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CUCURBITACIN E AND I IN *IBERIS AMARA*: FEEDING INHIBITORS FOR *PHYLLOTRETA NEMORUM*

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Key Word Index—*Iberis amara*; Cruciferae; cucurbitacins; cucurbitacin E; cucurbitacin I; glucosinolates; *Phyllotreta nemorum*; Coleoptera; Chrysomelidae; flea beetle; feeding inhibitors.

Abstract—Cucurbitacin E and cucurbitacin I have been isolated from green parts of *Iberis amara* and identified by TLC, UV and MS. It is shown that cucurbitacins act as feeding inhibitors for the flea beetle *Phyllotreta nemorum*. The most potent feeding inhibitors in green parts of *I. amara* towards *P. nemorum* are cucurbitacin E and I, and the concentrations of these compounds in the plant are found to be high enough to prevent feeding of the flea beetle.

INTRODUCTION

It is well known that Cruciferae and some other plant families contain glucosinolates [1, 2] and products thereof [3]. Glucosinolates are feeding stimulants for a number of insect species feeding on this family [4] including *Phyllotreta cruciferae* Goeze (Coleoptera; Chrysomelidae) [5]. The volatile hydrolysis products, isothiocyanates, are powerful attractants for *Phyllotreta* species [6, 7] as well as for some other species feeding on Cruciferae [4].

It is evident that glucosinolates play an important role in the host plant selection of a number of specialized insect species associated with this plant family. However, not all species containing glucosinolates are equally acceptable. This could be due to differences in concentration and structure of glucosinolates or it could be due to other substances. One possibility is that feeding inhibitors are present in non-acceptable species. The role of feeding inhibitors in host plant selection of phytophagous insects has been pointed out repeatedly [8–10].

The responses of crucifer feeders to secondary substances in Cruciferae not related to the glucosinolates have scarcely been investigated. Seeds of *Iberis* species are known to contain cucurbitacins [11, 12], a group of substances with powerful physiological activities [13].

In field and laboratory studies it was shown that *P. nemorum* L. accepts a number of glucosinolate-containing plants, the most attractive being radish, *Raphanus sativus* var. *radicula* and turnip, *Brassica campestris* var. *rapifera*, but totally ignores *Iberis amara* L. [14]. This suggests that *Iberis amara* contains substances which are detected by the beetles' sense organs and which inhibit the feeding response.

In seeds of *Iberis amara* cucurbitacin E (**1**) and I (**2**) are the most abundant cucurbitacins. It has been shown that these substances are potent feeding inhibitors for *P. nemorum* [14]. The present study was undertaken to investigate whether cucurbitacins are present in green parts of *I. amara* in sufficient amount to prevent feeding of flea beetles, and to investigate whether cucurbitacins are the most important feeding inhibitors in green parts of this plant.

Table 1. R_f values of cucurbitacins on Si gel plates

Compounds	Solvent* 1	2	3	4
Cucurbitacin E (1)	0.62	0.59	0.67	0.68
Cucurbitacin I (2)	0.46	0.26	0.42	0.46

*For solvent systems see Experimental

RESULTS

Preliminary results have indicated that cucurbitacins are present in green parts of *I. amara*. R_f values for **1** and **2** are presented in Table 1, the relative figures being in agreement with lit. values [15]. From green parts of *I. amara* $CHCl_3$ and H_2O extracts were prepared and investigated for feeding inhibitory activity Q′ (see Experimental). The results (Table 2) show that the $CHCl_3$ extract contained the largest amount of feeding inhibitory activity. Fig. 1 shows the results obtained for different

Table 2. Feeding inhibitory activity Q′ ± s.e. of extracts of green parts of *Iberis amara*

Extract	Dilution factor	Q′ ± s.e.	Number of experiments
$CHCl_3$	1	1.00 ± 0.000*	8
$CHCl_3$	2	1.00 ± 0.000*	8
$CHCl_3$	4	1.00 ± 0.000*	6
$CHCl_3$	8	0.94 ± 0.034*	6
$CHCl_3$	16	0.90 ± 0.024*	10
$CHCl_3$	32	0.67 ± 0.070†	8
H_2O	1	0.71 ± 0.039	8

*Q′($CHCl_3$) significantly higher than Q′(H_2O), $P < 0.001$.
†Q′($CHCl_3$) not different from Q′(H_2O), $P > 0.05$.

