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BIOCHEMICAL MECHANISMS OF RESISTANCE TO INSECTS IN SOYBEAN: EXTRACTION AND FRACTIONATION OF ANTIFEEDANTS

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Abstract—Leaf extractables/fractions obtained from the leaves of soybean PI 227687 showed greater antifeedant activity towards the third-instar larvae of the cabbage looper, *Trichoplusia ni* (Hub.). Extraction of leaves with ethyl acetate followed by 60% methanol, and counter-current partitioning of leaf extractables between ethyl acetate, water and hexane was not convenient for isolation of antifeedants. Methanol (60%) in water extracted most of the antifeedants from leaves, and this extract when partitioned between ethyl acetate and water (the latter repeatedly extracted with the former), concentrated the antifeedants from soybean leaves for further processing on thin layer chromatography or high performance liquid chromatography.

There were quantitative differences in ethyl acetate fraction of PI 227687 (6%) and Davis (3.2%), and in flavonoids resolved at Rf 0.10–0.20 in benzene: ethyl acetate: methanol (4:3:3). Most of the flavonoids with antifeedant activity resolved at Rf 0.10–0.80. The antifeedant activity of flavonoid bands from PI 227687 was greater than the corresponding bands of Davis.

Key Words Soybean, antifeedants, flavonoids, cabbage looper, host-plant resistance

Résumé—Les extraits ou fractions foliaires obtenus a partir des feuilles de PI 227687 ont montre une activite phagodissuasive elevee sur les larves du troisieme stad de l'arpenteuse du chou, *Trichoplusia ni* (Hub.). L'extraction des feuilles effectuee avec l'acetate d'ethyle suivie du méthanol a 60%, et la separation à contre-courant des extraits foliaires entre l'acetate d'ethyle, l'eau et l'hexane n'ont pas facilite l'isolement des phagodissuadents. Le méthanol (60%) dans l'eau a permis d'extraire la plupart des phagodissuadants àpartir des feuilles. Separé entre l'acétate d'éthyle et l'eau (la fraction aqueuse se separé a plusieurs reprises de celle d'acetate d'éthyle) cet extrait a permis de concentrer les phagodissuadants a partir des feuilles de soja pour un deuxieme traitement par la chromatographie sur couchemince ou la chromatographie liquide a haute performance.

On a constaté des différences quantitatives dans la fraction d'acétate d'éthyle de PI 227687 (6%) et celle de Davis (3,2%), ainsi que dans les flavonoides résolus entre Rf 0,10 et 0,20 dans le systeme benzène: acétate d'éthyle: méthanol (4:3:3). La plupart des flavonoides ayant une activitéphagodissuasive se sont résolus à Rf 0,10–0,80. De plus, l'activité phagodissuasive des bands de flavonoids de PI 227687 était plus élevée que les bands correspondantes de Davis.

Mots Clés Soja, phagodissuadants, flavonoides, arpenteuse du chou, résistance variétale

INTRODUCTION

Flavonoids of varying solubilities commonly occur in plants. Non-polar flavonoid aglycones such as isoflavones, flavanones, dihydroflavonols, flavones, and flavonones are usually extracted with chloroform, ether, ethyl acetate, or benzene, while the more polar flavonoids such as hydroxylated flavones, flavonols, aurones and chalcones are extracted with acetone, ethanol, methanol, water, or a combination of these (Markham, 1975). The polar solvents also extract large amounts of sugars, polysaccharides, and other water soluble components, while the non-polar

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solvents extract chlorophyll and fats, which may interfere in biological evaluations and in resolving the components by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC)(Sharma and Norris, 1991a).

Various solvent systems have been used to extract flavonoids from soybean leaves to evaluate their biological activity towards insects. Methanol extract exhibited higher antibiotic activity than the petroleum ether or dichloromethane extracts (Smith and Fischer, 1983) against *Plusia includens* Wlk. Chiang et al. (1986) used 60% methanol to extract antifeedants from the insect resistant soybean cultivar PI 227687, and then subjected it to sequential extraction with hexane, ether, ethyl acetate, methanol and water. This led to distribution of biological activity in several fractions. Khan et al. (1986) used ethyl acetate for extracting flavonoids from the insect-resistant soybean cultivar PI 227687.

