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INSECTICIDAL ACTIVITY AND DITERPENE CONTENT OF *PERSEA INDICA*

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Key Word Index—*Persea*; Lauraceae; *Macaronesia*; Lymantriidae; *Heliothis*; Noctuidae; diterpenes; ryanodol; cinnceylanol.

Abstract—Extracts of *Persea indica* were toxic against *Macaronesia fortunata* (Lepidoptera: Lymantriidae) and *Heliothis armigera* (Lepidoptera: Noctuidae). Two insecticidal diterpenes, ryanodol and cinnceylanol, were present in the plant's petrol, methanol and water extracts. The methanol extract produced the highest larval mortality and growth reduction against *M. fortunata*, and also had a negative effect against *H. armigera* larvae. The reduction in both larval weight and development brought about by the components of the petrol extract can be partially attributed to the presence of the two diterpenes, but these products alone, however, do not explain the high toxicity of the methanol extract. The differential effect of the methanol extract and the potential use of *P. indica* for pest control are discussed.

INTRODUCTION

The search for natural pest control agents among secondary plant metabolites has been extended to many different plant families. The current resurgence of interest in plant-derived insecticides has focused on tropical floras [1], but locally adapted native plants are less likely to acquire serious new pest problems [2] and that is of particular importance for island ecosystems. The flora of Macaronesia (Canary Islands, Madeira and Azores) is an ancient remnant of the Tertiary flora and affords us a graphic example of what the flora of the Tethyan region was like in the first half of the Tertiary period [3]. The Canarian Laurel forest is a unique plant community whose potential chemical richness remains largely unexplored. The dominant tree species are *Laurus azorica*, *Persea indica*, *Apollonia barbusana*, *Ocotea foetens* and *Ilex canariensis*.

The fact that *P. indica* is toxic to mice [4] and that the traditional use of its leaves was to protect stored potatoes from pests, led us to select this plant species for insecticidal activity screening. The target organisms for the bioassays included: *Macaronesia fortunata* B. *et al.* (Lepidoptera: Lymantriide), an endemic insect that preferentially feeds on *Pinus canariensis* and *Spartocitysus supranubius* and can cause considerable damage during outbreaks in the Canarian pine forest, and *Heliothis armigera* Hb. (Lepidoptera: Noctuidae), a polyphagous crop pest.

RESULTS AND DISCUSSION

Studies with M. fortunata

The chronic larval growth bioassay with treated artificial diets showed that the methanolic fraction from *Persea* produced the highest mortality to neonate larvae of *M. fortunata* after 12 days of treatment. This extract gave over 10 times higher mortality than the petrol extract (Table 1).

The number of second-instar larvae found among the treatments (as percentage of total second-instar larvae) was significantly lower than the control ($p<0.05$), with the *P. indica* methanolic extract being the most active at the 0.01% level. We did not find any moulted larvae after 12 days of treatment.

We have already isolated two toxic polyhydroxy pentacyclic diterpenes, ryanodol (**1**) and cinnceylanol (**2**), from the stems of *P. indica* [4]. Compound **1** is known in nature in the form of the α-pyrrolecarboxylate ester derivative ryanodine [5]. This insecticidal and toxic alkaloid was obtained from the plant species *Ryania speciosa* (Flacourtiaceae), and it is used as a commercial insecticide in the U.S. to control codling moth, thrips, European corn borer and several other insects (Agrisystems International, Wind Gap, PA, U.S.A.). Ryanodine is highly toxic to mammals [6]. In contrast, ryanodol has a low toxicity to mice and it is a potent knockdown agent for injected houseflies and cockroaches [7]. Compound **2** has been previously isolated from the bark of *Cinnamonum zeylanicum* (Lauraceae) [8, 9] as an insecticidal compound causing abnormal ecdyses to *Bombyx mori* larvae [8].

