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Composition and antimicrobial activity of the essential oils from invasive species of the Azores, *Hedychium gardnerianum* and *Pittosporum undulatum*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

The compositions of the essential oils from the leaves and flowers of *Hedychium gardnerianum* and from the leaves of *Pittosporum undulatum* growing on San Miguel Island (Azores) were investigated, and the compounds were identified by GC–MS analyses. The oils in the leaves and flowers of *H. gardnerianum* were rich in α-pinene, β-pinene and α-cadinol, whereas that from *P. undulatum* was found to contain monoterpenes, sesquiterpenes, diterpenes and alkanes, of which the sesquiterpenes, calamenene (41.4%), farnesol (10.9%), spathulenol (5.6%) and β-selinene (5.2%) and the diterpene (8β,13β)-kaur-16-ene (10.7%) were the major components. Their potential antimicrobial activities were tested against *Staphylococcus aureus*, *S. epidermis* and *Pseudomonas aeruginosa*, and those with the highest activities against *S. aureus* and *S. epidermis* were from *H. gardnerianum*; none had activity against *P. aeruginosa*. Additionally, the essential oils from *Pittosporum undulatum* had good antithrombin activity whereas that from *H. gardnerianum* did not.

Keywords: *Pittosporum undulatum*; Pittosporaceae; *Hedychium gardnerianum*; Zingiberaceae; Antimicrobial; Antithrombin; Calamenene; Farnesol; Kaur-16-ene; Pinene

1. Introduction

As part of a study whose main objective is the discovery of potential commercial uses of the forestry biomass (and its associated plants), the essential oils from the leaves of two highly invasive plants of the Azores, *Hedychium gardnerianum* and *Pittosporum undulatum*, were chemically studied, and their potential bioactivities evaluated.

The first invasive species, *H. gardnerianum* Sheppard ex Ker-Gawler is a rhizomatous perennial herb of the Zingiberaceae family and is typically named "Conteira". It has a stalk which can extend up to 2 m long, with oblong leaves reaching 30 cm and several yellow-orange flowers in a spike of 20–30 cm in length (Fig. 1A). It was introduced into the Azores from its native Himalayas in the middle of the 19th century, and is widespread on all of the Azorean islands except Corvo. It is extensively distributed throughout San Miguel Island with large areas being overtaken. It also spreads rapidly wherever the native forest becomes degraded, as well as being scattered in the dense laurel forest of the island (Sjögren, 1984). While the composition and characteristics of the essential oils of various *Hedychium* species have been reported (Gottlieb and Magalhães, 1959; Haggag and El-Shamy, 1980), only the oils from *H. gardnerianum* rhizomes have been examined (Weyerstahl et al., 1998).

The second invasive species described here is *P. undulatum* Vent., a tree or shrub of the Pittosporaceae family, and named "incenso" in the Azores. It has white flowers and lanceolate, acute, glabrous leaves (10×3 cm) with undulated margins; which are yellow-green in

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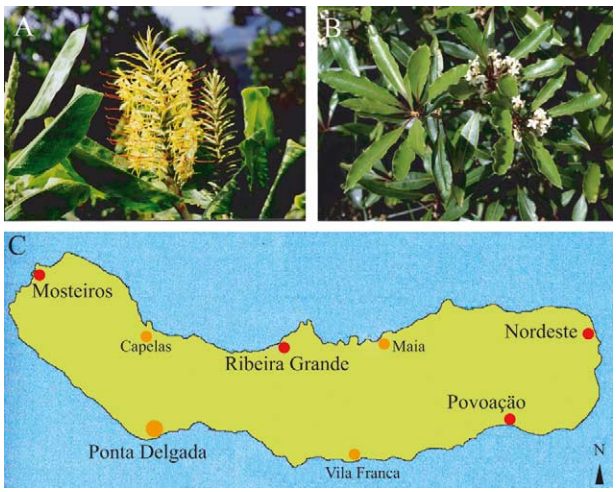

Fig. 1. (A) *Hedychium gardnerianum*, (B) *Pittosporum undulatum* and (C) *H. gardnerianum* sample collection sites on S. Miguel Island, Azores.