Flavonoids in soybean contribute to genotypic resistance to plant pathogens (Keen et al., 1972; Keen and Paxton, 1975; Ingham et al., 1981; Ebel, 1986) and insects (Chiang et al., 1986; Khan et al., 1986). Glyceollins inhibit the feeding by Mexican bean beetle, *Epilachna verivestis* Muls. (Hart et al., 1983). Coumestrol, phaseol and afromosion possess antifeedant properties towards *P. includens* (Caballero and Smith, 1986). Diadzein, flavonoid X2, sojagol, coumestrol and glyceollins obtained from leaf extractables of PI 227687 are antifeedant and/or antibiotic towards cabbage looper, *Trichoplusia ni* Hb. (Sharma and Norris, 1991a).

The present studies were conducted on extraction of antifeedants from the insect-resistant (PI 227687) and the susceptible (Davis) soybean genotypes with polar and non-polar solvents, and counter-current/TLC partitioning of the leaf extractables to locate and evaluate the biologically active fractions against *T. ni*.

MATERIALS AND METHODS

Plants

Plants of the insect-resistant (PI 227687) and susceptible 'Davis) genotypes were grown in the greenhouse at the United States Department of Agriculture (USDA), Dairy Forage Research Center, University of Wisconsin, Madison. Seedlings germinated in moistened vermiculite were transplanted in 20-cm-dia. earthen pots containing a sterilized mixture of soil, sand and vermiculite (2:1:1). Plants were fertilized with 50 ml solution of 1% Miracle-Gro (Stern's Nursery Inc., Geneva, NY, USA) every fortnight. Potted plants were kept under Metalarc high intensity light (1000 Watt) for 16-h photophase. Plant growing conditions were the same as described by Sharma and Norris (1991b). Leaves from the 8-week-old plants (V8 stage of development) (Fehr et al., 1971) were removed and stored at -5°C or lyophilized and stored in a desiccator.

Insects

Insects were reared under laboratory conditions (27 ± 2°C and 60 ± 5% relative humidity). The cabbage looper, *T. ni* larvae were reared on a pinto bean-based artificial diet (Shorey and Hale, 1965) under laboratory conditions (27 ± 2°C, 60 ± 5% r.h.). Freshly moulted third-instar larvae, starved for 4 h, were used to monitor antifeedant activity of different leaf extractables/fractions.

Extraction and fractionation

Two procedures involving 60% methanol and ethyl acetate were used for extracting flavonoids from soybean leaves. The residue was extracted reciprocally with a polar or non-polar solvent.

Ethyl acetate-methanol (60%) extraction system

In the first procedure, green leaves (100 g) were homogenized with 850 ml of ethyl acetate in an ice-cooled Waring blender for 5 min. The homogenate was filtered through Whatman filter paper no. 1. The residue was re-extracted with 500 ml of ethyl acetate on an automatic shaker for 8 h and then filtered. Each leaf sample was re-extracted five times. After extraction with ethyl acetate the residue was extracted three times with 500 ml of 60% methanol as described above. Ethyl acetate and 60% methanol extractables were rotoevaporated at 50 ± 1°C.

Ethyl acetate and 60% methanol extractables were dissolved in 200 ml of ethyl acetate and 100 ml 60% methanol (1:1), and partitioned with 200 ml distilled water + 50 ml ethanol in a separatory funnel six times. Aqueous and ethyl acetate fractions were rotoevaporated to dryness at 50 ± 1°C. Ethyl acetate and aqueous fractions were then re-dissolved in benzene: ethyl acetate: methanol (4:3:3), and 40% ethanol, respectively, as 10% stock solutions and stored at 4°C.

Another lot of leaf extractables was dissolved in 100 ml each of ethyl acetate, *n*-hexane and 60% methanol in a separatory funnel. The aqueous phase was separated. Hexane soluble components were partitioned with 200 ml 60% methanol six times. Methanol soluble components (60%) were reduced by rotoevaporation to about 200 ml, and then

partitioned between ethyl acetate and water + 50 ml ethanol. The volume of aqueous phase was maintained at about 200 ml, and it was repeatedly partitioned with 200 ml ethyl acetate three times. All fractions were rotoevaporated to dryness at 50 ± 1°C. The hexane fraction was dissolved in *n*-hexane, the ethyl acetate fraction in benzene: ethyl acetate: methanol (4:3:3), and the water fraction in 40% ethanol as 10% stock solutions; and stored at 4°C.

