Antiproliferative and cytotoxic effects of bioactive compounds isolated from *Onosma bourgaei*

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Abstract

Onosma species have been used commonly for traditional medicine for years due to their bioactive compounds content. *Onosma bourgaei* aerial part was extracted with hexane and methanol successively. The methanol extract was subjected to chromatographic techniques to isolate allantoin (**1**), 3,4-dihydroxybenzaldehyde (**2**), luteolin-7-*O*-glucoside (**3**), apigenin-7-*O*- β -glucoside (**4**), diosmetin-7-*O*- β -glucoside (**5**), rosmarinic acid (**6**), globoidnan A (**7**). The structure of isolated compounds were identified by spectroscopic techniques such as 1D-NMR, 2D-NMR, FTIR, and LC-TOF/MS/MS. Antiproliferative activity of extract and natural compounds were carried out using HeLa (human epithelial cervix adenocarcinoma, ATCC® CCL-2™), HT-29 (human colorectal adenocarcinoma, ATCC® HTB38™), MCF-7 (human mammary gland adenocarcinoma, ATCC® HTB22™) and A549 (human lung carcinoma, ATCC® CCL185™) cancerous cells and normal cells, FL (human epithelial amnion cell, ATCC® CCL62™). Lactate dehydrogenase (LDH) was performed for cytotoxicity. The compounds, **4**, **6**, and **7** displayed the strong antiproliferative activity against corresponding cell lines. Apigenin-7-*O*- β -glucoside (**4**) revealed the excellent activity on HeLa, HT29, A549, MCF6 cancer cell lines with the values of (IC₅₀, μ M) 167.3, 196.8, 181.1, 203.5 respectively compared standard compound, cisplatin.

1. Introduction

Natural products act a substantial role in drug innovation stage as raw materials due to revealing wide range of biological effects as well as containing bioactive secondary metabolites [1–5]. *Onosma* genus which is the leading of Boraginaceae family consists of 230 species, distributed thorough Mediterranean, Europe, and Asia [6]. It is represented by almost 103 species in Turkey flora [7, 8].

Phytochemical research on this genus revealed isolation of bioactive compounds such as alkaloids, flavonoids, naphthazarins, aliphatic ketones, and naphthoquinones [9]. Naphthoquinones isolated from *O. paniculatum* had anti-inflammatory potential [10] and shikonins and alkanins generated from *O. echinoides* revealed the wound healing activity [11]. Naphthoquinones isolated from *O. visianii* was reported to exhibit noticeable antibacterial and cytotoxic activities [12]. In addition, naphthoquinones belonging to the shikonin family isolated from *O. visianii* roots extract could be a promising candidate for acaricidal products to be used in crop protection [13]. The scientific reports on *Onosma* species presented a large variety of biological effects including cholinesterase and tyrosinase inhibition effects with antioxidant activity, antibacterial, antitumor [14, 15], hypoglycemic [16], parasitocidal, antifungal activities [17].

Onosma species have been employed widely in food and medicine. Furthermore, these species have been exploiting as traditional medicine such as purgative and anthelmintic as well as in bronchitis, abdominal pain, strangury, urinary calculi, fewer, wounds, burns and blood disease. Moreover, flowers of this genus have been employed as stimulants, and cardiotonic while the leaves have been used for purgative and cutaneous outbursts [18].

Cancer is the unusual increase of cells that proliferate uncontrollably. It is the third chief source of death in the world after cardiovascular and infectious diseases. This illness is recognized as a main public health challenge all worldwide. Breast cancer is the prominent reason of death among the women in many nations. Due to increasing cancer cases in developing and developed countries, the demand for new chemotrophic compounds is inevitable. Owing to the significant side effects of synthetic compounds, interest in natural products has been increasing rapidly lately. The compounds found in plants have a potential to defend against cancers types such as lung, prostate, breast and colon cancers [19].

In this study, six compounds were isolated and identified, in addition, their antiproliferative activity was carried out on HeLa, HT29, A549, and MCF-7 cancerous cells, and FL (human amnion cells) which is a normal cell.

2. Materials Methods

2.1. General experimental procedures

NMR spectral analyses were executed on a spectrometer at 400 MHz (Bruker), 600 MHz (Varian) for ^1H and 100 MHz, 150 MHz for ^{13}C NMR, respectively. UV analysis was executed by UV-2600 spectrophotometer (Shimadzu). Sephadex LH-20 was employed for column chromatography as a stationary phase. Analytical alumina plates (60 F₂₅₄) were employed for TLC analysis. Agilent 6210 LC-TOF/MS spectrometer was used for HRMS analysis. The cell proliferation ELISA, BrdU kits and 5-fluorouracil were supplied from Roche Diagnostics GmbH (Germany).

2.2. Plant material

Onosma bourgaei was obtained from Tokat, Turkey and identified Dr. Ozgur Eminagaoglu, Artvin Coruh University, Artvin, Turkey in which a voucher sample was deposited (ARTH-5600).

