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Original Research Article

In vitro Assessment of Cytotoxic Potential of *Ajuga orientalis* L. Methanol Extract on MCF-7and MRC-5 cells

Arwa R. Althaher*

Department of Pharmacy, Faculty of Pharmacy, Al-Zaytoonah University of Jordan, 11733, Amman, Jordan

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ABSTRACT

Cancer is a significant public health concern both globally and in Jordan. Modern cancer therapies are associated with multidrug resistance, severe side effects, and high costs. There has recently been a lot of interest in screening for plants that can be used in cancer prevention and treatment. The current study is intended to assess the cytotoxic activity of methanolic extract of aerial parts of *Ajuga orientalis* L. on a human breast cancer cell line (MCF-7). The cytotoxic activity of the extract against human breast cancer cells (MCF-7) and normal fibroblast (MRC-5) was assessed using the (3-(4,5- dimethylthiazol-2-yl) - 2,5- diphenyltetrazolium bromide (MTT) assay. The cells were treated with different concentrations of methanol extract of *Ajuga orientalis* (3.125-200 µg/mL) for 24, 48, and 72 hrs. The extract demonstrated inhibition in the proliferation of the MCF-7 cell line in a dose-dependent manner, without any toxic impact on normal fibroblast cells. Furthermore, the extract showed significant cytotoxic activity after 72 hrs of incubation (IC_{50} 30.5±0.05 µg/mL). In conclusion, the methanol extract of *A. orientalis* showed a potent reduction in the proliferation of MCF-7 cells and could be considered a potential source of the anticancer compound.

Keywords: *Ajuga orientalis* L, Lamiaceae, Cytotoxic activity, MTT, MCF-7, MRC-5.

Introduction

Medicinal plants have played an essential part in the discovery and development of medications during the past few decades. Many medicinal plants provide unique treatments with high efficacy and few side effects compared to the known standard pharmaceuticals used to treat the most debilitating diseases, such as cancer.[1]
Medicinal plants make up roughly 20% of Jordan's flora.[2,3] As a result, many are used in traditional medicine and pharmaceutical production.[4,5]
The Lamiaceae family includes the genus Ajuga as one of its members. Ajuga s a genus of 50 species of evergreen, clump-forming rhizomatous, annual, or perennial herbs that grow to a height of 5-50 cm and are found primarily in Asia, Africa, Australia, and North America, in addition to Europe.[6]
Ajuga orientalis L. (also known as Eastern bugle) is a 20-40cm tall annual herbaceous flowering plant with opposite leaves that grows in humid areas (Ajloun, Salt, Amman, and Karak).[2] *A. orientalis* is a medicinal plant with diverse biological activities. It is used in traditional medicine worldwide to treat rheumatism, gout, diabetes, asthma, malaria, and gastrointestinal disorders.[7,8] *A. orientalis* has antibacterial, antitumor, antioxidant, and anti-inflammatory properties.[6-11] This study aimed to assess the cytotoxic activity of methanolic extract of the aerial part of *Ajuga orientalis* L. on the human breast cancer cell line (MCF-7).

*Corresponding author. E mail: a.althaher@outlook.com
Tel: +962-6-4291511

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Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Materials and Methods

Plant materials
Ajuga orientalis L. aerial parts were collected in April 2021 from Ajloun County, Northern Jordan (32°23'13.3" N 35°46'18.2" E). Prof. Dr. Sawsan Oran identified the plant taxonomically (University of Jordan, Amman, Jordan). The voucher specimen of the collected plant (voucher no. 14) was deposited in the herbarium of the Department of Biological Sciences, University of Jordan, Amman.

Preparation of the methanolic extract
The aerial parts of *A. orientalis* were air-dried at room temperature in the dark for approximately six weeks, then ground to a fine powder. Twenty grams were extracted for 72 hrs at room temperature with frequent agitation in 200 mL of methanol (1:10 w/v sample-to-solvent ratio). The extracts were then filtered with Whitman filter paper (No. 1), and the solvents were evaporated using a rotary evaporator under reduced pressure. The crude extract was then collected and kept at -20°C.[12]

Cell culture
Human breast adenocarcinoma MCF-7 (HTB-22, Breast Adenocarcinoma), and MRC-5 (CCL-171) (normal human fibroblast) cells were obtained from the American Type Culture Collection (ATCC; USA). The cells were grown in Dulbecco's modified eagle medium (DMEM) (Euroclone, Italy). All media were supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest, South America), 1% (v/v) penicillin-streptomycin (Euroclone, Italy), and 1% (v/v) L-glutamine (Euroclone, Italy). The cells were maintained at 37°C in 5% carbon dioxide and 95% humidity. The cells were removed from the flask surface using 1X trypsin-EDTA solution (PAN, Germany) and washed in phosphate buffer saline (PBS, Euro-clone S.p.A; Pero, Milan, Italy) once they had reached 80% confluence. To obtain the cell suspension, the cells were then resuspended in a growth medium. The number of viable cells was also determined using the trypan blue dye exclusion technique.[13]
Methanol extracts of *Ajuga orientalis* were tested for cytotoxicity in breast cancer cells (MCF–7) and normal fibroblast cells (MRC-5).