Methanol (60%) ethyl acetate extraction system

Lyophilized leaf powder (50 g) was homogenized in an ice-cooled Waring blender for 5 min in 850 ml 60% methanol. The homogenate was filtered through Whatman filter paper no. 1. The residue was re-extracted five times with 500 ml of 60% methanol. The mixture was agitated on an automatic shaker for 8 h. The residue was extracted thrice with 500 ml ethyl acetate as described above. Methanol (60%) and ethyl acetate extracts were rotoevaporated to dryness at 50 ± 1°C.

Methanol extractables were then dissolved in 400 ml of 60% methanol in water and ethyl acetate (1:1) + 50 ml ethanol in a separatory funnel. Ethyl acetate and aqueous phases were separated. The aqueous phase was partitioned with 200 ml of ethyl acetate + 50 ml of ethanol six times. The volume of aqueous phase was maintained at about 200 ml. Ethyl acetate and aqueous fractions were rotoevaporated at 50 ± 1°C and dissolved in ethyl acetate: ethanol: water (3:1:1) and 40% ethanol, respectively as 10% stock solutions; and stored at 4°C.

TLC

Different extracts/fractions were chromatographed on 250 µm-thick Silica gel (Sigma Chemicals, St. Louis, Mo.) fluorescent plates to resolve, especially, the flavonoids (Sharma and Norris, 1991a). Fifty µg of each extract/fraction were spotted as a thin spot with a micro-capillary tube. The spots were dried under an air stream from a hair drier. The prepared plates were then developed in benzene: ethyl acetate: methanol (4:3:3) solvent system in a TLC chamber containing 250 ml solvent mixture. The chamber was lined with a filter paper to aid saturation of the enclosed atmosphere. When the solvent front reached 80% of the plate length, the plates were removed, and allowed to dry at room temperature. Resolved components were visualized under 254 nm UV light (Mineralight lamp, UV SL-25, Ultra-violet Products San Gabriel, California) to mark the flavonoid bands. The relative to front (Rf) value of each spot was calculated in relation to the solvent front.

Ethyl acetate fraction was resolved on TLC plates. One ml of 10% stock solution was applied as a thin, uniform band on each TLC plate using a 23 cm long Pasteur pipette. The tip of the Pasteur pipette was drawn into a thin capillary over the gas burner to enable precise application of the extract. The band during application was constantly dried under an air stream. Plates were then developed, as described earlier. Silica gel bearing resolved components was scraped from the TLC plates with clean microscope slides (7.5 x 2.5 cm), and three fractions (Rf 0.1–0.2, 0.2–0.5, and 0.5–0.8) were collected individually. The silica gel of each fraction was extracted with ethanol. Each fraction was stirred for 5 minutes on a test tube shaker (Vortex - Genie, Scientific Industries Inc., Bohemia, NY, USA); and then was filtered through Whatman filter paper no. 1. The residue was washed thrice with 10 ml ethanol. Solvent was removed under a stream of nitrogen, and the contents were re-dissolved in ethanol as a 1% solution, and stored at 4°C.

The aqueous fraction of the leaf extractables, as partitioned between ethyl acetate and water, was also resolved on TLC plates. The plates were developed as described above. From each plate, the silica gel was scraped into 5 bands (Rf 0.0–0.1, 0.1–0.2, 0.2–0.4, 0.4–0.55 and 0.55–0.8). Resolved components were extracted from each band, as described above, and were stored as 1% solutions in ethanol at 4°C.

Bioassay for antifeedant activity

Leaf extractables were bioassayed using double- or multi-choice disc assays. Reciprocal testing of ethyl acetate extractables from PI 227687 and Davis was conducted using leaf discs from trifoliate no. 7 (from the stem bottom) at the V10 stage of soybean development (Fehr et al., 1971). Leaf discs were cut with a no.7 cork borer and kept between the folds of water soaked filter paper. A treatment disc received 4000 µg of leaf extractables (40 µl of 10% solution) by a micro-pipette. Control discs were treated only with 40 µl of solvent. Solvent was dried under vacuum in a desiccator for 1 h.

Soybean leaf extractables obtained by the 60% methanol-ethyl acetate extraction system were also tested on snap bean, *Phaseolus vulgaris* leaf discs. These leaf discs were prepared and treated with leaf extractables, as described above.

