

A Toxicological and Phytochemical Study on the Iran's *Ecballium elaterium* (L.) A. Rich.

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Abstract

Background: *Ecballium elaterium* is endemic to the northwestern Iran and its fruit is traditionally used for therapeutic purposes in Iran. The toxicological and phytochemical characteristics of the Iran's *Ecballium elaterium* have not been investigated so far.

Objective: Study on some toxicological and phytochemical features of the Iran's *Ecballium elaterium* fruit 90% ethanol extract.

Method: The antioxidant capacity in the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and total phenol and flavonoid contents were determined by spectrophotometry. The rutin (phytomyelin) content was measured by HPLC. Moreover, oral and intra-peritoneal LD₅₀ (median lethal dose) after 72 hours of the extract administration in rat were determined by the graphical method of Miller and Tainter. The cytotoxicity against several cancer and non-cancer cell lines was determined by the MTT (mitochondrial tetrazolium) method.

Results: The IC₅₀ (inhibitory concentration 50%) in the DPPH assay was 3.57±0.006 mg/mL. The total phenol in terms of mg Gallic acid/0.05 g extract was 17.84±0.92. The total flavonoid in terms of mg rutin/0.1 g extract was 33.35 ± 1.06. The extract contained 1 mg/g rutin. The oral and intra-peritoneal LD₅₀ of the extract were 472 mg/kg and 425 mg/kg respectively. The extract showed strong cytotoxicity in ACHN and ASPC1 cell lines, which was followed by Caco2 and PC12 cells. Non-neoplastic cells were much less affected by the extract in comparison with the cancerous cells.

Conclusion: The extract has low antioxidant activity. The oral and intra-peritoneal toxicities of the extract in the LD₅₀ test are moderate. The extract has considerable selective toxicity against cancer cells.

Keywords: *Ecballium elaterium*, Phytochemistry, Toxicity, Toxicology

Introduction

Ecballium elaterium (L.) A. Rich. (*E. elaterium*) (squirting or wild cucumber) is a perennial vine of the Cucurbitaceae family endemic to the Mediterranean region including the Azarbaijan and Guilan provinces of Iran [1]. In the areas where the plant grows, its fruit is traditionally used orally and topically for the treatment of various diseases such as sinusitis, fever, cancer, hepatic diseases, jaundice, constipation, hypertension, edema, rheumatic diseases and fungous infections [1-3]. Pharmacological studies have demonstrated that the plant's fruit has anticancer [4-6], antibacterial [3], antifungal [3], analgesic [7], antipyretic [7], anti-inflammatory [8], positive inotropic [9], hepatoprotective [10], blood bilirubin reducing [11], anti-leukemic [12] and anti-sinusitis [13, 14] effects, among others. The plant contains many bioactive compounds including phenols, flavonoids such as rutin (phytomelin) and triterpenoids (cucurbitacins) [15]. All parts of the plant especially fruits are considered as toxic [16]. Toxic and allergic reactions and even death in humans due to the use of the plant's fruit have been reported [16]. Considering the pharmacological effects and traditional uses of the plant, it is a potential source for development of new therapeutic agents [17]. However, little research regarding the plant's toxicity has been conducted. Moreover, the biological effects including toxicity and phytochemical characteristics of the Iran's *E. elaterium* have not been evaluated so far. Therefore, some toxicological and phytochemical parameters of the Iran's *E. elaterium* were investigated in the present study.

Materials and Methods

Plant collection

The fruits of *E. elaterium* were collected from the Moghan region of the Iran's Ardebil Province in July 2017 and a voucher specimen of the plant (number 5238) was deposited in the Central Herbarium of the Tabriz University (Iran).

Plant extraction

The plant fruits were dried, powdered (100 g) and macerated with a 90% ethanol solution for 3 days with three changes of the solution. The resulting extract was filtered and evaporated under vacuum into a dried powder extract. Then, the extract was dissolved in methanol and filtered through 0.45 μ membrane filter (Millipore), and injected directly for HPLC analysis.

