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Original article

Bioassay Guided Fractionation of *Allium austroiranicum* by Cytotoxic Effects against Ovary and Cervical Cancer Cell Lines

Masoud Sadeghi Dinani[1], Narges Zakeri Tehrani[1], Fatemeh Shafiee[2*]

[1]Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.
[2]Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Abstract

Background and objectives: Cancer is a major health problem in the world. The aim of this study was to extract the flowers of *Allium austroiranicum* used by Iranian people as a condiment or for its medicinal effects followed by bioassay guided fractionation of the extracts and fractions, using anti-proliferative effects against ovarian and cervical cancer cells. **Methods:** The air-dried flowers of *Allium austroiranicum* were extracted in a four-step extraction method, resulting hexan, chloroform, chloroform: methanol (9: 1), butanol and aqueous extracts. Anti-proliferative effects of the extracts were evaluated by MTT assay against OVCAR-3, HeLa, and HUVEC cell lines. The most potent cytotoxic extract was then subjected to fractionation by MPLC method on a RP-18 silicagel column. Finally, the cytotoxic effects of resulted fractions were analyzed again and the most potent cytotoxic fraction and its IC_{50} were determined. **Results:** Statistical analysis showed that butanol extract of *A. austroiranicum* showed the most potent cytotoxic effects against OVCAR-3, HeLa and HUVEC cell lines with IC_{50} values of 38±2, 56±1.4, and 60±3.5 µg/mL, respectively. On the other hand, for 7 fractions resulting from fractionation of the butanol extract, MTT assay results showed that 6th fraction (F) was the most cytotoxic fraction with IC_{50} of 2.7±0.26 and 7.5±0.5 µg/mL for OVCAR-3 and HeLa cancer cell lines, respectively. Primary evaluation of the fraction by TLC and NMR analysis suggested the steroidal saponins as the main constituents. **Conclusion:** *Allium austroiranicum* showed significant cytotoxic effects against ovarian cancer cell line especially fractions assumed to contain steroidal saponins. The fraction constituents have the potential of being strong cytotoxic agents and the isolation and identification of compounds are suggested.

Keywords: *Allium austroiranicum*; cytotoxicity; extraction; fractionation; OVCAR-3

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Introduction

Cancer is a major health problem and the second leading cause of death in the world [1]. In 2018, 1,735,350 new cancer cases and 609,640 cancer deaths has occurred in the United States [1] among which approximately 22,240 new cases of ovarian cancer were diagnosed and 14,070 deaths occurred [2]. Although ovarian cancer accounts for 2.5% of malignancies among females, it involves about 5% of female cancer deaths because of low survival rates, largely driven by late diagnosis. So, improving prevention and early detection is a research priority, since the diagnosed disease at the local stage has a 5-year relative survival rate of 93% [3].

Given the dangerous side effects of chemotherapy, nowadays, the using of alternative treatments, especially natural products, have attracted much attentions in order to reduce the

*Corresponding author: f_shafiee@pharm.mui.ac.ir

side effects of conventional chemotherapeutic agents [4]. Successful examples in this context include the anti-leukemic alkaloids, vincristine and vinblastine, extracted from *Catharanthus roseus*, and taxol from *Taxus brevifolia*, and camptothecin from *Camptotheca* species [5].

Among the valuable plants with anticancer effects, *Allium* species (Amaryllidaceae) which contain about 750 medicinal plants [6], have been used by humans for centuries due to their aroma, taste and medicinal properties [7]. *Allium* species have several beneficial therapeutic effects including antimicrobial, antifungal, antioxidant, antitumor, antiinflammatory, antithrombotic, hypocholesterolemic hypolipidemic, and hypoglycemic effects [7]. Studies also showed that herbs of this family demonstrate protective effects on gastric and colorectal cancer [8]. These therapeutic effects of garlic is due to the presence of vitamins, flavonoids, saponins, tannin, carbohydrates, proteins, and sulfur-containing compounds [9]. Flavonoids are among the most important natural compounds found in *Allium* species with strong antioxidant, free radical elimination, lubricant, and lipid peroxidase inhibitory effects [10]. On the other hand, steroidal saponins are valuable cytotoxic compounds, with the inhibitory effects on the growth of human cancer cell lines such as PC12, 3T3-L1, SF-268, NCI-H460, MCF-7, HEP-G2, and HeLa [11].

