

## Insecticidal Effect of Cucurbitacin E Glycoside Isolated from *Citrullus colocynthis* Against *Aphis craccivora*

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**Abstract:** *Citrullus Colocynthis* fruits were extracted by different solvents (n-hexane, methylene chloride, Chloroform and ethanol). Each extract was tested against *Aphis craccivora*. The highest insecticidal effect (LC<sub>50</sub> 11003 ppm) was obtained from the ethanol extract. The residue remaining after evaporation of ethanol extract was re-extracted by different solvents with increasing polarity. Each fraction was tested against *Aphis craccivora*. The butanol extract showed the maximum insecticidal effect. HPLC analysis of this fraction showed presence of six compounds, the most abundant of them was that having retention time (Rt.) 4.8 min. (69.3%). This compound was purified by using florisil column stepwise eluted with different mixtures of methanol: chloroform. Mass analysis of the isolated and purified compound showed the molecular ion peak at m/z 719. The elemental analysis (C, H and N) suggesting the molecular formula to be C<sub>38</sub>H<sub>55</sub>O<sub>13</sub>. I.R., proton and <sup>13</sup>C NMR analysis identified this compound as 2-O-β-D-glucopyranosylcucurbitacin E.

**Key words:** *Citrullus Colocynthis*; *Aphis craccivora*; 2-O-β-D-glucopyranosylcucurbitacin E.

### INTRODUCTION

Intensive use of insecticides could develop resistant insects to these chemicals and also may leave hazardous residues in the fruits and pollute the agro ecosystem. So plant extract may be better substitutes of synthetic ones. Aphid is one of the most common insect pests attacking a wide spectrum of economic plants, causing great loss in their yield. The problems of *Aphis craccivora* are not only due to direct damage but also its capability to transmit viruses (Blackman and Eastop, 1984).

*Citrullus colocynthis* (L.) (*Cucurbitaceae*), commonly known as bitter apple, colosynth or wild gourd. *C. colocynthis* has gained increasing attention as a natural insecticide and its activity has been evaluated against many economically important insect species. *C. colocynthis* has deterrent, antifeedant, growth-regulating and fertility – reducing properties on insects (Prabuseenivasan, *et al.*, 2004). Also, it is used as an abortifacient, cathartic, purgative and vermifuse, and for the treatment of fever, cancer, amenorrhea, jaundice, leukemia, rheumatism, tumour and as an insect repellent (Duke, 2006).

Several active chemical constituents of this plant were recorded. These including the bitter substances (colocynthin and colocynthetin), cucurbitacins A, B, C, D, and E (*α*-elaterin) (Adam, *et al.*, 2001), cucurbitacines E, I, J, K, and L (Sturm, *et al.*, 2009), cucurbitacin glycosides (Hatam, *et al.*, 1989, Seger, *et al.*, 2005 and Abbas, *et al.*, 2006), flavonoids and flavone glycosides (Maatooq, *et al.*, 1997 and Abbas, *et al.*, 2006). The cucurbitacins (tetracyclic triterpens contains cucurbitane skeleton) are of great interest because of the wide range of biological activities they exhibit in plants and animals. They are predominantly found in the Cucurbitaceae family, but are also present in several other families of the plant kingdom. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, cardiovascular, and antidiabetic effects (Jayaprakasam, *et al.*, 2003), antioxidant activity of cucurbitacins B and I (Jayaprakasam, *et al.*, 2003) and the glucosides of cucurbitacin I and L (abbas, *et al.*, 2006). Cucurbitacin B was also shown to exhibit anti-inflammatory activity (Peters, *et al.*, 1999 and Yesilada, *et al.*, 1988). Additionally, several studies indicated that different cucurbitacin species inhibit the proliferation of cancer cells through different mechanisms (Duncan, *et al.*, 1996, Blaskovich, *et al.*, 2003 and Sun, *et al.*, 2005).

