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Original Article

Screening of Some Rare and Endemic Plants Growing in Iran for Their Cytotoxic Effects on Cancer Cell Lines

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ABSTRACT

Khorasan Razavi Province is one of the most significant regions of plant diversity of the flora of Iran. Numerous rare indigenous plant species are grown in this province, which would be suitable potential candidates for assessing cytotoxic activities and apoptosis induction. In this study, we intended to explore the cytotoxic and apoptotic properties of the methanol extract of the root and aerial parts of 10 rare and endemic plants of Khorasan Razavi Province, namely *Dorema kopetdaghense*, *Chaerophyllum khorassanicum*, *Seseli staurophyllum*, *Seseli transcaucasicum*, *Haplophyllum obtusifolium*, *Crinitaria grimmii*, *Handelia trichophylla, Rheum turkestanicum, Eremostachys boissieriana*, and *E. macrophylla,* on PC-3, MCF-7, MDA, and B16F10 cancer cells using resazurin and flow cytometry assays. 100 µg/ml of *D. kopetdaghense* root extract on PC3 (p <0.001), MCF-7 (p <0.001), MDA (p <0.001), B16F10 (p <0.001) cells significantly decreased cell viability. 100 µg/ml of *H. obtusifolium* root extract on PC3 (p <0.001), MCF-7 (p <0.001), MDA (p <0.05), B16F10 (p <0.001) cells, and 50 µg/ml of *H. obtusifolium* root extract on MCF-7 (p <0.01), B16F10 (p <0.001) exhibited cytotoxic effects. The histogram obtained from the PI staining and flow cytometry indicated induction of apoptosis by 25 and 50 µg/ml *D. kopetdaghense* and *H. obtusifolium* root extracts in the PC3 and MCF-7 cell lines. The root extracts of *D. kopetdaghense* and *H. obtusifolium* had significant cytotoxic effects and could induce apoptosis. It suggests that plants with low IC_{50} values are considered potent cytotoxic species for further evaluation as candidate anti-cancer agents.

Keywords: *Dorema kopetdaghense*, *Haplophyllum obtosifolium*, Cytotoxic, Apoptosis

Abbreviations: BSL, Brine shrimp lethality; CAM, Complementary and alternative medicine; DPPH, Diphenyl-1-picrylhydrazyl; IC, Inhibitory concentration; iNOS, Inducible nitric oxide synthase; MDA, Malondialdehyde; PBS, Phosphate-buffer saline; PI, Propidium iodide; level, ROS, Reactive oxygen species.

INTRODUCTION

Cancer is the most well-known name utilized for more than 100 diverse types of malignancies that affect various organs of the body. Cancers are continually spreading with increased incidence and are considered the second leading cause of death globally in the 21st century [1-3]. Chemotherapeutic resistance of the tumor cells is one of the major problems in cancer treatment. This event results in the establishment of a more aggressive cell phenotype, which may have metastatic potential to spread to other tissues [4]. Forthermore, the chemotherapy components' side effects persuade researchers to achieve new approaches, such as the use of natural medicinal resources [5, 6]. As complementary and alternative medicine (CAM) develops for cancer treatment, herbal and traditional remedies are becoming more widely used [6]. The screening of plants with potential cytotoxicity can lead to the development of new alternatives with minimal side effects and acceptable efficacy [6, 7]. Due to the rich diversity in climate in different regions of Iran, the Iranian flora consists of various species, especially rare plants [8]. Therefore, Iran's unique climatic conditions are an appropriate environment for the growth of more than 8000 diverse plant species, which can be a valuable source to screen and discover novel therapeutic drugs from plants [9].

Although there are few reports about different Iranian endemic and rare plant species, some biological and therapeutic properties may be predicted since plants of the same family have some similar biological activities because of their similar components. In addition, local communities in different parts of the country have a deep knowledge about various therapeutic effects of plants throughout their long history [10]. In a study, Emami and colleagues evaluated the cytotoxicity of thirteen rare and endemic plants from Chaharmahal and Bakhtiari Province in Iran on breast (MCF-7), prostate (PC-3), ovary (CHO), liver (HepG2), and melanoma (B16-F10) cancer cell lines. They determined that the methanol extracts of *Stachys obtusicrena*, *Dionysia sawyer,* and *Cicer oxyodon* on CHO cells (p <0.05) and *Linum album* and *D. sawyer* on B16/F10 cells (p <0.05) displayed noteworthy cytotoxicity and apoptosis induction [11]. In another study, Behzad and colleagues assessed the cytotoxicity of 11 herbal species from different sites of Hamedan Province in Iran on HepG2, MCF-7, human lung adenocarcinoma (A549) and human colon carcinoma (HT-29) cells. They demonstrated that the methanol extracts of *Primula auriculata* showed the highest cytotoxic activity against HepG2, MCF7 and HT- 29 cells [12]. In a recent study, Soheili and