Sulfur containing compounds are the third group of natural compounds found in *Allium* species which have various therapeutic properties including cytotoxic effects [12]. These compounds have shown inhibitory effects on esophagus, colon, mammary gland and lung cancer in laboratory animals and in various cell lines [12].

Considering steroidal saponins, researchers have shown potent anticancer activity against various cell lines [13,14]. In this regard, it has been shown that a steroidal saponin named as zingiberensis extracted from *Dioscorea zingiberensis* exhibited cytotoxic effects against various cancer cells and induced apoptosis via caspase 3 and caspase 9 activation [15].

Being endemic to Iran, *Allium austroiranicum* is an edible plant, which grows widely in the west Provinces (Zagros Mountainous region) and is used by native people as a condiment and also for medicinal purpose [16].

Considering the importance of cancer as one of the most important causes of death in most countries, and *Allium* species as plants with various medicinal effects including cytotoxic effects this project was conducted to study the cytotoxic effects of *A. austroiranicum* extracts against OVCAR-3, HeLa, and HUVEC cell lines as the first study related to this medicinal plant.

Material and Methods

Ethical considerations

The Ethics Committee of Isfahan University of Medical Sciences approved this research with the code of IR.MUI.RESEARCH.REC1398.179 on 2019-07-05.

Materials

Trypsine, fetal bovine serum, culture media and DMSO were purchased from Biosera Company (France).

Plant material

Allium austroiranicum was collected from Zagros Mountains (Chaharmahal and Bakhtiari Province, Iran) during May 2017, and scientifically identified by the botanist, Mohammad Reza Joharchi. A voucher specimen was deposited at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Iran (herbarium No. 3999).

Extraction

The air-dried flowers of *A. austroiranicum* were powdered by means of a mill and extracted at room temperature using maceration method in a four-step extraction procedure for a week respectively by following solvents; hexane, chloroform, chloroform-methanol (9:1), and methanol. In order to extract the saponins, that are soluble in n-butanol, from other water-soluble compounds, the methanol extract was then concentrated under vacuum, dissolved in water and by adding n-butanol, the constituents were partitioned between water and butanol [17]. All extracts were primarily evaluated by TLC (SiO_2, butanol:Acetic acid:Water (BAW); 60:15:25) [17] and analyzed by MTT assay for anti-proliferative activity against OVCAR-3, HeLa and HUVEC cell lines in the final concentrations of 12.5, 25, 50, 100, and 200 µg/mL, to find the extract with the most potent cytotoxic effects [18].

The most potent cytotoxic extract against tumor cell lines, was subjected to fractionation by MPLC on a RP-18 silicagel column (LiChroprep® RP-18, 36×460 mm), using a linear gradient solvent system of water: methanol (90:10) to methanol (100%) [6]. Fractions were analyzed by TLC (SiO_2, BAW, 60:15:25), visualized by 1% cerium sulfate in H_2SO_4 2 N, and similar fractions were pooled together to prepare the final fractions, namely A-G. Final A-G fractions were dissolved in 1 mL DMSO and diluted by PBS to produce final concentrations of 3.125, 6.25, 12.5, 25 and 50 µg/mL for biological tests [18].

Cell lines and chemicals

HeLa (human cervical malignant cell line), OVCAR-3 (ovarian carcinoma cell line), and HUVEC (human umbilical vein endothelial cell line) were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran).

Cytotoxicity assay

In order to determine the cytotoxic effects of extracts and fractions, MTT assay was used. Cell suspension with 2×10^4 cells/mL of HeLa and OVCAR-3 in RPMI 1640, and equal concentration of HUVEC cells in DMEM culture medium in final volume of 180 µL, was seeded to each well of a 96 well-plate and incubated at 37 ºC in CO_2 incubator. Next day, 20 µL of various concentrations of each extract or fraction were added to the wells. After 48 h of incubation, 20 µL of MTT solution (5 mg/mL) was added to the wells and the plate was further incubated for 3 h. Finally, the resulted formazan crystals were dissolved in 150 µL of DMSO and the plate was subjected to determine the absorbance at 570 nm using a microplate reader (BioRad, USA) [15].

