*Journal of Chromatography A*, 658 (1994) 69–76
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2825

# Phytoecdysteroids in *Kochia scoparia* (burning bush)

Laurence Dinan

*Department of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter, Devon EX4 4QG (UK)*

ABSTRACT

Seed and plants of *Kochia scoparia* have been analyzed for phytoecdysteroids by high-performance liquid chromatography coupled to ecdysteroid-specific radioimmunoassay (RIA) or bioassay. Relatively low levels of RIA-positive material are found in seed and throughout the plant. Evidence is presented that the major phytoecdysteroids present in seed are 20-hydroxyecdysone and polypodine B (5β,20-dihydroxyecdysone), but significant amounts of other, as yet unidentified, phytoecdysteroids also occur. Extracts of whole plants contain a similar mix of phytoecdysteroids, but also contain a large amount of an apolar conjugate of ecdysone which is not present in seed. Concentrations of RIA-positive material vary throughout the plant, with higher concentrations being present in the root and leaves. Results are discussed in relation to the phytoecdysteroid distribution in other members of the Chenopodiaceae and with regard to the relationships between *K. scoparia* and its insect predators/pollinators.

## INTRODUCTION

Insect steroid hormone analogues (phytoecdysteroids) are detectable in many, but not all, species of plant. The concentrations occurring in phytoecdysteroid-positive species are often 100- to 1000-fold higher than those typically found in insects. While it has been suggested that ecdysteroids might have a phytohormonal rôle, it seems more probable that they act to deter insect predation, either by acting as antifeedants or by creating a hormonal imbalance in predatory insects, resulting in developmental disruption or even death [1]. While there is some supporting evidence for this latter rôle, it has not been conclusively proven and the issue is clouded by (1) certain polyphagous insect species being able to tolerate very high levels of ingested phytoecdysteroids without impaired development, (2) relationships between plants and insects being complex, since insects may be beneficial as well as detrimental to plant survival and (3) the diversity of allelochemicals elaborated by plants which probably provides several options for deterring a predator species and provides potential for synergism with regard to defence chemicals.

In order to resolve the question of the function(s) of phytoecdysteroids we have initiated a study focussed on one family of plants, the Chenopodiaceae which include many species of agronomic importance. Consideration is being given to the quantitative and qualitative differences in phytoecdysteroids between species of the family, within individual plants and between individuals within a population. Ultimately, the intention is to relate the findings to the phylogenetics of the Chenopodiaceae, the biology of the plants, the susceptibility of the plants to insect predation and the co-occurrence of other allelochemicals in each species.

To date, most work has concentrated on the genera *Chenopodium* [2–7], where phytoecdysteroids occur in about one-third of the species, and *Spinacia* [8–10]. Rapid and sensitive micro-analytical methods have been developed for the quantification of phytoecdysteroids in small plant samples (*ca.* 50 mg) using radioimmunoassay (RIA) or bioassay and using high-performance liquid chromatography (HPLC)

0021-9673/94/$07.00 
*SSDI* 0021-9673(93)E0417-S

coupled to RIA or bioassay for the characterization of the phytoecdysteroid profile in the extract [5,6]. The phytoecdysteroids of *C. album* comprise predominantly 20-hydroxyecdysone (20E: 69%) and polypodine B (PolB; 5β,20-dihydroxyecdysone: 28%) and a complex mixture of minor ecdysteroids (together 3%) [6]. Phytoecdysteroid concentration varies throughout the plant and during development. A concentration gradient occurs within aerial portions rising from the bottom of the stem [*ca.* 0.16 mg ecdysone (E) equivalents/g dry mass] and the lowest leaves (*ca.* 0.1 mg E eq./g dry mass) and such that the highest concentrations are present in the growing tips (1 to 3 mg E eq./g dry mass) and youngest leaves of non-flowering plants (*ca.* 1 mg E eq./g dry mass) [5]. Even higher levels are associated with the flowers, especially the anther tissue (5.4 mg E eq./g dry mass), but not the enclosed pollen (<0.2 mg E eq./g dry mass) [6]. Fluctuating levels are found in root tissue during development [4]. This pattern is common to the other members of the genus *Chenopodium* which contain phytoecdysteroids [6] and to *Spinacia oleracea* [8,10]. This has led us to suggest that phytoecdysteroids help to deflect insect predators from the nutritionally attractive tender young growths and to protect flowers of wind-pollinated species against predation. On this basis, one might predict at the simplest level that higher phytoecdysteroid levels might correlate with anemophilous pollination and low levels with entomophilous pollination. Unfortunately, the mode of pollination in the Chenopodiaceae has not been extensively studied [11], so it is not currently possible to assess the validity of this hypothesis.

