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Original article

Phytochemical Constituents and Biological Activities of *Salvia suffruticosa*

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Abstract

Background and objectives: *Salvia suffruticosa* is a perennial plant from Lamiaceae family. Many *Salvia* species have been employed as medicinal plants; despite the medicinal potentials of *S. suffruticosa*, there is limited studies regarding its phytochemical profile or biological properties. The aim of the present study was to investigate the chemical constituents of the essential oil and extract of the plant and evaluate its biological activities. **Methods:** Essential oil from the aerial parts of the plant was extracted by hydrodistillation and analyzed using gas chromatography/mass spectroscopy. Isolation of compounds from methanol and petroleum ether fractions was achieved by using column chromatography with different stationary phases. The structures of the isolated compounds were elucidated by NMR techniques. Cytotoxicity potentials were evaluated using MTT assay and acridine orange/ethidium bromide staining method. Antioxidant activity was assessed by DPPH method. **Results:** Hydrocarbon sesquiterpenes were identified as the predominant components of the oil, with β-caryophyllene (27.35%), bicyclogermacrene (22.15%), germacrene-D (9.49%) and β-farnesene (9.08%) as the major constituents. Phytochemical analysis of the extract resulted in isolation of lupeol (**1**), β-sitosterol (**2**), stigmasterol (**3**), caffeic acid (**4**) and 1-feruloyl-β-D-glucopyranose (**5**). Among the tested samples, lupeol demonstrated the most potent inhibitory activity toward breast cancer cell lines including MCF-7, T-47D and MDA-MB-231 with IC_{50} values equal to 33.38±2.6, 36.70±3.1 and 23.66±1.4 μg/mL, respectively; caffeic acid with IC_{50} value of 12.1±1.2 μg/mL showed the most potent radical scavenging activity. **Conclusion:** The results of this study suggested *S. suffruticosa* as a promising source of bioactive compounds useful in prevention and treatment of cancer.

Keywords: antioxidant; GC-MS; Lamiaceae; MTT assay; *Salvia suffruticosa*

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Introduction

The genus *Salvia* comprises about 900 species distributed all over the world and is one of the major genera belonging to Lamiaceae family [1]. *Salvia* species have been used since ancient times for different purposes including perfumery industry and for culinary and therapeutic applications. These plants have been traditionally employed for their cerebrovascular and cardiac benefits, anti-inflammatory, antirheumatic, antimicrobial, tranquilizing, anticancer, antidiabetic, hepatoprotective and many other medicinal properties [2-4]. Numerous phytochemical and biological studies have been carried out on a number of *Salvia* species.

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Flavonoids and terpenoids have been found to be the main secondary metabolites. Triterpenoids are the major terpenoids in aerial parts, while diterpenoids are dominant in the roots [4].

Salvia suffruticosa Montbret & Aucher ex Benth., as its name implies, is a suffruticose and perennial plant which grows natively in the west and north-west of Iran. It also grows in Turkey, Armenia, Iraq and Syria [5]. *Salvia suffruticosa* aerial parts are used in some salads and foods in Turkey [6]. To the extent of our knowledge, phytochemical studies on this species have been confined to essential oil analysis and camphor (48.5%), 1,8-cineol (18.6%) and camphene (7.9%) have been reported as the main components. Antibacterial potential of the essential oil has also been assessed on some Gram-positive and Gram-negative bacteria which has shown antibacterial activity against *Staphylococcus saprophyticus*, *Salmonella typhi*, *Shigella flexneri* and *Escherichia coli* with no effects on *Staphylococcus aureus* and *Staphylococcus epidermidis* [7]. In a previous study, dichloromethane and ethyl acetate extracts of *S. suffruticosa* at concentrations up to 100 µg/mL have shown no inhibitory activity on acetylcholinesterase enzyme [8].

In the present study, we have reported the isolation and structure elucidation of five compounds as well as essential oil composition, cytotoxic activity and antioxidant properties of the *S. suffruticosa* aerial parts have been evaluated.

Material and Methods

Plant material

The aerial parts of *S. suffruticosa* were collected at full flowering stage in May 2013 from its habitat in southern slopes of Mishu-dagh mountains located in East-Azerbaijan (northwest of Iran). The plant was authenticated at the Central Herbarium of Tehran University of Medical Sciences and a voucher specimen was deposited under the number TUH 44274.

