In vitro Cytotoxic Screening of Different Parts from Ornithogalum bungei on Selected Cancer Cells

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Abstract
Background: Natural products comprise a large section of pharmaceutical agents in the field of cancer therapy. In the present study, the organic extracts and fractions of various parts of Ornithogalum bungei were investigated for in vitro cytotoxic properties on three human cancer cell lines, hepatocellular carcinoma (HepG2), prostate cancer (PC3), and leukemia (K562) cells.

Methods: The present experimental study was conducted at Tehran University of Medical Sciences (Tehran, Iran) during 2017-2019. Separately extracted plant materials, including bulbs, stems, and flowers of O. bungei were assessed by the tetrazolium dye-based colorimetric assay (MTT). The selected extracts were submitted to fractionation using vacuum liquid chromatography and after MTT assay, the half maximal inhibitory concentration (IC50) value for each fraction was determined. The data were analyzed using One-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered statistically significant.

Results: The cytotoxicity of the bulb’s methanol extract and the dichloromethane extract of aerial parts increased in a concentration-dependent manner. Additionally, cell viability decreased in a dose-dependent manner. In the HepG2 cell line, the best IC50 values of fractions from DCM extracts of aerial parts were determined to be 19.8±10.2 µg/mL after 24 hours of exposure and 19.39±6.4 µg/mL following 48 hours of exposure. In the PC3 cell line, after 48 hours of exposure, the IC50 values of fractions were unaccountable, while the percentage of inhibition for A6 to A11 in 24 hours of exposure was more than 40 µg/mL.

Conclusion: O. bungei growing in Iran showed significant potentials as a cytotoxic agent with selective effects on different cancer cell lines.

Keywords
● Ornithogalum bungei Boiss ● Hyacinthaceae ● Biological products ● HepG2, PC3, K562 cells

Introduction
Cancer is known to be one of the significant public health problems in most countries and is considered as one of the leading causes of death.1 Medicinal plants have historically been used to treat various diseases in different parts of the world.2 The main reason behind their use in medical aspects is their secondary metabolites production.3 Natural products have been recognized
as potential sources for anti-cancer candidate molecules. Over half of all the pharmaceuticals or new chemical entities entering the market or clinical trials are still of natural origin.4

Ornithogalum L. genus belongs to the subclass Monocotyledonae and is classified in the Hyacinthaceae family. Certain species of the Ornithogalum genus have exhibited anti-cancer and anti-inflammatory effects in Chinese folk medicine.5 Although here are a few findings concerning the folkloric medicinal background, some other species are used in floriculture and even gardens as cut flowers.6 In addition, a previous report showed some poisonous species.7 Several cardenolide glycosides have been isolated from a number of species of the Ornithogalum genus. In 2012, Mimaki and colleagues isolated a cholestane glycoside OSW-1 (3β,16β,17α-trihydroxycholesta-5-en-22-one16-O-(2-O-4-methoxybenzoyl-β-D-xlyopyranosyl)-(1→3)-(2-O acetyl-α-L-arabinopyranoside)) from the bulbs of O. saundersiae with significant cytostatic activities against various malignant tumor cells, such as HL-60 (human promyelocytic leukemia) and MOLT-4 (human T lymphoblast; acute lymphoblastic leukemia) through the induction of apoptosis.8-11

Another investigation on the bulbs of O. saundersiae demonstrated some rearranged cholestane glycosides with a glycosidic moiety at C-16. These important findings resulted in the synthesis of OSW-1 and its active analog, which acts by damaging the mitochondria and apoptosis induction through an increase in cytosolic calcium.12, 13 In addition, investigation on the ethanol (EtOH) extract of O. saundersiae showed its protective effect against acute hepatic failure by apoptosis of hepatocytes and anti-inflammatory activity.13 Some other studies on O. saundersiae presented the OSW1 activity against some resistant anti-cancer agents, such as fludarabine-resistant chronic lymphocytic leukemia cells.14, 15 The aerial parts of O. cuspidatum were used as a food additive and sore throat calming agent in Iranian traditional medicine. The flowers and bulbs of O. cuspidatum have been reported to have saturated hydrocarbons, and the leaves are believed to contain oxygenated hydrocarbons as well as terpenoid compounds.16 Bio-screening investigations of the methanol (MeOH) extract of the bulbs of O. thyrsoides introduced high to moderate cytotoxic cholestane bisdesmosidic, spirocyclic glycosides, and cholestane glycosides against leukemia HL-60 cells.17, 18 In the present work, the anti-cancer property of MeOH extract obtained from the shoots of O. naronense was investigated on human colon cancer (DLD-1), endometrium cancer (ECC-1), and embryonic kidney cancer (HEK-293) cells. The results indicated that O. naronense extract caused an increase in the amount of intracellular free radicals leading to associated DNA damage as well as concentration-related apoptosis in cancer cells.19

