

## Divergence of Secondary Metabolism in Cell Suspension Cultures and Differentiated Plants of *Piper cernuum* and *P. crassinervium*

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Os principais metabólitos secundários das folhas de *Piper cernuum* são derivados de ácidos cinâmico e di-hidrocinâmicos, além da lignana cubebina. No caso de *P. crassinervium*, há um predomínio de flavonóides, ácidos benzóicos e hidroquinonas preniladas. As culturas celulares de *P. cernuum* produziram as feniletilaminas dopamina e tiramina enquanto que, no caso de *P. crassinervium*, foram isoladas quatro alcâmidas, incluindo as novas 2,3,4-trimetoxi-*N*-metil-aristolactama e 3-hidroxi-2-metoxi-*N*-metil-aristolactama.

The secondary metabolism in the leaves of *P. cernuum* produces cinnamic and dihydrocinnamic acid derivatives and the lignan cubebin. In case of *P. crassinervium* flavonoids and prenylated hydroquinones were characterized as major compounds. The cell cultures showed the production of the phenylethylamines dopamine and tyramine in *P. cernuum*, while in case of *P. crassinervium* four alkamides were isolated as major compounds, including the new 2,3,4-trimethoxy-*N*-methyl-aristolactam and 3-hydroxy-2-methoxy-*N*-methyl-aristolactam.

**Keywords:** Piperaceae, *P. cernuum*, *Piper crassinervium*, cell suspension, dihydrocinnamic acid derivatives, cubebin, tyramine, dopamine, aristolactams

### Introduction

The *Piper* species are mostly tropical plants of worldwide occurrence and are represented by ca. 700 species.<sup>1</sup> Their phytochemical investigation have led to the isolation of a number of physiologically active compounds *viz.* alkaloids/amides, propenylphenols, piperolides, chalcones, dihydrochalcones, flavonoids, prenylated benzoic acid derivatives and lignan/neolignans.<sup>2-12</sup> *Piper cernuum* Vell., an imposing, very large-leave shrub up to 6 m in height, is widely spread in Amazon, South-eastern and North-eastern regions of Brazil.<sup>13</sup> *Piper crassinervium* is a shrub frequently found in Atlantic Forest (Brazil), besides Peru, Colombia and Ecuador.<sup>13</sup> Although there is no report for medicinal use of *P. cernuum* and *P. crassinervium* their phytochemical investigation were undertaken as part of our study of Piperaceae species looking at the search for bioactive compounds, chemosystematic study and also the study of

regulatory process of the secondary metabolism that takes place during the differentiation process.

The regeneration or micropropagation of *Piper* species have been found in literature only for few species such as *P. longum*,<sup>14-16</sup> *P. colubrinum*,<sup>17</sup> and *P. nigrum*.<sup>18</sup> Nevertheless, the establishment of cell suspension cultures and investigation of associated secondary metabolism in Piperaceae species have so far remained untouched. Previous analysis of leaves of *P. crassinervium* have showed the occurrence of antifungal flavanones, benzoic acids and prenylated hydroquinones.<sup>10,19</sup> The only studies carried out on *P. cernuum* were addressed to investigate the composition of its essential oil<sup>20,21</sup> and its antimicrobial activity.<sup>22</sup> Herein we wish to describe the occurrence of four cinnamic acid derivatives (**1-4**) together the lignan cubebin (**5**) in the leaves of *P. cernuum*. Additionally, its callus and suspension cultures were showed to contain phenylethylamines tyramine (**6**) and dopamine (**7**) as major compounds. In case of *P. crassinervium* the major compounds were identified as the aristolactams (**8-11**), including the new natural product 2,3,4-trimethoxy-*N*-

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methyl-aristolactam (**10**) and 3-hydroxy-3-methoxy-*N*-methyl-aristolactam (**11**).

