

Original Article**Evaluation of the Antioxidant Activities and Cytotoxicities of Selected Medicinal Herbs Using Human Hepatoma Cell Line (HepG2)**Ali Bashiri Dezfouli¹, Jamileh Salar-Amoli*², Mina Yazdi³, Tahereh Ali-Esfahani⁴, Abbas Barin⁵

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ABSTRACT

Background: The aim of this study was to evaluate and compare the hepatotoxicity as well as antioxidant activities of hydromethanolic extract of six common traditional species (*Origanum vulgare*, *Pterocarya fraxinifolia*, *Ferula assafoetida*, *Artemisia dracunculoides*, *Rosmarinus officinalis*, and *Valerian officinalis*) in order to find a safe antioxidant source.

Methods: This research project was performed at Toxicology and Animal Poisoning Research Center, University of Tehran (Tehran, Iran), in 2016. The HepG2 cells viability was examined by LDH and MTT techniques following treatment with different concentrations of selected herbal hydroethanolic extracts for 72 h. Furthermore, assessment of antioxidative properties of the extracts was carried out by various scavenging models including DPPH and FRAP.

Results: The highest cytotoxicity was displayed by *F. assafoetida* extract (IC₅₀= 67.3 µg/ml). *R. officinalis* and *A. dracunculoides* extracts were noted as non-toxic due to the high effective dose. Interestingly, *V. officinalis* extract indicated stimulating effects on cell growth/ proliferation with ED₅₀ values of 20.9 µ/ml. The highest and lowest antioxidant capacities refer to *R. officinalis* and *V. officinalis*. In DPPH assay, the IC₅₀ value of *R. officinalis* and *V. officinalis* extracts was found to be 39.82 and 371.77 µg/ml, respectively. FRAP values of *R. officinalis* and *V. officinalis* were 2754.07 and 561.14 µM/g, respectively.

Conclusion: This study identified *R. officinalis* extract as a natural non-toxic agent with remarkable antioxidant potential in phytomedicine.

Keywords: Antioxidant Activity, Cell Viability, HepG2 Cells, Hydromethanolic Extracts, Medicinal Herbs.

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INTRODUCTION

About 65% of people use traditional medicine, mostly medicinal plants, and related active ingredients, in their primary health care approach to maintain good health as much as to treat of illness [1]. Most of the traditional plants are cheap and accessible source of natural antioxidants and therapeutic products. Due to the lack of the scientific evidence to date, there are always concerns about the safety and efficacy of such plant species. Various bioactive substances of plants such as alkaloids, cyanogens, saponins, tannins, and

phenolic compounds can threaten organisms' health depending on the amount, frequency and exposure pattern [2], hence, the potential toxicity of plant products should be assessed via in-vitro and in vivo.

A lot of researches referred to adverse and side effects of medicinal plants like nervous upset, problem associated with renal and circulatory systems and especially hepatotoxicity [3]. Mechanisms of hepatotoxicity may result in enzyme failures, necrosis, metabolization inability etc. Since the HepG2 cells show several characteristics of hepatocytes (e.g. releasing of albumin and expressing cytochrome P450), HepG2 cell line,

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despite some limitations, serve as a representative model to bioassay the toxicity of natural products and to study biotransformation of xenobiotics in laboratory experiments [4].

Free radicals are the most important factor for induction pathological responses like inflammation, aging, cardiovascular diseases, diabetes and Alzheimer's disease. Accordingly, oxidative stress is becoming acceptable as a serious risk factor for life-threatening complications. Antioxidants are among the best agents rendering free radicals inactive because they can scavenge the reactive oxygen species (ROS) prior to attack to biological targets [5]. Although it is common to use synthetic antioxidants in food and medicine industries, concerns about their safety have propelled the application of natural resources as alternatives [6]. Many research groups are currently studying the chemical structures and functions of natural antioxidants present in fruits, vegetables, grains and so forth. Most of plants have polyphenolic compounds such as flavonoids, tannins, trinoids and cathepsins in addition to A, C and E vitamins in their compositions [7].