The HPLC analysis of the *P. indica* extracts revealed that the two insecticidal diterpenes are present in both extracts in different ratios (**1**/**2**=0.87 and 1.29 in the petrol and methanol fractions respectively). The differences in **1** or **2** content between the petrol and methanol extracts do not explain the higher toxicity of the latter. The presence of other compounds along with **1** and **2** in the methanol extract (González-Coloma, unpublished data) could modify its biological action. Other potentially active compounds have been found in the

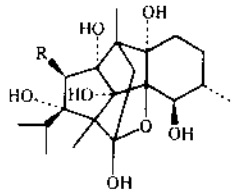
Table 1. Effects of *P. indica* hexane and methanol extracts incorporated into artificial diets on growth, survival and development of *M. fortunata* larvae (12 days of treatment)

Extract	1 (ppm in diet)	2 (ppm in diet)	% Extract in diet (wt:vol)	Growth* (% control)	Survivorship† (% total)	Second-instar larvae† (% total)
Hexane	0	0	0.0	100 a	96 a	58.3 a
	187	214	0.1	75.23 b	90 a	33.3 b
	935	1069	0.5	62.94 bc	100 a	8.0 c
	1871	2139	1.0	57.52 c	92 a	8.7 c
(F, p)‡				(13.61, <0.0001)		
MeOH	0	0	0.0	100	94 a	29.8
	32	25	0.01	83.57	34 b	0
	129	100	0.04	0	0	0

* Mean values followed by the same letter are not statistically different. Tukey's studentized range (HSD) test, $p = 0.05$.

† Differences respect to the control evaluated by Chi-square tests, $p = 0.05$.

‡ F value and probability level of one-way ANOVA analysis of log transformed larval weights.



R
1 OH
2 H

genus *Persea*. For example, estragole in the insecticidal essential oil of *P. americana* [10].

The diversity of chemical protectants may be essential for broad spectrum evolutionary protection against insects [11–13] and could lead to synergistic effects [14].

To test for the potential use of the active extracts of this endemic Canarian Lauraceae in the field, we have run a preliminary test with polar fractions of *P. indica* by spraying *M. fortunata* host plants with its aqueous extract. We used second- and third-instar larvae, and the results are shown in Fig. 1. We observed a 100% and an 80% larval mortality 23 days after treatment with dosages of 8 and 4% (w/v) respectively, as well as a significant reduction in the number of pupae formed. This aqueous extract had a long-lasting effect given that the larvae were in contact with the treated food for only 48 hr, suggesting either slow metabolism or slow detoxication of the allelochemicals ingested by *M. fortunata*.

Studies with H. armigera

The bioactivity of these *P. indica* polar fractions is not restricted to *M. fortunata* larvae. *Heliothis armigera* larvae fed diets dipped in extract plus Tween had their survivorship, growth and number of fourth-instar larvae reduced with increasing concentrations of extract (Table 2). We selected this dipping method because it is the best laboratory simulation of the surface application of an insecticide on a host plant, and the Tween was added to the carrier solvent to improve the penetration of the test solution into the diets.

The effects were less dramatic on this noctuid lepidoptera than on the lymantrid *M. fortunata*. It is possible that the differences observed are related to the different methods used in the application of the extracts to the diets (mixing it with the dry portion versus dipping the diet cubes in the extract) and/or because the generalist *H. armigera* deals with the toxic substances earlier and faster than the specialist *M. fortunata*. There are many examples of the differential effects that active plant substances have on specialist versus generalist insect species [15–17] which have been attributed to differences in the insects' metabolic adaptations [18].

The topical treatment had a significant effect on the percentage of fifth-instar *H. armigera* larvae found without affecting larval growth and survivorship (Table 2). The results of linear regression analyses performed on the amounts of **1** and **2** administered and the biological effects observed are shown in Table 3. For *M. fortunata* larvae, these compounds explained part of the effect that the petrol extract had on larval growth and development (70–77%, $p = 0.12$ and 0.16). For *H. armigera*, the dietary treatment effects observed had little relationship to the presence of such products in the diets (30–33%), while the topical treatment effect on larval development is strongly related to both chemicals ($r^2 = 0.85$, and $p = 0.008$ in both cases, Table 3).

These differences between the dietary and topical treatments could be due to the different concentrations of toxicants that reached the insect's system under these experimental conditions and/or to the fact that the topical application of the extract circumvented its contact with the insect gut's enzymes.

We conclude that extracts from *P. indica* have the potential to be used for local pest control, probably in the same way ryania powder is. We have shown that the activity of the extracts is not only due to the presence of the two known insecticidal diterpenes isolated from this plant [4]. More research is needed in order to identify other insecticidal compounds present in this plant species as well as to understand the specific mechanism of action of each toxicant.

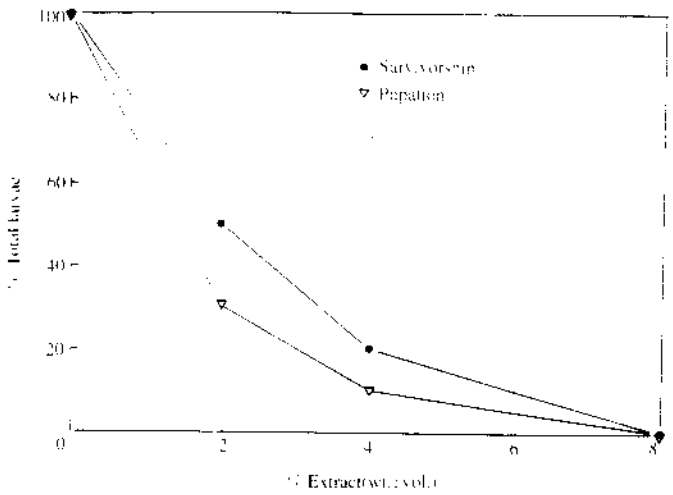


Fig. 1. Effect of a 48 hr dietary exposure to an aqueous *P. indica* extract on second third-instar *M. fortunata* larval survivorship and pupation 23 days after the treatment. *Significantly different from the control, Fisher's exact probability test, $p=0.05$.

Table 2. Dietary and topical effects of a *P. indica* methanol extract on *H. armigera* larval survivorship, growth and development (7 days of treatment)

			Extract on diet			Extract on larvae		
% Extract (wt:vol)	1 (ppm)	2 (ppm)	Surv.* (% total)	Growth† (% control)	L4‡ (% total)	Surv.* (% total)	Growth (% control)	L5* (% total)
0.0	0	0	94 a	100 a	40.4 a	86 a	100	86.0 a
0.03	97	75	90 a	136.47 b	72.4 b	74 a	107.89	78.4 ab
0.07	226	175	88 a	127.25 ab	41.0 a	67 a	101.3	76.3 ab
0.1	324	251	12 b	57.29 cd	0	64 a	95.88	77.7 ab
0.3	971	753	36 c	40.17 c	0	88 b	93.24	65.9 b
0.5	1619	1255	60 d	69.76 d	16.6 c	100 c	96.08	64.0 b
(F,p)‡				(8.22, <0.0001)			(0.18, 0.967)	

*Differences with respect to the control evaluated by Chi-square tests, $p=0.05$.
†Mean values followed by the same letter are not statistically different. Tukey's studentized range (HSD) test, $p=0.05$.
‡F value and probability level of one-way ANOVA analysis of log transformed larval weights.

EXPERIMENTAL

Plant extracts. Oven-dried (60°, 48 hr) terminal branches (avg. 20 cm, 1.5 g dry wt) from *P. indica* plants (a voucher specimen is deposited in the Instituto de Productos Naturales y Agrobiologia, Tenerife) collected on the island of Tenerife were ground and extracted with EtOH for 6 days in a Soxhlet apparatus. The cold extract was filtered and concd *in vacuo* to give a syrup gum.

The recovered EtOH extract (166 g, 33% w/w yield) was sequentially extracted (×3) with petrol, $CHCl_3$ and MeOH. A second crude extract was washed (×3) with H_2O to give the aq. fr.

Macaronesia fortunata *bioassays.* (i) *Petrol and* MeOH *extracts.* Three concns of the petrol extract (0.1, 0.5 and 1.0% w/v), and five of the methanolic fraction (0.1, 0.08, 0.06, 0.04 and 0.01% w/v) were assayed with petrol and MeOH controls. The extracts were admixed with the dry portion of the artificial diet (Bioserv Inc., Frenchtown, New Jersey; No. F9640) and the carrier solvent was removed under low pres.