2.3. Extraction and isolation process

Onosma bourgaei aerial part was dried at shade and dried material (750 g) was macerated in hexane (1.5 L) for 4 days, filtered, the solvent was evaporated under reduced pressure to generate the hexane extract. The solid part was subjected to re-extraction with methanol (1.5 L) for 4 days. After filtration, solvent was removed under reduced pressure to yield methanol crude extract. The column chromatography was subjected for methanol crude extract (3.0 g) and methanol was used as a mobile phase, Sephadex LH-20 and silica gel were used as stationary phase. A total 20 fractions were collected; each was 10 mL. The chromatographic process was repeated four times to obtain more products. The fractions including the same compounds were combined after checking the TLC application. Allantoin (2,5-dioxo-4-imidazolidinyl)urea (**1**), 3,4-dihydroxybenzaldehyde (**2**), and luteolin-7-*O*-glucoside (**3**) were purified. The fractions including mixture compounds were applied to preparative HPLC, yielded the isolation of pure compounds, apigenin-7-*O*- β -glucoside (**4**), diosmetin-7-*O*- β -glucoside (**5**), rosmarinic acid (**6**), globoidnan

A (7). The structure of isolated compounds was identified by spectroscopic techniques including 1D-NMR, 2D-NMR, and LC-TOF/MS.

Allantoin (1)

(42 mg), LC-TOF/MS m/z 157.0338 $[M-H]^-$ (calcd for $C_4H_6N_4O_3$, 157.0362). 1H NMR (600 MHz, DMSO- d_6) δ 10.47 (s, 1H), 8.02 (s, 1H), 6.97 (d, J = 8.1 Hz, 1H), 5.80 (s, 2H), 5.22 (d, J = 8.1 Hz, 1H). ^{13}C NMR (150 MHz, DMSO- d_6) δ 174.0 (C-7), 157.8 (C-2), 157.2 (C-5), 62.9 (C-3).

3,4-dihydroxybenzaldehyde (2)

(15 mg), 1H NMR (600 MHz, DMSO- d_6) δ 9.65 (s, 2H, CHO), 7.23 (d, J = 7.9 Hz, 1H, H6), 7.19 (s, 1H, H2), 6.84 (d, J = 7.9 Hz, 1H, H5). ^{13}C NMR (150 MHz, DMSO- d_6) δ 191.4 (C-7), 166.5 (C-5), 153.0 (C-4), 146.8 (C-3), 129.8 (C-1), 125.3 (C-6), 115.2 (C-2).

Luteolin-7-O- β -glucoside (3)

(45 mg) LC-TOF/MS/MS m/z 447.0946 $[M-H]^-$ (calcd for $C_{21}H_{19}O_{11}$, 447.0927). 1H NMR (600 MHz, DMSO- d_6) δ 13.0 (s, 1H, 5-OH), 7.43 (dd, J = 7.2, 1.9 Hz, 1H, H-6'), 7.40 (d, J = 1.9 Hz, 1H, H-2'), 6.87 (d, J = 7.2 Hz, 1H, H-5'), 6.76 (d, J = 1.9 Hz, 1H, H-8), 6.71 (s, 1H, H-3), 6.42 (d, J = 1.9 Hz, 1H, H-6), 5.06 (d, J = 7.3 Hz, 1H, H-1"), 3.72 (m, 1H, H-6 β "), 3.49 (m, 1H, H-6 α "), 3.44 (m, 1H, H-3"), 3.28 (m, 1H, H-5"), 3.26 (m, 1H, H-2"), 3.17 (m, 1H, H-4").

^{13}C NMR (150 MHz, DMSO- d_6) δ 182.3 (C-4), 165.0 (C-2), 163.4 (C-7), 161.6 (C-5), 157.4 (C-9), 150.7 (C-4'), 148.4 (C-3'), 121.6 (C-1'), 119.6 (C-2'), 116.5 (C-5'), 114.0 (C-6'), 105.8 (C-10), 103.5 (C-3), 100.4 (C-1"), 99.9 (C-6), 95.2 (C-8), 77.6 (C-3"), 76.9 (C-5"), 73.6 (C-2"), 70.0 (C-4"), 61.1 (C-6"),

Apigenin-7-O- β -glucoside (4)

(17 mg), 1H NMR (600 MHz, DMSO- d_6) δ 13.0 (s, 1H, 5-OH), 7.99 (d, J = 8.5 Hz, 2H, H-2', H-6'), 6.86 (s, 1H, H-3), 6.93 (d, J = 8.5 Hz, 2H, H-3', H-5'), 6.86 (d, J = 1.5 Hz, 1H, H-8), 6.46 (d, J = 1.5 Hz, 1H, H-6), 5.06 (d, J = 7.3 Hz, 1H, H-1"), 3.60 (1H, m, H-6 α "), 3.40 (1H, m, H-6 β "), 3.33 (1H, m, H-5"), 3.29 (1H, m, H-3"), 3.17 (1H, d, J = 9.2 Hz, 1H, H-4"), 3.15 (1H, m, H-2").

^{13}C NMR (150 MHz, DMSO- d_6) δ 182.5 (C-4), 164.8 (C-2), 163.4 (C-7), 161.6 (C-5), 157.4 (C-9), 129.1 (C-2', C-6'), 121.4 (C-1'), 116.5 (C-3', C-5'), 105.8 (C-10), 103.6 (C-3), 100.4 (C-1"), 99.9 (C-6), 95.3 (C-8), 77.6 (C-5"), 76.9 (C-4"), 73.5 (C-3"), 69.9 (C-2"), 61.0 (C-6"),

Diosmetin-7-O- β -glucoside (5)

(21 mg), 1H NMR (600 MHz, DMSO- d_6) δ 12.9 (s, 1H, 5-OH), 7.52 (dd, J = 8.4, 2.4 Hz, 1H, H-6'), 7.38 (d, J = 2.4 Hz, 1H, H-2'), 6.80 (d, J = 2.4 Hz, 1H, H-8), 6.77 (d, J = 8.4 Hz, 1H, H-5'), 6.55 (s, 1H, H-3), 6.45 (d, J = 2.4

Hz, H-6), 5.07 (d, $J = 7.8$ Hz, 1H, H-1"), 3.94 (m, 1H, H-6 β "), 3.88 (s, 3H, OMe), 3.73 (m, 1H, H-6 α "), 3.46 (m, 1H, H-3"), 3.27 (m, 1H, H-5"), 3.24 (m, 1H, H-2"), 3.15 (m, 1H, H-4").