The potential cytotoxicity of methanolic extracts of *A. orientalis* on MCF–7 and MRC-5 was done with the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to Althaher's method.[14] At a density of $1x10^4$ cells per well, each cell line was seeded in triplicate on 96-well plates (Corning, USA). After 24 hrs, cells were treated with 100 µL of the tested extract at different concentrations (200- 3.125 µg/mL) were added into each well containing the cells. After 24, 48, and 72hrs post-extract addition, 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated at 37°C for 3 hours. After that, the supernatant containing MTT was removed carefully, and 200 µL DMSO was added to each well to dissolve the formed insoluble formazan crystals. The absorbance was measured at 570 nm and 630 nm using a microplate reader (Bio-Tek, USA), and the difference between the readings was used for the analysis of the results. Doxorubicin (4.25- 0.085 µg/mL) was used as a standard anticancer drug. The half-maximal inhibitory concentration (IC_{50}) values were determined as a concentration of extract that demonstrated 50% inhibition of proliferation in any examined cell line.

Statistical analysis

All of the experiments were carried out in triplicate, and the findings are presented as mean± SD. The data was analyzed using one-way analysis of variance (ANOVA), and differences were considered significant at a p-value less than 0.05. The IC_{50} was calculated using the GraphPad Prism9.0.2 statistical software (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Ajuga orientalis is a member of the Lamiaceae family that grows abundantly in Jordan's humid regions. Many countries have used the Ajuga species in traditional medicine. Drug discovery from active herbal ingredients is a significant challenge nowadays.

The cytotoxic activity of *A. orientalis* methanolic extract was assessed by MTT assay against human breast cancer cells (MCF-7) and a normal human fibroblast cell line (MRC-5) for 24, 48, and 72 hrs (Figures 1-A and B).

Overall, dose-dependent inhibition of MCF-7 cancer cell viability by *A. orientalis* methanolic extract was observed compared to control cells (untreated). Also, at all incubation durations, the highest concentration (200 µg/mL) proved cytotoxic. Furthermore, at 72 hrs, the extract dramatically slowed the proliferation of MCF-7 cells (3.125-200 µg/mL) (Figure 1-A). After 48 hrs, the extract (6.25-200 µg/mL) effectively inhibited the proliferation of MCF-7 cells compared to the incubation time (24 hrs) to the effective concentrations (50-200 µg/mL) (Figure 1-A). In contrast, the results revealed that the methanolic extract of *A. orientalis* had no cytotoxic activity against the normal fibroblast cell line (MRC-5) (Figure1-B and IC_{50} in Table 1). Moreover, the results were compared to (Doxorubicin) the standard cytotoxic drug. Doxorubicin showed a significant cytotoxic effect on cancerous and non-cancerous cells (Figure 1- C and D, Table 1).

Previously, the chemical composition of methanol extract of *A. orientalis* have been reported, [11] where the major components were 9-octadecenoic acid, methyl ester, (E) - (27.2%), hexadecanoic acid, methyl ester (12.8%), and methyl stearate (9.6%).[11]

Table 1: Comparison of IC_{50} values between *Ajuga orientalis* methanolic extract and doxorubicin (standard anticancer drug) on MCF-7 and MRC-5 cell lines after 24, 48 and 72 hrs of incubation. Values are mean of three replicates ± Standard deviation SD.

	***A. orientalis* methanol Extract (IC_{50} ± SD µg/ml)**	**Doxorubicin (IC_{50} ± SD µg/ml)**
MCF-7 (24hrs)	222.5 ± 0.18	0.410 ± 0.07 [a]
MCF-7 (48hrs)	182.1 ± 0.0.06 [a]	0.215 ± 0.10 [a]
MCF-7 (72hrs)	30.5 ± 0.05[a]	0.101 ± 0.015 [a]
MRC-5 (24hrs)	751.2 ± 0.30	0.652 ± 0.06 [a]
MRC-5 (48hrs)	552.3 ± 0.51	0.389 ± 0.12 [a]
MRC-5 (72hrs)	492.6 ± 0.09	0.258 ± 0.05 [a]

