

Evaluating Cytotoxic Potential of the Fruit and the Leaf Extracts of *Sambucus ebulus* (L.) on MCF7 and AGS Cell Lines



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ABSTRACT

Background: Breast and stomach cancers are the most common malignancies in Iranian females and males, respectively. Enriching with phytochemicals that have antioxidant and cytotoxic activities, extracts from dwarf elder (*Sambucus ebulus* L.) holds promises to be used for alternative medication.

Materials and Methods: We investigated the cytotoxic and antiproliferative activities of the leaf and the fruit ethyl-acetate (EA), as well as the methanolic (MeOH) extracts of dwarf elder upon treatment of the MCF-7 and AGS cells. Twenty-seven concentration series ranging from 10 to 2000 µg/mL were administered to the cells, and their growth inhibitory potential was assessed using MTT assay. The potential anticancer compounds of the extracts were quantified applying an improved high-performance liquid chromatography (HPLC).

Results: All extracts showed positive dose-dependent cytotoxic activities on both cell lines. The EA extracts demonstrated more cytotoxicity compared to those of the MeOH ones ($P < 0.0001$). The leaf EA extract showed IC50 values of 65 and 50 µg/mL, while those of the fruit were estimated as 58 and 50 µg/mL on the MCF-7 and AGS cells, respectively. The AGS cell line showed more susceptibility to all extracts tested compared to the MCF-7. MeOH extracts caused only a maximum of ~20% reduction in cell viability at 2000 µg/mL concentration. According to the HPLC analysis, leaf extracts contained phenolic compounds, including p-coumaric acid (0.10 mg per g powder), rutin (0.07 mg per g powder), and quercetin (0.02 mg per g powder).

Conclusion: EA extract of the fruit shows the highest cytotoxicity: reducing 35.3% viability of the AGS cells with 10 µg/mL concentration. It can be considered a potential chemopreventive agent for cancer therapies. However, MeOH extracts with far low cytotoxicity or non-cytotoxic at some concentrations would be an appropriate candidate for preventing tumor growth without affecting neighboring normal cells.

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1. Introduction

With 13776 new cases and 3526 deaths every year, cancer accounts for the second main chronic disease and the third leading cause of mortality in Iran [1]. It is a complex disease influenced by genetic and epigenetic abnormalities, as well as environmental risk factors [1, 2]. Breast and stomach cancers are the most common malignancies of Iranian females and males, respectively. They have heterogeneous geographical distributions in the country [3-7]. Despite significant progress on the molecular and cellular mechanisms of cancer etiology, it still constitutes a serious health problem, especially in developing countries like Iran, where the cancer is mainly attributed to lifestyle changes, obesity, smoking habit, and pesticide exposure [8-10]. Different approaches such as surgery, radiotherapy, and chemotherapy or a combination of them applying to treat cancers are costly and often cause adverse effects. Exploring new therapeutic compounds with targeted efficacy on neoplastic cells and decreased side effects on normal healthy tissues have priority in tumor medications.

Medicinal plants were used in folk medicine to treat various diseases, and they have shown promising results to be used against neoplastic growth [11, 12]. Medicinal plants are usually rich resources for bioactive compounds with antineoplastic properties along with other accompanying compounds. They mostly have no cytotoxic side effects on normal cells [13]. Dwarf elder, also known as elderberries (*Sambucus ebulus* L.), belonged to the family Adoxaceae has a long history in folk medicine. Different parts of *S. ebulus* have been used as the therapeutic and the nutritional regimen in different world regions, from Western Europe to the Middle East [14]. A variety of phytochemical compounds have been detected in different parts of this plant, such as rhizomes, leaves, fruits, and flowers. Biochemical analyses confirmed differential nutritional and therapeutic values of different parts of the plant. Nutritional compounds of the plant are attributed to its minerals, fibers, vitamins, and sugars. *Sambucus* species are also rich with important secondary metabolites such as anthocyanin, flavonoids, polyphenols, triterpenes, tannins, and lectins, most of which have medicinal values, including antioxidant, anti-inflammatory, analgesic, antimicrobial, anticancer, neuroprotective, and antidepressant activities [15-19]. Quercetin, a polyphenolic compound, can inhibit tumor growth through induction of apoptosis in a variety of cancer cell lines [20-22]. It can inhibit the development of gastric, esophageal, and ovarian cancers [23]. 3,3',4',5,7-Pentahydroxyflavone-3-rhamnoglucoside (rutin) is a diglyco-

