

CHEMICAL ECOLOGY OF CANARIAN LAUREL FOREST: TOXIC DITERPENES FROM *Persea indica* (LAURACEAE)¹

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Abstract—The tree species *P. indica* (Lauraceae) is an important endemism in the Canary Islands laurel forest and can readily be distinguished by its defoliated appearance due to the seasonal action of wild rats (*Rattus rattus*), which eat the plant and become intoxicated. These observations and the phytochemical interest of this plant species led us to study the potentially toxic chemicals responsible for such action. We found that an ethanolic extract of *P. indica* and its water fraction were toxic when injected into laboratory mice. The mice also died after ingestion of the stems and showed a significant preference for those extracted and rehydrated with an 8% aqueous extract solution when compared with the water control. Two compounds that have been isolated from the toxic fraction and identified by spectroscopic methods are the polyhydroxy pentacyclic diterpenes ryanodol and cinnceylanol. Possible ecological implications are discussed.

Key Words—*Persea indica*, Lauraceae, ryanodol, cinnceylanol, X-ray analysis, *Rattus rattus*, toxicity, mice feeding trials.

INTRODUCTION

We began the study of the chemical ecology of the Canarian laurel forest because of its importance as a relict flora and the potential presence in this plant species

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of unknown chemical products with ecological and economic relevance. Moreover, plant species are being lost at a much faster rate than rates of evolutionary replacement (Wilson, 1988), and loss of a species means loss of chemicals potentially unique in nature (Eisner, 1989).

The flora of Macaronesia (Canary Islands, Madeira, and Azores) is an ancient remnant of the Tertiary flora, which has been preserved to the present day and affords us a graphic example of what flora of the Tethyan region was like in the first half of the Tertiary period. Towards the end of this period, the subtropical forest flora was displaced because of changes in the climatic zones. The probable southern migration of a cooler, drier climate led to the development of the modern Mediterranean sclerophyllous vegetation (see Bramwell, 1976).

For this project, the tree species *Persea indica* was chosen for two reasons: (1) Its present day distribution represents only a part of its former range, so it can be considered a good example of paleoendemism (Bramwell, 1972), which has never been studied from a phytochemical point of view. (2) This tree has a characteristic defoliated appearance resulting from the seasonal action (April, May) of a wild forest rat (*Rattus rattus*) that obtains cortical tissue by selecting terminal twigs from larger branches and biting through the stem at an average distance of 20 cm from the tip of the branch. The rough outer bark is then removed to expose the phloem, cambium, and some xylem. It also has been observed that these wild rates become intoxicated from eating the plant, occasionally falling from the trees.

In the study of plants and animals that feed on them, the notion that secondary chemicals play an important role in food selection by vertebrate herbivores has become widely accepted (Harborne, 1982). These products have been used to explain herbivore preferences at many levels: colubus monkeys that avoid the phenolic barrier present in the abundant tree species of their environment by eating low-tannin seeds and leaves of relatively rare deciduous trees (McKey and Gartland, 1981); squirrels that select low-monoterpene ponderosa pines (Farentinos et al., 1981); browsing mammals that choose palatable shrubs based on leaf structure and phenolic compounds (Simonetti et al., 1984; Cooper and Owen-Smith, 1985); Canada geese avoiding herbs with high concentrations of tannins (Buschbaum and Valiela, 1987); elephants avoiding species with high total phenols, steroidal saponins, and lignin (Jachmann, 1989); or desert wood rats that avoid feeding on creosotebush (*Larrea tridentata*) phenolic resin to the point of energy imbalance (Meyer and Karasov, 1989).

We selected this plant-herbivore system to demonstrate that *P. indica* contained secondary chemicals that could interfere with its selection as a food source by rodents having negative effects on them and to isolate and identify the active products of this plant due to its potential phytochemical importance.

METHODS AND MATERIALS

Plant Material and Extraction. *P. indica* branches were collected at El Moquinal, Monte de Las Mercedes, Tererife, in October 1988. Air-dried, chopped, terminal twigs (≥ 20 cm, 627 g) were extracted with EtOH for six days in a Soxhlet apparatus. The cold extract was filtered and then concentrated in vacuo to give a syrup gum.