*Kochia scoparia* is a highly insect-resistant member of the Chenopodiaceae. It has rapid growth potential and will grow on arid, saline soils. It has become a serious weed species in North America [12,13], but it is also of interest as a potential forage crop for livestock [14,15]. A number of potential allelochemicals have been identified from *K. scoparia*: saponins, flavonoids, oxalate, alkaloids and phenolic acids [14–19].

Rangeland grasshoppers (*Melanoplus* spp.) avoid eating *K. scoparia* if given a choice of foods [20], but, if given no choice, they eat the older, less nutritious leaves rather than the tender, younger leaves [21]. Grasshoppers fed on *K. scoparia* experience reduced survival, impaired ovarian development and low fecundity [22], reminiscent of effects one might expect from the application of exogenous ecdysteroids. If a concentration gradient of phytoecdysteroids exists in the aerial portions of *K. scoparia* similar to that found in *C. album*, this might be related to the feeding pattern of the insects of this plant. Phytoecdysteroids have not been reported to be present in *K. scoparia*, although there is a brief report of the presence of "moulting hormone activity" in fruits of *Kochia* [23]. The aims of this research were thus (1) to determine whether ecdysteroids are present in *K. scoparia*, (2) to use chromatographic means to begin to identify which ecdysteroids are present and (3) to quantify ecdysteroid concentrations in various portions of the plant. *K. scoparia* presented some interesting methodological problems in the analysis and quantification of phytoecdysteroids because of the relatively low levels present and because of severe interference from other compounds present in apparently all parts of the plant.

## MATERIALS AND METHODS

### *Source of seed and growth of plants*

Seeds of *K. scoparia* were purchased from Mr. Fothergill's Seeds, Kentford, Suffolk, UK. Plants were grown in coir (ICI) to a height of 30–40 cm, at which point they had initiated flowering.

### *Extraction of plant material*

*Seed.* Saponins were selectively extracted from seed by a modified procedure of Kernan *et al.* [17]. Seed were immersed in 0.1% (w/v) NaOH solution for 15 min with gentle shaking. Seed were then washed with distilled water and freeze-dried for 24 h.

*Plant portions.* Plants were removed from the pots and the roots cleaned of compost. Plants were dismembered with a sharp scalpel blade.

The fresh weight of each portion was determined and then freeze-dried to constant mass (4 days) before the dry mass was determined.

*General extraction procedure.* Seed or freeze dried plant material was ground with a pestle and mortar. Samples (25 mg) were extracted three times with 1-ml aliquots of methanol for 3 h at 55°C. The three extracts from each sample were pooled, 1.3 ml water added and partitioned twice against 2 ml hexane to remove pigments and non-polar lipids. The aqueous methanol fraction was used for ecdysteroid determination.

*High-performance liquid chromatography*

HPLC equipment and general procedures have been described previously [24]. Columns and separation systems were as follows:

*RP1.* Spherisorb ODS-2 (250 × 4.6 mm I.D., 5-$\mu$m particle size) eluted at 1 ml/min with a linear gradient from methanol–water (3:7, v/v) to methanol over 30 min and then isocratically with methanol for a further 10 min.

*RP2.* The above reversed-phase column eluted at 1 ml/min with a linear gradient from methanol–water (45:55, v/v) to methanol–water (7:3, v/v) over 30 min and then isocratically with methanol–water (7:3, v/v) for a further 10 min.

*NP1.* Apex II DIOL column (150 × 4.6 mm I.D., 5-$\mu$m particle size) eluted isocratically at 1 ml/min with methanol–dichloromethane (4:96, v/v).

All separations were monitored at 242 nm and fractions of 30-s (NP1) or 1-min (RP1 and RP2) duration were collected for further analysis by RIA and/or bioassay.