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colleagues screened the methanol-dichloromethane extracts of some Iranian medicinal plants for cytotoxic activities on MCF-7, A-549, and PC-3 cells. In this study, *Hypericum scabrum* L. exhibited cytotoxic activity against cancer cell lines with the half maximal inhibitory concentration (IC_{50}) values of 14.21-39.77 mg/ml [13]. Overall, it is suggested that the plant extracts with low IC_{50} values are likely to be considered as anti-cancer agents in diminishing cancer progression in scientific studies.

To the best of our knowledge, there is a lack of published research about the biological properties of rare and endemic plant species. Developing alternatives that have minimal side effects and acceptable efficacy can be achieved by screening plants with potential cytotoxicity. In this study, we assessed cytotoxicity and apoptosis induction of methanol extract from 10 rare or endemic plants, including species from the Apiaceae, Rutaceae, Aristolochiaceae, Lamiaceae, Polygonaceae, and Asteraceae families, on the PC-3, MCF-7, breast (MDA), and B16-F10 cancer cell lines, as well as NIH as normal cells. All plants were collected from Khorasan Razavi Province, Dargaz mountains, which is located at 37.4455° N (latitude), 59.1099° E (longitude).

MATERIALS AND METHODS

Plant Materials

Ten species of rare or endemic plants from different families from the north and northwest of Dargaz, in Khorasan Razavi Province, located in the northeast of Iran, were collected in spring and summer 2105 and identified by Dr. M.S. Amiri [14]. Voucher specimens of each plant species were maintained in the herbarium of the School of Pharmacy, Mashhad University of Medical Sciences, Iran (Table 1).

Preparation of Extracts

The plants dried in shadow and away from sunlight. Then, the root and aerial parts of each species were separately powdered, and 100 g of each powder was macerated at room temperature (25 °C) for 48 h using methanol. Afterward, macerated powder samples were extracted with pure methanol by the percolation method. Finally, the methanol extracts were concentrated by a rotary evaporator, and the subsequently obtained extracts were dried via a freeze dryer. All powder extracts were kept at -20 °C until further use. The yield percentage of the obtained extracts was accessible in Table 2.

Cell Culture and Treatment

PC-3 (prostate cancer), MCF-7 (breast cancer), B16/F10 (melanoma cancer), MDA (breast cancer), and NIH (mouse embryonic fibroblast) cells were provided by the Cell Bank at the Pasteur Institute (Tehran, Iran). Cells were grown in RPMI 1640 medium (Bio-IDEA) plus 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin and were incubated in a humidified atmosphere (95%) containing 5% CO_2 at 37 °C. Every concentration and time course was repeated three times and compared with an untreated control sample that received an equal volume of the culture medium [10].

Table 1 Voucher specimens, localities, and the collected places of ten medicinal plants from Khorasan Razavi Province of Iran

Species	State	Family	Voucher number	Location
Caerophyllum khorassanicum	Rare	Aristolochiaceae	13156	Zarin kooh- north of Dargaz- 1207 m height
Crinitaria grimmii	Rare	Asteraceae	13154	Zarin kooh- northwest of Dargaz- 960 m height
Dorema kopetdaghense	Rare	Apiaceae	13220	Zarin kooh- north of Dargaz- 886 m height
Eremostachys boissieriana	Rare	Lamiacea	13221	Zarin kooh- north of Dargaz- 817 m height
Eremostachys macrophylla	Rare	Lamiacea	13222	Zarin kooh- north of Dargaz- 1104 m height
Handelia trichophylla	Rare	Asteracea	13157	Dargaz to Kalat road- Zanlanloo - 1463 m height
Haplophyllum obtusifolium	Rare	Rutaceae	13155	Zarin kooh- northwest of Dargaz- 985 m height
Rheum turkestanicum	Rare	Polygonaceae	13223	Zarin kooh- north of Dargaz- 879 m height
Seseli staurophyllum	Endemic	Apiaceae	13158	Dargaz to Ghoochan road- Jolfan- 1205 m height
Seseli transcaucasicum	Endemic	Apiaceae	13???	Dargaz

