Biocidal Activity of Some Spanish Mediterranean Plants

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We have screened 80 plant species of the Spanish Mediterranean flora for ovicidal, JH-mimic, anti-JH, and toxic activity against insects. Fractions of 12 species have shown ovicidal activity against *Leptinotarsa decemlineata*. Twenty-one species have given evidence of JH-mimic activity by alteration of *Tribolium castaneum* metamorphosis. Thirty-two extracts or fractions had toxic effects on *Oncopeltus fasciatus* nymphs, and seven have produced some alterations in the fertility of the treated insects as to be considered as prospective anti-JH effects. A compound has been isolated from *Chrysantemum coronarium* D4 fraction inducing precocious metamorphosis on *O. fasciatus*. Tentative NMR, ME, and IR spectroscopy identification gives no molecular resemblance to the precocenes molecular structures, so it may present reasonable leads for development of new insect growth regulators.

Chemical plaguicides have been relevant in the gross increase of agricultural products. There is a great demand for chemicals to control insects, weeds, and plant diseases in order to improve the crop output in many countries. However, research on new chemical structures able to overcome the disadvantages of the pesticides actually in use is harder every day, since the growing concern about specificity and risk to humans increases steadily as do the requirements to register a new chemical as a pest control agent (Ware, 1983; McLaren, 1986; Horn, 1988).

Only recently have the subtleties of insect–plant interactions become apparent, especially an appreciation of chemically mediated interactions between insects and their host plants. Many botanical species possess secondary metabolic products, which in many, if not most cases, play one major function in the defense of the plant against insect attack (Ware, 1983; Bell, 1986; Horn, 1988). It has been suggested (Bowers, 1976; Bell, 1986) that some of these secondary plant compounds may furnish the bases of new molecular structures able to interfere with either metabolic pathways or enzymatic systems of the pests to be controlled.

In the last few years some of these substances have shown strong biological activity against pest insects as antifeedants (Meinwald et al., 1978; Kubo and Nakanishi, 1978; Vigneron, 1981), insect growth regulators (Bowers et al., 1976; Bowers and Nishida, 1980; Saxena, 1986), fungicides (Cojocarn et al., 1986; Schlumbaum et al., 1986), and even insecticides (Klocke et al., 1986).

The Spanish Mediterranean flora have many species of Lamiaceae, Asteraceae, Cistaceae, and other botanical families cited as possessing the biological activities referred to above. So, a research program (Simón et al., 1982) was started in order to investigate the biological capabilities against insects of some autochthonous plants.

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We report here the results of biocidal activity of the dichloromethanic and methanolic extracts of 80 species included in 25 botanical families of the Valencian community flora.

MATERIALS AND METHODS

Insects. *Leptinotarsa decemlineata* was obtained from a culture maintained at 25 °C, 50–60% relative humidity (rh), 16 h/8 h (day/night) photoperiod on fresh potato leaves. Eggs that were 0–24 h old were used for the experiences.

The strain of *Tribolium castaneum* was reared in 500-cm^3 glass jars on a diet of wheat flour and yeast (4:1). The culture and test insects were maintained at 30 ± 1 °C and 70 ± 5% rh in complete darkness. Pupae 0–24 h old were collected for the experiments.

The stock of *Oncopeltus fasciatus* was maintained at 27 ± 2 °C, 45–50% rh, 16 h/8 h (day/night) photoperiod on sunflower seeds. Groups of 15 third-instar nymphs were used for the experiments.

Plant Materials. The aerial parts of the plant to be studied were collected in the spring, dried in the shade to avoid any side effects due to sunlight, and crushed to a fine powder. Given amounts (400–500 g) of plant material were extracted in a Soxhlet apparatus, successively with dichloromethane and with methanol. The extracts were then concentrated at temperatures below 40 °C and high vacuum, until 10 mL of residue was obtained. After concentration, the extracts were separated by open-column chromatography (silica gel, Merck 60 mesh) in the following fractions. (1) Dichloromethane extracts: fraction D1 eluted with dichloromethane; fraction D2 eluted with dichloromethane–acetone (9:1); fraction D3 eluted with acetone; fraction D4 eluted with methanol. (2) Methanol extracts: fraction M1 eluted with dichloromethane–acetone (1:1); fraction M2 eluted with acetone; fraction M3 eluted with acetone–methanol (9:1); fraction M4 eluted with acetone–methanol (50:50); fraction M5 eluted with methanol.