Statistical analyses

Cytotoxicity assay was performed in three independent experiments and four replicate wells for each concentration of extracts or fractions. PBS-treated cells were considered as negative control and doxorubicin as the positive one; the results were expressed as percent of cell viability (% ± SD). SPSS 23 software was used for statistical analysis. Analysis of variance (ANOVA) followed by tukey HSD post hoc test was used to distinguish the differences between groups, while significance was assumed as $p < 0.05$. Finally, the IC_{50} of each extract or fraction was determined by preparing the graph of cell survival (%) against concentration using GraphPad Prism 7.0 software and reported as IC_{50}± SD.

Results and Discussion

Cytotoxic effects of *A. austroiranicum* various extracts on HeLa, OVCAR-3, and HUVEC cells were measured in vitro by MTT assay. The analyzed data showed that the cytotoxic effects of extracts, except for the aqueous extract, against three investigated cell lines were concentration-dependent, i.e. increasing concentration of compounds led to increased cytotoxicity.

For all cell lines, there were significant differences between the cytotoxic effects of aqueous extract versus three other extracts, in a way that the aqueous extract showed significant lower cytotoxicity (p value < 0.05). For OVCAR-3 cells, there was significant difference between the cytotoxic effects of butanol and chloroform extract (p value = 0.017), whereas this difference in the case of butanol and chloroform-methanol was not significant (p value = 0.088).

For Hela cells, the results showed significant toxicity at concentrations of 25 µg/mL and higher for butanol, and 50 µg/mL and higher for chloroform-metanol extract in comparison to the negative control. For OVCAR-3 cells treated with various concentrations of different extracts, significant toxicity was observed at concentrations of 12.5 µg/mL and higher for butanol, and 25 µg/mL and higher for chloroform-metanol extract in comparison to PBS as the negative control. HUVEC cells as the normal cell line, with the same extracts showed significant toxicity at concentrations of 12.5 µg/mL and higher for butanol, and 25 µg/mLl and higher for chloroform-metanol extract in comparison to PBS as the negative control without significant differences with cancer cells in these concentrations.

The IC_{50} of butanol extract was calculated as 38 ± 2, 56 ± 1.4, and 60 ± 3.5 µg/mLfor OVCAR-3, HeLa, and HUVEC cells, respectively. However, the calculated IC_{50} of butanol extract against both cancer and normal cell lines was less than other extracts; so, it was determined as the most potent extract and selected for further fractionation. The IC_{50} of all extracts against mentioned cell lines were summarized in table 1.

The results obtained from the cytotoxicity assay of *A. austroiranicum* are consistent with the

results of other studies on *Allium* species. For example, in Kazemi et al. study [18], the cytotoxic effects of butanol extract of *Allium affine* Ledeb was investigated against MCF-7, MDA-MB-231 and OVCAR-3 cells. The results of this study revealed that OVCAR-3 cell line was most affected by butanol extract when compared with two other cell lines with IC_{50} of about 7 µg/mL [18], slightly less than the calculated IC_{50} for these cells in the present study. On the other hand, In Mahmoudvand et al. study [19], evaluation of anti-leishmanial and cytotoxic effects of methanol and aqueous extracts of *A. sativum* showed that these extracts had low cytotoxic effects against murine macrophage cells with IC_{50} calculated as 291.4 and 348.2 µg/mL, respectively [19]. These results were similar to our study for aqueous extract with IC_{50} of about 190±10 for OVCAR-3. In addition, in the study of Bakht et al., it was showed that butanol extract of *A. sativum* revealed the highest antibacterial and antifungal effects, while petroleum ether, methanol and aqueous extracts did not show any inhibitory effect against the tested organisms [20].

In another study, the aqueous extract of *A. sativum* showed cytotoxic effects in higher concentration (even more than 10 mg/mL) against human larynx carcinoma cell line (Hep-2) and this effect was attributed to the organosulphoric compounds in this extract [21]. According to the fact that organosulphoric compounds are mainly changed and destroyed during extraction and fractionation, it seems that low cytotoxic effects of *Allium* species extracts is related to the destruction of this compounds, leading to the higher value of calculated IC_{50} in comparison to the IC_{50} resulted in the present study while more stable steroidal saponins and flavonoids could be the main effective constituents [22].

