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Original Article

Chemical composition, *in vitro* assessment of antioxidant properties and cytotoxicity activity of ethanolic and aqueous extracts of *Ajuga orientalis* L. (*Lamiaceae*)

[Composición química, evaluación *in vitro* de propiedades antioxidantes y actividad citotóxica de extractos etanólicos y acuosos de *Ajuga orientalis* L. (*Lamiaceae*)]

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Abstract

Context: *Ajuga orientalis* L. (*Lamiaceae*) is an aromatic herb used in traditional medicine in Jordan and neighboring countries.

Aims: To evaluate the cytotoxic potential of ethanolic and aqueous extracts from the aerial parts of *A. orientalis* against three cell lines MCF-7, Caco-2, and HDFa. In addition to assessing the total phenolic and flavonoid contents, antioxidant activity. Further to analyze the phytochemical constituents.

Methods: The phytochemical analysis was performed using gas chromatography/mass spectrometry. The total phenolic (TPC) and flavonoid (TFC) contents were assessed using colorimetric methods. The antioxidant properties of both extracts were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power ability. Cytotoxicity was investigated using methyl thiazol tetrazolium (MTT) assay.

Results: The most abundant compounds in the extract were 9-octadecenoic acid, methyl ester, (E)- (27.2%), hexadecanoic acid, methyl ester (12.8%), and methyl stearate (9.6%). The ethanolic extract demonstrated higher TPC and TFC than the aqueous extract. Also, the ethanolic extract showed higher antioxidant activity than aqueous extract using DPPH and the reducing power ability. Furthermore, the aqueous extract of *A. orientalis* has a potent cytotoxic effect against the Caco-2 cell line (IC_{50} = 2.059 ± 0.10 µg/mL). In contrast, the ethanolic extract demonstrated a cytotoxic effect against the MCF-7 cell line (IC_{50} = 59.32 ± 0.04 µg/mL). Both extracts did not exhibit a toxic effect against normal dermal fibroblast cell line (HDFa). Compared to non-cancerous cells, the ethanolic extract of *A. orientalis* demonstrated high selectivity against MCF-7 cells and limited selectivity against Caco-2 cells. In comparison, the aqueous extract was highly selective against both cancerous cells.

Conclusions: *A. orientalis* demonstrated antioxidant properties and significant antiproliferative potential against breast and colon cancer. Therefore, additional investigations are needed to study the mechanism of the cytotoxicity for this plant.

Keywords: *Ajuga orientalis*; antioxidant properties; cytotoxic activity; GC-MS; MTT.

Resumen

Contexto: *Ajuga orientalis* L. (*Lamiaceae*) es una hierba aromática utilizada en la medicina tradicional en Jordania y países vecinos.

Objetivos: Evaluar el potencial citotóxico de extractos etanólicos y acuosos de partes aéreas de *A. orientalis* frente a tres líneas celulares MCF-7, Caco-2 y HDFa. Además de evaluar el contenido total de fenoles y flavonoides, la actividad antioxidante. Además de analizar los componentes fitoquímicos.

Métodos: El análisis fitoquímico se realizó mediante cromatografía de gases/espectrometría de masas. Los contenidos de fenoles y flavonoides totales se evaluaron mediante métodos colorimétricos. Las propiedades antioxidantes de ambos extractos se evaluaron utilizando la actividad de eliminación de radicales 2,2-difenil-1-picrilhidrazilo (DPPH) y la capacidad de poder reductor. La citotoxicidad se investigó mediante el ensayo de metiltiazol tetrazolio (MTT).

Resultados: Los compuestos más abundantes en el extracto fueron ácido 9-octadecenoico, éster metílico, (E)- (27,2%), ácido hexadecanoico, éster metílico (12,8%) y estearato de metilo (9,6%). El extracto etanólico demostró mayor TPC y TFC que el extracto acuoso. Además, el extracto etanólico mostró mayor actividad antioxidante que el extracto acuoso usando DPPH y la capacidad de poder reductor. Además, el extracto acuoso de *A. orientalis* tiene un potente efecto citotóxico frente a la línea celular Caco-2 (IC_{50} = 2,059 ± 0,10 µg/mL). En cambio, el extracto etanólico demostró un efecto citotóxico frente a la línea celular MCF-7 (IC_{50} = 59,32 ± 0,04 µg/mL). Ambos extractos no exhibieron un efecto tóxico contra la línea celular de fibroblastos dérmicos normales (HDFa). En comparación con las células no cancerosas, el extracto etanólico de *A. orientalis* demostró una alta selectividad frente a las células MCF-7 y una selectividad limitada frente a las células Caco-2. En comparación, el extracto acuoso fue altamente selectivo contra ambas células cancerosas.

