

RESEARCH ARTICLE

Purification of Cucurbitacins D, E, and I from Ecballium Elaterium (L.) A. Rich Fruits and Study of Their Cytotoxic Effects on the AGS Cell Line

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Abstract

Background: The plant *Ecballium elaterium* (L.) A. Rich, belongs to the Cucurbitaceae family which occupies an important position in traditional medicine prescriptions. It has been reported that a freeze-dried aqueous extract of *E. elaterium* fruits has cytotoxic effects on the AGS human stomach adenocarcinoma cell line. We here focused on anticancer effects of the main chemicals purified from *E. elaterium* fruits. **Materials and Methods:** We isolated cucurbitacins D, E, and I from chloroform, and ethyl acetate fractions of a methanolic extract of *E. elaterium* fruits and assessed their cytotoxic effects on the AGS cell line by MTT assay. The methanolic extract was fractionated to petroleum ether, chloroform, and ethyl acetate fractions. The compounds isolated by column chromatography were identified by NMR spectroscopy. **Results:** After 24 h of incubation with AGS cells, the IC₅₀ values were 0.3, 0.1, and 0.5 µg/ml for cucurbitacins D, E, and I respectively. **Conclusions:** This finding suggests that because of its cucurbitacins, *E. elaterium* fruit may have some cytotoxic effects on gastric cancer cells. Also, compared with D and I, cucurbitacin E showed greater potency in this regard.

Keywords: Ecballium elaterium- chromatography- cucurbitacins- gastric cancer- MTT assay

Asian Pac J Cancer Prev, 17 (10), 4631-4635

Introduction

There is a growing interest in the use of herbs as source of therapeutics because of wide variety of biologically effective chemicals in medicinal plants (Sporn and Suh, 2000). The *Ecballium elaterium* (L.) A. Rich, also known as squirting cucumber, is a wild medicinal plant that belongs to the Cucurbitaceae family. It is grown abundantly in the West Asia and Mediterranean region.

The most studied effective chemicals in Cucurbitaceae family are cucurbitacins, which are highly oxygenated tetracyclic triterpenes (Chen et al., 2005). These chemicals and their glycosylated derivatives exhibit a variety of biochemical activities such as anti-inflammatory, antifertility, anticancer, and antimicrobial functions (Ríos et al., 2005). Among the studied roles of cucurbitacins, their function as JAK/STAT inhibitor, MAPK modulator and cytoskeleton disruptor, propose their excellent potency for cancer treatment and prevention investigation (Lee et al., 2010).

It is reported previously that the *E. elaterium* juice contains cucurbitacins D, E, I, B, L, and R and cucurbitacins derivatives such as glycosylcucurbitacins and hexanorcucurbitacins (Attard and Scicluna-Spiteri,

2001; Seger et al., 2005a, 2005b). The probable potency of *E. elaterium* in the treatment of cancer has drawn increasing attention recently (Attard and Cuschieri, 2004; Attard et al., 2005; Bohlooli et al., 2012; Jacquot et al., 2014). Cytotoxic effect of freeze-dried extract of *E. elaterium* fruit on gastric adenocarcinoma and esophageal squamous cell carcinoma cell lines has been shown (Bohlooli et al., 2012). Also, cytotoxicity of cucurbitacin E extracted from *E. elaterium* and its induced morphological effects on ovarian cancer cells have been studied in vitro (Attard and Cuschieri, 2004; Attard et al., 2005). It is shown that cucurbitacin D purified from *E. elaterium* induce CDK1-mRNA up-regulation and causes proliferation arrest of a non-small cell lung carcinoma cell line (Jacquot et al., 2014).

Gastric cancer is considered as the fifth most common cancer in the world and the third leading cause of cancer mortality and morbidity (Ferlay et al., 2012). Bohlooli et al., (2012) reported that the freeze-dried extract of *E. elaterium* fruit has cytotoxic effects on human stomach adenocarcinoma cell line AGS. So, we decided to improve the anticancer studies of this plant by setting a purification method for some cucurbitacins (D, E, and I) of the endemic Mugan variety of *E. elaterium* and investigation of their

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effects on AGS cell line viability.