Field collected eggs laid on *Spartocitysus supranubius* shrubs (Las Cañadas, Tenerife, June 1990) were kept in the laboratory until hatching, and five neonate *M. fortunata* larvae were placed on *ca* 6 g (wet wt) aliquots of diets in 30 ml plastic cups. Ten cups per treatment were randomized in clear plastic boxes at room temp. and a 16:8 hr light photoperiod.

The insects were counted, weighed after 12 days of treatment and the number of second-instar larvae recorded. For the tabulated presentation, larval growth was measured as the percentage of the controls based on live larval weights. For the analysis, log transformed larval weights (to normalize the data) were used in a one-way ANOVA analysis to test for treatment effect and the mean larval weights were separated using the Tukey's studentized range (HSD) test ($p<0.05$). Survivorship was measured as the percentage of total larval survivors, and the data were analysed as contingency tables using Chi-square tests. Extracts were considered active when the probability level for the Chi-square statistic was <0.05.

(ii) *Aqueous extract.* Three concns of the extract (2, 4, and 8% w/v) and a distilled H_2O control were sprayed on the plant's

Table 3. Parameters of the linear regressions (r^2, r squared and p, probability level) of *M. fortunata* and *H. armigera* biological responses on the ryanodol and cinnceylanol content of the diets and extracts used for treatments

Insect species	Biological response	r^2 (%)	p	r^2 (%)	p
M. fortunata	Ln larval wt.	77.26	0.12	77.0	0.12
	development	70.01	0.16	7.01	0.16
H. armigera	Ln larval wt.*	33.46	0.23	33.48	0.23
	development*	30.05	0.26	30.06	0.26
	Ln larval wt.†	38.76	0.18	38.77	0.18
	development†	85.84	0.008	85.84	0.008

*Dietary treatment.
†Topical treatment.

branches until they were dripping. The branches were placed in two sets of 2 l glass jars with 5 either second- or third-instar larvae per treatment and set. The larvae were allowed to feed on treated branches for 2 days and then transferred to freshly collected untreated branches. Larvae and pupae were counted and the food was changed every 2 days for 23 days. The survivorship values and number of pupae were calculated as percentage of the total larval survivors. The environmental conditions and data analysis were as described above.

Heliothis armigera *bioassays.* (i) *Dietary treatment. Heliothis armigera* larvae from a laboratory colony (CIB, CSIC, Madrid) were reared on a general noctuid diet [19]. Second-instar larvae were fed *ad libitum* with diets dipped in either increasing concentrations of a *P. indica* MeOH extract (0.03, 0.07, 0.1, 0.3 and 0.5% w/v) or the corresponding solvent alone (MeOH–1% Tween 20, 1:1) for 15 sec.

Fifty larvae per treatment (except for the 0.03% concentration, where $n = 32$ because of a shortage in larvae) were individually placed in 30 ml plastic cups and the cups randomized in a growth chamber at 21° and a 16:8 hr light photoperiod. The diet was changed every other day, the insects counted, weighed and the number of fourth-instar larvae recorded after 7 days of treatment. For the tabulated presentation, the data analysis was as described for *M. fortunata*.

(ii) *Topical treatment.* Second-instar *H. armigera* larvae were dipped for 15 sec in either the same MeOH extract concns described above or the solvent alone, and then transferred to control diets. Fifty larvae per treatment were individually placed in 30 ml plastic cups and randomized in a growth chamber with similar environmental conditions to that of the dietary treatment. The insects were counted, weighed after 7 days of treatment and the number of fifth-instar larvae recorded. For the tabulated presentation, the data analysis was as described above.

Chemical analysis of compounds **1** *and* **2**. The two insecticidal diterpenes, ryanodol and cinnceylanol, present in *P. indica* were quantified by reverse-phase HPLC following a sample clean-up procedure. Aliquots of 0.5 g of the petrol, 1.0 g of the MeOH and 0.118 g of the H_2O frs were chromatographed on a silica gel column eluted with a petrol–EtOAc–MeOH gradient. A total of 10 fractions were eluted (petrol–EtOAc, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9, 0:1; EtOAc–MeOH, 19:1 and 1:1) and monitored by TLC using $CHCl_3$–MeOH–H_2O (7:13:8), with visualization by H_2O–EtOAc–H_2SO_4 (16:3:1, oleum reagent).