*Ecdysteroid radioimmunoassay and bioassay*

Ecdysteroids present in extracts or in HPLC fractions were quantified by radioimmunoassay using the DBL-1 antiserum (bleed F; generously provided by Professor Dr. J. Koolman, Universität Marburg, Marburg, Germany) as described previously [5]. Ecdysone (E) was used as the radiolabelled and reference ligands and results are expressed in ecdysone equivalents. Cross-reactivity factors for 20-hydroxyecdysone and polypodine B with this batch of serum were 0.48 and 2.87, respectively (E = 1). Samples were also assessed using the microplate-based $B_{II}$ cell bioassay for ecdysteroid receptor agonists/antagonists [25,26]. Briefly, cells of the *Drosophila melanogaster* $B_{II}$ cell line [27] are grown in the wells of sterile 96-well plates and their densities measured turbidometrically using a microplate reader. In the presence of ecdysteroid agonists, absorbance values (cell densities) are depressed relative to controls. Results are expressed as 100 $(A_{control} - A_{test})$, where $A_{control}$ = the absorbance at 405 nm of wells containing cells grown for 6 days in the absence of ecdysteroid and $A_{test}$ = the absorbance of wells grown in the presence of the test substance. The microplate reader was zeroed on wells containing Schneider's *Drosophila* medium but no cells. There is a logarithmic relationship between the bioassay response and the amount of agonist, such that ≤0.05 ng 20E eq. produces no response, 1 unit = 0.25 ng 20E eq., 2 units = 0.375 ng 20E eq., 3 units = 0.5 ng 20E eq., 4 units = 1.25 ng 20E eq. and 5 units (maximal response) ≥ 5ng 20E eq. Ecdysteroid antagonists may be assessed by determining the ability of test compounds to prevent the reduction in absorbance brought about by $5 \cdot 10^{-8}$ *M* 20-hydroxyecdysone.

*Enzymic hydrolyses*

Fractions for enzymic hydrolysis were dissolved in 10 $\mu$l ethanol and 200 $\mu$l *Helix pomatia* gut hydrolases (Sigma, Type H1; 10 mg/ml 0.1 *M* sodium acetate buffer, pH 5.4) and incubated at 37°C for 5 days [28]. Protein was precipitated by the addition of ethanol (1 mL) and aliquouts of the supernatant after centrifugation were assessed by RIA.

*Solid-phase extraction*

A portion of the aqueous methanol phase (1 ml) deriving from the extract of whole plants was diluted with water (6 ml) and applied to an activated $C_{18}$-cartridge (Sep-Pak, Millipore). The cartridge was sequentially eluted with 5 ml each of 10, 25, 60 and 100% methanol in water [29].

## RESULTS

### *Initial studies*

Previous studies on other members of the Chenopodiaceae have established generally suitable procedures for the extraction of phytoecdysteroids from small plant samples and their analysis and quantification by RIA, bioassay and HPLC [5,6,30]. However, when these methods were applied to extracts of aerial portions or seed of *K. scoparia*, the presence of low levels (relative to *C. album*) of RIA-positive material (*ca.* 40 μg E eq./g dry mass) was indicated, but there was not a linear relationship between RIA response and aliquot size in the RIA (see Fig. 1 for an example), preventing accurate quantification of ecdysteroid levels. The deviation from linearity appeared to derive from the presence of interfering substances co-occurring in the extracts (saponins being the most probable candidate).

A number of chromatographic and partition approaches were attempted to alleviate this interference, but these failed to separate the interfering substances adequately from the RIA-positive material. It has, however, been reported that saponins may be selectively extracted from seed of *K. scoparia* with 1% (w/v) NaOH [17] and thus attention was initially concentrated on seed.

