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Anti-carcinogenic effects of *Satureja khuzistanica* and *Zataria multiflora* essential oils on K562 cell line proliferation

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A B S T R A C T

The aim of this study was to investigate cytotoxic effect of *Satureja khuzistanica* and *Zataria multiflora* essential oils on PBMC and K562 cell lines growth inhibition. The plant essential oils were extracted by hydro-distillation and analyzed by GC-MS. In addition, the total phenol content and antioxidant activity of oils were assessed. The PBMC and K562 cell lines were cultured in RPMI medium containing 10% FBS supplemented with 0, 12.5, 25, 50 and 100 (µg/ml) of essential oils for 24, 48 and 72 h. The MTT assay revealed anti-proliferative effect of essential oils on both cell lines in a dose dependent manner. These effects became more prominent after 72 h. Both essential oils contained high amounts of phenolic compounds such as thymol and carvacrol. Due to the higher percentage of carvacrol, which has more toxicity than thymol, *S. khuzistanica* had a greater antioxidant activity as well as inhibitory effect on cell growth. In conclusion, the *S. khuzistanica* and *Z. multiflora* essential oils have the potential to be used in the production of anticancer medicines.

Keywords: Anti-proliferation, Antioxidant activity, Carvacrol, Cytotoxicity, GC-MS

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1. Introduction

Cancer is an uncontrolled cell proliferation leading to second cause of mortality worldwide (Hassanpour & Dehghani, 2017). The chemicals, radiations, unhealthy diet, environmental factors, infection and tobacco smoke are risk factors for cancer (Anand et al., 2008). The environmental carcinogenic substances influence directly or indirectly the cell cytoplasm and nucleus (Poon et al., 2016). Cancer occurs when mutations in vital genes lead to dysfunction of genes, disruption of cell cycle and abnormal proliferation (Aizawa et al., 2016). Overall, the cancer prevalence worldwide has raised over the past decade; for instance; 1,665,540 and 585,720 Americans were diagnosed and died from cancer in 2014 (Hassanpour & Dehghani, 2017).

Traditional herbal medicine has become center of attention during the last decades and number of member state regulating herbal medicine has increased from 65 (1999) to 119 (2012). Moreover, the research institutes for herbal medicine has been upgraded from 19 (1999) to 73 (2012) (W.H.O, 2013) . There are hundreds of literature data revealing the importance and utilization of herbal medicines to cure diseases like cancer (Rafieian-Kopaei et al., 2013). According to latest WHO reports, traditional system of herbal treatment has even been adapted in developed countries including Belgium (31%), Australia (48%), France (49%), Canada (70%) and Germany (77%) (WHO, 2013).

Natural products are pharmacologic agents and have been traditionally used for prevention and cure of chronic diseases (Sefidkon et al., 2013). Today, blood cancer is one of the most important diseases and plants are potential candidates for cancer treatment (Zali et al., 2011). Aromatic plants contain essential oils with chemo-preventive properties due to volatile extracts comprising of a wide variety of active phytochemicals, such as monoterpenes, polyphenols and flavonoids (Lai & Roy, 2004).

Satureja khuzistanica Jamzad (Persian name, 'marzeh khuzistani') belongs to the family Lamiaceae, and is an endemic plant of Iran with wide distribution in the southern part of this country. The plant has commercial and medicinal values and has been widely used in pharmaceutical, food, cosmetics and perfumery industries due to its high essential oil, which mainly contain carvacrol and terpinene compounds (Kouravand et al., 2018; Sefidkon et al., 2013). In traditional medicine, *S. khuzistanica* has been used as an antimicrobial, spasmolytic cicatrisant, diuretic (Gohari et al, 2005), analgesic, antiseptic and anti-inflammatory agent (Naghibi et al., 2005). Ghazanfari et al. (2006) demonstrated that oral consumption of *S. khuzistanica* essential oil has remarkable impact in the treatment of inflammation and diabetes.

