

ANTIFUNGAL DERIVATIVES FROM *Piper mollicomum* AND *P. lhotzkyanum* (PIPERACEAE)

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Bioguided fractionation of the extracts from leaves of *Piper mollicomum* and *Piper lhotzkyanum* against the fungi *Cladosporium cladosporioides* and *C. sphaerospermum* afforded seven bioactive compounds, four being chromenes: methyl 2,2-dimethyl-2H-chromene-6-carboxylate, methyl 8-hydroxy-2,2-dimethyl-2H-chromene-6-carboxylate, 2-methyl-2-[4'-methyl-3'-pentenyl]-2H-1-benzopyran-6-carboxylic acid, 2,2-dimethyl-2H-chromene-6-carboxylic acid, one dihydrochalcone: 2',6'-dihydroxy-4'-methoxydihydrochalcone, and two flavanones: 7-methoxy-5,4'-dihydroxy-flavanone and 7,4'-dimethoxy-5-hydroxy-flavanone. The structures of the bioactive isolated derivatives were elucidated by interpretation of their NMR data [ $^1\text{H}$  and  $^{13}\text{C}$  (BBD, DEPT 135°)], and mass spectral data as well as by comparison with data described in the literature.

Keywords: *Piper mollicomum*; *Piper lhotzkyanum*; antifungal derivatives.

## INTRODUCTION

Phytochemical studies on *Piper* species describe the isolation of several classes of metabolites including amides, flavonoids, chromenes, lignans, benzoic acids, and phenylpropanoids.<sup>1</sup>

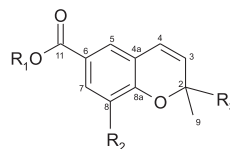
*Piper mollicomum* Kunth. and *P. lhotzkyanum* Kunth. belong to the Piperaceae family and are distributed in Brazil, in the Amazon region and the Atlantic Forrest.<sup>2</sup> A previous investigation of *P. lhotzkyanum* afforded one chromene, two prenylated benzoic acids, six sesquiterpenoids, and phytol.<sup>3</sup> Another study with this plant described the isolation and characterization of a C-glucosylflavone, four phenylpropanoid derivatives, and one flavanone.<sup>4</sup> No phytochemical studies were found for *P. mollicomum*, in exception the volatile oil composition.<sup>5</sup> However, no report about the antifungal potential to these species has been described in literature.

In this paper we describe the isolation, characterization and evaluation of antifungal potential of two chromenes (**1** and **2**) and one dihydrochalcone (**5**) from *P. mollicomum*, and two chromenes (**3** and **4**) and two flavanones (**6** and **7**) from *P. lhotzkyanum*.

## RESULTS AND DISCUSSION

The structures of compounds **1** – **7** were deduced by interpretation of their NMR data [ $^1\text{H}$  and  $^{13}\text{C}$  (BBD and DEPT 135°)] and mass spectral data as well as by comparison of the data described in the literature.

The  $^{13}\text{C}$  NMR spectra of **1** – **4** showed aromatic carbon peaks at  $\delta$  115.9 – 156.9 and carbinolic carbons at  $\delta$  77.2 – 79.9 (C), which in association to intense peak at  $\delta$  28.4 (2 X CH<sub>3</sub>) and sp<sup>2</sup> carbons at  $\delta$  127.9 – 131.0 (CH) and 121.5 – 121.4 (CH) suggested the occurrence of chromene derivatives.<sup>6</sup> The presence of an 1,3,4-trisubstituted aromatic ring to compounds **1** and **4** was defined by the presence, in their  $^1\text{H}$  NMR spectra, of three signals at  $\delta$  6.80 (d,  $J$  = 8.7 Hz),  $\delta$  7.70 (d,  $J$  = 1.5 Hz) and  $\delta$  7.85 (dd,  $J$  = 8.7 and 1.5