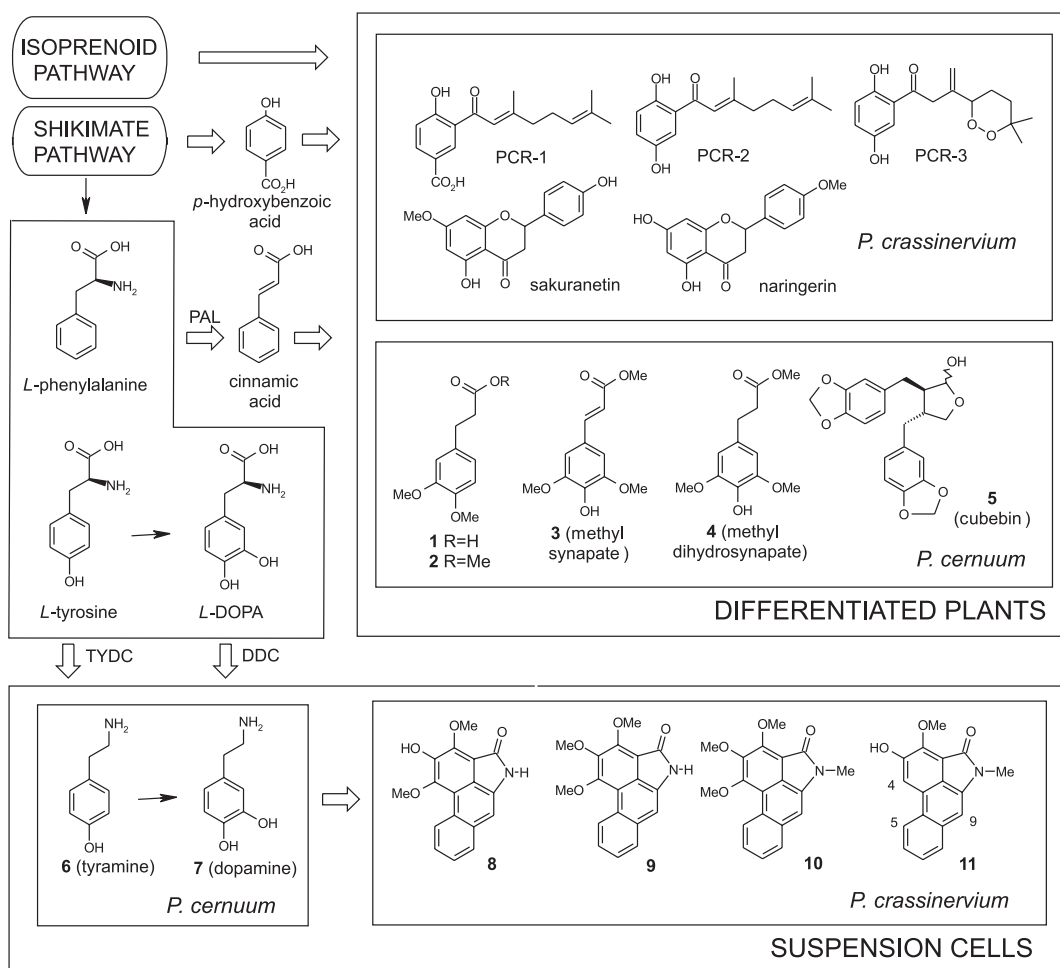
## Results and Discussion

Major secondary compounds in leaves of *P. cernuum* were isolated by chromatographic fractionation and were identified as methyl 3,4-dimethoxy-dihydrocinnamate (**1**),<sup>6</sup> 3,4-dimethoxy-dihydrocinnamic acid (**2**),<sup>6</sup> methyl 3,5-dimethoxy-4-hydroxy-dihydrocinnamate (methyl dihydrosynapate, **3**)<sup>23</sup> and methyl 3,5-dimethoxy-4-hydroxy-cinnamate (methyl sinapate, **4**).<sup>24</sup> Cubebin (**5**)<sup>3</sup> was identified as the sole lignan (Figure 1). The occurrence of dihydrocinnamic acid derivatives, have been previously recorded from other *Piper* species such as in *P. lhotzkyanum*<sup>6</sup> while cubebin is quite common.<sup>3</sup>

The callus cultures of *P. cernuum* was initiated from leaves explants in MS media supplemented with 2,4-D (0.2 mg mL<sup>-1</sup>) and GA<sub>3</sub> (0.5 mg mL<sup>-1</sup>) as growth regulators. In case of *P. crassinervium* the best callus initiation was

obtained from shoots using ANA (1.0 mg mL<sup>-1</sup>) and BAP (0.2 mg mL<sup>-1</sup>). In both cases the cell suspension developed nicely in liquid media having the same nutrients composition and growth regulators and reached a maximum growth at 26/34 days (Figures 2 and 3), respectively.