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The aim of the present study is to test different extracts of *Citrullus colocynthis* fruits against *A. craccivora*, and to separate and identify the active compound from the active extract.

## MATERIALS AND METHODS

### **Extraction of the Plant Material:**

*C. colocynthis* (L.) fruits were collected from El-wadi El-geded and identified by Flora & Plant Classification Research Department, Agricultural Research Centre, Egypt. The fruits were cleaned from the dust and debris then dried separately under room temperature. The fruits were grind separately by electric mill. Samples of milled plant were soaked in the solvents (n-hexane, methylenechloride, chloroform and ethanol) separately for 24-hr with shaking. The extract was then evaporated to dryness under vacuum using a rotary evaporator at 40°C. The crude extract was transferred quantitatively to a clean and weighted flask, then kept in the refrigerator until used.

### **Purification of the Ethanol Extract:**

The active extract (the ethanol extract) was evaporated to dryness under vacuum at 40°C using rotary evaporator. The remaining residue was extracted three times by each of the following solvents with increasing polarity: n-hexane, methylene chloride, n-butanol, n-propanol, chloroform, ethyl acetate, acetone, methanol and water. The remaining residue after evaporation of each solvent was weight and tested for insecticidal effect against (*A. craccivora*). The active fraction (the butanol extract) was analyzed by HPLC to identify the number of included compounds.

### **Separation of the Active Compound from the Butanol Extract:**

The major compound in the butanol extract was purified by glass column technique as follows:

The butanol extract was evaporated to dryness under vacuum by rotary evaporator and the remaining residue was dissolved in 2-ml of methanol. Each one ml of this alcoholic solution was fractionated by two glass columns containing Florisil (60-100 mesh, ACROS) or silica gel 60 (70- 230 mesh, Macherey-Nagel). A stepwise elution with 25-ml portions of different mixtures of methanol: chloroform was used. The mobile phases which were used for florisil column were (methanol: chloroform) 10: 90, 25:75, 50: 50, 75: 25, 100: 0 and 100: 0 (six fractions). The mobile phases which were used for silica gel column were (methanol: chloroform) 10: 90, 25: 75, 25: 75, 25: 75 and finally 50: 50 (five fractions). Each obtained fraction was analyzed by HPLC.

### **HPLC Analysis:**

The HPLC analysis was carried out by using HPLC Hewlett Packard 1100, equipped with G1322A degasser, G1311 quaternary pump, G1313A auto sampler, G1316A column thermostat and G 1314A variable wavelength detector. The analysis was performed by using ODS C18 column, 25-cm X 4.6 X 5µm, mobile phase: methanol, flow rate: 0.5-ml/min and 275nm detection wavelength. This detection wavelength was decided from spectrophotometer scanning which was carried out using UV-Visible Shimadzu spectrophotometer.

### **GC Analysis:**

Glucose content in the pure cucurbitacin E glycoside was determined by using GC Hewlett Packard 6890 instrument. The separation conditions were: ZB 1701Column 30m X 0.25 mm X 0.25 µm. Mobile phase: hydrogen 17.3 ml/min. Injection chamber temperature: 250 °C. Oven temperature starts at 180 °C to 200 °C (2 °C/ min.). FID detector temperature: 270 °C. ~ 10 mg of the pure compound was hydrolyzed by 1N HCl at 100°C for 6hr. The hydrolysate was evaporated to dryness at 50°C under stream of nitrogen. The remaining residue was derivatized by TMCS as described by Ronald Kirrk and Ronald Sawyer, (1991).

### **Instruments Used:**

C, H and N elements analysis was carried out by using Perkin Elmer 2400 C, H, N elemental analyzer. Mass analysis was performed by Shimadzu QP-2010 plus mass analyzer operated at 70eV. The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. <sup>1</sup>H spectrum was run at 300 MHz and <sup>13</sup>C spectrum was run at 75.46 MHz in deuterated methanol (CD<sub>3</sub>OD). I.R. analysis was performed by Buck Scientific model-500 IR spectrophotometer.