[a] *p*-value<0.05

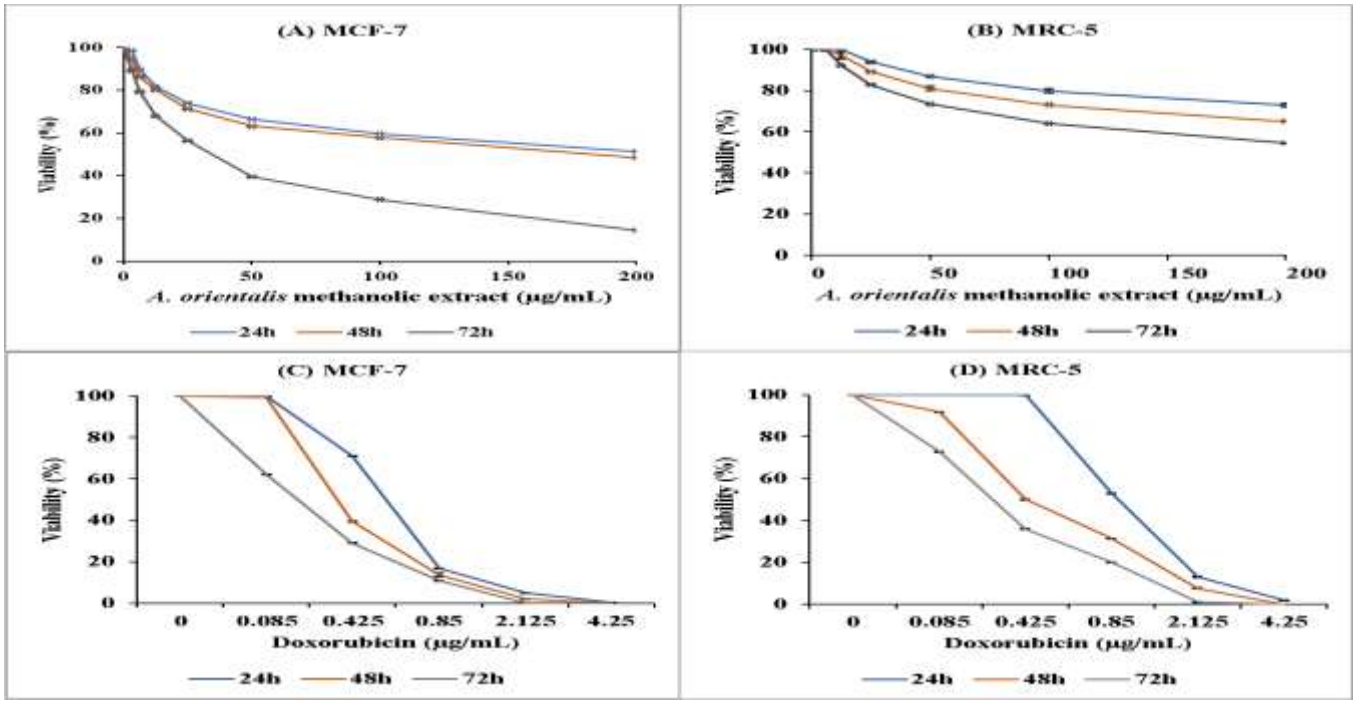

Figure 1: Cytotoxic activity of methanolic extract of *Ajuga orientalis* (3.125-200 µg/ml) on (A) human breast cancer cell line MCF-7 (B) normal human fibroblast cell line MRC-5 after 24, 48, and 72hrs of incubation. The cytotoxicity of doxorubicin (0.085-4.25 µg/ml) on (C) MCF-7 cells (D) on MRC-5 cells after 24, 48, and 72 hrs of incubation. Data were presented as means ± SD (n = 3).

Also, Oran *et al*. (2022) assessed the antioxidant and cytotoxic activity of the ethanolic and aqueous extracts of *A. orientalis* and total phenol and flavonoid contents. The results showed that the ethanolic extract had a higher content of phenol and flavonoids than the aqueous extract. Additionally, the antioxidant properties of both extracts were evaluated using DPPH and the reducing power ability. Moreover, *A. orientalis* aqueous extract has a strong cytotoxic effect on the Caco-2 cell line (IC_{50} of 2.059 ±0.10 g/mL). On the other hand, the ethanolic extract was cytotoxic to the MCF-7 cell line (IC_{50} 59.32 ± 0.04 g/mL). Both extracts had no toxic effect on the normal dermal fibroblast cell line (HDFa). [11] Sadati and others (2012) reported the n-hexane extract of *A. chamaecistus* ssp. *Tomentella* showed significant cytotoxic activity against colon carcinoma (HT-29), colorectal adenocarcinoma cells (Caco-2), breast ductal carcinoma (T47D), and Swiss mouse embryo fibroblasts (NIH-3T3), while the diethyl ether fraction was moderately cytotoxic against HT-29.[15]

Furthermore, the antiproliferative activity of Ajugalide-B (ATMA) from *A. taiwanensis* was tested against hepatoma (HepG2), lung adenocarcinoma (A549), gastric carcinoma (AGS), and colon carcinoma (HT29). ATMA disrupted the focal adhesion complex and reduced phosphorylation by paxillin and focal adhesion kinase (FAK). Also, it lowered the tumorigenic and metastatic abilities of the human lung cancer cell line (A549).[16] Rauca and others (2019) also investigated the antiproliferative potential of ethanolic extracts of *A. genevensis*, *A. chamaepitys*, and *A. laxmannii* on murine colon carcinoma cell lines (C26) and murine melanoma cell lines (B16.F10) on C26 and B16.F10 cells, the ethanolic extract of *A. laxmannii* demonstrated the most potent cytotoxicity (IC_{50} 176.3 and 236.8 µg/mL respectively).[17] Regardless of the plant species, cell type, or dosages used, the results in this study were compatible with the previous studies.

Conclusion

The methanolic extract of *A. orientalis* was found to have a considerable cytotoxic effect on human breast cancer cell lines (MCF-7). Future research is required to determine the mechanism of action that could be attributed to the cytotoxic activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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