side of quercetin, which is the predominant flavonol type of flavonoid in foods. Even though rutin and quercetin share several biological activities, the antiproliferative effects of rutin and quercetin on cancer cell lines were significantly different [24]. Chlorogenic acid (CGA) has antioxidant and anticancer properties based on its biological and pharmacological activities [25]. Caffeic acid (3,4-dihydroxy cinnamic acid) is another essential phenolic compound with prooxidant activity that shows anticancer properties similar to proapoptotic compounds [26]. Ethanolic and methanolic (MeOH) extracts of *Sambucus* plants are rich in polyphenolic compounds like anthocyanin and flavonoids capable of scavenging reactive molecules like hydrogen peroxide, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and other free radicals, thus prevent the formation of DNA adducts and likely neoplastic growth [27]. Additionally, these compounds could prevent the teratogenicity of albendazole as studied in a mouse model [28].

Sambucus spp. contains several lectin glycoproteins stored in different parts of the plant. Some lectin proteins known as ebulin toxins have inhibitory activity against protein synthesis and have been isolated from different parts of *S. ebulus*, such as fruits (ebulin f), leaves (ebulin l), and rhizomes (ebulin r1 and r2). *S. ebulus* also contains other lectin isoforms (B-B type) with potential cell-agglutinating activity, like SELld in leaves, SELlm in roots, and SELfd in fruits [29, 18]. The cell agglutination activities of the lectins can potentially be used for the agglutination of the neoplastic cells, especially in liquid tumors like leukemia and lymphoma [30]. Here, we studied cytotoxic and antiproliferative activities of a broad concentration range of the fruit and the leaf ethyl-acetate (EA) and MeOH extracts of *S. ebulus* on AGS and MCF-7 cell lines. Also, high-performance liquid chromatography (HPLC) was used to quantify the secondary metabolites from the extracts with potential anticancer activity.

2. Materials and Methods

Plant materials

Dwarf elder (*Sambucus ebulus* L.) plants were collected from the Southern coastal areas of the Caspian Sea (Sari, Mazandaran Province, Iran) during April-May of 2019. They were authenticated by a pharmacognosy expert at the Pharmacology Department (Mazandaran University of Medical Sciences), and a voucher thereof (No. 16-93) was deposited at the herbarium in Sari School of Pharmacy. Leaves and fruits of the plants were air-dried in the shade and grounded into a fine powder using a

grinder. MeOH and EA extracts were obtained using the maceration method [19, 27]. Briefly, the dried powder of the leaves and the fruits were individually mixed with MeOH and EA solvents (1:1.5 W/V) and incubated at room temperature for 24 h with occasional stirring. The solvents were removed, and the step was repeated three times. The solution was subjected to evaporation in a vacuum rotary evaporator at 35°C and then was placed in a freeze drier for complete solvent removal. The crude powder of the extract was kept at 4°C away from light. An integrative concentration ranges of the plant extracts, including 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1300, 1400, 1500, 1600, 1700, 1800 and 2000 µg/mL were prepared by dissolving crude extracts in RPMI-1640. Cells treated with 0.5% (v/v) Dimethyl sulfoxide (DMSO) was used as solvent and volumetric concentration was considered as control (zero concentration of extract).

Cell lines and culture media

MCF7 and AGS cells (Pasteur institute, Iran) were grown in Roswell Park Memorial Institute (RPMI-1640) culture medium containing 10% (v/v) fetal bovine serum (FBS) 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, US). Culture flasks were maintained at 37°C in 5% CO₂ and 95% humidified atmosphere at a CO₂ incubator. Subcultivation of the cells was organized upon 80% confluency. The culture medium was poured off, and the cells were trypsinized (500 µL of 0.025% trypsin in PBS/ EDTA solution) to detach from the flask. The trypsin was inactivated by the addition of the fresh culture medium. The cells were washed two times with phosphate-buffered saline (PBS) and were collected by centrifugation at 1500 rpm for 15 min (4°C). Cell counting was done using the hemocytometer counter and trypan blue method. Approximately, 1×10⁶ cells were subcultured into a fresh growth medium and were maintained at 37°C under 5% CO₂ and 95% air.