Extraction and fractionation

Powdered air-dried aerial parts of the plant (1 kg) were macerated with methanol 80% v/v (5×4 L) at room temperature. Evaporation of the solvent under reduced pressure at 40 °C provided the total extract. The obtained dried extract was fractionated using solvents of increasing polarity namely petroleum ether, chloroform and methanol [9].

Isolation and purification of compounds

Petroleum ether fraction (40 g), with the highest cytotoxic activity among the fractions, was subjected to column chromatography over silica gel (35-70 mesh, Merck). Mixtures of hexane and ethyl acetate (100:0 to 0:100) were used stepwise as eluents to give seventeen fractions (A-Q). The fraction D (1 g) was washed once with n-hexane, and then the precipitates were moved on silica gel column (230-400 mesh, Merck) and eluted by n-hexane-ethyl acetate (8:2) to afford six fractions (D1-D6). Purification of compound 1 (130 mg) was achieved via chromatography of fraction D2 on a silica gel column (230-400 mesh) eluted with n-hexane-ethyl acetate (9:1). Fraction G (750 mg) was moved on a silica gel column (230-400 mesh) eluting with chloroform-ethyl acetate (96:4) to give fractions G1-G8. A mixture of compounds 2 and 3 (34 mg) was obtained from the fraction G5 by chromatography on a silica gel column (230-400 mesh) washed by n-hexane-ethyl acetate (85:15). The methanol fraction, having the highest free radical scavenging activity among the fractions, was chromatographed on a Sephadex LH-20 column by methanol 80% as the eluent to give six fractions. Chromatography of fraction M4 (1.2 g) on a reversed phase (RP-18) column (230-400 mesh, fully endcapped, Fluka) using a step gradient mixture of acetonitrile-water (10:90 → 40:60) yielded five fractions (M4a-4e). Fraction M4a (25.8 mg) was purified on a Sephadex LH-20 column (methanol 80%) to get compound 4 (3.7 mg). Repeated chromatography of fraction M5 (38 mg) on Sephadex LH-20 by methanol 80% resulted in isolation of compound 5 (2.2 mg).

Identification of compounds

The progress of separation was monitored by thin layer chromatography (Pre-coated Silica gel GF254 plates, Merck, Germany). The TLC plates were visualized under UV lights (254 and 366 nm) or by spraying anisaldehyde/sulfuric acid reagent followed by heating.

The structures of the isolated compounds were elucidated by ^{1}H-NMR and ^{13}C-NMR spectral analysis and also by comparison with data previously reported in the literature. The NMR spectra were recorded on a Bruker DRX-500

instrument (500 MHz for 1H and 125 MHz for 13C) using tetramethylsilane (TMS) as the internal standard and CDCl3 and DMSO-d6 as solvents.

Essential oil extraction

100 g of dried powdered aerial parts of *S. suffruticosa* was hydrodistilled for 3 hours using a Clevenger type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and kept in refrigerator until analysis.

GC-MS and GC-FID analyses

Gas chromatography was performed with a Hewlett-Packard 6890 gas chromatograph equipped with a HP-5MS capillary column (30 m×0.25 mm id, 0.25 μm film thickness) coupled to a Hewlett-Packard model 5973 mass selective detector operated in the electron impact mode at 70 eV. The initial oven temperature was 40 ºC and was raised to 250 ºC at constant velocity of 3º C/min. The GC injector temperature was 250 ºC and 1 μL of the diluted essential oil was injected with a split ratio of 1:90. The flow rate of helium, as carrier gas, was 1 mL/min. Identification of components was based on GC retention indices and computerized comparison of their mass spectra with those in Wiley7n.l library as well as collation of the mass spectra with those reported in the literature [10]. The essential oil was also analyzed for relative quantification of components using an Agilent 6890 gas chromatograph coupled to a FID detector. The FID detector temperature set at 290 ºC and the operation was conducted under the same condition as described for GC-MS analysis.

Cell lines and cell culture

Human breast cancer cell lines including MCF-7, T-47D and MDA-MB-231 were obtained from the National Cell Bank of Iran (NCBI). The cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 U/mL penicillin and incubated at 37 ºC in air/$_{CO2}$ (95:5 v/v) atmosphere.

