



Evaluation of the Cytotoxicity Activity of *Gypsophila ruscifolia* by Bioassay-Guided Fractionation

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Abstract

Background and objectives: The second cause of death in the world and the third cause in Iran is cancer which requires special attention for treatment. The previous reports of *Gypsophila* genus show significant toxic effects on different cancer cell lines. In this study, bioassay-guided fractionation was applied to determine the cytotoxic activity of root and aerial parts extracts and fractions of *Gypsophila ruscifolia*. **Methods:** n-Hexane, chloroform, and methanol: H₂O (8:2) extracts of root and aerial parts were prepared. Inhibition of cell growth determined by MTT assay in MCF-7, HT-29, A-549, and AGO-1522 cell lines. The most effective extract was fractionated by column chromatography. The cytotoxic effect of fractions was evaluated by MTT assay and apoptotic property of the cytotoxic fraction was determined by annexin V/PI assay in MCF-7 cell line. **Results:** The chloroform root extract of *G. ruscifolia* showed cytotoxicity in MCF-7 cells with IC₅₀ value of 111.6 ± 11.78 µg/mL. MTT assay of five of fractions demonstrated that F3 and F4 with IC₅₀ values of 73.09 ± 14.22 and 67.98 ± 15.31 µg/mL on MCF-7 cell line, respectively showed cytotoxic effects. Also, F4 demonstrated apoptotic potential in MCF-7 cell line. **Conclusion:** Considering the results of cytotoxicity and apoptosis studies, isolation and identification of responsible compounds in the chloroform root extract of *Gypsophila ruscifolia* can be useful in cancer research studies.

Keywords: apoptosis; cell line; cell survival; *Gypsophila ruscifolia*; medicine plant

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Introduction

The uncontrolled cell division that is influenced by environmental factors and genetic disorders result in cancer [1]. There are many types of cancer; among them, cancer of lung, stomach,

liver, rectum, and breast have the highest mortality rates. According to the World Health Organization reports, 70% of cancer deaths worldwide are in Africa, Asia, Central, and South

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America [2]. In Iran, due to the changing of people's lifestyles, cancer is a third cause of death. Based on 2017 reports, age-standardized mortality rate was 81.9 per 100000 that will increase each year [3]. After isolation of morphine with painkilling and hypnotic properties from *Papaver somniferum* L in 1803, natural compounds were widely used for different diseases [4]. About 50% of anticancer drugs are comprised of isolated compounds of natural origin and semisynthetic or synthetic compounds with templates of natural products. Taxol, camptothecin, *Vinca* alkaloids, and podophyllotoxins are effective anticancer examples of natural origin [2]. The Caryophyllaceae family with 85 genera and 2,630 species exhibits various biological activities. A large number of plants in this family have shown anticancer properties [5]. *Gypsophila* is one of the largest genera of this family with around 150 species, 35 of which are found in Iran [6]. Triterpenoid saponins are characteristic compounds in the roots of *Gypsophila* [7]; besides, sterols, flavonoids, phenolic acids, oligosaccharides, cyclic peptides, fatty acids, and alkaloids have been also reported from this genus. Studies have demonstrated hypocholesterolemic, anti-inflammatory, antiviral, alpha-glucosidase inhibitory, immunomodulatory, spermicidal and cytotoxic activities as well as being used as adjuvants in vaccines [8-10]. Considering the availability and distribution as well as the previous cytotoxic reports about the genus, *Gypsophila ruscifolia* Boiss. a perennial herbaceous plant growing in East and West Azarbaijan provinces of Iran [11], was selected for cytotoxicity and induction apoptosis evaluations.

Material and Methods

Ethical considerations

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved this research with the code of IR.SBMU.REC.1398.037 on 2019-07-24.

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), (Gibco, New Zealand), RPMI 1640 medium, Penicillin-Streptomycin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Phosphate-buffered saline (PBS), 5-Fluorouracil

and DMSO (Dimethyl Sulfoxide) (Merck, Germany), (Sigma, USA), methanol, hexane, chloroform, ethyl acetate (Dr.Mojallali, Iran) were used in this study. Invitrogen Bioscience annexin V Apoptosis Detection Kit FITC was used to flow cytometry.

