0014-4754/94/090840-03$1.50 + 0.20

Identification of a plant growth inhibiting iridoid lactone from *Duroia hirsuta*, the allelopathic tree of the 'Devil's Garden'

J. E. Page*, S. Madriñán[a] and G. H. N. Towers

Botany Department, University of British Columbia, Vancouver (British Columbia, Canada V6T 1Z4) and [a]The Harvard University Herbaria, Harvard University, Cambridge (Massachusetts 02138, USA)
Received 13 April 1994; accepted 16 June 1994

Abstract. Bioactivity-directed fractionation of a root extract of *Duroia hirsuta* (Rubiaceae), a toxic and potentially allelopathic understory tree from the western Amazon, has led to the isolation of the tetracyclic iridoid lactone, plumericin (**1**). Bioassays showing plumericin strongly inhibited lettuce radicle elongation at a concentration (IC_{50}) of 35.8 μm/ml (123 μM). The isolation of a highly potent inhibitor of plant growth from *Duroia hirsuta* supports the hypothesis that the lack of vegetation surrounding this tree is the result of allelopathy.
Key words. *Duroia hirsuta*; Rubiaceae; allelopathy; plumericin; iridoid lactone; plant growth inhibition.

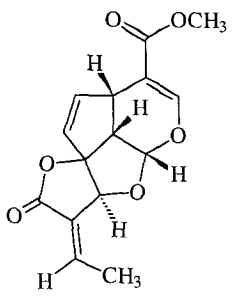
The heavily shaded understory of the tropical forest is a competitive environment where plants struggle for available resources such as light, space and nutrients. For plants growing in such habitats, the biosynthesis and release of toxic allelochemicals which inhibit the germination and growth of competitors may be an important evolutionary strategy[1,2]. While a study by Campbell et al.[3] has hinted at the importance of allelopathy as a determinate of plant community structure in the tropical forest, little is known of the chemistry of the toxic agents involved in mediating plant–plant (allelopathic) interactions in these diverse ecosystems.

An intriguing but uninvestigated example of a possibly allelopathic species from the forests of the western Amazon is *Duroia hirsuta* (Poeppig & Endl) Schumann (Rubiaceae). The forest floor beneath the canopy of this small understory tree is devoid of nearly all vegetation, and where *Duroia hirsuta* grows in groups of twenty or more individuals, anomalous clear zones are formed in the otherwise dense tropical forest[4]. These open areas, called 'jardín del diablo' (Devil's Garden) in Colombia[4] and 'limpo de canelo de vehlo' (Clearing of the Shinbone of an Old Man) in Brazil[3], are viewed with fear and superstition by the native people of the region. Native beliefs hold that the roots of *Duroia hirsuta* exude a poison that kills the surrounding forest, an explanation supported by the finding that soil samples from beneath *Duroia hirsuta* inhibited the germination and growth of lettuce seeds in a field study of allelopathy[3]. *Duroia hirsuta* is myrmecophilous, and the ants associated with this tree may contribute to its allelopathic effect by removing seeds and seedlings from around their host plant. Other myrmecophilous species such as *Cordia nodosa* (Boraginaceae), *Tococa guianensis* and *Maieta guianensis* (Melastomataceae), may be found growing in association with *Duroia hirsuta*, usually on the margin of a 'Devil's Garden'. Although no previous chemical investigation of *Duroia hirsuta* has been performed[5], its ethnobotanical uses suggest it is a highly toxic plant: Schultes[4,6] reported that Indians of the Putumayo bind the caustic bark of *Duroia hirsuta* to their skin, a practice that produces raised blisters which recede leaving a blue-brown 'tattoo'. Its leaves have been used as a fish poison[7]. As well, the mashed leaves, bark and roots of *Duroia hirsuta*, suspended in baskets in a river, have been used to make the water poisonous to enemies drinking it downstream[8]. In this paper we report the isolation of plumericin (**1**), a plant growth inhibiting iridoid lactone, from the roots of *Duroia hirsuta* and the evaluation of its inhibitory activity.