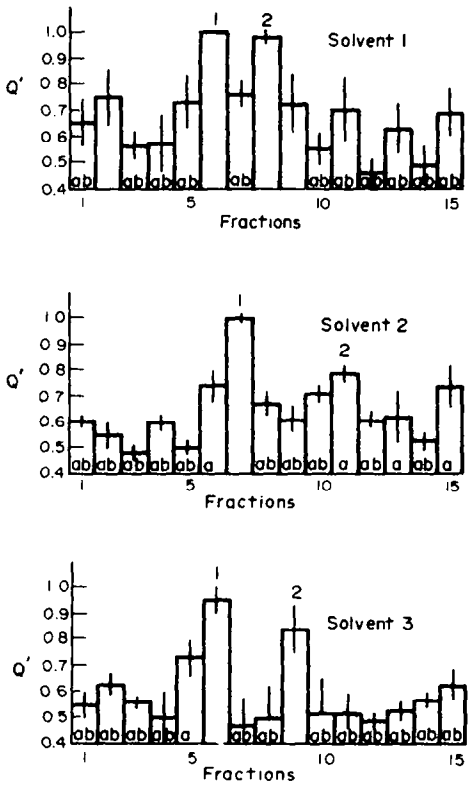

Fig. 1. Feeding inhibitory activity Q' ± s.e. of different fractions of $CHCl_3$ extract of *I. amara* separated by TLC. For details see Experimental. Number of experiments for each solvent: 6. (a) Fraction has significantly lower Q' value than fraction with R_f value as **1**, $P < 0.05$. (b) Fraction has significantly lower Q' value than fraction with R_f value as **2**, $P < 0.05$.

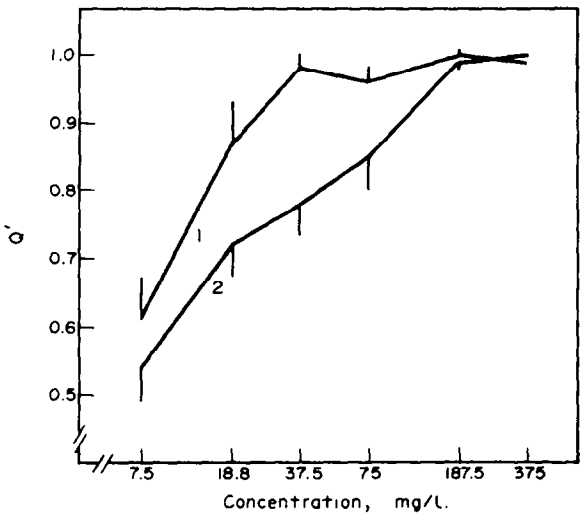

Fig. 2. Feeding inhibitory activity Q' ± s.e. of different concentrations of cucurbitacin E (**1**) and cucurbitacin I (**2**). For details see Experimental. Number of experiments normally 8, in a few cases 4, 9, 11.

TLC fractions of the $CHCl_3$ extracts (see Experimental). The largest feeding inhibitory activity was found in the fractions with R_fs as **1** and **2**.

The fractions with R_fs as **1** have in most cases significantly higher Q'-values than all other fractions except the fractions with R_f values as **2**. These fractions have the second highest activity with Q'-values significantly higher than most of the other fractions. It is thus evident that the most potent feeding inhibitors are compounds with properties as **1** and **2**.

The compounds in the fractions corresponding to **1** and **2** showed UV spectra characteristic for diosphenol cucurbitacins [11], with λ_{max} at 232 and 270 nm and shift of the 270 nm peak to 314 nm in the presence of 0.01N NaOH. The MS fragmentation pattern for the isolated compounds were almost identical with that indicated in the lit. [16, 17] and with that obtained for authentic **1** and **2**. The isolated compounds were indistinguishable from authentic **1** and **2** by co-chromatography in four solvents (Table 1), colour reactions with $FeCl_3$, *p*-dimethylaminobenzaldehyde, and UV light [11].