Plant material

Gypsophila ruscifolia was collected from Yam village (East Azarbaijan province, Iran) in June 2018 and identified by Dr. Maryam Malekmohammadi, botanist at the Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical sciences, Tehran, Iran. A voucher the specimen was deposited at the Herbarium of TMRC (TMRC-4479).

Extraction

Two hundred g of the air-dried aerial parts and roots of *G. ruscifolia* were separately powdered and extracted by maceration method with hexane, chloroform, and methanol: water (8:2). The extracts were concentrated and dried.

Fractionation

The most effective extract was subjected to column chromatography (230-400 mesh, 20×700 mm) with a gradient system of hexane-ethyl acetate (100:0 →0:100). The fractions evaluated by thin-layer chromatography (TLC) and similar fractions were combined. Finally, five fractions obtained.

Cell lines

MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), A-549 (non-small cell lung carcinoma), and AGO-1522 (human dermal fibroblast) were provided from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran).

Cytotoxicity assay

One of the most common assays to monitor viable cells in multi-well plates is the MTT assay [12]. MTT assay was performed according to the method by Mosmann [13]. In brief, the cells in the exponentially growing phase were seeded into 96 well microplates (5 × 10³ cells/well). After 24 h, they were exposed to variant concentrations (12.5, 25, 50, 100, 200, 300 µg/mL) of extracts and fractions. Finally, MTT solution (0.5 mg/mL in PBS) was added to each well and incubated for 4 h. The formed formazan crystals were dissolved in 200 µL of DMSO and the absorbance was

measured at 570 nm by a microplate reader. 5-Fluorouracil was applied as the positive control.

Annexin V/PI assay

Flow cytometry is a useful tool for the concurrent evaluation of necrosis and apoptosis in population of cells [14]. MCF-7 cells were cultured into the six-well plate 1×10^5 cells/well. The cells were treated with fraction 4, the most cytotoxic fraction of chloroform root extract of *G. ruscifolia*, with the concentrations of 25, 50 and 100 $\mu\text{g/mL}$. After 48h, cells were washed with tissue culture medium and 5 $\mu\text{g/mL}$ of the annexin V conjugate was added. After 45 min at room temperature, again cells were washed and 1 $\mu\text{g/mL}$ of PI was added [15]. DMSO 1% was used as the negative control.

Statistical analyses

Cytotoxic activity of extracts and fractions were evaluated by the concentration-response curve (Graph Pad Prism 7.0) and expressed as IC_{50} value \pm SD. The significant difference between each group and the negative control group (DMSO) was analyzed by Student's *t*-test and ANOVA; p value < 0.05 .

Results and Discussion

MTT assay was applied to measure the cytotoxic activity of n-hexane, chloroform, methanol:water 8:2 extracts of *Gypsophila ruscifolia* against MCF-7, HT-29, A-549, and AGO-1522 cell lines. The n-hexane extract did not dissolve in DMSO;

even with adding 10% PG. The methanol extracts of the root and aerial part did not show toxic effects at tested concentration. There was significant difference between cytotoxic effects of the chloroform root and aerial extract ($p < 0.05$). The cytotoxic behavior of the chloroform root extract against MCF-7, HT-29, A-549, and AGO-1522 cell lines has been exhibited in figure 1. Treatment with chloroform root extract (50, 100, 200 and 300 $\mu\text{g/mL}$) compared to the control, showed significant decrease in cell viability for the MCF-7 cell line ($p < 0.0001$). However, cell viability showed no significant change at concentrations below 50 $\mu\text{g/mL}$. For A-549 cell line at 25 $\mu\text{g/mL}$ and higher concentrations, the extract demonstrated toxicity ($p < 0.05$). As shown in figure 1, chloroform root extract at 100, 200 and 300 $\mu\text{g/mL}$ concentrations on HT-29 a human colon cell line and AGO-1522 as a normal cell line displayed significant decrease in cell viability. The IC_{50} value of the chloroform root extract was calculated as 111.6 ± 11.78 , 179.2 ± 9.59 , 141.7 ± 11.02 , and 165.4 ± 14.29 for MCF-7, HT-29, A-549, and AGO-1522 cell lines, respectively. Regarding more considerable cytotoxic effects of this extract on three human cell lines, the chloroform extract of root was selected to be fractionated. The IC_{50} of the extracts and positive control have been demonstrated in table 1.