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Zataria multiflora Boiss (vernacular name of Avishan Shirazi, in Iran) belongs to the family Laminaceae and geographically grows in Iran, Pakistan and Afghanistan (Hosseinzadeh et al., 2000). The plant has traditionally been used as an antiseptic, anesthetic and anti-spasmodic drug (Ali et al., 2000), also extensively used as a flavor ingredient in Iranian foods. The flowered browses of the plant contain essentials oil with various pharmaceutical, antimicrobial, and antioxidant effects (Saeedi-Saravi et al., 2008). The main constituents of its essential oil are phenolic compounds such as carvacrol and thymol (Zandi-Sohani & Ramezani, 2015). The cytotoxicity and antibacterial capacity (Amin et al., 2010) as well as radioprotectivity and chemoprotectivity (Hosseinimehr et al., 2011) effects of *Z. multiflora* essential oils have been reported.

To the Knowledge of authors, no research has been conducted on the cytotoxic effects of *S. khuzistanica* and *Z. multiflora* plant extracts on cell viability of K562 leukemia cell line. Therefore, we aimed to investigate the apoptotic effects of these two species extract on cell viability of PBMC and K562 cells.

2. Material and Methods

2.1. Extraction and analysis of essential oil

The aerial parts of *S. khuzistanica* were collected from Khuzestan province in the southwest of Iran during full flowering stage in 2017. The aerial parts of *Z. multiflora* were harvested in pre-flowering stage from wild grown plants in the Mountain regions of Fars province located in the south of Iran. The harvested plants were dried at room temperature for two weeks, then, air-dried plants (in each habitat) were ground and powdered with mixer for essential oil extraction and other experiments. The powdered plants aerial parts (250 g) were hydro-distilled using a Clevenger type apparatus for 3 h, according to the method recommended in the European Pharmacopoeia (Council of Europe, 1997). The resultant essential oil was dried over anhydrous sodium sulfate and stored at 4ºC until analyzed and tested. Dilutions of the oil in the various assays were then made from the parent oil stock by addition of dimethyl sulfoxide (0.1%; DMSO) to yield the desired final test concentrations (Hojjati & Barzegar, 2017).

2.2. GC-MS analysis

The chemical analysis of the essential oils was performed using Agilent technology GC-6890A and GC-MSD 5975 fitted with a HP-5MS column (30 mm × 0.25 mm i.d, 0.25 mm film thick). 0.5 µL of essential oils were injected for analysis, and Helium was used as carrier gas at 1 mL/ min. The oven temperature was programmed from 60ºC to 250ºC at the 5ºC/ min rate, and was finally hold for 1 min. Injector and detector temperatures were 230ºC and 300ºC, respectively. The compounds of essential oils were identified according to GC-MS Retention time, Kovats index and Mass spectra NIST, 2013 (National Institute of Standards and Technology, 2013).

2.3. Total phenolic content and antioxidant activity of essential oils

Total phenolic content of the essential oils was measured according to the absorbance values of the oil reacted with Folin-Ciocalteu reagent at 765 nm and compared the results with Gallic acid as standard solution according to the method described previously with minor modifications (Hojjati & Barzegar, 2017). Total phenolic content was expressed as milligrams of Gallic acid equivalent per gram of essential oil (mg GAE/g). Antioxidant activity of the essential oils was assessed using DPPH and ferric reducing antioxidant power (FRAP) assays based on original methods by Hojjati and Barzegar (2017) and Saidi (2014) with some modifications, respectively.

2.4. Cell culture

One type of cancer cells (K562) and one type of normal cells (PBMCs) were used to determine the cell viability against essential oils exposure. The human myeloid leukemia K562 cells were obtained from Pasture Institute of Iran, Tehran, Iran. Fresh peripheral blood was taken with informed consent from three healthy volunteers into sterile falcon tubes containing heparin (200 IU/ml). Human peripheral blood mononuclear cells (PBMC) were isolated by using 1.077 g/ml Ficoll/ Hypaque as previously described (Boyum, 1968).

Culture medium used for the growth of K562 was as follows: RPMI 1640 (Gibco, Life technologies GmbH, Frankfurt, Germany) enriched with Fetal Bovine Serum-FBS 10%, Streptomycin (100 µg/ml, Sinagen, Tehran, Iran) and Penicillin (100 µg/ml, Sinagen, Tehran, Iran). The cells were grown in incubator (holding British Standard) with 5% CO2, 95% humidity and 37ºC (Aslani et al., 2014).

