Chromatographic and Mass Spectral Analysis of Cucurbitacins of Three *Cucumis sativus* Cultivars

Cotyledons from three *Cucumis sativus* seedlings [(1) Eversweet, a nonbitter cultivar resistant to the cucumber beetle, (2) Palomar a bitter cultivar susceptible to the cucumber beetle, and (3) a plant introduction No. 173889 (PI), a resistant bitter type] were analyzed for their major bitter principles known as cucurbitacins (Ct). Ct's A, B, C, D, E, and I were identified by using chromatographic and mass spectral data. The presence of cucurbitacin C was confirmed in Palomar and PI 173889. A nonbitter compound similar in chromatographic and mass spectral behavior to the bitter compound Ct C was found in Eversweet extracts. This compound differed from any of the other Ct's examined in this study as well.

The Cucurbitacins (Ct) were reported to have been first isolated from some members of the plant family Cucurbitaceae (Rehm, 1960; Lavie and Glotter, 1971). Also called bitter principles, these compounds are closely related tetracyclic triterpenes (Figure 1). Later, Ct's were found to occur in certain Cruciferae (Bull and Norton, 1970), Scrophulariaceae (Gmelin, 1967), Begoniaceae (Fokina and Belova, 1971), Liliaceae (Kupchan et al., 1978), and Euphorbiaceae (Paris and Tesseer, 1972). Medicinal and toxic properties of these compounds have stimulated a continuing interest in them (Kupchan et al., 1978; Shrobia, 1976).

It was found that Ct in bitter cucurbits were attractants to spotted cucumber beetles (Chambliss and Jones, 1966). Cucurbits lacking Ct were less susceptible to cucumber beetle damage. These studies were extended to striped and banded cucumber beetles (Da Costa and Jones, 1971). Spotted cucumber beetle feeding was correlated with Ct's, total sugars, and fatty acid content in *Cucurbita pepo* L (Sharma and Hall, 1971a,b). The major Ct in the cotyledons of *C. pepo* L was found to be Ct C (Sharma and Hall, 1973).

Most seedlings of commercially desirable cultivars contain Ct as glycosides, which make them susceptible to insect attack at the vulnerable cotyledon stage. However, the plant introduction 173889 (PI), introduced to the United States from India by the Federal–State plant introduction system, although bitter, was observed in screening tests to be relatively resistant to the banded cucumber beetle. The present study was concerned with the identification of the Ct's of PI 173889, a bitter cultivar Palomar, and a nonbitter cultivar Eversweet. A second objective of this research was to obtain mass spectral data on Ct extracted from cucumber seedlings as well as all the Ct known to occur in the cucurbitaceae. Due to the very small amounts of Ct present, only qualitative data were obtained. Many of the members of Cucurbitaceae contain less than 0.01% Ct (Rehm and Wessels, 1957).

MATERIALS AND METHODS

Seed and Plant Sample Production. Palomar seed were obtained from Ferry Morse Seed Co. Seeds of the PI and Eversweet were produced from self-pollinated plants grown at the Auburn University horticulture greenhouses and farm. Seeds of the three varieties were planted in flats of soil mix (1 part of perlite, 1 part of peat moss, and 2 parts of sterilized sandy loam) and grown in a growth chamber (12 h of daylight at 34 °C and 12 h of dark at 16 °C). Seedlings were harvested in 3–5 days after germination as they reached the fully expanded cotyledon stage which corresponded to the stage at which cucumber beetle damage in the field is at a maximum. Cotyledons were pinched off at the junction of the cotyledon and hypocotyl and kept frozen (−12 °C) until analyzed.

Table I. Thin-Layer Chromatography[a] of Crystalline Cucurbitacins

cucurbitacin	R_f value
A	0.347
B	0.510
C	0.394
D	0.328
E	0.591
I	0.450

[a] Silica gel plates, 0.5 mm, with a fluorescent indicator developed with ethyl acetate–benzene, 75:25.

Plant Extracts. Reagent-grade solvents (Mallinckrodt) were used for extraction procedures and thin-layer chromatography (TLC). The ethanol for the final TLC elutions was redistilled in a 100-cm all-glass still. Frozen samples were ground in a blender with 0.1 N orthophosphate buffer, pH 5.4. The cotyledon–phosphate slurry was held at 49 °C for 24 h to allow the naturally occurring enzyme elaterase (β-glycosidase) to hydrolyze the cucurbitacin glycosides to the aglycons. After hydrolysis, 95% ethanol was added (7 parts of ETOH to 3 parts of slurry), the mixture was filtered, and the filter was washed with ethanol. The ethanol solution was extracted with chloroform ($3 \times {}^1/_3$ volume) and the chloroform extracts were reduced to dryness and stored at −12 °C.