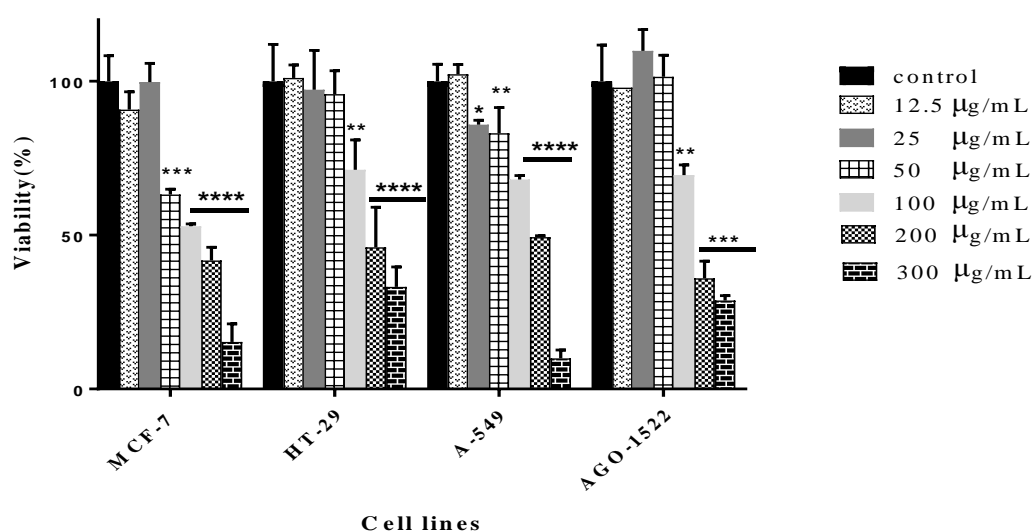


Figure 1. Concentration-dependent manner of chloroform root extract cytotoxicity on MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), A-549 (non-small cell lung carcinoma), and AGO-1522 (human dermal fibroblast) cell lines. Experiments were performed in triplicate. Significant difference between each concentration and control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Table 1. Cytotoxicity of extracts and chloroform fractions of *Gypsophila ruscifolia* extract

Samples	IC ₅₀ (µg/mL)			
	MCF-7	HT-29	A-549	AGO-1522
Aerial parts: n-hexane extract	*	*	*	*
Roots: n-hexane extract	*	*	*	*
Aerial parts: chloroform extract	194.3 ± 11.32	222.2 ± 10.27	**	195.7 ± 18.90
Roots: chloroform extract	111.6 ± 11.41	179.2 ± 9.59	141.7 ± 11.02	165.4 ± 14.29
Aerial parts: 80% methanolic extract	**	**	**	**
Roots: 80% methanolic extract	**	**	**	**
Fraction 1	**	**	**	**
Fraction 2	**	**	**	**
Fraction 3	73.09 ± 14.22	**	**	103.7 ± 10.71
Fraction 4	67.98 ± 15.31	**	184.4 ± 7.80	80.3 ± 11.44
Fraction 5	195.3 ± 8.25	**	108.3 ± 14.7	87.56 ± 12.32
Positive control 5-Fu	3.1 ± 1.02	3.99 ± 1.01	3.1 ± 1.03	14.2 ± 1.02

*in the investigated concentrations, the extracts did dissolve in DMSO.