Conclusiones: *A. orientalis* demostró propiedades antioxidantes y un importante potencial antiproliferativo contra el cáncer de mama y colon. Por lo tanto, se necesitan investigaciones adicionales para estudiar el mecanismo de citotoxicidad de esta planta.

Palabras Clave: actividad citotóxica; *Ajuga orientalis*; GC-MS; MTT; propiedades antioxidantes.

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Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl-hydrated; ATCC: American Type Culture Collection; MCF-7: Breast adenocarcinoma; Caco-2: Colorectal adenocarcinoma; HDFa: Primary dermal fibroblast normal cells; DMEM: Dulbecco's Modified Eagle Medium; RPMI: Gibco Roswell Park Memorial Institute (RPMI) 1640 Medium; DMSO: Dimethyl sulfoxide; FBS: Heat-inactivated fetal bovine serum; PBS: Phosphate buffer saline; EDTA: ethylene diamine tetraacetic acid; MTT: (3-(4,5-dimethylthiazol-2w-yl)-2,5-diphenyl tetrazolium bromide).

INTRODUCTION

Despite the many benefits of modern synthetic drugs, people still prefer natural herbs to synthetic drugs (Yuan et al., 2016). Due to various valuable components in different plants, many biologically active secondary metabolites in various plant species exhibit pharmacological effects such as lessening the risk of diseases induced by reactive oxygen species (ROS) (Zilani et al., 2017) via diverse mechanisms of action like scavenging free radicals, quenching ROS, hindering oxidative enzymes (Pisoschi and Pop, 2015), as well as they exhibit antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidant properties (Konappa et al., 2020; Sunoqrot et al., 2021).

Alkaloids, flavonoids, tannins, glycosides, saponins, volatile oils, phenolic compounds, steroids, and other phytochemical elements contribute to medicinal plants' therapeutic potential (Tungmunnithum et al., 2018). Many anti-cancer drugs, such as colchicine, vincristine, vinblastine, and paclitaxel, are derived from medicinal plants in some form (Lichota and Gwozdzinski, 2018).

Medicinal plants account for about 20% of Jordan's total flora (Oran and Al-Eisawi, 1998). Several of them have been used in traditional medicine and the pharmaceutical industry (Afifi and Abu-Irmaileh, 2000). Although many herbal remedies have a well-known composition and certain biological effects, some of them are still used solely on the basis of traditional medicine, with no validation of their safety and efficacy.

The investigation of medicinal plants used in folk medicine may result in novel herbal formulations with significant biological activities. Because of their considerable pharmacological effects, natural compounds are effectively used to create new phytomedicines.

Ajuga orientalis L. (also known as Eastern bugle) belongs to the *Lamiaceae* family. It is an annual herbaceous flowering plant with opposite leaves that grows 20 to 40 cm tall. In Jordan, it is distributed in humid areas such as Ajloun, Salt, Amman, and Karak (Oran and Al-Eisawi, 1998). *A. orientalis* is a plant that has medicinal properties. It is used in folk medicine worldwide to treat rheumatism, gout, asthma, diabetes, malaria, and gastrointestinal disorders (Sajjadi and Ghannadi, 2004; Cocquyt et al., 2011). *A. orientalis* has antibacterial, antitumor, antioxidant, and anti-inflammatory properties (Gautam et al., 2011; Zengin et al., 2018). The potential antioxidant and cytotoxic activity of the *A. orientalis* may contribute to better therapeutic outcomes compared to the administration of synthetic anticancer drugs.

In Jordan, there is not enough data on the biological activities for *A. orientalis*. Therefore, this study aims to screen the phytochemical constituents and to investigate the antioxidant capacity and potential antiproliferative activity of the ethanolic and aqueous extracts of *A. orientalis* against human breast adenocarcinoma (MCF-7), colorectal adenocarcinoma (Caco-2), as well as normal dermal fibroblast cell line (HDFa). In addition to determining the content of phenols and flavonoids.

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteu phenol reagent, ascorbic acid, HPLC grade methanol, quercetin, gallic acid were obtained from (Merck Co, Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from (Sigma Chemical Co, St. Louis, MO, USA). Other solvents and reagents in this study were purchased from commercial sellers and were dealt with by standard methods before use.

Plant material collection

The aerial parts of *Ajuga orientalis* were collected from Ajloun county (Northern of Jordan) at GPS (31.9494964, 35.9342189) in April 2021. The plant materials have been taxonomically identified by Prof. Sawsan Oran, Department of Biological Sciences, University of Jordan. Voucher specimens (voucher no. AO-012) of the collected plants were deposited at the herbarium of the Department of the Biological Sciences, University of Jordan, Amman.

