Antitumor Activity of β-glucan Extracted from *Pleurotus Eryngii*

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Abstract

Pleurotus eryngii, a type of edible mushroom that exhibit various pharmacological properties, including antioxidant and anticancer effects. In the present study, extracted β-glucan from the *P. eryngii* was tested as an antioxidant and anti-tumor factor. β-glucan was extracted and analyzed by HPLC and FT-IR. Analytical results showed more than 90% similarity in chemical structure and purity. Potential antioxidant activity of β-glucan was examined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and compared with ascorbic acid.β-glucan confirmed a potential scavenging activity. The anticancer activity of theβ-glucan was assessed using different concentrations (6.25 to 400 μgmL^{-1}) on MCF-7 and HepG2 cell lines. *P. eryngii*β-glucan exerted a dose-dependent reduction in MCF-7 and HepG2 cell viability with an IC_{50} of 280.00 and 539.5 $\mu g\ mL^{-1}$, respectively. At the same time, no significant effect was recorded on normal cell line WRL 68. The obtained results are expected that could be used to develop *P. eryngii*β-glucanas an antitumor drug.

Keywords: *MTT, pleurotus eryngii, Cytotoxicity, β-glucan, Antioxidant, MCF-7, HepG2.*

Introduction

Mushrooms have attracted a great deal of interest in many areas of food and bio-pharmaceutical research and are well known for their nutritional and medicinal values [(1)]. Several major components with immunomodulatory and/or antitumor activity have been isolated from mushrooms. These include mainly polysaccharides, such as β-glucans, polysaccharo-peptides, polysaccharide-protein conjugates, and proteins. β-glucans have a wide range of biological activities. Mushroom β-glucan polysaccharides are fibers that mostly present as linear and branched chains with different types of glycosidic linkages, such as (1-3), (1-6)- β-glucans and (1-3)-α-glucans, others are heteroglycans containing glucuronic acid, xylose, galactose, mannose, arabinose or ribose[(2)].

Pleurotus eryngii is an edible mushroom,considered to be a health food not only for low fat and calories but also being rich in amino acids, vitamins, and dietary fiber[(3)]. *In addition,* β-glucans of *P. eryngii* received an increasing interest for its bioactive properties including antitumor, immunomodulator, antioxidant and antiallergic activities. Studies have indicated that the polysaccharides isolated from *P. eryngii* were mostly β-glucans, which exhibited potential activities[(4)].

Chemically, β-glucans are heterogeneous, non-starch polysaccharides, which form the structural compounds of the cell wall of certain microorganisms, including yeast and algae, mushrooms, and grains, such as oats and wheat. β-glucans may be insoluble or soluble. Insoluble β-glucans fibers consist of β-(1-3/1-4)-D-linked glucose units, whereas soluble viscous β-glucans fibers consist of β-(1-3)/1-6)-D-linked glucose [(5)].

Many chemical compounds identified as specific agents for inhibiting cancer cell proliferation were also showed significant toxicity toward normal cells, as well

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as their side effects. Many potential anticancer drugs have considerable side effects[6]. Therefore, discovery of new safer drugs with potential activity against tumor has become an important goal of research in biomedical sciences. Polysaccharides from mushroom sources can stimulate immune cells, including macrophages, granulocytes, nature killer cells and monocytes to trigger cytokine production and thus stimulating the immune system[7].

In same point, this study was conducted first to extract β-glucans from *P. eryngii, and* evaluate the cytotoxic activity of the extracted β-glucanagainst tumor cell lines HepG2 and MCf-7, as approach in developing a mushroom polysaccharides to use either individually or in combination with medicinal drugs combination in cancer treatment.

Materials and Method

Mushroom Strain: *Pleurotus eryngii* strain was kindly provided and authenticated by Dr. Ahmed A. Kareem, Department of Organic Farming, Ministry of Agriculture, Baghdad, Iraq.

β-Glucan Extraction from *P. eryngii:* β-glucan was extracted using a water extraction method[8]. In brief: dried fruit bodies of *P. eryngii*were powdered. The powder was mixed with ddH_2O in ratio of 1:20 (wt/v). The pH of the mixture was adjusted to 7.0 using 20% Na_2Co_3. Mixture heated to 90°C for 6 h with shaking (100 rpm). After heating process, the mixture was centrifuged at 8000 rpm for 10 min at 4°C. The pellet was discarded, and the supernatant was transferred to new container and the pH was further adjusted to 4.5 using 2M HCl. The solution was centrifuged at 8000 rpm for 30 min at 4°C. Pellet which contained proteins was discarded and supernatant was mixed with absolute ethanol in a ratio of 1:1 and left for 12 h at 4°C to precipitate the beta-glucan. The mixture was centrifuged at 3000 rpm for 10 min at 4°C. Finally, the pellet was homogenized with absolute ethanol and then oven-dried at 60°C.