Ornithogalum bungei Boiss., which is locally called “Shir-morgh”, is an endemic species distributed in the Golestan forests of Iran.20, 21 Flowers of this bulbous plant are white with a green stripe on the midrib of the petals.22 The biological activities of O. bungei have not been reported in previous papers. Thanks to pharmacological screening, such as cytotoxic activity investigations, scientists could obtain important preliminary data to select plant extracts with potential anti-tumor properties. This study aimed to evaluate the anti-tumor effects of the extracts and fractions from different parts of O. bungei on HepG2, PC3, and K562 cell lines.

Materials and Methods

The Ethics Committee of Tehran University of Medical Sciences approved the study protocol under the code IR.TUMS.PSRC. REC.1396.2242, Tehran, Iran. The experiments were performed at the School of Pharmacy, Tehran University of Medical Sciences (Tehran, Iran), during 2017-2019.

Plant Collection

The plants were collected by the authors in May 2016 from Golestan province, Iran. Taxonomic identification was carried out scientifically, and a voucher specimen has been deposited at the herbarium of the School of Sciences, University of Tehran (voucher number: 48680-TUH).

Extraction

Different parts of O. bungei, including bulbs, stems, flowers, fruits, and seeds, were carefully separated, air-dried at room temperature, and grounded by a mill into fine powder. The extraction was performed stepwise with dichloromethane (DCM) and MeOH in triplicate according to the following procedure: the dried plant samples were placed in separate flasks, mixed with proper amounts of solvent, sonicated in an ultrasonic water bath at 40 °C for 30 minutes, and then mechanically stirred for 24 hours (three times). The solutions of the obtained extract were filtered and concentrated under a vacuum using a rotary evaporator at 40 °C (table 1).
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**Cell Culture Preparation**

HepG2, PC3, and K562 (obtained from Pasteur Institute of Iran, Tehran) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 100 units/mL penicillin (Biosera, UK), and 100 mg/mL streptomycin (Biosera, UK). The cells were cultured in a humidified atmosphere of 5% CO2 at 37 °C. An equal volume of FBS-containing medium (<0.5% DMSO as solvent) was used as the control for all the measurements.

**Cell Viability Assay**

Cytotoxicity was evaluated utilizing a colorimetric assay method.23 Proliferation test was based on the color reaction of mitochondrial dehydrogenase in living cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, UK). The cells (1×10^4 cells/well) were seeded into the 96-well plate in quadruplicate. After 24 hours of incubation at 37 °C, the cells were treated with various concentrations of extracts. MTT with the final concentration of 5 mg/mL was added at the end of the treatment period to each well, and the plate was incubated for additional four hours. The formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm on a microplate reader (Anthos, UK). Cell proliferation was calculated as percent control and presented as mean±SD.

**Screening Procedure**

For preliminary screening, the polar and nonpolar extracts were examined on the three cell lines at the final concentration of 200 µg/mL, and the most effective ones were forwarded for further investigation. The cytotoxicity assays were repeated at the concentrations of 100, 150, and 200 µg/mL. The plant extracts, which indicated dose-dependent cytotoxic properties were selected for further evaluation. The third step of cytotoxicity assay was performed for fractions at the concentrations of 25, 50, and 100 for HepG2 cells and 5, 10, 25, 50, and 100 for PC3 cells.

**Carbohydrate Existence Test**

Two mL of MeOH extract solution was examined for carbohydrate existence using Molisch’s method.24 Three drops of α-naphtol (Molisch’s reagent) and 2 mL of concentrated H₂SO₄ (sulfuric acid) were added along the walls of the test tube. Carbohydrate becomes dehydrated in contact with concentrated H₂SO₄ to form furfural derivatives, which react with Molisch’s reagent resulting in a violet-colored ring at the junction of the two liquids; this indicated the presence of carbohydrate in MeOH extract of *O. bungei*.

