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## Foliar epicuticular wax of *Arrabidaea brachypoda*: flavonoids and antifungal activity

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### Abstract

The epicuticular wax of the leaves of *Arrabidaea brachypoda* was analyzed for its flavonoid content and four compounds (**1**–**4**) were isolated. They are known flavonoids and showed antifungal activity against *Cladosporium sphaerospermum*. They were identified by mass spectrometry, proton nuclear magnetic resonance spectroscopy and nuclear Overhauser effect spectroscopy (NOESY) as 3',4'-dihydroxy-5,6,7-trimethoxyflavone (**1**), cirsiliol (**2**), cirsimaritin (**3**) and hispidulin (**4**). Two major chemical features resulting from this work are the first report of **1** as a natural product and the first finding of cirsiliol in Bignoniaceae. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** *Arrabidaea brachypoda*; Bignoniaceae; Flavonoids; Epicuticular wax; Antifungal activity; 3',4'-Dihydroxy-5,6,7-trimethoxyflavone; Cirsiliol; Cirsimaritin; Hispidulin

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## 1. Introduction

*Arrabidaea brachypoda* (DC) Bur. is a bignoniaceous vine shrub native from the Brazilian cerrado, 1.0–2.0 m high, abundantly branched, with coreaceous leaves and pink–purple flowers in terminal inflorescences (Lorenzi and Souza, 1995). Its local name is “cipó-una” or “tintureiro”, the latter meaning “those which tint”, although this species is not known for this property. Rather, this is attributed to one of its relatives, *A. chica* (H. & B.) Verlot (Harborne, 1967; Scogin, 1980), which presents a red dye, carajurin (3-desoxyanthocyanidin), extracted from its leaves (Chapman et al., 1927).

Investigating the foliar epicuticular wax of several dicotyledons from the cerrado, Amaral et al. (1985) found that the thickness of the lipophilic cuticular layers is not perfectly correlated with the contents of epicuticular wax. Representing one extreme, *A. brachypoda* showed a very thin lipophilic cuticular layer but an exceptionally high content of epicuticular wax. Wax consists of a complex mixture of many compounds, mainly a homologous series of hydrocarbons, esters, acetones, alcohols and carboxylic acids (Baker, 1982). Terpenoids and flavonoids also occur, both being related to protection of the plant against pathogens and the latter to protection against ultraviolet (UV) radiation. In a chemosystematic study of five Bignoniaceae species from the cerrado Blatt et al. (1998) reported the presence of apigenin and luteolin in their aglycone forms in *A. brachypoda*. This result led us to investigate the flavonoids of the wax, since aglycones are known to occur on the surface. The main goal of this work was to quantify and identify the flavonoids of the wax present in *A. brachypoda* and to verify their antifungal activity.

## 2. Materials and methods

### 2.1. Plant material

Leaves of *A. brachypoda* were collected in the Ecological Reserve of Mogi-Guaçu, state of São Paulo, Brazil, in June, September, November and December of 1997. A voucher specimen (*T. Alcerito 01*) has been deposited in the Herbarium of Botanical Institute of São Paulo (SP).

### 2.2. General experimental procedures

The UV spectra were obtained using a Beckman DU-70 spectrophotometer. Proton nuclear magnetic resonance ( $^1\text{H}$  – NMR) and nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded in dimethyl sulfoxide (DMSO) on a Bruker Advance DPX-300 MHz spectrometer, using tetramethylsilane (TMS) as internal standard. Chemical shifts are given as  $\delta$  values (ppm) and coupling constants ( $J$ ) in Hertz. Electrospray ionization mass spectra (EIMS) were obtained by gas chromatography–mass spectrometry (GC–MS) on HP5890II/5989B equipment. For the GC–MS analysis, a 25 m  $\times$  0.32 mm HP Ultra-1 (film thickness 25  $\mu\text{m}$ ) fused silica