Elderberry, *Sambucus canadensis* pith discs (400 µm thick) impregnated with sucrose (400 µg/disc) were used as a non-soybean substrate to evaluate the antifeedant activity of leaf extractables as described by Sharma and Norris (1991b). Elderberry pith discs were sized with a no. 7 cork borer (18 mm dia.) and

treated with 400 µg of sucrose (40 µl of 1% sucrose solution) applied with an automatic pipette. Pith discs were dried on a filter paper under a slow stream of air from a table fan and then mounted 2 cm above a thin wax layer in a Petri dish arena on insect pins. The pith discs were then treated with 40 µl of respective leaf extractables/fractions at the same dosage or different dosages of the same fraction. The control discs were treated with 40 µl of respective solvents. The solvent was dried from discs under vacuum in a desiccator for 1 h. The treated leaf/elderberry pith discs were offered to the cabbage looper larvae with control discs in a double- or multi-choice assay.

Double-choice assay. Two discs, one treated with sucrose + leaf extractables and the other treated with sucrose + solvent only, were positioned 5 mm apart in an appossed arrangement on a thin wax layer in the centre of a 9-cm-dia. Petri dish arena. The wax layer was covered with a filter paper. A filter paper attached to inner surface of each dish cover was moistened with 2 ml water to keep the leaf discs turgid, and the elderberry-pith discs moist. One 4-h-starved, but water-satiated third-instar larva was released in each Petri dish arena. A larva was confined with the assay discs for 12 h, or as otherwise indicated in a given experiment. The remaining discs were passed through a leaf area meter (LI 3100 LI-COR, Inc., Lincoln, NE) to record the unconsumed disc area.

Multi-choice assay. Discs treated with different extracts/fractions, or different dosages of the same extract/fraction were arranged in a circular pattern in a 9-cm-dia. Petri dish arena. The discs were pinned to the wax layer as described above. One untreated disc, receiving sucrose + solvent only, was centered in the Petri dish as a positive control. Third instar larvae, starved for 4 h but water-satiated, were released in the Petri dish arena. The number of larvae released in each dish were the same as the number of discs offered in a Petri dish. The larvae were confined with the discs for 12 h. The unconsumed area of each disc was measured using the leaf area meter as described above.

Statistical analysis

Data were subjected to analysis of variance, and the treatment means were compared using least significant difference (LSD) at $P < 0.05$. Antifeedant activity of different leaf extractables/fractions was computed in relation to the control discs as follows:

$$\text{Antifeedant activity (\%)} = \frac{\text{Unconsumed area of the control disc} - \text{Unconsumed area of the treated disc}}{\text{Total disc area}} \times 100$$

RESULTS AND DISCUSSION

Antifeedant activity of ethyl acetate extractables from PI 227687 and Davis leaves

PI 227687 leaf discs were less preferred than the Davis leaf discs (Fig. 1). Leaf discs from PI 227687 treated with ethyl acetate extractables of the same cultivar showed the highest antifeedant effect for the third-instar larvae of the cabbage looper. Davis leaf discs treated with ethyl acetate extractables from PI 227687 were also less preferred than the untreated Davis leaf discs. These results confirm the resistance and antifeedant activities of leaf extractables from PI 227687 reported by Leudders and Dickersen (1977) and Khan et al. (1986). Ethyl acetate extractables from Davis increased insect feeding on PI 227687 leaf discs. However, leaf discs from Davis treated

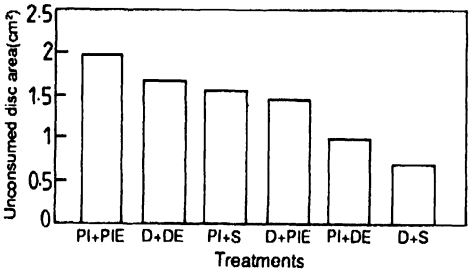

Fig. 1. Antifeedant activity of ethyl acetate leaf extractables from PI 227687 and Davis to cabbage looper *T. ni* under multi-choice conditions (4000 µg of leaf extractables/leaf disc). (PI = PI 227687, D = Davis, PIE = PI 227687 ethyl acetate, DE = Davis ethyl acetate extract, and S = solvent).

with ethyl acetate extractables from the same cultivar were relatively less damaged than the untreated discs. Presence of some antifeedant activity in extracts from Davis leaves may be due to resistance induced by mild damage by the thrips in the greenhouse (Chiang et al., 1987; Ebel, 1986).

Antifeedant activity of ethyl acetate-methanol extracts/fractions (60%)

Partitions of leaf extractables between ethyl acetate and aqueous phases (the former being repeatedly extracted by the latter) led to accumulation of antifeedant activity in the aqueous fraction (Fig. 2). Larval feeding decreased with increasing dosage of the water fraction. At lower dosages (< 400 µg/disc), it showed phagostimulant activity in combination with sucrose. Leaf discs treated with the ethyl acetate fraction showed phagostimulant activity.

Fractionation of ethyl acetate-methanol 60% extractables between hexane, ethyl acetate, and water,

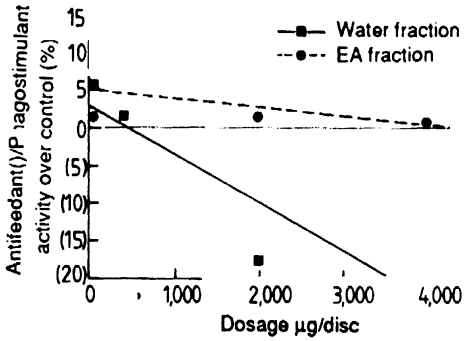

Fig. 2. Dosage-response relationship for ethyl acetate (EA) and water fractions of ethyl acetate extractables of PI 227687 leaves.

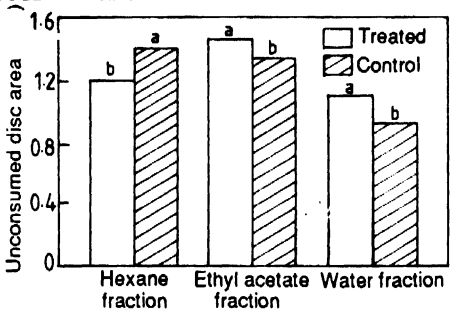

Fig. 3. Antifeedant activity of hexane, ethyl acetate and water fractions of PI 227687 leaves to cabbage looper, *T. ni* under double-choice conditions (4000 µg/disc). Treated and control bars with the same letter are not significantly different at $P < 0.05$.

lead to distribution of antifeedant activity in the ethyl acetate and aqueous fractions (Fig. 3). The hexane fraction in combination with sucrose increased leaf disc feeding.

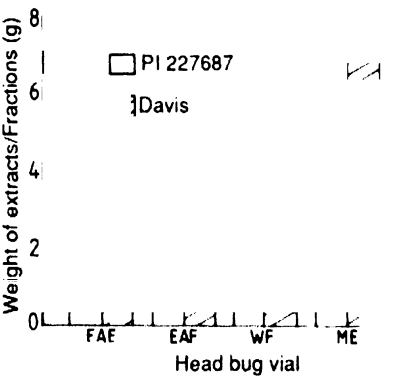

Fig. 4. Yield of different extracts/fractions (g) from PI 227687 and Davis leaves. EAE = ethyl acetate extract, EAF = ethyl acetate fraction of methanol extract, WF = water fraction of methanol extract and ME = Methanol (60%) extract.

Antifeedant activity of methanol (60%) ethyl-acetate extracts/fractions

Yield of 60% methanol and ethyl acetate extractables were almost the same in PI 227687 and Davis (Fig. 4). However, the quantity of the ethyl acetate fraction, which exhibited greatest antifeedant activity (Table 1), was nearly twice in PI 227687 than in Davis. However, the aqueous fraction was greater in Davis, which did not show any antifeedant activity. Aqueous extracts from Davis leaves have been shown to exhibit phagostimulant activity towards the Mexican bean beetle (Smith et al., 1979). Aqueous fraction from PI 227687 showed some antifeedant activity on snap bean discs. Leaf extractables and their fractions obtained from Davis leaves did not show antifeedant activity. Ethyl acetate extractables obtained after 60% methanol extraction also did not

Table 1. Antifeedant activity of different fraction of PI 227687 and Davis leaf extracts to third-instar larvae of the cabbage looper, *T. ni*