**Fractionation**

Fractionations of the selected extracts was initiated by normal phase vacuum liquid chromatography (VLC). 200 g silica (mesh: 230-400 ASTM; Merck, Germany) was packed as the stationary phase under reduced pressure on an 80×5 cm column. Then, 43 g of the selected extracts were applied to 40 g silica and loaded on top of the column. While the vacuum was applied, the mobile phase was eluted gradient consisting of n-hexane-CHCl₃ (100:0, 80:20, 60:40, 40:60, 0:100 v/v, each 500 mL), and CHCl₃-MeOH (95:5, 85:15, 80:20, 75:25, 50:50, 25:75, 20:80, 10:90, 0:100 v/v, each 500 mL), and 15 fractions were collected. The similar ones were combined based on thin-layer chromatography (TLC), and 11 fractions were obtained (A1-A11).

**Statistical Analysis**

One-way analysis of variance (ANOVA) followed by Tukey’s *post hoc* test was employed to compare the differences between various treatment groups using GraphPad Prism version 5.01 (San Diego, CA). P<0.05 was considered statistically significant.

**Results**

The cytotoxic effects of different extracts were primarily evaluated on HepG2, PC3, and K562 cells at the final 200 µg/mL concentration. In HepG2 cells, the MeOH extract of bulbs and the DCM extract of stems inhibited the growth of cells over 50% within 24 hours. With the increase in the time of cell incubation, the extracts’ cytotoxicity was markedly influenced, and the effect of the DCM extract of the flowers increased. The DCM extract of bulbs and MeOH

<table>
<thead>
<tr>
<th>Substance</th>
<th>Bulb (g)</th>
<th>Stem (g)</th>
<th>Flower (g)</th>
<th>Fruit (g)</th>
<th>Seed (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried material</td>
<td>124.01</td>
<td>172.00</td>
<td>53.00</td>
<td>20.00</td>
<td>5.00</td>
</tr>
<tr>
<td>DCM extract</td>
<td>2.73</td>
<td>31.32</td>
<td>12.00</td>
<td>0.81</td>
<td>0.38</td>
</tr>
<tr>
<td>MeOH extract</td>
<td>24.00</td>
<td>131.20</td>
<td>20.00</td>
<td>3.34</td>
<td>0.42</td>
</tr>
</tbody>
</table>

DCM extract: Dichloromethane extract; MeOH extract: Methanol extract
extract of stem induced cytotoxicity over 20% within 48 hours (figure 1). In PC3 cells and following 24 hours of incubation, the bulb MeOH extract and the DCM extract of flowers showed cytotoxic effects over 30% (figure 2). In addition, after 48 hours of incubation, the cytotoxic effect of the DCM extract of stems increased. The MeOH extract of bulbs and the DCM extracts of stems and flowers were active against K562 cells at 24 hours, while no more activity was observed at 48 hours (figure 3). The other extracts exhibited no significant cytotoxicity in the cell lines.

Three selected extracts, including the MeOH extract of bulbs and the DCM extracts of stems and flowers, were assayed at concentrations of 100, 150, and 200 µg/mL. Figure 4 illustrates the cytotoxic effect of the extracts on HepG2 cells. The MeOH extract of bulbs and DCM extract of stems showed the maximum inhibition in 200 µg/mL. With the increase in the incubation time, the cytotoxicity of these extracts remained stable, yet the effect of the DCM extract of flowers began to increase up to 48 hours. In PC3 cells, the cytotoxicity effects of the DCM extracts of stems and flowers were initiated after 48 hours of incubation, whereas the effect of MeOH extract of bulbs was stable in different times of exposure and inhibited the growth of cells over 30% (figure 5).

The highest cytotoxic effect of the MeOH extract of bulbs was observed on K562 cells. The other extracts had no significant cytotoxicity in 24 hours and no more activity up to 48 hours (figure 6). Based on the cytotoxic results, the MeOH extract of the bulbs and the DCM extracts of stems and flowers demonstrated better activities than the others.

No significant activities of the fractions were observed on K562 cells neither in 24 hours nor 48 hours. Therefore, further investigation was carried out with two cell lines of HepG2 and PC3. Regarding the patterns of the equal constituent of the DCM extracts of stems and flowers on TLC and their similar cytotoxic effects, they were combined (named A) and applied on the VLC column, resulting in 11 fractions (A1-A11), which
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were screened by the cytotoxic assay.