^{13}C NMR (150 MHz, DMSO- d_6) δ 182.5 (C-4), 167.1 (C-2), 163.7 (C-7), 161.8 (C-5), 160.5 (C-3'), 157.8 (C-9), 151.0 (C-4'), 122.4 (C-6'), 117.8 (C-5'), 116.9 (C-1'), 109.5 (C-2'), 105.7 (C-10), 102.4 (C-3), 101.4 (C-6), 101.1 (C-1"), 96.3 (C-8), 62.2 (C-6"), 55.8 (4'-OMe).

Rosmarinic acid (6)

(45 mg), ^1H NMR (400 MHz, DMSO- d_6) δ 9.67 (s, 1H, 9'-OH), 9.21 (s, 1H, OH), 8.83 (s, 1H, OH), 8.76 (s, 1H, OH), 8.31 (s, 1H, OH), 7.47 (d, $J = 15.8$ Hz, 1H, H-7), 7.07 (d, $J = 1.4$ Hz, 1H, H-2), 7.03 (dd, $J = 8.1$; 2.0 Hz, 1H, H-6), 6.77 (d, $J = 8.1$ Hz, 1H, H-5), 6.23 (d, $J = 15.8$ Hz, 1H, H-8), 6.69 (d, $J = 1.4$ Hz, 1H, H-2'), 6.64 (d, $J = 7.9$ Hz, 1H, H-6'), 6.53 (d, $J = 7.9$ Hz, 1H, H-5'), 5.05 (dd, $J = 14.1$, 8.4 Hz, 1H, H-8'), 3.01 (dd, $J = 14.1$, 8.4 Hz, 1H, H-7 β '), 2.92 (dd, $J = 14.1$, 8.4 Hz, 1H, H-7 α '). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.3 (C-9'), 166.3 (C-9), 149.0 (C-7), 146.3 (C-3), 146.1 (C-4), 145.3 (C-4'), 127.7 (C-1'), 125.8 (C-1), 144.4 (C-3'), 120.5 (C-5'), 122.1 (C-6), 117.1 (C-2'), 116.2 (C-5), 115.9 (C-6'), 115.3 (C-2), 113.7 (C-8), 73.3 (C-8'), 36.5 (C-7').

Globoidnan A (7)

(65 mg), ^1H NMR (400 MHz, DMSO- d_6) δ 8.23 (s, 1H, H-4), 7.51 (s, 1H, H-2), 7.36 (s, 1H, H-5), 7.27 (s, 1H, H-8), 6.92 (d, $J = 8.00$ Hz, 1H, H-5'), 6.85 (s, 1H, H-2'), 6.77 (s, 1H, H-3"), 6.73 (dd, $J = 1.7$, 8.0 Hz, 1H, H-6'), 6.64 (s, 1H, H-2"), 6.68 (s, 1H, H-6"), 5.23 (dd, $J = 4.4$, 7.6 Hz, 1H, H-8"), 3.05 (m, 1H, H-7"). ^{13}C NMR (100 MHz, DMSO- d_6) δ 171.3 (C-9"), 166.2 (C-9), 149.9 (C-7), 147.9 (C-7), 145.4 (C-4'), 145.3 (C-3'), 144.6 (C-5"), 144.5 (C-4"), 138.6 (C-8a), 131.6 (C-1'), 130.2 (C-4a), 128.8 (C-1), 128.1 (C-4), 123.5 (C-3), 122.9 (C-2), 120.7 (C-2"), 117.3 (C-2'), 117.1 (C-3"), 116.2 (C-5'), 115.8 (C-6"), 112.0 (C-5), 108.5 (C-8), 73.8 (C-8"), 36.6 (C-7").

2.4. Preparation of cell cultures

The antitumor capacity of isolated compounds was investigated using the cell lines, HeLa, HT29, MCF-7, A549 and FL. The trials were executed in a laminar cabinet. The incubation was carried out in T75 cell culture flask, fetal bovine serum (10%), penisilin-Streptomisin solution (2%), NaHCO_3 (0.026 M) at 37 °C, 5% CO_2 for 4 days. Due to the confluent of the cells, the medium was substituent with the Tripsin-EDTA (10 mL) solution, then the flask was incubated for 8 min. The flask was shaken to remove the cells from surface and the suspension cells were added to the falcon tubes (50 mL) sterilised. The fresh medium (DMEM) was added to the cells to neutralise the Trypsin and centrifugation was carried out at 2000 $\times$ g for 15 minutes. The supernatant was aspirated, the cell tablets were suspended by addition of medium (2.0 mL). The trypan blue (10 μL) was combined with suspended cells (10 μL), afterward, the mixture (10 μL) was added to the Thoma cell counting chamber to define the cell concentration. After the preparation of cell stock suspension, it was pipetted to the cell plates (5×10^3 cells/well)[20].