HPLC analysis and <sup>1</sup>H NMR of extracts from callus and cell suspension cultures (cells and media) of *P. cernuum* showed the presence of two major compounds that were isolated and identified as tyramine (**6**)<sup>25</sup> and dopamine (**7**).<sup>3</sup> Their content as dansyl derivatives was determined during the cell growth and increased along the development (Figure 4). Dopamine has been previously identified in *Piper amalago*<sup>3</sup> and also in suspension cultures of *Mucuna pruriens*.<sup>26</sup> The analysis of the extracts by HPLC indicated that no other alkaloids was produced either in cell, excreted into the liquid media or in the differentiated tissues of the *P. cernuum* plants. Both phenylethylamines have been frequently found in plants as conjugates such as *N-trans* (-coumaroyl)-tyramine in *P. sanctum*,<sup>27</sup> as *N-feruloyl*-tyramines in *P. argyrophyllum*,<sup>28</sup> and as *N-benzoyl*tyramine



**Figure 1.** Major secondary metabolites in differentiated tissues and suspension cells of *P. cernuum* and *P. crassinervium* and their biosynthetic relationship. PAL: phenylalanine ammonia lyase; DDC: dopamine decarboxylase; TYDC: tyrosine decarboxylase; isolated compounds: **1-11** sakuranetin, naringerin, PCR1-3.

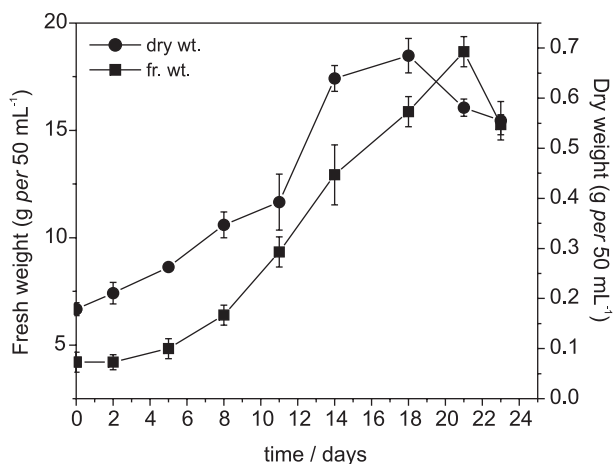


Figure 2. Growth cycle of *P. cernuum* cell suspension.

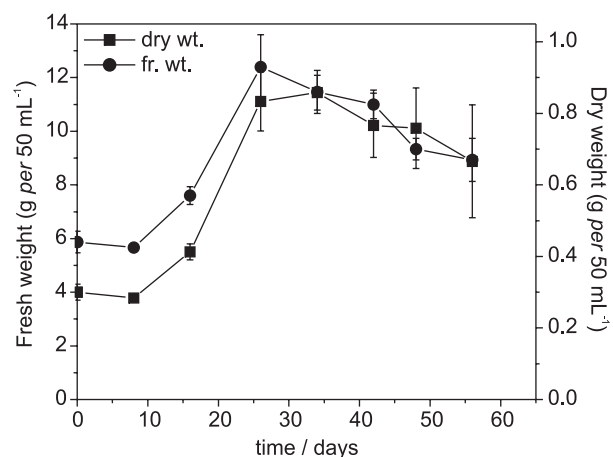


Figure 3. Growth cycle of *P. crassinervium* cell suspension.

in *Aniba riparia*.<sup>25</sup> Tyramine amide is wound inducible phytoalexin on tobacco (*Nicotiana tabacum*)<sup>29</sup> and is directly involved in the suberization process of wounded potato (*Solanum tuberosum*).<sup>30</sup> Together dopamine are important intermediates in the biosynthesis of benzylisoquinoline alkaloids.<sup>31</sup>

The extract from cell suspensions of *Piper crassinervium* was analyzed by <sup>1</sup>H NMR whose signals showed the presence of several methoxyl and aromatic hydrogens. The extract was fractionated by several chromatographic steps yielding four compounds (8-11). Compounds 8 and 9 were identified as goniopedaline C and piperolactam C, which were previously isolated from *Goniothalamus sesquipedalis*<sup>32</sup> and *P. argyrophyllum*, *P. boehimerifolium*, *P. longum*, *P. wightii* besides *P. acutisleginum*,<sup>33</sup> respectively.