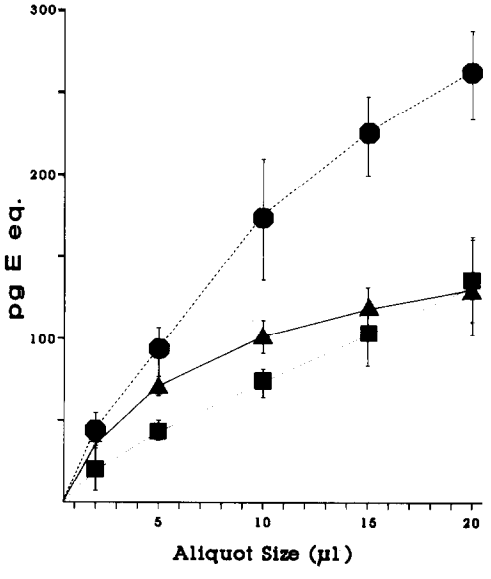


Fig. 1. Radioimmunoassay response in relation to aliquot size for methanolic extracts of *Kochia scoparia* seed: control seed (▲), seed pretreated with 1% (w/v) NaOH (■) and seed pretreated with 0.1% (w/v) NaOH (●). Error bars indicate the standard deviation ($n = 4$).

### *Analysis of seed*

Kernan *et al.* [17] found that *K. scoparia* seed contained 1 to 2% of the dry mass as saponins and demonstrated that washing seed with 1% NaOH selectively extracted 96% of these saponins. Ecdysteroids are, however, alkali-labile [31]. Ten-fold dilution of extracts from seed which had been pre-treated with 1 or 0.1% NaOH were assessed for linearity of RIA response and compared to an extract of untreated seed (Fig. 1). It is clear that pre-extraction with NaOH improves the linearity of the response considerably, and that more than twice as much RIA-positive material is recovered after treatment with 0.1% NaOH than with 1% NaOH. In addition to extracting saponins, 1% NaOH is extracting or degrading a portion of the RIA-positive material. The tangential nature of the initial linear portion of the data for the extract after 0.1% NaOH treatment and the curve for the control extract indicate that ecdysteroids in the seed are not being extracted or degraded by treatment with 0.1% NaOH.

A portion of the extract from 0.1% NaOH-pretreated seed was separated by RP-HPLC (system RP1) and monitored by RIA and bioassay (Fig. 2). RIA-positive material elutes in the region in which many phytoecdysteroids elute and the largest peak elutes at the retention time of 20E and PolB. Significant amounts of less polar RIA-positive material and smaller amounts of more polar RIA-positive material are also present. Bioassay revealed fractions 17 and 23 to be highly active in the ecdysteroid agonist assay; none of the extracts or HPLC fractions showed any antagonistic activity. The breadth of the apolar peak in Fig. 2 indicates that it consists of several components. The seed extract was therefore separated on system RP2, revealing two regions of non-polar RIA-positive material, the first eluting at the retention time of E, and the second with a retention time intermediate between that of E and ponasterone A (PoA). The

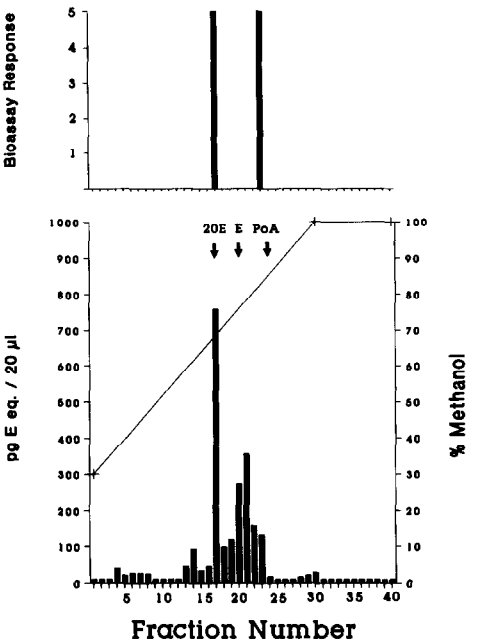


Fig. 2. Reversed-phase HPLC–RIA–bioassay of a methanolic extract of *Kochia scoparia* seed which had been pretreated with 0.1% NaOH to remove saponins. A portion of the extract (equivalent to 4 mg seed) was separated on HPLC system RP1 and fractions of 1 ml were collected. Aliquots (20 µl) of each fraction were subjected to RIA and bioassay: lower panel HPLC–RIA results and upper panel HPLC–bioassay results.