young tissue and dark green when mature (Fig. 1B). *P. undulatum* was introduced into the Azores from Australia long ago, primarily for protection of orange tree plantations, and exists on all nine islands of the archipelago. It is very firmly established from the sea level up to about 500 m, especially on lava flows. Its spontaneous spreading and encroachment has significantly transformed the Azorean landscape in a most severe way (Sjögren, 1984), and even in its native Australia, it is one of an increasing number of native plants that now function as environmental weeds in colonized habitats, i.e. by extending outside their initial natural ecological ranges (Mullet and Simmons, 1995; Rose, 1997,1998; Rose and Fairweather, 1997). Although the oils from fruits and leaves of another *Pittosporum* species have been examined recently (Ramanandraibe et al., 2000), as well as the oils from fruits and seeds of *P. undulatum* (Balasubrahmanyam and Rawat, 1990; Yaacob and Ariffin, 2000), this is the first report of the characterization of its leaf oil. The biological activities of the oils were also preliminarily evaluated against *Staphylococcus aureus*, *S. epidermis* and *Pseudomonas aeruginosa*, as well as for antithrombin activity (Medeiros et al., 2000).

2. Results and discussion

H. gardnerianum leaf and flower tissues from several sources on San Miguel Island were collected (Fig. 1C) from September to October 2000. Hydrodistillation of each sample was carried out in a modified Clevenger apparatus with a water cooled oil receiver to reduce the potential of hydrodistillation over-heating artifacts. Table 1 shows the components of the essential oils, whose compositions were determined by GC and GC–MS (Adams, 1995). Forty-seven compounds were identified in the oils, all of which were either mono- or sesquiterpenes: 41 were identified in both the leaf and flower extracts with each accounting for ~93–96% and 87–94% of the total oil fractions, respectively. It is evident

Table 1
Percentage composition (w/w) of the essential oils of *H. gardnerianum* leaves and flowers

Peak	Compound	Leaves				Flowers		
		N[a]	P	R	M	N	P	R
1	α-pinene	17.51	14.19	8.38	18.13	9.75	18.37	4.43
2	camphene	t[b]	t	t	t	t	t	t
3	β-terpinene	0.92	0.91	0.59	0.91	0.34	t	t
4	β-pinene	9.93	11.99	5.06	11.00	6.40	14.53	3.12
5	β-myrcene	0.20		0.44	0.24	0.38	0.78	t
6	γ-phellandrene	t	t	t	t	1.16	2.60	t
7	2-carene	–	–	–	–	0.85	1.79	t
8	*p*-cymene	0.21	–	0.14	t	7.40	8.16	3.85
9	limonene	0.75	0.97	0.44	0.77	1.01	1.92	t
10	eucalyptol	t	t	t	t	t	t	t
11	γ-terpinene	–	–	0.08	t	6.70	14.43	3.15
12	α-pinene oxide	–	t	–	t	t	–	–
13	linalool	t	t	t	t	0.57	1.89	t
14	thujol	–	t	–	–	t	t	–
15	(E)-3(10)-caren-4-ol	–	t	–	–	–	–	–
16	α-cubebene	0.20	t	0.11	–	t	t	t
17	α-copaene	0.22	t	0.13	t	t	t	t
18	β-elemene	0.15	t	0.43	0.18	–	–	–
19	α-gurjunene	0.25	t	0.18	t	–	–	–
20	caryophyllene	8.89	8.18	7.04	7.66	4.17	5.49	4.95
21	aromadendrene	1.88	1.71	1.62	1.32	0.87	0.84	t
22	γ-cadinene	0.74	0.92	0.66	0.69	0.45	0.65	t
23	EBCP[c]	1.23	t	1.90	1.30	0.91	t	t
24	γ-muurolene	0.38	t	0.36	–	0.26	–	t
25	γ-elemene	–	–	–	–	1.19	0.87	1.55
26	patchoulene	3.91	1.41	9.81	4.42	–	–	–
27	valencene	1.42	2.05	1.48	1.50	1.14	0.73	1.94
28	α-farnesene	5.67	1.25	7.92	7.14	2.72	2.97	2.74
29	isocaryophyllene	–	t	–	–	0.39	t	t
30	β-cubebene	2.58	2.85	2.86	2.26	1.47	0.76	2.35
31	β-cadinene	0.94	0.93	0.96	0.91	7.50	4.81	9.20
32	δ-cadinene	7.88	4.89	8.76	7.68	t	t	t
33	calamenene	0.21	0.56	0.47	0.21	t	–	–
34	α-muurolene	0.40	0.48	0.49	0.39	t	t	t
35	nerolidol	–	–	–	–	1.24	0.74	t
36	γ-gurjunene	0.14	t	0.19	t	t	t	t
37	β-guaiene	6.11	5.49	4.94	4.84	3.39	1.04	4.20
38	germacrene B	2.93	3.04	4.94	2.39	1.90	0.93	2.68
39	eudesmene	0.92	1.52	1.34	0.84	0.77	–	t
40	dihydro-*cis*-α-copaene-8-ol	0.93	1.28	1.23	0.87	0.40	–	–
41	cubenol	0.83	1.08	1.35	0.88	0.38	–	–
42	longifolenaldehyde	1.51	1.86	1.68	1.43	1.40	0.80	2.13
43	spathulenol	0.57	0.41	0.39	0.27	0.32	t	t
44	τ-cadinol	3.16	5.76	5.20	4.06	3.28	1.26	7.05
45	τ-muurolol	2.64	5.86	4.90	3.84	4.11	1.78	8.72
46	δ-cadinol	0.93	1.93	1.80	1.54	1.29	t	2.66
47	α-cadinol	6.42	14.59	12.54	8.73	12.54	5.76	26.22
	Total	93.56	96.10	95.36	96.40	86.65	93.90	90.94