Compound 10 was showed to be has similar spectrometric data as compound 9, but was recognized as its *N*-methyl derivative since the <sup>1</sup>H and <sup>13</sup>C NMR data showed a singlet at  $\delta$  3.48 and 26.4, respectively. The molecular ion in the mass spectrum at  $m/z$  323 Da was compatible with the molecular formula C<sub>19</sub>H<sub>17</sub>NO<sub>4</sub>.

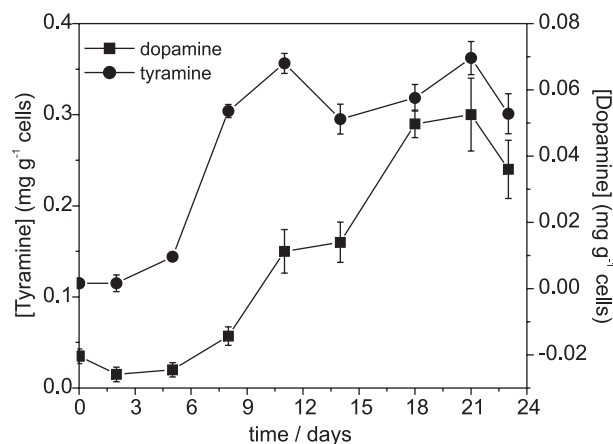


Figure 4. Changes in the content of tyramine and dopamine (determined as dansyl derivatives) during the growth cycle of *P. cernuum* cell suspension.

All <sup>13</sup>C NMR data could be assigned based on the similarities with 9, thus 10 could be determined as 2,3,4-trimethoxy-*N*-methyl-aristolactam.

Compound 11 showed a molecular ion at 279 Da. Its <sup>1</sup>H NMR showed a *N*-methyl signal at 3.49 and a methoxyl group at 4.13 and could be determined as *N*-methyl-3-methyl-cepharanone B by comparison of spectrometric data<sup>31</sup> and it has not been previously described elsewhere. The determination of two major aristolactams in the suspension cells during the growth curve showed that 9 is converted to its *N*-methyl derivative 10 (Figure 5).

Such type of alkaloids groups are relatively restrict in plant kingdom and has been found in Aristolochiaceae, Piperaceae, Annonaceae, Menispermaceae and Monimiaceae species.<sup>34</sup>

The production of phenylethylamines or aristolactams in cell suspension cultures of *P. cernuum* and *P. crassinervium*, respectively, is a metabolic drift that requires the predominance of tyrosine decarboxylase (TYDC) over

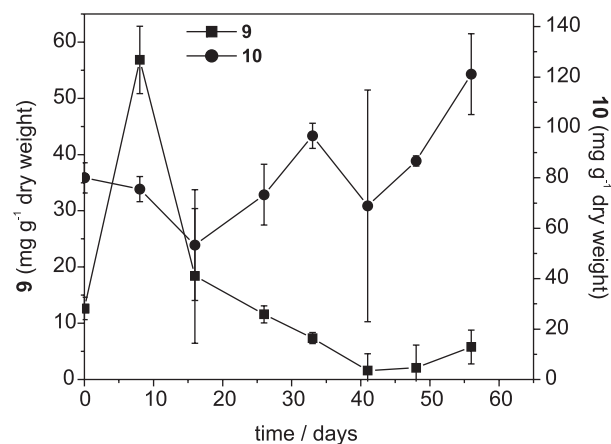


Figure 5. Changes in the content of aristolactams 9 and 10 during the growth of cell suspension of *P. crassinervium*.

phenylalanine ammonia lyase (PAL). This branch point in the differentiated tissue is followed by additional steps of general phenylpropanoid pathway including the oxidation and methylations steps and/or hydrogenation yielding the cinnamic and dihydrocinnamic acid derivatives in *P. cernuum*. Since only one lignan (cubebin) was isolated, further reductions of cinnamoyl-S-CoA esters to the cinnamyl alcohols followed by oxidative coupling to yield lignans are not fully expressed such as in other *Piper* species.<sup>3</sup> Such divergence was also observed in suspension cells of *P. crassinervium*, but in this case, phenylethylamines are supposedly consumed in the formation of aristolactams **8-11**. Apparently, the Mannich condensation is the limiting step in *P. cernuum* and no aristolactams could be detected in its suspension cells. The modulation of alkaloid metabolism in plant suspension culture of alkaloid-free species have been previously observed.<sup>35</sup>