Determination of the extract total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu colorimetric method according to a modification of the procedure described by Obanda [18]. In this method, the plant extract solution (1mL) was diluted with 5 mL distilled water and mixed with 500 μ L of the Folin-Ciocalteu reagent in a volumetric flask. 1 mL of 15% sodium carbonate solution was added to the mixture and allowed to stand for 30 min. Then, the absorbance was determined at 725 nm using a spectrophotometer (Human, USA). Gallic acid was used as standard to produce the calibration curve. The total phenolic content was expressed as mg of gallic acid equivalents per 100 g of

extract. All samples were analyzed in triplicates.

Determination of the extract total flavonoid content

Total flavonoid content was estimated by aluminum chloride colorimetric assay [19]. 1 ml of extract and standard solution of rutin (250, 500, 750, 1000 and 1250 mg/L) was added to 10 mL volumetric flask, containing 4 mL of distilled deionized water (dd H₂O). After 5 min, 0.3 mL 10% AlCl₃ was added and the total volume was made up to 10 mL with dd H₂O. The solution was mixed well and the absorbance was calculated against prepared reagent blank at 420 nm with a UV-Vis spectrophotometer. Data of total flavonoid content of dry extract was expressed as milligrams of rutin equivalents (Ru) per 0.1 g dry extract (mg Ru/0.1 g extract). All samples were analyzed in triplicates.

DPPH (2, 2- diphenyl – 1 - picrylhydrazyl) assay of the extract

The DPPH test was used to estimate the antioxidant capacity of the extract [20]. 1 ml of various concentrations (250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL) of the extract in ethanol was added to 4 mL of 0.004% methanol solution of DPPH. After a 60 min incubation stage at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I %) was calculated in following way:

$$I\% = \left[\frac{A \text{ blank} - A \text{ sample}}{A \text{ blank}} \right] * 100$$

A blank = Absorbance of the control solution (containing all reagents except the test extract).

A sample = Absorbance of the test extract.

Extract concentration providing 50% inhibition (IC₅₀%) was calculated from the graph of percent inhibition against extract concentration. IC₅₀% values were compared to IC₅₀% value of a “standard” antioxidant, in this case ascorbic acid, obtained by the same procedure.

Determination of the extract rutin content Preparation of standard solutions

10.0 mg of rutin was dissolved in 2 mL of methanol and diluted to 10.0 mL with 50% methanol. Also, 2 mL of this was diluted to 10 and 100 mL with 50% methanol. All sample solutions were filtered through 0.45 µm membrane filter (Millipore), and injected directly [15].

HPLC Analysis

The HPLC system was a Waters 600E with a UV-Vis detector (Waters 486) and a 50 µL sample loop. A discovery reversed phase YMC Triart-C18 (250 mm×4.6 mm i.d., particle size 5 µm) column was used. The mobile phase consisted of water with 1% glacial acetic acid (solvent A), and methanol (solvent B). The gradient was used for determination of rutin in the extract [15] with some modifications: 68% A/32% B, 0-5 min; 50% A/50% B, 5-20 min; 100% B, 20-35 min. The flow rate was 1.3 mL/min, and the injection volume was 20 µL. The monitoring wavelength was 350 nm.

Quantitative determination

Calibration curve of rutin was obtained by triplicate measurements on methanolic solutions of the standard at increasing concentration. The calibration curve was found by using the simple equation:

$$A = mC + b$$

Where C is the rutin concentration and A is the peak area, calculated by HPLC software.

Evaluation of the extract toxicity in rat

Different doses of the extract dissolved in normal saline were gavaged or intraperitoneally injected once to groups each consisting of 10 rats (5 males and 5 females) in a volume of 5 mL/kg. Then, the numbers of dead animals within 72 h after gavage or intraperitoneal injection were counted and LD₅₀ was calculated according to the graphical method of Miller and Tainter described previously [21, 22]. The extract doses were 5, 25, 50, 100, 250, 500, 750, 1000, 3000 and 5000 mg/kg.