**in the investigated concentrations, the IC₅₀ could not be determined.

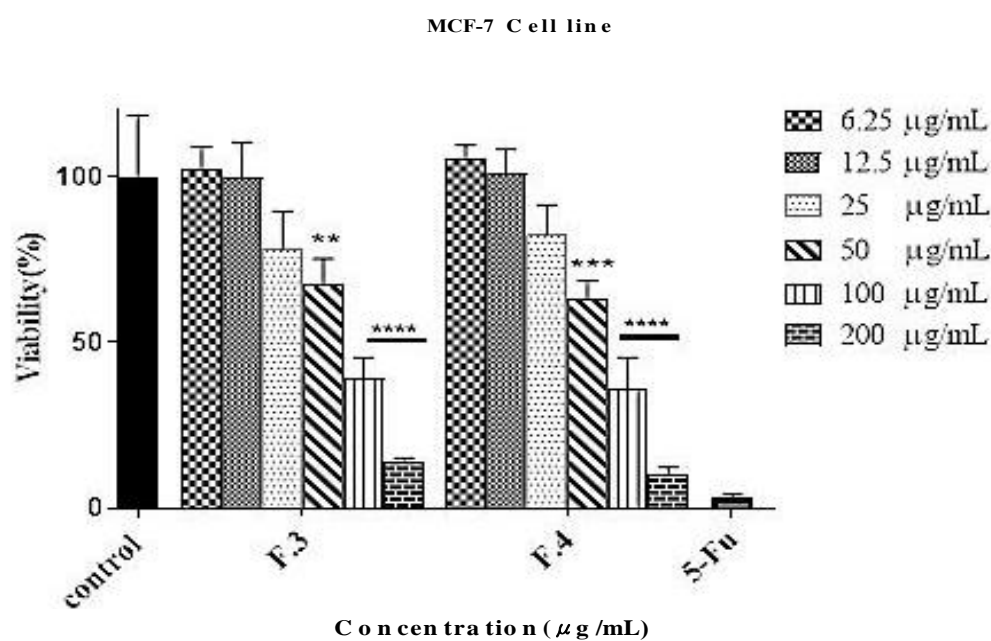
Further investigation was applied to detect the cytotoxicity compounds of the chloroform roots extract. So, five fractions of chloroform extract (6.25, 12.5, 25, 50, 100, 200 µg/mL) were tested again with MTT assay. IC₅₀ values of F 1-5 in table 1 showed that MCF-7 cell line was the most susceptible cell line treated with with F3 and 4; (IC₅₀ of 73.09 ± 14.22 and 67.98 ± 15.31 µg/mL, respectively). F5 exhibited significant inhibition of cell growth on the A-549 cell line; 108.3 ± 14.7 µg/mL (p value < 0.001). None of the fractions displayed effects on HT-29 cell line and there was no significant difference between F 3, 4, 5 on normal cell (AGO-1522). Based on NCI protocols [16], F3, 4, 5 of *G. ruscifolia*, fractions obtained from column chromatography, displayed moderate cytotoxicity on MCF-7 and A-549 cell lines. As shown in figure 2, F3 and F4 at concentrations 50, 100 and 200 µg/mL exhibited inhibition in cell growth in MCF-7 cell line; however, AGO-1522, which is a normal cell, treated with F3 at 50; 100; 200 µg/mL and F4 at 25; 50; 100; 200 µg/mL indicated significant decline in cell viability compared to control.

To detect the underlying basis for the toxic effect of the most effective fraction in breast cancer cell, the percentage of cell apoptosis occurred by fraction 4 was analyzed in MCF-7 cells. The results reveal that F4 in concentrations of 25, 50 and 100 µg/mL against MCF-7 cell line showed increase induced apoptosis in a concentration-dependent manner compared with negative control after 48 h. As shown in figure 3, percentage of cell apoptosis of F4 in concentrations of 25, 50, 100 µg/mL was reported 20.94%, 28.87% and 52.44%,

respectively. Also, there was significant difference between F4 and the control group (p < 0.05).

Earlier evaluation about the cytotoxic effects of *G. ruscifolia* in TMRC supported the result of this study, as the aerial parts of the chloroform extract of this species demonstrated cytotoxic effect against MCF-7 cell line, whereas methanolic extract was not toxic [17,18]. *Gypsophila* is mainly known as a triterpenoid saponins source with considerable cytotoxic effects. These group of compounds are mostly present in polar the fractions such as n- butanol or methanol fractions [7,19-21]. However, bioassay-guided fractionation of *G. oldhamiana* resulted in isolation and identification of triterpenoids from the EtOAc fraction that showed antiangiogenic activities and notable cytotoxicity against lung cancer (H460 cell line) [8]. In a previous research, inhibition of cell growth by the methanol extract of several species of *Gypsophila* against A-549, HT-29, HepG-2, MCF-7, and MDBK cell lines revealed that up to concentration 100 µg/mL they did not effect on MCF-7, A-549, and HT-29 cell lines, only *G. bicolor* showed cytotoxic effect on MDBK cell line with IC₅₀ = 7.82 µg/mL [22]. On the other hand, IC₅₀ values of methanol extract of *G. spheerocephala* against A-549, HT-29 and MCF-7 cell lines were obtained to be than 700 µg/mL [23]. The result of the present study revealed that chloroform root extract and fractions demonstrated toxic effect against MCF-7 cell lines. Mixtures of compounds in botanical extracts can enhance the activity of each other (synergism) or decrease the effects (antagonism) [24].