Fractionation of the butanol extract was performed by MPLC which resulted in final 7 fractions, named A to G. Analyzing A-G fractions by MTT assay showed that fractions E and F had significant cytotoxic effects against all cell lines in comparison to other fractions (p value = 000). The most potent cytotoxic effect of E and F was observed for OVCAR-3 cells (p value = 0.001), while the difference between the cytotoxic effects of E and F fractions was not significant for this cell line (p value =0.866).

Finally, the IC_{50} of E and F fractions for HeLa and OVCAR-3 cells were calculated as 9.6 ± 1.2 and 7.5 ± 0.5 µg/mL for HeLa, and 4.67 ± 1.15 and 2.7 ± 0.26 µg/mL for OVCAR-3, respectively. However, these amounts for HUVEC cells were determined as 19.66 ± 5 µg/mL and 6.5 ± 3.9 µg/mL, respectively. Table 1 is a summary of calculated IC_{50} of each fraction against various cell lines.

Several studies have been conducted on cytotoxic effects of steroidal saponins as one of the most important categories of compounds available in *Allium* plants. In this regard, the cytotoxic effects of steroidal saponins isolated from *A. vavilovii* and *A. umbilicatum* on J-774 and WEHI-164 cell lines with IC_{50} value of 3-5 µg/mL [23] and 90-160 µg/mL [24], respectively, have been established. In the present study, the cytotoxic effect of *A. austroiranicum* was more considerable against OVCAR-3 cell line. There are some examples of studies for using this cell line in order to evaluate the cytotoxic effects of saponins. For example, in one study, the IC_{50} of *Tribulus terrestris* against this cell line was calculated as 157 µg/mL [25].

Table 1. The calculated IC_{50} ± SD (µg/mL) of various extracts or fractions of *Allium austroiranicum* against cell lines

	Positive control	**Extracts**			
Cell lines	doxorubicin	Chloroform-methanol	Butanol	Chloroform	Water
OVCAR-3	0.5±0.1	55.0±5.0	38.0 ± 2.0	80.0±10.0	190.0±10.0
HeLa	0.2±0.0	60.0±13.0	56.0 ± 1.4	120.0±31.0	Unaccountable[1]
HUVEC	0.6±0.0	61.0±1.0	60.0± 3.5	170.0±30.0	Unaccountable

	Fractions						
Cell lines	A	B	C	D	E	F	G
OVCAR-3	Unaccountable	Unaccountable	Unaccountable	20.4±4.2	4.6±1.2	2.7±0.2	28.3±4.6
HeLa	Unaccountable	Unaccountable	Unaccountable	Unaccountable	9.6±4	7.5±4.7	44.3±3.2
HUVEC	Unaccountable	Unaccountable	Unaccountable	Unaccountable	19.66±8	6.5±3.9	Unaccountable

[1]in the investigated concentrations, the IC_{50} could not be determined.

Also, it was showed that extracts rich in a saponin, named as protodioscin, has the most cytotoxic effects [25]. In another study, a saponin extracted from the *Xanthoceras sorbifolia* Bunge showed the IC_{50} of about 2 µg/mL against these cells [26], which was a little less than the calculated IC_{50} in our study. In the present work, it was shown that the cytotoxic effects of F fractions E and were more considerable compared to the first fractions. According to the roles of extraction and separation in reverse phase chromatography and the results of primary TLC and NMR analyses, the butanol extract and its derived fractions have been considered to be rich in steroidal saponins and the observed cytotoxic effects could be related to the presence of these compounds. The results are in agreement with studies published on steroidal saponins and confirm the cytotoxic activity of steroidal saponins extracted from *A. austroiranicum*. This work was the first study about the biological activity of *A. austroiranicum* as an endemic edible plant of Iran. The present study showed cytotoxic effects of the plants butanol extract and its derived fractions, containing much amounts of steroidal saponins, suggesting its potential to be considered as a suitable candidate for isolation and identification of active ingredients.

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Author contributions

Masoud Sadeghi Dinani supervised the study and conducted the experiments related to the extraction and fractionation. Narges zakeri Tehrani performed practical experiments in both plant related and cell culture parts. Fatemeh Shafiee supervised the study and conducted the experiments related to cell culture and analyzed the data.

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

HUVEC: human umbilical vein endothelial cell; IC_{50}: 50% inhibitory concentration; MPLC: Medium performance liquid chromatography; OVCAR-3: ovarian carcinoma; TLC: Thin-layer chromatography