		Unconsumed disc area (cm^2)					
		Snap bean discs			Elderberry pith discs		
Fraction	Cultivar	Treated	Control	% Reduction in disc feeding	Treated	Control	% Reduction in disc feeding
Methanol extract (60%)							
Ethyl acetate fraction	PI 227687	1.01 ± 0.12 a	0.53 ± 0.13 b	-34.3	1.40 ± 0.04 a	0.93 ± 0.08 b	-33.6
	Davis	0.85 ± 0.11 a	0.80 ± 0.14 a	-3.6	1.09 ± 0.09 a	1.11 ± 0.05 a	1.4
Water fraction	PI 227687	1.28 ± 0.04 a	0.82 ± 0.13 b	-32.9	1.21 ± 0.07 a	1.06 ± 0.06 a	-10.7
	Davis	1.13 ± 0.03 a	1.11 ± 0.05 a	-1.4	1.13 ± 0.07 a	1.02 ± 0.06 a	-7.9
Ethyl acetate	PI 227687	1.00 ± 0.08 a	0.90 ± 0.07 a	-7.1	1.15 ± 0.03 a	1.30 ± 0.03 a	10.7
	Davis	1.00 ± 0.07 a	0.75 ± 0.13 a	-17.9	1.08 ± 0.05 a	1.13 ± 0.09 a	3.6

Each disc was treated with 4000 µg of respective extracts/fractions.
Experiment was conducted as a double-choice assay and there were 10 replications.
Larvae were confined for 8 h with snap bean and for 12 h with elderberry pith discs.
Figures followed by the same letter in row for snap bean or elderberry discs are not statistically different at $P < 0.05$.

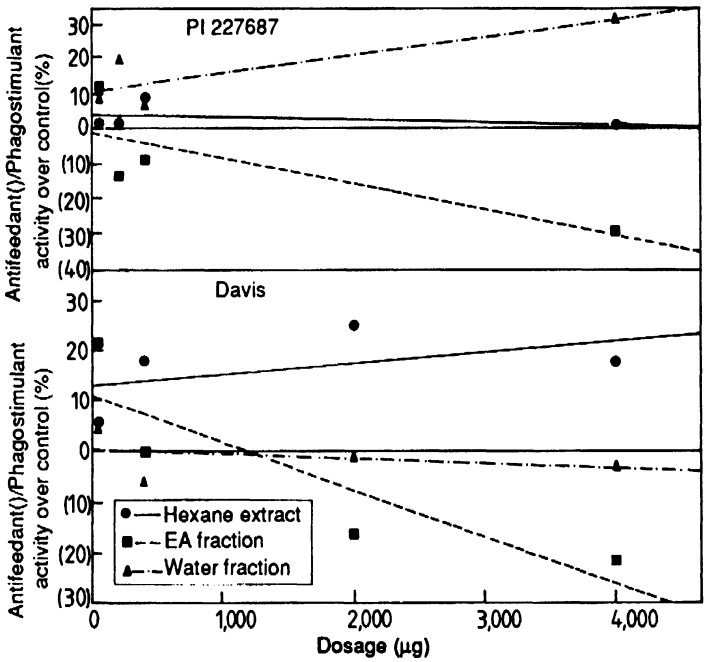

Fig. 5. Dosage response relationship of ethyl acetate and water fractions of 60% methanol extract and ethyl acetate extract of PI 227687 and Davis leaves. Figures in parenthesis on vertical axis for Antifeedant.

show any antifeedant effects. Antifeedant activity of the ethyl acetate fraction was also confirmed in dosage response studies under multi-choice conditions (Fig. 5). Ethyl acetate fraction from Davis leaves showed some antifeedant activity at 2000–4000 µg/disc. Aqueous fraction of the 60% methanol extract and ethyl acetate extract obtained after 60% methanol extraction increased the larval feeding in combination with sucrose.

Leaves extracted with ethyl acetate had high levels of chlorophyll. The samples thus, need to be cleared of chlorophyll and fatty materials before being processed in TLC or HPLC. Partitioning between ethyl acetate and water leads to the accumulation of antifeedant activity in the latter. However, the aqueous fraction contains large amounts of sugars, polysaccharides, and other water soluble components, which also makes the processing of samples on TLC and HPLC relatively difficult. Extraction with 60% methanol elutes most of the antifeedants from the leaves (Table 1). Partitioning of 60% methanol extractables between ethyl acetate and water can be carried out to concentrate the antifeedants in ethyl acetate fraction, which is

Table 2. Rf values of ethyl acetate and water fraction of PI 227687 and Davis leaf extracts resolved on Silica Gel TLC plates in benzene:ethyl acetate:water (4:3:3)

	Rf value			
	Ethyl acetate fraction		Water fraction	
S. No.	PI 227687	Davis	PI 227687	Davis
1	0.08 (F)	0.07 (F)	0.09	0.09
2	–	–	0.21	0.21
3	0.36 (F)	0.36 (F)	0.38	0.36
4	–	–	0.46	0.46
5	–	–	0.58	0.60
6	0.64	0.64	–	–
7	0.71	0.71	0.71 (F)	0.71 (F)
8	0.78	0.78	–	–
9	0.81	0.81	0.81 (F)	0.81 (F)
10	0.88	0.88	0.88 (F)	0.88 (F)

F = Faint spot.