Evaluation of the extract cytotoxicity in vitro

Caco-2 (colon adenocarcinoma), ACHN (renal adenocarcinoma), ASPC1 (pancreas adenocarcinoma) and PC12 (pheochromocytoma), and NIH3T3 (Swiss mouse embryo fibroblast) were obtained from Pasteur Institute of Iran (Tehran, Iran). RPMI (Roswell Park Memorial Institute medium) 1640, DMEM (Dulbecco's Modified Eagle's medium) and FBS (fetal bovine serum) were purchased from Biosera (UK). Pen-strep and trypsin- EDTA were purchased from Gibco (UK). MTT (3-[4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl tetrazolium bromide) was purchased from sigma (Germany).

Mitochondrial tetrazolium test (MTT) assay (23) was conducted to assess the viability of NIH3T3, ACHN, ASPC1, Caco2 and PC12 against various concentrations of the extract. The above-mentioned cell lines were maintained as exponentially growing cultures in either DMEM or RPMI 1640 cell culture medium supplemented with 10-15% FBS and antibiotics, under culture conditions. The plant extract was used at concentrations ranging from 1.953-1000 µg/mL doubling dilutions, and the cells were incubated for 24, 48 and 72 h. By 24 h intervals, cells were treated by MTT solution (5 mg/mL) and the absorbance was measured. Each experiment was performed in 4 repetitions and cells with no treatment were considered as control. The concentration required for 50% inhibition of cell viability (IC₅₀) were determined by a nonlinear regression analysis and expressed in mean ± standard error of mean (SEM).

Statistical analysis

Statistical differences between treated and control groups were examined by one-way analysis of variance followed by Turkey's tests (Stats-Direct version 3.1.18). Difference was considered statistically significant at P<0.05.

Results

Phenolic and flavonoid contents

The total phenolic compounds of the extract with gallic acid as standard and the flavonoid content of the extract in terms of rutin equivalent (the standard curve equation: $y = 0.001x + 0.0588$, $r^2 = 0.999$) are given in the table 1.

Antioxidant activity

The results of free radical scavenging capacity of the extract, measured with DPPH assay, and the ratios ($IC_{50\%}$) AA/ ($IC_{50\%}$) extract are presented in the table 1. They represent the ascorbic acid equivalent of the extract antioxidant capacity, i.e. the amount of ascorbic acid in milligrams equivalent to one milliliter extract.

Rutin content

Replicate injections of single standard at different concentrations were used to obtain calibration curve. The UV detection wavelength was set at 350 nm. The equation $y = 101.45 \cdot x + 6.132$ ($R^2 = 0.99$) with the applied rutin concentration (x) and the area (y) was obtained (Fig. 1). 1 mg/g of rutin was determined in the extract.

LD₅₀ of the extract in rat

The oral and intra-peritoneal LD₅₀ of the extract in rat were 472 mg/kg and 425 mg/kg respectively. There was no significant

difference between mortality of the male and female rats.

The extract cytotoxicity

The IC_{50} values presented in the table 2 show that the extract was different from control group ($P < 0.05$) and demonstrated cytotoxic activity on all experimental cell lines. Interestingly, the extract showed strong inhibitory activity against ACHN and ASPC1 cell lines after 48 h and 72 h followed by Caco2 and PC12 cells. NIH3T3 cells (as non-neoplastic cells) were less affected by the extract in comparison to other cell lines. This can suggest that the extract may be less toxic for the noncancerous cell lines.

Data expressed as IC_{50} values ($\mu\text{g/mL}$) represent means of four determinations. Abbreviations: Caco-2 (colon adenocarcinoma), ACHN (renal adenocarcinoma), ASPC1 (pancreas adenocarcinoma) and PC12 (pheochromocytoma), and NIH/3T3 (Swiss mice embryo fibroblast).

