ORIGINAL ARTICLES

Cucurbitacin Glucosides and Biological Activities of the Ethyl Acetate Fraction from Ethanolic Extract of Egyptian Ecballium elaterium

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ABSTRACT

Column chromatography of the ethyl acetate extract afforded four compounds were isolated for the first time from the ethanolic extract of the whole plant Ecballium elaterium (L.) A. Richard, these compounds were identified as 2-O-β-D-glucopyranosylcucurbitacin E (1), 2-O-β-D-glucopyranosyl cucurbitacin I (2), rutin (3) and isorhamnetin 3-O-β-D-glucopyranosyl-β-D-glucopyranoside -7-O-glucosyl (4). Their structures were elucidated by different spectroscopic methods (UV, IR, MS, 1H NMR), direct comparison with authentic samples and /or literature data. Biologically, the ethyl acetate extract showed cytotoxic effect (IC₅₀ 19.12 µg/ml). The extract also, demonstrated significant antioxidant effect (SC₅₀ > 8.4µg/ml) compared with vitamin C (SC₅₀>7.4 µg/ml). The fungicidal activity of the extract was also studied and a significant result was obtained.

Key words: Ecballium elaterium (L.) A. Richard; Cucurbitaceae ; Cucurbitacin glycosides; flavonol glycosides; cytotoxicity; anti-oxidant, fungicidal activities.

Introduction

The plants of the family Cucurbitaceae commonly known as Gourd or Melon family. They are distributed through out the world; many of them are edible, while some are medicinal or even ornamental plants (Rizk, 1986). In Egypt, some of the cucurbitaceous species and varieties are widely distributed and have been cultivated since decades. Among these plants is Ecballium elaterium (L.) A. Richard, known as the (squirtng cucumber) or (spitting cucumber). It is a decumbent, perennial herb restricted to the Mediterranean Basin and cultivated in central Europe and England. It is common throughout the Mediterranean area as a medicinal plant (Kloutsos et al., 2001; Rust et al., 2003; Rios et al., 2005 and Kavalci et al., 2007). Ecballium elaterium is of interest today because its fruits extracts are still used in Mediterranean region in different medicinal system (Rust et al. 2003; Uslu et al., 2006). The diluted aqueous extract of the fruits is a traditional anti-inflammatory and analgesic for chronic sinusitis. It also possesses other uses especially the treatment of fever, cancer, liver disorders, jaundice, constipation, hypertension, dropsy, rheumatic diseases, and fungicidal (Rios et al., 2005; Latté, 2009; Mazokopakis et al., 2009 and Chan et al., 2010). Cucurbitacins seem to be responsible for the major pharmacological and biological effects of this plant (Rios et al., 2005; Balbaa et al., 1979; Everaldo and Anthony, 2001; Ahmnae et al., 1999; Attard et al., 2005; Abou-Khalil et al., 2009). All parts of the squiring cucumber are toxic, particularly the ovoid green fruits. Several toxicity and allergic reactions have been described if used undiluted (Kloutsos et al., 2001; Kavalci et al., 2007; Raikhlun-Eisenkraft and Bentur, 2000, Satar et al., 2001; Eken et al., 2008; Alcoceba et al., 2010 and Ghaleb Adwan et al., 2011).

Using plants for medicinal purposes is an important part of the culture and the tradition in Egypt. Indeed, Ecballium elaterium may be a great natural source for the development of new drugs and may provide a cost-effective mean of treating cancers and other diseases in the developing world. Reviewing the current literature, it was found that nothing was reported dealing with phytochemical and biological study of the whole plant cultivated in Egypt. Hence, the main goal of the present study was to investigate chemical content and the biological activities of the ethyl acetate extract obtained from the ethanolic extract of the whole plant cultivated in Egypt.

Here, it has been reported that isolation and structure elucidation of four compounds identified as 2-O-β-D-glucopyranosylcucurbitacin E (1), 2-O-β-D-glucopyranosyl cucurbitacin I (2), rutin (3) and isorhamnetin 3-O-β-D-glucopyranosyl-β-D-glucopyranoside -7-O-glucosyl (4) for the first time from whole plant. Biologically, cytotoxicity, antioxidant and anti fungal effect of the extracts have been investigated and significant results were pronounced.