Fractionation of Extract. The recovered EtOH extract (29 g, yield 4.6%) was chromatographed on a silica gel column and eluted with a light petroleum-EtOAc-MeOH gradient. A total of 16 fractions were eluted (light petroleum-EtOAc, 85:15 to 0:100; EtOAc-MeOH, 95:5 to 50:50) and bioassayed for mice toxicity. Part of the toxic fraction (eluted with light petroleum-EtOAc, 55:45) gave a white precipitate (A) and a yellow supernatant (B). By dissolving A in aqueous MeOH, allowing the solvent to evaporate slowly at room temperature, and repeating the process, pure compound I was obtained.

Fraction B (1.2 g) was analyzed by droplet countercurrent chromatography (DCCC) in a model A apparatus (Tokyo Rikakikai Co., Japan). The sample was dissolved in a mixture of both mobile and stationary phase and injected into the apparatus using a 20-ml sample chamber. The flow rate was 8-10 ml/hr, and the eluates were collected in 3 to 6-ml fractions.

The fractions were monitored by TLC on silica-gel sheets (Schleicher-Schull). The solvent system was CHCl_3 -MeOH- H_2O , 35:65:40 and the compounds were detected with a mixture of H_2O -AcOH- H_2SO_4 , 80:15:5 (oleum reagent).

Thirteen fractions were obtained and the active ones (6-9) combined and rechromatographed by DCCC (625 mg). Pure crystals of compound II were obtained from recrystallization on MeOH of the active fractions collected.

Physical Data. Melting points were taken on a Kofler hotplate apparatus and are uncorrected. [^1H] NMR spectra were recorded on a Bruker P 200 SY spectrometer, py-d_5 and CD_3OD were used as solvents. Mass spectra were taken in a Hewlett-Packard 5995 and high-resolution mass spectra (HRMS) in a VG Micromass Zab-2F spectrometer.

Ryanodol (I). m.p. 345-347°C (from methanol-water) [lit. synthetic I, 266-267°C (Wiesner et al., 1967), 267-268°C (Belanger et al., 1979)], [^1H] NMR (CD_3OD , 200 MHz) δ : 3.80 (1H, d, $J = 10$ Hz, 10-H), 3.74 (1H, s, 3-H), 1.34 (3H, s, 17-H), 1.05 (3H, d, $J = 6$ Hz, 21-H), 1.00 (3H, d, $J = 6$ Hz, 19-H), 0.95 (3H, d, $J = 6$ Hz, 18-H), 0.89 (3H, s, 20-H); [^1H] NMR (py-d_5 , 200 MHz) 4.57 (1H, d, $J = 7$ Hz, 10-H), 4.26 (1H, s, 3-H), 2.15 (3H, s, 17-H), 1.50 (3H, d, $J = 6$ Hz, 21-H), 1.28 (6H, d, $J = 6$ Hz, 18-H and 19-H), 1.14 (3H, s, 20-H). MS, m/z (rel. int.): 400 (M^+) (7), 382 (2), 357 (16), 339 (100), 321 (63), 303 (49), 293 (36), 261 (25), 257 (14), 247 (18), 243

(20), 229 (11), 195 (10), 165 (9), 155 (12). HRMS, calcd. for $C_{20}H_{32}O_8$ 400.2097, found 400.2090.

Cinnceylanol (II). m.p. 140–141°C (from methanol) [lit. m.p. 139–142°C (Yagi et al., 1980)]; [1H] NMR (CD_3OD , 200 MHz) δ : 3.80 (1H, d, $J = 10$ Hz, 10-H), 2.40, and 1.76 (each 1H, d, $J = 15$ Hz, 3-H), 1.31 (3H, s, 17-H), 1.00 (6H, d, $J = 6$ Hz, 18-H and 19-H), 0.95 (3H, d, $J = 6$ Hz, 21-H), 0.86 (3H, s, 20-H). MS, m/z (rel. int.) 384 (M^+) (2), 366 (5), 349 (5), 348 (17), 341 (14), 331 (16), 330 (69), 323 (100), 315 (17), 305 (24), 289 (75), 287 (20), 271 (22), 259 (20), 223 (13), 217 (11), 195 (15). HRMS, calcd. for $C_{20}H_{32}O_7$ 384.2166, found 384.2157.

X-Ray Analysis of Ryanodol (I). The molecular formula was $C_{20}H_{32}O_8$ and the molecular weight 400.468 g/mol. Accurate cell parameters of the unit cell [$a = 16.283$ (1) Å, $b = 13.065$ (1) Å, $c = 9.112$ (1) Å, $V = 1936.6$ (3) Å³] were determined on a crystal measuring 0.30 × 0.26 × 0.20 mm, by least-square analysis of setting angles of 35 reflections ($10 < \theta < 36$) using graphite monochromated CuK_{α} radiation ($\mu = 8354$ cm⁻¹), automatically located and centered on a Philips PW-1100 diffractometer. The space group was $P2_12_1$, and $z = 4$. Two reflections were measured every hour to ascertain crystal stability, and no significant variation was observed, with $\omega/2\theta$ scan and scan width 1.50. The intensities of 3534 reflections were collected and 3449 reflections were considered in accordance with $I \geq 2\sigma(I)$. They were subsequently corrected for Lorentz and polarization effects but no absorption corrections were applied.