Preparation of ethanolic and aqueous extracts

The aerial parts of *A. orientalis* were air-dried at room temperature in the dark for approximately five weeks, then ground to a fine powder. Ten grams of the milled aerial parts were soaked in the 100 mL of the solvent (10:1 v/w ratio) for extract preparation: distilled water and ethanol (absolute). For ethanolic and aqueous extracts, the suspensions were placed at 20°C, under frequent agitation for 72 h. Afterward, the extracts were filtered through Whitman No. 1 filter paper, and the solvents were evaporated using a rotary evaporator under reduced pressure. Then the

crude extract was formed and stored at −20°C in an airtight container for later analysis (Freshet et al., 2017). The ethanolic extract obtained was 2.5 g, and the percentage yield was 0.25% compared to the aqueous extract was 1.2 g, and the percentage yield was 0.12%.

Gas chromatography/mass spectrometry (GC/MS) analysis

The phytochemical investigation of *A. orientalis* was performed on GC-MS equipment (Shimadzu QP 2010, USA) linked to Shimadzu 2010 mass spectrometer system, operating in electron impact mode at 70 eV. Experimental conditions of the GC-MS system were as follows: DP-5 capillary standard non-polar column, dimension: 30 m, ID: 0.25 mm, Film thickness: 0.25 µm. The flow rate of the mobile phase (carrier gas: He) was set at 1.0 mL/min, and an injection volume of 1 µL (1 g of powdered sample dissolved in 5 mL methanol) was employed in splitless mode at injection temperature 280ºC. The oven's temperature was programmed at 45°C for 1 min then raised to 300°C at 15°C/min. The total running time for the sample was 30 min at a range of 50-550 m/z. The chemical constituents of the plant sample were identified by comparing the retention indices and mass spectra with those in the NIST17-1 library website. The relative proportion of each component in the extract was computed by comparing the average peak area of each component in the extract to the total areas.

Total flavonoid content (TFC)

The total flavonoid content in the ethanolic and aqueous extracts of *A. orientalis* aerial parts was determined using Benslama et al. (2017). Briefly, 500 µL of $AlCl_3$ (2% in methanol) was mixed with 500 µL of each extract (1 mg/1 mL methanol). Quercetin (1 mg quercetin/1 mL methanol) was made in a series of standard concentrations (0-100 µg/mL) and used as a standard. Methanol was used as blank. All reaction mixtures were then incubated for 10 min at room temperature, protected from the light. The absorbance was measured at 430 nm using a UV-vis spectrophotometer. The samples were measured in triplicate. The results were represented as mg quercetin equivalents (QE) per mg extract.

Total phenolic content (TPC)

TPC of *A. orientalis* ethanolic and aqueous extracts was determined using the Folin-Ciocalteu method as described by Tambe and Bhambar (2014). Gallic acid (0-100 µg/mL) was used as standard. Briefly, 1 mL of each extract (1 mg/mL) dissolved in ethanol was mixed with 1 mL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min. Then, 10 mL of 7% (w/v) sodium carbonate (Na_2CO_3) was added to the mixture, followed by the addition of 13 mL distilled water and mixed gently. After standing at room temperature for 90 min, the absorbance was read at 760 nm using a UV-vis spectrophotometer. Ethanol was used as blank. The results were expressed as mg gallic acid equivalent (GAE)/ g of extract. All samples were measured in triplicate.

Antioxidant activity

DPPH free radical scavenging capacity

The assessment of antiradical capacity of ethanolic extract *A. orientalis* was performed according to Chen et al. (2016) using 2,2-diphenyl-1-picrylhydrazyl-hydrated (DPPH) assay. The antioxidant capacity was expressed as IC_{50} (µg/mL). Ascorbic acid was used as positive control (10-100 µg/mL). One milliliter of DPPH solution (0.1 mM, in ethanol) was combined with 1 mL of each extract at different concentrations (10-100 µg/mL). The reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was measured at 517 nm against the blank (ethanol) using a spectrophotometer. All samples were measured in triplicate. The percentage inhibition of the DPPH radical by the ethanolic and aqueous extract was calculated based on the equation [1]:

$$\text{DPPH scavenging capacity (\%)} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad [1]$$

Where A: absorbance

The IC_{50} value was calculated to determine the concentration of the plant extract needed to inhibit 50% of DPPH radical.

Reducing power ability

The reducing power ability of both extracts of *A. orientalis* was estimated using the method described by Althaher et al. (2020). One milliliter of each concentration of each plant extract (10-1000 µg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was promptly cooled, mixed with 2.5 mL of 10% trichloroacetic acid (TCA), and centrifuged at 3000 rpm for 10 min after incubation at 50°C for 20 minutes. After that, 2.5 mL of supernatant was mixed well with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride before being allowed to stand for 10 min. A visible/UV spectrophotometer was used to evaluate the mixture's absorbance at 700 nm against a blank (phosphate buffer at pH 6.6). The experiment was carried out three times, and the results were expressed as mean (IC_{50} µg/mL) ± SEM. The reducing

power of the ethanolic and aqueous extracts of *A. orientalis* was also compared to that of ascorbic acid (standard).