β-Glucan Analysis by High Performance Liquid Chromatography (HPLC): The samples and standard were analyzed by HPLC (SYKAM, Germany) supplied with S2100 quaternary gradient pump andfluorescence detector RF-20A (UV280). The condition analysis of β-glucan; mobile phase: dH_2O and orthophosphoric acid (90:10 v/v); column: C18–ODS (25cm x 4.6 mm);Flow rate = 0.7 mL min^{-1}. Preparation of sample: 1 mg dissolved in 25 mL dH_2O and then 20 µLwas injected into HPLC column for analysis. The separation occurred on liquid chromatography and, the eluted peaks were monitored by UV-Vis 10 A-SPD spectrophotometer.

FT-IR (Fourier Transformed Infrared) Analysis: The chemical structure of β-glucan was analyzed using FT-IR spectrometry (Shimadzu IR Prestige-21– Japan). The FTIR spectrum was utilized to detect the functional groups of glucan structure compared with the standard. This was done under FT-IR spectrometry in wavelength range 4000-400 cm^{-1} and at a resolution of 8 cm^{-1}. This test involved mixing an equal volume of glucan sample and standard (2 mg) with potassium bromide (KBr) (100 mg), then grinding the mixture by special grinder until soft and fine powder obtained. The sample was loaded in target mold and analyzed [9].

Determination of Carbohydrate Content: Carbohydrate content was calculated by multiplying the reducing sugar content which was determined depending on Fehling's reducing method[10]. Briefly, 10 g sample was mixed with 20 mL sulphuric acid (0.5 M). Reflux was then performed in a sand bath for 2.5 hours. The residue was washed after filtration (Whatman filter No. 1) with warm dH_2O. The solution was then neutralized with Na_2CO_3 powder and the mixture's volume was completed to 100 mL with dH_2O. Titrations were performed using 5 mL Fehling's solution (equal volumes of solution A and B) pipetted into a conical flask and aliquot of 5 mL dH_2O was added. The solution was then boiled for 15 seconds. Methylene blue indicator (a few drops) was then titrated with the solution until the color changed from blue to green. The carbohydrate content was then calculated according to following equation:

$$\text{Carbohydrate Content (\%)} = \frac{5 \times 0.005 \times 100 \times 100}{V \times 10 \times W} \times 0.9\%$$

Where V = volume of sample solution (titration volume) and W = weight of powdered sample.

Antioxidant Activity

Antioxidant activity of extracted β-glucanwas detected using DPPH (Sigma Aldrich, USA) for free radical scavenging assay[11]. Scavenging potential of β-glucan against DPPH radicals was determined spectrophotometrically (Aquarius, Cecil, Italy). Colour change (from deep- violet to light- yellow) when DPPH reduced was measured at 517 nm. In our experiment, set of concentrations (12.5, 25, 50, 100 and 200 µg mL^{-1})

were used. Ascorbic acid was used as positive control. The inhibition (%) of radicals by β-glucan was calculated according to the formula:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of } -\text{ve control} - \text{Absorbance of the sample}}{\text{Absorbance of the } -\text{ve control}} * 100$$

Cytotoxicity Assay

Cell Culture and Maintenance: Human breast carcinoma cells (HepG2), human breast adenocarcinoma cells (MCF-7) and one non-carcinogenic liver cell line (WRL68) were kindly provided from Biotechnology Research Center, Al-Nahrain University. Cells were maintained and cultured in RPMI-1640 (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin G and 100 µg mL^{-1} streptomycin. Cells (3 x 10^4 cell mL^{-1}) were seeded into tissue culture flasks and allowed to grow at approximately 80 to 90% confluence monolayer (24 to 48 h). Cultures were maintained at 37ºC in CO_2incubatorwith humidified atmosphere. Gentle trypsinization (50 mg mL^{-1} of trypsin) was used for harvesting the cells[12].