MTT assay

Antiproliferative activity of the extracts were studied using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) colorimetric method. In MTT assay, mitochondrial enzyme reduces soluble MTT into an insoluble color formazan product in viable tumor cells, which may be measured spectrophotometrically. Briefly, 200 µL of the cell suspension (5×10³ cells/mL) were seeded in 96-well plates and incubated for 24 h to adhere to the plate and hereafter subjected to various concentration ranges of the extracts. The cells were incubated with different concentrations of the plant extracts rang-

ing from 10 to 2000 µg/mL for 48 h. Ten µL MTT (5 mg/mL in phosphate buffer solution) was mixed into the wells, and incubation continued for another 4 h in the dark. MTT solution was gently replaced with 150 µL DMSO to dissolve formazan crystals. The optical density of the chromogenic purple formazan was recorded by an ELISA apparatus (Bio-Rad) at 570 nm. IC₅₀ (50% inhibitory concentration) of the extract was estimated using Equation 1:

$$1. \text{Cytotoxicity (\%)} = 100 \times (\text{OD of untreated cells} - \text{OD of treated cells}) / \text{OD of untreated cells}$$

Statistical analysis

Each treatment was scheduled as three technical and two biological controls using a completely randomized design (CRD), and the results were analyzed using 1-way ANOVA (analysis of variance). Statistical analyzes were carried out using SAS Software Ver. 6.12, and P<0.05 was considered the significant level. Additionally, the Quest Graph™ IC₅₀ Calculator was complementarily done to precisely find the points needed to draw best-fitting regression line, particularly around the inflection point [31], using Equation 2:

$$2. Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{X}{\text{IC}_{50}}\right)^{\text{Hill coefficient}}}$$

High-performance liquid chromatography

Flavonoid or phenolic acid contents of the extracts with potential anticancer activities were resolved using an improved HPLC method. The HPLC machine had a solvent delivery system (K- 1001) equipped with a Rheodyne injection valve (20 µL sample loop inserted) and a model K-2600 UV-vis spectrophotometric detector set at 275 nm [24].

3. Results

Cytotoxicity of the leaf and the fruit MeOH, as well as EA extracts of dwarf elder plant ranging from 10-2000 µg/mL, was investigated on MCF-7 and AGS cell lines using MTT assay. The leaf EA extract showed positive dose-dependent cytotoxic activities on both cell lines, with more cytotoxicity on the AGS cell line. A significant decrease in cell viability was predominantly appeared in concentrations between 0 and 100 µg/mL with the IC₅₀ values of 65 and 50 µg/mL for MCF-7 and AGS cell lines, respectively. The least proliferation rates of 22.9% and 7.2 % were detected following treatment of MCF-7 and AGS cell lines with 2000 µg/mL of the extract, respectively. Similarly, the fruit EA extract showed posi-