Several common medicinal plants including *Origanum vulgare*, *Pterocaryan fraxinifolia*, *Ferula assafoetida*, *Artemisia dracuncululus*, *Rosmarinus officinalis* and *Valeriana officinalis* were chose for this study. To date, little information is available regarding the toxic effects of the above-mentioned plants on hepatocytes and their antioxidant activities. Therefore, in this study, we evaluated the toxicity effect and antioxidant activities of hydroethanolic extracts of above-mentioned herbs.

MATERIALS AND METHODS

Growth Media and Chemicals

This experiment was conducted at Toxicology and Animal Poisoning Research Center, University of Tehran, Tehran, Iran in 2016. All the materials were provided from the best available grades to perform the study. Growth medium (Dulbecco's modified Eagle's medium; DMEM) containing 10% fetal calf serum (FCS), 25 mg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 µg/ml amphotericin B, essential amino acids and L-glutamine was obtained from Gibco (Germany). Cytotoxicity detection kit for measuring lactate dehydrogenase (LDH) was purchased from Roche (Germany). Dimethyl sulfoxide (DMSO), 1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH), diphenyltetrazolium bromide (MTT) and

trypsin/EDTA were purchased from Sigma (USA) and solvents were provided from Merk (Germany).

Cell Line and Cell Culture

HepG2 cell line was provided by the Iranian Biological Research Center (Iran). The cells were grown in DMEM (4.5 g/lit D-glucose) supplemented with 10% FCS, 1% antibiotic/antifungal and incubated at 37 °C under a humidified atmosphere with 5% CO₂. The media was changed every three days and the cells were passaged by trypsinization.

Extract Preparation

All plants were purchased from a well-known sale center in Tehran and confirmed by botany experts in Toxicology and Animal Poisoning Research Center, University of Tehran, Iran. Hydromethanolic extracts were prepared from 40 gr of plant leaves/ stem in 200 mL of hydroalcoholic solution (20% water and 80% methyl alcohol) [8]. After 24 h in room condition, the extracts were filtered several times and then concentrated using a rotary evaporator (HeidolphLaborota 4003, Germany) at 50 °C. All the semi-solid extracts were dried by a freeze-dryer in order to obtain the final product. The extraction yield expressed as the percentage of the crude extract weight to raw material weight.

MTT Cytotoxicity Assay

MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assay was used to measure the viability of HepG2 cells after exposure to different concentrations of herbal extracts [9]. The cells were cultured in 96-well plates (2x10⁴ cells in each well) and allowed adhering for at least 24 h. Following treatment with different concentration of each dried extract (25, 50, 100 and 200 µg/ml in medium plus 0.2% DMSO) for 72 h, cells were washed twice with phosphate-buffered saline (PBS; Gibco, Germany). Then, 15 µl MTT stain was added to each well and incubate for 4 h. To dilute the formazan crystals formed, the MTT working solution was replaced by 100 µl of DMSO followed by a 15 min shaking at room temperature. The absorption rates were read by an ELISA plate reader (Awareness Technology Inc., USA) in wavelength of 570 nm, with 630 nm as reference wavelength.

The viability of cells (in each well) was calculated with the following formula:

Viability= the average absorption rate of cells/ the average absorption rate of the control cells × 100

The half-maximal inhibitory concentration (IC50) or the median effective dose (ED50) was calculated for each extract.

LDH Cytotoxicity Assay

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme released upon membrane damage. The lactate dehydrogenase release assay kit (Roche, Germany) for evaluation of HepG2 cell cytotoxicity was used based on the manufacturer's protocol. The absorbance at 490 nm was measured an ELISA plate reader (Awareness Technology Inc., USA) to determine the level of LDH released. The results are expressed as percent of control.