Materials and methods

Collection and extraction of plant material. Samples of *Duroia hirsuta* were collected from the site of a 'Devil's Garden' near Mitú, in the Columbian Comisaria of Vaupés, in April 1993. Voucher specimens (Madriñán, S. et al. 1193) are deposited at the Herbarium of the University of British Columbia and the Harvard University Herbaria. Air-dried roots (47 g) and leaves (164 g) were extracted repeatedly with methanol at room temperature. The filtered extracts were concentrated by rotary evaporation and their biological activity evaluated using a lettuce radicle elongation bioassay.

Lettuce radicle elongation bioassay. Bioassays were conducted in 6 cm plastic Petri dishes lined with two Whatman No. 1 paper disks. For testing, extracts and fractions were dissolved in HPLC-grade methanol (10 mg/ml) while plumericin (**1**) was dissolved in HPLC-grade dichloromethane (1 mg/ml). Aliquots of test solutions were added to each Petri dish and the solvent allowed to evaporate before the addition of 1.25 ml of distilled water. Final test concentration was 500 μg/ml.



1

The inhibitory activity of **1** was evaluated at concentrations of 200, 100, 50, 25, 12.5, 6, 3 and 1 µg/ml. Control plates received identical volumes of the appropriate solvent containing no test substance. Twenty commercial lettuce seeds (*Lactuca sativa* cv. 'Grand Rapids') were evenly distributed over the surface of the moistened paper and the sealed Petri dishes incubated in the dark for 48 h at 24 °C. Growth was terminated by freezing prior to measurement of radicle length. Ungerminated seeds were not included in the analysis, since there was no significant difference between treatment and control groups. All treatments were performed in triplicate.