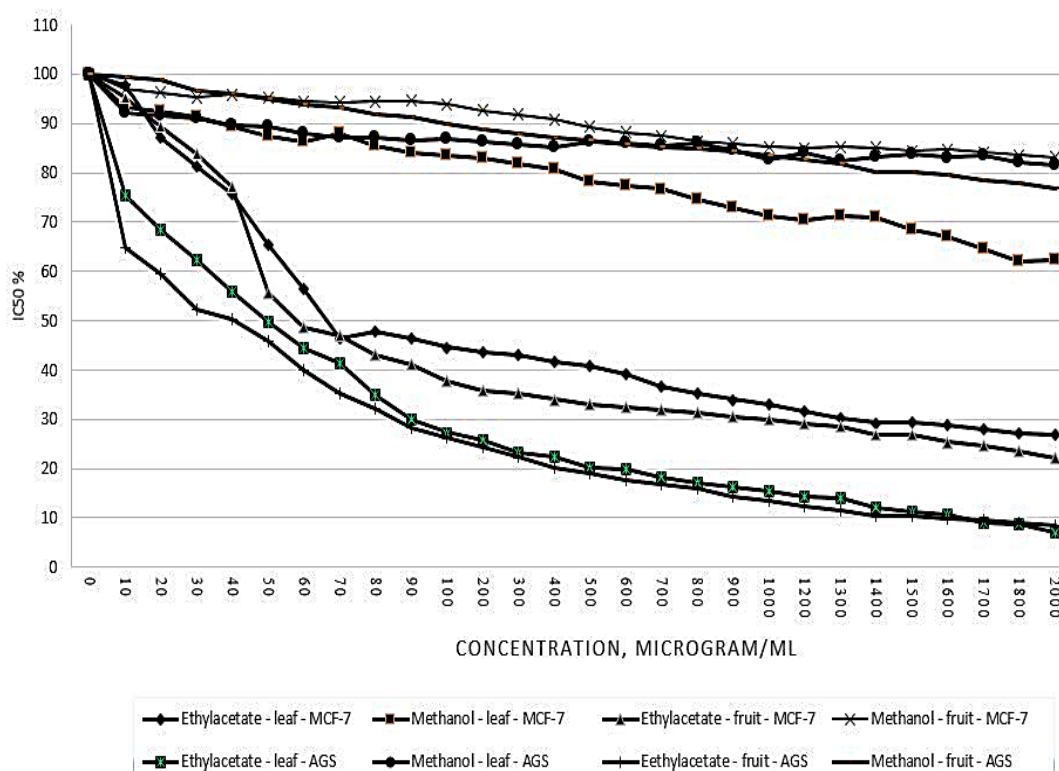


Figure 1. Growth inhibition rate of the AGS and MCF-7 cells following treatment with different concentrations of the leaf and the fruit MeOH and EA extracts of the dwarf elder plant

The x-axis shows the concentration of the extract in $\mu\text{g/mL}$, and the y-axis shows the percentage of IC50. Zero concentration of the extract is considered the control contains RPMI-1640 (Roswell Park Memorial Institute) with 0.5% DMSO. * $P < 0.0001$ compared to control.

tive dose-dependent cytotoxic activities, with greater growth arrest on the AGS cell line. The toxicity graph is composed of an inflection point at $50 \mu\text{g/mL}$, a gentle slope at concentration ranges of $>50 \mu\text{g/mL}$, but a sharp slope at the concentrations of $<50 \mu\text{g/mL}$. The IC50 values of the fruit EA extract were $58 \mu\text{g/mL}$ and $50 \mu\text{g/mL}$ for MCF-7 and AGS cells, respectively. Minimum viability percentages of 22.05% and 8.6% were detected following treatment of the MCF-7 and AGS cell with the $2000 \mu\text{g/mL}$ concentration. The MeOH extract of the fruits had much lower cytotoxicity compared to the EA one. This extract could cause the maximum growth reaches to 20% following treatment with the $2000 \mu\text{g/mL}$ concentration. Comparisons between the leaf extracts following treatment of the MCF-7 cells revealed that the EA extract is more cytotoxic than the MeOH one yielding a cytotoxic difference of 56% as recorded following treatment with $2000 \mu\text{g/mL}$ concentration ($P < 0.0001$). When treated on the AGS cell line, the EA extract was even more cytotoxic than MeOH one, caus-

ing 69.9% more cell death at $2000 \mu\text{g/mL}$ concentration ($P < 0.0001$). Results of the comparative cytotoxic effects of the fruit MeOH and EA extracts were somehow similar to those of the leaf extracts. However, the fruit EA extract was even more cytotoxic than the leaf EA extract. The fruit EA extract caused 56.03% and 64.7% more cell death of the MCF-7 and AGS cell lines, respectively, at $2000 \mu\text{g/mL}$ concentration compared to the fruit MeOH extract ($P < 0.0001$). The $10 \mu\text{g/mL}$ concentration of the fruit EA extract caused 35.3% death of the AGS cells.

Likewise, the comparative analysis showed that the EA extracts of the leaf and the fruit are more cytotoxic than those of the MeOH one, with the leaf EA extract IC50 values of 65 and $50 \mu\text{g/mL}$ and the fruit EA extract IC50 values of 58 and $50 \mu\text{g/mL}$ both on MCF-7 and AGS cells, respectively ($P < 0.0001$) (Table 1). The MeOH extracts of the leaf and the fruit had low cytotoxicity, causing an approximately 20% reduction in cell viability at $2000 \mu\text{g/mL}$ concentration.