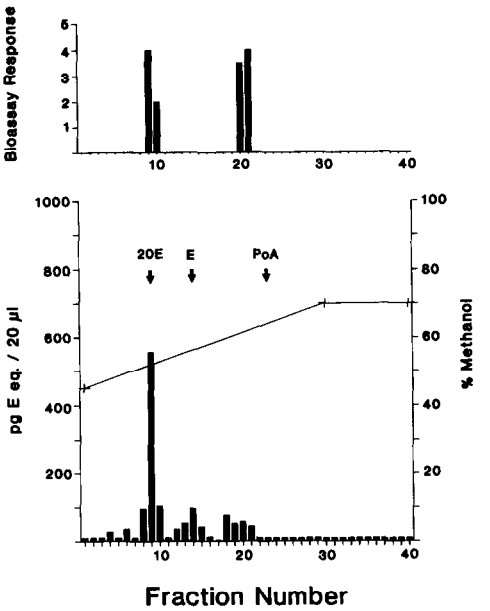


Fig. 3. Reversed-phase HPLC–RIA of an extract of *Kochia scoparia* seed (pretreated with 0.1% NaOH) on system RP2. Details as for Fig. 2.

latter peak is still broad and probably consists of at least two components. This is reinforced by biological activity being associated with fractions 20 and 21, but not with fractions 18 and 19. Hydrolysis of portions of the HPLC fractions with *Helix* enzymes prior to RIA revealed no significant increase in RIA response for any of the fractions, indicating that *Helix*-hydrolysable ecdysteroid conjugates are not present in *K. scoparia* seed.

20E and PolB, which have been identified as the major ecdysteroids in other chenopods, generally co-elute on RP-HPLC. The possibility that *K. scoparia* seed contain both 20E and PolB was assessed by separating fraction 9 (RP2: Fig. 3) on system NP1 (Fig. 4). RIA-positive peaks are detected co-chromatographing with both ecdysteroids. Fraction 8–10 and 15 are bioassay-positive. While PolB is less RIA-positive with the DBL-1 antiserum than 20E, it is biologically

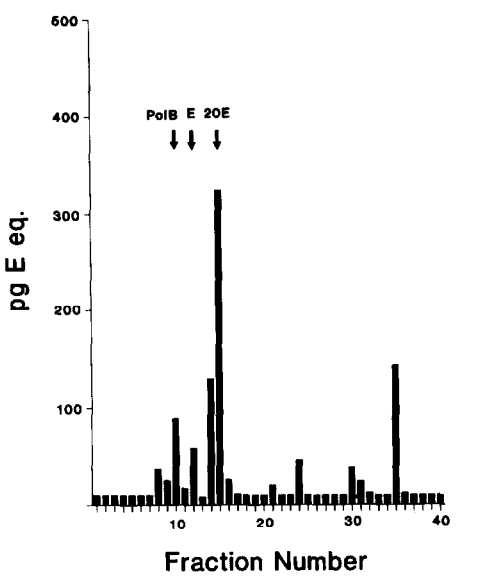


Fig. 4. Normal-phase HPLC–RIA of the RIA-positive material from fraction 9 of the RP-separation (system RP2, Fig. 3) of an extract of *Kochia scoparia* seed. The column was eluted isocratically with 4% methanol in dichloromethane (1 ml/min). Fractions of 0.5 ml were collected and 50 µl aliquots were subjected to RIA.

more potent in the $B_{II}$ bioassay. Several other peaks of RIA-positive material are also detected after normal-phase separation, but these do not have significant biological activity. The identity of these compounds is currently unknown.

*Analysis of plants*

Partitioning plant extracts between either $CHCl_3$–water or butanol–0.1 *M* NaOH was not effective at removing interfering substances from the ecdysteroids, nor were attempts to hydrolyse saponins with either hesperidinase [32] or *Helix* enzymes. Chromatographic separation on silica was also not effective, but separation on reversed-phase cartridges was partially so. When an extract of whole plants was applied to a $C_{18}$ Sep-Pak and eluted sequentially with 10, 25, 60 and 100% methanol in water, 53 and 33% of the RIA-positive material eluted in the 60 and 100% methanol fractions, respectively. These were analyzed by RP-HPLC (system RP1; Fig. 5); revealing four peaks of RIA-positive material: I–IV. All four peaks were found in the 60% fraction, while the 100% fraction contained only the two non-polar peaks, III and IV. Peak I co-chromatographs with 20E and peak II with E. Peak III has the same retention time as the apolar peak in seed. Peak IV was not observed in extracts of seed. Peaks I and III are biologically active in the $B_{II}$ bioassay. Peak IV is susceptible to hydrolysis by *Helix* enzymes, showing a three-fold increase in RIA-response and being converted to a compound which co-chromatographs with E on systems RP1 and NP1 (data not shown).