Cell culture

Human breast adenocarcinoma carcinoma MCF-7 (ATCC® HTB-22, breast adenocarcinoma), Caco-2 (ATCC® HTB-37, colorectal adenocarcinoma), and Primary Dermal Fibroblast Normal cells (ATCC® PCS-201-012, HDFa, normal from skin) cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were grown in a vented cell culture flask as an attached monolayer in commercially defined Gibco Roswell Park Memorial Institute (RPMI) 1640 Medium (Euroclone, Italy) (for MCF-7) and Dulbecco's modified eagle medium (DMEM) (Euroclone, Italy) for Caco-2 and fibroblast cells, 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 incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity. The cells were handled in sterile conditions under a class II biological safety cabinet, using commercially pre-sterilized and disposable equipment. Upon reaching 80% confluence, the old medium was aspirated, and the surface of the cell culture flask (SPL, Korea) was washed with 1× phosphate buffer saline PBS (Euroclone, Italy). Then, the cells were detached from the flask surface by using 1× trypsin/ ethylene diamine tetraacetic acid (EDTA) (PAN, Germany) after an incubation period of 5 min. Consequently, trypsin was neutralized with FBS supplemented media to deactivate its action. The cell suspension was then passed into new culture flasks or separated for further experiments (Belkacem et al., 2021; Althaher et al., 2022).

Cell viability and counting

The number of viable cells was counted using the trypan blue dye exclusion staining assay. One hundred microliter aliquot of cells was taken and diluted with 100 µL of trypan blue (Sigma, USA) in a 1:1 ratio. Furthermore, the mixture was then loaded onto a hemocytometer and examined under an inverted light microscope (Leica, USA).

Cell proliferation assay (MTT)

The CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit® (MTT) (Promega, Madison, USA) was proceeded according to the manufacturer's instructions to assess the ani-proliferative activity of ethanolic and aqueous extracts of *A. orientalis* and doxorubicin (Adriamycin, USA). It is a colorimetric, non-radioactive, and fast test that reduces 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the reduction of yellow MTT substrate to insoluble purple formazan by the mitochondrial enzymes in a viable cell. Then spectrophotometry was used to quantify the intensity of the color resulting by dissolving the formed crystals via the solubilization solution supported with the kit. Approximately 7×10^3 cells were seeded into each well of tissue culture treated 96-well plate (SPL, Korea) in triplicate in plain medium and incubated for 24 h at 37°C; then, the media were aspirated from the wells. Afterward, the cells were treated with ethanolic and aqueous extracts of *A. orientalis* separately in serial dilutions as the following (1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL in DMSO) at a volume of 100 µL per well. Media with 0.02% DMSO was used as a negative control, while doxorubicin (standard anticancer drug) (0.05-100 µg/mL) was used as a positive control (Jarrar et al., 2020). After 72 h of incubation, the old media was aspirated, then 15 µL of MTT reagent was added to 100 µL of new media added to each well. The plate was kept in an incubator for 4 h; then, 100 µL of solubilizing/stop solution was added to each well. An hour later, the absorbance was measured using a 96-well plate reader (Biotek Elx808, USA) at a 570 nm wavelength.

The half-maximal inhibitory concentration (IC_{50}) values, the drug concentration at which 50% of cells are viable, were calculated from the logarithmic trend line of the cytotoxicity graphs. Moreover, the results were presented as the mean ± SD.

The selectivity index (SI) for tested cell line was determined by dividing the IC_{50} value primary dermal fibroblast normal cells (HDFa) by the IC_{50} value on cancerous breast cells (MCF-7), also on the IC_{50} value of colorectal adenocarcinoma Caco-2 [2].

$$SI = \frac{IC_{50} \text{ of extract on normal cells (HDFa)}}{IC_{50} \text{ of extract on cancerous cells (MCF-7, Caco-2)}} \quad [2]$$

The SI value represents the sample's selectivity for the cell lines that were examined. SI > 3 samples were thought to have a high selectivity for cancerous cells (Chothiphirat et al., 2019).

Statistical analysis

The data obtained were represented as mean ± SEM of triplicate analyses using GraphPad Prism 9.0.2 (GraphPad Software, San Diego, USA). Moreover, the significant differences between the groups were determined using one-way ANOVA and Tukey's post hoc test, where p-value < 0.05 was considered statistically significant.