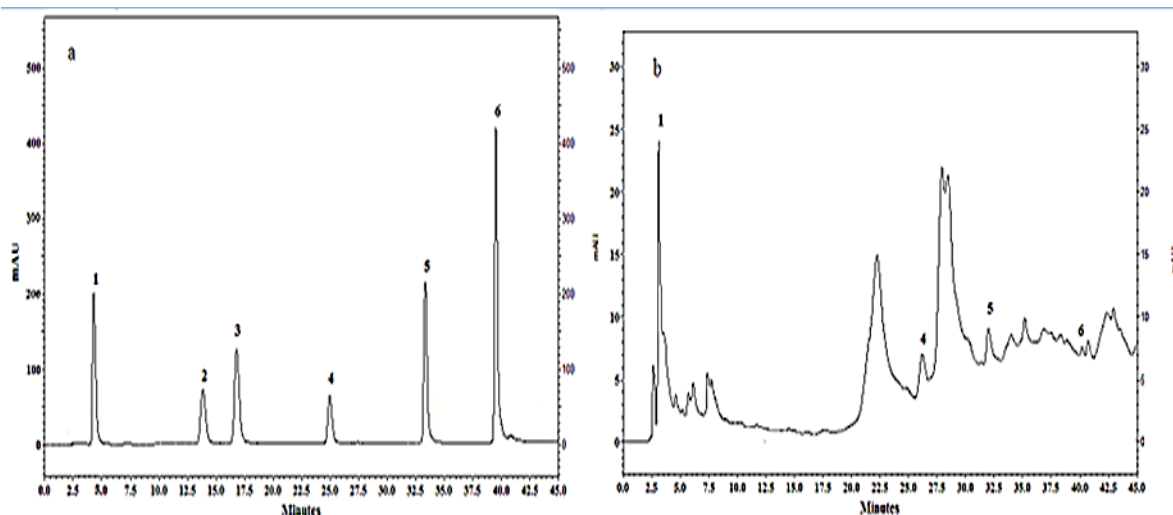



Figure 2. HPLC chromatograms of the phenolic compounds from the standard samples and determined in the extracts at 275 nm. The x-axis represents retention time (minutes) and the y-axis represents mAU (milli-Absorbance Unit). A: Standards; b: Extract; 1, gallic acid; 2, chlorogenic acid; 3, caffeic acid; 4, p-coumaric acid; 5, rutin; 6, quercetin.

The HPLC analysis was conducted at 25°C with a flow rate of 1 mL/min for quantitative separation of the phenolic compounds from the extracts. The retention times and the UV spectral peaks of the authentic standard compounds were intended to detect the contents of the extracts (Figure 2 A, B). The standard samples included gallic acid, caffeic acid, p-coumaric, rutin acid, and quercetin provided from Sigma-Aldrich company. Phenolic content from the sample extracts was collected during the time 0 up to 45 min and resolved considering the peak area of corresponding concentrations of the sample and standard. Three phenolic compounds, including p-coumaric acid (0.10 mg/g of powder), rutin (0.07 mg/g

of powder), and quercetin (0.02 mg/g of powder), were detected in the extracts.

Results of the MTT test were further analyzed using the Quest Graph™ IC50 Calculator¹, and the resulting graph and its line equation were presented in Figure 3.

4. Discussion

Breast and stomach cancers are the leading malignancies in Iranian females and males, respectively. Epidemiological studies show that the incidence and mortality rates of these two cancers are increasing in Iran, specifically

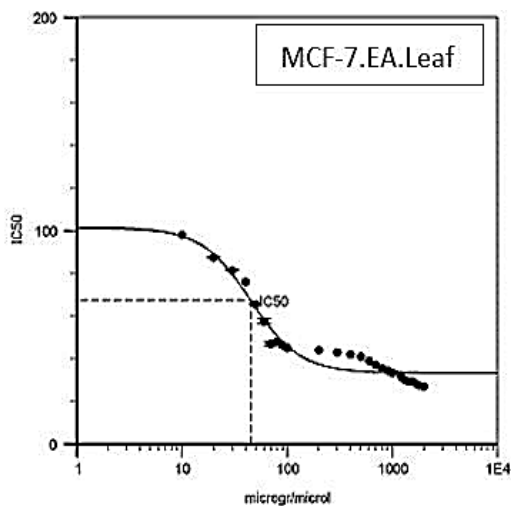
1. <https://www.aatbio.com/tools/ic50-calculator>

Table 1. Statistical analysis of the cytotoxicity of the leaf and the fruit ethyl-acetate and methanolic extracts of *Sambucus ebulus* L. on MCF7 and AGS cell line

Extracts	Mean Square	F Ratio	P*
Leaf ethyl-acetate extract on MCF-7	1475.943	913.745	<0.0001
Leaf ethyl-acetate extract on AGS	1778.138	624.064	<0.0001
Leaf methanol extract on MCF-7	128.155	53.070	<0.0001
Leaf methanol extract on AGS	139.409	514.287	<0.0001
Fruit ethyl-acetate extract on MCF-7	1615.202	700.440	<0.0001
Fruit ethyl-acetate extract on AGS	1403.948	1123.881	<0.0001
Fruit methanol extract on MCF-7	130.351	464.208	<0.0001
Fruit methanol extract on AGS	137.982	580.539	<0.0001