In vitro cytotoxicity assay

Cytotoxicity potentials of samples from aerial parts of *S. suffruticosa*, including extracts, fractions and isolated compounds from the most effective fraction, were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [11]. In brief, 195 μL of each three types of breast cancer cell lines (MCF-7, T-47D and MDA-MB-231) was seeded in wells of 96-well microplates at density of 10000 cells/mL and incubated overnight in the above mentioned conditions. Afterward, 5 μL of the sample solutions containing various concentrations of the test samples in DMSO and media was added per well in triplicate and incubated for 48 h (final concentrations for extract and fractions were 20, 100, 250 and 500 μg/mL and for pure compounds were 5, 10, 15, 30, 50 and 65 μg/mL); the final concentration of DMSO was 0.1%. After treatment, the medium was removed and 200 μL fresh medium containing 20 μL MTT (0.5 mg/mL) was added to each well, then returned to the same condition for an additional 4 h. Thereafter, the remaining MTT solution was carefully removed and insoluble formazan crystals were dissolved in 200 μL DMSO and the absorbance of each well was detected by microplate reader at 492 nm. Etoposide was used as the positive control and there were also three control wells in each plate containing cells without test samples and three blank wells containing culture medium with 0.1% DMSO. IC_{50} values, the samples concentration causing 50% cell growth inhibition, were determined by nonlinear regression analysis and expressed in mean±SD compared with the control.

Acridine orange/ethidium bromide (AO/EB) staining assay

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine orange (AO) and ethidium bromide (EB) staining [12]. In brief, MCF-7 cells were cultured in 6-well plates at seeding density of 4×105 cells and then treated with the most potent sample at its IC_{50} concentration for 24 h. After centrifuging and decanting the supernatant, the cells washed with phosphate buffered saline (PBS) followed by staining with 100 μL of a 1:1 mixture of acridine orange and ethidium bromide (100 μg/mL) solution. The cells were immediately analyzed and photographed by a fluorescent microscope (Axioskop 2 plus, Zeiss, Germany). AO permeates all cells and makes the nuclei green when bound to double stranded DNA in living cells, whereas EB is only taken up by cells when the cellular membrane integrity is lost, and

then intercalate into DNA and fluoresce red, so makes the nuclear condensation and fragmentation in apoptotic cells visible.

DPPH free radical-scavenging assay

Free radical-scavenging potentials of samples from *S. suffruticosa*, including extracts, fractions and isolated compounds from the most effective fraction, were evaluated using DPPH assay method [13]. In brief, the sample stock solution (1.0 mg/mL) was diluted to give final concentrations of 500, 250, 100, 50, 20 and 10 µg/mL, in methanol. A total of 1 mL of a freshly prepared 80 µg/mL DPPH in methanol solution was added to 1 mL of each diluted sample solution and were kept in dark place at room temperature and allowed to reaction for 30 minutes. Butylated hydroxytoluene (BHT) was used as the positive control. Absorbance of the solutions was measured at 517 nm on a Cecil CE 7250 spectrophotometer and the IC_{50} was determined.

Results and Discussion

Phytochemical analysis of petroleum ether fraction of *S. suffruticosa* aerial parts resulted in isolation of 3 compounds including pentacyclic triterpenoid lupeol (compound 1) [14] and a mixture of two steroids in proportion of 93:7 including β-sitosterol (compound 2) and stigmasterol (compound 3) [15]. Purification processes on methanol fraction resulted in isolation of two phenolic acids including caffeic acid (compound 4) [16] and ferulic acid glucosyl ester or 1-feruloyl-β-D-glucopyranose (compound 5) [17]. The structures of these compounds have been determined by 1H-NMR and 13C-NMR spectral analysis and confirmed by comparison with those data reported in the literature (figure 1). The essential oil yield was 0.2% (v/w) on dry weight basis. Sixteen compounds, representing 97.6% of the oil, were identified as a result of GC-MS and GC-FID analyses of the essential oil from the aerial parts of *S. suffruticosa* (table 1). The essential oil was rich in hydrocarbon sesquiterpenes (73.21%) with β-caryophyllene (27.35%), bicyclogermacrene (22.15%), germacrene-D (9.49%) and β-farnesene (9.08%) as the main constituents. A review of a previous study on essential oil from the aerial parts of *S. suffruticosa* collected from east of Iran revealed that sesquiterpenes constituted only a small portion of the oil and monoterpenes such as camphor and 1,8-cineole were the dominant compounds. Variations in essential oil composition of a single species have been previously studied in several studies and have shown significant differences in essential oil profile due to existence of chemotypes or variation in climatic and environmental conditions [18-22].