*Quantification of ecdysteroids*

RIA was used to quantify ecdysteroid levels in seed and throughout mature plants (Table I). Seed were pre-extracted with 0.1% NaOH. Ecdysteroid levels in plant extracts were determined by dilution (100-fold) of the extract and assessment of the RIA response with 2- to 20-$\mu$l aliquots. At these levels (<100 pg), linearity of RIA response was observed and concentrations were calculated from the gradients of plots of aliquot size *vs.* RIA response.

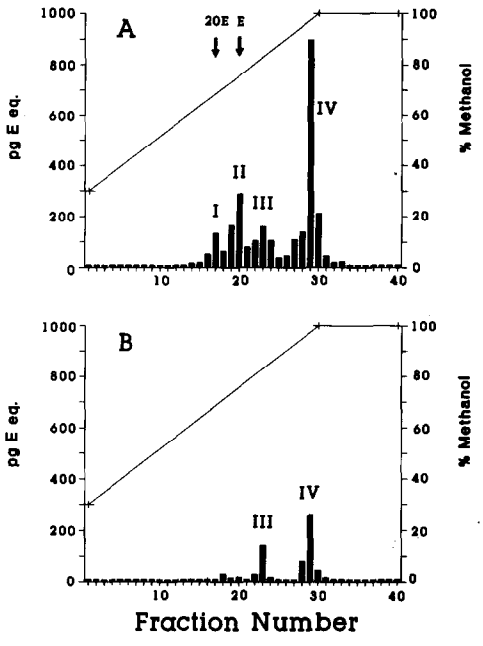


Fig. 5. Reversed-phase HPLC–RIA of fractions deriving from an extract of whole plants of *Kochia scoparia*. Plants were extracted with methanol and partitioned against hexane as described in "Materials and Methods" and then a portion (equivalent to 6 mg dry mass of plant) was separated on a $C_{18}$ Sep-Pak cartridge. The RIA-positive fractions [(A) 60% methanol in water and (B) methanol] were separated on HPLC system RP1, 1-ml fractions collected and aliquots (50 $\mu$l) subjected to RIA.

TABLE I

ECDYSTEROID LEVELS IN *KOCHIA SCOPARIA* AS DETERMINED BY RADIOIMMUNOASSAY (DBL-1 ANTISERUM)

| | RIA response ($\mu$g ecdysone equivalents/ g dry mass) |
|---|---|
| Seed | 30 |
| Roots | 143 |
| Lower stem | 26 |
| Middle stem | 21 |
| Upper stem | 37 |
| Lowest leaves | 102 |
| Middle leaves | 112 |
| Uppermost leaves | 44 |
| Flowers | 56 |
| Senescing (red) leaves | 41 |

## DISCUSSION

The combined use of RIA, bioassay and HPLC provides an effective means of identifying from very small samples which plant species contain phytoecdysteroids and for determining ecdysteroid profiles to identify those species which may contain novel ecdysteroid agonists or antagonists. Since analyses can be performed on such small samples, many samples may be processed rapidly and simultaneously. This approach has often been used in the analysis of ecdysteroids in insects, but has not been extensively used for phytoecdysteroids. In our studies on the Chenopodiaceae, these procedures have allowed the rapid analysis of *ca.* 100 species using seed samples of 50 mg or less. Only with species of the genera *Bassia*, *Corispermum* and *Kochia* were problems encountered [30], owing to interference in the RIA. Since *K. scoparia* provides a rare opportunity to relate phytoecdysteroid levels and distribution to insect feeding preference data, it was worth persevering with the analysis of this species.

