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Article

Piperamides from *Piper ottonoides* by NMR and GC-MS Based Mixture Analysis

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The species *Piper ottonoides* Yuncker (Piperaceae), known as "joão-brandim", is a shrub that occurs in the Brazilian Amazon rainforest. Its roots and leaves are used in traditional medicine as local anesthetic to treat toothache and sore throat. In this study, the structural characterization in mixture of isobutyl amides present in semi-purified fractions from fruits, leaves, stems and roots of *P. ottonoides* was achieved by employing gas chromatography-mass spectrometry (GC-MS) and a combination of nuclear magnetic resonance (NMR) techniques (^{1}H, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, TOCSY and ^{1}H-^{1}H *J*-RES). The MS fragmentation patterns and the NMR chemical shifts, multiplicities, coupling constants and the signal correlations in the two-dimensional spectra were carefully analyzed also taking into account some key elements of differentiation among the compounds. The data set allowed identifying unequivocally the new amide *N*-isobutyl-7-(4'-methoxyphenyl)-2*E*,4*E*-heptadienamide, which we named ottonoidenamide as well as piperovatine and chingchengenamide A in all parts of the plant, the additional presence of pipercallosine and pipercallosidine in roots and of dihydropiperlonguminine in fruits and leaves. The presence of piperovatine and pipercallosine in *P. ottonoides* should be associated to its traditional use.

Keywords: *Piper ottonoides*, Piperaceae, isobutyl piperamides, mixture analysis

Introduction

The botanical family Piperaceae currently contains five genera: *Manekia* Trel., *Peperomia* Ruiz & Pav., *Piper* L., *Verhuellia* Miq. and *Zippelia* Blume.[1] The genus *Piper* comprises approximately 1000 species of herbs, shrubs, small trees and vines, distributed among Asia, South Pacific and America,[2,3] which are present in the daily life of many people as medicines and food.[4-6] The chemical composition of the genus *Piper* has been widely investigated revealing amides, lignoids, chromenes, phenylpropanoids, pyrones, terpenoids, steroids and flavonoids.[4,7-10]

The species *Piper ottonoides* Yuncker, known in Brazil as "joão-brandim", "joão-brandinho" and "jamburandi", is a shrub that occurs in the Brazilian Amazon rainforest. Its roots and leaves are used in traditional medicine as local anesthetic to treat toothache and throat pain. The essential oil from its leaves contains mainly mono- and sesquiterpenoids[11] and the ethanol extracts from its leaves, stems and roots show toxicity against two insect caterpillars found in corn and soy crops.[12]

The structural elucidation of natural compounds traditionally involves the separation, purification and

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then the structural analysis of the pure compounds. This methodology is certainly important and has allowed producing an unprecedented knowledge about the chemical composition of plants and other organisms as well as the discovery of bioactive compounds. Nowadays, the accumulation of structural compound data and the improvement of analytical techniques have enabled a great progress in quickly and efficiently accessing the metabolite profiles and/or active principles from the exceptionally complex natural matrices. The advances in hyphenated analytical tools in combination with library databases have allowed access structural information and rapid identification of known compounds in different matrices.[13] On the other hand, nuclear magnetic resonance (NMR) spectroscopy can both be coupled to a separation technique and used to directly analyze compounds in mixture.[14,15]

The aim of this study was to unequivocally achieve the structural characterization of the piperamides present in *P. ottonoides* without their previous isolation by using integrative gas chromatography-mass spectrometry (GC-MS) and NMR techniques (^{1}H, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, TOCSY and *J*-RES). The approach has allowed characterizing in fruits, leaves, stems and roots of the species a new piperamide (**2**) and other five known piperamides (**1**, **3**-**6**). Their distribution in all parts of the plant was also visualized.

Experimental

General experimental procedures

The thin layer chromatography (TLC) analyses were performed in pre-coated silica gel 60 F254 (Merck), with mobile phase $CHCl_3$/EtOAc 7:3 v/v (previously optimized condition) and UV irradiation (254 nm) to visualize the spots. The column chromatography (CC) experiments were performed in silica gel 60 ASTM (Merck): 230-400 mesh for the root extract and 70-230 mesh for the fruit, leaf and stem extracts. The GC-MS analyses were carried out on a Shimadzu GC-2010 chromatograph coupled to a Shimadzu GCMS-QP2010s mass spectrometer fitted with a 30 m × 0.25 mm HP-5 ms column coated with 0.25 μm film thickness. Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1}, 1 μL of CH_2Cl_2 solutions (1 mg mL^{-1}) was injected into the injection port using a split ratio of 1:10. Compound separation was achieved following a linear temperature program of 100 °C (3 min), 100-280 °C (10 °C min^{-1}), 280 °C (10 min). MS parameters were: ionization voltage (EI) 70 eV, peak width 2 s, mass range 30-650 Daltons and detector voltage 1.5 V. The NIST05 MS database (National Institute of Standards and Technology, USA) was used when necessary. The 1D and 2D NMR spectra were recorded at 25 °C on a Varian VNMRS500 (500 MHz) spectrometer in 5 mm NMR tubes and 0.6 mL of $CDCl_3$ (Tedia) and tetramethylsilane (TMS) as internal reference. Chemical shifts (δ) were given in ppm and coupling constants (*J*) in Hz. All 2D experiments were performed using field gradient and the heretonuclear correlation experiments (HMBC and HSQC) with adiabatic pulses. The chemical shifts of the carbon atoms were taken from the HSQC and HMBC spectra. The spectra were processed with MestreNova v. 6.0.2 software. Positive ion electrospray high resolution mass spectral analysis (HRESITOF) was made with a Bruker Daltonics micrOTOF instrument (Bruker Co., Fremont, CA, USA).