2.5. Assessment of cytotoxicity based on MTT

Growth of cells was quantitated by the ability of living cells to reduce the yellow dye 3- (4, 5-dimethyl- 2-thiazolyl)-2, 5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. MTT assay is an accurate method to evaluate the survival of cells. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living cells (Sylvester, 2011). In this study, to evaluate the effects of Z. multiflora and S. khuzistanica essential oils on the morphology of PBMC and K562 cell line, 104 cells were positioned in 96-well plates (Surface, Denmark). The concentrated plant essential oil was dissolved in dimethyl sulphoxide (DMSO) (Sigma Aldrich, Germany) to obtain appropriate solutions of the essence. The substock solutions of 12.5, 25, 50 and 100 µg/ml was prepared by diluting the stock solution into serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 1).

After 24 h, the cells were treated with different concentrations of *S. khuzistanica* and *Z. multiflora* essential oils (12.5, 25, 50, 100 µg/ml), prepared separately. The normal cells and K562 cell line were incubated for 24, 48 and 72 h with different concentrations of essential oils (12.5, 25, 50, 100 µg/ml). Then, 20 µl MTT solution 0.5 mg/ml was added into the wells and incubation continued at 37ºC for 3 to 5 h. After this time, the supernatant was removed and 200 µl of DMSO solution was added at room temperature for 15 min. Finally, the percentage of cell viability was determined by ELISA reader at 570 nm. The percentage of cell viability was calculated using the following equations:

[mean optical density (OD) of treated cells/mean OD of control cells] × 100. Percent of cytotoxicity = 100 – percent of Viability

(Zamanian-Azodi et al., 2012). The effects of essential oil were expressed by IC50 values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Table 1. Volatile composition of *S. khuzistanica* and *Z. multiflora* essential oils.

Compound	*RT (min)	*KIexp	% *Satureja*	% *Zataria*
α- thujene	5.253	927	2.34	0.65
α- pinene	5.420	934	2.67	3.32
Camphene	5.742	947	0.27	0.25
β- pinene	6.375	972	1.81	0.66
beta myrcene	6.664	984	2.44	1.74
3- octanol	6.80	993	-	0.19
α- phellandrene	7.009	997	0.70	0.24
delta 3 carne	7.153	1005	0.16	0.07
α- terpinene	7.331	1011	4.56	2.28
p- cymene	7.575	1019	9.55	7.34
limonene	7.642	1022	1.42	0.83
1,8 cineole	7.775	1026	0.29	0.68
β-ocimene	8.097	1037	0.20	0.07
γ- terpinene	8.486	1050	22.72	6.66
(z)-sabinene hydrate	8.742	1058	0.13	0.21
cis linalool oxide	8.845	1072	-	0.10
α- terpinolene	9.175	1081	0.19	0.25
trans linalool oxide	9.267	1088	-	0.08
linalool	9.697	1090	0.06	5.26
hotrienol	9.825	1098	-	0.18
borneol	11.830	1168	0.57	0.25
terpinene-4-ol	11.941	1176	0.59	1.13
α- terpineol	12.108	1184	0.06	1.01
cis dihydrocarvone	12.555	1200	-	0.07
thymyl methyl ether	13.111	1224	-	1.27
carvacrol methylether	13.352	1227	0.21	1.88
carvone	13.852	1243	1.12	-
anethole	14.497	1258	0.39	-
thymol	14.674	1279	1.82	30.61
carvacrol	15.563	1300	38.33	22.18
piperitenone oxide	16.741	1363	0.11	-
carvacryl acetate	16.830	1370	0.80	1.13
carvacryl acetate	16.844	1380	-	1.13
caryophyllene	18.029	1418	0.79	2.56
β-gurjenene	18.232	1432	-	0.07
alloaromadendrene	18.507	1449	0.21	0.11
aromadendrene	18.521	1449	-	1.17
α- humulene	18.803	1481	0.06	0.17
ledene	19.874	1500	0.13	0.83
β-bisabolene	20.163	1509	1.70	-
α- amorphene	20.318	1516	0.05	-
delta cadinene	20.518	1520	0.05	-
spathulenol	21.873	1570	0.12	1.03
caryphyllene oxide	22.04	1579	0.47	1.06
isospathulenol	23.243	1640	-	0.13
bisabolene epoxide	23.965	1699	-	0.14
total			97.12	99.14

*RT: Retention Time; KIexp: Kovats ndex in this experiment; %: Relative percentage obtained from peak area.