Solvents of each of the fractions were eliminated by vacuum evaporation at temperatures below 40 °C.

Biological Assays. Each of the above fractions was run for biological activity with the following tests:

(1) Ovicidal Activity. This assay was carried out with the test described by Cuñat et al. (1981) on *L. decemlineata*. A 10-μg portion of the fraction was applied topically to newly laid eggs (less than 24 h old). If the fraction proved active (inhibition of more than 90% hatching of the assayed eggs), the assay was repeated with a lower dose until hatching of about 25% of

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Table I. Fractions from Plant Extracts Showing Ovicidal Activity against *L. decemlineata* L.

family	species	active fraction	dose, μg/egg	act. level[a]
Apiaceae	*E. maritimum*	M3	10.0	+++*
			1.0	+
Asteraceae	*A. squamatum*	M3	10.0	+++
	C. coronarium	M3	10.0	+++
	I. viscosa	M4	10.0	+++
	X. spinosum	D4	10.0	++
Cupresaceae	*J. thurifera*	D2	0.01	++
Lamiaceae	*B. hispanica*	D1	10.0	+++
	O. basilicum	D1	1.0	++
	S. acinos	D2	10.0	+++*
			1.0	+
Leguminosae	*A. cytisoides*	M3	10.0	+++
	G. tinctorea	D4	10.0	+++
Rutaceae	*F. xantoxiloides*		0.1	++

[a] Key: (*) dose cited for comparative pourposes only; (+) hatching between 50 and 75%; (++) hatching between 25 and 50%; (+++) hatching below 25%.

Table II. Fractions from Plant Extracts Showing JH Activity against *T. castaneum* Herbst.

family	species	active fraction	dose, μg/pupa	act. index[a]
Amarantaceae	*A. patulus*	D1	10.0	1.72
Apiaceae	*E. maritimum*	D1	10.0	3.74
Asterceae	*C. coronarium*	D1	10.0	1.26
	X. spinosum	M5	10.0	2.30
Brassicae	*L. graminifolium*	D1	10.0	3.30
Cistaceae	*C. monspeliensis*	D1	10.0	2.23
Cupresaceae	*J. thurifera*	M2	10.0	3.95
Chenopodiaceae	*A. articulata*	M4	10.0	3.10
	S. kali	D1	10.0	1.91
Ericaceae	*E. multiflora*	D1	10.0	2.01
Lamiaceae	*B. hispanica*	D1	10.0	1.99
	M. suaveolens	D2	10.0	2.60
		M5	10.0	1.90
	O. basilicum	D1	10.0	2.36
	S. acinos	D2	1.0	1.16
	S. hirsuta	D4	10.0	2.18
	T. buxifolium	D1	1.0	0.89
Leguminosae	*A. phoetida*	M3	10.0	1.00
	C. patens	M5	10.0	0.98
	G. tinctorea	D4	10.0	1.85
Malvaceae	*A. officinalis*	D1	10.0	2.63
Scrophulariaceae	*V. sinuatum*	D1	10.0	3.03

[a] Activity index: 0 = no effect; 1 = small gin traps present or retention of short urogomphi with genitalia essentially adultoid (or both); 2 = several well-developed gin traps or intermediate genitalia (or both); 3 = well-developed gin traps on each abdominal segment, nearly pupal genitalia, and patches of pupal cuticle on abdomen; 4 = virtually a second pupa.

the treated eggs was reached. This dose was considered the minimal active dose. Each experiment was carried out with 30 eggs and was repeated once.

