and AsPC-1 human cell lines. Cells have been grown in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum and subjected to ethanolic extract of *Agaricus brasiliensis* mushroom at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg/ml for 24h. In each experiment was replicated three times and two different experiments were conducted. Mean ± SD (n=3) are values shown.

In our experiments, the cytotoxic of the ethanolic extract *A. brasiliensis* mushroom on WRL68 and AsPC-1 human cell lines were shown to be a dose-responsive manner which found significant variation compared to that control (Fig 1). These results indicated that ethanolic extract *A. brasiliensis* mushroom significantly and specifically inhibited the proliferation of human embryonic liver WRL68 and pancreatic AsPC-1 cell lines relative to control.

Our data have shown that the maximum dose responses (IC₅₀) of WRL68 and AsPC-1 reported of 172.6 µg/ml and 158.2 µg/ml respectively at 2.23 and 2.1 µg/ml ethanolic mushroom concentrations compared to control. Although this is the first work on the effect of ethanolic extract *A. brasiliensis* mushroom was recorded to inhibit the viability of human embryonic liver WRL68 and pancreatic AsPC-1 cell lines. Simply, although it was not related to *A. brasiliensis*, another mushroom extract from *Cyathus striatus* has been discovered that growth inhibition against HPAF-II and PL45 human pancreatic adenocarcinoma cells can be reached even in small doses and short exposure time (Sharvit et al., 2012). Furthermore, the MMH01 compound obtained from *Antrodia cinnamomea* mushroom was successful in inhibition toward BXP3 pancreatic cancer cells

(Chen et al., 2009). Another work reported in (Yu et al., 2012) registered as anti-proliferative effect toward PANC-1 and AsPC-1 pancreatic cancer cells by an anthraquinone derivative collected from *Antrodia camphorata* mushroom. Additionally, Cheng et al., (2013) demonstrated that triterpenes extracted from *Poria cocos* acted as anti-proliferative agents against four pancreatic PANC-1, MIAPaCa-2, AsPC-1 and BxPC-3 carcinoma cell. Also, Ghosh and Sanyal, (2020) observed the ethanolic extract of *Calocybe indica* mushroom inhibited proliferation of AsPC-1 pancreatic cell line.

Figure(2): The effect of various concentrations of *A. brasiliensis* mushroom ethanolic extract on growth inhibition of two human embryonic liver WRL68 and human pancreatic AsPC-1 cancer cells after 24hr incubation time.

In figure(2), Cytotoxicity was determined at a high rate of growth inhibition 97.9% and 95% in AsPC-1 and WRL68 at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ concentrations respectively as compared to the cell control.

In data study, *A. brasiliensis* mushroom extract detected that improving anticancer activities through existence several of the bioactive compounds.

Antitumor activity of mushroom derivatives on AsPC-1 and WRL68 cell lines. Till now, no study has been performed about the anticancer effect of *A. brasiliensis* mushroom on AsPC-1 and WRL68 cell lines, however, there are a few experimental data supporting potential anti-tumor activity of this mushroom against another human cell lines.

Ziliotto., (2014) was observed antitumor activity of dichloromethane/ methanol and hexanic *A. brasiliensis* extracts mushroom at 250 $\mu\text{g/ml}$ for K-562, NCI-ADR, NCI-460, UACC62, OVCAR, HT-29 and 786-0 human cancer lines. Similarly, Shimizu et al., (2016) explained the cytotoxic effects of Agarol from *A. brasiliensis* mushroom against human carcinoma cell lines A549, MKN45, HSC-3 and HSC-4. Another study cytotoxic effect of *A. brasiliensis* extracts on human myeloma cells and leukemic cells (Tangent et al., 2017). Other study has shown that the hot water extraction of *A. brasiliensis* mushroom observed inhibition toward human leukemia Jurkat cell (Kozarski et al., 2014).

Phytochemical profile analysis

Phytochemical profile of the ethanolic extract of *Agaricus brasiliensis* achieved the occurrence of flavonoids, glycosides, phenols, saponins, alkaloids, tannins as shown in table(1), the mean of pH extracts was (5.5-6).

Table(1): phytochemical profile of the ethanolic extract of *A. brasiliensis*.

Effective compounds	<i>A. brasiliensis</i> mushroom extract
Flavonoids	+
Glycosides	+
Saponins	+

Phenols	+
Alkaloids	+
Tannins	+

Several studies on many substances of *A. brasiliensis* such as sterol, sesquiterpenes, riboglucan, anthraquinones, glucomannan, sodium pyroglutamate, derivatives of benzoic acid, lectins, RNA-protein complex, organic acids, amino acids, lactic and fumaric acid, steroids, quinolones, phenolic compounds and polysaccharides such as β -glucans have been reported as potential bioactive substances (Motoi, 2012; Da Silva et al., 2017; Moukhaet al., 2020; Venkateshgobiet al., 2021).