The fractions previously eluted were combined to give a total of 3 frs: (1) petrol–EtOAc (7:3 to 3:2); (2) petrol–EtOAc (1:1) to EtOAc–MeOH (19:1); and (3) EtOAc–MeOH (1:1). Compounds **1** and **2** were present in fr. 2, after washing each fr. (× 3) in 500 µl 30% aq. MeOH and performing a TLC and an HPLC analysis of each wash.

HPLC analysis of the washes. The samples were resolved on a 2.5 × 15 cm ultrasphere C-18 column using an isocratic system of 30% aq. MeOH for 20 min at a flow rate of 1 ml min^{-1}, in which **1** and **2** were detected with a refractive index detector having R_ts 13.0 and 16.0 min respectively.

For the quantification of these products, 2 injections were averaged for each extract wash (3 washes per extract) and their respective refractive index values for the three washes added. Pure samples of **1** and **2** previously isolated from *P. indica* [4] were used as external standards. The relationships between the amounts (in ppm) of **1** and **2**, the larval weights and the percentage of total moulted larvae obtained from the different treatments were analysed with linear regression models. Larval weight values were log transformed to assure random distribution of residuals.

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REFERENCES

1. Jacobson, M. and Crosby, D. G. (1971) *Naturally Occurring Insecticides*. Marcel Dekker, New York.
2. Berenbaum, M. R. (1989) in *Insecticides of Plant Origin*, ACS Symp. Ser. 387, p. 11. American Chemical Society, Washington, DC.
3. Bramwell, D. (1976) in *Biogeography and Ecology of the Canary Islands* (Kunkel, G., ed.), p. 207. D. R. W. Junk, The Hague.
4. Gonzalez-Coloma, A., Hernandez, M. G., Perales, A. and Fraga, B. M. (1990) *J. Chem. Ecol.* **16**, 2723.
5. Wiesner, K., Valenta, Z. and Findlay, J. A. (1967) *Tetrahedron Letters* 221.
6. Sutko, J. L., Ito, K. and Kenyon, J. (1985) *Fed. Proc.* **44**, 2984.
7. Waterhouse, A. L., Pesaah, I. N., Francini, A. O. and Casida, J. E. (1987) *J. Med. Chem.* **30**, 710.
8. Isogai, A., Murakoshi, S., Suzuki, A. and Tamura, S. (1972) *Agric. Biol. Chem.* **41**, 1779.
9. Isogai, A., Suzuki, A., Tamura, S., Murakoshi, S., Ohasi, Y. and Sasada, Y. (1976) *Agric. Biol. Chem.* **40**, 2305.
10. Stein, U. and Klingauf, F. (1990) *J. Appl. Entomol.* **110**, 160.
11. Feeny, P. (1977) *Ann. Missour. Bot. Garden* **64**, 221.
12. Jermy, T. (1983) in *Natural Products for Innovative Pest Management* (Whitehead, D. L. and Bowers, W. S., eds), p. 223. Pergamon Press, Oxford.
13. Fu-Shun, Y., Evans, K. A., Stevens, L. H., Van Beck, T. A. and Schoonhoven, L. M. (1990) *Entomol. Exp. Appl.* **54**, 57.
14. Berenbaum, M. (1985) in *Chemically Mediated Interactions between Plants and other Organisms* (Cooper-Driver, G. A., Swain, T. and Conn, E. E., eds), *Recent Advances in Phytochemistry*, Vol. 19, p. 139. Plenum Press, New York.
15. Breuer, M. and Devkota, B. (1990) *J. Appl. Entomol.* **110**, 128.
16. Bloem, K. A., Kelley, K. C. and Duffey, S. S. (1989) *J. Chem. Ecol.* **15**, 387.
17. Ramachandran, R., Mukherjee, S. N. and Sharma, R. N. (1989) *Entomol. Exp. Appl.* **51**, 29.
18. Krieger, R. I., Feeny, P. P. and Wilkinson, C. F. (1971) *Science* **172**, 579.
19. Poitout, S. and Bues, R. (1970) *Ann. Zool. Ecol. Anim.* **2**, 79.