MTT: The cytotoxic effect of β-glucan against HepG2, MCF-7 and WRL68 was estimated using 3-(4,5-Dimethylthyiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13]. Briefly, cells were cultured in 96-well plates and incubated until cells reach to 80% confluence. Medium was removed and 200 µL of different β-glucanconcentrations (25 to 400 µgmL^{-1} RPMI-1640 serum free medium)were added to the respective wells containing the cells. Wells with untreated cells were used as the negative control. After 24 h, 10 µL of MTT (Sigma Aldrich, USA) was added to each well.Plates were further incubated at 37ºC, 5% CO_2 for 4 h. Themedium was then carefully removed and 100 µL of dimethyl sulfoxide was added per well and incubated for5 min.Absorbance at 540 nm was measured using an ELISA microplate reader. The percentage of viability (%) was calculated according to the following formula:

$$\text{Viability (\%)} = \text{OD control} - \text{OD sample}/\text{OD Control} \times 100$$

Cytotoxicity of each sample was expressed as IC_{50} value.

Statistical Analysis: Data were expressed as means±standard deviation (SD) and analyzed by a one-way analysis of variance (ANOVA) followed with Dunn's test using GraphPad Prism(Graph Pad Software Inc.). A $p \leq 0.05$ was considered to indicate a statistically significant difference between groups.

Results and Discussions

Dried fruiting bodies of *P. eryngii* was subjected to β-glucan extraction which depended on heating-acid extraction steps. This method is characterized by its ability to extract glucan from mushrooms with significant quantities, limited use of organic solvents and time saving. The total yield of extracted glucan was 7.9%. Previously reported that the total yield of glucan extracted from *P. eryngii*was 6% [14]. The advantages of this procedure were heating, and extensive acid treatment followed byethanol application which leads to β-glucan precipitation and dissolve or remove most the proteins, mannan, nucleic acids and others. The impurities affect the physical and chemical properties of β-glucanand may cause reducing in its ability to be soluble in water [15]. Furthermore, the carbohydrate content for the extracted β-glucanwas 54%, indicating purity and method of choice for β-glucan extraction.

Regarding HPLC analysis, results in Fig. (1) revealed one major peak in sample of the extracted β-glucan at retention time 3.16 min (487.633 mAU) with an overall area percentage of 90%, which indicated the purity of the extracted β-glucan by comparing with the standard which exhibited almost the same retention time at 2.94 min (377.215 mAU). Purity of 90% gave an indication for the successful β-glucan extraction method. HPLC was used for detecting the purity of polysaccharides including β-glucan extracted from mushrooms and yeasts [16].

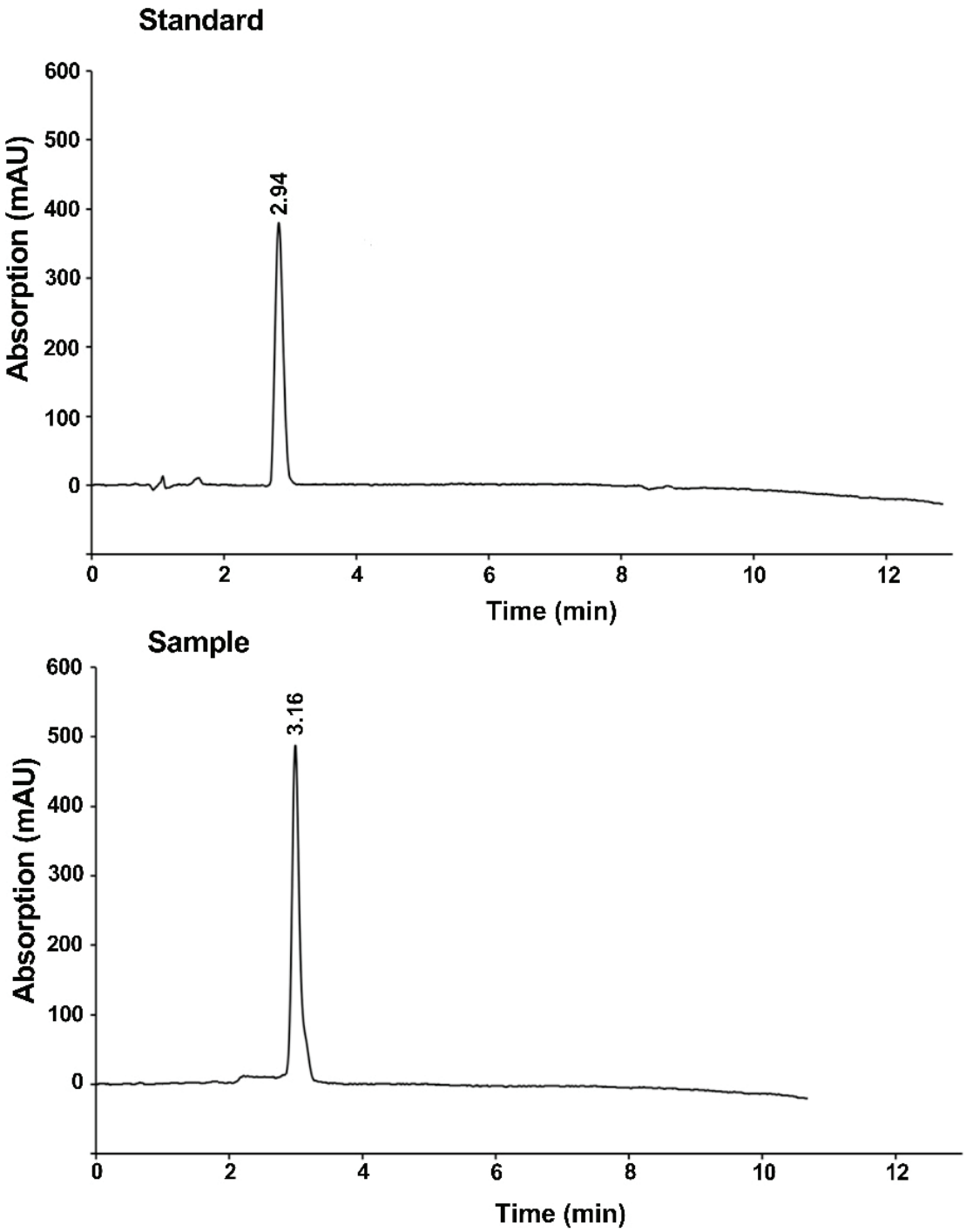

Fig. 1: HPLC chromatogram for (Top) β-glucan standard and (Down) the test sample.

FT-IR analysis of *P. eryngii*β-glucan with absorption range of 4000-400 cm^{-1} to was compared with the resulted functional groups of the standard. Fig. (2A), shows that the band range of ~1027.99 cm^{-1} is a characteristic feature of polysaccharides and assigned for β-1,4 glucans[17], the absorbance peak at this band represent the existence of C-O-C group[18]. In addition, hydroxyl groups and carboxyl groups were detected at band 2923.88 cm^{-1}, these groups are features of carbohydrate structure[19]. Moreover, both sample and standard showed high degree of similarity with respect to overall spectra abortion.

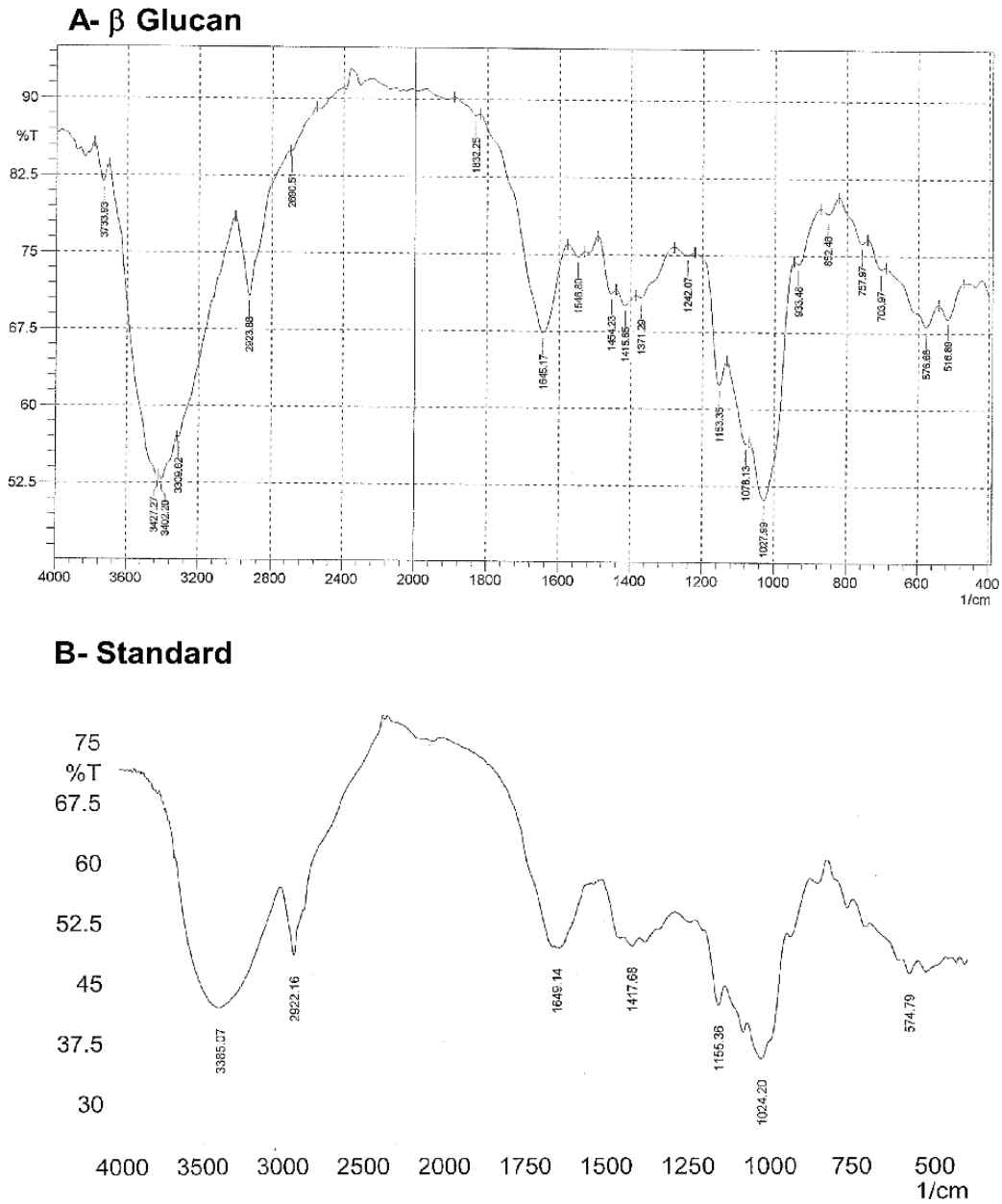

Fig. 2: FT-IR Spectra of (A) extracted *P. eryngii* β-glucan sample (B) standard.

The scavenging activity of *P. eryngii*β-glucan was estimated using increasing concentrations of β-glucan. Results in Fig. (3) demonstrate a potential free radical scavenging capability of *P. eryngii*β-glucan with calculated IC_{50} value of 39.3 µg mL^{-1}. By comparing with ascorbic acid (IC_{50} 27.47 µg mL^{-1}), *P. eryngii*β-glucan showed no significant differences in the pattern of free radicles reduction among all the tested concentrations. β-glucanare the most abundant forms of polysaccharides which display many biological activities including antioxidant[20]. Antioxidant activity of β-glucan is highly dependent on mushroom source and method of extraction. Our results are in agreement with Roncero-Ramos*et al*.,[21] which described the antioxidant activity of β-glucan from different mushroom sources including *P. eryngii*. Another study involving edible mushrooms revealed that β-glucan exhibited antioxidant activity 64 to 93% reduction of DPPH [22].

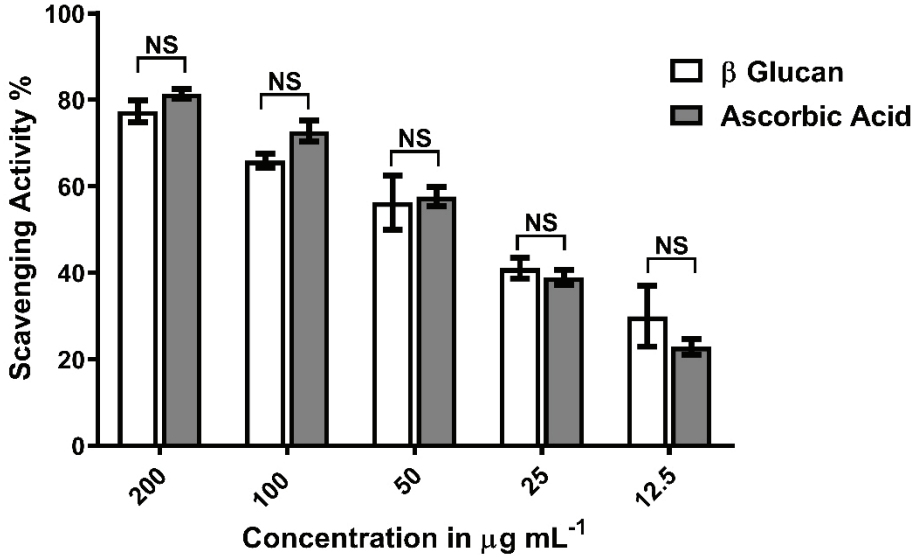

Fig. 3: Mean (%) DPPH scavenging activity of *P. eryngii* β-glucan with respect to ascorbic acid. NS: Non-Significant.