Table 3. Antifeedant activity of different bands of the ethyl acetate fractions from PI 227687 and Davis leaves to third-instar larvae of cabbage looper, *T. ni*

	PI 227687		Davis	
Rf value	UCDA*	% Reduction in disc feeding	UCDA*	% Reduction in disc feeding
0.1–0.2	2.32 a*	-16.6	2.06 a	-7.6
0.21–0.5	2.98 b	-39.3	2.23 a	-13.4
0.51–0.8	2.30 a	-15.9	2.00 a	-5.1
Untreated control	1.84 a	–	–	–
SE	± 0.207			

+Figures followed by the same letter are not statistically significant at $P < 0.05$.
*UCDA = Unconsumed disc area (cm^2).

Table 4. Weight and antifeedant activity of different bands of aqueous fraction of PI 227687 and Davis leaf extracts against third-instar larvae of cabbage looper, *T. ni*

	PI 227687			Davis		
Rf value	Weight (mg/100 mg of aqueous fraction)	UCDA*	% Reduction in disc feeding	Weight (mg/100 mg of aqueous fraction)	UCDA*	% Reduction in disc feeding
<0.01	4.0	1.42 b+	-30.3	4.1	1.25 b	-16.2
0.1–0.20	6.8	1.42 b	-30.3	3.7	1.34 b	-22.4
0.20–0.40	23.1	1.35 b	-29.8	18.5	1.41 b	-27.5
0.40–0.55	0.3	1.32 b	-23.3	2.8	1.34 b	-22.5
0.55–0.80	2.0	1.26 b	-19.0	2.0	1.30 b	-19.7
Control	–	0.99 a	–	–	1.02 a	–
SE		± 0.087	–	–	± 0.058	–

*UCDA = Unconsumed disc area (cm^2).

+Figures followed by the same letter within a column are not statistically different at $P < 0.05$.

Aqueous fraction of ethyl acetate extract resolved in benzene:ethyl acetate:methanol (4:3:3).

relatively free from large amounts of chlorophyll, sugars and other water soluble components. This fraction is quite amenable to processing on TLC or HPLC for preparative or quantitative purposes (Sharma and Norris, Unpubl. data).

Antifeedant activity of TLC fractions

Resolved flavonoid spots tended to partition in the counter-current separation into relatively polar (Rf < 0.50) and less polar (Rf > 0.50) components on TLC plates in benzene:ethyl acetate:methanol (4:3:3) (Table 2). Thus, counter current partitioning was quite effective in separating the flavonoids, which can be used in TLC or HPLC separation and quantification of biologically active compounds.

Compounds resolved between Rf 0.10 to 0.80 in the ethyl acetate fraction showed antifeedant activity (Table 3). Antifeedant activity of the flavonoid bands from PI 227687 was greater than the corresponding bands from Davis. Mass of the flavonoid bands from PI 227687 resolved at Rf 0.10 to 0.40 was higher than in Davis (Table 4). Compounds resolved at Rf 0.40 to 0.55 were relatively less in PI 227687. Antifeedant activity of bands resolved between Rf 0.10 to 0.55 from PI 227687 was greater than the corresponding bands from Davis. Thus, both quantitative differences and the greater biological activity of flavonoid bands from PI 227687 appears to contribute towards its resistance to *T. ni* larvae. Several compounds isolated from these fractions have been shown to possess antifeedant and/or antibiotic properties towards *T. ni* larvae (Caballero and Smith, 1986; Khan et al., 1986; Sharma and Norris, 1991a).

When studying the chemical basis of resistance to insects in soybeans, leaves can be extracted with 60% methanol and the leaf extractables partitioned between ethyl acetate and water (the latter repeatedly partitioned by the former). The ethyl acetate fraction contains most of the antifeedants, and this can be used for isolating and quantifying the antifeedants using TLC or HPLC. Differences in amounts of biologically active fractions and biological activity *per se* of the active fractions account for the resistance of PI 227687 towards insect herbivores.

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