Table 2 The part used and the extraction yield of ten medicinal plants from Khorasan Razavi Province of Iran

Species	Part of plant	Extraction yield (%)
C. khorassanicum	Aerial part	10.4
C. grimmii	Aerial part	11.79
D. kopetdaghense	Aerial part	5.66
	Root	5
E. macrophylla	Aerial part	12.88
	Root	21.38
E. boissieriana	Aerial part	10.41
	Root	9.1
H. obtusifolium	Aerial part	10.15
	Root	9.57
H.trichophylla	Aerial part	6.69
R. turkestanicum	Aerial part	3.60
	Root	20.78
S. staurophyllum	Aerial part	9.68
	Root	10
S.transcaucasicum	Aerial part	18.31

Cell Viability

To determine the cytotoxicity of various methanol extracts, we used the resazurin agent as an indicator of cell viability [15]. For assessment of cell viability, 10^4 cells per well for each of the 5 cell lines were seeded in 96-well plates and grown with 50 and 100 µg/ml of methanol extracts of each species for 48 h. Doxorubicin (1 and 5 µg/ml) was considered the positive control. Afterwards, 10 µl resazurin reagent (10 mg/dl) was added to each well and incubated for 4 h. Then, cell viability was measured with an ELISA microplate reader according to the absorbance at 570 and 600 nm and compared with the control group absorbance (Awareness, Palm City, FL, USA) [16].

Flow Cytometry Analysis of Apoptosis

Apoptosis was evaluated by flow cytometry of propidium iodide (PI) stained cells and by assessment of the sub-G1 peak of treated PC3 and MCF7 cells. 5×10^4 cells per well in 12-well plates were incubated for 24 h. Then, cells were grown with 50 and 100 µg/ml of root methanol extracts of *D. kopetdaghense* and *H. obtusifolium* root, which had significant toxicity based on viability test, after 48 h. Then, after washing with phosphate-buffer saline (PBS), cells were trypsinized and harvested. Afterward, cells were incubated with 400 µL of hypotonic buffer (50 µg/mL PI in 0.1% sodium citrate and 0.1% Triton X-100) for 30 min in the dark at 4 °C. Finally, flow cytometric analysis was carried out (BD Biosciences, CA, USA) [17, 18].

Statistics Analysis

Statistics were carried out using GraphPad Prism Version 8.0. One-way analysis of variance (ANOVA) followed by Tukeys-Kramer *post hoc* test was used for data analysis. All results were presented as mean ± SEM, and p-values <0.05 were considered statistically significant.

RESULTS

Cytotoxicity

According to the results, 100 µg/ml of the methanol extracts of *D. kopetdaghense* root exhibited significant cytotoxic effect on PC3 (p <0.001), MCF-7 (p <0.001), MDA (p <0.001), B16F10 (p <0.001) and NIH (p <0.001) cells, and 50 µg/ml of *D. kopetdaghense* root on MCF-7 (p <0.05), B16F10 (p <0.05) and NIH (p <0.05) significantly decreased cell viability. Also, 100 µg/ml of the methanol extracts *H. obtusifolium* root on PC3 (p <0.001), MCF-7 (p <0.001), MDA (p <0.05), B16F10 (p <0.001) and NIH (p <0.001) cells and 50 µg/ml of *H. obtusifolium* root extract on PC3 (p <0.05), MCF-7 (p <0.001), MDA (p <0.01), B16F10 (p <0.001) and NIH (p <0.001) significantly decreased cell viability in a dose dependent manner after 48 h (Table 3 & Fig. 1).