Table 1- The amounts of total phenolic and flavonoid compounds and antioxidant capacity of the extract ^a

Phenolic compounds (mg GA ^b /0.05 g extract)	Flavonoids compounds (mg Ru ^c /0.1 g extract)	DPPH radical scavenging activity $IC_{50\%}$ (mg/mL)
17.84±0.92	33.35 ± 1.06	3.57±0.006

^a Values represent means±S.D. of triplicate measurements.

^b GA, gallic acid.

^c Ru, rutin.

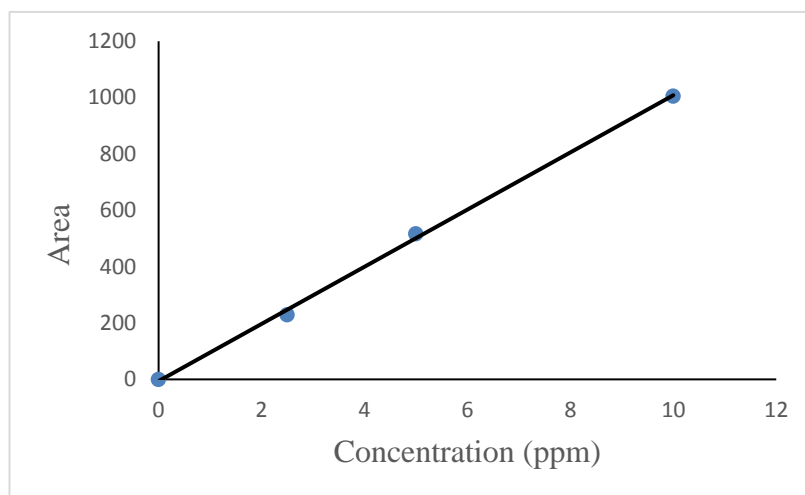


Figure 1- Calibration curve of rutin at 350 nm wavelength.

Table 2- IC₅₀ values of the extract effect on various cancerous and non-cancerous cells.

Cell lines	IC ₅₀ values (µg/mL)		
	24 h	48 h	72 h
Caco2	537.841	233.539	2.633
ACHN	419.183	<1.953	2.192
ASPC1	433.307	11.058	<1.953
PC12	702.122	88.817	139.429
NIH3T3	418.580	280.836	249.902
control	100	100	100

Discussion

The total phenol, flavonoid and rutin contents as well as antioxidant activity of the extract were measured. The extract LD₅₀ in rat and in vitro cytotoxicity were also evaluated. The IC₅₀ of the extract in the DPPH assay shows that it has low antioxidant activity. LD₅₀ of the extract administered orally and intraperitoneally were 472 mg/kg and 425 mg/kg. The oral and intra-peritoneal LD₅₀ of the extract seems to be comparable in this study. The mortality of male and female rats was not significantly different. Substances having LD₅₀ values in the range of 50-500 mg/kg are regarded as moderately toxic [22]. Thus, the 90% ethanol extract of the plant is

orally and intraperitoneally moderately toxic in the rat. The LD₅₀ tests conducted previously on the *E. elaterium* fruit are cited as follows. The intra-peritoneal LD₅₀ of the fruit extracted sequentially by chloroform, ethylacetate and 96% ethanol was 129.2 mg/kg in mice [7]. The intra-peritoneal LD₅₀ of the plant fruit petroleum ether extract was 3.34 g/kg in rat [24]. The intravenous LD₅₀ of the fresh fruit juice was 2.5 mg/kg in rat [25]. Oral LD₅₀ of fruit juice was 61 µL in mice [26]. The fruit juice seems to be more toxic than the fruit extracts in the LD₅₀ test. Besides, toxicity of the fruit extract appears to depend on the solvent used for extraction and rout of administration.