capillary column was used. The conditions utilized were: mass selective detector, with He as a carrier gas, linear velocity 32 cm/min, split ratio 1:10, temperature program 100–300°C at 10°C/min, hold 15 min at 300°C, injector and detector temperature 300°C. High-performance liquid chromatography–mass spectrometry (HPLC–MS) was performed on an HP1090 Plus 59987A/5989B instrument. For the HPLC analyses, the fractions were subjected to reversed-phase chromatography using a gradient solvent of MeOH:H<sub>2</sub>O:AcOH. The column used was HP Spherisorb ODS2 (5 µm, 125 m × 4 mm) and the interface was the electrospray. Bioautography was performed on thin layer chromatograms (silica gel, pre-coated plates, Art. 5715-Merck).

### 2.3. *Micro-organisms*

*Cladosporium sphaerospermum* Pemzig has been maintained at Section of Physiology and Biochemistry, Botanical Institute of São Paulo under voucher number SPC 491.

### 2.4. *Rinsing the wax*

The epicuticular wax of the air-dried leaves was obtained through a 10 s dip (three times) in CHCl<sub>3</sub> (Silva Fernandes et al., 1964). The pooled solvent was dried under reduced pressure.

### 2.5. *Content of the wax*

After removing the wax, the foliar area of 50 leaves was determined using the Leaf Area Skye Instruments Ltd. program. The dry weight of the wax was divided into the total area, giving the results in µg/cm<sup>2</sup>. Four measurements were taken (June, September, November and December of 1997).

### 2.6. *Extraction of the flavonoids from the crude wax (chloroform extract)*

Two techniques were used to isolate the flavonoids from the crude wax. (1) One-dimensional paper chromatography (PC) on chromatography paper (Whatman 1Chr), using 15% acetic acid as solvent, was left to run overnight. The flavonoids were detected under UV and eluted from the paper strips with MeOH. (2) Sephadex LH-20 (Pharmacia) column chromatography (CC) was monitored by UV (80% MeOH) (Markham, 1982). The fractions obtained were examined under GC–MS and HPLC–MS as described above.

### 2.7. *Content of the flavonoids in the wax*

Five grams of the crude wax was deposited on four paper chromatography sheets. The resulting flavonoid fraction was eluted in MeOH until no purple/yellow (with NH<sub>3</sub> stream) color change was detected under UV. This fraction was deposited on

preparative silica gel 60 PF<sub>254</sub> (Merck) thin layer chromatography (TLC) plates (manually prepared), using CHCl<sub>3</sub>:MeOH (99:1) as solvent, to obtain five bands corresponding to flavonoids (Markham, 1982).

### 2.8. Isolation and identification of the flavonoids

Preliminary silica gel 60 (70–230 Mesh, Merck) CC [CHCl<sub>3</sub>:MeOH (99:1, 98:3, 97:3 and 95:5)] of the crude wax yielded 300 fractions of 15 ml each, which, after monitoring on TLC, resulted in five dark spots under UV (336 nm). These spots served as a guideline to preparative TLC of the flavonoid fraction obtained by PC. In this step four flavonoids were isolated. The identification of flavonoids was based on UV, MS and NMR spectral data and confirmed by comparison with published spectral data.

### 2.9. Antifungal assay

Antifungal activity of the crude CHCl<sub>3</sub> extract and of the isolated compounds was determined by direct bioautography method on TLC plates (Homans and Fuchs, 1970). One hundred µg of the crude wax diluted in CHCl<sub>3</sub>:MeOH (99:1) were applied on pre-coated silica gel 60 F<sub>254</sub> TLC plates (Aldrich, 0.2 mm). After evaporation of the solvents, suspensions of *C. sphaerospermum* spores were sprayed and incubated at 25°C in the dark. After 48 h, clearly visible inhibition zones indicated the minimum inhibitory activities. After the positive answer of the crude wax, different volumes of a CHCl<sub>3</sub>:MeOH (99:1) solution (10 mg/ml) of the isolated compounds were submitted to the steps above.