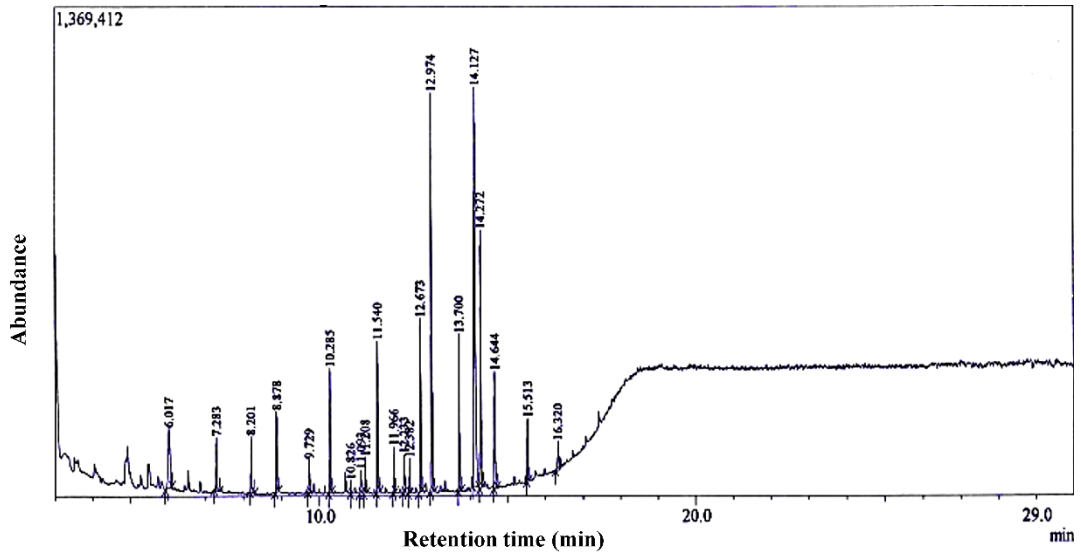

Figure 1. GC-MS chromatogram of methanolic extract of the aerial parts of *A. orientalis*

RESULTS AND DISCUSSION

Oxidative stress has been associated with cancer initiation and progression by causing cells injury, DNA damage, enhancing gene mutations rate, genome instability, and altering cell metabolism and signaling (Kruk and Aboul-Enein, 2017; Liguori et al., 2018). Natural antioxidants protect against oxidative stress by protecting cellular components from free radical damage. Furthermore, they can block the generation of free radicals or disrupt the propagation of free radicals, reducing oxidative stress and improving immunological function (Khlebnikov et al., 2007). Finding natural antioxidants from natural sources has received much interest recently. Natural antioxidants found in plants, such as phenolic compounds and flavonoids, have antioxidant properties that target significant amounts of reactive oxygen intermediates, resulting in effective therapeutic strategies (Forni et al., 2019).

Phytochemical analysis

The GC-MS analysis of the methanolic extract of *A. orientalis* aerial parts revealed 21 compounds, accounting for 94.5% of the total. The GC-MS spectrum of the extract is shown in Fig. 1. Moreover, 9-octadecenoic acid, methyl ester, (E)- (27.2%), hexadecanoic acid, methyl ester (12.8%), and methyl stearate (9.6%) were the predominant compounds. Minor compounds were also found, such as 3,4-dihydroxyphenylglycol (0.89%) and 2-octylcyclohexanone (0.32%). The compounds present in the extract belong to organic compounds, fatty acids methyl ester, long-chain fatty aldehyde, aromatic fatty acid, fatty alcohol, and alcohol. The retention time, molecular formula, molecular weight, and the peak area percentage from the NIST17-1 library are shown in Table 1.

The chemical composition and biological properties of different *A. orientalis* extracts differed significantly. Even within the same species, the chemical composition of plants can differ. These variations are associated with several factors that may have a significant impact on the yield, composition, and biological activities, such as genetic background, environmental conditions, habitat, developmental stage, harvesting time, and extraction methods (Zouari et al., 2012).

Compared to other studies that reported the chemical composition of *A. orientalis*, there is a significant variation in the chemical composition. The main compounds of the volatile oil of the aerial parts of *A. orientalis* in Iran were germacrene-D (24.2%), β-cubebene (18.3%), β-caryophyllene (16.9%), and α-cubebene (5.3%) (Sajjadi and Ghannadi, 2004). On the other hand, β-pinene (23.5%), α-pinene (6.9%), limonene (10.8%), linalool (8.3%), and eugenol (7.7%) were the primary constituents of the essential oils of *A. chamaecistus* subsp. *scoparia* (Haghir Ebrahimabadi et al., 2016), aromatic fatty acid, fatty alcohol, and alcohol.

Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The flavonoid content of both extracts of *A. orientalis* was evaluated by colorimetric assay, using quercetin as a standard flavonoid compound. TFC of *A. orientalis* in ethanolic and aqueous extracts was shown in Table 2. The total flavonoid content of the extracts was calculated from the regression equation of the calibration curve ($Y = 0.0124x + 0.5158$; $R^2 = 0.991$) and

expressed as mg quercetin equivalents (QE) per mg of extract where TFC in the ethanolic extract was higher than in the aqueous extract. On the other hand, total phenolic contents in both extracts of *A. orientalis* were determined by Folin-Ciocalteu assay using gallic acid as the standard. The absorbance values from various concentrations of gallic acid were used to generate the calibration curve. The total phenolic content was calculated from the regression equation of the linear calibration curve ($Y = 0.0042x + 0.1195$; $R^2 = 0.994$) and expressed as mg gallic acid equivalents (GAE) per gram of extract. As shown in Table 2 the ethanolic extract had higher TPC than the aqueous extract.

Plants contain many phytochemical compounds, including phenolic acid and flavonoid compounds. Because of their antioxidative and possibly anticarcinogenic properties, the effect of these phytochemicals is currently of great interest. In addition to their metal-chelating abilities, phenols and flavonoids act as free radical scavengers, reducing agents, and singlet oxygen quenchers. Because of their antioxidant activities in chelating redox-active metal ions, halting lipid-free radical chains, and reversing hydroperoxide conversions into reactive oxyradicals (Swallah et al., 2020).

Ajuga species contain a wide range of phenolic and flavonoid compounds, which may improve the antioxidant activities of extracts. The variation in phenolic and flavonoid content could also be attributed to the status of various secondary metabolites in other growing locations, genetic factors, or ecological factors (Toiu et al., 2019). Rauca et al. (2019) reported that the total phenolic and flavonoid contents of *A. genevensis*, *A. chamaepitys*, and *A. laxmannii* ethanolic extracts were higher than methanolic extracts. Furthermore, *A. reptans* and *A. genevensis* ethanolic and methanolic extracts showed higher phenolic than flavonoid contents (Toiu et al., 2019). The results of the previous studies were much higher than our study's phenolic and flavonoid contents. In another study, the total phenolic content from the ethanolic extract of *Ajuga chamaecistus* subsp. *scoparia* was 2.64 mg GAE/g DW (Movahhedin et al., 2016), which is lower than the *Ajuga* species considered herein.

Table 1. Gas chromatography mass spectrometry (GC-MS) analysis of methanolic extract of the aerial parts of *A. orientalis.*

Peak No.	RT	Compound	Relative area (%)	M.W	M.F
1	6.0	Benzoic acid, methyl ester	5.3	136	$C_8H_8O_2$
2	7.3	Unknown			
3	8.2	Cyclohexasiloxane, dodecamethyl-	1.9	444	$C_{12}H_{36}O_6Si$
4	8.9	Benzeneacetic acid, 3-methoxy-.alpha.,4-bis[(trimethylsilyl)oxy]-, ethyl ester	2.9	370	$C_{17}H_{30}O_5Si_2$
5	9.7	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	1.3	430	$C_{12}H_{36}O_6Si$
6	10.3	Mollugin, trimethylsilyl ether	4.1	356	$C_{20}H_{24}O_4Si$
7	10.8	2-Octylcyclohexanone	0.3	210	$C_{14}H_{26}O$
8	11.1	3,4-Dihydroxyphenylglycol, 4TMS derivative	0.9	458	$C_{20}H_{42}O_4Si_4$
9	11.2	Pentadecanal-	1.2	226	$C_{15}H_{30}O$
10	11.5	4-(Trimethylsilyl)oxy-3-methylbenzoic acid, trimethylsilyl ester	5.4	296	$C_{14}H_{24}O_3Si_2$
11	12.0	Hexadecanal	1.3	240	$C_{17}H_{34}O$
12	12.2	Unknown			
13	12.4	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, (2E,7R,11R)-	1.1	338	$C_{22}H_{42}O_2$
14	12.7	Benzeneacetic acid, alpha,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester	7.4	472	$C_{20}H_{40}O_5Si_4$
15	13.0	Hexadecanoic acid, methyl ester	12.8	270	$C_{17}H_{34}O_2$
16	13.7	Cyclononasiloxane, octadecamethyl-	5.4	666	$C_{18}H_{54}O_9Si_9$
17	14.1	9-Octadecenoic acid, methyl ester, (E)-	27.2	296	$C_{19}H_{36}O_2$
18	14.3	Methyl stearate	9.6	298	$C_{19}H3_8O_2$
19	14.6	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane	4.8	443	$C_{13}H_{40}O_5Si_6$
20	15.5	Unknown			
21	16.3	Ethyl 4-hydroxyphenylacetate, TBDMS derivative	1.7	294	$C_{16}H_{26}O_3Si$

RT: retention time; M.W: molecular weight; M.F: molecular formula.