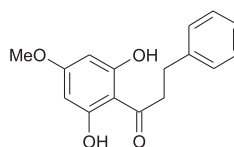


**1** R<sub>1</sub> = Me; R<sub>2</sub> = H; R<sub>3</sub> = Me (C-10);

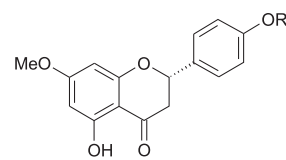
**2** R<sub>1</sub> = Me; R<sub>2</sub> = OH; R<sub>3</sub> = Me (C-10);

**3** R<sub>1</sub> = H; R<sub>2</sub> = H; R<sub>3</sub> =

**4** R<sub>1</sub> = H; R<sub>2</sub> = H; R<sub>3</sub> = Me (C-10)



**5**



**6** R = H

**7** R = Me

Figure 1. Chromenes, dihydrochalcone and flavanones isolated from *Piper mollicomum* and *P. lhotzkyanum*

Hz) assigned to H-8, H-5 and H-7, respectively. As in the  $^1\text{H}$  NMR spectrum of **1** was observed an intense peak at  $\delta$  3.87 (OCH<sub>3</sub>), this compound was elucidated as the methyl ester of **4**. Thus, compounds **1** and **4** were identified as methyl 2,2-dimethyl-2H-chromene-6-carboxylate<sup>6</sup> and 2,2-dimethyl-2H-chromene-6-carboxylic acid,<sup>7</sup> respectively. Similarly, the  $^1\text{H}$  NMR spectrum of **3** indicated the same 1,3,4-trisubstituted aromatic ring. The additional signals at  $\delta$  1.68 (s, 3H, H-5'), 1.58 (s, 3H, H-6'), 2.10 (m, 2H, H-2'), 1.80 (t,  $J$  = 7.4 Hz, H-1') and 5.10 (m, 1H, H-3') suggested a 4'-methyl-3'-pentenyl substitution. As the singlet at  $\delta$  1.44 (3H) was assigned to H-9, it is in the carbinolic carbon C-2. The comparison of  $^1\text{H}$  and  $^{13}\text{C}$  NMR data with those reported in the literature<sup>3,8</sup> allowed the

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identification of **3** as 2-methyl-2-[4'-methyl-3'-pentenyl]-2H-1-benzopyran-6-carboxylic acid. The <sup>1</sup>H NMR of **2** showed two doublets at δ 7.33 (*J* = 1.8 Hz) and 7.48 (*J* = 1.8 Hz), which were indicative of a 1,2,3,5-tetrasubstituted aromatic ring. The remaining NMR data, in association to LREIMS, which showed the molecular ion-peak at *m/z* 234, suggested an additional hydroxyl group. Since the aromatic hydrogens were positioned in *meta*, this group was attached at C-8, which was confirmed by signal at δ 143.4 (C) in the <sup>13</sup>C NMR spectra, characteristic of an aromatic carbinolic carbon. Therefore, this compound was identified as methyl 8-hydroxy-2,2-dimethyl-2H-chromene-6-carboxylate.<sup>9</sup>

The <sup>1</sup>H NMR spectrum of **5** showed two triplets at δ 3.02 (*J* = 7.3 Hz, 2H) and 3.40 (*J* = 7.3 Hz, 2H). These signals associated to multiplet at δ 7.1 – 7.3 (5H) and a peak at δ 5.91 (2H) suggested the occurrence of a dihydrochalcone. Signals referring to carbonyl group at δ 204.5 (C=O), aromatic group at δ 94.5 – 165.6 despite of aliphatic carbons at δ 45.6 (CH<sub>2</sub>), 30.5 (CH<sub>2</sub>) and 55.5 (CH<sub>3</sub>), in the <sup>13</sup>C NMR spectra (BBD and DEPT 135°), confirms the structural arrangement of **5** as 2',6'-dihydroxy-4'-methoxydihydrochalcone.<sup>10</sup>