Cell proliferation assay

BrDU (5-bromo-2'-deoxyuridine) Cell ELISA (Roche) kit enabling the determination of amount of cellular DNA was applied to establish the antiproliferative activity and IC₅₀ concentration of the compounds to be tested[21]. When BrdU was added to cell culture, dividing cells included BrdU in their DNA as if they included thymidine. Hence, measurement of cellular division in suspended and monolayer cells could be achieved. Cultured cells were pipetted into 5000 cells per well. The compounds and extract dissolved in DMSO (5, 10, 25, 50, 75, 100, 150 and 200 µg/mL) were added to the cells in the plate and final volume of the well was completed to 200 µL with the medium and incubated for 24 hours at 37 °C. Dulbecco's Modified Eagle's Medium (DMEM) was added in one well, DMSO used for negative control was added into a well at the highest concentration (5%), the anticancer compound, cis-platinum was used as positive control and it was added to the cells at the same concentrations as the compounds. After the incubation, 10 µL/well of BrDU labelling solution was added on the cells and the cells were re-incubated at 37 °C for 4 hours. The medium on the plates was aspirated and the marking solution was removed, the liquid residue was removed by blotting on the filter paper, the FixDenat solution was added to the plate as 200 µL / well and incubated at 25°C for 30 minutes. FixDenat denatured DNA by fixing cells, so anti-BrdU-POD antibodies could penetrate to the DNA and reached to the BrdU easily. After the incubation, FixDenat solution was removed by aspiration. Anti-BrdU-POD solution was added to the cells as 100 µL / well and incubated at 22 °C for 90 minutes. The plates were then blotted to remove antibody conjugate and the wells were washed three times as to be 200 to 300 µL / well. The washing solution was removed by pouring and blotting and substrate solution was added as 100 µL / well. It was incubated for 30 minutes at 22 °C until the color developed. Finally, sulphuric acid (25 µL, 1.0 M) was added to each well and microplate was incubated for 2 minutes. The ELISA reader was used for proliferation at 450–650 nm. The result was calculated as % inhibition using the Eq. (1).

$$\% \text{ inhibition} = \left[\frac{A_1 - A_2}{A_2} \right] \times 100 \quad (1)$$

In which A₁ is the absorbance of the treated cells and A₂ is the absorbance of the untreated cells [22].

2.5. Cytotoxicity assay

The compounds isolated were subjected to the cytotoxicity using the LDH technique. HeLa, HT-29, MCF-7 and A549 and FL cell lines were used for cytotoxicity. LDH method indicates the cytotoxic or cytostatic of the tested compounds[23, 24]. LDH is the stable cytoplasmic enzyme that serves the conversion of sugar to energy. The cells were incubated in sterile T75 cell culture flask including medium (DMEM), 10% FBS and 2% PenStrep solution for 5 days at 37 °C in 5% CO₂. When the cells became confluent, the medium in the flasks was replaced with Trypsin-EDTA solution (10 mL) and mixed for 5 minutes. After the incubation, the flask was gently shaken to separate the cells from the surface, the resulting cell suspension was transferred to falcon tubes. Neutralization of trypsin was performed by adding 10 ml of fresh medium (DMEM) to the flask and then centrifuged at 1500 x g for 10 minutes. After the aspiration

of supernatant, the cell pellets were suspended with a sterile Pasteur pipette by adding fresh medium (2 mL). For cell counting, 10 µl of cells taken from the cell suspension was mixed with trypan blue solution, this mixture (10 µl) was added onto a Thoma slide, cells were counted under microscope and cell concentration was determined. After counting, cell stock suspension (5000 cells/well) was prepared in a sterile 15 ml Falcon tube and pipetted into 96 cell culture plates and incubated with compounds at different concentrations. After the incubation, the cell-free culture supernatant was collected and 100 µL of this supernatant was transferred to another microplate and the reaction mixture in the kit was added to each supernatant as 100 µL / well and incubated for another half hour. LDH passed to the supernatant during incubation, reduced the tetrazolium salt INT to form formazan with a coupled enzymatic reaction. Thus, LDH passing to the supernatant is directly related to the amount of formazan formation at this step. As a result, the amount of formazan dye in the wells was determined with ELISA plate reader measuring the absorbance at 500 nm. The equation was used to calculate the cytotoxicity (2).

$$\text{Cytotoxicity (\%)} = [1 - A1 / A2] \times 100 \text{ (2)}$$

In which, A1 is the tested sample, A2 is solvent control.

2.6. Cell imaging

The phase contrast microscope was used for evaluation of morphological changes of cells treated with the compounds. The cells in a well plate (5000 cells / well) were left for 24 h as attachment. The record of image of the compounds was executed with inverted microscope [25].

Statistical analysis

SPSS software with one-way ANOVA was used for the statistical analysis. The results were determined as the mean value $\pm$ standard deviation. The trials were carried out with three times. The differences were considered as a significant at $P < 0.05$.

3. Results And Discussion

3.1. Isolation and identification of natural compounds

The methanol extract of *Onosma bourgaei* was subjected to TOF-MS/MS analysis to observe the compounds peaks to be isolated (Fig. 1).

After the extensive chromatographic techniques seven compounds were isolated and their structures were elucidated by spectroscopic analyses (Fig. 2). The first compound isolated from *O. bourgaei* was the allantoin (1). In ^1H NMR, the signal observed at δ 10.47 belonged to the H-1. Due to the electron withdrawing effect of two carbonyl groups and nitrogen atom, the hydrogen bonded to N-1 atom exposed to deshielded effect, so it gave the signal at downfield region. The other proton bonded to N-4 gave the signal at δ 8.02 as a singlet. The signals at δ 6.97 ($J = 8.1$ Hz) and δ 5.80 (s) were assigned to H-6 and H-

8 respectively. The signal at δ 5.22 as a doublet with coupling constant 8.1 Hz attributed to H-3 proton. In ^{13}C -NMR and DEPT spectra revealed 4 signals, of which 3 were assigned to the carbonyl carbons and one to a C-3 carbon [26].