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Materials And Methods

General:

UV spectra were measured in methanol by Schimadzu UV-260 Spectrophotometer (Japan); IR spectra were done on Jasco FT/IR 6100 Spectrophotometer; FAB and EIMS were carried out on Joel JMS-AX 500, 70 ev and Shimadzu GC/MS-QP5050A, 70 ev; 1H- spectra were run in DMSO-d6 and CD3OD at 300 using Varian Mercury-VX-300 NMR Spectrometer; Chemical shifts were reported in δ units relative to TMS; Column chromatography was carried out using silica gel (70-230 mesh, Sigma- Aldrich), silica gel HF254 and Sephadex LH-20; TLC was performed on silica gel coated aluminum plates (Merck kieselgel 60 F254, Germany) and paper chromatography (Whatman No. 1). Developed chromatograms were visualized under UV light and by spraying with anisaldehyde/sulphuric acid reagent followed by heating at 100°C for 10 min. For TLC analysis, the following solvent systems were used, chloroform-methanol-water (65: 35:10, lower phase, system 1), Benzene - ethyl acetate - formic acid - water (3: 5: 1:6: 0.4, system 11), Butanol-acetic acid - water (4: 1:5 system 111).

Plant material:

_Ecballium elaterium_ (L.) A. Richard was collected in the flowering stage on May 2006 from Sahel El-Arish, Sinai, Egypt. The plant was kindly identified by Dr. Ali M.A., Faculty of Environmental Agriculture science, Suez Canal University, North Sinai, El-Arish, Egypt. Voucher specimen is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University.

Extraction and isolation:

The air dried powdered whole plant (750g) was extracted by cold percolation using ethyl alcohol 90% till exhaustion. The combined extract was evaporated under reduced pressure at 45-50°C to give about 100 g greenish viscous residues. The obtained residue was dissolved in distilled water and then successively extracted by the following solvents with increasing polarities; petroleum ether, chloroform and ethyl acetate. Different fractions were combined, dried (anhydrous sodium sulphate) and concentrated to afford residues of 18 g, 2g and 6 g, respectively.

The active EtOAc fraction (6g) was subjected to vacuum column chromatography over silica gel HF254, eluting with gradient EtOAc to MeOH, to give three main fractions that showed a positive reaction to AlCl3 and or anisaldehyde/sulphuric acid reagent followed by heating at 100°C on TLC.

**Fraction 1** was purified over Sephadex LH-20 by elution with MeOH to give 32 sub-fractions, where sub-fractions 12-24 were pooled and further purified over Sephadex LH-20 by elution with MeOH to give compounds 1(35mg) and 2(26mg).

**Fraction 2** was purified over Sephadex LH-20 by elution with MeOH to give 16 sub-fractions, where sub-fractions 5-12 were further purified on a vacuum column packed with silica gel HF254 by elution with EtOAc: MeOH 8:2 which afforded compound 3 (75mg).

**Fraction 3** was purified over Sephadex LH-20 by elution with MeOH to give 34 sub-fractions, where sub-fractions 21-30 were pooled and further purified over Sephadex LH-20 by elution with MeOH:H2O 7:3 to give compound 4 (22mg).

**Compound 1:**

Yellowish- white cubic crystals (methanol), Rf 0.7 (system 1); IR (KBr, umax cm-1): 3410(OH), 1725 and 1271(acetate group), 1675, 1645 and 1630(C=O conjugated with double bond, β-unsaturated ketone, 2930, 2658 and 1073. FABMSm/z (% rel. abund.): 719 (M+1, C38H54O13,16%), 674(M+ - 43,35%), 659 (M+ - 60,35%), 557 (M+ - hexose, 40%), 495(10), 477 (8), 403 (8), 382(15), 230(75), 205 (19), 165(16), 164 (45), 113 (33), 112(33), 111 (25), 96 (100), 95(27), 60 (68) and 43(22). The 1H-NMR (300 MHz): δH0.88 (3H, s, C-18), 0.91 (3H7, s, C-19), 1.03(3H,s,C-28), 1.06(3H,s,C-29), 1.16 (3H, s, C-30), 1.24(3H,s,C-26), 1.29 (3H, s,C-27), 1.63(3H,s,C-21), 2.0 (3H, s25-OAC), 2.41(1H, d, J=6.3 Hz,C-17), 2.65 (1H', d, J=14 Hz,C-12 β), 3.05(1H,d,J=14Hz,C-12α), 3.28 (1H,br.,C-10), 3.33-4.1(sugar protons, m), 4.52(1H,br,m,C-16), 4.9(1H,d,J=6.3Hz,H1'), 5.15(1H,m,C-6), 5.71(1H,d,J=3.3Hz,C-1), 6.58 (1H,d,J=15.3Hz,C-23), 7.01 (1H,d,J=15.3Hz,C-24).
Compound 2:

Yellowish –white cubic crystals (methanol), $R_f$ 0.45 (system1); IR (KBr, $\nu$ cm$^{-1}$): 3424 (OH), 2985, 1635 and 1619 (C=O conjugated with double bond, $\beta$-unsaturated ketone), 1420, 1273, 2930 and 1077. FABMS$^+$ m/z (% rel. abund.): 677 (M$^+$ + 1, C$_{36}$H$_{52}$O$_{12}$,16%), 659 (M$^+$ - 18,15%), 515(M$^+$ - hexose,7 %), 498(118), 477(18), 403(25), 381(35), 232(50), 203(19), 165(48), 164(19), 113(43), 112(7), 96(33), 95(70), 68 (100) and 43(18). The $^1$H NMR (300 MHz): 8H0. 82 (3H, s,C -18), 0.84 (3H, s,C -19), 0.86(3H, s ,C-28), 1.20 (3H, s,C-29), 1.25 (3H, s,C-30), 1.29 (3H, s,C-26), 1.31 (3H, s,C-27), 1.34 (3H, s,C-21), 2.49 (1H, d, $J$=8.4 Hz,C-17), 2.54 (1H, d, $J$=14 Hz,C-12 $\beta$), 3.1 5 (1H, d, $J$=14 Hz,C-12 $\alpha$), 3.35 (1H, br., C-10), 3.38-4.09 (sugar protons, m), 4.58 (1H,br.,m,C-16), 4.93 (1H,d,$J$=6.3,H1'), 5.0 5 (1H,m,C-6 ), 5.91 (1H, d,$J$=3.3Hz,C-1), 6.88 (1H,d,$J$=15Hz,C-24).

Compound 3:

Yellow powder (methanol), $R_f$ 0.40 (system2); The UV (Table 1); EIMS m/z (% rel. abund.: 610(M$^+$,3%), 302(M$^+$ of aglycone,100%) for C$_{15}$H$_{10}$O$_{7}$, 286(44), 165(7), 162(7), 161(6), 154(19), 153(26), 152(18), 151(22), 147(11), 146(5), 137(86), 134(7), 124(10), 123 (13),118(5). $^1$HNMR (Table 2).

Compound 4:

Yellowish green powder (methanol), $R_f$ 0.28 (system2); The UV( Table1); FABMS$^+$ m/z (% rel. abund.: 803 (M$^+$ + 1, C$_{34}$H$_{42}$O$_{22}$, 10%), 789(60), 641 (35), 479(30), 317(57), 303(19), 287(45), 163(45), 153(18), 152(25), 149(63), 139(54), 124(47), 123(33), 103(35). $^1$HNMR (Table 2).

Acid Hydrolysis:

Compounds isolated (7mg each) were refluxed, separately with 7% aqueous sulphuric acid (10 ml) for 2hrs on boiling water bath. Then water was added and the mixture was extracted with chloroform. The aqueous layer in each case was neutralized with BaCO$_3$ and subjected to PC (solvent system111), investigation against authentic sugars, visualized by aniline phthalate spray reagent. All compounds gave reddish brown spot for glucose ($R_f$0.25). Compound 3showed beside glucose another spot for rhamnose ($R_f$0.38).