Materials and Methods

Chemicals

All solvents and reagents used for chromatography were purchased from Merck (Germany). MTT powder was obtained from Sigma. The human cancer cell line AGS and Human ForeSkin Fibroblast normal cell line Hu02 was provided from Iranian Biological Resources Center's Cell Bank (Tehran, Iran). All reagents and medium were prepared just before use. Cucurbitacins D, E, and I used as standards were obtained from Extra synthese, Genay, France. AGS cells were cultured in Ham's F-12 nutrient mix with L-glutamine and sodium bicarbonate (Cat. No. 10-FN1-500, G. Innovative Biotech Co, Iran) medium supplemented with 10% FBS (Cat. No.FB-st 500, Pasteur Institute of Iran). Hu02 cells were cultured in DMEM with L-glutamine medium (Cat. No. 12,800-058, Gibco) supplemented with 10% FBS (Cat. No.FB-st 500, Pasteur Institute of Iran). The cells were incubated at 37 °C in a water-saturated atmosphere of 5% CO₂ and 95% air until confluence.

Plant Material

Mature fruits of *E. elaterium* were collected from the Mugan region of Iranian Azarbaijan by Naser Jafargholizadeh (Figure 1) and the plant specimen authenticated by Professor Farideh Attar (Department of Botany, School of Biology, College of Science, University of Tehran) and deposited in Central Herbarium of Tehran University-Faculty of Science (TUH), Tehran, Iran with voucher N0 48500.

Isolation Procedure

The juice was squeezed from the fruits. Equal volumes of *E. elaterium* juice, and methanol were mixed by shaking at room temperature for 24 hours. The supernatant was collected and air-dried at room temperature (total extract). The total extract was re-extracted with petroleum ether and solvent was collected and air-dried at room temperature (petroleum ether fraction). Fractionation of the total extract was continued with chloroform and ethyl acetate separately and the collected samples were air-dried at room temperature (chloroform and ethyl acetate fractions).

Standards of the cucurbitacins D, E, I and petroleum ether, chloroform and ethyl acetate fractions were dissolved in methanol and developed on thin layer chromatography (TLC) with a chloroform-methanol (9:1) solvent system. TLC was performed on silica gel GF254. Spots were detected under UV light (254 nm) and spraying with anisaldehyde-sulfuric acid reagent by heating for 5-10 min at 120°C. The spots matching with standards were detected in chloroform and ethyl acetate fractions.

The chloroform fraction was chosen for further purification with column chromatography. A 5 cm × 60 cm column was used as the first column and filled with Silicagel 230-400 mesh as adsorbent. The column was eluted with a gradient of methanol and chloroform (0:100, 60:40) to give 8 fractions (CM1-CM8) and fractionation was monitored by TLC. The CM3 fraction was evaporated

and applied to a second column same as first one. The column was eluted with 80% chloroform: 20% ethyl acetate to 100% ethyl acetate and 8 fractions were achieved (CE1-CE8). The collected fractions monitored with TLC and discovered that fractions CE4, CE6 and CE7 are matched with cucurbitacin E, I and D respectively. For further purification these substances were subjected to thick layer chromatography on silica gel plate and isolated compounds were identified by NMR spectroscopy.

MTT assay

Cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5 biphenyltetrazoliumbromide (MTT) assay. The MTT assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure in vitro cytotoxic effects of drugs on cell lines or primary patient cells (van Meerloo et al., 2011).

AGS and Hu02 cells (1×10^4 cells/well) were seeded into a 96-well plate and were grown to 80% confluency. Isolated compounds were solubilized in DMSO and diluted with medium to the desired final concentration (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 1.5 or 2.0 µg/ml) and cells incubated for a further 24 h. The final concentration of DMSO showed no interference with the growth of the cell line. As a positive control, AGS and Hu02 cells were also incubated with the anticancer drug mitoxantrone for 24 h. After incubation, 100 µl of 1.0 mg/ml MTT was added to the cells for 3 h at 37 °C, followed by the addition of 100 µl DMSO as the solubilizing agent. The absorbance at 570 nm was read using a microplate reader (Rayto Life and Analytical Sciences Co., Ltd. China) and the proportion of cell survival was calculated by dividing the average absorbance of the treated cells by the average absorbance of untreated cells. All experiments were performed ten times in duplicate and data was analyzed with the Table Curve 2D/Ver 5.0 automated curve fitting and equation software.