A



B

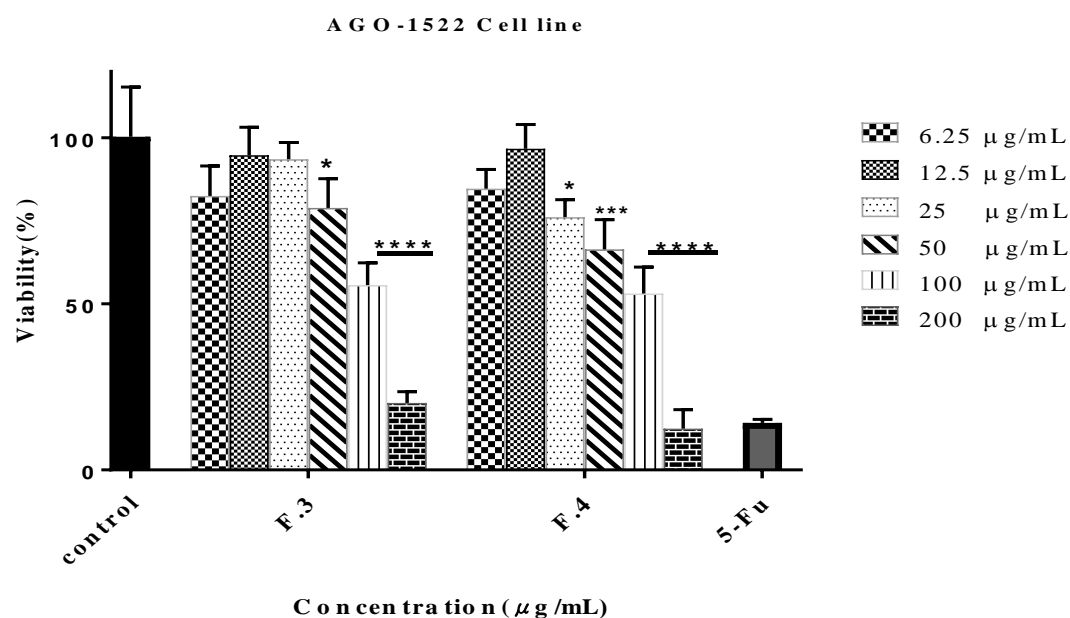


Figure 2. A) Concentration-dependent manner of cytotoxic activities of F3, 4 with the same toxic effect on MCF-7(human breast adenocarcinoma) cell line. B) AGO-1522(human dermal fibroblast) cell line was treatment with F3, 4 in different concentrations. Experiments were performed in triplicate. Significant difference between each concentration and control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 5-Fu was applied as the positive control.