Allantoin is a significant natural product synthesised by plants, animals and bacteria, moreover, it is derived from the degradation of purines through uric acid [27]. Allantoin has revealed the significant wound healing activity through fibroblast growth stimulation. Therefore, the commercially available cosmetic products (1376) consist of allantoin at a concentration of 2% [28]. The other compounds isolated from *Onosma bourgaei* were 3,4-dihydroxybenzaldehyde (**2**) [29], luteolin-7-*O*-glucoside (**3**) [30], apigenin-7-*O*- β -glucoside (**4**) [31], diosmetin-7-*O*- β -glucoside (**5**) [32], rosmarinic acid (**6**) [33], globoidnan A (**7**) [34]. The spectral analyses and comparison of the spectral values with the reported data confirmed the proposed structures.

3.2. Antiproliferative effect of isolated natural compounds

The IC_{50} values and the tumor specificity (TS) ratios of compounds were determined by Elisa BrdU test, which is a very sensitive proliferation test technique, and is available in the Table. According to Elisa BrdU measurement, compounds **4**, **6**, and **7** showed a significantly higher anti-proliferation against the tested cell lines than the control compound cisplatin (Fig. 3).

The compounds **4**, **6**, and **7** revealed the strong antiproliferative activity against HeLa cell lines with the values of 167.3 μM , 249.8 μM and 164.0 μM respectively compared to cis platin (422.5 μM). In concern to the HT29 cell lines, 196.8 μM , 277.9 μM , 188.8 μM IC_{50} values were found for the compounds, **4**, **6** and **7** respectively. In A549 cell lines, the activity of compounds, **4**, **6** and **7** were determined as 181.1 μM , 241.5 μM , and 176.3 μM (IC_{50}) respectively with comparison of cis platin (436 μM). These compounds (**4**, **6** and **7**) displayed excellent antiproliferative effect on MCF7 with the values of 203.5 μM , 220.3 μM , 158.4 μM respectively in comparison to cisplatin (471.5 μM). the same trend was observed for the FL cell lines. In accordance with the literature, it is presented that apigenin-7-*O*- β -glucoside (**4**), rosmarinic acid (**6**), and globoidnan A (**7**) have an antiproliferative and cytotoxic effect.[34–36] In addition, luteolin-7-*O*-glucoside (**3**) showed a significantly greater anti-proliferative effect on HeLa and HT29 cancer cells than the control substance cisplatin (IC_{50} ; 305.7 and 367.6 μM , respectively) (Table). This compound **3** was reported to have significant antiproliferative activity[37] as well. It was confirmed here that compound **3** is a potent molecule against HeLa and HT29 cells, but it could not reveal any activity against MCF7 and A549 cell line (Fig. 3). Though **4**, **6**, and **7** molecules have exhibited substantially antiproliferative effects on human cancer cells, the tumour-selectivity index values of these molecules in normal human FL cells are also important. The tumour-selectivity index (TS) was calculated by the following Eq. (1).

$$\text{TS} = \text{A} / \text{B} \quad (1)$$

In which A is the mean of IC_{50} concentration of each agent against normal cells and B is the mean of IC_{50} concentration of each agent against tumor cells.

All compounds showed excellent selectivity ($TS > 1$) for the HeLa cells over the FL cells while compound **6** displayed good selectivity for the HT29, A549 and MCF7 cells (Table). The compound **7** exhibited good selectivity for only MCF7 cells (Table). The compounds **1**, **2**, and **5** failed to show any anti-proliferative activity against the tested cell lines (Table). Compounds **1** and **5** were found to be non-toxic in accordance with the literature [38]. The non-toxic properties of these molecules suggest that they may have protective effects. Indeed, these two molecules can in particular mitigate the damage caused by chemotherapy.

3.3. Cytotoxicity of compounds

The cytotoxicity of isolated compounds on corresponding cancer cells and normal cells were executed by LDH cytotoxicity assay and were presented by treatment of cells with various concentrations of the compounds (50, 100, 150 and 250 $\mu\text{g/mL}$), that revealed a concentration dependent damage of cell membranes. The cytotoxicity results demonstrated that treatment of the cells with high concentrations ($> 150 \mu\text{g/mL}$) of the compound **6** and **7** led to noteworthy corruption of cell membrane yet compounds **6** and **7** did not have the same effect at low or mid concentrations (Fig. 4).

HeLa, HT29 and FL cell lines displayed no cytotoxicity in response to compound **3** up to 150 $\mu\text{g/mL}$ concentrations. Same concentrations ($< 20 \mu\text{g/mL}$) of the compounds **1**, **2**, **4** and **5** did not lead to any cytotoxic effect towards cells, inferring that the high toxicity determined was intensely ascribed to the compound **6** and **7**. Indeed, according to another observation, cytotoxicity of extract using 100 $\mu\text{g/mL}$ concentrations was close to that of the compound **6** and **7** against of the HeLa, HT29 and FL cell lines. When proliferation, tumour-selectivity index and toxicity tests were evaluated together, it was clear that compound **4** was a very good treatment option against cervix (HeLa) cancer (Fig. 4).

