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Original article

Evaluation of Antioxidant, Antibacterial and Cytotoxic Activity of Methanol Extract from Leaves and Fruits of Iranian Squirting Cucumber (*Ecballium elaterium* (L.) A. Rich)

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Abstract

Background and objectives: *Ecballium elaterium* (L.) A. Rich (squirting cucumber) has been used traditionally as a remedy for different disorders such as fever, sinusitis, and rheumatic disease. In the present study, antibacterial, antioxidant, and cytotoxic activities of leaves and fruits were evaluated. Also, total flavonoid and phenolic contents were measured were measured. **Methods:** The fruits and leaves of *E. elaterium* were extracted by percolation method with methanol. The antioxidant activity was assessed by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) methods. Then, the total phenolics and flavonoids contents were measured. The cytotoxicity was tested against three cancerous cell lines (MCF-7, MDA-MB-468 and MKN-45) and a normal cell line (HDF). Antibacterial activity was investigated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. **Results:** The total phenolics content of leaves and fruits were 39.97 and 30.90 mg gallic acid equivalents/g of dry extract, respectively. Also, the total flavonoids content in leaf extract was 49.17 mg quercetin as equivalents/g of dry extract while flavonoids were not detected in the fruit extract. In the DPPH assay, the IC_{50} values were 1.15 and 1.18 mg/mL for leaves and fruits, respectively. The fruit extract showed the most considerable antibacterial activity (MIC 37.5 mg/mL) against *P. aeruginosa*. Both extracts indicated cytotoxicity on MDA-MB-468 cells (IC_{50} 264 and 50 µg/mL, respectively). **Conclusion**: The antioxidant, antibacterial, and cytotoxicity of extracts may be due to some secondary metabolites like phenolic compounds and flavonoids. This study suggests that this plant could be considered for further investigations as a natural source of biological compounds.

Keywords: anti-bacterial; antioxidants; cytotoxicity; *Ecballium elaterium*; flavonoids

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Introduction

Ecballium elaterium (L.) A. Rich, commonly called squirting or spitting cucumber, is a wild plant in Cucurbitaceae family [1]. It is distributed in the western Asia and Mediterranean area, as well as Moghan, Ardabil province in Iran [2]. It is also cultivated in central Europe and England [1]. The fruits are ovoid, fleshy, roughly 4 cm in length, comprising black seeds and a liquid known as "juice of *E. elaterium*" [1,2].

The fruit extract of *E. elaterium* has been traditionally used in different medicinal conditions such as fever, sinusitis, cancer, liver

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cirrhosis, hypertension, rheumatic disease, jaundice, constipation, and dropsy [1]. The fruit juice has been used to cure sinusitis by dwellers of Moghan area in Iran [2]. Also, the fruits are generally used for the treatment of fever or flu in Tunisia [3]. In addition, the plant roots have been used as analgesic and to ameliorate hemorrhoids in Turkish folk medicine [4]. So far, various pharmacological studies have been performed on *E. elaterium* [5]. The anti-inflammatory property of *E. elaterium* water extract has been demonstrated in a rabbit model [6]. It has been suggested that the anti-inflammatory effect of this plant is due to production of chemotaxis factors such as TNF-α, IL-1, and IL-6 [7]. *Ecballium elaterium* extract can inhibit nitric oxide synthase which leads to decrease of nitric oxide (NO) metabolites as shown in rhinosinusitis patients [7,8].

Also, cytotoxic effect of freeze-dried extract from *E. elaterium* fruits has been demonstrated on gastric adenocarcinoma (AGS) and esophageal squamous (KYSE30) cell lines [2].

The juice of *E. elaterium* fruit has been known as a major source of lipids, proteins, sugars, triterpenoides (cucurbitacins), gum, carbohydrates, peptides, tannins, and minerals [3]. Generally, cucurbitacins and their glycosylated derivatives display a wide range of pharmacological activities like anti-inflammatory, antifertility, anticancer, and antimicrobial functions [9].