As represented in figure 7, fractions A6 to A10 showed the maximum inhibition dose and time-dependent cytotoxicity effect on HepG2 cells (P<0.01). Fraction A6 inhibited the growth of hepatocellular cells by over 70% after 48 hours of exposure. Fractions A1 to A5 did not show any significant effects after 24 and 48 hours of incubation. Fraction A11 inhibited the growth of the cells up to 30% with the increase in the time of exposure. In PC3 cells, although fractions A6 to A11 demonstrated dose- and time-dependent cytotoxicity in comparison to the control group, they did not show any significant effects in higher concentrations compared to the extract (A). Fractions A1 to A5 had no significant efficacy on PC3 cells (figure 8).

Generally, a time-dependent MTT viability reduction after 24 and 48 hours was observed in the treatment of HepG2 and PC3 cells with different concentrations of the DCM extracts of stems and flowers and the MeOH extract of bulbs. The cytotoxic effect of fractions concluded from the DCM extracts of stems and flowers is expressed as IC$_{50}$ (half maximal inhibitory concentration) values (presented in table 2). In HepG2 cell line, the fractions concluded from DCM extracts of stems and flowers showed moderate to good cytotoxic activity after 24 hours of treatment at different concentrations of 25, 50, and 100 μg/mL (IC$_{50}$ values for A6, A7, A8, A9, A10, and A11 <50 μg/mL). High activities were observed in fractions of A6, A7, A8, A9, and A10 against HepG2 cell line (IC$_{50}$ <25 μg/mL) after 48 hours of exposure. On the other hand, fractions A6 to A10 moderately decreased the viability of PC3 cancer cell lines following 24 hours of exposure. These fractions did not display a remarkable cytotoxic activity on PC3 cell lines after 48 hours of exposure (not shown). The lowest fraction IC$_{50}$ values (20.09
μg/mL and 19.84 μg/mL) were observed with A6 and A7, respectively in HepG2 cells (table 2).

**Discussion**

To date and to the best of our knowledge, the present study was the first evaluation of the cytotoxic potential of *Ornithogalum bungei* on human cancer cells. We reported that the Organic extracts of stems, flowers, and bulbs of *O. bungei*, collected from northern parts of Iran, had cytotoxic activities against three human cell lines, namely HepG2, PC3, and K562. In this study, it was observed that the MeOH extract of bulbs and the DCM extracts of stems and flowers are of a profound effect on HepG2 cell lines. Moreover, they showed varying degrees of response to PC3 cell lines and K562. The MTT assay determined the IC50 concentration of the extract fractions. It could be observed that the DCM fractions of stems and flowers (A6-A8) for HepG2 and PC3 were the most potent fractions in different times, concentrations, and selectivity (table 2). These reports are consistent with previous studies on the cytotoxic effects of other species of *Ornithogalum*. The earlier studies on

<table>
<thead>
<tr>
<th>Incubation</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
<th>A7</th>
<th>A8</th>
<th>A9</th>
<th>A10</th>
<th>A11</th>
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<tbody>
<tr>
<td>IC50 (μg/mL) in HepG2 cells</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>24 hours</td>
<td>22.3±2.3</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>20.0±2.8</td>
<td>19.8±10.0</td>
<td>20.6±13.0</td>
<td>23.1±8.3</td>
<td>24.3±19.3</td>
</tr>
<tr>
<td>48 hours</td>
<td>21.7±5.6</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>19.3±4.6</td>
<td>22.9±3.5</td>
<td>22.2±1.09</td>
<td>20.5±9.72</td>
<td>23.6±7.2</td>
</tr>
<tr>
<td>IC50 (μg/mL) in PC3 cells</td>
<td></td>
<td></td>
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<tr>
<td>24 hours</td>
<td>49.0±0.8</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>71.6±9.8</td>
<td>53.4±6.3</td>
<td>53.8±10.5</td>
<td>50.3±8.2</td>
<td>50.6±3.1</td>
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<tr>
<td>48 hours</td>
<td>&gt;50</td>
<td>&gt;50</td>
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Ornithogalum genus resulted in carbohydrate-based compounds, such as polysaccharides, in the polar extract of bulbs. Four water-soluble polysaccharides were separated and identified from the polar extracts of *O. caudatum* bulbs, which exhibited strong anti-tumor activities against Sarcoma 180 solid tumors implanted in BALB/c mice in vivo.25, 26 On the other hand, the Molisch’ test indicated a high content of carbohydrate-based compounds in the MeOH extract of the bulbs. Thus, it was assumed that the cytotoxic effect of the bulbs is on account of the polysaccharides. Consequently, the MeOH extract of the bulbs was used for specific isolation and characterization of the polysaccharides in a separate study, and the investigation continued with the DCM extracts of stems and flowers.