(2) *JH-Mimic Activity.* The juvenilizing effects of the fractions were studied according to a 1–4 graded-score test (Martínez-Pardo et al., 1974) on *T. castaneum*. A dose of 10 μg of the fraction was applied topically to the ventral side of the abdomen of newly molted pupae (less than 24 h old). Active fractions were diluted and tested again until the average activity index was below 1.0 ± 0.2. Each experiment was performed with 30 pupae and was repeated once.

(3) *Toxicity and Anti-JH Activity.* This was done, basically, according to Bowers et al. (1976). Third-instar *O. fasciatus* nymphs were confined to a 10-cm Petri dish coated with 500 μg/cm^2 of the fraction. Later (72 h), the surviving nymphs were transferred to a clean 500-cm^3 glass jar and held at standard conditions. Toxicity effects were considered according to the number of insects dead during exposure to the chemical. Each experiment was carried out with 15 nymphs and was repeated twice.

Table III. Fractions from Plant Extracts Showing Toxicity against *O. fasciatus* Dallas

family	species	active fraction	dose, μg/cm^2	toxicity, %
Amarantaceae	*A. patulus*	D1	100	50
Asteraceae	*A. caerulescens*	D1	100	57
	C. sonchifolia	D4	100	40
	I. critmoides	M5	100	90
		D	100	55
	I. viscosa	M4	100	50
Brassicaceae	*L. graminofolium*	M4	100	40
		M5	100	45
	S. irio	M	500	90
Capparaceae	*C. espinosa*	D	100	89
Chenopodiaceae	*A. articulata*	M5	100	46
	A. halimifolium	M	500	82
	S. kali	D1	100	63
Cistaceae	*C. monspeliensis*	D4	100	73
Cupresaceae	*J. thurifera*	D2	100	67
Globulariaceae	*G. alypum*	M5	100	50
Lamiaceae	*B. hispanica*	D1	100	40
	M. suaveolens	D2	100	90
		D3	100	90
		D4	100	90
		M5	100	60
	S. hirsuta	M5	100	55
	T. beliom	D2	100	56
	T. piperella	D3	100	40
Leguminosae	*A. phoetida*	M5	100	50
	A. onobrychioides	D3	100	45
	C. patens	D1	100	43
	G. tinctorea	D1	100	67
		D4	10	47
	O. natrix	D1	100	40
Poaceae	*E. grus-galli*	D2	100	63
	H. hirta	D4	100	50

After methamorphosis occurred and reproduction was successful with the production of viable offspring, the tests were finished. The tests were considered positive for JH antagonistic activity, either when precocious metamorphosis occurred or when sterility of the resulting adults was detected.

Toxicity over 50% and/or detection of anti-JH activity warranted further assays at lower doses, until 25% toxicity or absence of anti-JH activity was observed.

Controls were run in parallel and received the same amount of solvent as treated insects. The solvents used were acetone, cyclohexane, or acetone–cyclohexane (1:1).

RESULTS

In Table I the results of the ovicidal test of the assayed fractions that showed the higher activities appears. Most of the species that proved active are from the Asteraceae or Lamiaceae families, although other plant species have shown ovicidal activity as well. It is noteworthy that the active fractions from Asteraceae are the more polar ones, whereas in Lamiaceae it is just the opposite, the less polar ones being most active.

The strongest effect was obtained from the second dichloromethane fraction of the Cupresaceae *Juniperus thurifera*, that inhibit 50% hatching when a 0.01 μg/egg dose was applied.

Table II shows the results obtained in the juvenilizing test. The family Lamiaceae presented the highest number of active species as well as the fractions with higher activity. D2 from *Satureja acinos* gave an average grade of 1.16 at 1 μg/pupa. On the other hand, fraction M2 of *J. thurifera* induced molting to supernumerary pupae of over 90% of the treated insects at 10 μg/pupa.

It has to be noted, also, that, in most of the active plant species, the alteration of the metamorphosis is found in the D1 fraction, which has shown Tritiacontane as the major and almost exclusive component.