Table 2. Total phenolics, and flavonoid contents of ethanolic and aqueous extracts of *A. orientalis.*

Sample	Total phenolics (mg GAE/g of extract)	Total flavonoid (mg QE/g of extract)
A. orientalis ethanolic extract	7.7 ± 0.11	7.9 ± 0.24
A. orientalis aqueous extract	0.4 ± 0.21	0.2 ± 0.22

Values are represented as mean ± SEM (n = 3); GAE: gallic acid equivalent EQ: quercetin equivalent.

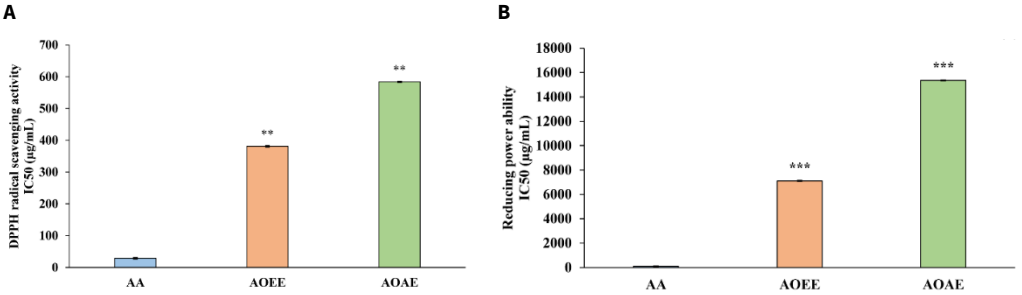

Figure 2. The antioxidant activity of *A. orientalis* ethanolic extract (AOEE), *A. orientalis* aqueous extract (AOAE), and ascorbic acid (AA) (standard).

(A) DPPH radical scavenging activity; **(B)** reducing power ability. Values are presented as mean (IC50 µg/mL) ± SEM of a triplicate measurement. *P<0.05, **p<0.01, ***p<0.001 *vs*. AA (ANOVA, Tukey post hoc test).

Antioxidant capacity

The antioxidant capacity of both extracts of *A. orientalis* were determined using DPPH radical scavenging assay and reducing power ability. The results were presented in Fig. 2A. In general, the results revealed that the ethanolic extract had a higher antioxidant activity than the aqueous extract. On the other hand, both extracts had lower antioxidant activity than ascorbic acid (standard).

The antiradical capacity of both extracts was assessed using DPPH free radical scavenging activity. The results were expressed as mean ± SEM of IC_{50} (µg/mL). The data showed that the antioxidant activities of both extracts were significantly lower than that of ascorbic acid (standard). For the DPPH scavenging activity (ethanolic extract 381.4 ± 0.61; aqueous extract 584.1 ± 0.23 *vs*. ascorbic acid 28.7 ± 0.51, p<0.01) as shown in Fig. 2A. On the other hand, the reducing power activity was higher in ethanolic extract (7110.0 ± 0.36) than aqueous extract (15356.0 ± 0.41) and significantly lower than ascorbic acid (91.50 ± 0.12) (p<0.001), Fig. 2B.

The plants containing abundant phenols and flavonoid compounds are a probable source of natural antioxidants. The ability of phenolic compounds to act as reducing agents, electron donors, oxygen quenchers, or metal chelators is mainly attributable to their redox characteristics (Al-Mansoub et al., 2021). The antioxidant activity of *A. ciliata* Bunge (PAB) was reported by Zhang et al. (2019). Polyphenols from the mentioned plant showed considerable hydroxyl radical scavenging, superoxide anion radical scavenging, and DPPH free radical scavenging abilities (Zhang et al., 2019). Additionally, the methanolic extract of *A. avi* exhibited significant phenolic compounds responsible for antioxidant and antidiabetic activities (Fettach et al., 2019). In our study, both extracts of *A. orientalis* showed lower antioxidant activities compared to the previous studies. The environmental conditions, particularly the plant's habitat, are factors affecting the formation of secondary active components in the plant and the antioxidant activity (Toiu et al., 2019).

Cytotoxic activity

In vitro assessment of antiproliferative activity of the ethanolic and aqueous extracts of *A. orientalis* against human cancer cell lines (MCF-7 and Caco-2) and normal dermal fibroblast cell line (HDFa) using the MTT assay showed that the aqueous extract of *A. orientalis* was very toxic against colon cancer line (Caco-2) compared to MCF-7 cell line and selective towards the HDFa (Table 3). The ethanolic extract of *A. orientalis* demonstrated a higher cytotoxic effect against the MCF-7 cell line than the Caco-2 cell line. Treatment of the ethanolic and aqueous extracts of *A. orientalis* for 72 h revealed no cytotoxic activity towards HDFa normal dermal fibroblast cell line with IC_{50} values 227.2 ± 0.02 and 119.3 ± 0.03 µg/mL, respectively. In addition, the results were compared to the standard cytotoxic drug (doxorubicin) (Table 3). Moreover, compared to non-cancerous cells, the ethanolic extract of *A. orientalis* demonstrated high selectivity against MCF-7 cancer cells and low selectivity against Caco-2 cells. On the other hand, the aqueous

Table 3. Cytotoxicity and selectivity index (SI) of ethanolic and aqueous extracts of *A. orientalis* and doxorubicin on MCF-7, Caco-2 and HDFa cell lines at 72 h of treatment.