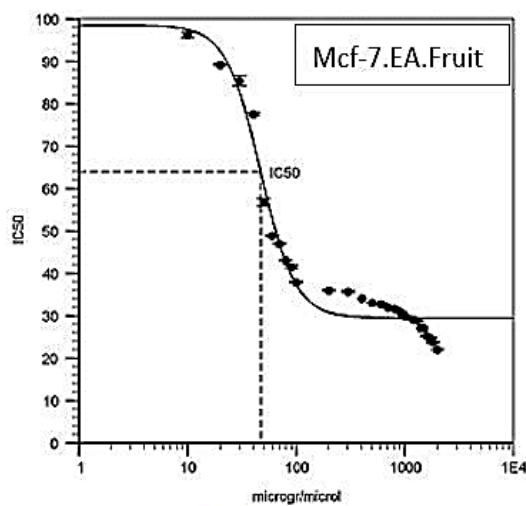
*P<0.05 is considered statistically significant.





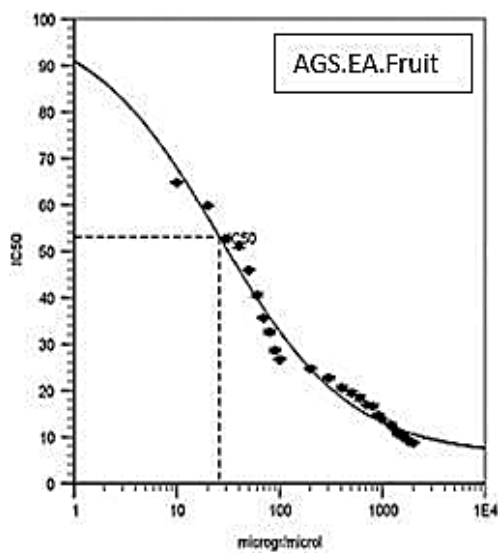
IC₅₀ = 47.208

$$y = 29.431 + \frac{(98.440 - 29.431)}{1 + \left(\frac{X}{47.208}\right)^{2.719}}$$



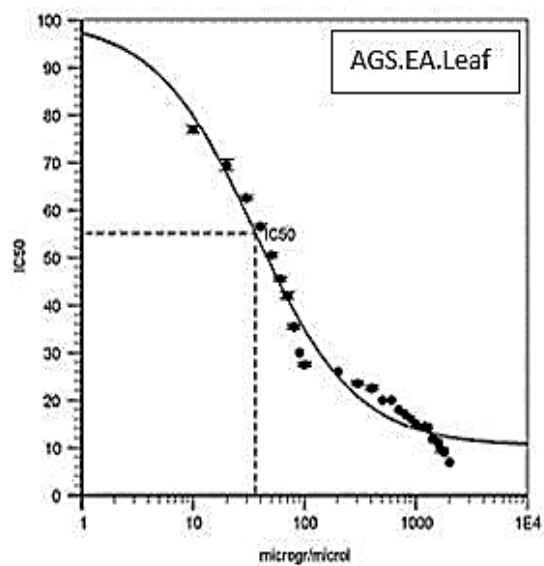
IC₅₀ = 45.098

$$y = 33.221 + \frac{(101.314 - 33.221)}{1 + \left(\frac{X}{45.098}\right)^{1.848}}$$



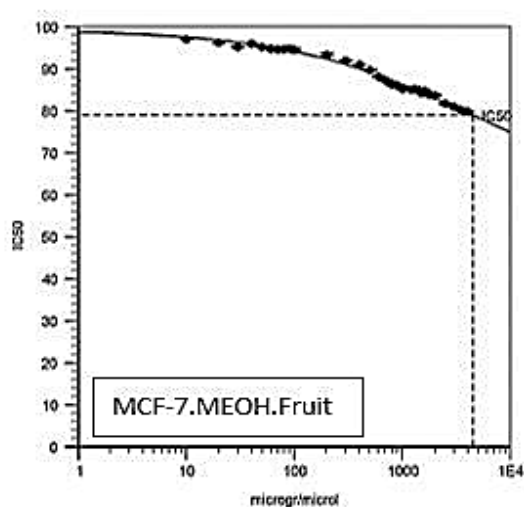
IC₅₀ = 26.159

$$y = 6.019 + \frac{(99.930 - 6.019)}{1 + \left(\frac{X}{26.159}\right)^{0.686}}$$



