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IX. სტატიები შეიარაღებენ ზრდასხული ლარკურები, ინტეგრალურად გამოწვეული შეთვალება, დამინიჭებული გამოწვეული შეთვალება, საქართველო, საქართველო, საქართველო, საქართველო, საქართველო, საქართველო, საქართველო, საქართველო, საქართველო.

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Abstract.

In this study, β-glucan was extracted by the hot water extraction method followed by ethanol precipitation and purified using ion and gel filtration chromatography, then evaluate the anticancer effects of β-glucan that purified from Phoenix dactylifera on cancer cell line. Ahmed Nahi Glioblastoma Multiform (ANGM) cancer cell line was used in the in vitro study. Cell line exposure times were calculated after 24, 48, and 72 hours in a micro titration plate under absolutely sterile conditions. High molecular weight β-glucans can be obtained using the hot water extraction method without having to use strong agents to change their structure, like alkalis or acids. Anti-cancer property of β-glucans can be obtained using the hot water extraction method followed by ethanol precipitation and purified using ion and gel filtration chromatography. The structural characteristics of β-glucan were determined. Further, the cytotoxic effects of date fruits-derived β-glucan on malignant cell line were investigated.

Materials and Methods.

1. Extraction and Purification of β-glucan from Phoenix dactylifera

Water Extraction Method: A water extraction method was used to extract β-glucan from date fruits [5]. Dates (500 g) were mechanically crushed, cut into smaller pieces, and dried. The powder was mixed with double distilled water in ratio of 1:20 (wt/v). The mixture was heated to 90°C for 6 hours in a shaker water bath. At 4°C, the mixture was centrifuged for 20 minutes at 5000 rpm. The particle was discarded, and the supernatant was obtained. The suspension was centrifuged at 5000 rpm for 20 minutes at 4°C after the supernatant solution (500 mL) was diluted with ethanol absolute (500 mL) in a ratio of 1:1. The supernatant was removed, and the pellet was collected. The pellet was washed with acetone before the suspension performed a 20-minute centrifugation at 5000 rpm at 4°C, then it was dried. An ethanol 100% solution was used to dilute a 200 mL solution of the polysaccharide in water, and centrifugation was used to collect the precipitate (5000 rpm for 15 min). Four times of this procedure were done to precipitate β-glucan (glucan-p1). The phenol-sulfate acid method was used to determine the polysaccharide content, and protein content was measured according to Bradford, (1976) by using a UV, Visible Spectrophotometer that measure the absorbance at 595nm.

2. Purification of β-glucan by DEAE cellulose- 52 column

Glucan-P1 was dissolved in distilled water, and a dropper was used to add the solution gradually along the balanced DEAE cellulose-52 column wall. The phenol-sulfate acid method was used to determine the polysaccharide content of each fraction by using a UV, Visible Spectrophotometer that measured the absorbance at 490 nm, and protein content was measured at 595 nm. The eluent's main sugar-containing fractions were collected, mixed, and dried in an oven at 60 C. (glucan-p2).

3. Gel filtration chromatography by using Sephadex G-100 Column

The previous step's glucan-P2 fraction was re-dissolved in deionized water and gradually added to the Sephadex G-100 column which had been set up for equilibration. Deionized water was used for elution at a flow rate of 18 ml/hr with a yield of 5 ml for each tube. At 490 nm and 595 nm, the absorbance of the samples that were collected and the elution gradient curve were both calculated. The sample with the highest concentration...
of glucan was chosen, and it was dried in an oven (at 60 °C) to produce pure glucan (glucan-P3).

4. Determination of Carbohydrate Concentration:
As described by Dubois and his colleagues [6] the phenol-sulphuric acid method was carried out as mentioned in subsequent steps. From glucose stock solution (100 μg/ml) different concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 μg/ml) were prepared with a final volume of 1 ml. One milliliter of solvent and one milliliter of 5% phenol were mixed in a test tube. With vigorous mixing, 5 mL of strong sulfuric acid was added, and it was then allowed to cool at room temperature. It was allowed for the reaction mixture to cool to room temperature The blank contained 1 milliliter of distilled water, 1 milliliter of a 5% phenol solution, and 5 milliliters of H2SO4. Measurements were made of the absorbance at 490 nm. The glucose concentrations were plotted against the corresponding absorbance on a standard curve (Figure 1). Using a solution of glucan at a concentration of 1 mg/ml, the amount of carbohydrate in glucan was determined (glucan-p1, glucan-p2 and glucan-P3). and put through the same prior addition, then the absorbance at 490 nm was measured. The standard curve was used to calculate the carbohydrate concentration (Figure 1).