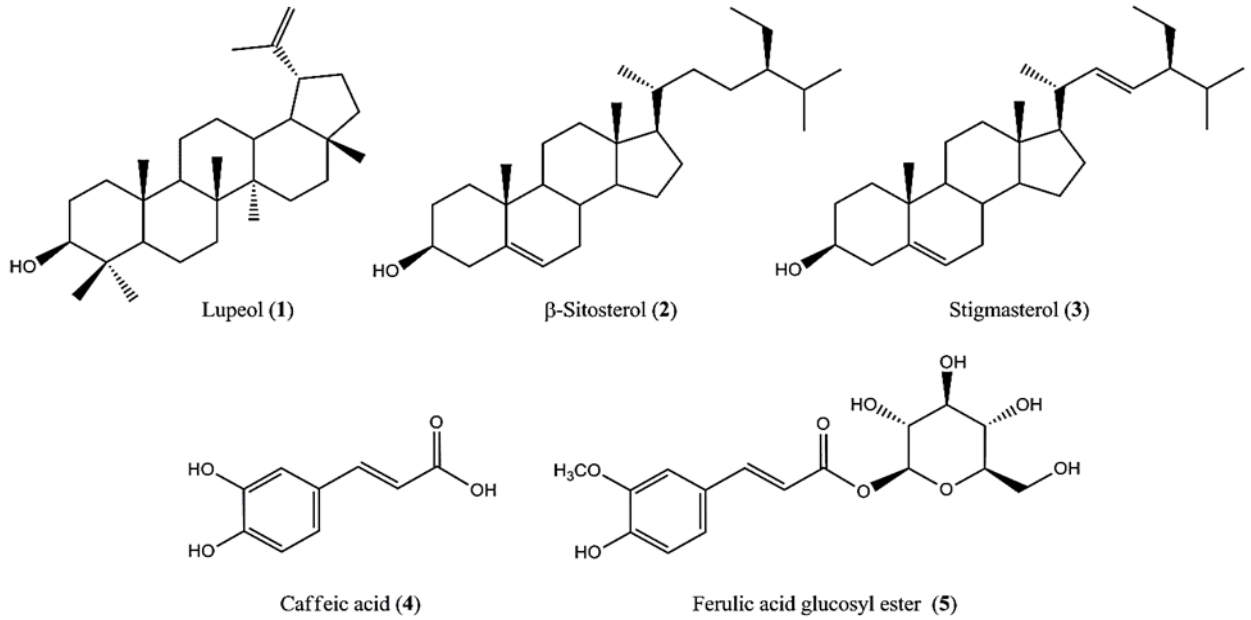

Figure 1. Structures of the isolated compounds from *Salvia suffruticosa* aerial parts

Table 1. Chemical composition of the essential oil from *Salvia suffruticosa* aerial parts

No.	Compounds[a]	RI[b]	%	RI[c]
1	α-Pinene	935	0.75	932
2	Myrcene	991	1.18	988
3	Linalool	1098	1.92	1095
4	Bornyl acetate	1287	1.42	1284
5	β-Bourbonene	1392	1.31	1385
6	β-Caryophyllene	1422	27.35	1417
7	β-Farnesene	1457	9.08	1440
8	Aromadendrene	1466	1.82	1464
9	Germacrene-D	1489	9.49	1484
10	Bicyclogermacrene	1510	22.15	1500
11	Germacrene-B	1562	2.01	1559
12	Spathulenol	1584	3.99	1577
13	Caryophyllene oxide	1591	5.32	1582
14	α-Cadinol	1657	1.62	1652
15	Phytol	1950	2.52	1949
16	Hexadecanoic acid	1965	5.68	1959
	Hydrocarbon monoterpenes		1.93	
	Hydrocarbon sesquiterpenes		73.21	
	Oxygenated monoterpenes		3.34	
	Oxygenated sesquiterpenes		10.93	
	Oxygenated non-terpenes		5.68	
	Diterpenes		2.52	
	Total identified		97.6	

Note: a Compounds listed in order of elution from HP-5MS column, b Retention indices to C8-C24 n-alkanes on HP-5MS column, c Reported retention indices on DB-5 column