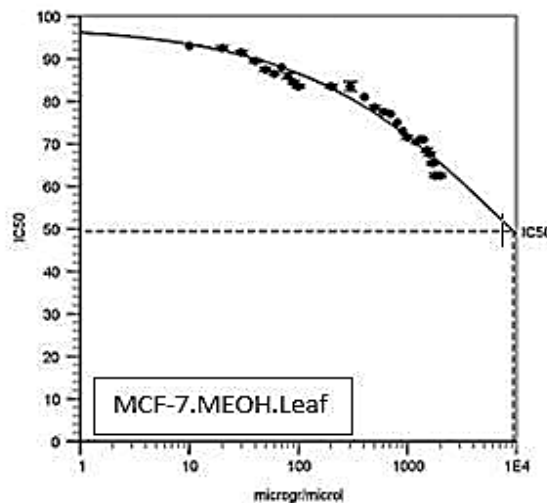
IC₅₀ = 36.345

$$y = 10.354 + \frac{(99.930 - 10.354)}{1 + \left(\frac{X}{36.345}\right)^{0.959}}$$



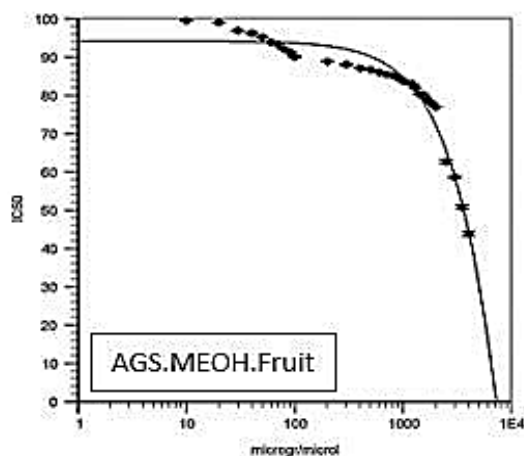
IC₅₀ = 4536.220

$$y = 58.493 + \frac{(99.310 - 58.493)}{1 + \left(\frac{X}{4536.220}\right)^{0.500}}$$



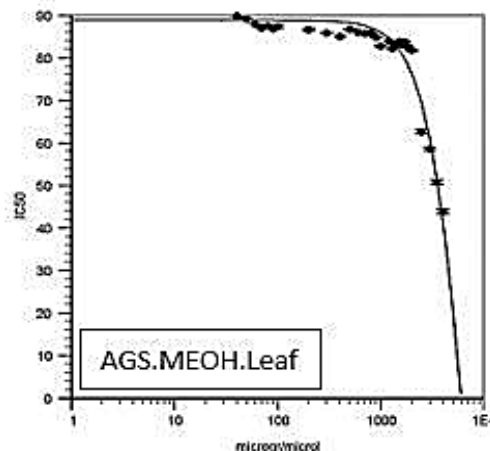
IC₅₀ = 9314.467

$$y = 1.247 + \frac{(97.700 - 1.247)}{1 + \left(\frac{X}{9314.467}\right)^{0.448}}$$



IC₅₀ = 7297.321

$$y = -132.617 + \frac{(88.891 + 132.617)}{1 + \left(\frac{X}{7297.321}\right)^{2.178}}$$



IC₅₀ = 24988.840

$$y = -440.933 + \frac{(94.142 + 440.933)}{1 + \left(\frac{X}{24988.840}\right)^{1.249}}$$

Figure 3. The results of the Quest Graph™ IC50 Calculator show the regression diagram of the concentration of each extract and the percentage of IC50 and line equation

The x-axis shows the concentration of the extract in micrograms per milliliter, and the y-axis shows the percentage of IC50.

cally in the northern and the northwestern parts [1, 4-6, 25]. Preliminary studies conducted by different research groups show that an increase of the proven risk factors such as lifestyle and environmental xenobiotics like pesticide exposure might be involved in tumorigenesis mainly through epigenetic modifications of essential genes regulating cell homeostasis [2, 10, 32, 33]. Dwarf elder contains antioxidant phytochemicals helping to stabilize cell homeostasis. Here, we report cytotoxicity of an integrative concentration range (10- 2000 $\mu\text{g/mL}$) of the fruit and the leaf EA and MeOH extracts of *S. ebulus* on MCF-7 and AGS cells. The leaf EA extract showed IC₅₀ of 65 and 50 $\mu\text{g/mL}$ for MCF7 and AGS cells, respectively. However, the MeOH extract of the leaves had low cytotoxic activity, causing a maximum growth arrest of 20% with the 2000 $\mu\text{g/mL}$ concentration.