Biological study:

Cytotoxic activity (Hensen et al., 1989):

Cytotoxicity of ethyl acetate extracts was measured against Human hepatocarcinoma cell line (Hep- G2) using MTT Cell Viability Assay. The percentage viability was plotted against the extract concentrations and the 50% cell viability (IC$_{50}$) was calculated from the curve. The results are presented in Fig. (1).

Antioxidant activity (Van Amsterdam et al., 1992):

Antioxidant activity of the ethyl acetate extract was measured using DPPH method, in a flat bottom 96 well-microplate, a total test volume of 200 µl was used. In each well, 20µl of different concentrations (0-100µg/ml final concentration) of tested extract were mixed with 180µl of ethanolic DPPH (0.1mM) were mixed and incubated for 30 min at 37ºC.TriPLICATE wells were prepared for each concentration and the average was calculated. Then photometric determination of absorbance at 515 nm using microplate ELISA reader. The half maximal scavenging capacity (SC$_{50}$) values for tested extract and ascorbic acid was estimated via two competitive dose curves. Abs$_{50}$ of ascrobic acid= (Abs$_{100}$ – Abs$_{0}$)/2. SC$_{50}$ of ascrobic acid was calculated using the curve equation.SC$_{50}$ of ethyl acetate extract was determined using the curve equation utilizing Abs$_{50}$ of ascrobic acid.

Anti-fungal effect (El-Shawaf and Gomaa, 2000):

Aspergillus orizea, Aspergillus niger, Penicillium sp., and Saccharomyces cerevisiae were obtained from Dept. of Microbiology, Fac. of Agriculture Mansoura Univ., Egypt. Aspergillus orizea, Aspergillus niger and Penicillium sp. were plated with Potato Dextrose Agar, While Saccharomyces cerevisiae was plated with nutrient agar media. The ethyl acetate extract was injected into paper discs (6mm diamters) in amount of 20µl to determine its effect on microorganisms using minimum inhibition concentration technique during their growth at 30ºC for 5 days. Discs injected with 20µl of pure ethanol and ethyl acetate served as negative control.
The inhibition zones were measured in millimeter. The sensitivity of the organisms for the extract was recorded on in Table (3).

### Table 1: UV spectral data (nm) of the isolated compounds 3 and 4.

<table>
<thead>
<tr>
<th>Isolated compound</th>
<th>MeOH</th>
<th>NaOMe</th>
<th>AlCl₃</th>
<th>AlCl₃ + HCl</th>
<th>NaOAc</th>
<th>NaOAc/H₃BO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 3</td>
<td>256, 370</td>
<td>272,325,405</td>
<td>270, 458</td>
<td>265, 428</td>
<td>274, 390</td>
<td>258, 388</td>
</tr>
<tr>
<td>Aglycone of Compound 3</td>
<td>256, 296, 371</td>
<td>283, 330, 428</td>
<td>270, 306, 432</td>
<td>266, 298, 354, 428</td>
<td>270, 328, 382</td>
<td>261, 301, 387</td>
</tr>
</tbody>
</table>

### Table 2: ¹HNMR spectrum (ppm, CD₃OD, 300 MHz) of the isolated compounds 3 and 4.

<table>
<thead>
<tr>
<th>Isolated compound</th>
<th>δ ppm, CD₃OD, 300 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 3</td>
<td>6.18 (1H, d, J=2.1Hz, H-6), 6.38 (1H, d, J=2.1 Hz, H-8), 6.8 (1H, d, J=8.4Hz, H-5'), 7.7 (1H, d, J=2.7, 8.4 Hz, H-6'), 7.62 (1H, d, J=2.7Hz, H-2'), 5.1 (1H, d, J= 2.2 Hz, C1'''), 4.5 (1H, d, J= 8.4 Hz, C1''), 3.32 -3.92 m and 0.94 (3H, CH₃)</td>
</tr>
<tr>
<td>Compound 4</td>
<td>6.9 (1H, d, J=2.4 Hz, H-6), 7(1H, d, J=2.4 Hz, H-8), 7.85 (1H, dd, J=1.5, 8.7 Hz, H-6'), 7.52 (1H, d, J=1.5 Hz, H-2'), 7.22(1H, d, J= 8.7 Hz, H-5'), 5.4 (1H, d, J= 8.4Hz, H-1''), 3.7 (1H, Br, s), 3.85 (3H, s), 3.26-3.69 (sugar protons, m)</td>
</tr>
</tbody>
</table>