[a] Place of sampling: N = Nordeste; P = Povoação; R = Ribeira Grande ; M = Mosteiros.
[b] t = traces.
[c] EBCP = epibicyclosesquiphellandrene.

(Table 1) that there are large quantitative differences in the oil compositions between the leaves and the flowers, as well as from the locations on San Miguel Island where the plants were obtained. The main components in the leaf oils were the monoterpenes, α-pinene (8.38–18.13%), and β-pinene (5.06–11.99%), and the sesquiterpenes α-cadinol (6.42–14.59%), caryophyllene (7.04–8.89%), β-guaiene (4.84–6.11%), δ-cadinene (4.89–8.76%), τ-cadinol (3.16–5.76%) and τ-muurolol (2.64–5.86%). Although there are quantitative differences in the composition of the oils of the leaves collected from different locations of the island, the overall monoterpene contents were between 28.05-31.05% except for Ribeira Grande, which was 15.08%.

The main components in the flower oils were also the monoterpenes, α-pinene (4.43–18.37%) and β-pinene (3.12–14.53%), and the sesquiterpenes, α-cadinol (5.76–26.22%), τ-cadinol (1.26–7.05%), τ-muurolol (1.78–8.72%) and β-cadinene (4.81–9.20%). The amounts of several other monoterpenes was also significant, i.e. *p*-cymene (3.85–8.16%) and γ-terpinene (3.15–14.43%). Interestingly, the monoterpene content varied from 14.55 to 64.47% for plant specimens collected at Ribeira Grande and Povoaçäo, respectively.

In comparison with the published data on the natural population of *Hedychium coronarium* oils (Haggag and El-Shamy, 1980), the leaf and rhizome oils of *H. gardnerianum* have as their major components, α-pinene and β-pinene. Additionally, myrcene, limonene, *p*-cymene, camphene and γ-terpinene were present in all samples of *Hedychium* species examined. Sesquiterpenes (30%) were also present in the essential oil from the rhizomes of *H. gardnerianum* (Weyerstahl et al., 1998), which were mainly cadinane derivatives. Thus, it can be concluded that the results obtained compare well with those obtained in other reports on the essential oils of other *Hedychium* species, as well as with that from the rhizomes of this plant.

Analyses of the essential oils from the leaves of *P. undulatum* were carried out using plants collected in September–October 2000 in Ribeira Grande on San Miguel Island, with hydrodistillation carried out as before. Table 2 shows representative data for the major constituents in its leaf essential oils, these being identified using GC and GC-MS, respectively. Accordingly, 21 compounds were identified of which 17 accounted for ~96.8% of the total (Table 2). Of the mixture of monoterpenes, sesquiterpenes, diterpenes and alkanes, the main constituents present were the sesquiterpenes calamenene (41.4%), farnesol (10.9%), spathulenol (5.6%) and β-selinene (5.2%) with (8β,13β)-kaur-16-ene (10.7%) being the major diterpene. The remaining constituents, e.g., alkanes, such as docosane, and monoterpenes, such as 4-terpineol, were present in lower amount.