Table 3 Cytotoxicity (% of cell viability) of methanol extracts of plants on PC3, MCF-7, MDA, B16F10 and NIH cells

Plant name↓	PC3		MCF7		MDA		B16-F10		NIH	
	50[1]	100	50	100	50	100	50	100	50	100
C. khorassanicum- A	93.6 ± 2.5	88.8 ± 2.1	86.3 ± 8.8	88.1 ± 2.9	90.1 ± 2.3	87.8 ± 1.4	95.4 ± 6.2	93.2 ± 6	96.7 ± 4.3	83.8 ± 2.5
C. grimmii- A	90.9 ± 2.9	100 ± 7.9	91.1 ± 2.1	89.0 ± 3.2	111.1 ± 3.7	94.1 ± 1.8	96 ± 6.4	77.9 ± 4	99.4 ± 3.8	99.6 ± 5.5
D. kopetdaghense- A[2]	100.8 ± 5.6	95.3 ± 6.1	85.2 ± 4.3	86.8 ± 4.1	88.4± 4.8	69.5 ± 7.6	84.1 ± 8.2	81.5 ± 6.6	85.2 ± 2.0	94.7 ± 4.0
D. kopetdaghense- R[3]	97.4 ± 4.7	1.6 ± 3.5 ***	72 ± 7.6 *	0.5 ± 2.3 ***	92.5 ± 6.5	3.7 ± 4.6 ***	66.7 ± 7.3 *	3.9 ± 11.6 ***	70.2 ± 6.6*	31 ± 9 ***
E. macrophylla- A	86.5 ± 4.9	96.4 ± 1.3	71.8 ± 5.5 *	88.8 ± 6.5	102 ± 4.5	99.2 ± 4.6	98.6 ± 7.6	100.8 ± 2.8	88.6 ± 3.0	88 ± 2.6
E. macrophylla- R	87.0 ± 3.5	87.7 ± 2.9	96.4 ± 4	83.3 ± 10	103.9 ± 1.7	93.6 ± 7.1	108 ± 7.1	105.5 ± 13	98.7 ± 2.6	87.3 ± 3.0
E. boissieriana- A	86.1 ± 4.8	100.2 ± 6.2	77.0 ± 2	80.8 ± 3.4	101.7 ± 3.6	95.6 ± 3.7	105.5 ± 13	91 ± 10.6	87.6 ± 3.5	87 ± 3.6
E. boissieriana- R	90.5 ± 5.7	90.7 ± 7.6	85.5 ± 2	66.6 ± 1 *	108 ± 2.2	99.7 ± 6.6	98 ± 15.2	104.6 ± 11.6	94.6 ± 4.6	69.4 ± 4.5 *
H. obtusifolium- A	93.3 ± 4	78.6 ± 3	77.0 ± 0.8	63.1 ± 1.2 *	77.3 ± 3.6	66.2 ± 2 *	76.3 ± 8	66.5 ± 2 *	94.5 ± 5.6	79.7 ± 9.7
H. obtusifolium- R	71.5 ± 3.2 *	19.2 ± 5.3 ***	55.6 ± 1.9 ***	22.2 ± 7.5 ***	69.6 ± 0.8 **	60.7 ± 2.5 ***	47.2 ± 4.5 ***	2.4 ± 9 ***	55.4 ± 2.7 ***	44.3 ± 6.6 ***
H. trichophylla- A	100.3 ± 5.5	103.8 ± 4.1	83.0 ± 4	79.9 ± 3.3	109.7 ± 3.5	108.2 ± 2.7	95.2 ± 4	90 ± 3.2	101.2 ± 4.6	104.4 ± 1.2
R. turkestanicum- A	76.2 ± 1.4	97.5 ± 4.3	75.9 ± 5.6	82.4 ± 0.6	96.5 ± 4.9	95.7 ± 2.7	102 ± 11.4	104.9 ± 11.3	86.7 ± 3.4	85.3 ± 5.4
R. turkestanicum- R	82.5 ± 3.8	74.5 ± 4.7	90.3 ± 1.2	71.3 ± 3.5 *	74.1 ± 2.1	69.9 ± 3 *	98.7 ± 7.4	90.2 ± 9.1	91.3 ± 5.3	76 ± 4.7
S. staurophyllum- A	80.3 ± 2.5	97.9 ± 6	87.9 ± 1.3	90.0 ± 1.3	95.2 ± 3.6	103.7 ± 2.7	89.1 ± 15	89.8 ± 16	77.2 ± 7.4	100 ± 3
S. staurophyllum- R	88.8 ± 6.5	77.0 ± 4.1	85.1 ± 4	86.3 ± 4.1	86.3 ± 1.6	90.1 ± 1.7	105.3 ± 3	104.6 ± 8.1	100.4 ± 3	84.5 ± 1.7
S. transcaucasicum -A	77.3 ± 2.2	79.3 ± 5.2	86.2 ± 1.8	76.0 ± 1	88.3 ± 7.9	90.6 ± 3.7	104.5 ± 7	89.8 ± 3	73.8 ± 7.9	67.8 ± 4.7 *