### Table 3: The antimicrobial activity of ethyl acetate extract of Ecballium elaterium.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Inhibition zone (mm/20 µl)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus orizea</td>
<td>13</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>10</td>
</tr>
<tr>
<td>Penicillium sp</td>
<td>8</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>6</td>
</tr>
</tbody>
</table>

*This value determined by difference from control (6 mm) of each. (15-20mm): very high sensitive; (10-15): high sensitive; (5-10): moderate sensitive; (<1-5): resistant

!(IC₅₀ 19.12 µg/ml; EtOAc extract against Hep-G2 cells; IC₅₀ = 19.12 µg/ml)

**Fig. 1:** Cytotoxic activity of ethyl acetate extract against Hep-G2 cells.

### Results And Discussion

Column chromatography of the ethyl acetate extract and repeated chromatographic methods on sephadex afforded four compounds (1, 2, 3, 4). This finding represents the first isolation of these compounds from *Ecballium elaterium* (L.) A. Richard cultivated in Egypt.

**Compound 1:**

A positive FAB mass spectrum of Compound (1) exhibited the [M+H]⁺ ion peak at m/z 719 , suggesting (M⁺)=718 and solving for *C₃₈H₅₄O₁₃* , the IR spectrum at 1725 and 1271 cm⁻¹ and the singlet at δ 2.0 ppm integrated for 3 protons confirmed the presence of acetate group. The β configuration of the carbon C-1'' is deduced by the chemical shift and the coupling constant of the anomeric proton H-1'' (δ 4.9, d, J=6.3 Hz). The ¹HNMR spectrum displayed signals comparable to the published data for 2-O-β-D-glucopyranosylcucurbitacin E (Maatooq et al., 1993; Seger et al., 2005 and Seger et al., 2004). Its structure was determined on the basis of the spectral data and by comparison of NMR data with those reported for closely related cucurbitacins (Maatooq et al., 1993; Seger et al., 2005; Seger et al., 2004). The IR, Ms and ¹HNMR confirmed the identity of this compound as 2-O-β-D-glucopyranosylcucurbitacin E.
Compound 2:

The FAB mass spectrum of compound (2) exhibited \([\text{M+H}]^+\) (positive ion mode) ion peak at m/z 677 solving for \(C_{36}H_{52}O_{12}\). The absence of absorption band at 1725 cm\(^{-1}\) in IR spectrum, as well as, the singlet at 8.2 ppm integrated for 3 protons in the \(^1\)HNMR spectrum confirmed the absence of acetate group from compound (2). The IR,Ms and \(^1\)HNMR of compound (2) were in good agreement with the reported data of 2-O-\(\beta\)-D-glucopyranosyl cucurbitacin I (Maatooq et al., 1993; Seger et al., 2005; Seger et al., 2004). Both Compounds (1) and (2) and their aglycones as well as various other cucurbitacins are well distributed in the family cucurbitaceae. To the best of our knowledge, this is the first report on the isolation of 2-O-\(\beta\)-D-glucopyranosyl cucurbitacin E and 2-O-\(\beta\)-D-glucopyranosyl cucurbitacin I from Ecballium elaterium L.

![Fig. 2: Structures of isolated compounds.](image)

Compound 3:

It was identified as rutin by UV (Markham, 1982; Mabry et al., 1996; Mabry et al., 1970 and Harborne et al., 1975) (Table 1) and \(^1\)HNMR (Vilatersana et al., 2000) (Table 2). It was further identified by co-spotting with standard rutin on TLC (solvent system11). Acid hydrolysis gave quercetin which was identified by TLC and UV (Markham, 1982; Mabry et al., 1996; Mabry et al., 1970 and Harborne et al., 1975) (Table 1). Glucose and rhamnose were confirmed by PC (system111). This is the first time to isolate and identify rutin from this plant. But it has been previously determined quantitatively from the leaves by UV (Tokr et al., 1997).