2.6. Statistical analysis

All analyses were reported as means ± SD (standard deviation). The SPSS Software v. 24 (SPSS Inc., Chicago, IL, USA) and analysis of variance (ANOVA) were used to investigate significant differences between the average values (95% confidence). Significant differences between the means were calculated using Duncan test at a significant level of $p < 0.05$.

3. Results and Discussion

3.1. Chemical composition of essential oils

The essential oil obtained by hydro-distillation of the aerial parts of *S. khuzestanica* and *Z. multiflora* was subjected to GC-MS to identify composition. Qualitative and quantitative analytical results of *S. khuzestanica* and *Z. multiflora* essential oils were shown in Table 1. According to gas chromatography–mass spectrometry (GC-MS) analysis, 35 and 40 volatile compounds were identified from *S. khuzestanica* and *Z. multiflora* essential oils representing 97.12% and 99.14% of the total oil, respectively. Based on the Table 1, carvacrol (38.33%), γ terpinene (22.72%) and p-cymene (9.55%) were the main compounds in the S. khuzestanica oil. Altough Saeidi (2014) reported only carvacrol as the main constituent of *S. khuzistanica* essential oil; Alizadeh (2017) found carvacrol, γ-terpinene, β-bisabolene and pcymene to be the major constituents of the oil of *S. khuzistanica*, which was consistent with our findings. As observed in Table 1, thymol (30.61%), carvacrol (22.18%), Linalool (9.57%), γ terpinene (8.44%) and p-cymeme (7.34%) identified as the main components in the essential oil of *Z. multiflora*. It was in close agreement with the previous results (Raiesi et al., 2019: Zandi-Sohani & Ramezani, 2015). Comparison of our findings with others suggests that differences in the components of the essential oils may be attributed to various factors such as the genetic, geographic, cultivar, species, ripening stage, cultural practices, extraction methods, soil and climate conditions (Hojjati & Barzegar, 2017; Saeidi, 2014).

Table 2. Antioxidant activity of *Satureja khuzistanica* and *Zataria multiflora* essential oils.

Essential oil	Total Phenolic Content (mg GA eq/g)	DPPH (%)	FRAP (µmol/g)
Zataria	216.27±17.40^{b*}	39.22±4.15^{b}	25.74±2.29^{b}
Satureja	249.53±16.59^{a}	47.19±3.26^{a}	30.02±3.11^{a}

*Values represent mean ± Standard Deviation of three replicates and values with different letters in each column were significantly different at the level of $p < 0.05$.

3.2. Total phenol content and antioxidant activity of essential oils

The results of total phenol content and antioxidant activity of essential oils are shown in Table 2. The level of total phenolic compounds in *S. khuzestanica* and *Z. multiflora* essential oils were 249.53 and 216.27 mg GA eq/g, respectively. In addition, the data showed that in both methods, the antioxidant activity of *S. khuzestanica* oil was significantly higher than that *Z. multiflora* essential ($p < 0.05$). The antioxidant activity of *S. khuzestanica* essential oil in the two methods of DPPH and FRAP assays were 47.19% and 30.02 (µmol/g), respectively, which were significantly higher than the level of antioxidant activity of *Z. multiflora*. The results of the total phenolic content and the antioxidant activity of the essential oils were similar to those of some researchers and were in contrast to some others. The amount of phenolic compounds of *Z. multiflora* essential oil in this study was higher

than the values reported by Mojaddar-Langroodi et al. (2019) and Alizadeh and Shaabani (2014), whereas Raeisi et al. (2019), observed more phenolic compounds. Total phenol content and antioxidant potential of *S. khuzistanica* oil studied in this work was higher than the amount reported by Saidi (2014) and Alizadeh (2017).