DPPH Radical Scavenging Assay

Free radical scavenging activity of the hydroalcoholic herbal extracts was measured by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) kit according to Mensor method with some modifications [10]. A solution of 1ml of methanolic solution of DPPH (0.3mM) was added to 2.5ml of sample extracts and then left in the dark at room temperature for 30 min. The absorbance of the mixture was read at 517 nm against methanol as blank by a spectrophotometer (Beckman Du-650). Each experiment was carried out in triplicate and DPPH-radical scavenging activity is calculated as percent using the equation below:

$$\text{DPPH} = \frac{[(\text{control absorbance} - \text{sample absorbance}) * 100 / \text{control absorbance}]$$

The antioxidant activity was expressed as IC50 that referred to the concentration of sample required for scavenging 50% of DPPH free radicals. Finally, percentage of inhibition was plotted against

concentration and IC50 was calculated from the graph.

FRAP Radical Scavenging Assay

Ferric reducing ability of plasma (FRAP) assay of the hydroalcoholic herbal extracts was measured according to a slightly modified protocol [11]. An aliquot of 50 µl of plant-extracted samples was mixed with 1.5 ml reagent solution. The FRAP reagent solution consisted of 300 mM acetate buffer (pH= 3.6), 10 mM 2, 4, 6-Tripyridyl-s-Triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃.6H₂O (10:1:1, v/v/v). The prepared samples were incubated for 30 min at 37 °C. The absorbance of the samples was read at 593 nm by a spectrophotometer (Beckman Du-650). The antioxidant potential of the sample was determined against a standard curve of ferrous sulfate (500-5000 µM). All measurements were carried out in triplicate and the results were reported as µM of FeSO₄ equivalents/g of extract.

Statistical Analysis

The data are presented as the average of the results of three replicates ± standard deviation. One-way ANOVA analysis was used for statistical comparisons. The *P*-value <0.05 was considered to be statistically significant. Simple linear regression analysis was performed to study the correlation between the results of antioxidant capacity assays.

RESULTS

Yield of Extract

The yields of extracts of the selected plants are expressed as dry matter content of per plant in Table 1. The highest and the lowest yield were belonged to *A. dracunculus* (12.61%) and *V. Officinalis* (1.84%), respectively.

Table 1. Extraction yields of selected plant species.

Scientific Name	Common Name	Family	Plant Parts	Yield (%)
<i>Artemisia dracunculus</i>	Tarragon	Asteraceae	Leaves	12.61
<i>Ferula asafoetida</i>	Devil's Dung	Apiaceae	Stems	11.61
<i>Origanum vulgare</i>	Oregano	Lamiaceae	Leaves	7.19
<i>Pterocarya fraxinifolia</i>	Caucasian wingnut	Juglandaceae	Leaves	4.17
<i>Rosmarinus officinalis</i>	Rosemary	Lamiaceae	Leaves	3.95
<i>Valeriana officinalis</i>	Valerian	Caprifoliaceae	Stems	1.84

Cytotoxicity

The effects of different concentrations of prepared extract (25, 50, 100 and 200 µg/ml) on the inhibition and/or proliferation of HepG2 cells were studied in 72 h using LDH release and MTT colorimetric assays. The statistical analysis showed no meaningful difference between the results from both assays. Thus, the results of LDH release method are reported as representative in this paper (Figure 1).

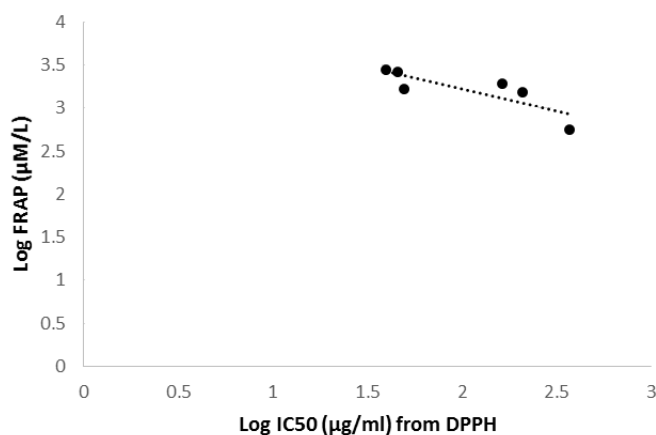


Figure 1. Viability rate of HepG2 cells following 72-h exposure to different concentrations of tested hydroalcoholic extracts.