## Experimental

### Instrumental and chromatographic materials

Silica gel (Merck 230-400 mesh) and Sephadex LH-20 (Pharmacia) were used for column chromatographic separation. Silica gel PF254 (Merck) was used to TLC preparative purification. The NMR spectra (300 and 200 MHz) and <sup>13</sup>C NMR (75 and 50 MHz) were recorded on Bruker DRX-300 using CDCl<sub>3</sub> as solvents or otherwise stated and TMS as internal standard. IR spectra were obtained on a FT-IT 510 Nicolet spectrometer. LREIMS were measured at 70 eV on a HP 5990/5988A spectrometer. IR: KBr; EIMS 70 eV. HPLC analysis was performed on a HP-1050 using C-18 Econosil, (Alltech) (5 μm, 250 X 4.6 mm column with UV detection at 254 nm.

### Plant material

The specimens of *P. cernuum* and *P. crassinervium* were collected in February 1998, in Núcleo de Picinguaba (Ubatuba), São Paulo, Brazil. Voucher specimens (Cordeiro-1888 and Kato-0084) are deposited at Herbarium of Instituto de Botânica, São Paulo – SP, Brazil.

### Isolation of secondary compounds from leaves of *P. cernuum*

Air-dried and powdered leaves (942 g) were extracted with MeOH. Removal of residual solvent under reduced pressure furnished 137.8 g of crude extract. Around 90 g of this extract was suspended in MeOH:H<sub>2</sub>O (20%) and then filtered through celite. The filtered soln was extracted

with hexane and then with CH<sub>2</sub>Cl<sub>2</sub>, yielding CH<sub>2</sub>Cl<sub>2</sub> extract (8.4 g). Part of this fraction (3.0 g) was submitted to silica gel (260 g) CC. The column was eluted with CHCl<sub>3</sub> and CHCl<sub>3</sub>-MeOH 99:01. Frs. 6 to 12 (195 mg) were pooled and further purified by silica gel (20 g) CC with hex-EtOAc gradient furnishing **1** (80 mg). From the first column, frs. 14-15 (378 mg) were combined and submitted into silica gel (40 g) CC eluted with hex-EtOAc gradient. The resulting frs. 17-27 (74.8 mg) were subsequently purified by prepared TLC with CHCl<sub>3</sub>-MeOH (99:01) and yielded **2** (8 mg), **4** (17.0 mg), **3** (5.6 mg) and **5** (5 mg).

*3,4-Dimethoxy-dihydrocinnamic acid (1)*. Amorphous solid C<sub>12</sub>H<sub>16</sub>O<sub>4</sub>; IR ν<sub>max</sub>/cm<sup>-1</sup>: 3500-2500, 1701, 1505, 1464. LREIMS *m/z* (rel. int.): 210 M<sup>+</sup> (25), 151 (100), 107 (14), 91 (14), 77 (20).

*Methyl 3,4-dimethoxy-dihydrocinnamate (2)*. Amorphous solid C<sub>12</sub>H<sub>16</sub>O<sub>4</sub>; IR ν<sub>max</sub>/cm<sup>-1</sup>: 1736, 1518, 1465, 3000, 2952, 2836. LREIMS *m/z* (rel. int.): 224 M<sup>+</sup> (25), 151 (100), 121 (9), 91 (16), 77 (20).

*Methyl 3,5-dimethoxy-4-hydroxy-dihydrocinnamate (methyl dihydrosinapate) (3)*. IR ν<sub>max</sub>/cm<sup>-1</sup>: 1730, 2947, 2843, 1571, 1432. LREIMS *m/z* (rel. int.): 238 M<sup>+</sup> (39), 167 (100), 134 (10), 121 (9), 77 (15).

*Methyl 3,5-dimethoxy-4-hydroxy-cinnamate (methyl sinapate) (4)*. IR ν<sub>max</sub>/cm<sup>-1</sup>: 1730, 1571, 1432. LREIMS *m/z* (rel. int.): 240 M<sup>+</sup> (39), 207 (8), 167 (100), 134 (10), 121 (9), 77 (15).

*(-)-Cubebin (5)*. [α]<sub>D</sub><sup>27</sup>-78° (CHCl<sub>3</sub>; c 0.2). <sup>1</sup>H NMR: δ 6.47-6.50 (m, 6H, ArH), 5.90 (s, 2H, O<sub>2</sub>CH<sub>2</sub>), 5.23 (brs, 1H, H9), 3.82-3.55 (m, 2H, H9'), 2.64-2.40 (m, 4H, 2H7, 2H6), 2.20-2.10 (m, 2H, H8, H8').