Singh et al., have revealed the correlation between essential oil composition of *Ocimum americanum* L. and micronutrient content of soil, temperature and soil moisture [21]. As an example, availability of iron had a considerable influence on aliphatic hydrocarbons biosynthesis in *O. americanum*. The main components of the essential oil from *S. suffruticosa* have been reported among major constituents of oils from other *Salvia* species. For example β-caryophyllene and bicyclogermacrene, two main components identified in our oil sample, have also been reported from many other *Salvia* species such as *S. virgata* (β-caryophyllene 46.6%) [23], *S. aethiopis* (bicyclogermacrene 29.54-41.48%) [24], *S. millitorhiza* (β-caryophyllene 12.2-31.7%) [25], *S. aethiopis* (β-caryophyllene 24.6%), *S. hypoleuca* (β-caryophyllene 22.0% and bicyclogermacrene 15.1%) [26], *S. sharifii* (bicyclogermacrene 15.7%) [27], *S. tomentosa* (β-caryophyllene 11.2%) [28] and *S. triloba* (β-caryophyllene 11.8%) [29].

The results of free radical scavenging and in vitro cytotoxicity assays have been summarized in table 2. As the results show, the petroleum ether fraction was found to be the most effective among other fractions against the three breast cancer cell lines in MTT assay. Among the isolated compounds from this fraction, lupeol showed a marked inhibitory activity against MCF-7, T-47D and MDA-MB-231cell lines with IC_{50} values of 33.38, 36.70 and 23.66 μg/mL respectively, which were comparable to positive control etoposide (IC_{50} values of 25.27, 27.92 and 28.18 μg/mL).

Apoptosis was determined morphologically by staining MCF-7 cells with acridine orange/ethidium bromide after treatment with negative and positive controls and lupeol. Analysis of the AO/EB staining revealed that lupeol reduced cell viability and induced apoptosis in the MCF-7 cell line. Nuclear condensation and fragmentation were evident (figure 2).

The IC_{50} values for free radical (DPPH) scavenging activity of extract and fractions ranged from 64.1 to 232.3 μg/mL. The highest DPPH radical scavenging activity was found in methanol fraction (IC_{50}: 64.1 μg/mL), therefore it was decided to determine free radical scavenging capacity of compounds 4 and 5 purified from this fraction. Caffeic acid with an IC_{50} of 12.1 μg/mL exhibited the most pronounced antioxidant activity, while that of synthetic antioxidant BHT was equal to 18.7 μg/mL. 1-Feruloyl-β-D-glucopyranose (compound 5) exerted a weaker scavenging ability on DPPH with IC_{50} equals to 28.7 μg/mL.

Numerous radical scavengers or antioxidants have proven beneficial in protection against destructive effects of reactive oxygen species thus reducing the risk of many chronic diseases related to oxidative cell damages such as hyperglycemia and diabetes mellitus, cancer, rheumatoid arthritis, cardiovascular diseases, atherosclerosis, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [30-32]. Beside free radical scavenging activity of caffeic acid, its inhibitory effect on some enzymes involved in cancer invasion and metastasis has been shown [33]. Growth inhibitory effects of lupeol have been previously investigated on some cancerous cell lines. Moriarityl et al., have estimated its IC_{50} on Hep-G2, A-431 and H-4IIE cell lines to be 32.86, 43.1 and 331.13 μg/mL, respectively [34].

The cytotoxicity of lupeol on three prostate cancer cell lines, including PrEC, LNCaP, and CWR22Rv1 has been determined in a mechanistic study and has shown considerable cytotoxic effect on LNCaP (IC_{50}=8.96 μg/mL) and CWR22Rv1 (IC_{50}=7.89 μg/mL) [35].

Table 2. DPPH free radical scavenging and cytotoxic activities of the total extract, fractions and isolated compounds from *Salvia suffruticosa* aerial parts

Sample	DPPH assay IC_{50} (µg/mL)	MTT assay, IC_{50} (µg/mL)		
		MCF-7	T4-7D	MDA-MB-231
Total extract	156.2 ± 4.1	>500	137.39±5.3	249.15±6.6
Methanol fraction	64.1 ± 2.8	>500	>500	>500
Chloroform fraction	147.8 ± 6.9	270.72±10.7	216.14±8.5	99.7±7.3
Petroleum ether fraction	232.3 ± 5.7	90.29±5.1	91.39±6.0	54.95±3.8
Lupeol (1)	-	33.38±2.6	36.70±3.1	23.66±1.4
β-sitosterol and Stigmasterol (2,3)	-	>65	>65	>65
Caffeic acid (4)	12.1 ± 1.2	-	-	-
Ferulic acid 7-O-glucoside (5)	28.7 ± 3.1	-	-	-
BHT (Positive control)	18.7 ± 0.8	-	-	-
Etoposide	-	25.27±2.3	27.92±3.1	28.18±3.6

(-): Not evaluated

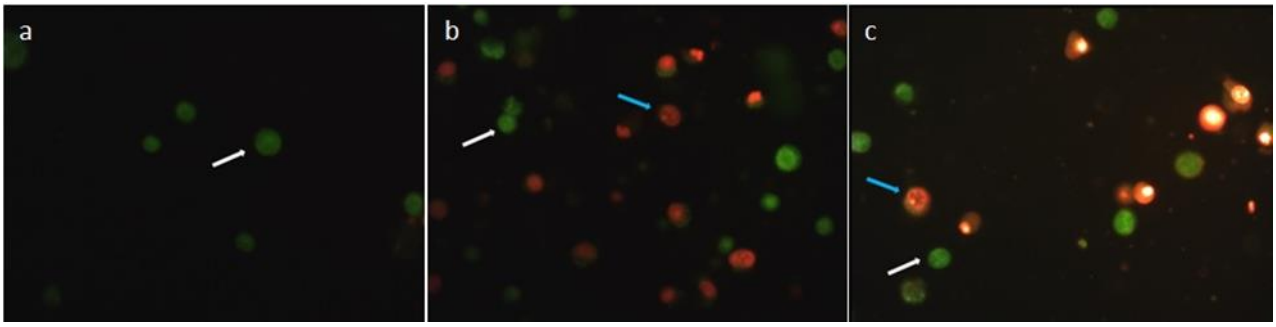

Figure 2. Morphological and nuclear changes of MCF-7 cells treated with DMSO 1%, lupeol and etoposide for 24 h as determined by acridine orange/etidium bromide double staining (400× magnification); a: DMSO 1% as control; b and c: cells treated with IC_{50} concentrations of lupeol and etoposide respectively. White arrow indicates live cells and blue arrow indicates apoptosis

Moreover, its antiproliferative activity on some other cell lines such as melanoma cell lines, i.e., 451-Lu (IC_{50}=16.21 µg/mL) and WM35 (IC_{50}=14.5 µg/mL), promyelocytic leukemia HL60 (IC_{50}=8.49 µg/mL), pancreatic adenocarcinoma As-PC1 (IC_{50}=14.94 µg/mL), colorectal adenocarcinoma DLD-1 (IC_{50}=53.34 µg/mL) and some other cell lines have been proved earlier [36]. Furthermore, several studies have exhibited anti-inflammatory, carcinogenesis inhibition and anti-mutagenic activities of lupeol and lupeol-rich extracts; and a number of reports have indicated no sign of toxicity or adverse effects from lupeol [37]. The results of our study also affirmed that lupeol and lupeol rich fraction showed significant antiproliferative activities toward the breast cancer cell lines. In addition to radical scavenging ability, cytotoxic potentials of *S. suffruticosa* make it useful in both cancer prevention and therapy. This plant could be subjected to further studies for evaluation of other biological potentials and finding more bioactive components.

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Author contributions

Arezoo Rustaie performed the experiments, analyzed data and wrote the manuscript; Abbas Hadjiakhoondi helped supervise the project; Tahmineh Akbarzadeh and Nasrin Samadi supervised biological experiments and data analyses; Maliheh Safavi and Reyhaneh Sabourian participated in cytotoxicity assay; Mahnaz Khanavi supervised the project and revised the manuscript critically.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

AO/EB: Acridine Orange/Ethidium Bromide; BHT: Butylated Hydroxytoluene; $CDCl_3$: Deuterated Chloroform; DMSO: Dimethyl Sulfoxide; DMSO-*d6*: Deuterated Dimethyl Sulfoxide; DPPH: 2,2-diphenyl-1-picrylhydrazyl; eV: Electron Volt; GC-MS: Gas Chromatography-Mass Spectrometry; FBS: Fetal Bovine Serum; FID: Flame Ionization Detector; IC_{50}: half maximal inhibitory concentration; MHz: Megahertz; MTT: 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR: Nuclear Magnetic Resonance; RP: Reversed Phase; TLC: Thin Layer Chromatography; UV: Ultra Violet