Table IV. Fractions and/or Extracts with Antijuvenile Hormone Activity against *O. fasciatus*: Precocious Metamorphosis of Third-Instar Nymphs or Alteration of the Newly Ecdysed Adults Fertility

family	species	active fraction	dose, $\mu g/cm^2$	toxicity, %	act. shown[a]	
					fert alt	prec metam
Asteraceae	*C. bourgeanus*	M	500	33	*	
	B. pilosa	M	500	27	*	
	C. coronarium	D4	100			++
Brassicaceae	*S. irio*	M	500	90	*	
Capparaceae	*C. espinosa*	D1	100	89	*	
Chenopodiaceae	*A. halimifolium*	M	500	82	*	
Leguminosae	*D. pentaphillum*	M	500	30	*	
Malvaceae	*A. officinalis*	M	500	42	*	

[a] Key: (*) qualitative alteration of the fertility of the resulting adults; (++) precocious metamorphosis shown by about 50% of the treated insects.

The results about toxicity are reflected in Table III. The families Leguminosae, Lamiaceae, and Asteraceae presented the highest number of active species and, also, the most active fractions.

The Leguminosae *Genista tinctorea* showed the highest degree of toxicity in the fraction D4 which, at 10 $\mu g/cm^2$, killed almost 50% of the insects.

Among the Lamiaceae, *Mentha suaveolens* had the most promising activity, with four active fractions at the level of 100 $\mu g/cm^2$; three of them, D2, D3, and D4, killed 90% of the insects. Asteraceae had four active species. Fraction M5 of *Inula chrithmoides* is the most active, killing 90% of the insects.

Some plant extracts have shown considerable effects in the alteration of the fertility of the insects surviving the treatments. According to Bowers et al. (1976), this could be considered potential anti-JH activity.

In Table IV the fractions that were active in the toxicity and anti-JH tests are reported. The observed results gave qualitative reduction in fertility together with some degree of toxicity. The highest level was shown by *Caparis spinosa* D1 fraction at 100 $\mu g/cm^2$, followed by *Sysimbrium irio* and *Atriplex halimifolium* extracts at 500 $\mu g/cm^2$. Since several factors are involved, such as length of the preoviposition period, number of eggs laid per female, total number of eggs per female, total length of the fertility period, successful hatching of the eggs, etc., experiments are in progress to determine quantitatively the observed reductions in fertility.

The most startling discovery has been the precocious metamorphosis induced by fraction D4 of *Chrysantemum coronarium*, that was active at 10 $\mu g/cm^2$. The chemical responsible has not been identified yet, but tentative NMR, ME, and IR spectrometry has given MW 250 and a chemical structure of an unsaturated chain with a spiroketal moiety. This compound has been, in comparative tests, as effective as precocene I against *O. fasciatus*. Experimental work is under way to determine the compound potential against some other insect species.

DISCUSSION

As noted earlier, many species of green plants possess secondary metabolic products, allelochemicals. A few of these chemicals have been extracted for use as insecticides for over a century (nicotine, pyrethrum, rotenone, ...) (Ware, 1983; Horn, 1988). The Spanish Mediterranean flora have been known to have many species, cited by their content of allelochemicals, influencing various physiological processes. These have been the reasons for the screening study whose results are reported here.

The ovicidal activity found in some of the tested fractions is better than that reported by Sehnal and Skuhravy (1976), for the same insect species, using similar methods and 30 very active juvenoids. In our assays, 0.01 μg of *J. thurifera* D2 fraction prevents hatching of 50% of the treated eggs, a 10-fold improvement over the most active juvenoid mentioned above [see Sehnal and Skuhravy (1976)]. On the other hand, *Fagara xantoxiloides* extract presented the same level of activity than the referred juvenoid, since 0.1 μg of the extract/egg prevented 50% hatching. Other fractions tested gave activity levels of the same order that average juvenoids (1 μg/egg prevents hatching of about 25%), such as *S. acinos* D2 and *Eringium maritimum* M3, whereas *Ocimum basilicum* at this same dose prevented hatching of 50% of the treated eggs. It has to be noted that a commercial distillate of this plant (Sweet basil, *O. basilicum*) has been reported to have two compounds with very high JH-mimic activity, the juvocimenes (Bowers and Nishida, 1980).