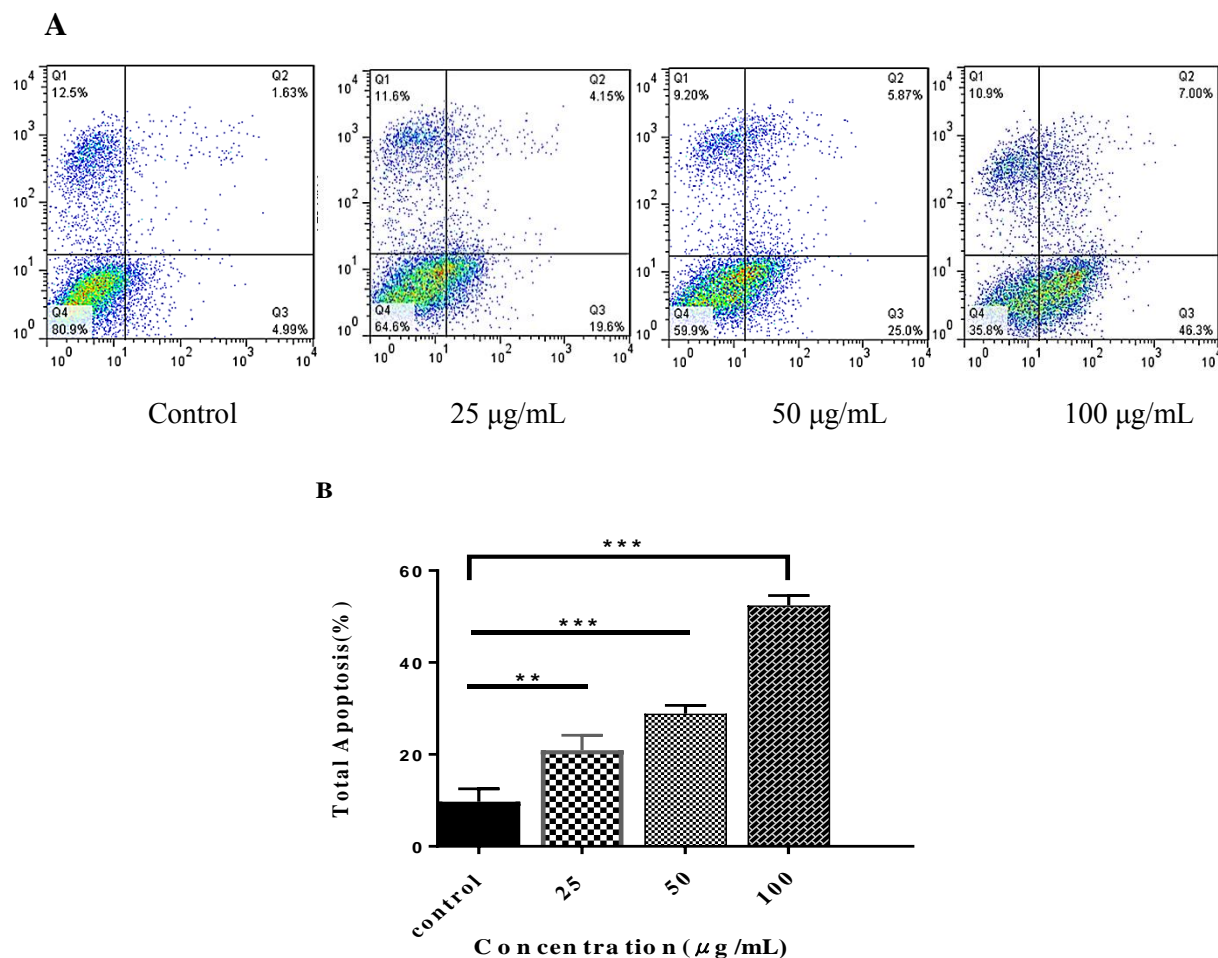


Figure 3. **A)** AnnexinV/PI assay with F4 at 25, 50, 100 µg/mL concentration on MCF-7 (human breast adenocarcinoma) cell line. DMSO was used as the negative control. **B)** Comparison of induced apoptosis of each concentration with negative control. Experiments were performed in triplicate. Significant difference between each concentration and control group: (** $p < 0.01$, *** $p < 0.001$).

Gypsophila trichotoma methanol extract at 100 µg/mL concentration was non-active on NR8383 macrophage [25], however new triterpenoid saponin isolated from the species showed cytotoxicity activity below 100 µg/mL of on leukaemic (NB-4, EOL-1) cell lines [19]. As cytotoxic compound can operate various pathways of cell death, it is essential to find how toxic compound act to determine the side effects of this [14]. Analysis of flow cytometry displayed that fraction 4 might induce apoptosis in MCF-7 cells. Previous reports of *G. oldhamiana* root extract showed apoptotic effects in human hepatoma compared to normal human hepatic cell [26] and *G. trichotoma* root extract induced apoptosis in a macrophage cell line [27]. Considering the inhibitory cell growth results of the chloroform root extract and its fractions on cancer cells, additional phytochemistry

investigations should be applied to identify responsible compounds. Also, the evaluation of the molecular mechanisms of anticancer effects is needed.

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Author contributions

Mahmoud Mosaddegh and Maryam Hamzelo-Moghadam supervised the study; Mohammad Reza Delnavazi designed phytochemistry part; Maryam Malekmohammadi collected and identified the specimen; Roksana Shahrestani

was involved in cell culture experiments; Marzieh Kamali performed practical experiments; Maryam Hamzeloo-Moghadam and Marzieh Kamali analyzed the data and prepared the manuscript.

Declaration of interest

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

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Abbreviations

MCF-7: human breast adenocarcinoma; HT-29: human colon adenocarcinoma; A-549: non-small cell lung carcinoma; AGO-1522: human dermal fibroblast; TLC: thin-layer chromatography; IC₅₀: 50% inhibitory concentration; MTT: 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PG: propylene glycol; NCI: National Cancer Institute