The fruit juice of spitting cucumber contains different structures of cucurbitacins such as cucurbitacins B, D, E, I, L, and R and cucurbitacins derivatives like hexanorcucurbitacins and glycosylcucurbitacins [9]. Among these compounds, cucurbitacin B is the most plentiful and active compound [10].

In the present study, some biological activity of the leaves and dried fruit extracts of *E. elaterium*, growing in North of Iran has been investigated.

Material and methods

Ethical considerations

This study was approved by the Ethical Committee of Guilan University of Medical Sciences (ID: IR. GUMS. REC. 1396. 247, Date: 6.10.2018).

Plant material

The aerial parts of *E. elaterium* were collected from Ardebil province in North-West of Iran, in September 2017. The plant was identified and given herbarium specimen number (HGUM-302). The voucher specimen was deposited at the herbarium of school of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran.

The leaves and fruits of plant (300 g each) were shade dried and crushed to a powder and then extracted by percolation with methanol, at room temperature for 24, 48 and 72 h. The solvent was evaporated by rotary evaporator to obtain methanol extract of leaves (61.8 g) and fruits (26.5 g). The extracts were stored in a refrigerator until required.

Total phenolics content

Total phenolics content of extracts were measured by the Folin-Ciocalteu method. One mL of each extract (1 mg/mL) was mixed with 5 mL of Folin-Ciocalteu reagent (formerly diluted tenfold with distilled water) and stand at room temperature for 10 min. Next, 4 mL sodium bicarbonate solution (75 g/L) was added. Then the mixture was permitted to stand for a further 30 min in the dark at room temperature. The absorbance was measured at 765 nm using a UV/VIS spectrophotometer UV/VIS spectrophotometer (Lambda 25 PerkinElmer, USA). Four known concentrations of gallic acid (GA) standard (25, 50, 70, 100, and 200 µg/mL) were used to plot the calibration curve. Finally, total phenolic contents of samples were quantified by using the calibration cure. The concentrations were expressed as mg of gallic acid equivalents (GAE)/ g of dry extract [11,12]. All the experiments were performed in triplicate

Total flavonoids content

Total flavonoid content was measured as described by Saeidnia and Gohari. [13]. Five mL of aluminum trichloride ($AlCl_3$) (2% in methanol) was added to 5 mL of extract (1 mg/mL). Absorbance of the mixture was measured at 415 nm after 10 min. Blank sample consisted of 5 mL extract and 5 mL methanol without $AlCl_3$. Total flavonoids content was measured using a standard curve of quercetin (0-100 mg/L). Total flavonoid content was expressed as mg of quercetin as equivalents (QE)/ g of extract [14].

DPPH radical scavenging activity

The antioxidant activity of samples was evaluated using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay [12]. Briefly, 1 mL of different

concentrations of each extract was added to 2 mL DPPH methanol solution (40 µg/mL). The absorbance was measured at 517 nm after 30 min. The test was repeated three times for each concentration. Vitamin E was used as the positive control [15]. Percentage of radical scavenging activity of the fruits and leaves extracts was calculated by using the equation: inhibition% = $[(A_0-A_s)/A0]\times100$, where A_0 is the absorbance of the control and A_s is the absorbance of the sample. IC_{50} values (indicating the concentration of the extract (mg/mL) providing 50% radical scavenging) were calculated from the graph-plotted scavenging percentage against extract concentration [16].

Antibacterial activity

Antibacterial activity of fruit- and leaf extracts were assessed against one Gram-positive (*Staphylococcus aureus* ATCC25923) and three Gram-negative bacteria (*Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC27853, and *Escherichia coli* ATCC25922) by broth micro dilution (BMD) method according to Clinical and Laboratory Standards Institute (CLSI) guideline and the minimum inhibitory concentration (MIC) was measured [17].

Bacterial strains were provided from Bahar Afshan company, Tehran, Iran. The bacteria were maintained in nutrient agar (Merck, Germany) slants at 4 °C and sub-cultured in Petri plates prior to use. Bacterial suspension was matched with concentration of 0.5 McFarland standards (10^6 CFU/mL).