By means of the intensity of the UV absorption peaks at 270 nm and 314 nm [18], the amount of **1** and **2** isolated by preparative TLC was calculated to 150 mg **1** and 80 mg **2** per kg fresh plant material. Feeding inhibitory activity Q' of different concentrations of **1** and **2** was estimated and the results are presented in Fig. 2. The amount of solution taken up by the radish leaf discs used in the assay was established to be a third of the leaf weight. The amount of **1** and **2** on the radish leaf discs corresponds then to a concentration of about a third of the concentrations used in the solutions. The concentration of **1** and **2** in *I. amara* is thus high enough to prevent feeding of *P. nemorum*. Other compounds with properties like cucurbitacins, but not yet identified, are present in lower concentration in green parts of *I. amara* Compounds which behave as glycosides of cucurbitacins are also present in the green parts of the plant.

DISCUSSION

Our results indicate that **1** and **2** are important defences in *Iberis amara* against at least one phytophagous insect, *P. nemorum*. It has been shown that cucurbitacins also inhibit feeding of the leaf beetles *P. undulata*, *P. tetrastigma* and *Phaedon cochleariae* [14] and that *Iberis amara* is not acceptable to *P. cruciferae* [6] and only slightly acceptable to *Pieris* species [19]. The method used in the separation of the $CHCl_3$ extract does not allow us to conclude whether or not there are other feeding inhibitors present in weak concentration or with weaker activity, especially in regions near **1** and **2**.

Even though glucosinolates and isothiocyanates make crucifers more acceptable to a number of adapted species, it is important to think of them as part of a defence system against non-adapted species [9, 20]. Further elaboration of the glucosinolate system in plants is perhaps not likely to be successful in the defence against adapted species which apparently have mechanisms to detoxify glucosinolates. A more successful defence would occur when different kinds of substances are synthesized [9]. Since most *Iberis* species contain cucurbitacins and only a few lack them [11], it is likely that the ability to synthesize these substances arose early in the evolution of the genus.

Cucurbitacins are widely distributed in the Cucurbitaceae [13]. They are specific feeding stimulants for at least 2 species of leaf beetles feeding on this plant family [21, 22]. Moreover, they are known to offer protection

against a polyphagous mite, *Tetranychus urticae*, which is often damaging to non-bitter cucurbits [23].

Cucurbitacins in Cucurbitaceae and glucosinolates in Cruciferae serve much the same function: they are inhibitors for non-adapted species and stimulants for adapted species. The function as inhibitors are considered to be primary; at present no other function of cucurbitacins in Cruciferae has been reported.

EXPERIMENTAL

Plant material. Seeds of *Iberis amara* var. grandiflora and radish (*Raphanus sativus* var. radicula) purchased from J. E. Ohlsens Enke, Copenhagen. The *I. amara* plants were grown outdoors, harvested when flowering and kept at −20° until isolation was performed. Radish plants were grown in the greenhouse and used in the assay.

Insects. The flea beetles used in the assay were from a laboratory culture reared on radish or turnip plants.

Assay. A leaf disc test was used, 4 discs (d = 14 mm) were cut from radish leaves with a cork borer. Two discs were dipped in the solvent (control discs) and two in the soln or extract to be tested (test discs). After evapn of the solvent the leaf discs were placed alternatingly in plastic vials (h = 7 cm; V = 400 ml) with a bottom layer of gypsum-charcoal. Discs were fastened to the bottom layer with pins. The bottom layer, prepared from $CaSO_4$ and charcoal (9:1 by vol.), was mixed in the vial and wetted until a homogeneous viscous mass with a smooth surface was formed, which hardened on drying. It can keep a high humidity without appearing wet. The bottom layer was satd with H_2O before each test, excess H_2O was evapd in an air stream. Under these conditions leaf discs appeared fresh and their diameter did not change during the 24 hr test period. When the discs were fastened the vial was closed with a plastic lid. Adult beetles (3–6) were introduced through a hole (d = 13 mm) in the lid, the hole was closed with a plug of cotton wool. The beetles were allowed to feed for 24 hr. Tests were made at 25 ± 1° and a photoperiod of 18 hr light and 6 hr darkness. Test vials were illuminated from above. Only beetles less than 10 days old were used in the assay. They were fed *ad libitum* with radish leaves for at least 2 days prior to a test. The amount eaten of control and test discs was determined visually. Discs, mounted between two microscope slides, were placed above a disc of graph paper (d = 14 mm, cut with a cork borer) and the number of mm^2 eaten was determined under a preparation microscope.

Calculations. The feeding inhibitory activity Q′ = C/(T + C). C is the amount eaten of the 2 control discs, T the amount eaten of the 2 treated discs. The statistical significance was evaluated by means of the Mann–Whitney U Test [24].