### Cell suspension cultures of *P. cernuum*

Leaves obtained from seedlings growing in basic medium Murashigue and Skoog (MS)<sup>36</sup> were used as explants to initiate callus culture in solid medium containing MS (10.0 g L<sup>-1</sup>) supplemented with myo-inositol (100.0 mg L<sup>-1</sup>), thiamine hydrochloride (1.0 mg L<sup>-1</sup>), antioxidant mixture (Sigma A 1179, 50.0 mg L<sup>-1</sup>), sucrose (2%), 2,4-D (0.2 mg L<sup>-1</sup>), gibberellic acid GA<sub>3</sub> (0.5 mg L<sup>-1</sup>) and agar (0.17%). The petri dishes were maintained at 26 °C under photoperiod of 16/8 h. The callus thus obtained were subcultivated every three weeks and transferred to the liquid medium containing MS (10.0 g L<sup>-1</sup>), myo-inositol (100.0 mg L<sup>-1</sup>), thiamine hydrochloride

(1.0 mg L<sup>-1</sup>), antioxidant mixture (50.0 mg L<sup>-1</sup>), sucrose (3%), 2,4-D (0.2 mg L<sup>-1</sup>), GA<sub>3</sub> (0.5 mg L<sup>-1</sup>), pyridoxine hydrochloride (0.5 mg L<sup>-1</sup>), nicotinic acid (0.5 mg L<sup>-1</sup>), glycine (2.0 mg L<sup>-1</sup>), casein enzymatic hydrolysate (250.0 mg L<sup>-1</sup>). Cell suspensions were established after 2 or 4 subcultures and have been subcultivated every three weeks.

#### *Isolations of secondary compounds from cells of P. crassinervium*

30.0 g of fresh cells were extracted with MeOH yielding 170 mg of crude extract. The extract was suspended in H<sub>2</sub>O and extracted with *n*-BuOH. The *n*-BuOH fraction (82.3 mg) was dissolved in MeOH and filtered over silanized silica gel to retain the apolar compounds and the yielding fraction (46 mg) was subjected to prepared TLC with CHCl<sub>3</sub>-MeOH (7:3) yielding **6** (23 mg) and **7** (15 mg).

*Tyramine (6)*. LREIMS *m/z* (rel. int.): 137 (21), 107 (75), 91 (15), 77 (100), 51 (24). <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD): δ 7.02 (2H, d, 8.5, H2, H6), 6.68 (2H, d, 8.5, H3, H5), 2.90 (2H, t, 6.5, H7), 2.70 (2H, t, 6.5, H8). <sup>13</sup>C NMR (CD<sub>3</sub>OD): δ 128.5 (C1), 130.8 (C2), 116.70 (C3), 157.7 (C-4), 116.7 (C-5), 130.8 (C-6), 33.89 (C-6), 42.3 (C-7).

*Dopamine (7)*. <sup>1</sup>H NMR (300 MHz, DMSO): δ 6.65 (1H, d, *J*=8.5, H5), 6.59 (1H, d, *J*=2.1, H2), 6.47 (1H, dd, *J*=2.1, 8.5, H6), 2.90 (2H, t, 6.5, H7), 2.78 (2H, t, 6.5, H8).

#### *Quantification of tyramine and dopamine*

2.0 g of fresh cells were extracted and derivatized to dansyl derivatives according to procedure described<sup>37</sup> but with dihydrochloride putrescine as internal standard. The dansyl derivatives were determined by HPLC as described.<sup>38</sup> Standard curves of tyramine and dopamine hydrochloride (Sigma) were prepared similarly.

#### *Cell suspension cultures of P. crassinervium*

Its seedlings were cultivated in Gamborg B5 media supplemented with mio-inositol (100 mg L<sup>-1</sup>), thiamine (1 mg L<sup>-1</sup>), sucrose (30 g L<sup>-1</sup>), indolacetic acid (AIA) (0.02 mg L<sup>-1</sup>), AG<sub>3</sub> (0.02 mg L<sup>-1</sup>) and agar (0.7%). The shoots (*ca.* 1 cm) were inoculated in Gamborg B5 media supplemented similarly as before but supplemented with ANA, (1.0 mg L<sup>-1</sup>), BAP (0.2 mg L<sup>-1</sup>), and 0.7% agar. The subculturing of the callus was carried out by regular intervals of 20 days. Cell suspensions cultures of *P. crassinervium* were established by transferring friable

callus to the same basal supplemented medium but without addition of agar. The subculturing was performed at intervals of 15-20 days by 2-fold dilution of the cells into fresh medium.