Similarly, the EA extract of the fruits was more cytotoxic compared to its MeOH extract, with IC₅₀ values of 58 and 50 $\mu\text{g/mL}$ on MCF-7 and AGS cells, respectively. The fruit MeOH extract showed low toxicity, causing approximately 20% growth arrest against both MCF7 and AGS cells at the highest concentration. The EA extracts are more cytotoxic than the MeOH extract yielding cytotoxic activity of 56% on MCF-7 cells with the leaf extract and 69.9% on AGS cells with the fruit extract as recorded following treatment with the 2000 $\mu\text{g/mL}$ concentration. In another study, the EA extracts of the fruits showed the highest cytotoxicity, reducing 35.3% cell viability at 10 $\mu\text{g/mL}$ concentration.

Cytotoxicity of the fruit EA extract from *S. ebulus* was investigated on HepG2 and CT26 cancer cell lines and compared with those of the normal CHO and fibroblast cells [33]. IC₅₀ Mean \pm SD values of 97.03 \pm 1.52 and 152.70 \pm 3.36 $\mu\text{g/mL}$ were recorded against HepG2 and CT26 cells, respectively that are greater than those reported for the normal cells. Likewise, the antioxidant and anticarcinogenic activities of the MeOH extracts of flowers, fruits, and leaves of *S. ebulus* were evaluated on mouse fibroblast (L929) and HeLa human cervix adenocarcinoma cell lines [34]. A maximum growth inhibition rate of 33.81% was documented following treatment of HeLa cells with 10 $\mu\text{g/mL}$ of the leaf extract [33]. The cytotoxic effects of 16 medicinal plants extracted with n-hexane, dichloromethane, MeOH, and water were tested against AGS, HT-29, and MDA-MB-435S cells. All extracts showed differential cytotoxic activities, among which the MeOH extract of *Blumea lacera* showed IC₅₀ of 30 and 80 $\mu\text{g/mL}$ on the AGS and breast cancer cells [30, 33, 34].

Similar to the present research results, the AGS cells had more susceptibility than the breast cancer cells. Polar

solvents show the best antioxidant activity for trapping free radicals, DPPH, and nitric oxide, as well as in reduction tests. They allow preferential extraction of phenolic compounds. By contrast, non-polar solvents such as chloroform, n-butanol, and EA are suitable for the extraction of flavonoids. EA is an adequate solvent for the extraction of higher amounts of flavonoids either solely in a single step or in successive steps together with other solvents [9]. This effect may explain the higher cytotoxic values of the EA extracts as documented in the present research. The main outlook in this project was to search for the concentration range/s which would decrease or stop the proliferation of the cells without killing them. Keeping in mind that, as a side effect in most pharmacotherapy methods, the therapeutic drug would affect both the normal and cancerous cells, treatment with the concentration of the plant extract inhibiting proliferation of the tumor is the most priority in cancer treatment. The MeOH extracts showing low or negative cytotoxicity at most tested concentrations would be the promising compounds to prevent cell proliferation without killing them. However, there are some shortcomings in this research, as the absence of the anticancer drug as positive control and comparing the behavior of the cancer cells with that of the normal one. It is particularly recommended that the effects of the purified phenolic compounds potentially responsible for the anticancer activity of *S. ebulus* extracts be studied on normal and cancerous cell lines to gain inclusive results.

5. Conclusion

Our findings suggest that the *S. ebulus* extracts are particularly rich with phenolic compounds, including p-coumaric acid, rutin, and quercetin, possibly modulating their beneficial use for breast and gastric malignancies.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences (Code: IR.MAZUMS.REC.1398.574).

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Authors' contribution

Conceptualization and supervision: Ali Barzegar; Methodology: Ali Barzegar and Mohammad Ali Ebrahimzadeh; Investigation, writing- original draft, and writing- review & editing: Ali Barzegar, Mohammad Ali Ebrahimzadeh, and Farzaneh Rasouli Asl; Data collection: Farzaneh Rasouli Asl; Data analysis: Mohammad Ali Ebrahimzadeh, Ali Barzegar, Farzaneh Rasouli Asl; Funding acquisition and resources: Farzaneh Rasouli Asl, Ali Barzegar, Mohammad Ali Ebrahimzadeh.

Conflict of interest

The authors declared no conflict of interest.

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