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ISOLATION AND PURIFICATION OF CUCURBITACIN D AND I FROM *ECBALLIUM ELATERIUM* (L.) A. RICH FRUIT JUICE

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The objective of this study was to develop a rapid, economic, and efficient method for simultaneous selective isolation, separation, and purification of cucurbitacin D and I from *Ecballium elaterium* (L.) A. Rich fruit juice via reversed-phase flash chromatography combined with HPLC. The chloroform extract of the fruit juice was fractionated with flash chromatography using a chloroform, acetone and methanol solvent combination at a 5 ml/min flow rate. Then, a validated HPLC method was utilized for purification of the two targeted cucurbitacins. Cucurbitacin D and I were collected automatically by the fraction collector. The fractions containing the same compounds were pooled and lyophilized. The purified cucurbitacin D and I compounds were identified by NMR, LC-MS, and UV spectra analysis. The results suggest that the applied procedure is simple, quick, and highly efficient. The HPLC method was found to be linear, accurate, precise and rugged for the quantification of the cucurbitacins studied.

Keywords: cucurbitacin D; cucurbitacin I; *Ecballium elaterium* (L.) A. Rich*;* isolation; method validation

ИЗОЛАЦИЈА И ПРЕЧИСТУВАЊЕ НА КУКУРБИТАЦИН D И I ОД ОВОШЕН СОК НА *ECBALLIUM ELATERIUM* (L.) A. RICH

Целта на ова истражување беше да се развие брз, економичен и ефикасен метод за истовремено селективно изолирање, раздвојување и пречистување на кукурбитацин D и I од овошен сок на *Ecballium elaterium* (L.) A. Rich преку реверзно фазна флеш-хроматографија комбинирана со HPLC. Хлороформскиот екстракт беше фракциниран со флеш-хроматографија со употреба на раствор со комбинација од хлороформ, ацетон и метанол со брзина на проток од 5 ml/min. Потоа беше применет валидиран HPLC метод за пречистување на двата целни кукурбитацини. Кукурбитацини D и I беа автоматски собрани со фракционен колектор. Фракциите што ги содржеа истите соединенија беа обединети и лиофилизирани. Пречистените кукурбитацини D и I беа идентификувани по пат на NMR, LC-MS, и UV спектрална анализа. Беше утврдено дека со методот HPLC е линеарен, точен, прецизен и робустен за квантификација на испитуваните кукурбитацини.

Клучни зборови: кукурбитацин D; кукурбитацин I; *Ecballium elaterium* (L.) A. Rich*;* изолација; валидација на метод

1. INTRODUCTION

Ecballium elaterium (L.) A. Rich (*E. elaterium*), which grows naturally in the Mediterranean region, is known as the squirting cucumber [1, 2]. It has numerous biological activities in vitro such as analgesic, antipyretic, anti-hepatotoxic, anti-cancer, anti-inflammatory and cytotoxic [3–5]. The plant extract and the fruit juice have been widely

used for long time as a folk medicine to specifically treat sinusitis [6, 7].

E. elaterium is a rich source of cucurbitacin type secondary metabolites. The cucurbitacins are a group of highly oxygenated tetracyclic triterpenes having a unique 19-(10→9β)-abeo-10-lanost-5-ene (cucurbitane) skeleton (Fig. 1).

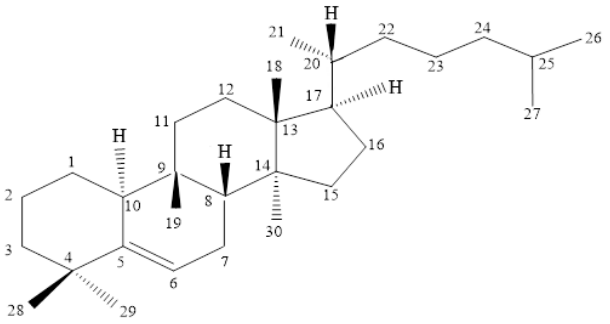

Fig. 1. General structure of cucurbitacins

Cucurbitacins are generally crystalline substances at room temperature. Most of them are slightly soluble in water but very soluble in petroleum ether, chloroform, benzene, ethyl acetate, methanol and ethanol. Their absorption maxima for ultraviolet light (UV) is between 228 and 234 nm depending on the type of cucurbitacin [2, 8–10]. The cucurbitacins exhibit a wide range of biological activities in living beings. They have been investigated for their cytotoxic, hepatoprotective, anti-diabetic, anti-bacterial, anti-inflammatory, antimicrobial, cardiovascular, and antioxidant activities. Additionally, some cucurbitacin species inhibit the proliferation of cancer cells [5, 8–15]. Currently, 12 classes of cucurbitacins are known which contain 16 cucurbitacin types, and they are grouped according to the variations in their molecular structure [8, 15]. Cucurbitacins B, E, D and I are the main cucurbitacins in the *E. elaterium* fruit [7]. Cucurbitacins B, D, E, I, L, R and their derivatives have been previously identified in *E. elaterium* fruit juice [4, 6].

Some studies report procedures for the extraction and isolation of cucurbitacins from different plants of Cucurbitaceae family [16–18]. The extraction of cucurbitacins is generally applied to the plant material or to the dried fruit juice using methanol or ethanol. Chloroform is mostly used for the purification of cucurbitacins from the alcoholic extracts because of their partition between water and chloroform [9, 19]. To our knowledge, there is no study on the extraction of cucurbitacin D (Cu D) and cucurbitacin I (Cu I) directly with chloroform from the juice of the fruit. Isolation can also be accomplished with different chromatographic protocols such as column chromatography and TLC. TLC has been utilized for the purification of cucurbitacins from plant extracts [9, 15, 19–22]. However, these techniques are time and solvent consuming.

Different techniques such as Flash Chromatography (FC) and HPLC may be used for the isolation and purification of plant extracts. FC is a rapid and economical method for the separation of mixtures at relatively high flow rates. FC offers good separation and can be used in both normal phase and reverse phase separations, but to the best of our knowledge, the use of FC has barely been studied to separate cucurbitacin species [23, 24].

HPLC is a good instrument for separating individual cucurbitacins from mixtures [19]. The use of HPLC coupled to a diode-array (HPLC-DAD) detector has undoubtedly made things much easier, allowing for quick and efficient characterization of crude extracts [9].

An HPLC method using gradient elution of Acetonitrile (ACN) in water has been documented for the analysis of a number of the main cucurbitacin types commonly found in plants [20, 21, 25–28]. HPLC purification may be achieved by separating the target compound from the other compounds. For an optimum purification, each compound should give a characteristic peak under the chosen chromatographic conditions such as the proper mobile phase, flow rate, detector, and column type [29]. Table 1 summarizes different analytical and preparative methods for the analysis of cucurbitacins by HPLC in the literature.

The aim of the present study was to develop and validate a simple, rapid, economical, and efficient method to simultaneously isolate, separate, purify and identify Cu D and Cu I from *E. elaterium* fruit juice with high yield.

T a b l e 1

Analytical and preparative methods for the analysis of cucurbitacins by HPLC

Stationary phase (Column)	**Mobile phase**	**Flow rate (mL/min)**	**Elution type and program**	**Determined cucurbitacin**	**Wavelenght (nm)**	**Reference**
(A) Analytical; Nucleosil 100-5 C18 (250 mm × 4 mm i.d; 5 μm) protected by a precolumn (B) Semipreparative; C18 (250 mm × 10 mm i.d; 5 μm)	(A) A: H_2O: formic acid (99.5:0.5) B: Methanol: acetonitrile (50:50) (B) A: H_2O: formic acid (99.5:0.5) B: Methanol: acetonitrile (50:50)	(A) 0.8 (B) 2	(A) Gradient; starting at 10% B, 50% B at 16 min, 80% B at 30 min, 100% B at 33 min, B was maintained at 100% for another 2 min. (B) Isocratic	Cucurbitacin B, D, E, I and glycosides	230	[4]
(A) Preperative; Econosil C18 250 × 22 mm, 10 μm (B) Analytical; Alltima C18 250 × 4.6 mm i.d., 5 μm	(A) A: Acetonitrile, B: Water or A: Methanol, B: Water (B) A: Acetonitrile, B: Water or A: Methanol, B: Water	(A) 13 (B) 1	(A) Gradient; Acetonitrile (20-55% in 50 min.), or methanol (60-75% in 50 min.) (B) Acetonitrile (30-70% in 57 min.), and methanol (60-75% in 50 min.)	Cucurbitacins	unspecified	[20]
Eurospher C_{18} (250 mm × 4 mm i.d; 5 μm)	A: Acetonitrile, B: Water	1	Gradient; 0–35 min linearly from 20% A to 60% B, 35–40 min linearly from 60% to 20%, then held for 5 min.	Cucurbitacin E; Cucurbitacin I; Cucurbitacin E glycoside; Cucurbitacin I glycoside	230	[26]
Hewlett Packard 20 cm × 4.6 mm i.d., 10 μm	A: Acetonitrile-water (2:8) B: Acetonitrile-water (45:55)	2	Gradient, starting 100% A, 0% A (0-35 min.)	Cucurbitacin B, D, E, I, 3-epi-iso-cucurbitacin D and two glycosides	229	[27]
Kromasil C8 (150 mm × 4.6 mm ID, 5 μm)	Acetonitrile - 2% Acetic acid	1	Linear gradient	23, 24 – dihydrocucurbitacin F;23, 24- dihyrocucurbitacin D; Cucurbitacin B; Cucurbitacin E	215	[28]
(A) Analytical; Supelco®, 150 × 4.6 mm, 5 μm (B) Preperative; Shimadzu®, ODS, PREP-ODS, 20 × 250 mm	(A) Acetonitrile-Water (40:60) (B) Acetonitrile-Water (42:58)	(A) 1.2 (B) 6	Isocratic	Dihydrocucurbitacin B; Cucurbitacin B	230	[30]
TSK Gel ODs 120A 150 × 4.6 mm	Acetonitrile-water (2:3)	1	Isocratic	Cucurbitacin B	230	[31]
Bio-Sil C18 HL 90-5S; 250 × 4.6 mm i.d., 5 μm	A: Acetonitrile B: Water	2	Gradient; starting 20% A, 45% A (0-35 min.)	Cucurbitacin E	229	[32]
LiChrospher 100 RP-18e (4 mm i.d.×250 mm, 5 μm) Column temperature 40 ℃	0.05% Trifluoroacetic acid-methanol (42:58)	1	Isocratic	Cucurbitacin E; Cucurbitacin I; Cucurbitacin E glycoside; Cucurbitacin I glycoside	236	[33]
Analytical C18	A: Water, B: Acetonitrile	1	Gradient; starting with 100% of water and minutes A 80%, B 20%, after 10 min A 60%, B 40% and after 5 min A 40%, B 60%.	Cucurbitacin E; Cucurbitacin I	229, 254	[34]

2. MATERIALS AND METHODS

2.1. *Materials, instruments, standards and reagents*

Ripe fruits of *E. elaterium* were collected in September from Adana province, Turkey and stored at 18 ℃ until processed. The plant was identified by Dr. Turan Arabacı (Faculty of Pharmacy, İnönü University). The voucher specimens were deposited in the Herbarium of İnönü University (INU), Malatya, Turkey.

A Nüve EV 018 vacuum oven (Nüve, Turkey), a Büchi Rotavapor R-210 (Büchi, Switzerland), and an Alpha 1-2/LD Plus freeze dryer (Martin Christ, Osterode Germany) were used for sample preparation.

The FC separation was performed on a Büchi (Switzerland) FC system with dual C-601 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, and silica gel filled (Merck, 70 – 230 mesh) 21.0 × 129 mm polypropylene cartridges.

A Shimadzu LC-20AD Prominence HPLC system (Shimadzu Corp., Kyoto, Japan) was used

for purification/separation tests. It had a DAD detector (SPD-M20A) equipped with a pump system, an auto sampler (SIL-20A HT), a column heater (CTO-20A), a fraction collector (FRC-10A), and a degasser unit (DGU-20A5).

An Agilent 1100 LC/MSD SL single quadruple mass spectrometer (Agilent Technologies, Palo Alto, CA) was used for measuring ESI-MS. ^{1}H NMR spectra were recorded at 600 MHz on a Bruker Avance III 600 spectrometer (Bruker, Rheinstetten, Germany).

Cu D and Cu I standards (purity of ≥95%) were purchased from Extrasynthese (Genay, France). HPLC grade solvents (acetone, ACN, chloroform, hexane and methanol) were obtained from Carlo Erba (Milan, Italy). All mobile phase solvents were ultrasonically degassed before use. Ultrapure water used for all experiments was produced by a Millipore Synergy UV purification system (Molsheim, France).

2.2. *Extraction and isolation of cucurbitacins*

The ripe fruits were pressed. The juice was collected and strained. The juice was filtered through a double layer cheesecloth, then through filter paper, and dried in the vacuum oven at 23 ºC. To prevent degradation of secondary metabolites, the residue was stored at about −18 ºC prior to utilization for extraction.

Five grams of dried residue was dissolved in 50 ml deionized water. This aqueous solution was extracted three times with 50 ml hexane at 40 ºC for 6 h to remove waxes, pigments, high boiling terpenes, apolar fatty acids, and lipids. After extraction, the phases were separated and stored at −18 ℃ until analysis.

The remaining aqueous phase (49 ml) was extracted with chloroform (50 ml), which has high affinity for cucurbitacins. The extraction with chloroform was performed three times at room temperature for 6 h. The extract was then filtered and concentrated to 10 ml by the rotary evaporator under reduced pressure at 40 ºC. This organic phase contained a mixture of partially purified cucurbitacins.

FC was used for the fractionation of the chloroform extract to obtain pure cucurbitacins in target fractions. The solvent system used for FC was chosen from TLC separation experiments. For this purpose, the optimum solvent system ratio giving the best separation of Cu D and Cu I was determined from several solvent systems. Standards and test samples were spotted on TLC silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany). The solvent system which gave the best separation was a chloroform-acetone-methanol (77:10:13; v/v/v) solution. The Rf values for Cu D and Cu I were found to be 0.61 and 0.69, respectively.

The flash column was first equilibrated with chloroform, and then the chloroform extract (total of 10 ml) was eluted with isocratic elution using the above solvent system at a constant flow rate of 5 ml/min. The detection wavelength was 254 nm. In total, 42 fractions of varying volumes were collected based on absorbance values. The elution solvent of each fraction was evaporated and the residues were dissolved in 2 ml of ethanol. Then, the solution was filtered through a 0.45 μm filter and placed in HPLC vials for testing the presence of Cu D and Cu I by HPLC-DAD. This procedure was repeated for each fraction. Cu D and Cu I identification was performed by comparing retention times and UV spectra of the compounds present in each fraction and the pure standards. The presence of Cu D and Cu I (in varying amounts) was determined in eleven fractions. These fractions were pooled and concentrated by evaporation of ethanol. The remaining solution was about 5 ml, and it was placed in HPLC vials for further separation and purification.

A Nucleosil RP-C_{18} column (250 mm × 4.6 mm id, 5 μm, pore size, 100 Å) was used for HPLC analysis. The chromatographic conditions were set by a slight modification of a previously described method [27, 32]. ACN/water (20:80, v/v)) and ACN/water (45:55, v/v) were used as mobile phases A and B, respectively. The gradient elution started at 10 % solvent B, gradually increased to 100 % solvent B in 60 min, isocratic for 10 min, then decreased to 10 % solvent B in 5 min at a flow rate of 0.75 ml/min for a total period of 75 min. After each test, the column was equilibrated for 5 min.

HPLC analysis was carried out at constant column temperature (40 ºC), and a 10 μl injection volume was taken for qualitative/quantitative analysis. Analyte absorbance was recorded in the range of 190–800 nm by using a DAD detector. Maximum Cu D and Cu I peak absorbances were at 230 and 235 nm, respectively. Most cucurbitacins give UV-absorbance at 229 nm because of their chemical structure [9]. Therefore, the cucurbitacins studied here were fractioned at 229 nm.

For the purification step, the injection volume was increased to 50 μl and the rest of the settings were kept as above. Fractions of Cu D and Cu I were collected automatically by the fraction collector. The time based collection of the peaks was performed at about 36.50–38.50 minute intervals for Cu D and 44.00–45.50 min interval for Cu

I. Cu D and Cu I fractions were pooled separately at room temperature and stored at −18 ºC for further processing. The collected fractions were divided into 5 ml portions for lyophilization and kept at –18 ºC overnight. The frozen samples were lyophilized to obtain solid purified Cu D and Cu I crystals.

2.3. *Quantification and identification of the isolated cucurbitacins*

Cu D and Cu I standards were used for the quantification and identification of both cucurbitacin compounds. A stock solution of each standard was prepared in 5 ml ethanol at a concentration of 1 mg/ml for Cu D and 0.22 mg/ml for Cu I. Calibration standards were prepared at concentrations ranging from 10 to 500 μg/ml for Cu D and from 5 to 100 μg/ml for Cu I. The calibration curves were separately drawn by plotting the peak area against the concentration of Cu D and Cu I. The curves were analyzed using linear regression equations and correlation coefficients.

Quantification of individual cucurbitacins was directly determined by HPLC-DAD according to the above described HPLC method using external standard curves of the authentic standards. Cu D and Cu I concentrations were determined from the direct correlation between the peak area and amount.

The isolated compounds were further identified by spectroscopic methods including UV, LC-MS and NMR. Isolated Cu D and Cu I samples were dissolved in ethanol and determined by HPLC-DAD according to the method described above on the basis of retention time and by comparison of UV spectra of the standards. The mass spectrometer was operated in positive ionization mode for both Cu D and Cu I with an ESI source and mass range set to *m/z* 100–1500. The mobile phase ACN/water/methanol (33:34:33, v/v/v) with 0.1% formic acid (CH_2O_2) was delivered at a constant flow rate of 0.8 ml/min. ^{1}H NMR fingerprints were established to identify the Cu D and Cu I obtained by HPLC fractionation.

2.4. *Method validation*

The developed method was fully validated according to the International Conference on Harmonization guidelines (ICH Q2R1) [35]. Validation tests were performed for the limit of detection (LOD), limit of quantification (LOQ), linearity range, accuracy, precision (intra- and inter-day), ruggedness and stability.

The linearity of the method was determined at five different concentration levels (measurements were triplicated) for each cucurbitacin. The calibration curve for each cucurbitacin with the respective correlation coefficient was calculated by least-squares linear regression analysis of the peak area. The LOD and LOQ values were determined at signal-to-noise ratios of 3.3 and 10, respectively. The precision of the method was demonstrated by inter-day (reproducibility) at four different days and intra-day (repeatability) at three times in a day operation under the same conditions, both expressed as variation studies and relative standard deviation (RSD %). The accuracy of the method was determined by calculating the percent recovery of the compounds by the standard addition method. Known amounts of standard solutions of Cu D and Cu I (50, 100, and 150 %) were added to pre-quantified sample solutions, and the quantity of each cucurbitacin was subsequently determined from the corresponding calibration curve. The accuracy was evaluated by calculating the mean recovery values which were calculated according to the following formula:

$$\text{Recovery (\%)} = ((\text{detected amount} - \text{original amount})/\text{amount spiked}) \times 100.$$

The ruggedness of the method was determined by carrying out experiments on two different instruments (Shimadzu and Agilent HPLC, Agilent Technologies, Waldbronn, Germany) and by different operators using different columns (Nucleosil RP-C_{18} column and Inertsil ODS 3V C_{18}, 4.6 × 250 mm, 5μm, GL Sciences Inc., Tokyo, Japan). In order to demonstrate the stability of the standard and sample solutions during analysis, the solutions were analyzed at times 0, 24, 48 and 72 h.

3. RESULTS AND DISCUSSION

Cucurbitacins exhibit a wide array of in vitro and in vivo pharmacological effects, including anti-tumor activity. Target compounds (Cu D and I in present study) should be at high purity for studies such as cancer research. High purity isolated cucurbitacin compounds are being investigated for their anti-cancer activities in recent studies. For this purpose, we established a simple and reliable method for simultaneous isolation and purification of cucurbitacins starting from the fruit juice.

Extraction solvent type is the most important variable affecting extraction efficiency. The physical state of the starting material is also significant in terms of the progress of the extraction. This condition indirectly affects the amount of the target compound. According to the literature, the basic components of *E. elaterium* fruit juice are cucur-

bitacin B, D, E and I [36]. A high ratio of Cu D and I are obtained when the fruit juice is extracted directly with chloroform. This is an effective and selective solvent, and it was used for cucurbitacins for the first time. The extraction efficiency with chloroform was about 97 % for both cucurbitacins. In contrast with methanol, the chloroform extract does not contain polar components; so there is no need for a further step to remove polar components. Since chloroform is highly volatile, the extraction was performed at room temperature. The FC separation yield was 91 % for Cu D and 90 % for Cu I.

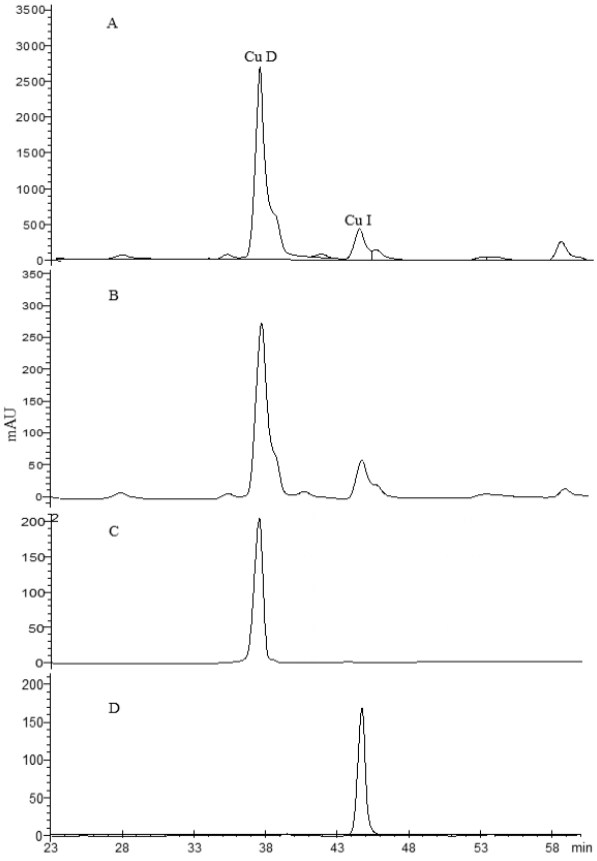

Fig. 2. HPLC chromatograms of (A) chloroform extract of *E. elaterium* fruit juice; (B) an FC fraction; (C) standard Cu D; (D) standard Cu I

The HPLC chromatograms of the chloroform extract of *E. elaterium* fruit juice, the FC fraction, and the authentic Cu D and Cu I standards are given in Figure 2. The HPLC peaks of the chloroform extract and the FC fraction were similar, and the peaks of Cu D and Cu I in these samples were in good agreement with those of the standards. Cu D and Cu I were the major compounds in both the chloroform extract and the FC fractions. The peaks were sharp and symmetric. The intense peaks of Cu D and Cu I in the chloroform extract indicated that Cu D and Cu I were present in higher amounts than the other components. The retention times were 37.7 ± 0.03 min for Cu D standard and 44.7 ± 0.04 min for Cu I standard.

The optimal analytical HPLC conditions such as the composition of the mobile phase, elution mode, flow rate, and column temperature were investigated by using a Nucleosil RP-C_{18} column (250 mm × 4.6 mm i.d., 5 μm).

Three mobile phase combinations tested for HPLC separation were (1) 100 % ACN (phase A) - 100 % water (phase B), (2) 2 % acetic acid in water (phase A) – 100% ACN (phase B) and (3) 0.1 % TFA in water (phase A) – 100 % ACN (phase B). The retention times of the components decreased and the resolution of the peaks increased in the presence of acetic acid and TFA phases. Although, this situation seems to be an advantage, the removal of the components from acetic acid and TFA phases at the final step requires an extra separation process. Therefore, ACN and water mixtures of different ratios were used as the mobile phase. The gradient elution program gave better peak shapes and resolution than the isocratic elution program. The retention time of Cu D and Cu I decreased with increasing column temperature. Optimum separation and fractionation of Cu D and Cu I was obtained at a 0.75 ml/min flow rate. HPLC injection volume was found to be an important factor affecting the separation efficiency. Good separation resolution was obtained with small injection volumes (50 μl). Large injection volumes (>50 μl) caused broadened peaks and poor separation of the target compounds.

Cu D and Cu I peak fractions of the automatically collected samples were identified by comparing their retention times and UV spectra of HPLC-DAD with those of the standards. The retention times of the isolated Cu D and Cu I were 37.6 ± 0.03 min and 44.7 ± 0.04 min, respectively. The retention times of the isolated compounds were very close to the standard's retention times. The use of the DAD detector facilitated easy identification and determination of the purity of the compounds. The UV spectra of the standards and isolated Cu D and Cu I are shown in Figure 3.

The peaks of the isolated Cu D and Cu I were in good agreement with those of the authentic standards. Peak purity index values were close to 1.0 for each cucurbitacin type. HPLC peak identification was determined by overlapping the chromatograms of the isolated Cu D and Cu I with their standards as shown in Figure 4. Rapid and exact identification and determination of peaks in this way may be used to directly identify bioactive compounds in the mixtures.

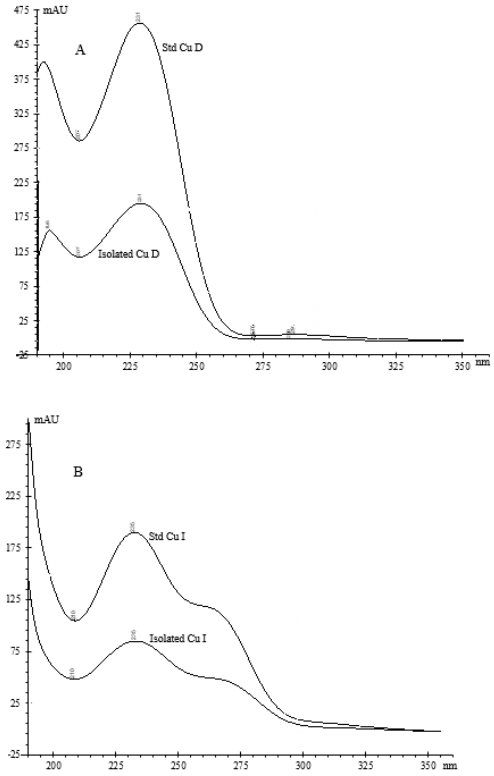

Fig. 3. Overlapping UV spectra of (A) standard and isolated Cu D; (B) standard and isolated Cu I

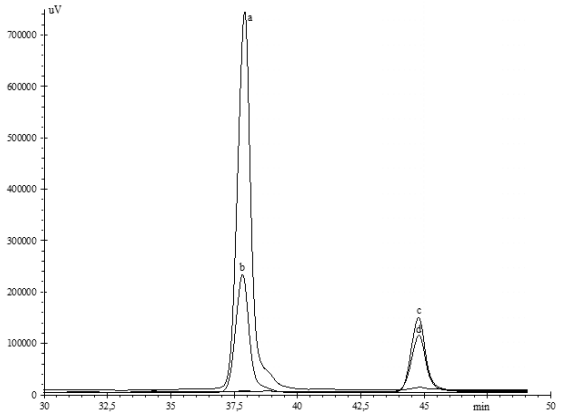

Fig. 4. Overlapping chromatograms of standard and isolated Cu D and Cu I (a: Standard Cu D; b: Isolated Cu D; c: Standard Cu I; d: Isolated Cu I)

LC-ESI-MS analysis was performed in positive ionization mode to obtain information on the composition of the standard Cu D and Cu I as well as isolated Cu D and Cu I. The LC/MSD SL chromatograms are shown in Figure 5. The following interpretation of the major ion formation may be proposed: the characteristic protonated ion at *m*/*z* 499.4 $[M+H-H_2O]^+$ corresponds to $C_{30}H_{44}O_7$ with molecular mass 516.7 for the isolated Cu D. The difference in the *m*/*z* ratio was due to the facile loss of a molecule of water from Cu D (Fig. 5(A)). This is also demonstrated with the mass chromatogram of the Cu D standard (Fig. 5(B)). The specific ion at *m*/*z* 497.3 $[M+H-H_2O]^+$ corresponds to the loss of a molecule of water from $C_{30}H_{42}O_7$ with molecular mass 514.7 for the isolated Cu I (Fig. 5(C)) [37–39]. The same mass chromatogram is observed for the Cu I standard (Fig. 5(D)).

Melting points of the isolated cucurbtacins were determined in open capillary tubes with a Branstead Electrothermal 9100 capillary melting point instrument and were uncorrected. The melting points of 151.2 ºC and 149.3 ºC were recorded for Cu D and Cu I, respectively. These results were quite close and within the range of the values specified by the suppliers [37, 38].

T a b l e 2

^{1}H-NMR (600 MHz, $CHCl_3$) of cucurbitacins D and I

	Cucurbitacin D	**Cucurbitacin I**
Position	**δ_H/ppm (J/Hz)**	**δ_H/ppm (J/Hz)**
1α	2.32 m	2.96 d (2.7)
1β	1.22 s	–
2	4.39 m	–
3	–	–
4	–	–
5	–	–
6	5.79 br d (6.0)	5.89 br s
7α	1.96 m	2.36 m
7β	2.40 d (14.4)	2.04 m
8	1.98 br d (7.8)	2.03 m
9	–	–
10	2.74 br d (12.6)	3.49 br s
11	–	-
12α	3.25 d (15.0)	3.23 d (14.4)
12β	2.70 d (14.4) / 2.40 d (14.4)	2.74 d (14.7)
13	–	–
14	–	–
15α	1.39 dd (1.8, 7.2)	1.64 m
15β	1.86 d (12.6)	1.54 ….
16	4.08 m	4.44 m
17	2.56 d (7.2)	2.55 d (6.8)
18	0.99 s	1.04 s
19	1.08 s	0.88 s
20	–	–
21	1.41 s	1.39 s
22	–	–
23	6.70 d (15.6)	6.68 d (15.0)
24	7.12 d (15.2)	7.09 d (15.0)
25	–	–
26	1.35 s	1.36 s
27	1.43 s	1.28 s
28	1.25 s	1.00 s
29	1.36 s	0.87 s
30	1.28 s	1.43 s

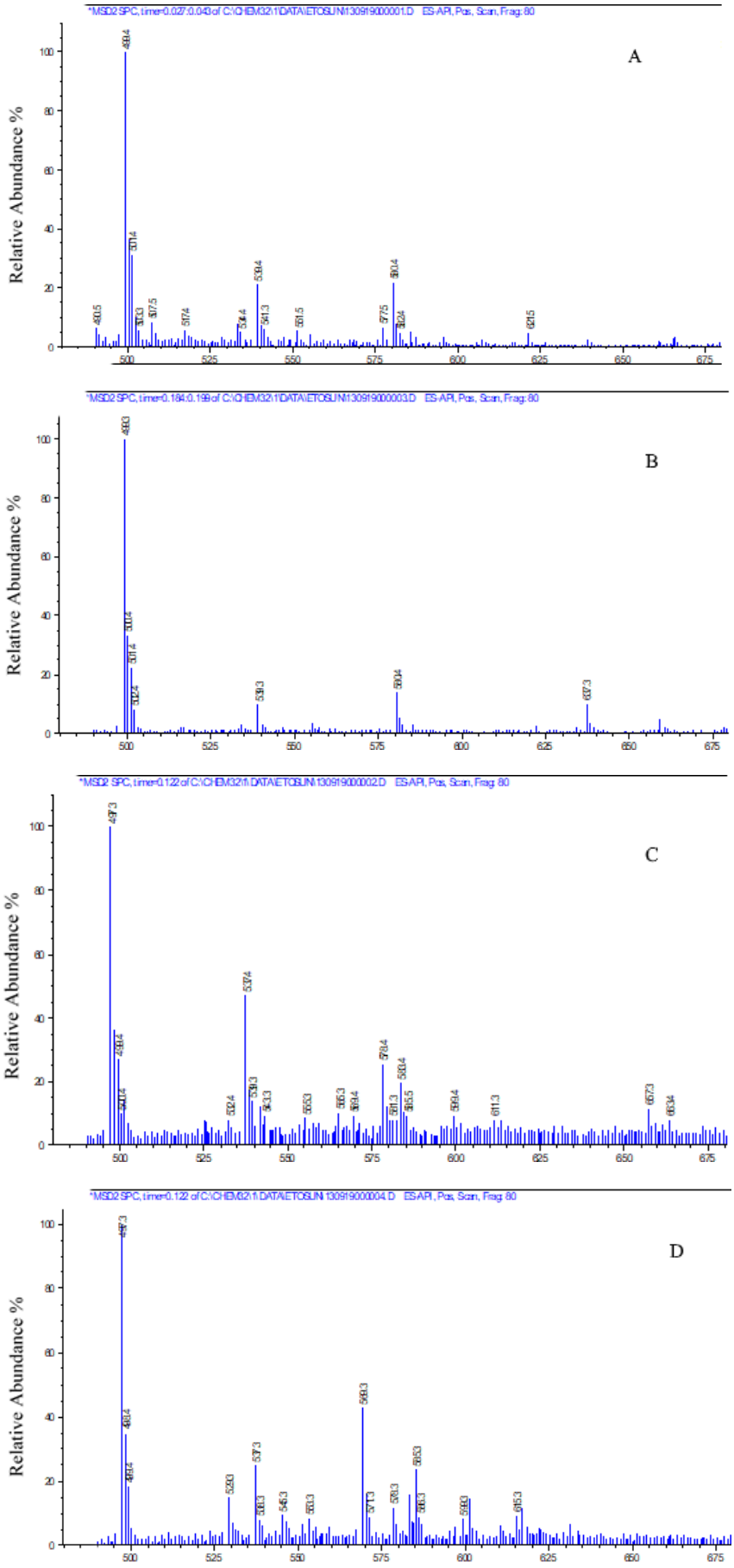

Fig. 5. The mass spectra of (A) isolated Cu D, (B) standard Cu D, (C) isolated Cu I, (D) standard Cu I

The ^{1}H spectral data of the purified Cu D and Cu I are listed in Table 2. The ^{1}H NMR spectral data of Cu D (IUPAC Name: (2*S*,8*S*,9*R*, 10*R*,13*R*,14*S*, 16*R*,17*R*)-17-[(*E*,2*R*)-2,6-dihydroxy-6-methyl-3-oxohept-4-en-2-yl]-2,16-dihydroxy-4, 4,9,13,14-pentamethyl-2,7,8,10,12,15,16,17-octahydro-1*H*-cyclopenta[a]phenanthrene-3,11-dione) (Fig. 6(A)) exhibited eight tertiary methyl group signals at δ_H 0.99 (3H, s, H-18); 1.25 (3H, s, H-28); 1.36 (3H, s, H-29); 1.28 (3H, s, H-30); 1.35 (3H, s, H-26); 1.43 (3H, s, H-27); 1.41 (3H, s, H-21); 1.08 (3H, s, H-19), an olefinic proton at δ_H 5.79 (1H, br d, J = 6.0 Hz, H-6), two *trans*-coupled olefinic protons on a side chain at δ_H 6.70 (1H, d, J = 15.6 Hz, H-23) and 7.12 (1H, d, J = 15.2 Hz, H-24), two hydroxymethine protons at δ_H 4.39 (1H, m, H-2) and 4.08 (1H, m, H-16), and a pair of doublets at δ_H 2.7,2.4 (1H, d, J = 14.4, 14.4 Hz, H-12β) and 3.25 (1H, d, J = 15.0 Hz, H-12α). The ^{1}H NMR spectral data of Cu I (IUPAC Name: (8*S*,9*R*,10*R*,13*R*,14*S*,16*R*,17*R*)-17-[(*E*,2*R*)-2,6-dihydroxy-6-methyl-3-oxohept-4-en-2-yl]-2,16-dihydroxy-4,4,9,13,14-pentamethyl-8,10, 12,15,16, 17-hexahydro-7*H*-cyclopenta[a]phenanthrene-3, 11-dione) (Fig. 6(B)) exhibited eight tertiary methyl group signals at δ_H 1.04 (3H, s, H-18); 1.00 (3H, s, H-28); 0.87 (3H, s, H-29); 1.43 (3H, s, H-30); 1.36 (3H, s, H-26); 1.28 (3H, s, H-27); 1.39 (3H, s, H-21); 0.88 (3H, s, H-19), an olefinic proton at δ_H 5.79 (1H, br s, H-6), two trans-coupled olefinic protons on a side chain at δ_H 6.68 (1H, d, J = 15.0 Hz, H-23) and 7.09 (1H, d, J = 15.0 Hz, H-24), a hydroxymethine protons at δ_H 4.44 (1H, m, H-16), and a pair of doublets at δ_H 2.74 (1H, d, J = 14.7, 14.4 Hz, H-12β) and 3.23 (1H, d, J = 14.4 Hz, H-12α). Cu D and Cu I NMR signals were compared with the literature values and a good agreement was observed [40–45].

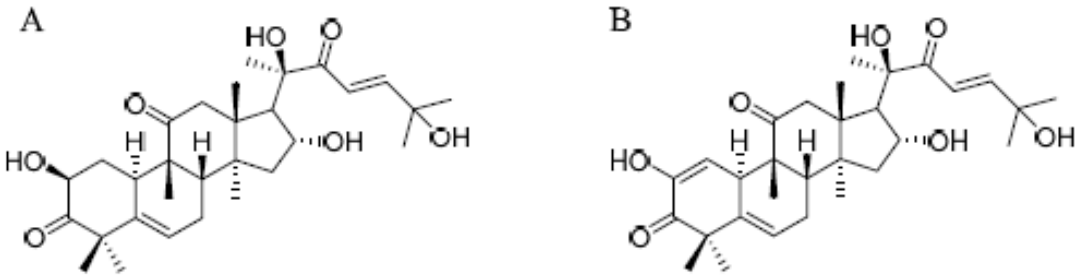

Fig. 6. The chemical structure of cucurbitacin D (A), cucurbitacin I (B)

The HPLC method was used to quantitatively determine the amount of Cu D and Cu I isolated from the fruit juice. The lyophilized Cu D and Cu I crystals were dissolved in ethanol and the concentration of the compounds was calculated by individual calibration curves. The mean concentration values for three parallel determinations were 418 ± 0.1 μM for Cu D and 296 ± 0.1 μM for Cu I. These values correspond to 86.4 μg/g dried residue for Cu D and 61 μg/g dried residue for Cu I, respectively.

Linear calibration plots for Cu D and Cu I at five different concentrations were triplicated for method validation. The linear equations for calibration plots were $y = 99480x + 279497$ (R^2 = 0.9995) for Cu D and $y = 87193x + 31473$ (R^2 = 0.9999) for Cu I. The LODs for Cu D and Cu I were 2.34 μg/ml and 1.79 μg/ml, respectively. The LOQs for Cu D and Cu I were 7.08 μg/ml and 5.98 μg/ml, respectively.

The recovery was 100.48, 97.35, and 100.45 for the known amount of 50, 100, and 150 % Cu D, respectively, and 99.90, 99.98 and 101.27 for the known amount of 50, 100 and 150 % Cu I, respectively. The data showed that the added recoveries of the standards were accurate.

Intraday and inter-day RSDs were 0.25 % and 1.58 % for Cu D and 0.21 % and 1.65 % for Cu I, respectively. Intraday RSDs were smaller than the inter-day RSDs for all tested samples. The HPLC method was found to be precise for intraday and inter-day RSD values. The relatively low RSDs for cucurbitacins in both intraday and inter-day experiments also indicated that the method was repeatable and reproducible. There were no marked changes in the chromatograms obtained from two different HPLC machines; thus, the developed HPLC method was rugged.

The standard and sample solutions were analyzed for stability at 0, 24, 48, and 72 h after preparation. The peak areas of Cu D and Cu I remained almost unchanged. Therefore, the standard and sample solutions were stable at least up to 72 h. The results of linearity, LOD, LOQ, precision, and recovery are summarized in Table 3.

An analytical HPLC purification method is much better than the other chromatographic methods to obtain high purity components. Subsequently, a simple, rapid and reliable HPLC method was developed for the simultaneous purification and quantification of the two cucurbitacins. The described HPLC method resulted in a good separation. The analytical method was also fully validated.

Several advantages of the developed method can be mentioned. First, the sample preparation is easy and only non-polar components like cucurbitacins are extracted from fruit juice with chloroform. Second, the fractions which contain cucurbitacins are better separated using FC. Third, the HPLC conditions, in which aqueous acetonitrile is used as the mobile phase, are not complicated. This is also an advantage for removal of solvents from the final product without any impurities. Fourth, the high purity final product is suitable for medical research. Other benefits of the developed method include low cost analysis and good performance of analytical HPLC in contrast to expensive preparative HPLC.

T a b l e 3

Summary of HPLC method validation for Cu D and Cu I

Parameters	**Cu D**	**Cu I**
Linearity		
Range (μg/ml)	10 - 500	4 - 100
Linear equation	y = 99480x + 279497	y = 87193x + 31473
Slope	99480	87193
Intercept	279497	31473
Correlation coefficient (R^2)	0.9995	0.9999
Precision (RSD, %)		
Intraday	0.25	0.21
Inter day	1.58	1.65
LOD (μg/ml)	2.34	1.79
LOQ (μg/ml)	7.08	5.98
Stability (RSD, %)		
0 h	0.44	0.98
24 h	0.43	1.19
48 h	0.40	0.53
72 h	0.48	1.07
Recovery (%)		
Level 1 (50%)		
Original mean (μg)	32.47 ± 0.61	32.58 ± 0.89
Spiked mean (μg)	16.00	16.00
Detected mean (μg)	48.69 ± 0.81	48.53 ± 0.89
Recovery (%)	100.48	99.90
RSD (%)	1.67	1.87
Level 2 (100 %)		
Original mean (μg)	32.47 ± 0.61	32.58 ± 0.89
Spiked mean (μg)	32.00	32.00
Detected mean (μg)	62.76 ± 0.53	64.56 ± 0.47
Recovery (%)	97.35	99.98
RSD (%)	0.85	0.72
Level 3 (150 %)		
Original mean (μg)	32.47 ± 0.61	32.58 ± 0.89
Spiked mean (μg)	48.00	48.00
Detected mean (μg)	80.83 ± 0.93	81.60 ± 0.99
Recovery (%)	100.45	101.27
RSD (%)	1.15	1.21

4. CONCLUSIONS

In the present study, a simple and effective procedure coupling FC and HPLC with a DAD detector has been developed for the separation, purification and quantification of Cu D and Cu I from a chloroform extract of *E. elaterium* fruit juice. The method was validated and quantified for Cu D and Cu I. The method showed good linearity, accuracy and precision within acceptable limits, and the LOD and LOQ values confirmed the efficiency of the method at low concentrations of cucurbitacins. The purity of each isolated cucurbitacin was the same as its standard. The method developed here seems to be quite efficient for the separation and quantification of the components present in plant extracts.

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