The structure was solved by direct methods (MULTAN 80), using the solution with the highest figure of merit from 36 different sets (2200 relationships, $E_{min} = 1.5$). Successive difference Fourier revealed the positions of all non-H atoms. The positional and thermal parameters were refined using full matrix least-squares. After several difference Fourier maps, which allowed the localization of all H atoms, several anisotropic least-squares cycles were carried out, but the H atoms were considered as fixed isotropic contributors. Function minimized $\sum w(|F0| - |Fc|)^2$, where w was empirically calculated, not to give trends in $\chi^2 w \Delta^2 F$ vs. $\langle F0 \rangle$ and vs. $\langle \sin \theta / \lambda \rangle$. Convergence was reached at $R = 3.6\%$ and $R_w = 3.8\%$.

The absolute configuration was determined by comparing the 40 (ΔFc 0.052) more relevant Bijvoet pairs, giving the following average Bijvoet differences: 0.156 for the right enantiomer vs. 0.395 for the wrong one.

Toxicity Bioassay. This bioassay was design to detect toxic compounds in the different fractions obtained during the purification and partitioning processes. Laboratory mice (18–22 g) were injected intraperitoneally with either the EtOH extract (10 mg), the silica-column fractions (10 mg), or the droplet countercurrent chromatography (DCCC) fractions (1 mg) with 1% Tween 60

(Merck) or physiological saline solution (9/00 ClNa) as the carrier vehicle, compared with appropriate controls, and their mortality recorded at 24 hr.

The reported LD_{50} s are based on plots of dose vs. probit percent mortality calculated according to Tallarida and Murray's procedure (1986). Three to five mice, caged together, were used for each concentration of product assayed, five concentrations of compound I were tested (13.37, 17.54, 18.78, 20.01, and 20.50 mg/kg) and six of compound II (3.61, 5.82, 8.39, 12.12, 26.83, and 35.58 mg/Kg).

Feeding Experiments. All the feeding experiments were run at room temperature and at a 12:12 light-dark photo-period.

The survivorship of mice feeding ad libitum on *P. indica* dried stems was calculated by comparing their performance with that of mice feeding ad libitum on mice chow for six days. One set of three mice for the control, and two more sets for the treatment were used after a 24-hr starvation period. Each set was kept in a 240 × 135 × 130-cm-high cage.

A second feeding experiment was designed to check for food preferences. Extracted *P. indica* twigs were oven dried (60°C, 48 hr) and rehydrated with increasing concentrations of an aqueous extract obtained by dissolving the EtOH residue in distilled water (10% w/v). The concentrations used were: 4, 6, and 8% w/v, and the results compared with 100% water-rehydrated twigs as control. Petri dishes containing weighed amounts of the differently treated stems were offered individually to the mice for 30 min. Three mice per cage and five cages per treatment were used in the three feeding trials. The cages were as previously described, and food consumption was calculated gravimetrically.

RESULTS AND DISCUSSION

From the field observations we suspected that *P. indica* was toxic to rodents. To check for this activity, we injected laboratory mice intraperitoneally with a crude ethanolic extract and its water fraction, observing that the injected mice suffered muscular convulsions prior to death. To check if *P. indica* stem tissue also was toxic to mice after ingestion, they were fed ad libitum on dried stems. The animals started showing similar symptoms to those observed after intraperitoneal injection of the toxic extracts after three days, reaching a 100% mortality rate on the sixth day of treatment (Figure 1). On the other hand, it is known that when animals encounter toxic foods, they will initially eat small quantities of it and then refuse to feed (Meyer and Karasov, 1989).