By contrast, a previous analysis of a natural population of *Pittosporum viridiflorum* (Ramanandraibe et al., 2000) revealed that the main constituents of its leaf oils were the sesquiterpenes, δ-cadinene (10.6%) and α-cadinol (18.3%). Interestingly, although γ-cadinene is present in *P. undulatum* leaf tissue (~3.3%), no δ-cadinene or α-cadinol were detected, this perhaps being an indicator of a molecular marker for *P. viridiflorum*.

Table 2
Main components (%) of the leaves of *Pittosporum undulatum*

Compound	%
4-terpineol	1.6
α-copaene	2.8
β-elemene	0.5
β-patchoulene	0.8
allo-aromadendrene	0.5
β-selinene	5.2
γ-elemene	4.0
α-muurolene	1.3
γ-cadinene	3.7
calamenene	41.4
1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-naphthalene	1.3
spathulenol	5.6
farnesol	10.9
(8β,13β)-kaur-16-ene	10.7
eicosane	2.0
docosane	2.7
tricosane	1.8
Total	96.8

All the oils obtained were subsequently tested on an antimicrobial bioassay against *Staphylococcus aureus*, *S. epidermis* and *Pseudomonas aeruginosa* using the paper disc diffusion method. The results obtained (Table 3) reveal good antimicrobial activity of the oils of *H. gardnerianum* against *Staphylococcus* strains but not against *Pseudomonas* species. By contrast, the oils from *P. undulatum* displayed no antimicrobial activity.

Finally the oils of the two plants were subjected to an antithrombin bioassay test (Medeiros et al., 2000). Under the conditions employed (see Section 3), the crude oil of *P. undulatum* gave ~93% antithrombin activity, this being more active than the control treatment using heparin (~74%), whereas *H. gardnerianum* oil was inactive.

Taken together, these data reveal a potential use for the oils of these invasive species. In conclusion, while

Table 3
Antimicrobial activities of the essential oil fraction from *H. gardnerianum* leaves (inhibition zone in mm)[a]

Essential oil origin	*S. aureus*	*S. epidermis*	*P. aeruginosa*
Nordeste	21	18	0
Povoação	15	21	0
Ribeira Grande	20	13	0
Mosteiros	21	21	0
tetracycline	22	21	7

[a] Note: essential oil (30 μl/dish); tetracycline (50 μg/dish).

H. gardnerianum with a higher composition of monoterpenes showed antimicrobial activity, the oils of the leaves of *P. undulatum* with higher content of sesquiterpenes and diterpenes only displayed antithrombin activity. Further testing will be carried out to establish the identity(ies) of the active components involved.

3. Experimental

3.1. Plant material

The fragrant red and creamy yellow flowers, held in dense spikes, of *H. gardnerianum*, appear towards the end of summer–beginning of autumn (Dallwitz, 1980; Watson and Dallwitz, 1991; 1992 onwards). For that reason, samples of leaves and flowers (8 kg) were collected in September–October 2000 from plants growing in natural stands in different locations on San Miguel Island (Nordeste, Povoação, Ribeira Grande and Mosteiros) (Fig. 1C). The leaves (8 kg) of *P. undulatum* were also collected in September–October 2000 from plants growing at Ribeira Grande on San Miguel Island. The plants were selected at random, and the leaves and flowers were collected from all sides of the plant. The samples were immediately placed in plastic bags and transported to the Institute of New Technologies of Azores. The flowers and leaves were separated and stored in plastic bags in a freezer at −20 °C. Two sets of voucher herbarium specimens were made: one is in the Museum Carlos Machado Herbarium (Azores), and the other is in the Institute of New Technologies of Azores (INOVA-78 for *H. gardnerianum* and INOVA-79 for *P. undulatum*)

3.2. Distillation of the essential oils

The essential oils from each sample of *H. gardnerianum* flowers (2 kg) and *P. undulatum* leaves (6 kg) were obtained by hydrodistillation for 3 h in a modified Clevenger type apparatus. These were collected in a lighter than water oil graduated trap and stored at −1 °C until analyzed.