Comparing IC₅₀ values indicated that *F. asafoetida* extract had the highest IC₅₀ (67.3 µg/ml) and its inhibitory effect increased in a dose-dependent manner. Consequently, *F. asafoetida* may have more potential that is toxic in its chemical composition than the others. In the following, IC₅₀s of *P. fraxinifolia* (107.6 µg/ml) and *O. vulgare* (164.3 µg/ml) extracts placed in the second and

third position. The IC₅₀ values of *R. officinalis* and *A. dracunculus* were higher than 200 µg/ml, which was above our selected range (42). Hence, the extracts of these medicinal plants did not show any significant cytotoxic effects on the hepatocytes and potentially considered as non-toxic agents. *V. officinalis* extract had a stimulatory effect on HepG2 cell line with the ED₅₀ of 20.9 µg/ml.

Antioxidant Capacity

The antioxidant capacity of hydro-methanol extracts of tested plants was assessed using DPPH and FRAP assays. The results from DPPH method were presented as IC₅₀s in Table 2. A lower IC₅₀ value corresponded to a higher free radical scavenging capacity of the plants. Therefore, *R. officinalis* and *V. officinalis* extracts showed the highest and the lowest DPPH free radical scavenging capacities with IC₅₀ of 39.82 and 371.77 µg/ml, respectively.

To confirm the results of DPPH method, the FRAP assay was also performed and the results were reported as µM of FeSO₄ equivalents/g of extract in the range of 561.14- 2754.07 (Table 2). Although *F. asafoetida* had a high antioxidant capacity (2629.37 µM/g), the greatest antioxidant capacity was observed in *R. officinalis* while *V. officinalis* extract had the lowest antioxidant capacity. With regard to FRAP results, the order of the antioxidant capacity of the tested extracts was as follows:

R. Officinalis > *F. asafoetida* > *P. fraxinifolia* > *O. vulgare* > *A. dracunculus* > *V. officinalis*

Our study established an agreement between the results of two assays except for *O. vulgar* and *P. fraxinifolia*. The natural logarithm was used to normalize the distribution ($R^2=0.68$) (Figure 2).

Table 2. Comparison of antioxidant and the cytotoxicity capacities of tested plant extracts and the after 72 h exposure in HepG2 cells.

Scientific Name	Cytotoxicity Capacity		Antioxidant Capacity	
	IC ₅₀ /ED ₅₀ * (µg/ml)		FRAP (µM/L)	DPPH IC ₅₀ (µg/ml)
<i>Artemisia dracunculus</i>	200>		1508.90 ± 8.26	207.85 ± 3.1
<i>Ferula asafoetida</i>	67.3		2629.37 ± 8.34	45.40 ± 0.09
<i>Origanum vulgare</i>	164.3		1670.75 ± 18.99	49.59 ± 0.41
<i>Pterocarya fraxinifolia</i>	107.6		1915.22 ± 13.07	163.02 ± 0.59
<i>Rosmarinus officinalis</i>	200>		2754.07 ± 7.44	39.82 ± 1.81
<i>Valeriana officinalis</i>	20.9*		561.14 ± 2.38	371.77 ± 2.79