To verify that the toxic fraction also was causing a feeding response, we designed the trials described in Methods and Materials. We found that mice ate significantly more ($P \leq 0.005$) of the 6% and 8% aqueous extract rehydrated

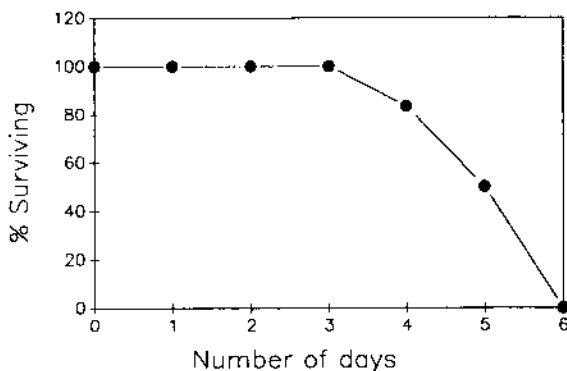


FIG. 1. Survivorship curve for laboratory mice fed on *P. indica* dried stems, expressed as percentage of control. $N = 9$ animals (3 for control and 6 for treatment).

stems than of the water controls, and they did not show any preference below the 4% aqueous extract treatment when compared to the control (Figure 2).

Besides the toxicity that the stems had on laboratory mice, preliminary field observations on the seasonal feeding preferences of the wild rats on this

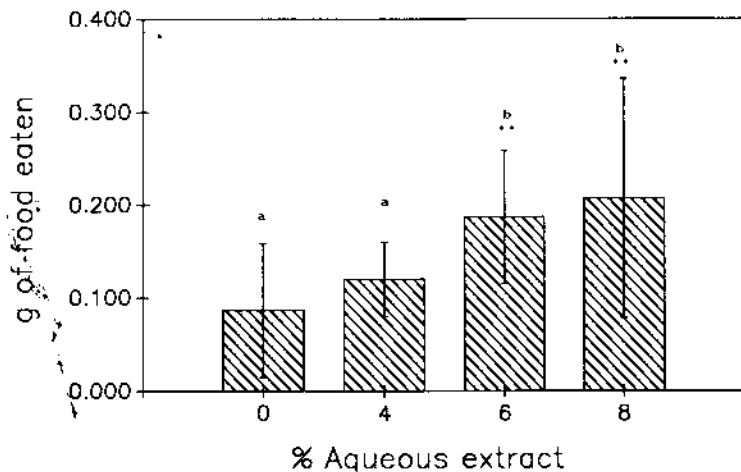


FIG. 2. Amount of *P. indica* stems eaten after rehydration with increasing concentrations of its aqueous extract. Treatments were as follows: 0, control with water and rehydration; 4, 4% aqueous extract; 6, 6% aqueous extract; 8, 8% aqueous extract ($N = 15$ animals per treatment). * $P < 0.05$, ** $P < 0.05$, t test significance level when different from the control. ^{a,b} $P < 0.05$ significance level for between-treatment comparisons.

tree and our laboratory data suggest that there are components in *P. indica* stems that induce a positive feeding response in rodents.

These preferences can be governed by a balance between beneficial nutritional components and toxic secondary chemicals. The aqueous extracts used in the food-choice experiment contained toxic products along with sugars, proteins, and minerals that could explain the response observed for this extract. There are many examples of herbivores' food choices governed by the ratio between beneficial nutritional components and toxic secondary chemicals: howler monkeys influenced by the protein-fiber ratio and secondary chemicals of their food (Milton, 1979), bullfinches preferentially feeding on seeds with a high fat-phenolic compounds ratio (Greig-Smith, 1988), etc. Allelochemicals are also likely to cause depletion of several mineral nutrients, causing herbivores to develop an appetite for minerals (Freeland et al., 1985).

Carbon-nutrient balance of the plants determined by resource availability in the environment must play an important role in determining levels and types of plant defense (Bryant et al., 1985; Bryant, 1987), and hence herbivore impact (Niemelä and Danell, 1988). Moreover, temporal variations in plant chemistry cause seasonal changes in the use of different species of plants by herbivores (Buchsbaum and Valiela, 1987; Cooper et al., 1988). This type of change could explain the seasonal feeding behavior of *Rattus rattus* on *P. indica* that we have observed in the field. Quantitative analysis of the seasonal variations of this plant's toxic compounds and beneficial elements (nitrogen, water, etc.) is needed to test this hypothesis.