For determination of MIC value, 96 U-shaped wells plates were used. Two-fold serial dilution of the stock solution of each extract (100 µL) was prepared using 100 µL of Mueller Hinton Broth (MHB) in ten wells. Finally, 2.5 µL of bacterial suspension equivalent to the 0.5 McFarland standards was added to each of the wells. DMSO and gentamicin were used as negative and positive controls, respectively. After incubation at 37 °C for 24 h, the lowest concentration of each sample that inhibited the visible growth of the tested strain was considered as MIC value [18,19].

Cell cultures and cytotoxicity assay

In this study, three cancerous cell lines including human breast cancer cell lines (MCF7 and MDA-MB-468) and Human poorly differentiated gastric cancer (MKN-45) and human dermal fibroblast (HDF) as a normal cell line were used to evaluate the cytotoxic activity of methanol extract from leaves and fruits of this plant. All cell lines were bought from Pasteur Institute, Tehran, Iran.

Three cancerous cell lines were cultured in RPMI1640 medium (Gibco, Germany), and the normal cells (HDF) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Germany), supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, Sweden), at 37 °C in humidified air containing 5% CO_2. The cytotoxicity of methanol extracts of leaves and fruits was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly 1×10^4 (cells of each cancerous cell line /well) was plated in 96-well plates. The cells were incubated at 37 °C, in 5% CO_2, for 24 h and a humidified atmosphere. Different concentrations of the extracts (25, 50, 100, 200, 300, 400, 500, 600, 800, and 1000 µg/mL) were added to cells and for 24 and 48 h, separately. Then, MTT (0.5 mg/mL, 100 µL) which was dissolved in phosphate buffer saline (PBS), was added per well and incubated for 4 h. Then MTT solution was removed and the cells were washed with PBS (2×100 µL). One hundred microliters of DMSO was added per well, and the plate was shaken at 100 rpm for 10 min to solubilize the formazan crystals.

The absorbance was measured at 570 nm using an ELISA reader apparatus (Stat Fax 2100, Awareness, USA) and the cell survival was assessed [13]. The IC_{50} values (median growth inhibitory concentration) were obtained from the IC_{50} of concentration-response curve in the Microsoft office excel (2010) software. Each data was the mean value of three independent experiments and presented as mean ± SD. All experiments were performed in triplicate.

Results and Discussion

In the present study the antibacterial, antioxidant and cytotoxic activities as well as total phenolics and flavonoids contents of *Ecballium elaterium*, collected from North-West of Iran were investigated.

The total phenolic content of leaf and fruit extracts have been reported in table 1, in reference to the standard curve for gallic acid ($y = 0.00088x - 0.0388$, $R^2 = 0.9975$).

Table 1. Total phenolics and flavonoids contents and antioxidant activity of methanol extract from fruits and leaves of *Ecballium elaterium*

Sample	Total phenolics	Total flavonoids	DPPH IC_{50} (mg/mL)
fruit	30.90±0.003	-[a]	1.18
leave	39.97±0.01	49.17±0.01	1.15
Vitamin E	-	-	0.014

Results have been expressed as mean±SD of 3 determinations; [a] not detected; total phenol expressed as: mg of gallic acid equivalents/g of dry extract; total flavonoid expressed as mg of quercetin as equivalents/g of extract

The total flavonoid content was measured with regarding to standard curve of quercetin ($y = 0.0173x - 0.3984$, $R^2 = 0.9933$) (table 1). Flavonoids were not detected in fruit extract.

Two similar studies were conducted on *E. elaterium*, growing in Tunisia. Felhi et al., reporting that the phenolics and flavonoids contents of the methanol extract from fruit peels were 107 ± 4 mg GAE/g and 18 ± 0.6 mg QE/g, respectively [20]. Furthermore, Abbassi et al. investigated the total polyphenol contents of different parts of this plant including fruits, leaves, roots, and flowers. The results showed that fruits and leaves contained 43.61±1.65 and 46.84±2.5 mg GAE/ g dry weight, respectively [4]. Also, the flavonoid content in fruits and leaves were 9.91±0.5 and 17.38±1.65 mg catechin equivalents CE/ g dry weight [4].