3.4. Morphological assessment of isolated compounds

A digital camera attached inverted microscope (Leica IL10, Germany) was used to present the effect of the compounds on the cell morphology. The picture shows microscopic images of the morphology of cell lines treated with different concentrations of compounds **2**, **3** and extracts compared to the controls. Compounds at various concentrations were treated with cells and cell death was determined from the morphology of the cells (Fig. 5). Compounds and extract were able to inhibit the growth of treated cells compared to control cells that displayed integrity confluent growth following 24 h in plate surface. In contrast to control cells, most of the treated cells were lost their astrocyte-like or fibroblast-like appearance. Generally, at concentrations of 150 $\mu\text{g/mL}$ and higher, the number of cells appears fewer, separate, and smaller. However, the reaction of the compounds with cells at low ($< 150 \mu\text{g/mL}$) concentrations did not reduce their cell numbers and appeared structurally like non-interacting cells.

Compounds treated cell lines also exhibited apoptotic indicator such as cell shrinkage, apoptotic bodies, and atypical shape depending on concentration. Thus, based on observations in this study, morphological changes of the cell membrane treated with compounds clearly associated with apoptosis.

Phytochemistry of *Onosma bourgaei* was investigated completely. The significant natural compounds were isolated and identified from this plant and their anticancer activity was carried out. Apigenin-7-*O*- β -glucoside (**4**) was found to have the most anticancer activity among the compounds. The corresponding products were isolated from this plant at first. Hence, *Onosma bourgaei* could be an essential source of these compounds. Due to the extensive usage of allantoin in cosmetic, this plant could be a raw material for cosmetic industry. In addition, agricultural techniques should be improved to increase the quantity of these compounds in *Onosma bourgaei*.

4. Conclusions

Bioactive compounds were isolated and identified from *Onosma bourgaei*. Due to the usage of the isolated compounds in pharmaceutical and food industries, this plant could be a good source for corresponding compounds. Apigenin-7-*O*- β -glucoside (**4**), rosmarinic acid (**6**), and globoidnan A (**7**) revealed the strong antiproliferative activity against HeLa (human epithelial cervix adenocarcinoma), HT-29 (human colorectal adenocarcinoma), MCF-7 (human mammary gland adenocarcinoma) and A549 (human lung carcinoma,) cancerous cell lines. Therefore, these compounds have a potential to be used in cancer drug discovery.

Declarations

Acknowledgement

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This is an observational study. Tokat Gaziosmanpasa Research Ethics Committee has confirmed that no ethical approval is required.

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Consent to participate

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Author contribution

Preparation of plant material, and extraction were carried out by Ilyas Yildiz. Spectroscopic analysis was carried out by Nusret Genc. Antiproliferative activity was executed by Ali Aydin. The execution of the project, the coordination between the team members, and the writing of the article were carried out by Ramazan Erenler.

References

1. Demirtas I, Erenler R, Elmastas M, Goktasoglu A. Studies on the antioxidant potential of flavones of *Allium vineale* isolated from its water-soluble fraction. Food Chem. 2013;136(1):34-40. <https://doi.org/10.1016/j.foodchem.2012.07.086>.
2. Sahin Yaglioglu A, Akdulum B, Erenler R, Demirtas I, Telci I, Tekin S. Antiproliferative activity of pentadeca-(8E, 13Z) dien-11-yn-2-one and (E)-1,8-pentadecadiene from *Echinacea pallida* (Nutt.) Nutt. roots. Med Chem Res. 2013;22(6):2946-2953. <https://doi.org/10.1007/s00044-012-0297-2>.
3. Erenler R, Telci I, Ulutas M, Demirtas I, Gul F, Elmastas M, Kayir O. Chemical Constituents, Quantitative Analysis and Antioxidant Activities of *Echinacea purpurea* (L.) Moench and *Echinacea pallida* (Nutt.) Nutt. J Food Biochem. 2015;39(5):622-630. <https://doi.org/10.1111/jfbc.12168>.
4. Elmastas M, Erenler R, Isnac B, Aksit H, Sen O, Genc N, Demirtas I. Isolation and identification of a new neo-clerodane diterpenoid from *Teucrium chamaedrys* L. Nat Prod Res. 2016;30(3):299-304. <https://doi.org/10.1080/14786419.2015.1057583>.
5. Topçu G, Erenler R, Çakmak O, Johansson CB, Çelik C, Chai H-B, Pezzuto JM. Diterpenes from the berries of *Juniperus excelsa*. Phytochemistry. 1999;50(7):1195-1199. [https://doi.org/10.1016/S0031-9422\(98\)00675-X](https://doi.org/10.1016/S0031-9422(98)00675-X).
6. Binzet R. *Onosma anatolica*, a new species of Boraginaceae from Turkey. PhytoKeys. 2016;(69):39-49. <https://doi.org/10.3897/phytokeys.69.8360>.
7. Davis PH, Mill RR, Tan K, (1988). *Flora of Turkey and the East Aegean Islands*. Edinburg University Press, Edinburgh.