Thin-Layer Chromatography. Silica gel plates (0.5 mm) with a fluorescent indicator (Quantagram, Fairfield, NJ) were used for preparative separations and qualitative determinations. Crystalline Ct's A, B, C, D, E, and I used as standards were obtained from the laboratory of Dr. David lavie, Weisman Institute, Israel. Standards and cotyledon extracts were applied in chloroform and developed with an ethyl acetate–benzene (75:25) solvent system. Separations were viewed with short-wave (180–300 nm) ultraviolet light.

Mass Spectrometry. A Du Pont 21-490 mass spectrometer was operated at 70 eV and a source temperature of 225 °C. All samples were introduced in the solid state with a probe temperature of 290–310 °C. Perfluorokerosene was used for mass marking.

RESULTS AND DISCUSSION

Plant Extracts. Though the seedlings of most *Cucumis sativus* varieties are quite bitter to the taste, the concentration of Ct in the cotyledon is very low, and accurate quantitative estimations are difficult. Several investigators have estimated the concentration from the relative size of paper chromatographic spots [0.38% in fruit (Enslin, 1954) and 0.001–0.009% in seedlings (Rehm and Wessels (1957)]. In extraction procedures, it was necessary to taste both the filtrates and residues at each step to be sure that the bitter compounds were not lost. The concentration of ethanol

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Table II. Mass Spectrometry[a] of Crystalline Cucurbitacins

A		B		C		D		E		I	
m/e	% abundance	*m/e*	% abundance	*m/e*	% abundance	*m/e*	% abundance	*m/e*	% abundance	*m/e*	% abundance
41	25	43	29	41	21	41	15	43	96	41	25
43	96	55	10	43	100	43	59	67	8	43	100
45	21	57	13	45	42	55	14	87	12	55	21
67	17	87	12	55	14	67	10	96	100	69	23
87	16	96	100	60	22	69	14	111	13	87	30
91	18	105	8	69	12	87	18	112	12	91	22
95	21	111	12	95	13	96	100	113	22	96	67
96	100	112	8	96	27	111	22	121	10	111	26
111	22	113	10	111	95	112	19	136	6	112	20
113	21	149	8	113	10	113	18	164	26	146	32

[a] Du Pont 21-490 operated at 70 eV.

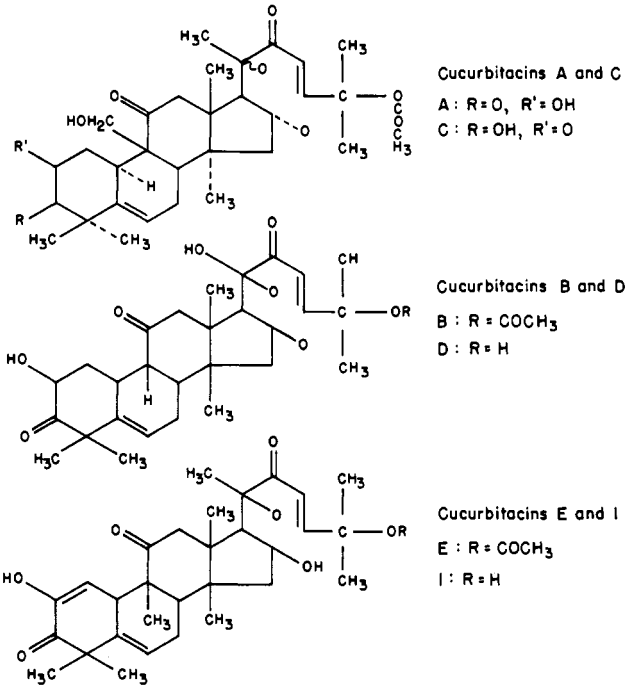

Figure 1. Bitter principles found in the plant family Cucurbitaceae. Structures: Reprinted with permission from the "Merck Index" (1976). Copyright 1976 by Merck & Co., Inc.

(7 parts of 95% ethanol to 3 parts of slurry) in the ethanol extraction was critical in retaining the Ct.

Thin-Layer Chromatography. Previous chromatography of Ct was conducted with paper impregnated with formamide (Enslin et al., 1945, 1956, 1957; Rehm et al., 1957); however, the authors were not able to reproduce this work. Reproducible R_f values were obtained with thin-layer plates as described above without formamide impregnation (Table I). Cochromatography with the Ct standards used for Table I established that the only Ct occurring in the fully expanded cotyledons of the cultivar Palomar and the PI was Ct C. Comparison of the size of the spots on the chromatogram and of the intensity of the spots when viewed under UV light indicated that there was less than half as much Ct C in the flesh of the PI as there was in the Palomar cotyledons. Though cotyledons of both plants tasted bitter, the reduced amount of Ct C in PI (a feeding stimulant to cucumber beetles) may have been below the detection threshold for banded cucumber beetles. This would account for the reduced feeding by that species noted in screening studies. While chromatograms of the Eversweet cultivar lacked a spot with a R_f value equal to Ct C, cochromatography showed two poorly separated spots, indicating that a compound in the Eversweet extract was chromatographically similar to the purified Ct C. For this reason, this compound was separated and eluded along with the spots corresponding to Ct C from the other two plant extracts for mass spectral analysis.