[1] Concentration in µg/ml; [2]A: Aerial part; [3]R: Root
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

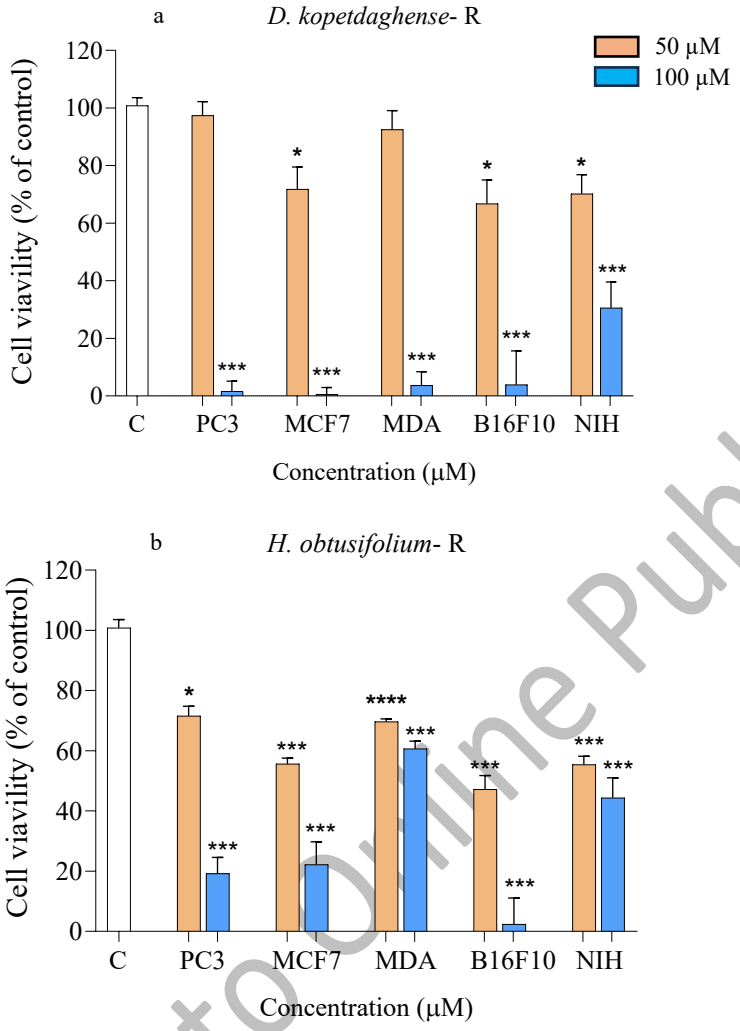

Fig. 1 Cytotoxicity (% of cell viability) of methanol extracts of A: *D. kopetdaghense*- R and B: *H. obtusifolium*- R on PC3, MCF-7, MDA, B16F10 and NIH cells. 100 µg/ml of the *D. kopetdaghense*- R extract revealed a notable cytotoxic effect on all these cell lines (p <0.001), and 100 µg/ml of the *H. obtusifolium*- R extract exhibited a significant cytotoxicity on PC3, MCF7, B16F10 (p <0.001), NIH (p <0.01), and MDA (p <0.05) cell lines. All data are shown as mean ± SEM of three independent assessments. Significance is shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group.

Flow Cytometry Analysis of Apoptosis

Our results showed that extracts of *D. kopetdaghense* and *H. obtusifolium* root on PC3 and MCF7 cells induce cell death through apoptosis. 25 and 50 µg/ml of the methanol extracts of *D. kopetdaghense* and *H. obtusifolium* root increased apoptotic cell numbers on PC3 and MCF-7 compared to the control group (Fig. 2).