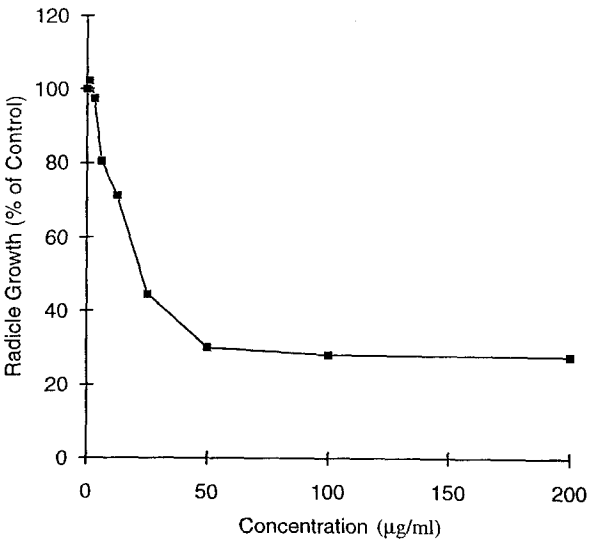
Results

Bioactivity-directed fractionation of *Duroia hirsuta* extracts. Initial tests using the lettuce radicle elongation bioassay showed significantly higher inhibitory activity (seedlings with short twisted radicles) in the root extract of *Duroia hirsuta* compared with the leaf extract (data not shown). The active root extract was partitioned between methanol–water (7:2) and dichloromethane, and yielded, upon drying with magnesium sulfate, 1.2 g of dichloromethane-soluble extract in which most of the biological activity was concentrated. A portion of the dichloromethane-soluble extract (98 mg) was separated by vacuum liquid chromatography (VLC, 5 g silica gel 60 GF_{254}), eluting with increasingly polar combinations of hexane, dichloromethane, ethyl acetate and methanol, to yield seven fractions of which fractions 5 and 6 exhibited biological activity. The active fractions were combined and applied to a preparative TLC plate (Merck silica gel 60 F_{254}, 20 cm × 20 cm, 0.5 mm precoated plates) for direct bioassay[9]. Briefly, after development in dichloromethane-ethyl acetate (9:1), the solvent was allowed to evaporate from the plate which was then covered with moistened Whatman No. 1 paper. Lettuce seeds were distributed evenly over the surface of the filter paper, and the plate incubated in a sealed transparent box for 42 h under diffuse fluorescent lighting to allow the seeds to germinate and grow. Moisture was kept constant by additional mistings. At the termination of the incubation, a zone of inhibited growth was apparent at Rf ~ 0.6. The silica from beneath the zone of inhibition was scraped off, extracted with dichloromethane and dried to yield a single compound as indicated by TLC. Additional amounts of the material of interest were obtained from the dichloromethane-soluble root extract by repeated VLC. Final purification was achieved by semi-preparative HPLC (Waters µBondapak C_{18} 7.8 × 300 mm column, UV detection at 230 nm) using acetonitrile-water (50:50) as a mobile phase to yield the tetracyclic iridoid lactone, plumericin (**1**). Plumericin was recrystallized from benzene and gave rectangular plates: mp 206–208 °C (lit.[16] 209–212 °C); high resolution EI mass spectrometry: $C_{15}H_{14}O_6$ (m/z 290.0791); low resolution EI mass spectrometry: m/z (relative intensity) 290 (M^+, 51), 272 (28), 261 (47), 258 (59), 230 (100), 213 (20), 201 (76), 193 (81), 173 (46), 160 (70), 139 (76), 77 (43), 53 (43). The UV, ^{1}H- and ^{13}C-NMR spectra of plumericin (**1**) were consistent with published data[10–12].

Evaluation of plant growth inhibitory activity of 1. The inhibitory activity of **1** was determined at concentrations of 200 µg/ml–1 µg/ml and expressed as a percentage of control (solvent only)-treated seedlings (fig.). Each treatment concentration was tested in triplicate. Plumericin inhibited the elongation of lettuce radicles in a dose-dependent manner. The concentration required to produce a 50% inhibition (IC_{50}) of radicle growth was 35.8 µg/ml (123 µM) with a 95% confidence interval of 27.6–48.2 µg/ml calculated using probit analysis[13].

Discussion

While direct evidence for the role of allelopathy in the 'Devil's Garden' phenomenon has yet to be obtained, the isolation of a potent inhibitor of plant growth from



Radicle growth of lettuce seedlings treated with different concentrations of plumericin (**1**). Values represent mean of three replicates.

the roots of *Duroia hirsuta* supports the hypothesis that *Duroia hirsuta* produces toxic allelochemicals that inhibit or kill the vegetation of the surrounding forest. The allelopathic principle, the highly functionalized iridoid lactone plumericin (**1**), has been reported to be extremely toxic to a wide range of organisms including bacteria[14], fungi[15], viruses[15], tumour cells[16,17], algae[18] and molluscs[19]. The array of toxicity and bioactivity exhibited by plumericin may be linked to the ability of the α-methylene γ-lactone moiety to undergo Michael addition with glutathione and other biological nucleophiles[20]. Plumericin has a restricted distribution within the plant and animal kingdoms and has been previously isolated from only three apocynaceous genera; *Plumeria*[17,21], *Allamanda*[16,21] and *Nerium*[22], as well as from the Caribbean sponge, *Cliona caribboea* (Clionidae)[12]. Although many iridoids are known from the Rubiaceae[23], this is the first report of the occurrence of plumericin in a member of this family.

The isolation of plumericin as an allelochemical is noteworthy for several reasons. The unusual structural features and limited distribution of plumericin contrast with the majority of plant secondary metabolites implicated as causative agents in allelopathic interactions, many of which are ubiquitous phenolic acids or other simple organic molecules[2]. Also, plumericin strongly inhibits plant growth at concentrations below that of many previously identified allelopathic agents[24].

Allelopathy has been described in numerous ecosystems such as the Californian chaparral, Florida scrub and Australian *Eucalyptus* communities[25,26]. However, the importance of allelopathy in the tropical forest, and the role of allelochemicals in mediating interactions between tropical forest species is a neglected area of research. Further investigations of the chemical ecology of *Duroia hirsuta* and the role of plumericin in the 'Devil's Garden' are underway.