In the current paper, we did not report the mechanism of action of *O. bungei*, yet the results regarding cytotoxicity of other species against similar cell lines, such as hepatocellular carcinoma (HCC) cells, showed the consistency of cytotoxic effects with possible mechanisms of action. Since the significant hallmarks of HCC are uncontrolled cell proliferation, resistance to cell death, immortality, invasion, metastasis, and inflammation induced by mediators, such as IL6, IKK-NFκB, and inflammatory-NFκB, the induction of 85–90% NFκB reporter activity inhibition in HEP3B (HCC-derived cells) was observed in the study on *O. saundersiae* NFκB signaling pathway.27 The total synthesis of the OSW-1, a compound identified in *O. saundersiae*, was accomplished in 1999. OSW-1 exhibited...
cytotoxicity against leukemia, ovarian, and brain cancer and represented varying degrees of sensitivity to liver cancer cell lines owing to wild-type p53 factor in SK-HEP-1 cells as well as p53-independent factor-induced apoptosis by *Ornithogalum* in HEP3B cells. This showed that in exhibiting cellular sensitivity to *Ornithogalum*, in addition to the NFκB pathway, other signaling pathways and targeting features are significant. In a study by Ramesh and colleagues, the cytotoxic effect of *Ornithogalum* in HEP3B cells through TNF-α and NFκB signaling pathways was reported. Additionally, the comparison of HEP3B to HCC-derived cell lines and the treatment of normal hepatocytes showed a specific and selective activity of *Ornithogalum* to liver cancer cells. In another study, OSW-1 damaged the mitochondrial membrane in both human leukemia (HL-60) and pancreatic cancer cells (AsPC-1), resulting in the transmembrane potential loss, increase in cytosolic calcium, and activation of calcium-dependent apoptosis in electron microscopy and biochemical analyses. *Ornithogalum* cell cycle analyses implied increases in the S and G2/M phases through inactivation of the cdc2-cyclinB2 complex in AsPC-1 cells. Moreover, *Ornithogalum* induced p21 expression as well as S-phase arrest with elevated expression of the glucose-regulated protein (grp) and related target genes in HCC. The results of another study revealed that sodium-calcium exchanger 1 (NCX1) inhibition by OSW-1 could induce calcium-dependent cell death. Knowledge about multiple-targeting pathways by *Ornithogalum*, such as NFκB activation, enrichment analysis of oxidative stress, inflammatory NFκB signaling, and inhibition of Nrf2/Nrf1 could be critical for the determination of targeted therapeutics for HCC.

Cogitating the overall activity of the extracts in this study and following the preliminary anti-tumor studies on the plant species, as well as possible mechanisms reported in previous investigations on Hyacinthaceae family, *O. bungei* could be considered as a potential anti-hepatocellular carcinoma plant. A limitation of this study was the lack of information about the genus of *Ornithogalum*; accordingly, further studies on cytotoxic isolated compounds seem to be necessary. This screening study was an advantageous primary level reporting the cytotoxicity effect of *O. bungei*. However, the discovery of the mechanisms of action needs to be investigated in more detail. Further research should focus on the structure-activity relationship of potent compounds from this species.

**Conclusion**

Overall, HepG2 cells were the most sensitive against the examined extracts and fractions. All of these results exhibited that *O. bungei* could be considered as a source of bioactive anti-tumor substances. Finally, the possibility of using *Ornithogalum* genus plants, such as *O. bungei*, as a therapeutic agent for hepatocellular cancer deserves further fundamental research, and phytochemical studies on this genus are of great significance to obtain further information about future anti-tumor compounds.

**Acknowledgment**

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**Conflicts of Interest:** None declared.

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