In comparison with Abbassi et al. study, the results of the present study also showed that both fruits and leaves contained approximately the same amount of phenolic compounds.

The antioxidant capacity of both extracts was determined by DPPH radical scavenging test and the results have been showed in table 1. Felhi et al., showed that the scavenging power of peels of fruit extract was 1.2± 0.1 mg/ml [17] which is comparable with the results of this study.

The results of antibacterial tests have been displayed in table 2.

Table 2. The minimum inhibitory concentration (MIC) of methanol extracts from fruits and leaves of *Ecballium elaterium*

	MIC(mg/mL)	
Bacteria	**Fruits**	**Leaves**
Staphylococcus aureus	75	75
Escherichia coli	75	150
Pseudomonas aeruginosa	37.5	75
Enterococcus faecalis	75	150

Note: MICs were expressed in mg/mL and measured by broth micro dilution (BMD) method

It was demonstrated that both fruit- and leaf extracts had antibacterial activities against tested bacteria. The range of the MIC was between 37.5 and 150 mg/mL. The fruit extract showed the highest antibacterial activity (MIC 37.5 mg/mL) against *P. aeruginosa* whereas its MICs against other tested strains including *S. aureus*, *E. coli* and *E. faecalis* were 75 mg/mL. The best antibacterial activity for leaves extract was reported against *S. aureus* and *P. aeruginosa* (MIC 75 mg/mL). This extract showed weaker activity against *E. coli* and *E. faecalis* (MIC 150 mg/mL).

In a study, effect of several extracts of *E. elaterium* were evaluated against some bacteria such as *S. aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *B. subtilis*, *Micrococcus luteus*, *E. coli*, and *Klebsiella pneumoniae* as well as some fungi like *Fusarium phyllophilum* and *Penicillium sp* [17]. It was demonstrated that acetone and diethyl ether extracts of fruit- peels had the highest antibacterial activity particularly against *Micrococcus luteus* (MIC 2 mg/mL, for both extract) while no antifungal effect was detected [17].

The results of another study displayed that the ethanol extract from fruits of this plant showed antibacterial activity against different strains of *S.aureus* (MIC ranged between 0.195-1.563 mg/mL). It also exhibited antifungal activity against *C. albicans* strains (ranged between 0.048- 6.25 mg/mL) [1]. In another investigation, the fruit extract of *E. elaterium* showed significant activity against number of isolated strains of *Klebsiella pneumonia* (MIC 32-64 µg/mL) [1]. The results of our study revealed that the effect of Iranian squirting cucumber on tested bacteria was lower compared to previous studies.

Several studies have indicated that natural chemical constituents of plant species are significantly influenced by different environmental agents like climate, geography, soil type and sun exposure. Consequently, the variation of these factors can lead to noteworthy changes in biological properties. Therefore, the results of antibacterial test in present study is different from plants that were grown in other countries [19,20].

The cytotoxic activity of extracts was investigated on three cancerous cell lines including MKN-45, MCF7, and MDA-MB-468 and a normal cell line (HDF). The results indicated that both leaf and fruit extracts showed cytotoxic activity on MDA-MB-468 cell line (IC_{50} 264 and 50 µg/mL, respectively). However,

the extracts did not show significant cytotoxicity on other cell lines in the range of evaluated doses. The results indicated that both leaf and fruit extracts showed cytotoxic activity on MDA-MB-468 cell line (IC_{50} 264 and 50 µg/mL, respectively). However, the extracts did not show significant cytotoxicity on other cell lines in the range of evaluated doses (25-1000 µg/mL).

The results of an investigation showed that aqueous extract from *E. elaterium* fruits showed cytotoxic activity on two human cancerous cell lines including human gastric carcinoma (AGS) and human esophageal squamous cell carcinoma (KYSE30) with the IC_{50} 2.5 and 500 µg/mL, respectively [2,4].