As seen in Table 2, the essential oil of *S. khuzistanica* has higher phenolic content and was highest antioxidant activity, compare to *Z. multiflora*. Thus, our results showed that, a correlation between the antioxidant activities and the total phenolic contents was revealed. On the other hand, there was a significant increase in antioxidant activity by increase in total phenolic content of samples. Otherwise, ferric reduction and DPPH radical scavenging occurred more powerfully with the increasing concentrations of phenolic compounds in essential oils. The antioxidant activity of medicinal plants is associated with the phenolic content of them. High redox potentials of phenolic compounds provided them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Alizadeh, 2017; Raeisi et al., 2018). Probably because of the different types of phenolic compounds in each essential oil, they showed different antioxidant activity.

3.3. *The Effect of essential oils on proliferation of PBMC normal cells and K562 cell line*

The results of the effect of four different concentrations (12.5, 25, 50 and 100 µg/ml) of *S. khuzistanica* and *Z. multiflora* essential oils on cell viability of PBMC and K562 cell line at 24, 48 and 72 h incubation were presented in Fig. 1. Evaluation of cell survival was measured using the MTT test. The 50% growth inhibitory concentration (IC50) of 28.21 and 18.06 (µg/mL) was obtained for *S. Khuzistanica* essential oil for the PBMC and K562 cell line, respectively, and it was found 64.97 and 42.82 (µg/mL) on normal PBMC and K562 cell line, respectively for Z. multiflora essential oil.

The absorption rate based on the different concentrations of essential oils at different times was significantly lower than that of the control group (did not receive any essential oil), indicating a toxicity effect of essential oil on PBMC and K562 cell line. According to our results, the amount of cell survival was dependent on the essential oil concentration and incubation time. Hence, the concentration of 100 µg/mL of essential oil and 72 h incubation was the most fatal with the lowest living. Incubation of PBMC from 12.5 to 100 (µg/mL) of *S. Khuzistanica* essential oil for 24 h reduced cell viability from 92.2 to 53.7%, respectively, however, at the same concentrations of essential oil after 72 h of incubation, cell viability was decreased from 78.3 to 47.1%, respectively (Fig. 1A and B). Similarly, at maximum concentration (100 µg/mL) and 72 h incubation, *Z. multiflora* essential oil showed the highest activity on PBMC and K562 cells (Fig. 1C and D). The results showed the effect of both essential oils on the growth of tested cells, although PBMC cell line was less susceptible to the cytotoxicity of both essential oils at the same essential oil concentrations and incubation times compared to the K256 cell line.