3.3. GC and GC–MS analyses

The GC analyses used a VARIAN model 3400 equipped with a flame ionization detector (FID) and a 15 m RT×5 column (15 m, 5% diphenyl, 95% dimethylsiloxane). The temperature regimen of the column was programmed to increase after 1 min from 45 to 130 °C at a rate of 1 °C/min and then to 220 °C at a rate of 2 °C/min, the latter temperature being held for 20 min, whereas the temperature of both the injector and detector was at 250 °C. The GC–MS analyses of the oils of *H. gardnerianum* were performed on a Varian Chrompack Saturn model GC–MS 2000, operated under the following conditions: mode, EI; EI energy, 1500 V; mass range, 0–2000 amu; ion range, 40–650. A column type, CP-sil 8 CB Lowbleed/MS (5% diphenyl, 95% dimethylsiloxane) with a length of 30 m and an inside diameter of 0.25 mm, an outside diameter of 0.39 mm and a film thickness of 0.25 μm was used. The temperature of the column was programmed as above, with the temperature of the injector and detector being 250 and 170 °C, respectively. The GC–MS analyses of the oils of *P. undulatum* were performed on a Hewlett Packard model 6890 Series GC System equipped with a HP 5973 MS detector (EI mode, 70 eV) and a HP-5 (5% phenylmethylsiloxane column, 30 m long with a 2.5 mm i.d. and a film thickness of 0.25 μm). The temperature of the column was programmed to increase after 1 min from 45 to 130 °C at a rate of 1 °C/min and then to 220 °C at a rate of 2 °C/min, whereas both injector and detector temperatures were at 250 and 170 °C, respectively. The components were identified by comparing their retention times to those of standard compounds, peak enrichment by co-injection with standards wherever possible, as well as by comparing their MS spectra with literature data. Kovats indices (Van den Dool and Kratz, 1963; Davies, 1990) were calculated by using a standard mixture of C_9–C_{16} *n*-alkanes and comparing with literature data (Davies, 1990; Choo et al., 1999; Couladis et al., 2000). Quantitative data were obtained by the peak normalization technique using integrated FID responses.

3.4. Antimicrobial activity

The antimicrobial activities of the essential oils were analyzed by the agar diffusion method (Tanaka, 1992). Filter paper discs (Oxoid, Basingstoke, England) (6 mm) were soaked in each essential oil (30 μl). The discs were then dried, and placed on agar plates, which had been uniformly spread with one of the microorganisms: *S. aureus* (ATCC25923), *S. epidermis* (ATCC12228) and *Pseudomonas aeruginosa* (ATCC27853). Positive controls used the antibiotic tetracycline at 50 μg/disc. Each test was carried out in triplicate. After 24 h incubation at 37 °C, plates were screened for growth inhibition zones.

3.5. Antithrombin activity

A chromogenic bioassay (Medeiros et al., 2000) was used to determine the antithrombin activity of the oil samples. Tris buffered saline, pH 8.0 (1 l, Sigma Chemical Company) was prepared according to the manufacturer's instructions, the chromogenic reagent, D-PHE-L-PIPECOYL-ARG *p*-nitroanilide (1.25 mg; Sigma Chemical Company) was dissolved in H_2O (1 ml) and the thrombin solution was obtained by reconstitut-

ing bovine plasma lyophilized powder (500 units, Sigma Chemical Company) in Tris buffered saline, pH 8.0 (900 μl). To each sample, prepared in Tris buffered saline, pH 8.0, at the final concentration of one part per thousand (1 ppt), was added an equal volume of thrombin solution. After thorough mixing (vortex), an aliquot (100 μl) of each sample was placed in a well of a 96 flat bottom Elisa plate (Falcon) and the chromogenic reagent (50 μl) was added. The absorbance at 405 nm (A^{405}) was next measured continuously over a 5 min period (t') in a microplate reader (Spectra Max 250, Molecular Devices). Pure methanol (50 μl) and heparin (1 ppt, Aldrich) were used as negative and positive controls, respectively. Three replicates were performed for each test and control samples, and the average percent activity ($A\%$) was calculated using the formula:

$$A\% = 1 - \frac{V_{\text{max sample}}}{V_{\text{max blank}}} \times 100, \quad \text{where}$$

$$V_{\max} = \frac{A^{405}_{t=t'} - A^{405}_{t=0}}{t'}$$

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