5. Determination of Protein Concentration
Bradford [7] described how to determine protein content as follows: In order to create a standard curve for bovine serum albumin, several concentrations of the BSA stock solution (0, 20, 40, 60, 80, and 100 µg/mL) were prepared. Coomassie Brilliant Blue G-250 dye were added to each concentration in a volume of 2.5 ml, stirred, and allowed to stand for two minutes at 37°C. The blank was made up of 2.5 ml of the dye reagent and 0.1 ml of Tris-HCl solution and used to measure the absorbance at 595 nm. A standard curve was created by comparing the BSA concentrations to the matching bovine serum albumin absorbance. Glucan sample (0.1 ml of 100 g/ml Tris-HCl) underwent the identical procedures as the preceding steps (Figure 2).

6. Anticancer effect of β-glucan
Preparation of β-glucan Concentrations: β-Glucan (glucan-P3) stock was prepared by dissolving 200 mg of prepared extract powder in 10 ml phosphate buffer saline and filtering through a sterile Millipore filter (0.22µm). Different concentrations were prepared starting from concentration (1000 µg/mL) ending with the concentration of (31.25 µg/mL) by using sterile serum free medium.

Cell Culture and Maintenance: Brain cancer cells (ANGM) was graciously provided by the Iraqi center for cancer and medical genetics research (ICCMGR), Mustansiriyah University. In RPMI-1640 (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum cells were maintained and cultivated. In tissue culture flasks cells were seeded and allowed to reach an 80–90% confluent monolayer (24 to 48 h). Cultures were kept at 37°C in a humid atmosphere using a CO2 incubator. Cells were harvested using mild trypsinization (50 mg trypsin mL⁻¹) [8].

MTT cytotoxicity assay: Using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cytotoxic effect of β-glucan on ANGM was calculated. In 96-well plates, cells were grown and incubated until they reached 80% confluence. 200 μL of various β-glucan concentrations (31.25, 62.5, 125, 250, 500 and 1000 µL/mL) were added to the corresponding wells containing the cells after the medium was discarded. Cells that had not been treated were used as the negative control in wells. 10 µL of MTT was applied to each well after 24 hours. Plates were incubated for an additional 4 hours at 37°C and 5% CO2. After carefully removing the media, 100 L of dimethyl sulfoxide was added to each well, and each was then given 5 minutes to incubate. A microplate reader for an ELISA was used to quantify absorbance at 540 nm [8]. The following formula was used to compute the inhibition percentage: (%) IR (%) = (ODc−ODt) / OD Control ×100

IR = Inhibitor rate, ODc = the optical density of control, ODt= the optical density of test

Statistical Analysis: Data were presented as means with standard deviations (SD) and evaluated using GraphPad Prism’s one-way analysis of variance (ANOVA) and Dunn’s test (Graph Pad Software Inc.). A statistically significant difference between groups was defined as p< 0.05.

Results.
Isolation and Purification of β- Glucan: Ion exchange chromatography and gel filtration were employed in two steps
to purify β-glucan from date fruits after it had been isolated using water extraction:

1. **Water extraction method.**

   The method of water extraction was used to extract glucan-P1. Yield of the glucan-P1 fraction was 62 g.

2. **Ion-exchange chromatography.**

   A DEAE cellulose-52 column was used to purify glucan-P1. The elution curve for glucan-P1 by the DEAE cellulose-52 column is shown in Figure (3). Elution peaks were observed in the tubes numbered 18–48, 59–74, and 94–105. Less protein impurity was found; it only appeared in the tubes 65–81 with a weak elution peak. The main sugar-containing fractions that were present in 18–48 tubes were combined to produce glucan-P2, and the yield was 7.25g.