Acknowledgments. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada to Dr. G. H. N. Towers. J. Page thanks the Natural Sciences and Engineering Research Council of Canada for a postgraduate fellowship. We gratefully acknowledge all those who provided assistance in collecting plant material including the staff of the National Herbarium of Colombia in Bogotá, Pedro Chequemara in Mitú and the Kubeo Indian Community of La Sabana, Rio Kubiya, Vaupés, Colombia. We thank Dr. R. E. Schultes and Dr. R. F. Raffauf for intellectual support and Dr. B. Bohm, E. Jovel and N. Page for reviewing the manuscript.

* To whom correspondence should be addressed.

1 Whittaker, R. H., and Feeny, P. P., Science *171* (1971) 757.
2 Harborne, J. B., Introduction to Ecological Biochemistry, p. 277. Academic Press, London 1988.
3 Campbell, D. G., Richardson, P. M., and Rosas, A., Biochem. Syst. Ecol. *17* (1989) 403.
4 Schultes, R. E., Econ. Bot. *41* (1987) 454.
5 NAPRALERT database, University of Illinois at Chicago, December 1993.
6 Schultes, R. E., and Raffauf, R. F., The Healing Forest, p. 380. Dioscorides Press, Portland, Oregon 1990.
7 Schultes, R. E., and Raffauf, R. F., Vine of the Soul, p. 122. Synergetic Press, Oracle, Arizona 1992.
8 Lamb, F. B., Rio Tigre and Beyond. North Atlantic Books, Berkeley, California 1985.
9 Li, H. H., Nishimura, H., Hasegawa, K., and Mizutani, J., J. chem. Ecol. *18* (1992) 1785.
10 Albers-Schonberg, G., and Schmid, H., Helv. chim. Acta *44* (1961) 1447.
11 Abe, F., Chen, R. F. and Yamauchi, T., Chem. pharm. Bull. *36* (1988) 2784.
12 Martin, G. E., Sanduja, R., and Alam, M., J. org. Chem. *50* (1985) 2383.
13 Finney, D. V., Probit Analysis. Cambridge University Press, Cambridge 1971.
14 Little, J. E., and Johnstone, D. B., Archs Biochem. *30* (1951) 445.
15 Sticher, O., in: New Natural Products with Pharmacological, Biological or Therapeutic Activity, p. 137. Eds H. Wagner and P. Wolff. Springer Verlag, Berlin 1977.
16 Kupchan, S. M., Dessertine, A. L. Blaylock, B. T., and Bryan, R. F., J. org. Chem. *39* (1974) 2477.
17 Kardono, L. B. S., Tsauri, S., Padmawinata, K., Pezzuto, J. M., and Kinghorn, A. D., J. nat. Prod. *53* (1990) 2477.
18 Coppen, J. J. W., Phytochemistry *22* (1983) 179.
19 Hamburger, M. O., Cordell, G. A., and Ruangrungsi, N., J. Ethnopharm. *33* (1991) 289.
20 Kupchan, S. M., Giacobbe, T. J., Krull, I. S., Thomas, A. M., Eakin, M. A., and Fessler, D. C., J. org. Chem. *35* (1970) 3539.
21 Coppen, J. J. W., and Cobb, A. L. Phytochemistry *22* (1983) 125.
22 Basu, D., and Chatterjee, A., Ind. J. Chem. *11* (1973) 297.
23 Inouye, H., in Methods in Plant Biochemistry, vol. 7. p. 99. Eds B. V. Charlwood and D. V. Banthorpe. Academic Press, San Diego, California 1991.
24 Einhellig, F. A., and Souza, I. F., J. chem. Ecol. *18* (1992) 1.
25 Williamson, G. B., in: Perspectives of Plant Competition, p. 143. Eds J. B. Grace and D. Tilman. Academic Press, San Diego, California 1990.
26 Lovett, J. V., in: The Science of Allelopathy, p. 75. Eds A. R. Putnam and C.-S. Tsang, Wiley Interscience, New York 1986.