Polysaccharides are the most well-known and have many tumor preventative constituents and immunomodulating effects. *A. brasiliensis* polysaccharide comprises of 57.7% glucose, 27.7% galactose, 7.3% mannose/ xylose, and 4% fucose (Martinet al., 2017). Among of these polysaccharides, β -glucan refers to a glucose molecule found in medical mushrooms. It shows immunomodulatory, antitumor, antiproliferative properties in cancer patients by activation of immune response (Cardozo et al., 2014). Moreover, antihyperglycemic, antihypertriglyceridemic, antihypercholesterolemic, and antiarteriosclerotic properties have been observed in β -glucans from *A. brasiliensis* (Wei et al., 2020).

Agaricus brasiliensis is abundant in certain natural compounds including polyphenols and polysaccharides which have antioxidant, antitumor, and immunomodulation (Hetland et al., 2020).

Lectins can be seen to be pharmaceutical sources with tumorcidal activities in animals and human trials. It causes cytotoxicity and apoptosis through attaching to cancer cell membranes which suppress tumor growth (Sendra et al., 2010).

Ergosterol is the vitamin D biosynthesis in the lipid fraction of *Agaricales* extracts. It affects human cancer cells through antitumor, antiproliferation, and antimigratory effects and inhibits angiogenesis (Shimizu et al., 2016).

Arginine was prescribed to cancer patients as a nutritional aid. It has been linked to a decrease in tumor growth and metastasis progression. Hence it has positive impacts to the immune response, weight gain, and cancer patient recovery period. (Tada et al., 2011).

Another report of *blazeispirol A*, collected from *A. brasiliensis*, reduces growth inhibition of Hep 3B hepatoma cells (Wang et al., 2013).

The agaritine component was found in *A. brasiliensis*. It has tumorcidal properties in leukemic cells (Nagaoka et al., 2006).

The blazein derivative occurs in *A. brasiliensis* which is considered as an anti-proliferative effect towards LU99 human lung cancer cells (Itoh et al., 2008).

Evaluation of Gas Chromatography-mass Spectrometry for Fungal Extracts

As shown in the figure 3, GC- MS of the chromatogram of methanolic extract of *A. brasiliensis* showed the presence of about five peaks. Their name, molecular formula, molecular weight, retention time (RT) and peak area (%) are listed in table (2).

The compounds in the ethanolic extract of *A. brasiliensis* mushroom were comprised mostly of Acetic acid ethyl ester (38.39%), followed by (3-Methyl-oxiran-2-yl)-methanol (34.71%), Chlorbromuron (22.86%), n-Hexadecanoic acid (3.85%) and Heptane, 1-(1-butenyloxy) (0.19%).

However, many of these identified constituents have been found to possess several pharmacological activities i.e. antitumor, antimicrobial, antioxidant, antiviral, anti-arthritis, antifungal, insecticidal and other therapeutic potentials (Mohamed, 2012; Gyorfiet al., 2013; Alshamma et al., 2017; Elkhateebet al., 2019; Adeoye-Isijola et al., 2021).

Table(2): Major phytochemicals obtained in the ethanolic extract of *A. brasiliensis*.

No. of peak	Compound name	Molecular formula	Molecular weight (g/mol)	Retention time (RT)	peaks area (%)
1	Heptane, 1-(1-butenyloxy)	C ₁₁ H ₂₂ O	170.29	1.99	0.19
2	Acetic acid ethyl ester	C ₄ H ₈ O ₂	88.1051	2.19	38.39
3	Chlorbromuron	C ₉ H ₁₀ BrClN ₂ O ₂	293.545	2.48	22.86
4	(3-Methyl-oxiran-2-yl)-methanol	C ₄ H ₈ O ₂	88.11	2.61	34.71
5	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.4241	19.46	3.85
Total identified					100

Figure(3): GC-MS Chromatogram of the ethanolic extract of *A. brasiliensis*.

Conclusions

This is first report in world on an ethanolic extract of mushroom *A. brasiliensis* which is effective in inhibiting cell growth of WRL68 and AsPC-1 cell lines, indicating that it may be used as an alternative therapy in the treatment of liver WRL68 and pancreatic AsPC-1 cell lines. Thus, it is suggested that the anticancer efficacy of the GC-MS detected compounds be evaluated in order to discover new therapeutic approaches for cancer treatment in future. Further studies are required to reveal the important chemical constituents responsible for anti-proliferative activity.

Conflict of interest

The author declares no conflict of interest.

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