8. Koyuncu O, Yaylacı ÖK, Özgüşi K, Sezer O, Öztürk D. A new *Onosma* (Boraginaceae) species from central Anatolia, Turkey. *Plant Syst Evol.* 2013;299(10):1839-1847. <https://doi.org/10.1007/s00606-013-0839-1>.
9. Kumar N, Kumar R, Kishore K. *Onosma* L.: A review of phytochemistry and ethnopharmacology. *Pharmacogn Rev.* 2013;7(14):140-151. <https://doi.org/10.4103/0973-7847.120513>.
10. Dong M, Liu D, Li Y-H, Chen X-Q, Luo K, Zhang Y-M, Li R-T. Naphthoquinones from *Onosma paniculatum* with Potential Anti-inflammatory Activity. *Planta Med.* 2017;83(07):631-635. <https://doi.org/10.1055/s-0042-120545>.
11. Nikita G, Vivek P, Chhaya G. Wound-healing activity of an oligomer of alkannin/shikonin, isolated from root bark of *Onosma echioides*. *Nat Prod Res.* 2015;29(16):1584-1588. <https://doi.org/10.1080/14786419.2014.986126>.
12. Vukic MD, Vukovic NL, Djelic GT, Popovic SL, Zaric MM, Baskic DD, Krstic GB, Tesevic VV, Kacaniova MM. Antibacterial and cytotoxic activities of naphthoquinone pigments from *Onosma visianii* Clem. *EXCLI journal.* 2017;16:73-88. <https://doi.org/10.17179/excli2016-762>.
13. Sut S, Pavela R, Kolarčík V, Cappellacci L, Petrelli R, Maggi F, Dall'Acqua S, Benelli G. Identification of *Onosma visianii* Roots Extract and Purified Shikonin Derivatives as Potential Acaricidal Agents against *Tetranychus urticae*. *Molecules.* 2017;22(6):1002-1016. <https://doi.org/10.3390/molecules22061002>.
14. Yildirim AB, Karakas FP, Turker AU. In vitro antibacterial and antitumor activities of some medicinal plant extracts, growing in Turkey. *Asian Pac J Trop Med.* 2013;6(8):616-624. [https://doi.org/10.1016/S1995-7645\(13\)60106-6](https://doi.org/10.1016/S1995-7645(13)60106-6).
15. Kretschmer N, Rinner B, Deutsch AJ, Lohberger B, Knausz H, Kunert O, Blunder M, Boechzelt H, Schaidler H, Bauer R. Naphthoquinones from *Onosma paniculata* induce cell-cycle arrest and apoptosis in melanoma Cells. *J Nat Prod.* 2012;75(5):865-869. <https://doi.org/10.1021/np2006499>.
16. Kumar N, Gupta A, Prakash D, Kumar P. Hypoglycemic activity of *Onosma hispidum* (Ratanjot). *Int J Diabetes Dev C.* 2010;30(4):213-216. <https://doi.org/10.4103/0973-3930.70862>.
17. Ahmad B, Ali N, Bashir S, Choudhary M, Azam S, Khan I. Parasitocidal, antifungal and antibacterial activities of *Onosma griffithii* Vatke. *Afr J Biotechnol.* 2009;8(19):5084-5087
18. Di Giorgio C, Delmas F, Tueni M, Cheble E, Khalil T, Balansard G. Alternative and complementary antileishmanial treatments: assessment of the antileishmanial activity of 27 Lebanese plants, including 11 endemic species. *J Altern Complement Med.* 2008;14(2):157-162. <https://doi.org/10.1089/acm.2007.7041>.
19. Acuña UM, Carcache PJB, Matthew S, de Blanco EJC. New acyclic bis phenylpropanoid and neolignans, from *Myristica fragrans* Houtt., exhibiting PARP-1 and NF-κB inhibitory effects. *Food Chem.* 2016;202:269-275. <https://doi.org/10.1016/j.foodchem.2016.01.060>.
20. Ökten S, Çakmak O, Erenler R, Yüce Ö, Tekin S. Simple and convenient preparation of novel 6,8-disubstituted quinoline derivatives and their promising anticancer activities. *Turk J Chem.* 2013;37(6):896-908. <https://doi.org/10.3906/kim-1301-30>.