The positive responses detected with both RIA and the $B_{II}$ bioassay demonstrate that seed of *K. scoparia* do contain phytoecdysteroids. The major ecdysteroids appear to be 20E and PolB, based on co-chromatography on RP- and NP-HPLC systems and on the presence of appropriate biological activity. The presence of E is indicated by RP-HPLC–RIA, but needs to be confirmed by other means. E has a very low affinity for the ecdysteroid receptor [26], so it is not surprising that this peak does not show activity in the $B_{II}$ bioassay. Several other peaks of RIA-positive material, presumably corresponding to other ecdysteroids, are also present. The identities of these are currently unknown. One of these compounds possesses significant biological activity and deserves to be investigated further.

Analysis of aerial portions of *K. scoparia* plants was hampered by difficulties in separating interfering substances from the ecdysteroids. However, it is clear that ecdysteroids are present in all parts of the plant. Qualitatively, there appears to be some difference between the ecdysteroid profiles of the plants and the seed. Most notable is the presence of relatively large amounts of a *Helix*-hydrolysable conjugate of ecdysone in plant material which is absent from seed. Its absence from seed is not an artefact deriving from the pre-treatment of seed with NaOH, since no evidence for this peak was seen in extracts of seed which had not been pre-treated. Since this conjugate was observed in extracts of whole plants, nothing can be concluded presently about its distribution within the plant. This would be worth investigating.

Quantitatively, levels of RIA-positive material in *K. scoparia* are low and fairly uniformally distributed when compared to *C. album*. Roots showed markedly higher ecdysteroid levels than the other samples and leaves contain higher levels than the stem. Roots may be the site of biosynthesis of phytoecdysteroids. Levels of RIA-positive material should relate directly to the levels of phytoecdysteroid, but is should be borne in mind that the measured RIA-response is a consequence of both the amount of each RIA-positive compound and their relative affinities for the antiserum. Thus, a similar RIA response can be obtained by a small amount of a high-affinity compound or a large amount of a low-affinity compound. Consequently, RIA assessments of complex situations such as might prevail in *K. scoparia*, with the ecdysteroid profile changing throughout the plant should be treated with some caution. However, it is unlikely that such an effect is masking a significant ecdysteroid concentration gradient in leaves of *K. scoparia*, but this possibility cannot be discounted until the phytoecdysteroids in different portions of the plant have been identified and separately quantified.

In conclusion, *K. scoparia* contains several phytoecdysteroids. There are some qualitative differences between ecdysteroids present in seed and in plants. Substantial evidence is presented for 20E and PolB being the major ecdysteroids in seed, as they are in almost all other chenopods which have been analyzed [30]. Although there are quantitative differences in ecdysteroid levels throughout mature plants these are not of the magnitude observed in *C. album*. Also, the distinct concentration gradient found in *C. album* is not present in aerial portions of *K.*

*scoparia*. Therefore, it seems unlikely that the phytoecdysteroids alone could account for the feeding behaviour of rangeland grasshoppers on leaves of *K. scoparia*. However, phytoecdysteroids may act synergistically with other secondary compounds (*e.g.* saponins) and a gradient of the other class of compound could engender a gradient of phytoecdysteroid potency. *Melanoplus* spp. Have been observed feeding on the stems of large plants of *K. scoparia* [21], and this may be associated with the lower levels of phytoecdysteroid there. Elevated ecdysteroid levels are not associated with flowering in *K. scoparia*. In this context, it is worth mentioning that *K. scoparia* is believed to be insect-pollinated [11].

ACKNOWLEDGEMENTS

This research was supported by a small grant (to L.D. and J. Roddick) from the University of Exeter Research Fund. I gratefully acknowledge the technical assistance provided by Pensri Whiting and thank Dr. Jim Roddick and Ms. Anna Rijnenberg for their helpful discussion.