In order to know whether *E. elaterium* extract is able to affect the proliferation procedures, we followed the growth of 4 cancerous cell lines and 1 non-cancerous cell line in the absence or presence of the extract. We found for all cell lines, the number of cells in the wells were reduced in presence of the plant extract in culture medium. The inhibitory effect on tumor cell proliferation was dose-dependent and the *E. elaterium* extract seems to be a potent inhibitor of tumor cell proliferation. IC_{50} values below 20 $\mu\text{g/mL}$ represent cytotoxic activity [27]. *E. elaterium* extract demonstrated cytotoxic effect on Caco2, ACHN, ASPC1 with the IC_{50} values 2.63, 2.192 and <1.953 $\mu\text{g/mL}$, respectively. The extract showed strong cytotoxicity in ACHN and ASPC1 cell lines, which was followed by Caco2 and PC12 cells. Non-neoplastic NIH3T3 cells were much less affected by the extract in comparison with the cancerous cells. The extract seems to have considerable selective toxicity on several cancerous cell lines versus noncancerous cells which is an important feature of the extract.

The bioactive compounds and mechanisms mediating the toxicity of the *E. elaterium* extract were not investigated in the present study. Several studies reported that either *E. elaterium* or its isolated constituents from this particular species and/or other members of cucurbitaceae family act as natural anti-proliferative agents against several cell lines such as gastric, esophageal [5], prostate, melanoma, ovary [28, 29], leukemia [30], glioblastoma [31], breast [28, 32], hepatocytes [33], colon [34], peripheral lymphocytes [35, 36] and pancreatic cancer cells [37]. In line with

our results, the *E. elaterium* seed oil inhibited growth of human colonic adenocarcinoma (HT29) and fibrosarcoma (HT1080) cell lines with the IC_{50} values 4.86 $\mu\text{g/mL}$ and 4.16 $\mu\text{g/mL}$ [38]. Our findings in renal adenocarcinoma ACHN and pancreas adenocarcinoma ASPC1 cell lines were also similar to those of *E. elaterium* fruit freeze-dried extract for growth of human gastric carcinoma cell lines (2.5 $\mu\text{g/mL}$). In addition, we found much higher toxicity in all our cancerous cell lines after 48 h than that surveyed case of human oesophageal squamous carcinoma cells (500 $\mu\text{g/mL}$) [5].

Molecular and cellular investigations have shown that cucurbitacins (17 main molecules from cucurbitacin A to T) may affect several intracellular pathways related to the cancer cell proliferation and survival. For instance, they can strongly inhibit the JAK/STAT3 (Janus kinase/signal transducer and activator of transcription 3) signaling pathway mainly via the suppression of STAT3 expression/activation and also are able to disrupt PKB/Akt (protein kinase B also known as Akt) and MAPK/ERK (mitogen-activated protein kinase/extracellular-signal-regulated-kinase) pathways [39, 40]. The molecular mechanisms of action of cucurbitacins in human cancer cells was well reviewed by Lee et al., 2010 [41], though it was discussed that these compounds may proceed their inhibitory properties via varied pathways in different cell lines.

Recently, it was shown that *E. elaterium* seed oil and cucurbitacin B purified from this species potently suppressed human microvascular endothelial cells angiogenesis, also the authors claimed they have inhibited the adhesion, migration and proliferation of U87

glioblastoma cancer cells most probably mediated by $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrins [30].

To sum up, the present study suggests that the Iran's *E. elaterium* extract has moderate toxicity when given orally to rat and it has remarkable selective toxicity on cancerous compared to noncancerous cells. These features indicate that Iran's *E. elaterium* may be a potential source for development of new therapeutic and especially anticancer agents. Additionally, the active constituents and

mechanisms responsible for toxicity of the Iran's *E. elaterium* should be identified.

Conclusion

The Iran's *E. elaterium* fruit 90% alcoholic extract has moderate toxicity when administered orally and intraperitoneally to rat. Moreover, it demonstrates considerable selective toxicity against several cancer cell lines compared to noncancerous cells.

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