3. **Gel filtration chromatography.**

   The glucan-P2 recovered via ion exchange chromatography was further purified using a Sephadex G-100 column. The results are shown in Figure 4. The eluted fractions (18 to 42 tubes) showed a single, symmetrical peak on the Sephadex G-100 column. A pure glucan was produced after this component’s collected solution was dried by oven at 60˚ C to obtain glucan-P3, a light-yellow refined product. The yield was 3.84g.

   Table (1) shows the residual protein content, Carbohydrate concentration and the recovery yield of β-glucan at each step of purification.

**Cytotoxic effect of β-glucan extract on Brain cell line.**

The effect of different concentrations of β-glucan (31.25, 62.5, 125, 250, 500, and 1000 µg/ml) on tumor cell line after (24, 48 and 72) hours of exposure in Figure 5. The results revealed significant cytotoxic effect at levels (P<0.05) for all concentration. β-glucan had highest inhibitory growth on ANGM at the highest dose (1000 µg/ml) for 48 hours of exposure. In addition, the morphology of ANGM cells was changed after treatment with β-glucan at higher concentration (1000 µg/ml) and cell density and adherent capacity decreased (Figure 6).
Discussion.

In the present result, we successfully isolated and purified β-glucan. The yield of extraction and purified β-glucan in the current investigation was 3.072%. Additionally, the waste residue from the extraction and purification process typically does not include poisonous and dangerous materials and may therefore be recycled. Additionally, the purified β-glucan's physicochemical characteristics were examined in light of their potential use in food and beverage applications. In comparison to alternative purification methods, the DEAE chromatography method was chosen because of its high sensitivity, and minimal sample loss [8]. The components of β-glucan extracted from date palm were determined using chromatography, which provided an effective approach for identifying the β-glucan [9]. β-Glucan had a concentration-dependent cytotoxic effect on ANGM cells. It significantly suppressed ANGM cell reproduction. The optical densities (OD) for the stained cell lines after treatment with various concentrations of the extracted β-glucan for 24, 48, and 72 hours showed that there were differences of (OD) between the concentrations, with the high concentration giving low value of OD, indicating maximum response, and the low concentration giving high value of OD, indicating minimum response in proportion to high percentage of viable cells. Previous studies have shown that β-glucans can

Table 1. Purification of β-glucan from date fruit.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein Concentration (mg/ml)</th>
<th>Carbohydrate concentration (mg/ml)</th>
<th>β-Glucan yield (% weight/ weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extraction (glucan-p1)</td>
<td>2.8 ±0.15</td>
<td>90 ±4.37</td>
<td>36 ±1.42</td>
</tr>
<tr>
<td>DEAE chromatography (glucan-p2)</td>
<td>0.9 ±0.08</td>
<td>120 ±7.03</td>
<td>19 ±0.86</td>
</tr>
<tr>
<td>Gel Filtration chromatography (glucan-p3)</td>
<td>0.04 ±0.007</td>
<td>210 ±11.57</td>
<td>5 ±0.26</td>
</tr>
<tr>
<td>LSD</td>
<td>0.661 *</td>
<td>43.72 *</td>
<td>5.77 *</td>
</tr>
</tbody>
</table>
modify biological responses and produce anti-tumor effects both \textit{in vitro} and \textit{in vivo} [10]. In agreement with our findings, Zhang, and his colleagues, [11] reported that water-soluble β-glucan from \textit{Poria cocos} inhibited cancer cell viability. The anticancer activity of β-glucans may be related to their ability to control inflammation through immune stimulatory patterns [12], and they may also have an effect on the regulation of gut hormones [13]. The bulk of anti-cancer medications work by putting tumor cells under oxidative stress, which is believed to be the cause of the majority of macromolecular changes in the cell. Among other macro-molecules, reactive oxygen species can damage DNA, membrane lipids, and proteins [14,15].

\textbf{Conclusion.}

In the present study, water soluble β-glucan was successfully isolated from \textit{Phoenix dactylifera} by water extraction and further fractionated by DEAE-52 cellulose and Sephadex G-100 chromatography, then characterized by HPLC. The obtained β-glucan exhibited a significant antitumor activity against Brain cell line (ANGM) in concentration dependent manner.

\textbf{Author Contributions.}

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hiba Mohammed Al-Khuzaay, Yasir Hussein Al-Juraisy and Ali Hussein Alwan. The first draft of the manuscript was written by Hiba Mohammed Al-Khuzaay and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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