REFERENCES

1 R. Lafont and D.H.S. Horn, in J. Koolman (Editor), *Ecdysone: From Chemistry to Mode of Action*, Georg Thieme Verlag, Stuttgart, 1989 pp. 39–64.
2 I. Tóth, M. Báthory, K. Szendrei E. Minker and G. Blazsó, *Fitoterapia*, 52 (1981) 77–80.
3 M. Báthory, I. Tóth, K. Szendrei and J. Reisch, *Phytochem.*, 21 (1982) 236–238.
4 L. Dinan, S. Riseborough, M. Brading, C.Y. Clément, D.J. Witts, J. Smith, S. Colombé, V. Pettitt, D.A. Wheeler and D.R. Greenwood, in I. Hrdy (Editor), *Insect Chemical Ecology*, Academia, Prague, and SPB Acad. Publ., The Hague, 1991, pp. 215–220.
5 L. Dinan, *Phytochem. Analysis*, 3 (1992) 132–138.
6 L. Dinan, *Experientia*, 48 (1992) 305–308.
7 L. Dinan, in S.B.J. Menken, J.H. Visser and P. Harrewijn (Editors), *Proceedings 8th International Symposium on Insect-Plant Relationships, Wageningen, 1992*, Kluwer Academic Publishers, Dordrecht, 1992, pp. 86–88.
8 R.J. Grebenok and J.H. Adler, *Phytochem.*, 30 (1991) 2905–2910.
9 R.J. Grebenok, P.V. Ripa and J.H. Adler, *Lipids*, 26 (1991) 666–668.
10 L. Dinan, unpublished results.
11 W.H. Blackwell and M.J. Powell, *Ann. Mo. Bot. Gard.*, 68 (1981) 524–526.
12 C.V. Eberlein and Z.Q. Fore, *Weeds Today*, 15 (1984) 5–7.
13 F. Forcella, *Weeds Today*, 16 (1985) 4–6.
14 E. Coxworth, D. Green and J.A. Kernan (Editors), *Saskatchewan Research Council Technical Report No. 221 R-814-4-B-88*, 1988.
15 M. Karachi, H.E. Kiesling, R.D. Pieper, G.M. Southward and R.E. Kirksey, *Bull. M. State Univ. Agric. Exp. Stn.*, 731 (1988) 52.
16 J. Souto and V.A. Milano, *Rev. Invest. Agropecu., Ser. 2*, 111 (1966) 367–383.
17 J.A. Kernan, E. Coxworth and S. Fleming, *J. Agric. Food Chem.*, 21 (1973) 232–234.
18 C.W. Dickie, M.L. Gerlach and D.W. Hamar, *Vet. Hum. Toxicol.*, 31 (1989) 240–242.
19 K. Drost-Karlowska and Z. Kowalewski, *J. Nat. Prod.*, 41 (1978) 289–290.
20 C.F. Hinks, M.T. Cheeseman, M.A. Erlandson, O. Olfert and N.D. Westcott, *J. Insect Physiol.*, 37 (1991) 417–430.
21 O. Olfert, C.F. Hinks, W. Craig and N.D. Westcott, *J. Econ. Entomol.*, 83 (1990) 2421–2426.
22 C.F. Hinks, O. Olfert, N.D. Westcott, E.M. Coxworth and W. Craig, *J. Econ. Entomol.* 83 (1990) 1338–1343.
23 T. Takemoto, S. Ogawa, N. Nishimoto, S. Arihara and K. Bue, *Yakugaku Zasshi*, 87 (1967) 1414–1418.
24 L. Dinan, *J. Chromatogr.*, 436 (1988) 279–288.
25 C.Y. Clément and L. Dinan, in I. Hrdy (Editor), *Insect Chemical Ecology*, Academia, Prague and SPB Acad. Publ., The Hauge, 1991 pp. 221–226.
26 C.Y. Clément, D.A. Bradbrook, R. Lafont and L. Dinan, *Insect Biochem. Mol. Biol.*, 23 (1993) 187–193.
27 E. Gateff, L. Gissman, R. Shrestha, N. Plus, H. Pfister, J. Schröder and H. zur Hausen, in E. Kurstak, K. Maramorosch and A. Dübendorfer (Editors), *Invertebrate Systems in Vitro*, Elsevier, 1981, pp. 517–533.
28 P. Whiting and L. Dinan, *J. Insect Physiol.* 34 (1988) 625–631.
29 R. Lafont, J.L. Pennetier, A. Andrianjafintrimo, J.C. Claret, J.F. Modde and C. Blais, *J. Chromatogr.*, 236 (1982) 137–149.
30 P. Whiting and L. Dinan, in preparation.
31 D.H.S. Horn, in G.A. Kerkut and L.I. Gilbert (Editors), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 7, Pergamon Press, Oxford, 1985, pp. 185–248.
32 H. Kohda, O. Takeda and S. Tanaka, *Chem. Pharm. Bull.*, 37 (1989) 3304–3305.