In order to isolate the toxic products involved in the interaction described here, we fractionated and purified the ethanolic crude extract. This procedure yielded two pure diterpenes. The less polar one was ryanodol (I, Figure 3), which was identified on the basis of the following: High-resolution mass spectrometry is in accordance with the formula $C_{20}H_{32}O_8$ and its $[^1H]$ NMR spectrum is in accordance to that described in the chemical literature for one of its esters, ryanodine, if the geminal proton to the esterified alcohol and the signals of the ester group are excluded (Waterhouse et al., 1985). We decided to submit

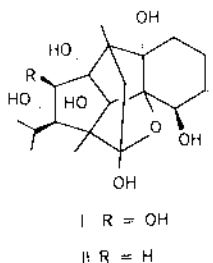


FIG. 3. Structures of ryanodol (I) and cinnceylanol (II).

this compound to X-ray analysis because only the *p*-bromobenzyl ether of ryanodol has been subjected previously to this technique (Srivastava and Przybylska, 1968), and the previous results obtained are not useful to study the interactions between this substrate and the enzymes involved in its metabolism. The crystallographic conformation of ryanodol (I) is illustrated in Figure 4, which also shows the atom numbering and the absolute stereochemistry.

Ryanodol (I) has been previously obtained synthetically (Wiesner et al., 1967; Wiesner, 1968; Belanger et al., 1979), and is isolated now for the first time from a natural source. This diterpene was known in nature in the form of its α -pyrrole carboxylate ester derivative known as ryanodine (Wiesner et al., 1967). This is an insecticidal and toxic alkaloid, which was obtained from the plant species *Ryania speciosa* (Flacourtiaceae), a shrub native to Trinidad and the Amazon basin, which is used as ryania powder to control agricultural and garden pests (Worthing, 1979). It is highly toxic because of an action on the transverse tubular system in muscles by reducing the release of calcium from the sarcoplasmic reticulum in cardiac muscle cells (Sutko et al., 1985). Ryanodol, in contrast to ryanodine, has low toxicity to mice and little activity at mammalian receptors, yet they are potent knockdown agents for injected houseflies and cockroaches, suggesting a possible difference in the target sites if mammals and insects (Waterhouse et al., 1987).

The more polar diterpene obtained was identified as cinnceylanol (II, Figure 3) on the basis of its physical data. This compound has been isolated previously from the bark of *Cinnamomum zeylanicum* (Lauraceae) (Isogai et al., 1976), as an insecticidal compound causing abnormal ecdyses to *Bombix mori* larvae at dosages lower than 16 ppm in artificial diets (Isogai et al., 1977). This

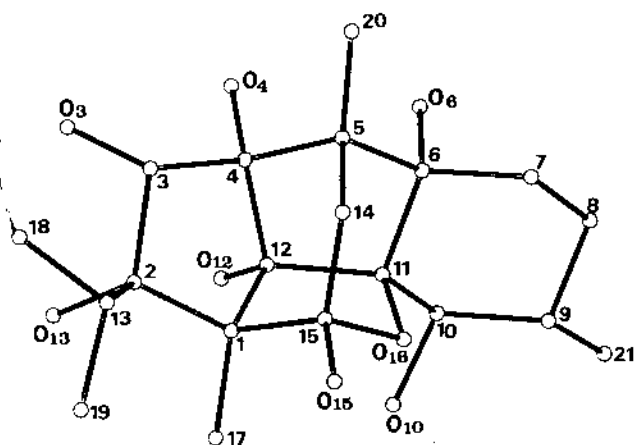


FIG. 4. Molecular structure of ryanodol (I).

TABLE 1. TOXICITY OF RYANODOL AND CINNCEYLANOL TO MICE

	LD ₅₀ (mg/kg)	95% limits	
		Lower	Upper
Ryanodol	17.918	16.227	19.786
Cinnceylanol	11.988	6.718	21.392

product also has been obtained from *C. cassia* as an anticomplement active component (Yagi et al., 1980). Cinnceylanol has not been described as being highly toxic to mammals, but from its structural similarities to ryanodol, it could share a similar mechanism of action.

The fact that we have found these insecticidal products in an endemic species of the island opens the possibility of exploiting their potential as natural pesticides to be used locally and emphasizes the importance of chemical screening of the biologically active products of this unique flora.

Both compounds, ryanodol and cinnceylanol, were toxic when injected intraperitoneally into laboratory mice (Table 1), showing similar symptoms to those previously described for the extracts. It is possible that *P. indica* contains toxic products other than I and II. The joint action of all these chemicals could result in a synergistic total effect since cooccurring synergists may be structurally optimized for intraspecific phytochemistry (Berenbaum, 1985). More experiments are needed to assess this possibility (i.e., comparisons of the effects of whole extracts against pure compounds at sublethal doses).

We plan to continue our search for other chemical and environmental factors governing the interaction discussed here, as well as the defensive role the above-mentioned chemicals play in the control of invertebrate herbivores.

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