General methods and instrumentation. MS were obtained at 70 eV. TLC was performed in trichloroethylene–*i*-PrOH (4:1) (solvent 1), C_6H_6–MeCOEt (2:1) (solvent 2), $CHCl_3$–EtOAc–HCOOH (12:12:1) (solvent 3), and diisopropyl ether–Me_2CO (5:2) (solvent 4), by ascent on Si gel plates (20 × 20 cm) containing a fluorescence indicator. The compounds were localized in UV light at 250 nm or with the reagents *p*-dimethylaminobenzaldehyde or $FeCl_3$ [11]. R_f values are presented in Table 1.

Isolation of feeding inhibitors for P. nemorum. Green parts of *I. amara* were dried by lyophilization. The dried plant material (5 g) were homogenized in $CHCl_3$ (100 ml) and filtered. The residue was washed twice with $CHCl_3$ (25 ml portions) and the combined filtrates were concd to dryness, *fraction C*. Plant material undissolved in $CHCl_3$ was homogenized in MeOH (100 ml) and filtered. The residue was washed twice with MeOH (25 ml portions) and the combined filtrates were concd to dryness, *fraction M*. The still undissolved plant material was homogenized in H_2O (100 ml) and filtered. The residue was washed twice with H_2O (25 ml portions) and the combined filtrates were taken to dryness, *fraction H*. The fractions M and H were dissolved in H_2O (50 ml), this soln was washed with 3 × 50 ml $CHCl_3$, then concd to dryness, *H_2O extract*. The $CHCl_3$ solns were combined with fraction C, concd to dryness, *$CHCl_3$ extract*. Prior to the assay the $CHCl_3$ extract was dissolved in 7.5 ml EtOH. From this solution samples of 750 μl were diluted with 250 μl H_2O. Similarly the H_2O extract was dissolved in 9 ml H_2O. From this soln samples of 900 μl were diluted with 100 μl 0.5% Na lauryl sulphate. Results from investigation of the H_2O extract and the $CHCl_3$ extract in the assay are presented in Table 2. Further separation of the compounds present in the $CHCl_3$ extract was performed by preparative TLC. The extract was dissolved in 10 ml $CHCl_3$–EtOH (1:1) and 500 μl of this soln was applied to 2 plates. This procedure is repeated with each of the solvents 1, 2 and 3 (see above). The solvents were allowed to move 15 cm from the starting line and after air drying of the chromatograms the compounds were localized in UV light. By this method the chromatograms were divided into 15 bands (fractions of about 16 × 1 cm) which were scraped off and treated on filters with $CHCl_3$–EtOH (1:1, 10 ml to each fraction). The filtrates No. 1 (at the solvent front) to No. 15 (at the starting line) and a corresponding reference filtrate from the band just below the starting line were taken to dryness, dissolved in EtOH (3 × 250 μl) and H_2O (250 μl). Results from investigations in the assay are presented in Fig. 1. The control discs were treated with the solns from the reference filtrates. Each fraction is tested 3 times. The whole procedure is repeated twice with each of the solvents.

Feeding inhibitory activity Q′ of **1** *and* **2**. Authentic **1** and **2** were dissolved in $CHCl_3$–EtOH (1:1) and subjected to preparative TLC in solvent 1. The appropriate bands were treated as before and the concentration in the final solns was determined by UV at 270 nm. Diluted series were assayed for feeding inhibitory activity, the results are presented in Fig. 2.

Isolation of **1** *and* **2**. $CHCl_3$ extracts prepared as described above and dissolved in 50 ml petrol (bp 50–70°) were washed with 10 × 50 ml MeOH–H_2O (1:9). The MeOH–H_2O solns were combined, concd to dryness, dissolved in 5 ml $CHCl_3$–EtOH (1:1), and purified by preparative TLC using solvent 1 and 3 (Table 1). The compounds **1** and **2** were isolated as described above. Identity of the compounds was established by TLC, and the colours obtained with *p*-dimethylaminobenzaldehyde, $FeCl_3$, and in UV light (Table 1). MS [16, 17] and UV spectra [11] of the compounds dissolved in EtOH and EtOH/NaOH were in agreement with corresponding spectra of authentic **1** and **2**. Isolation of **1** and **2** was also performed from the CH_2Cl_2 extract of 2.2 kg. *I. amara* using Si gel column chromatography as described in ref. [11].

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