Cell line	***A. orientalis* ethanolic extract** IC_{50} **(µg/mL)**	SI	***A. orientalis* aqueous extract** IC_{50} **(µg/mL)**	SI	**Doxorubicin (standard)** IC_{50} **(µg/mL)**	SI
MCF-7 breast cancer	59.32 ± 0.04*	4.67	29.37 ± 0.10**	4.06	0.375 ± 0.001**	0.096
Caco-2 colon cancer	239.8 ± 0.01	1.16	2.059 ± 0.01***	57.94	0.247 ± 0.02**	0.146
HDFa normal dermal fibroblast	277.2 ± 0.02		119.3 ± 0.03		0.036 ± 0.02***	

Data are represented as mean ± SD of three independent readings. Doxorubicin was used as standard cytotoxic drug. *p<0.05, **p<0.01, ***p<0.001. SI: selectivity index, a SI value > 3 indicates high selectivity (Chothiphirat et al., 2019).

extract exhibited remarkable selectivity against both tested cancer cells. While doxorubicin showed very low selectivity.

On the other hand, the antiproliferative activity of ajugalide-B (ATMA) from *A. taiwanensis* was tested against a variety of human cancer cell lines, including lung adenocarcinoma (A549) gastric carcinoma (AGS), hepatoma (HepG2), and colon carcinoma (HT29). ATMA disrupted the focal adhesion complex and decreased phosphorylation by paxillin and focal adhesion kinase (FAK) phosphorylation. Also, it reduced the tumorigenic and metastatic abilities of human lung cancer cell line A549 (Chiou et al., 2012). Furthermore, the cytotoxic effect of *A. chamaecistus* ssp. *tomentella* (using various extracts) against various cell lines, including colon carcinoma (HT-29), colorectal adenocarcinoma cells (Caco-2), breast ductal carcinoma (T47D), and Swiss mouse embryo fibroblasts (NIH-3T3) was investigated. The (n-hexane) extract was cytotoxic against all cancer cell lines tested, whereas the diethyl ether fraction had moderately cytotoxic against HT-29 (Sadati et al., 2012).

In addition, the antiproliferative potential of ethanolic extracts of *A. genevensis*, *A. chamaepitys*, and *A. laxmannii* on murine colon carcinoma cell line (C26) and murine melanoma cell line (B16.F10) was assessed by Rauca et al. (2019). The ethanolic extract of *A. laxmannii* demonstrated the most potent cytotoxicity (IC_{50} = 176.3 and 236.8 µg/mL) on C26 cells and B16.F10 cells, respectively. Our results in this study were consistent with the above research regardless of the plant species, type of cells, and the dosages used.

Furthermore, the selectivity index (SI) shows the high selective effects of the ethanolic extract of *A. orientalis* against MCF-7 cancer cells (SI > 3) and low selectivity against Caco-2 cells (SI < 3) *versus* non-cancerous cells (Chothiphirat et al., 2019). Additionally, *A. orientalis* aqueous extract demonstrated high selectivity (SI > 3) against both cancerous cells (MCF-7 and Caco-2), respectively. On the other hand, doxorubicin showed low selectivity against both cancerous cells (Table 3).

CONCLUSION

The findings of our study demonstrated that the ethanolic extract of *A. orientalis* has higher phenolic and flavonoid contents than aqueous extract. In addition to that, both extracts of the mentioned plant showed moderate *in vitro* antioxidant activity. *In vitro* antiproliferative effect of ethanolic extract of *A. orientalis* reported potent cytotoxic effect against the MCF-7 cell line. Compared with the aqueous extract of *A. orientalis* was very toxic against the colon cancer line (Caco-2). Also, both extracts did not exert any cytotoxic effect towards HDFa normal dermal fibroblast cell line, which indicates the potential chemopreventive role of *A. orientalis* against breast and colon cancer. Further studies should be carried out to study the underlying mechanism of cytotoxicity and isolate the bioactive compounds.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Oran SA	Althaher AR	Al Shhab MA
Concepts or ideas	x	x	
Design	x	x	x
Definition of intellectual content	x	x	
Literature search	x	x	x
Experimental studies		x	x
Data acquisition	x	x	x
Data analysis	x	x	x
Statistical analysis		x	x
Manuscript preparation		x	x
Manuscript editing	x	x	x
Manuscript review	x	x	x

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