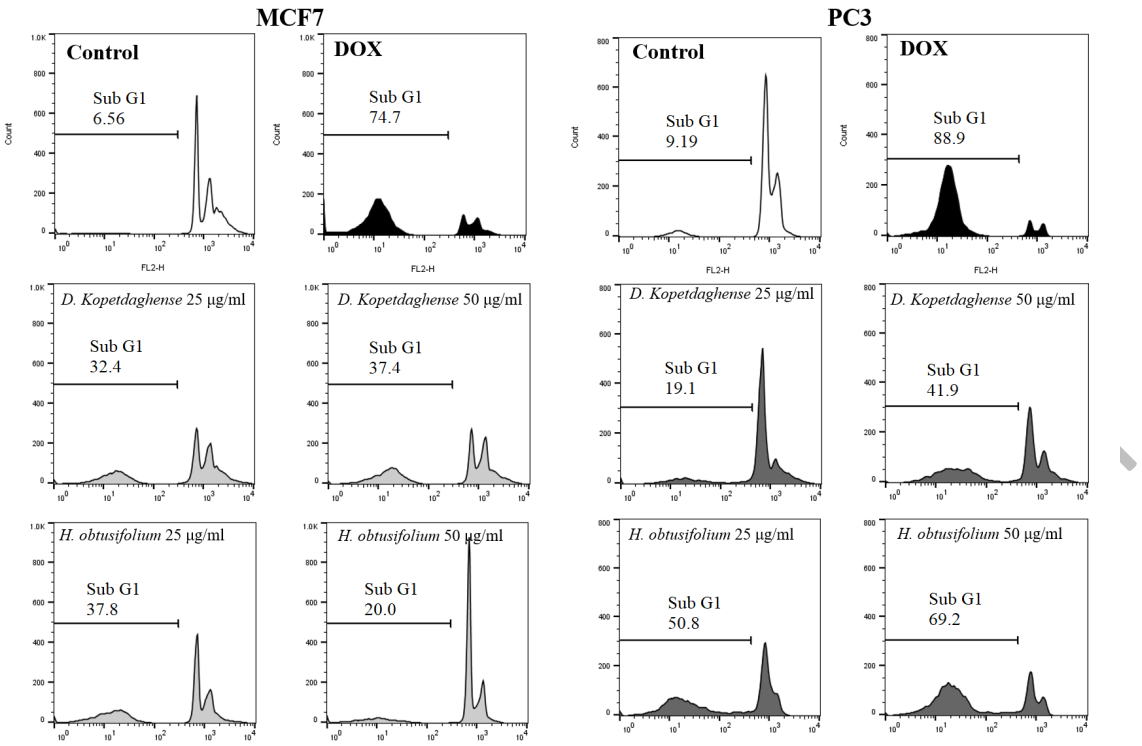

Fig. 2 Flow cytometry histograms of apoptosis assays by PI staining of MCF7 (A) and PC3 (B) cell lines. 25 and 50 µg/ml of the methanol extracts of *D. kopetdaghense* and *H. obtusifolium* root increased apoptotic cell numbers on PC3 and MCF-7 compared to control group.

DISCUSSION

Cancer is one of the serious concerns for human health, and the development of novel treatments with higher efficacy and minimal side toxicity is one of the main approaches in research [19]. Nowadays, herbal supplements and products are used as an accessible and effective cure, and tremendously attract both healthcare providers and the public to derive deep insights for the usage of medicinal plants [19-21]. Therefore, it is necessary to investigate the biological functions, properties, and side effects of unknown and rare plants. In our study, for the first time, the cytotoxic and anticancer potential of methanol extract from 10 rare and endemic plant species, namely *Dorema kopetdaghense*, *Chaerophyllum khorassanicum*, *Seseli staurophyllum*, *Seseli transcaucasicum*, *Haplophyllum obtusifolium*, *Crinitaria grimmii*, *Handelia trichophylla, Rheum turkestanicum, Eremostachys boissieriana*, and *E. macrophylla* from, Khorasan Razavi Province, were examined on PC3, MCF-7, MDA, B16F10, and NIH cells.

In accordance with our results, the methanol extract of *D. kopetdaghense* and *H. obtusifolium* root in all five cancer cell lines showed significant cytotoxic effects. In addition, the methanol extract of the aerial part of *H. obtusifolium* on MCF-7, B16F10, and the aerial part of *S. staurophyllum* on B16F10 cells displayed significant cytotoxic activity. Regarding the viability results, apoptosis induced by the extract of *D. kopetdaghense* and *H. obtusifolium* root was assessed with the PI method on MCF-7 and PC-3 cells. The outcome showed both extracts increased apoptosis compared to the control group dose-dependently which was in line with previous similar studies. An increase in the sub G1 peak in the flow cytometry histogram of treated cells confirms that at least a part of the cytotoxicity is exerted through apoptosis induction.