Results

Identification of compounds

¹H-NMR of cucurbitacins was achieved with 500 MHz NMR in CDCl₃ (Table 1) and the results were compared with published data (Velde and Lavie, 1983; Seger et al., 2005a, 2005b).

MTT assay

Cytotoxic effect of the *E. elaterium* fruits on AGS gastric cancer cell line and Hu02 normal cell line were analyzed with MTT assay. After 24 h of incubation of AGS cells, IC₅₀ values for cucurbitacins D, E, I, and mitoxantrone were 0.3, 0.1, 0.5 µg/ml, and 50 µM (25.9 µg/ml) respectively (Figure 2). After 24 h of incubation of Hu02 cells in aforementioned concentrations, cucurbitacins D, E, I, and mitoxantrone induced 23%, 31%, 30%, and 20% lethality respectively.



Figure 1. *E. Elaterium* Fruits Harvested from Mugan Region of Azarbaijan.

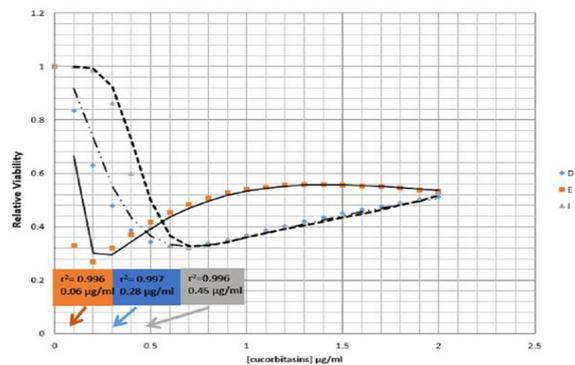


Figure 2. MTT Assay of Cytotoxic Activity of the Cucurbitacin E, D and I Purified from *E. Elaterium* Fruits on AGS Cell Line After 24 h, Compared to Control. D: 0.3 µg/ml; E: 0.1 µg/ml; I: 0.5 µg/ml

Discussion

The MTT assay of *E. elaterium* fruits cytotoxic effects on AGS gastric cancer cell line showed that cucurbitacin E has greater cytotoxicity in comparison with cucurbitacins D and I. The cytotoxicity of E were 4.7, 7.5 and 431.1 folds greater than D, I and mitoxantrone, respectively. IC50 values of cucurbitacins D, E, I, and mitoxantrone for AGS cells were 0.3, 0.1, 0.5 µg/ml, and 50 µM (25.9 µg/ml) respectively. However, in Hu02 normal cells the same concentration of cucurbitacins D, E, I, and mitoxantrone induced 23%, 31%, 30%, and 20% lethality respectively. Then, compared to normal cell line the cytotoxic effect of the cucurbitacins, especially cucurbitacin E, on cancerous cells was more drastic. This results could be considered as promising findings about application of cucurbitacins in gastric cancer therapy that is one of the most common cancers worldwide. There is a 10-fold variation in gastric cancer incidence internationally, with high rates seen in many countries of Eastern Asia, Central and Eastern Europe, and Central and South America, and much lower rates reported from North America, and Africa (Ferlay et al., 2012). Indeed, stomach cancer is the third leading cause of cancer death worldwide (Torre et al., 2015).

Herbal medicines are important sources of novel and potential pharmaceutical agents and natural therapies based on them are becoming increasingly important means of efficient treatment. Indeed, more than 70 percent