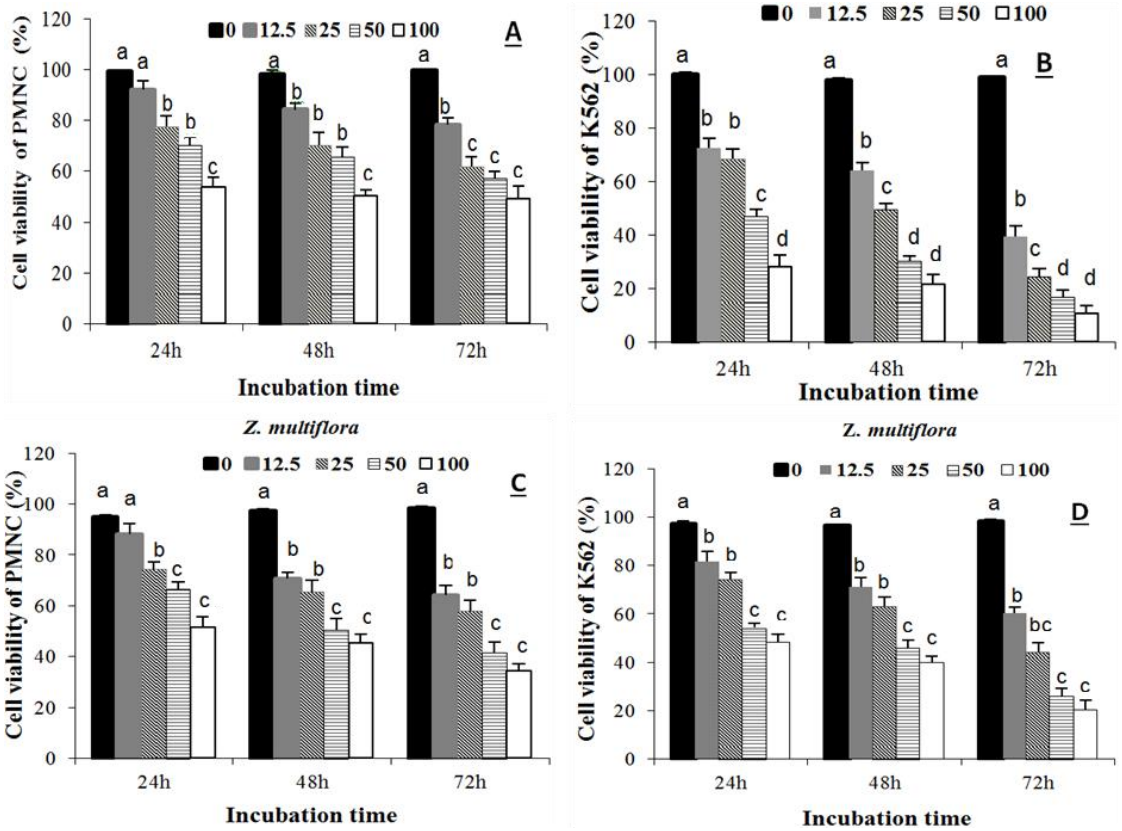

Fig. 1. Effects of *S. khuzistanica* (A-B) and *Z. multiflora* (C-D) essential oils on viability of PBMC and K562 cell line.

The presence of large amounts of carvacrol in both essential oils may be due to their anticancer effects because the cytotoxic potential of these monoterpens have already been proven by Horvathova et al. (2007) who reported that carvacrol and thymol significantly reduced the level of DNA damage induced in K562 cells by the strong oxidant H2O2. On the other hand, phenolic compounds such as thymol increase in production of reactive oxygen species (ROS) activity, increase in mitochondrial H2O2 production and depolarization of mitochondrial membrane potential (Dutta et al., 2011).

The strong antitumor activity of carvacrol has also been revealed by suppression of mouse B16 melanomas (He et al., 1997), lung tumors in rats (Zeytinoglu et al., 1998) and human larynx carcinoma Hep-2 cells (Stammati et al., 1999).

In addition, the results showed that essential oil of *S. Khuzistanica* had more significant effect on inhibition of cancer cell growth at different incubation times compared to Z. multiflora. The results in the present work are in agreement with the findings of Mohammadpour et al. (2015) who investigated the cytotoxicity potential of *S. bakhtiarica* and *Z. multiflora* oils on normal HEK cells compared to MDA-MB-231 and SKOV3 as cancerous cell lines. The results of the analysis of essential oils in this study also indicated that the presence of high levels of terpenoids such as carvacrol, γ-terpinene and p-cymene in *S. khuzistanica* oil.

The efficacy of the plant as an anti-cancer medicine may be related to terpenoid compounds which it could inhibit tumor cell proliferation in human breast and prostate (Yang & Dou, 2010), therefore, the reason for the greater effect of essential oil of *S. Khuzistanica* on cancer cells may be due to the higher amount of phenolic compounds as well as its antioxidant properties compared to *Z. multiflora* (Table 2).

4. Conclusion

Pharmaceutical plants with cytotoxic effect are good candidates to synthesis anticancer drugs. Moreover, due to lower side effects and pharmaceutical intervention, they attract pharmacists' attention to synthesis new drugs in order to treat refractory disease such as cancers (Mongelli et al., 2000). The data presented in this work provided scientific information that might be used for isolation of potentially active compounds from *S. khuzistanica* and *Z. multifora* medicinal plants in future. Based on our findings, both essential oils had high amount of terpenoids and cytotoxic effect on normal PBMC and K562 cell line due to the presence of high amounts of phenolic compounds such as thymol and carvacrol. However, due to the presence of higher amount of phenolic compounds and antioxidant activity, *S. khuzistanica* pronounced a greater inhibitory effect on cell growth. It is concluded that, the *S. khuzistanica* and *Z. multiflora* essential oils have the potential to be used in the production of anticancer drugs.

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