## 3. Results and discussion

Since the 1980s the number of reports relating free flavonoid aglycones to the cuticular wax has increased enormously. Aglycones of flavones and flavonols are the commonest flavonoids reported for the wax, although always as minor constituents. The exception occurs in ferns, where the flavonoids are always the major components (Wollenweber and Schneider, 2000). Wax is a potential source for rare flavonoid structures, rareness being an important factor in chemosystematics and also in bioactivity testings.

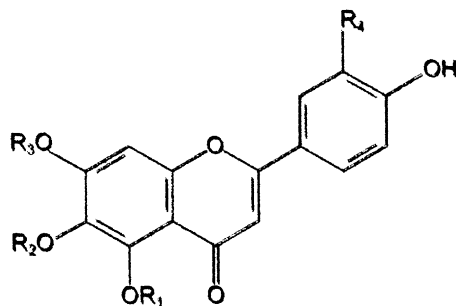
Non-polar, polymethylated or minimally hydroxylated flavonoid is correlated with plants' ability to accumulate lipophilic secondary products, especially terpenoids, either within specialized structures or externally. The occurrence of flavonoid aglycones is related to plants either living in or originating from arid or semi-arid habitats (Wollenweber and Dietz, 1981). *A. brachypoda* bears two of the characteristics related to the presence of flavonoid aglycones in the wax, these being a wax rich in terpenoid (Mimura, 2001) and its occurrence in the cerrado, a typical savanna ecosystem of Central and Southeast Brazil.

Until 1993, Bignoniaceae was not listed among the aglycone-accumulating families (Wollenweber, 1993), although Takemura (1993) had reported the occurrence of a

flavone, 7,4'-dihydroxy-5-methoxyflavone (thevetiaflavone), and later a new flavone, 6,7,3',4'-tetrahydroxy-5-methoxyflavone (carajuflavone), in the leaves of *Arrabidaea chica* f. *cuprea* (Takemura et al., 1995). Hase et al. (1995) identified 5,7-dihydroxy-6,4'-dimethoxyflavone (pectolarigenin) and related flavonoids from the leaves of *Millingtonia hortensis* and Azam and Ghanim (2000) reported circimaritin and circilioneol in *Tecomella undulata*. However, the localization of the flavonoids was not stated in these studies. Stermitz et al. (1992) and Wollenweber et al. (1996) were the first to relate the flavonoid aglycones of a bignoniaceous species, *Godmania aesculifolia*, to the leaf exudate.

We isolated and identified four flavones from the wax of *A. brachypoda* (1–4) by UV,  $^1\text{H}$  – NMR, NOESY, MS data and by comparison with data from the literature (Fig. 1). Among them, circilioneol (2), circimaritin (3) and hispidulin (4) are very commonly found as external flavonoids (Wollenweber, 1993). Circimaritin (3) and hispidulin (4) were previously found in Bignoniaceae (Hase et al., 1995; Azam and Ghanim, 2000), but this is the first report of circilioneol to this family. The fourth flavonoid, a 3',4'-dihydroxy-5,6,7-trimethoxyflavone (1), has only been reported as a synthesized compound, having inhibitory activity against arachidonate 5-lipoxygenase (Horie et al., 1986). This is the first report of 1 as a natural product.

The mass spectrum of flavonoid 1 afforded an  $[\text{M}]^+$  ion peak at  $m/z$  344 (100%,  $\text{C}_{18}\text{H}_{16}\text{O}_7^+$ ) and fragments at  $m/z$  329 (60%,  $\text{M}^+ - \text{CH}_3$ ) and  $m/z$  301 (10%,  $\text{M}^+ - \text{CH}_3 - \text{CO}$ ). In the  $^1\text{H}$  – NMR spectrum (DMSO/TMS), three methoxyl signals were observed at  $\delta$  3.73 (3H, s, 6-OCH<sub>3</sub>), 3.89 (3H, s, 5-OCH<sub>3</sub>) and 3.92 (3H, s, 7-OCH<sub>3</sub>). One olefinic signal at  $\delta$  6.91 (1H, s, H-3) was distinguished. The signals at  $\delta$  6.96 (1H, s, H-8), 7.58 (1H, s, H-2'), 6.95 (1H, d,  $J = 8.4$  Hz, H-5') and 7.60