Compound 4:

It was found to be a flavonol glycoside from its diagnostic UV absorption bands (\(\lambda_{\text{max}}\) 257 and 359). UV spectrum and its changes in the presence of diagnostic shift reagents pointed to the presence of free hydroxyl at C5 and C4' of a 3,7-disubstituted flavonoid glycoside framework (Markham, 1982; Mabry et al., 1996; Mabry et al., 1970 and Harborne et al., 1975).

The secondary ion mass(SIMS) gave the \([\text{M}^+1]\)-peak at m/z803 (\(C_{34}H_{42}O_{22}\)) and the following fragments at \([m+641 (m-hexose),479 (m-2 hexose),317 (m-3hexose)]\), suggested that it is a flavonol triglycoside, this is confirmed by the presence of the three anomic protons arising from the three sugar moieties were observed at \(\delta\) 5.4, 5.31 and 4.5 in its \(^1\)HNMR spectrum (Kenneth et al., 1994). The appearance of two anomic signal above \(\delta\) 5 pointed to the presence of two aglycoen-sugar linkages, the other anomic signal being located at \(\delta\) 4.5, more typical for sugar- sugar linkage. All anomic signals had coupling constants of ca 8.4 Hz, an usual value for \(\beta\)-glucopyranose ring (Marco et al., 1989). The aromatic part in the \(^1\)HNMR spectrum marked downfield shift in the signals of H-6 and H-8 (\(\delta 6.9, 7\) ppm). This suggests one glycosyl residue being bound to C-7. Moreover, The 3',4'-dioxygenated B-ring appeared as an AMX system with signals at \(\delta 7.85 (dd, J=8.7,1.5\) Hz), 7.52 (d, \(J=1.5\) Hz) and 7.22(d, \(J=8.7\) Hz) which were assigned to the protons H-6', H-2' and H-5' respectively. Sharp singlet at \(\delta 3.85\) was originated by the methoxyl group at C-3' (Oscar et al., 1986). The shift
produced in the δ values of isorhamnetin by glycosylation were more or less as expected (Kenneth et al., 1994; Marco et al., 1989 and Oscar et al., 1986). Total hydrolysis of this glycoside yield a sugar fraction which consisted only of glucose using PC (system111). The previous data besides comparison with published data for similar flavonol glycoside (Markham, 1982; Mahry et al., 1996; Mabry et al., 1970; Harborne et al., 1975; Kenneth et al., 1994; Marco et al., 1989; Oscar et al., 1986) confirmed that compound 4 has to be isorhamnetin 3-O-β-D-glucopyranosyl-β-D-glucopyranoside -7-O-glucosyl. To our knowledge, this is the first report on the presence of this compound in Ecballium elaterium L. However, 13CNMR assignment of these compounds will be addressed upon isolation of further quantity of them.

Biological investigation:

Cytotoxicity activity:

As shown in the Fig. (1), the treatment of Hep-G2 cells with the ethyl acetate extract lead to a high inhibition in the cell proliferation as concluded by the low IC50 value 19.12 µg/ml, which revealed a strong anti-tumor activity of the extract against hepatic carcinoma. This cytotoxicity was also noticed starting from the low tested doses of the extract (12.5 µg/ml, and 25µg/ml). The ethyl acetate extract was not yet studied before.

Antioxidant activity:

The DPPH assay showed the extract possessed a high potential scavenging activity with low SC50 value (8.4 µg/ml) compared with the scavenging activity of the well-known antioxidant (ascorbic acid, A.A), with SC50 value as low 7.4 µg/ml. This result suggested that there is promising potential for using Ecballium elaterium in treating human diseases that involve free radical and oxidative damage.

Anti-fungal effect:

As shown in Table 3, the ethyl acetate extract exhibited significant level of activity against Aspergillus orizea and Aspergillus niger, moderate activity against Penicillium sp. and Saccharomyces cerevisiae was completely resistant against the extract. In conclusion, the results presented in this report are encouraging. Therefore, further investigations are now needed to establish the exact mechanism of action and identify the active bio-ingredient(s) of the extract in order to explain their therapeutic efficacy and the possible toxic effect in vivo of these ingredients.

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References