21. Aydin A, Erenler R, Yilmaz B, Tekin Ş. Antiproliferative effect of *Cherry laurel*. J Turk Chem Soc, Sec A: Chem. 2016;3(3):217-228. <https://doi.org/10.18596/jotcsa.21204>.
22. Erenler R, Sen O, Aksit H, Demirtas I, Yaglioglu AS, Elmastas M, Telci İ. Isolation and identification of chemical constituents from *Origanum majorana* and investigation of antiproliferative and antioxidant activities. J Sci Food Agr. 2016;96(3):822-836. <https://doi.org/10.1002/jsfa.7155>.
23. Okten S, Erenler R, Koprulu TK, Tekin S. In vitro antiproliferative/cytotoxic activity of 2, 3'-biindole against various cancer cell lines. Turk J Biol. 2015;39(1):15-22. <https://doi.org/10.3906/BIY-1402-60>.
24. Erenler R, Pabuccu K, Yaglioglu AS, Demirtas I, Gul F. Chemical constituents and antiproliferative effects of cultured *Mougeotia nummuloides* and *Spirulina major* against cancerous cell lines. Z Naturforsch C. 2016;71(3-4):87-92. <https://doi.org/10.1515/znc-2016-0010>.
25. Yildiz I, Sen O, Erenler R, Demirtas I, Behcet L. Bioactivity-guided isolation of flavonoids from *Cynanchum acutum* L. subsp. *sibiricum* (willd.) Rech. f. and investigation of their antiproliferative activity. Nat Prod Res. 2017;31(22):2629-2633. <https://doi.org/10.1080/14786419.2017.1289201>.
26. Hawas UW, Abou El-Kassem LT, Shafer F, Al-Farawati R. In vitro inhibition of Hepatitis C virus protease and antioxidant by flavonoid glycosides from the Saudi costal plant *Sarcocornia fruticosa*. Nat Prod Res. 2019;33(23):3364-3371. <https://doi.org/10.1080/14786419.2018.1477153>.
27. Werner AK, Witte C-P. The biochemistry of nitrogen mobilization: purine ring catabolism. Trends Plant Sci. 2011;16(7):381-387. <https://doi.org/10.1016/j.tplants.2011.03.012>.
28. Becker LC, Bergfeld WF, Belsito DV, Klaassen CD, Marks JG, Shank RC, Slaga TJ, Snyder PW, Andersen FA. Final report of the safety assessment of allantoin and its related complexes. Int J Toxicol. 2010;29(3_suppl):84S-97S. <https://doi.org/10.1177/1091581810362805>.
29. Alvarenga TA, Bêdo TR, Braguine CG, Gonçalves UO, Magalhães LG, Rodrigues V, Gimenez VM, Groppo M, Silva MLA, Cunha WR. Evaluation of *Cuspidaria pulchra* and its isolated compounds against *Schistosoma Mansonii* adult worms. Int J Biotechnol Wellness Ind. 2012;1(2):121-127. <http://dx.doi.org/10.6000/1927-3037/2012.01.02.03>.
30. Chiruvella KK, Mohammed A, Dampuri G, Ghanta RG, Raghavan SC. Phytochemical and antimicrobial studies of methyl angolensate and luteolin-7-O-glucoside isolated from callus cultures of *Soymida febrifuga*. Int J Biol Sci. 2007;3(4):269-278
31. Erenler R, Yilmaz S, Aksit H, Sen O, Genc N, Elmastas M, Demirtas I. Antioxidant activities of chemical constituents isolated from *Echinops orientalis* Trauv. Rec Nat Prod. 2014;8(1):32-36
32. Elkattan A, Gohar A, Amer M, Naeem ZM, Ashour A, Shimizu K. Melanin synthesis Inhibitors from *Olea europeae*. Rec Nat Prod. 2019;14(2):139-143
33. Aksit H, Çelik SM, Sen Ö, Erenler R, Demirtas I, Telci I, Elmastas M. Complete isolation and characterization of polar portion of *Mentha dumetorum* water extract. Rec Nat Prod. 2014;8(3):277-280
34. Erenler R, Meral B, Sen O, Elmastas M, Aydin A, Eminagaoglu O, Topcu G. Bioassay-guided isolation, identification of compounds from *Origanum rotundifolium* and investigation of their antiproliferative

and antioxidant activities. Pharm Biol. 2017;55(1):1646-1653.

<https://doi.org/10.1080/13880209.2017.1310906>.

35. Smiljkovic M, Stanisavljevic D, Stojkovic D, Petrovic I, Vicentic JM, Popovic J, Grdadolnik SG, Markovic D, Sankovic-Babice S, Glamoclija J. Apigenin-7-O-glucoside versus apigenin: Insight into the modes of anticandidal and cytotoxic actions. EXCLI journal. 2017;16:795-807.
<https://doi.org/10.17179/excli2017-300>.
36. Erenler R, Sen O, Yildiz I, Aydın A. Antiproliferative activities of chemical constituents isolated from *Thymus praecox* subsp. *grossheimii* (Ronniger) Jalas. Rec Nat Prod. 2016;10(6):766-770
37. Park CM, Song Y-S. Luteolin and luteolin-7-O-glucoside protect against acute liver injury through regulation of inflammatory mediators and antioxidative enzymes in GalN/LPS-induced hepatitic ICR mice. Nutr Res Pract. 2019;13(6):473-479. <https://doi.org/10.4162/nrp.2019.13.6.473>.
38. Fernandes J, de Amorim GC, da Veiga TE, Cardoso J, Arruda AC, Arruda MSP, Castelo-Branco MT. Allantoin reduces cell death induced by cisplatin: possible implications for tumor lysis syndrome management. J Biol Inorg Chem. 2019;24(4):547-562. <https://doi.org/10.1007/s00775-019-01661-6>.

Table

Table. I

C₅₀ values and tumour specificity rate for isolated compounds

Compounds	IC ₅₀ (μM)					Tumour specificity			
	HeLa	HT29	A549	MCF7	FL	HeLa	HT29	A549	MCF7
1	ND	ND	ND	ND	ND	-	-	-	-
2	ND	ND	ND	ND	ND	-	-	-	-
3	305.73	367.62	ND	ND	341.75	1.12	0.93	-	-
4	167.31	196.84	181.13	203.48	175.07	1.05	0.89	0.97	0.86
5	ND	ND	ND	ND	ND	-	-	-	-
6	249.80	277.85	241.47	220.25	282.35	1.13	1.02	1.17	1.28
7	164.04	188.75	176.28	158.38	172.19	1.05	0.91	0.98	1.09
Cisplatin	422.49	399.55	436.52	471.48	478.95	1.13	1.20	1.10	1.02

ND; Not Determined

Figures

Fig. 1

Figure 1

LC-TOF/MS analysis of *Onosma bourgaei*

Fig. 2

Figure 2

Chemical structure of isolated compounds from *Onosma bourgaei*.

Figure 3

The Antiproliferative activity of isolated compounds on FL, HeLa, HT29, MCF7, and A549 cell lines. BrdU Cell Elisa Assay Kit was used for cell proliferation. Percent inhibition was reported as mean values $\pm$ SEM of three independent assays ($P < 0.05$).

Figure 4

Cytotoxic activity of isolated compounds on FL, HeLa, HT29, MCF7, and A549 cell lines. LDH Cytotoxicity Assay Kit was used for cytotoxicity. Percent cytotoxicity was reported as mean values $\pm$ SDs of three independent assays ($P < 0.05$).

Figure 5

Effect of the compounds and extract on the morphology of FL, HeLa, HT29, MCF7, and A549 cells.