Previously, the aqueous extract of *E. elaterium* was investigated on HepG-2 cell line by Ljubuncic et al. The IC_{50} value of this extract was 1000 µg/mL [2]. Their results were close to IC_{50} value of methanol extract of fruits and leaves on cancerous cell lines MKN-45, MCF-7, and normal cell lines in our study (>1000 µg/mL). However the cytotoxic effects of extracts on MDA-MB-468 cell line may be due to different compounds found in fruits and leaves. The fruits of *E. elaterium* are rich sources of bioactive compounds such as proteins, lipids, triterpenoides (cucurbitacins), and phenolic compounds [3,4]. Numerous amounts of these natural compounds such as phenolic compounds, triterpenoids, and flavonoids have antioxidant properties and scavenging activities [21]. Among them, phenolic compounds are powerful antioxidants and their significant roles in the prevention of medical conditions such as cardiovascular diseases, cancer, and inflammation have been demonstrated [22]. Antioxidants have been supposed to inhibit carcinogenesis during the beginning step because they exert radical scavenging effect, and are known as inducers, or inhibitors of xenobiotics metabolizing enzymes, including phase I and II enzymes [22,23]. They have also exhibited antimutagenic and anticarcinogenic effects [22,24]. The mechanism in which the phenolic compounds act as cytotoxic agents may be related to their pro-oxidant effects [22]. It is demonstrated that the polyphenols could be considered as both antioxidant and pro-oxidant, depending on the concentration and free radical source [25]. They are also well-known for their antimicrobial effects [26]. It is suggested that the antibacterial, antioxidant and cytotoxic properties of tested extracts may be due to this class of compounds. Moreover, cucurbitacins are a class of tetracyclic triterpenoids and have been firstly purified from plants of Cucurbitaceae family [27,28]. So far, significant cytotoxic activity has been reported from these compounds. For example, Cucurbitacin B was investigated for anti-proliferative activity against breast, pancreatic, laryngeal, and lung cancerous cell lines [10,27]. Similarly, cucurbitacin E showed high in vitro toxicity on Sarcoma Black, E0771 mammary adenocarcinoma, porostate human adenocarcinoma (PC-3) cells, breast (ZR-75-1), stomach (ST- 95-AT2), murine connective tissue (L929), and ovarian (OV-95-CC3) human carcinomas [28,29].

Jafargholizadeh et al., investigated the cytotoxicity of cucurbitacins D, E, and I, purified from aqueous extract of *E. elaterium*, on AGS cells and the IC_{50} values were 0.3, 0.1, and 0.5 µg/mL, respectively [9]. So, it is also suggested that the cytotoxic properties of fruits extract in our study, may be because of the existence of this group of natural compounds. In this study, Iranian *E. elaterium* which is named as squirting cucumber has been investigated for different biological activities. The fruit extract showed considerable antibacterial activity against *P. aeruginosa* while the leaf extract displayed the most activity on *S. aureus* and *P. aeruginosa*. Both extracts demonstrated significant cytotoxic activity on MDA-MB-468 cell line and also exerted approximately the same antioxidant activities. It is suggested that the antioxidant, antibacterial and cytotoxic activities of tested extracts may be due to numerous secondary metabolites such as phenolic compounds, triterpenoids, and flavonoids.

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Author contributions

Fatemeh Yousefbeyk and Masoud Hamidi designed and supervised the study; Saeed Ghasemi analyzed the data and was a consultant; Bahman Bavafa Bighdilou and Diba Eghbali Koohi performed the experimental parts; Fatemeh Yousefbeyk wrote the manuscript; all authors approved the final draft of the manuscript.

Declaration of interest

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

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Abbreviations

DPPH: 2,2´-diphenyl-1-picrylhydrazyl; MIC: minimum inhibitory concentration; BMD: broth micro dilution; IC_{50}: median growth inhibitory concentration, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide