

Rapid Detection of Cucurbitacins in Tissues and *in vitro* Cultures of *Ecballium elaterium* (L.) A. Rich.

E. Attard

Institute of Agriculture, University of Malta, Msida MSD 06, Malta.

E-mail: everaldo.attard@um.edu.mt

Introduction: Cucurbitacins are highly oxygenated compounds abundantly found in Cucurbitaceous genera such as *Citrullus*, *Cucumis*, *Cucurbita* and *Luffa* (11). A relatively common cucurbitacin found in Cucurbitaceous species is cucurbitacin E (CuE). Although cucurbitacins exhibit positive effects both pharmacologically (1-3, 6, 14, 17) and in protecting the plants from certain diseases (5), they have a high degree of bitterness even at concentrations lower than 10 ppb (10). With the emergence of new cultivars, the expression of the genes that favor the production of cucurbitacins can be enhanced leading to the production of an inedible cultivar. To determine the efficacy of a method that detects these cucurbitacins in plant tissues we have selected an “indicator plant” that stores an abundance of these compounds. Locally, the squirting cucumber (*Ecballium elaterium*) is also being used as a rootstock in general practice to graft cucurbitaceous crops, owing to the disease resistance of the squirting cucumber to several pests and diseases. The reference cucurbitacin, in this study, was CuE.

Materials and Methods: *Ecballium elaterium* tissue culture material was obtained from a callus stabilized on Murashige and Skoog (13) medium (MS, pH 5.7) containing 5 mg/l naphthalene acetic acid (NAA) and 1 mg/l benzylamino purine (BAP) and from shoot explants grown on 0.1 mg/l NAA and 1 mg/l BAP. Local *Ecballium elaterium* fruit were collected from Marsascala, washed, sliced and the juice strained off. The fresh callus material and fruit juice were dried in an oven at 40 °C for 24 hours. For the cucurbitacin determination, the dried material was ground in a mortar until pulverized completely, homogenized with absolute ETOH (5 ml), reduced to a volume of 2 ml on a water bath and then filtered through a 0.22 µm filter (Schleicher & Shuell, Germany). For CuE, the dried material was extracted by CHCl₃ (5 ml) and then mixed with an equal volume of petrol (12). The filtrate was dissolved in absolute ETOH (5 ml),

reduce likewise to 2 ml and then filtered through a 0.22 µm membrane pore size.

Standard CuE used to produce the standard curve was provided and authenticated by Prof. D. Lavie (Rehovot, Israel). The solvents used were all HPLC grade obtained from Sigma Co. Ltd. (U.S.A.). The water used for HPLC was passed through a 0.22 µm filter (Schleicher & Shuell, Germany) and degassed.

Chromatographic conditions. HPLC was performed using a Kontron Instruments HPLC system (Herts, U.K.) consisting of two HPLC pumps (Kontron 422), an Autosampler 465 and a Diode Array detector 440. The detection was recorded on a computer via the on line Microsoft Windows program for Kontron Instruments - KromaSystem 2000 Version 1.60. The column was a Bio-Sil C18 HL 90-5S column (Bio-Rad, CA, U.S.A., 250 x 4.6 mm i.d., 5 µm particle diameter, 90 Å pore size). Since the elution was of the gradient type, a mobile phase of acetonitrile:water starting at a ratio of 2:8 and ending with a ratio of 45:55 at 35 minutes. The flow rate was 2.0 ml/min and CuE was detected by UV absorption at 229 nm. Each sample was run in duplicates for 3 independent experiments.

Spectrophotometric conditions. All samples (100 µl, in duplicate), together with various concentrations of CuE standard (0.017 to 1.113 mg/ml), were mixed with 100 µl of a 2 % phosphomolybdic acid (PMA) solution (BDH, U.K.) in absolute ETOH (15) at room temperature, using a 96-well plate (NUNC, Denmark). The absorbance was measured at 492 nm after 5 minutes on a MTP reader (STATFAX 2100, U.S.A.). The results were expressed as percentage weight calculated on dry callus weight. Standard curves were plotted for both analytical methods. For the HPLC, dilutions of standard CuE were prepared in the range of 0.02 and 0.32 mg/ml, while for the spectrophotometric method dilutions ranged from 0.017 to 1.113 mg/ml were used. CuE was quantified

by HPLC by considering the peak area, while for the spectrophotometric method it was quantified by determining the optical density, and both extrapolated on the standard curve. All quantitative determinations were subjected to regression analysis and ANOVA (one-way analysis of variance) using the BMDP/DYNAMIC v. 7.0 (Cork, Ireland) statistical package to determine the significance of the results.

Results and Discussion: The retention time of CuE, when analyzed by HPLC, was 34.01 ± 0.12 min. Figure 1 shows that CuE is distinctively collected from the fruit extract as opposed to the other cucurbitacins and their glycosides. However, the analysis is time-consuming and therefore not suitable for processing large numbers of samples, as previously indicated (8). In the present study, the retention time for CuE goes in accordance with the value obtained (33.92 min) for the same compound extracted from *Cucurbita* species by Halaweish and Tallamy (9). The initial qualitative analysis led to the development of a more rapid but still sensitive technique.

According to Balbaa et al. (4), a triphenyl tetrazolium test gave consistent results in assaying cucurbitacins. Despite this, although there was a positive reaction between the tetrazolium salt and CuE, our results were inconsistent (unpublished). Another method (16) reported for measuring cucurbitacin content in *Hemsleya dolichocarpa* used the dimethylaminobenzaldehyde reagent for a spectrophotometric reaction, as described in the Chinese Pharmacopoeia (7). However, we obtained false positive results by this method in the determination of the cucurbitacin content. In the same research work, phosphomolybdic acid was mentioned as a spraying reagent for a thin-layer chromatography densitometric method, at wavelengths of 510 nm and 600 nm. An ethanol solution was also indicated by Stahl and Jork (15) for triterpene identification as a spraying reagent in TLC analysis.

A spectrophotometric reagent was prepared from a 2 % solution of PMA in absolute ETOH and used in the 96-well plate assay. The reaction between standard CuE and PMA after about 5 minutes was scanned over the 200 to 500 nm range to determine qualitatively the absorbance at different wavelengths (Fig. 2). Eventually 492 nm, one of the preset wavelengths in the ELISA readers, was selected as the test wavelength. The reaction was performed over a period of 10 minutes, measuring the absorbance at 492 nm every minute. The best reaction time was 5 min. The 96-well plate format was selected, as samples can be prepared at μ l volumes and tested within a short time period. Two calibration curves were set-up for CuE standard for the HPLC and spectrophotometric assays (Fig. 3). These showed highly significant correlation coefficients ($R^2=0.9942$ and 0.9994 , for PMA and HPLC, respectively, $v=4$). Fresh fruit and callus samples were extracted with ETOH and then subjected to both methods. The approximate time for the two investigations was 7 min and 11.25 h for the PMA and HPLC, respectively. The values obtained were calculated in mg/ml according to the respective standard curves and then plotted against each other to test for their correlation. In fact, there was a good fit ($R^2=0.9869$, $v=7$), between the two assays. Since the PMA assay is suitable for cucurbitacins, additional extraction steps were required to isolate CuE (12) and determine it using the 96-well plate assay.

Surprisingly, for tissue cultures treated with NAA/BAP, the sole cucurbitacin emerging in the crude ethanol extract was CuE (98.41 %). This was compared to the extracted CuE (98.24 %), with a yield of 100 % as detected by HPLC. This new assay has proven to be an alternative procedure to the HPLC procedure, achieving results in a shorter time but still with great consistency. The use of this assay for other plant extracts containing cucurbitacin E should be investigated further.

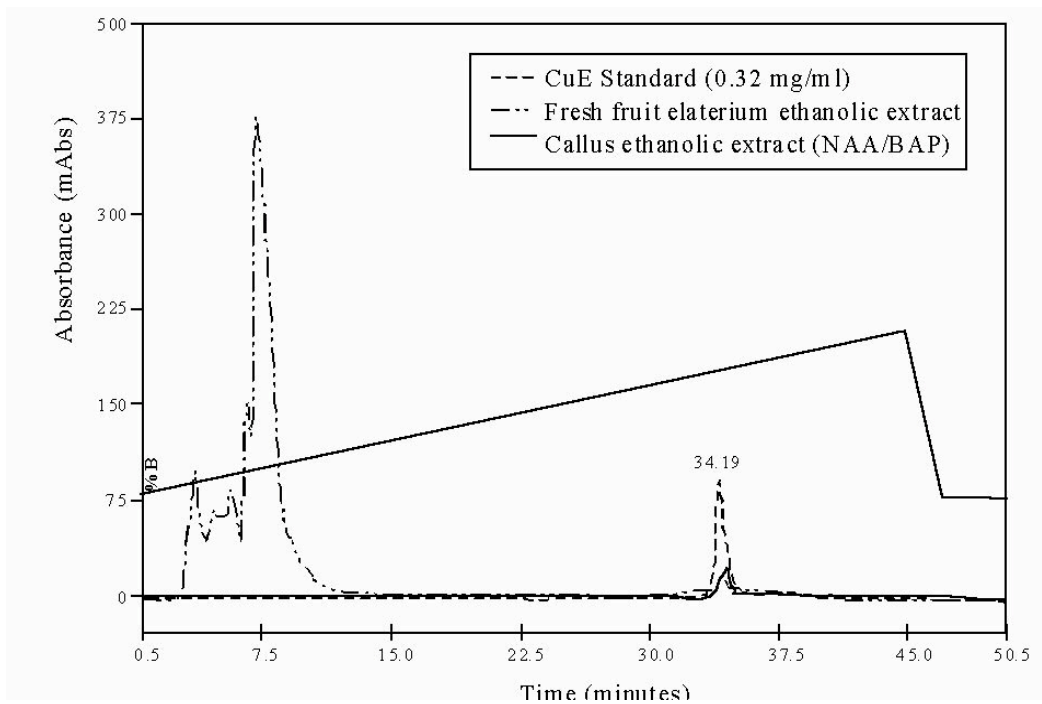


Figure 1: HPLC chromatograms of an authentic sample of Cucurbitacin E (0.32 mg/ml), a fresh fruit elaterium dried ethanolic extract and a callus ethanolic extract (NAA/BAP).

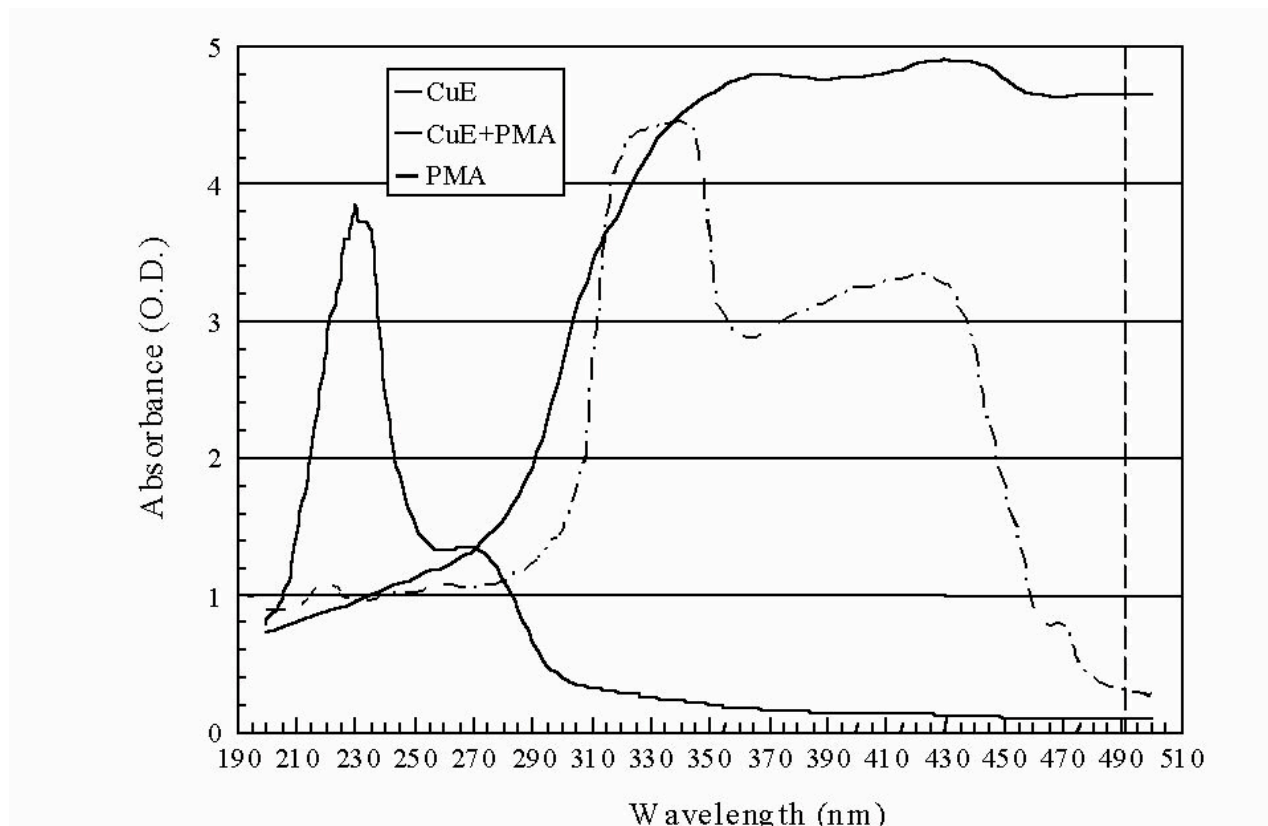


Figure 2: The U.V. profile for Cucurbitacin E, PMA and their reaction after 5 minutes at a wavelength scan from 200 - 500 nm on a GBC-UV/VIS spectrophotometer.

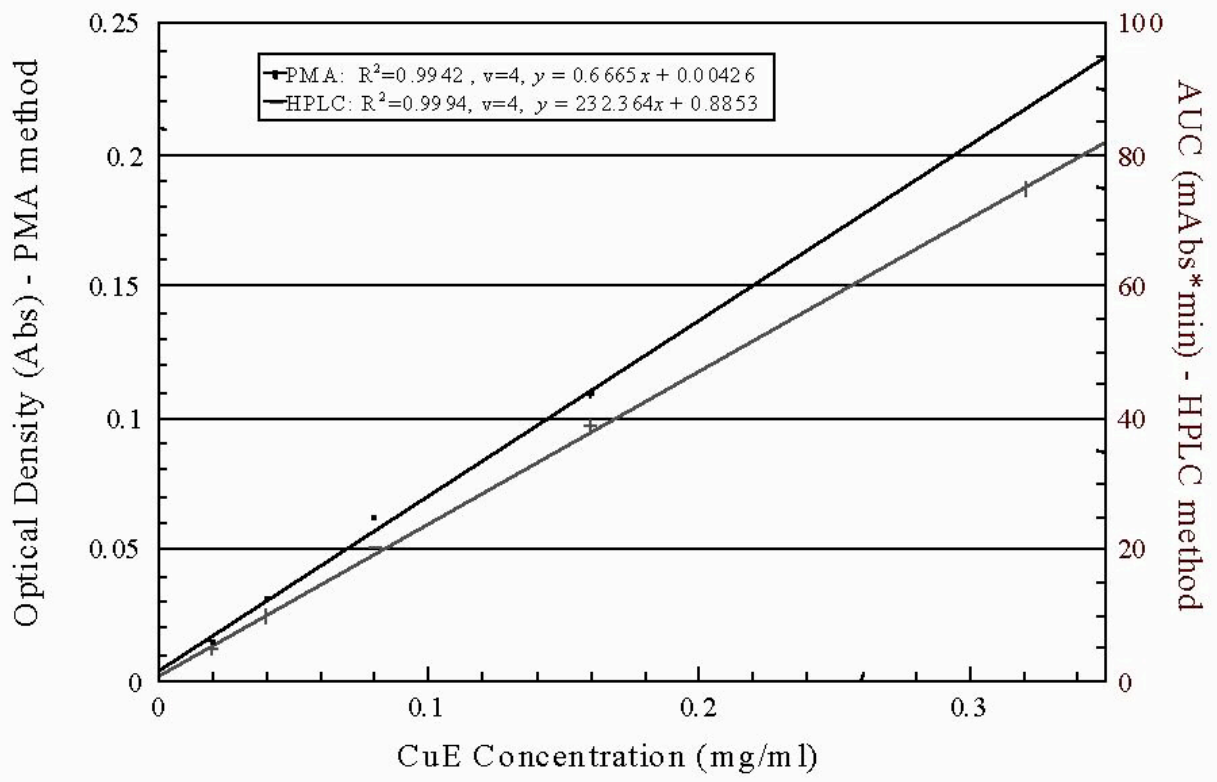


Figure 3: The standard curves for Cucurbitacin E with the CuE/PMA reaction and HPLC treatments.

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