The genus *Dorema* D. Don belongs to the Apiaceae (alternatively Umbelliferae) and is represented by seven species within the Iranian flora. These species are morphologically similar to one another and monocarpous, and possess thick roots with simple and large umbels. There is an important difference between Feula genera based on their corymbs [22]. In terms of the therapeutic effects of the genus *Dorema*, there exists a common belief and scientific observation, such as antispasmodic and expectorant properties and anti-microbial activity [22, 23]. In a study by Eskandani *et al.,* the cytotoxicity and apoptosis induction of the methanol extract from the seeds of *Dorema glabrum* Fisch were evaluated. Based on the results, diglucosyl caffeoyl ester from the seeds of *D. glabrum* Fisch disclosed growth inhibition and the occurrence of apoptosis in CAOV-4 cells (IC_{50}=99.7 µg/ml) [24]. Among these, *D. kopetdaghense* has shown anti-inflammatory and anti-inducible nitric oxide synthase (iNOS) enzyme effects in its non-toxic therapeutic concentrations (10–100 µg/ml, for 24 h), which kopetdaghins A, C and E isolated from the root were responsible for in our previous studies [25].

The genus *Haplophyllum* (Rutaceae) includes about 70 species, which are predominantly growing in warm temperate such as the northern tropical region of eastern Africa and the subtropical region of Eurasia. Iran possess the highest number of *Haplophyllum,* with 25 species and 13 of them are endemic [26]. In a study by Al-Muniri *et al.,* cytotoxic properties of different extracts of *H. tuberculatum* were investigated. Based on the results, some polyphenol compounds in this medicinal plant have drawn attention because they appear to have positive health benefits as a result of their possible anti-oxidant, and cancer-preventive actions. It has been established that different fractions of *H. tuberculatum* can cause cytotoxicity at a concentration of 500 µg/ml using brine shrimp lethality (BSL) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods [27]. Literature published about the cytotoxicity of the same plant genus has shown similarities in phytochemical content. Thus, it is predicted that the plants from the same genus have similar biological activity.

In some studies, the cytoprotective and a cytotoxic effects of *R. turkestanicum* have been reported. It has been shown that the ethyl acetate and *n*-hexane H_2O extracts (15–500 µg/ml) diminished the cell viability of MCF-7 and HeLa cells with no effect on non-

malignant cells dose and time dependently. Also, ethyl acetate (50–200 µg/ml) showed apoptosis induction in MCF-7 and HeLa cells [28]. Additionally, in another study dichloromethane and n-hexane extracts of *E. macrophylla* aerial parts represented cytotoxic effect on A549 and HT29 cells without any side effects on normal cells [29, 30]. Moreover, *R. turkestanicum* root extract (12-200 µg/ml) preserved PC12 and N2a cell lines against oxidative stress and cell death by diminishing malondialdehyde (MDA) level, reactive oxygen species (ROS) production and apoptotic cell death, which revealed the neuroprotective action of this plant [31]. Interestingly, in another study, *R. turkestanicum* exerted similar protective effects against doxorubicin-induced H9c2 cytotoxicity [32]. There is no evidence about the cytotoxicity of *S. Staurophyllum* and *S. transcaucasicum*. Taken together, screening the selected plants showed that *D. kopetdaghense* and the *H. obtusifolium* root methanol extracts have cytotoxic effects on all PC3, MCF-7, MDA, and B16F10 compared with the control group. Particularly, *H. obtusifolium,* the aerial part in the MCF-7, B16F10, and the methanol extract of the aerial part of *S. staurophyllum* in B16F10 cells showed significant cytotoxic activities. Also, the root extracts of *D. kopetdaghense* and *H. obtusifolium* showed a notable increase in apoptosis in PC3 and MC7 cell lines compared to the control group, which could highlight the anticancer potential therapeutic effects of these extracts.

CONCLUSION

Overall, Iran possesses a rich source of medicinal plants, which contain a variety of bioactive phytochemical compounds with pharmacological prominence. However, the characterization and exact underlying cellular and molecular mechanisms of cytotoxic effects of these compounds are unclear. This study offers a significant basis for further investigation to evaluate the regulatory pathways involved in the protective potential of natural bioactive components obtained from the flora of Iran on different human diseases.

Acknowledgment

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Conflict of Interest

We confirm that there are no conflicts of interest associated with this publication and there has been no financial support for this work that could have impressed its finding.

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Data and Material Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent for Publication

As this work is carried out in cell lines, there is no need for ethical clearance.

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