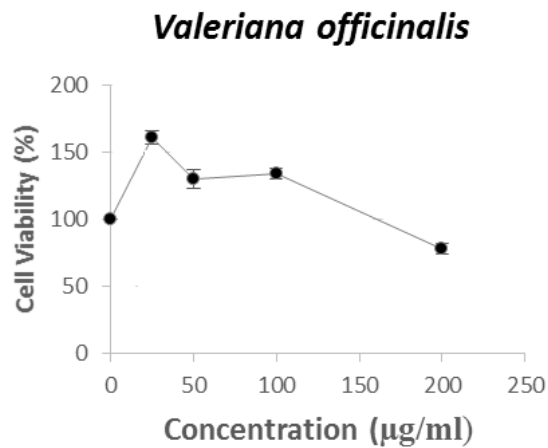
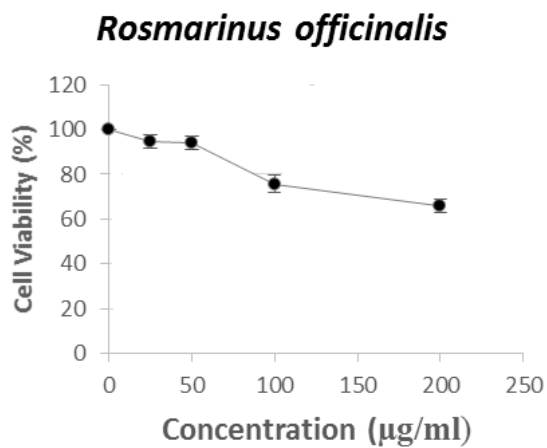
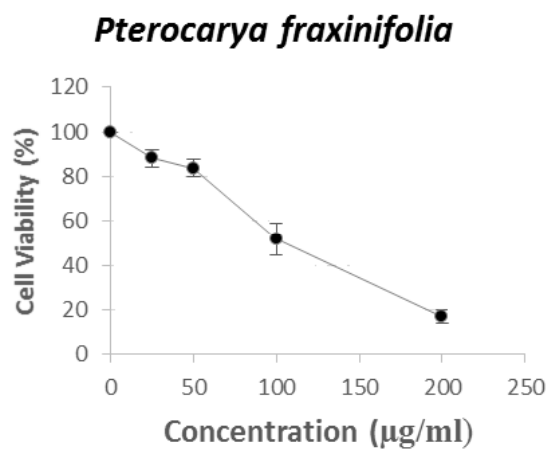
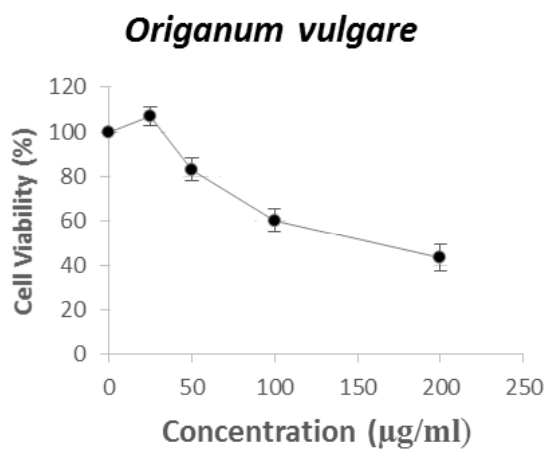
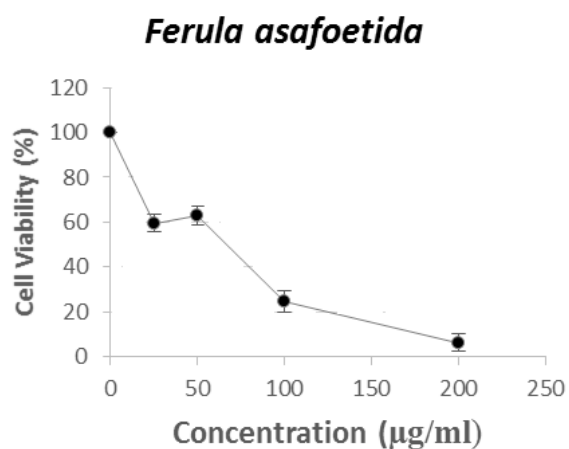
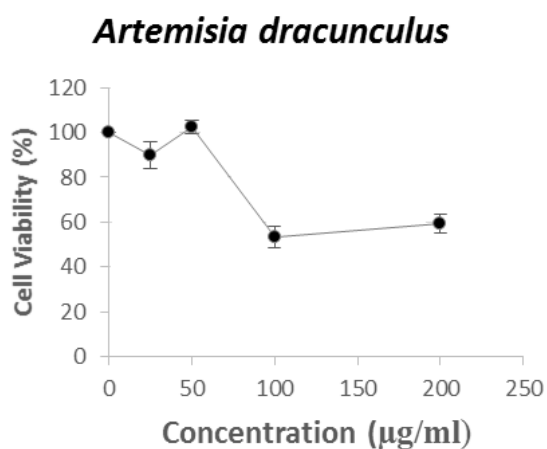