Table 1. ¹H-NMR (500 MHz, CDCl₃) of Cucurbitacins D, I and E

Compounds	Cucurbitacin D	Cucurbitacin I	Cucurbitacin E
Carbon No.	δ H(ppm), Hz	δ H(ppm), Hz	δ H(ppm)
1 a	2.3 m	5.9 d (2.68)	6.1 d
1 b	1.2 m	-	-
2	4.4 m	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	5.9 br d (5.6)	5.8 br s	5.7 m
7 a	1.9 m	2.4 m	
7 b	2.3 m	2.03m	
8	1.9 br d (8.1)	2.1 m	
9	-	-	-
10	2.7 br d (13.4)	3.5 br s	3.5 br s
11	-	-	-
12 a	3.2 d (14.7)	4.1 d (14.9)	
12 b	2.7 d (14.7)	2.7 d (14.9)	
13	-	-	-
14	-	-	-
15 a	1.4 d (12.7)	2.0 d (11.9)	
15 b	1.8 d (12.7)	1.4 d (10.0)	
16	4.3 m	4.4 m	
17	2.6 d (7.0)	2.6 d (7.1)	
18	0.9 s	1.0 s	0.9 s
19	1.1 s	1.0 s	1.1 s
20	-	-	-
21	1.4 s	1.4 s	1. s
22	-	-	-
23	6.7 d (15.0)	7.1 d (15.0)	6.3 d
24	7.12d (15.0)	6.6 d (15.0)	7.1 d
25	-	-	-
26	1.3 s	1.4 s	1.6 s
27	1.3 s	1.3 s	1.6 s
28	1.3 s	1.3 s	1.2 s
29	1.3 s	1.3 s	1.2 s
30	1.4 s	1.4 s	1.4 s
31	-	-	-
32	-	-	1.9 s

of new drugs that have been approved since 1981 have directly or indirectly been derived from natural products (Newman and Cragg, 2012). The *E. Elaterium* is a wild toxic herb from Cucurbitaceae family that produce cucurbitacin molecules and has medicinal importance in traditional treatment prescriptions (Attard and Scicluna-Spiteri, 2003).

Cucurbitacins are highly oxidized tetracyclic triterpenoids, which have been recognized as apoptosis

inducers in various cancer cell lines. Inhibition of JAK/STAT pathway and affecting MAPK pathway, PARP cleavage, expression of active caspase-3, decreasing pSTAT3 and JAK3 levels, as well as decreasing various STAT3 downstream targets such as Mcl-1, Bcl-2, Bcl-xL, and cyclin D3 were proposed as possible ways of cucurbitacins interference in cancerous cells (Rios et al., 2012).

Investigation of herb effects on gastric cancer has drawn more attentions in past decade. For example, the possibility of considering Phyllanthus urinaria extracts as chemopreventive agent for gastric cancer has been investigated (Lai et al., 2008). Methanolic extract of Momordica charantia exhibited cytotoxic effect against various cancer cell lines including AGS cell line (Li et al., 2012). The effects of aqueous extract of Sargassum pallidum (Turn.) C. on gastric cancer in rats has been reported (Zhang et al., 2012). And, induction of apoptosis in gastric cancer by the extracts or constituents of Citrus Reticulata Blanco was demonstrated (Kim et al., 2005). Anticancer potential of Hericium erinaceus extracts against human gastrointestinal cancers were studied (Li et al., 2014). Antitumor effect and apoptosis induction of Alocasia cucullata (Lour.) G. Don in human gastric cancer cells was reported (Wei et al., 2015). The Yiqi Jianpi Huaji Decoction (YJHD), a traditional Chinese medicinal formula composed of twelve herb ingredients, has recently been reported to act against human gastric cancer (Li et al., 2015). It is discovered that the extracts of endophytic fungus xkc-s03 from Prunella vulgaris L. spica inhibit gastric cancer (Tan et al., 2015) and the flavonoid rich extract of Myrcia bella induces cytotoxicity in gastric tumor cells (Serpeloni et al., 2015). And here our MTT assay showed that the cucurbitacins D, E, and I purified from E. elaterium fruit have cytotoxic effect on AGS cancer cell line. It would be interesting to investigate if mentioned cucurbitacins, especially E that showed more cytotoxicity, change the activities of cell cycle and/or ignite apoptotic genes and programmed cell death in AGS cells. These compounds may be used as adjuvant chemotherapy agents in gastric cancers. Nevertheless, the concentrations used in the current study were achieved by an in vitro study and the possibility to extrapolate these concentrations to clinical practice needs more investigations.

Acknowledgements

This research was supported by Iran National Science Foundation (INSF) grant number 90004329. The authors offer special thanks to Younes Aftabi from University of Mazandaran-Molecular and Cell Department, for his helpful suggestions and critical review of manuscript.

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