In the both <sup>1</sup>H NMR spectra of **6** and **7** were observed AB systems at δ 6.95 (d, *J* = 8.7 Hz) and 7.37 (d, *J* = 8.7 Hz), characteristic of *para*-disubstituted aromatic ring, and two doublets at δ 6.02 (*J* = 2.4 Hz, 1H) and 6.10 (*J* = 2.4 Hz, 1H) indicative of a 1,2,3,5-tetrasubstituted aromatic ring. These data, associated to the signals at δ 5.33 (dd, *J* = 13.0 and 2.9 Hz, H-2), 2.75 (dd, *J* = 17.0 and 2.9 Hz, H-3a), 3.07 (dd, *J* = 17.0 and 13.0 Hz, H-3b) and δ 12.6 (s, hydroxyl group chelated to carbonyl group) are characteristic of 5-hydroxy-flavanone derivatives.<sup>11</sup> LREIMS analysis showed molecular ion-peaks at *m/z* 286 (C<sub>16</sub>H<sub>14</sub>O<sub>5</sub> - compound **6**) and 300 (C<sub>17</sub>H<sub>16</sub>O<sub>5</sub> - compound **7**). These spectra showed also RDA fragmentation ion peaks at *m/z* 166 to both derivatives and peaks at *m/z* 120 and *m/z* 134 to **6** and **7**, respectively. This analysis confirms the presence of an additional methoxyl group at C-4' in **7**. Finally, compounds **6** and **7** were identified as 7-methoxy-5,4'-dihydroxy-flavanone and 7,4'-dimethoxy-5-hydroxy-flavanone, respectively.<sup>11-13</sup>

The occurrence of chromenes was described to *P. aduncum*,<sup>2,7,9,14</sup> *P. taboganum*,<sup>15</sup> *P. dilatatum*,<sup>16</sup> and *P. lhotzkyanum*,<sup>2</sup> while the occurrence of flavanones was reported to *P. hostmanianum*,<sup>6,17</sup> *P. hispidum*,<sup>18-20</sup> *P. carniconnectivum*,<sup>21</sup> *P. aduncum*,<sup>19,20,22</sup> *P. fadyenii*,<sup>20</sup> *P. steerni*,<sup>17</sup> *P. crassinervium*,<sup>4,23</sup> *P. lhotzkyanum*,<sup>3</sup> and *P. methysticum*.<sup>24,25</sup> Despite the high distribution of flavanones<sup>26</sup>, the presence of dihydrochalcones in the genus *Piper* has been restricted to *P. aduncum*,<sup>10,18-20,27-29</sup> *P. fadyenii*,<sup>20</sup> and *P. hispidum*.<sup>18-20</sup>

All of the isolated chromenes, dihydrochalcone, and flavanones are being reported here for the first time in *P. mollicomum*, since no phytochemical studies have previously been conducted with this species. Otherwise, although chromenes and flavonoids have previously been reported in *P. lhotzkyanum*,<sup>3,4</sup> the occurrence of **4** and **7** has been described at first time in this species.

The minimum quantity of compounds **1** – **7** necessary to inhibit growth of *Cladosporium cladosporioides* and *C. sphaerospermum* by means of direct bioautography assay,<sup>4,29</sup> showed that **3**, **4**, **5** and **6** were the most active (Table 1). As observed to **1** – **4**, the esterification of carboxyl group led to a decrease of the fungitoxic activity. Additional 4'-methyl-3'-pentenyl group in **3**, in comparison to **4**, did not modify the antifungal potential while the presence of a hydroxyl group at C-8 in **2**, in comparison to **1**, showed an enhancement of this potential. As showed in Table 1, the minimal amount of **6** to inhibit the growth both fungi were 1.0 µg, while to **7**, which differ by the presence of an additional methyl group in B ring, these values decreased to 25.0 µg to both fungi. These observations suggest important relationships between the structure of chromene or flavanone derivatives and the antifungal activity.

**Table 1.** Antifungal activity of compounds **1** – **7** against *Cladosporium cladosporioides* and *C. sphaerospermum*

Compound	Antifungal activity* (µg)	
	<i>C. cladosporioides</i>	<i>C. sphaerospermum</i>
<b>1</b>	50.0	50.0
<b>2</b>	10.0	10.0
<b>3</b>	5.0	5.0
<b>4</b>	5.0	5.0
<b>5</b>	5.0	10.0
<b>6</b>	1.0	1.0
<b>7</b>	25.0	25.0
Nystatin	1.0	1.0
Miconazole	1.0	1.0

\*minimum amount required for the inhibition of fungal growth on thin-layer chromatographic plates (TLC).