Figure 2. Correlation between DPPH and FRAP assays ($R^2= 0.68$).

DISCUSSION

Boosting antioxidant-related defenses is a key prevention strategy especially in high-risk populations like people suffering from cancer, hepatopathies and cardiovascular disorders [12]. The extensive use of synthetic antioxidants in multiple industries has raised concerns about human health and generating a trend toward the use of natural antioxidants [6]. Rich sources of natural antioxidants including grains, fruits, and vegetable in diet can decrease the level of oxidative damages significantly [13]. The notable characteristics of plant secondary metabolites e.g. antimicrobial, antifungal and antioxidant aspects make them increasingly applicable in many industries e.g. nutritional and pharmacological professions [14]. Although natural products are the basis of traditional medicine, being natural does not necessarily make a product safe. Accordingly, it is required to study the toxicity, risk assessment and the outcomes following administration of such plants in vitro and in vivo [2]

Due to the numerous hepatotoxic effects of phytochemical compounds, human hepatoma cells (HepG2 cell line) have been applied for this research. This cell line illustrates many metabolizing characteristics of hepatocytes extensively used in studies on kinetic, monitoring absorption, toxicity and effectiveness of various types of herb extracts [4].

The technique of plant extraction can affect the extraction yield, the percentage, and type of chemical composition in extract [8]. As regards to the possibility of toxicity of chemical solvents, the filtration and concentration processes of extracts should be performed accurately [15]. The structure of phenolic substance is often polar in character, but in some cases, it can be solved in low polar solvents by attachment of nonpolar groups. Thus, many researchers have used hydroethanolic solvents to achieve the highest extraction yield of polyphenolic compounds, flavonoids, and antioxidants [8].

The hydroalcoholic extract yields for leaves and stems of *O. vulgar* and for aerial parts of *R. officinalis* were reported 18.3% and 26.3% on dry matter basis, respectively [16]. In other studies, the yield of alcoholic extract of *R. officinalis* for aerial parts was 35% and for leaves was 6.1% [17, 18]. Meanwhile, in the present study, different values were proposed for these two species. The reported extraction yield of *Ferula gummosa* hydroalcoholic extract (11%) is almost same with *F. asafoetida* (11.6%) of the preset study [19]. The extraction

yield of *P. Fraxinifolia* leaves was obtained 23% by percolation metho [20]. The extraction yields of *V. officinalis* roots as 14% (with water solvent) and 3% (with petroleum solvents) [21]. Moreover, the yield of extraction for essential oil of *Russian tarragon* was 0.25%-2% [22]. A meaningful comparison of the yields of extraction is not possible maybe because of the varied extraction methods, type of solvents, the composition of the plant, part of the plants, type of soil, agriculture and climate conditions.

In our study, the antioxidant capacities are variable between the hydroalcoholic extracts of all plants. According to the line graph derived from the logarithms of the data, there was similarity in trends of both DPPH and FRAP results (except for *O. vulgura* and *P. fraxinifolia*). *R. officinalis* and *F. asafoetida* extracts had the highest antioxidant levels in their composition compared with other tested plants. The antioxidant capacity is directly associated with the phenolic compounds which mostly distributed in almost all parts of plants [7].