Various concentrations of *P. eryngii*β-glucan were tested againstMCF-7 and HepG2 tumor cells. In the present study we used the MTT assay, which is universally used to evaluate the cytotoxic potency of drugs *in vitro*[12]. Results in Fig. (4) shows that the more increasing in β-glucan dose the more reduction in MCF-7 and HepG2 viability. The effect of MCF-7 and HepG2 viability by *P. eryngii*β-glucanexhibited a dose dependent pattern of reduction with a calculated IC_{50} of 280.00 and 539.5 μg mL^{-1}, respectively. On the other hand, *P. eryngii*β-glucan hadslight toxic effect on the cell viability of normal cells WRL68.

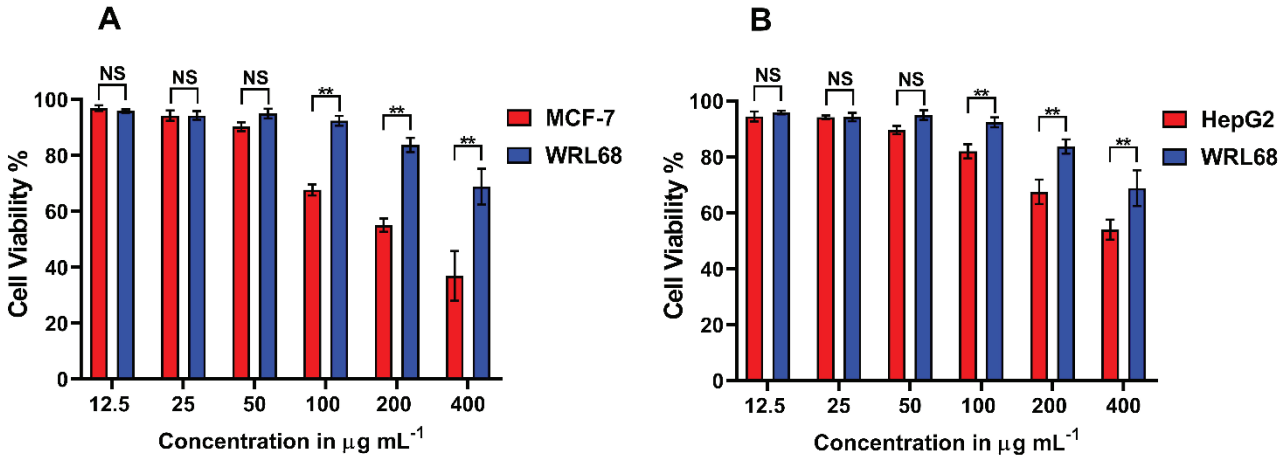

Fig. 4: MTT assay for *P. eryngii* β-glucan against (A) MCF-7 (B) HepG2 with corresponding normal cell line WRL68. Mean (±SD) of viability was detected after 24 h treatment. Differences considered significant: **$p \leq 0.01$. NS: Non-Significant, SD: Standard Deviation.

The biological, immunological and pharmacological activities of glucans extracted from edible mushroom are mainly attributed to β-glucan[23]. The cytotoxic activity of *P. eryngii*β-glucan against different types of tumor cells indicated a significant dose-dependent inhibition of cell proliferation and exerted direct cytotoxicity after 24 h. Many reports indicated the anti-proliferative effect of β-glucan extracted from different mushroom

sources. The effect of β-glucan*in vitro* against different tumor cell lines was well demonstrated [23]. In addition, pervious finding indicated that the toxicity of β-glucan on human pigmented malignant melanoma (Me45) cell line increased by increasing β-glucan concentration with viability reduction reached up to 19%. Moreover, it was proved that glucans in nature have low toxicity on normal cells and well tolerated by patients treated with glucan combination[24].

Conclusions

We can conclude that the extracted *P. eryngii*β-glucan exhibited strong antioxidant capabilities and promising anti-proliferative potential on tumor cells *in vitro*, which needed more investigations regard the mechanism of β-glucan in inducing tumor cell viability reduction.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq.

Conflict of Interest: Non

Funding: Self-funding.

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