Table III. Mass Spectrometry of Extracts[a] from Seedling Cotyledons of Three *C. sativus* Cultivars

Palomar		PI 173889		Eversweet	
m/e	% abundance	*m/e*	% abundance	*m/e*	% abundance
41	60	41	45	41	78
43	100	43	100	43	100
45	17	45	50	45	28
55	57	55	42	55	71
57	42	60	29	57	49
67	23	69	30	60	33
69	39	95	17	69	39
96	42	96	22	81	26
111	18	111	21	83	25
113	14	113	24	111	21

[a] Crystalline solids eluted from thin-layer fractions corresponding in R_f value to cucurbitacin C.

Table IV. High Mass-Low Intensity[a] Fragments of Crystalline Cucurbitacins and *C. sativus* Cotyledon Extracts

	m/e
cucurbitacins	
A	514 (M^+)
	496
	484
B	498 ($M^+ - 60$)
	485
	403
C	500 ($M^+ - 60$)
	482
	470
D	516 (M^+)
	403
	385
E	556 (M^+)
I	514 (M^+)
cotyledon extracts	
Palomar	500 ($M^+ - 60$)
	482
	470
PI 173889	500 ($M^+ - 60$)
	482
	470
Eversweet	499

[a] Highest mass peaks detectable in spectra.

Mass Spectrometry. At least three spectra of each standard and crystalline cotyledon extract were obtained and averaged to account for the inherent instrument variation occurring in most mass spectrometers. The low mass data (Tables II and III) and the high mass data (Table IV) are confirmatory of the mass spectrometry conducted previously with cucurbitacins (Audier and Das, 1966; Kupchan et al., 1970). The high mass spectra of the cotyledon extracts of Palomar and PI 173889 were identical

with the spectrum of the standard Ct C and relative intensities of the 10 most intense peaks were in good agreement, also, with m/e 43 being the base peak. While the overall spectrum of Eversweet cotyledon extract was similar to the spectrum of Ct C (base peak 43), the only peak found in the high mass region was m/e 499.

CONCLUSIONS

Thin-layer and mass spectral data confirmed the only Ct found in the fully expanded cotyledons of Palomar and PI to be Ct C. The amount of Ct C in PI was much less than in Palomar.

A compound from Eversweet cotyledons was shown to be similar but not identical in chromatographic and mass spectral data to Ct C. It was surprising to find a compound so similar to Ct C in the nonbitter cultivar Eversweet since all cucurbitacins impart a bitter taste to the plant. Further research may indicate that this compound is either a precursor or a derivative of a cucurbitacin.

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Condensed Tannins in Kernels of Thirty-one Pecan [*Carya illinoensis* (Wangenh) K. Koch] Cultivars

Thirty-one pecan cultivars or seedling nut meats were assayed for condensed tannin content. There was expected variation in total condensed tannin content of the different cultivars.

Tannins contain many phenolic hydroxyl groups that enable them to form stable cross-linkages with proteins and other macromolecules. Tannins characteristic of the pecan [*Carya illinoensis* (Wangenh) K. Koch] species are partly responsible for the kernel coloration; they are found in high quantities in the shuck and corky middle portion of the nut and to a lesser extent in the hull and kernel (Brison and Cain, 1957; Polles et al., 1979). Reports on the role of tannins or tannin-like compounds in the pecan are limited (Senter and Forbus, 1978).

Thirty-one cultivars or seedling nut meats were assayed for condensed tannin content to establish values that could be used in evaluating these pecan types in light of the aforementioned references.

MATERIALS AND METHODS

Fresh pecan meats of 31 types were extracted from the hull, separated from the packing material, and finely chopped. Pecan nut samples were collected from five trees for each variety in the summer of 1978. An aliquot meat sample was taken for each variety from a 227-g sample. Three 100-mg portions from each type of meat were weighed into separate 30-mL Corex centrifuge tubes, and 5 mL of 5% HCl in 1-butanol (Swain and Hillis, 1959) was added to each tube. The meats were then homogenized with a Polytron homogenizer for 30 s. After homogenization, the tubes were immersed in a boiling water bath for 1 h, removed, and centrifuged, and the contents were diluted 1:5 v/v with 1-butanol. The absorbance was read on a spectrophotometer at 550 nm, and the percent tannin was computed from a standard curve made from condensed tannin obtained from A. C. Waiss, Jr. The curve was linear in the range observed and had the equation mg of tannin = $0.0534A + 0.00187$. The variation among samples was determined by ANOV. Means of determinations were then separated by Duncan's multiple range ($p = 0.05$).

RESULTS AND DISCUSSION

Table I lists the percent condensed tannin found in 31