Cell suspensions of 15-20 days of age were used for determination of growth curves. In 50 mL flasks contained the medium prepared for the cell suspensions, were inoculated 5 g of fresh weight of *P. crassinervium* cells. This procedure was performed in triplicate. The cells were separated from cell suspensions by *vacuum* filtration and had their fresh weight determined. Then cells were kept at 50 °C, until constant weight.

#### *Isolation of aristolactams from cells of P. crassinervium*

Cells (100 g of fresh cell) obtained of cell suspensions by *vacuum* filtration, were extracted with MeOH under sonication bath (15 min). After elimination of solvent the residue was submitted to EtOAc partition, and concentrated under *vacuum* to yield the cell extract (40 mg). The extract was purified by preparative TLC (hexane: EtOAc 7:3) to yield four fractions. Fraction 2 was submitted to preparative TLC (CHCl<sub>3</sub>:MeOH 9:1), then by HPLC using a RP-18 column eluted with a gradient MeOH:H<sub>2</sub>O, yielding the aristolactams **8** (1.0 mg) and **11** (1.1 mg). Fractions 3 and 4 consisted of the pure aristolactams **9** (4.1 mg) and **10** (5.3 mg).

*3-Hydroxy-2,4-dimethoxy-aristolactam (goniopetaline, 8)*. LREIMS *m/z* (rel. int.): 295 (83), 280 (48), 266 (12), 252 (26), 237 (10), 209 (17), 164 (10), 153 (22). <sup>1</sup>H NMR: δ 9.19 (m, H5), 7.55 (m, 2H, H6, H7), 7.84 (m, H8), 7.19 (s, H9), 4.54 (s, MeO-2), 4.07 (s, MeO-4).

*2,3,4-Trimethoxy-aristolactam (piperolactam C, 9)*. <sup>1</sup>H NMR: δ 9.19 (m, H5), 7.52-7.58 (m, 2H, H6, H7), 7.82 (m, 9), 4.52 (s, 3H, MeO-2), 4.20(s, 3H, MeO-3) 4.00 (s, 3H, MeO-4). <sup>13</sup>C NMR: δ 167.2 (C1), 157.3 (C2), 154.3 (C3), 146.4 (C4), 116.5 (C4a), 126.6 (C4b, C5), 125.9 (C6), 126.8 (C7), 128.7 (C8), 133.3 (C8a), 106.1 (C9), 63.0 (MeO-2), 61.6, (MeO-3), 60.9 (MeO-4).

*2,3,4-Trimethoxy-N-methyl-aristolactam (10)*. LREIMS *m/z* (rel int): 323 (100), 308 (42), 265 (56), 250 (33), 194 (10), 139 (15). <sup>1</sup>H NMR: δ 9.18 (m, H5), 7.54 (m, 2H, H6, H7), 7.85 (m, H8), 7.00 (s, H9), 4.54 (s, MeO-2), 4.18 (s, MeO-3), 3.99 (s, MeO-4), 3.49 (s, MeN). <sup>13</sup>C NMR: δ 165.7 (C1), 156.8 (C2), 154.0 (C3), 146.4 (C4), 116.1 (C4a), 126.6 (C4b), 126.5 (C5), 125.7 (C6), 127.0 (C7), 128.7 (C8), 133.4 (C8a), 104.5 (C9), 63.0 (MeO-2), 61.6, (MeO-3), 60.8 (MeO-4), 26.4 (Me-N).

3-Hydroxy-3-methoxy-N-methyl-aristolactam (**II**). LREIMS *m/z* (rel int.): 279 (100), 264 (10), 236 (16), 221(10), 209 (11), 193 (18), 181 (15), 164 (23). <sup>1</sup>H NMR: δ 7.82 (s, H4), 7.82 (s, H4), 9.24 (m, H5), 7.56 (m, 2H, H6, H7), 7.81 (m, H8), 7.07 (s, H9), 4.13 (s, MeO-2), 3.49 (s, MeN).

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