For instances, in *R. officinalis*, rosmarinic acid derived from caffeoyl has the highest level of accumulation in plant and carnosic acid is the highest phenolic compounds exist in leaves with the best antioxidant activity among other phenolic compounds of the plant [23]. A range of other phenolic compounds like carnosol, rosmanol, isorosmanol, caffeic acid and methyl carnosate are also present in *R. officinalis* [23]. There is no report regarding the toxic effects of *R. officinalis* extract on HepG2 cells and even, in some cases, its hepatoprotective effect against the hepatotoxic agents such as carbon tetrachloride was reported in rats [24]. The toxic effect of the *R. officinalis* extract may be induced in doses higher than the effective doses in HepG2 cells. Therefore, the cytotoxic effects observed following treatment with high doses ($IC_{50} > 200$) is not notable and may be resulted from osmotic pressure and damages to the cell membrane.

Since the free radical reduction mechanism seemed to be related to the presence of hydroxyl groups in the structure of antioxidant molecules, the appropriate antioxidant activity of polar extract components can be because of the presence of phenolic/ non-phenolic hydroxyl groups [5]. The antioxidant capacity of *F. asafoetida* extract can be because of phenolic compounds in *Ferula* sp. and antioxidants such as ferulic acid and umbelliferone [25]. Besides, most of the antimicrobial, anti-inflammatory and cytotoxic activities are attributed to terpenoid coumarins in *F. asafoetida* [26]. Even

with the high capacity in scavenging free radicals, the potential of various fractions of *Ferula* sp. to induce hepatotoxicity in Hep3B and HepG2 cell lines was approved (IC50 range: 22.3-105.3 µg/ml) [26]. There is much research regarding the hepatotoxicity effects on Wistar rats following chronic to exposure to *F. asafoetida* and minor changes such as thrombosis and leukocytosis in mice because of oral administration [27, 28]. The toxicity of *F. asafoetida* can be attributed to control, a sesquiterpene coumarin, which it is cytotoxic has been previously confirmed.

The main antioxidant capacity of the leaves of *P. fraxinifolia* linked with the high content of phenolic compounds especially sesquiterpenes and monoterpenes [20]. High contents of rosmarinic acid methyl ester and hydroxycinnamic acid in the compositions of *Origanum* sp. makes this plant useful as food additives and skin color correctors [29]. Based on National Cancer Institute Standard (IC50>100), *P. fraxinifolia* and *O. vulgare* extracts can be considered as "non-toxic" to cells [30]. According to the published reports about the cytotoxicity of *O. vulgare* extract and also the minor difference between IC50 (107.6 µg/ml) and the toxicity threshold level for *P. fraxinifolia* obtained in this study, extreme caution should be taken before using these plants as natural antioxidants [31, 32].

The antioxidant capacity of *A. dracunculus* extract can be referred to various phenolic compounds. For example, sinapic acid is reported as the most abundant phenolic compounds of *A. dracunculus*[33]. Evaluating the toxicity of *A. dracunculus* total extract indicated that in order to induce toxicity in more than half of the cells, doses higher than 200 µ/ml is required. Consider *A. dracunculus* can as a non-hepatotoxic extract. Many researchers in have approved low toxicity of hydroalcoholic extract of *V. officinalis* in vitro and so far, no documented research is available regarding the genotoxic and epigenetic effects in in-vivo studies [34, 35]. In the present study, the growth promoting effect on HepG2 cells and low antioxidant capacity let the *V. officinals* not to be noticed as the first choice of an antioxidant supplement and should not be consumed incautiously.

CONCLUSION

The consumption rate of the medicinal plants is increasing due to the people imagination about their safety. There are diverse references to the side effects and life-threatening aspects of some

traditional plants species. Hence, evaluation the toxicity and safety of cosmetics, medicinal and food supplements containing plant additive prior to marketing is of main importance. Although the tested plants had different levels of antioxidant potential, *R. officinalis* extract had the greatest antioxidant capacity and can be safe for hepatocytes. Screening the herbal extracts in terms of their safety, effectiveness, and purification of their active ingredients can be an important step in phytomedicine.

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