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
<b>1</b>	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>	OH
<b>2</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	OH
<b>3</b>	H	CH <sub>3</sub>	CH <sub>3</sub>	H
<b>4</b>	H	CH <sub>3</sub>	H	H

Fig. 1. Structures of the flavonoids from *A. brachypoda*.

(1H, d,  $J = 8.4$  Hz, H-6') suggested the presence of the two-aromatic-ring system of a flavonoid structure. In this case, one signal was observed at  $\delta$  8.24 (2H, br s) which may be assigned to a phenolic proton. The presence of three methoxyl groups and the disubstituted B ring in this flavonoid structure is very clear; therefore the only structure possible is the 3',4'-dihydroxy-5,6,7-trimethoxyflavone (Horie et al., 1986).

5-Methoxylation seems to be a common feature in *A. chica* (Takemura, 1993; Takemura et al., 1995), while in *A. brachypoda*, 6-methoxylation is more widespread, followed by 7-methoxylation. We found 5-methoxylation only in **1**. Another factor raised from this result, together with the results obtained by Stermitz et al. (1992), Hase et al. (1995), Takemura et al. (1995), Wollenweber et al. (1996) and Azam and Ghanim (2000), is that hydroxylation or methoxylation at C<sub>6</sub> is very frequent in Bignoniaceae, in accordance with the first findings of Harborne (1967).

The chloroform extract of the leaf wax of *A. brachypoda* submitted to TLC and bioassay with the spore suspension of *C. sphaerospermum* showed three fungitoxic zones ( $R_f$  0.09, 0.18 and 0.24) when applying 100  $\mu\text{g}$  of the fraction. Cirsimaritin (**3**), cirsiliol (**2**) and 3',4'-dihydroxy-5,6,7-trimethoxyflavone (**1**), corresponding to the  $R_f$  values above, respectively, led to inhibition of *C. sphaerospermum* growth at 1  $\mu\text{g}$  of the pure compound. Hispidulin (**4**) ( $R_f$  0.03) was less active, responding as inhibitor of the fungal growth only at 10  $\mu\text{g}$ . A fifth flavonoid ( $R_f$  0.33), presenting the minimum amount required for the inhibition of fungal growth at 1  $\mu\text{g}$ , remains to be identified. It has been admitted for a long time that epicuticular wax can act as a physical or chemical barrier against attack by microorganisms (Juniper, 1983; Mendgen, 1996). Growth inhibition of the fungus *C. sphaerospermum* by the flavonoids isolated from *A. brachypoda* wax reinforces one of the ecological roles of these compounds.

Among the two methods tested here to isolate flavonoids from the wax, paper chromatography was proved to be best in isolating flavonoids from the other constituents of the wax (although not to obtain individual flavonoids, which was achieved through silica gel TLC), while the fractions obtained from CC were more contaminated, especially with terpenoids, which were detected after HPLC–MS.

The wax content varied from 633.9 (June 1997) to 800.4  $\mu\text{g}/\text{cm}^2$  (December 1997), with 664.8 (September 1997) and 789.9  $\mu\text{g}/\text{cm}^2$  (November 1997) as intermediate values. The lowest value (633.9  $\mu\text{g}/\text{cm}^2$ ) was obtained in the dry season and it is interesting to note that the values rise towards the wetter season (from September to December).

The flavonoid content measured by PC reached the average of 11.5% of the total wax weight. This fraction was further chromatographed via preparative TLC to obtain the flavonoids **1–4**.

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