**Bioassay:**

a) Lab-strain of bean aphid, *A. craccivora* was obtained from the Central Laboratory of Pesticides, Egypt. This strain was reared for several generations in the laboratory according to the method described by Norman and Sutton, (1967). Slid-dipping technique was used to evaluate the toxicity of the tested extracts against adult stage of *A. craccivora*. Serial concentrations of each fraction were prepared. By means of fine brush, ten adults were affixed to double face scotch tap and stuck tightly to slide on the dorsal part. The slides were then dipped in the prepared solutions for ten seconds. Each fraction tested at five different concentrations. Three replicates of ten adults were used for each concentration. Mortality was recorded two hours after treatment and all insects responded to touching with the fin brush were considered a live.

b) Statistical analysis and toxicity lines:  $LC_{50}$  obtained from each fraction were subjected to statistical analysis for evaluating the relative efficiency of each fraction as insecticide according to the method developed by Finney, (1971) and Ldp line software.

**RESULTS AND DISCUSSION**

**Extraction of the *C. colocynthis* Fruits by Different Solvents:**

The fruits of *C. colocynthis* were extracted by different solvents (n-hexane, methylenechloride, chloroform and ethanol). The ethanolic extract showed the highest insecticidal effect ( $LC_{50}$  11003 ppm) against *A. craccivora*. (table 1).

**Table 1:**  $LC_{50}$  of different *C. colocynthis* extracts against *A. craccivora*.

S.N.	Solvent	$LC_{50}$ (ppm)
1	n-hexane	23065
2	methylenechloride	19497
3	chloroform	17328
4	ethanol	11003

**Fractionation of the Ethanol Extract:**

The residue remaining after evaporation of ethanol, represented 9.1 g/ 100 dry fruit, was extracted with nine solvents with increasing polarity. The residue remaining after evaporation of each extract was weight and tested for its insecticidal effect against *A. craccivora* (table 2).

**Table 2:** The solvents which were used for extraction of the ethanolic extract residue and their extraction percentages

S.N.	Solvent	Extraction %
1	Hexane	0
2	Methylene chloride	5
3	n-Butanol	76
4	n-Propanol	12
5	Chloroform	0
6	Ethyl acetate	2
7	Acetone	0
8	Methanol	4
9	Water	1

The butanol fraction showed the highest insecticidal effect against *A. craccivora*. Therefore, it was chosen as a standard for determining the toxicity index. On the basis of the  $LC_{50}$  values, results indicated that the other fractions induced moderate to less toxic effect (table 3). This insecticidal potency of *C. colocynthis* extract can be attributed to the presence of specific extracted active ingredients of the tested plant such as saponin, alkaloids and glycosides (Yaniv, *et al.*, 1999).

**Table 3:**  $LC_{50}$  and toxicity index (TI) of the fractions obtained from the ethanol extract residue against *A. craccivora*

Fraction	$LC_{50}$ (ppm)	TI
Butanol	3123.10	100
Propanol	4553.39	68.58
Methanol	13816.78	22.60
Water	16641.29	18.76
Methylene chloride	20599.80	15.15
Ethyl acetate	73264.81	4.26

A similar work obtained by Abbas, *et al.*, (2006) that the butanol fraction of the methanolic extract residue of *C. colocynthis* contains three flavone glucosides and two cucurbitacin glucosides. Also Yoshikawa, *et al.*,

(2007) reported that the alcoholic extract of *C. colocynthis* fruits contains several compounds including colocynthosides A and B, cucurbitacin E 2-O- $\beta$ -D-glucopyranoside, and its aglycon, cucurbitacin E. Therefore, we suggested that our active compound will be one of -or very closed to- these compounds.

#### HPLC Analysis of the Butanol Extract:

Firstly, spectrophotometer scanning of the butanol extract was carried out to determine its detection wavelength ( $\lambda$ -max). The spectrophotometer scanning showed that this extract has a broad range of absorption from 300nm to 220nm. Therefore the first HPLC analysis of this extract was carried out at two different wavelengths (275 and 220 nm) to choose the best detection wavelength. HPLC analysis of the butanol fraction at 275 nm showed presence of six compounds that having retention times (Rt) values: 3.0, 3.2, 4.4, 4.8, 5.3 and 5.7 min. Their percentages were: 10.6%, 7.8%, 7.0%, 69.3%, 1.7% and 3.4% respectively, figure (1). While at wavelength 220 nm the compound that has Rt 3.2 was disappeared and the other five compounds were still appeared. Therefore, we selected the 275 nm to be the detection wavelength in the subsequent HPLC analyses.

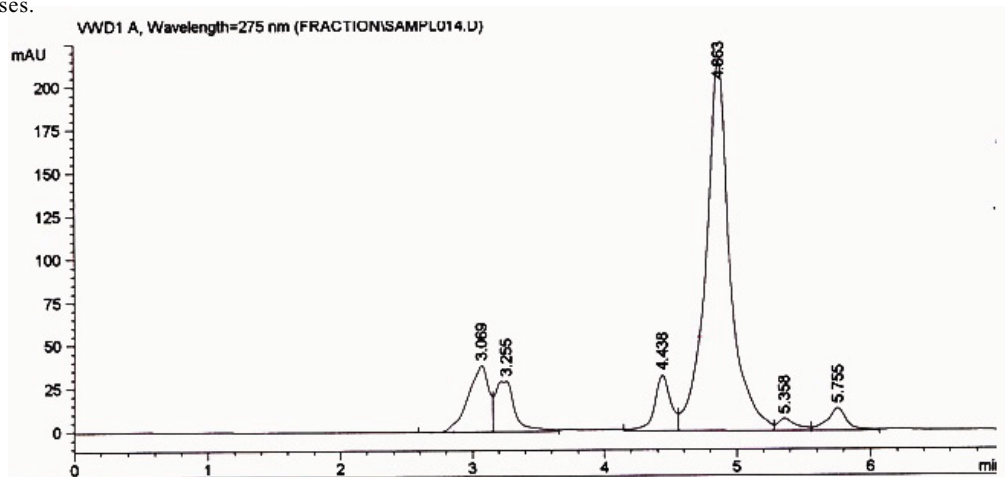


Fig. 1: HPLC chromatogram of the butanol fraction of the ethanolic extract residue. The analysis conditions were: ODS C18 column, Mobile phase: methanol, Flow rate: 0.5-ml/min. and detection wavelength: 275 nm.

#### Separation of the Active Compound from the Butanol Extract:

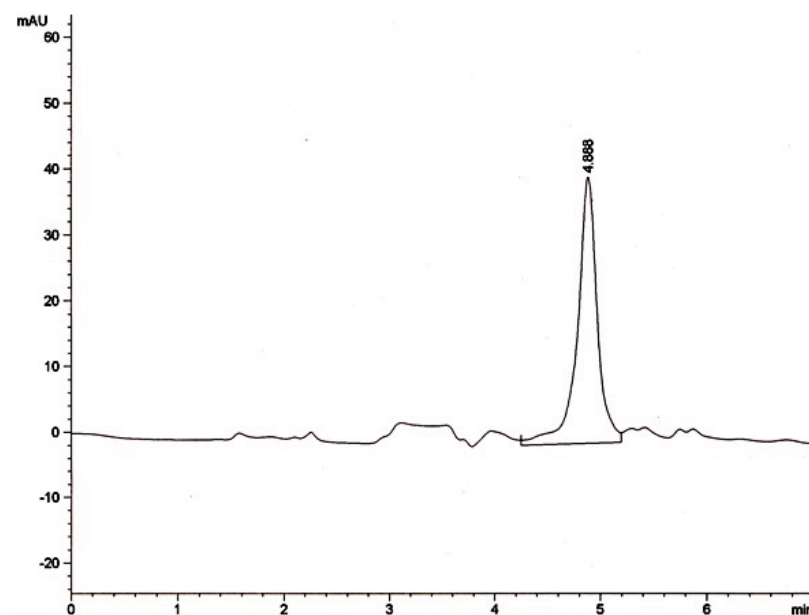
To separate the major compound (that has Rt. 4.8 min.) from the butanol extract, the residue remaining after evaporation of butanol was dissolved in 2-ml of methanol. This solution was then divided into two equal parts. Each part was tested to be purified by glass column technique.

The HPLC analysis of the fractions collected from the silica gel column showed that this column failed to separate the active compound, which has Rt 4.8 min, in a pure form. While the HPLC analysis of the fractions collected from florisil column showed that: The first two fractions mainly contained the compounds that having Rt values 5.3 and 5.7min. The remaining amount of them was then eluted with the third fraction with the first amount of the compound Rt 4.8 min. The fourth and the fifth fractions eluted all of the remaining amounts of the compound Rt. 4.8 min in a pure form. The sixth fraction eluted the compound that has Rt value 3.1 min. While the compound that has Rt. 4.4 min. was not eluted from the column.

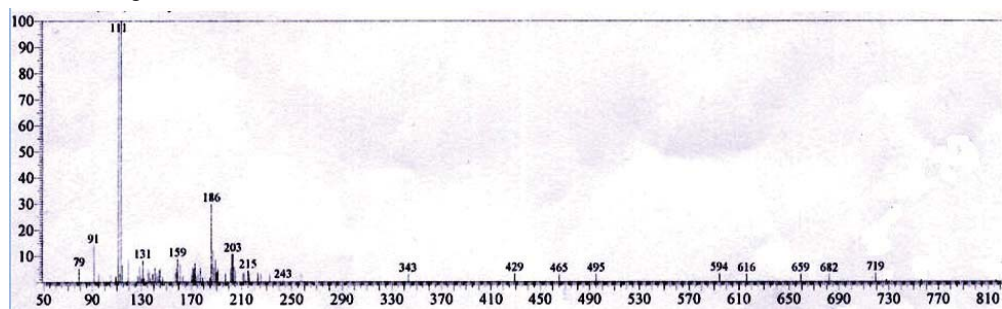
Therefore, the fourth and the fifth fractions, obtained from the florisil column, were combined and evaporated to dryness. HPLC analysis of the remaining residue showed presence of the pure compound that has Rt. value 4.8 min. (figure 2). This purified compound was analyzed by spectrophotometer, mass spectrometer and NMR for its chemical structure elucidation.

#### Mass Spectrometer and Elemental Analyses:

The C, H and N elemental analysis showed the empirical formula was approximately  $C_5H_4O$ . The mass spectrometer analysis showed the molecular ion peak ( $M^+$ ) at  $m/z$  719 (figure 3). These data suggested the molecular formula to be  $C_{38}H_{55}O_{13}$ . This is the molecular formula of cucurbitacin E glucoside.



**Fig. 2:** HPLC chromatogram of the active compound, purified from the butanol extract. The analysis conditions were: ODS C18 column, Mobile phase: methanol, Flow rate: 0.5-ml/min. and detection wavelength: 275 nm.



**Fig. 3:** Mass spectrum for the active compound, purified from the butanol extract.

#### **NMR Analysis:**

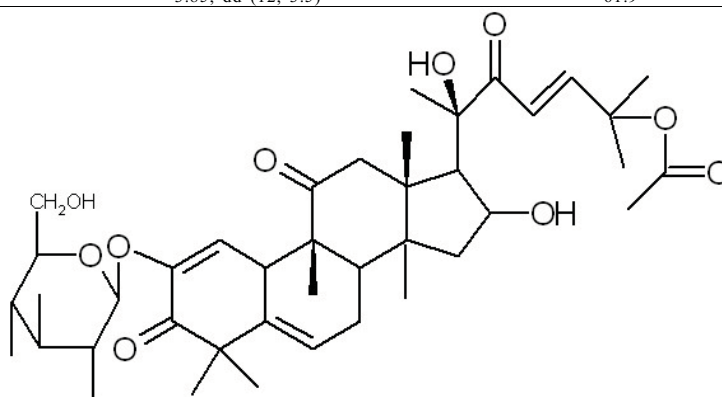
The chemical shift values for both  $^1\text{H}$  and  $^{13}\text{C}$  NMR and coupling constant ( $J$ ) were compared with those obtained by Pei-Lin, *et al.*, (2004) and Chun-Tao, *et al.*, (1985) for cucurbitacin E skeleton, and those obtained by Abbas, *et al.*, (2006) for 2-O- $\beta$ -D-glucopyranosylcucurbitacin I which differ from Cucurbitacin E in presence of acetyl group at C25 only. Chemical shift values of  $^{13}\text{C}$  NMR were also compared with that obtained by Yasuji, *et al.*, (1978) for cucurbitacin E. All of the chemical shift values were very similar to confirm that this compound is 2-O- $\beta$ -D-glucopyranosylcucurbitacin E (table 4). GC analysis of the acid hydrolyzed compound confirmed presence of glucose.

I.R. analysis showed a broad absorption peak at 3829  $\text{cm}^{-1}$  which indicates the hydroxyl group(s), and four absorption peaks at 3276, 3215, 3154 and 3124  $\text{cm}^{-1}$  which indicates presence of the =C-H groups. Also the absorption peaks at 1774 and 1713  $\text{cm}^{-1}$  for the carbonyl groups.

From the results obtained from mass spectrometer, elemental analysis, NMR and IR spectrophotometer analysis, we confirmed that the compound which isolated from *C. colocynthis* and has insecticidal effect against *Aphis craccivora* was 2-O- $\beta$ -D-glucopyranosylcucurbitacin E (figure 4).

**Table 4:**  $^1\text{H}$  (300 MHz, coupling constant  $J$  in Hz in parentheses) and  $^{13}\text{C}$  NMR (75.46 MHz) data of the pure active compound.

Carbon No.	Chemical shifts $\delta$ in ppm	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$
C1	6.12 d (2.2)	123.7
C2	-	147.2
C3	-	199.7
C4	-	46.7
C5	-	137.4
C6	5.8 (br., s)	122.3
C7	2.38 m, 2.05m	24.6
C8	2.07m	43
C9	-	50.3
C10	3.68 (br., s)	38.1
C11	-	216.5
C12	-	50.3
C13	-	50.1
C14	-	51.5
C15	-	46.6
C16	4.48 m	71.3
C17	-	59.4
C18	1.0 s	20.7
C19	1.01 s	20.5
C20	-	80.2
C21	141 s	24.9
C22	-	205
C23	6.83, d (15.5)	122.6
C24	6.97, d (15.5)	151.5
C25	-	80.7
C26	1.54	26.2
C27	1.57	26.4
C28	1.29	28.2
C29	1.28	20.6
C30	1.41	18.7
CH <sub>3</sub> C=O	2.05	21.9
171.8		
C1'	4.64, d (7)	101.1
C2'	3.42	71.8
C3'	3.44	78
C4'	3.53	70.8
C5'	4.02, dd (12, 2.2)	80.2
C6'	3.85, dd (12, 3.5)	61.9



**Fig. 4:** 2-O- $\beta$ -D-glucopyranosylcucurbitacin E

**Conclusion:**

2-O- $\beta$ -D-glucopyranosylcucurbitacin E is one of the cucurbitacins content of *Citrullus colocynthis* fruits. It was isolated and purified from *C. colocynthis* fruits collected from Egypt, by solvent extraction and glass column techniques. Its chemical structure was identified by I.R., mass spectrometry and  $\text{H}^1$  and  $\text{C}^{13}$  NMR analysis. This compound was found to have an insecticidal effect against *Aphis craccivora*.

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