These facts are in contrast with the low JH activity shown by the sweet basil fractions in our JH activity tests. Other plant species of the same family proved more active, such as *S. acinos* D2 fraction, which at 1 μg/pupa gave an average effect of 1.16, meaning that almost all treated insects presented urogomphal abnormalities. According to DeVries and Brown (1977), those abnormalities act as a direct barrier to the mating process.

At a dose 10-fold higher than that of *S. acinos*, five plants have had important JH activity, inducing severe metamorphical disturbances affecting over 75% of the treated insects (*E. maritimum*, *Lepidium graminifolium*, *J. thurifera*, *Verbascum sinuatum*).

In more than 50% of the fractions giving a positive response in the JH-mimic test, D1 has been responsible for the activity. We have been able to extract and characterize tritriacontane as the major compound in most of them. This hydrocarbon has been reported as a major component of the cuticular lipid extracts of some insect species (Armold and Regnier, 1975; Baker et al., 1979). The percentage of tritriacontane gradually increases for the first half of larval development of the black carpet beetle to become the predominant hydrocarbon in the older larvae. However, this alkane is only a minor component in adults (Baker et al., 1979). This could explain why a dose of 10 μg/pupa can alter the metamorphosis of the flour beetle (*T. castaneum*) in our test, probably by altering the synthesis of new cuticular lipids. Experiments are under way to determine this point.

The toxicity test used has allowed detection of strong knockdown toxicity to *O. fasciatus* of several of the fractions assayed. The most toxic fraction has been D4 of *G. tinctorea* that killed almost 50% of the exposed insects in 72 h at a dose of 10 $\mu g/cm^2$. Although this is not a very high toxicity level, it has to be noted that this is not a pure compound but a complex fraction (analysis by GLC revealed more than 16 peaks). A similar case is that of fractions D2, D3, and D4 of *M. suaveolens*.

In the studies with the anti-JH test, we found a plant extract that induced precocious metamorphosis in *O. fasciatus*. Our results were in agreement with those of Bowers and co-workers (1976). The effects of fraction D4 of *C. coronarium* on the treated nymphs were the same as those described for the precocenes (Bowers, 1976; Bowers et al., 1976), although the structural details of the molecule responsible bear very little resemblance to precocene ones. According to Staal (1986) this standard proof of anti-JH effects is their rescue by the concomitant administration of a juvenoid. Experiments are under way to determine the true nature of the above-mentioned compound activity.

Another point of interest is the fact that *C. coronarium* belongs to the same botanical family (Asteraceae) as *Ageratum houstonianum*, the natural source of precocenes.

On the basis of the preliminary biological data described here on the *Chrysantemum* compound, and if its biological activity can be improved and extended through chemical structure optimization studies, it may present reasonable leads for the development of new insect growth regulators.

Chemical defenses of plants through allelochemicals present all kinds of variation (Horn, 1988). In our study we have found 18 plants showing more than one type of activity. In such cases the observed effects are not the most apparent with the exception of *J. thurifera*, which has the highest ovicidal activity (Table I) and shows JH activity and toxicity too (Tables II and III). In almost 30% of the cases where more than one activity has been reported, it has been linked to fraction D1, which has Tritriacontane as the major component. As discussed earlier, these effects could be related to alterations in the contents of cuticular lipids (Armold and Regnier, 1975; Baker et al., 1979).

It is also important that toxicity has been found together with JH and anti-JH activity (Tables II–IV) in 11 species, which strongly supports one of the main goals set forth at the beginning of the study: looking for new natural chemicals (Slama, 1969; Bowers, 1976) that could provide new approaches to be used in a biorational management of insect pests, avoiding the major acute and environmental hazards of broad-spectrum insecticides (Horn, 1988).

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