## EXPERIMENTAL

### General procedures

Silica gel (Merck, 230-400 mesh) and Sephadex LH-20 (Sigma) were employed in the CC separations, whilst analytical TLC was performed using silica gel 60 PF<sub>254</sub> layers (Merck). <sup>1</sup>H and <sup>13</sup>C NMR spectra (BBD – broad band decoupled and DEPT 135° - distortionless enhancement by polarization transfer) were measured at 300 and 75 MHz, respectively, on a Bruker model DPX-300 spectrometer with samples dissolved in CDCl<sub>3</sub> (Aldrich). TMS was employed as internal standard: chemical shifts were recorded in δ (ppm) and coupling constants (*J*) in Hz. LREIMS (low resolution electronic impact mass spectrometry) spectra were measured at 70 eV on Finnegan-Mat INCOS 50 quadrupole spectrometer.

### Plant material

The plant material were collected at Ubatuba – SP, Brazil, on September, 2002 (*Piper mollicomum*) and at Poços de Caldas – MG, Brazil, on May, 2002 (*Piper lhotzkyanum*) and were identified by Dr. E. F. Guimarães (Jardim Botânico, Rio de Janeiro, Brazil). Voucher specimens (Kato-301 and Kato-226, respectively) have been deposited at the Herbarium of the Instituto de Botânica (SMA-SP).

### Extraction and isolation

Dried and powdered leaves of *P. mollicomum* (54 g) were extracted by maceration with MeOH (3 x 1 L) at room temperature. The resulting solutions were concentrated *in vacuo* to yield a crude extract (1.6 g) which was evaluated to detection of antifungal potential. As this extract showed activity, it was subjected to column chromatography over silica gel (gradient of hexane to EtOAc and from EtOAc to MeOH) yielding six fractions, in which fractions 2, 4 and 5 showed antifungal potential. Compound **1** was isolated in pure form from fraction 2 (16 mg). Fraction 4 was further chromatographed on a silica gel column using gradient mixtures of EtOAc in hexane yielding three sub-fractions (I-III). Sub-fraction II (10 mg) was composed by pure **2**. Fraction 5 was subjected to column chromatography over silica gel (gradient of EtOAc in hexane and MeOH in EtOAc) yielding three sub-fractions (I-III). Compound **5** was isolated from active sub-fraction II (89 mg). The <sup>1</sup>H NMR spectra of the remaining fractions showed a predominance of fatty material.

Dried and powdered leaves of *P. lhotzkyanum* (112 g) were extracted with MeOH (3 X 1.5 L) at room temperature to afford a

crude extract (5.2 g). This extract showed antifungal potential and was partitioned between MeOH/H<sub>2</sub>O (1:4) and CH<sub>2</sub>Cl<sub>2</sub> to give apolar phase (1.4 g). NMR analysis of this phase indicated a predominance of fatty material. The hydro-methanol solution was partitioned with EtOAc to afford an EtOAc phase (2.3 g). After evaluation of antifungal potential, which was detected in the EtOAc phase, this was subjected to column chromatography over silica gel (gradient of hexane to EtOAc and from EtOAc to MeOH) yielding five fractions. As the antifungal potential was detected in fraction 4 (490 mg), it was chromatographed on Sephadex LH-20 column, using MeOH as eluent to afford **3** (12 mg), **4** (23 mg), **6** (18 mg) and **7** (38 mg).

### Antifungal assay

The fungi used in this bioautographic assay *Cladosporium cladosporioides* and *C. sphaerospermum* have been maintained at the Instituto de Botânica, São Paulo – SP. Solutions corresponding to 100.0, 50.0, 25.0, 10.0, 5.0, 1.0 µg of pure compounds were applied to TLC plates which were sprayed with a spore suspension of fungi. After incubation for 48h in darkness in a moistened chamber at 25 °C, a clear inhibition zone appeared to indicate minimal amount of tested compounds.<sup>8,29</sup>

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