Plant material

Piper ottonoides was collected from wild specimen at the Experimental Field of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Rio Branco, Acre State, Brazil. A voucher specimen was deposited at the Herbarium Bradeanum, Rio de Janeiro, RJ, Brazil, under number HB 91291.

Extraction and semi-purification procedures

Dried and sieved fruits (7.7 g), leaves (9.4 g), stems (302.0 g) and roots (80.8 g) of *P. ottonoides* were extracted with ultra-sound assistance at room temperature, using 1.0 g of plant material and 100 mL of MeOH (6×) by 10 min each cycle. The supernatants were pooled and the solvents removed under low pressure yielding 1.05, 0.56, 22.91 and 7.54 g of the dried extracts respectively. The extracts were submitted to CC as described: 1.2 g of the root extract in 4.5 cm e.d. × 15.0 cm phase height, eluted sequentially with $CHCl_3$:EtOAc 9:1, EtOAc and MeOH yielding 87 fractions; 13.2 g of the stem extract in 4.5 cm e.d. × 30.0 cm phase height, eluted sequentially with $CHCl_3$:EtOAc 9:1, EtOAc, EtOAc:MeOH (7:3 and 1:1) and MeOH yielding 34 fractions; 561.3 mg of the leaf extract in 3.0 cm e.d. × 15.0 cm phase height, eluted sequentially with hexane, hexane:$CHCl_3$ (7:3 and 3:7), $CHCl_3$, $CHCl_3$:EtOAc (9:1 and 1:1), EtOAc and MeOH yielding 36 fractions; 207.1 mg of the fruit extract in 2.0 cm e.d. × 12.0 cm phase height, eluted sequentially with $CHCl_3$:EtOAc 9:1, EtOAc, EtOAc:MeOH 1:1 and MeOH yielding 20 fractions. The fractions were pooled together by TLC similarities, resulting in: 10 root fractions; 8 stem fractions; 7 leaf fractions and 5 fruit fractions. The fractions: 4-7 from roots; 4-6 from stems; 2-6 from leaves and 1-3 from fruits, that showed by TLC major spots with high UV absorption at 254 and Rf ≠ 0 were selected to be

further screened by GC-MS. The fractions: 6 (16.9 mg) from roots, 5 (67.9 mg) from stems, 2 (23.0 mg) from fruits, all eluted from the CC with $CHCl_3$:EtOAc 9:1 and 6 (6.5 mg) from leaves, eluted with $CHCl_3$:EtOAc 1:1 were selected for NMR analyses.

N-isobutyl-7-(4'-methoxyphenyl)-2*E*, 4*E*-heptadienamide (**2**) (ottonoidenamide)

HRESIMS $[M + Na]^+$ *m/z* 310.1645 (calculated 287.1879 for $C_{18}H_{25}NO_2$); for ^1H and ^{13}C NMR spectroscopic data, see Table 2; for GC-EIMS data see peaks with RRt = 1.03 in Table 1.

Results and Discussion

Many species of Piperaceae associated with the traditional use as an anesthetic have been reported to contain piperamides.[16-22] As *P. ottonoides* has similar properties, it should be expected that piperamides would be found in this species. The use of normal phase silica gel CC to isolate the compounds present in the MeOH extracts of fruits, leaves, stems and roots of *P. ottonoides* led to some minor semi-purified fractions which showed by subsequent GC-MS analysis, odd molecular ions characteristic of amide group. However the small amounts of these fractions associated with the significant number and similar chromatographic behavior of their constituents, as revealed by the TLC and GC chromatograms, have motivated us to analyze them as a mixture.

GC-MS of the piperamide enriched fractions

One fraction from each part of the plant that revealed by GC-MS representative amide peaks and less fatty compounds (by comparison to NIST05 MS database) was selected for further analyses.

Various studies have reported the MS fragmentation patterns of piperamides. These compounds usually present, among others, fragment ions related to the cleavage at the C–N bond and, when applied, ions containing the aromatic ring.[6,21,23-28]

The set of GC-MS chromatograms showed six different peaks with MS patterns compatible to piperamides (Table 1). The analyses of their MS fragments showed ions at *m/z* $[M - 72]^+$ corresponding to $[M\text{-}NHCH_2CH(CH_3)_2]^+$ and $[M - 100]^+$ corresponding to $[M\text{-}CONHCH_2CH(CH_3)_2]^+$, characteristic of some isobutylamides.[21,25,28] These fragments were present in most of the MS compound profiles of the four studied fractions (Table 1), strongly suggesting the presence of this kind of piperamides in *P. ottonoides*. The ions at *m/z* 121 and 135 that have been related to methoxy and methylenedioxy substituted tropylium ions, respectively,[6,21,24,26] were observed as base peak in most of the compounds present in all the fractions (Table 1). The ion at *m/z* 135 has been observed in the MS of piperamides with methylenedioxy substituted aromatic ring bearing either a benzylic methylene carbon or a double bond with no extending conjugation.[6,21,26] However, for the latter option, together with the ion at *m/z* 135, the MS should contain an ion at *m/z* 161 attributed to a benzodioxole-allylic fragment.[6,23,26,27] This ion was not observed in the MS profiles of the compounds present in *P. ottonoides* suggesting the absence of piperamides with methylenedioxy substituted aromatic ring bearing a conjugated double bond.

The overall analyses of the MS profiles allowed suggesting the number of carbon atoms and unsaturation in the carbon chains of these compounds. The peaks with relative retention times (RRt) 1.00 and 1.03 (Table 1), *m/z* $[M]^+$ 273 and 287, respectively, present in all fractions, might correspond to isobutyl piperamides with methoxy group in the aromatic ring (*m/z* 121) and carbon chains with five and six carbons, respectively, both containing two unsaturations (*m/z* $[M - 72]^+$ 201 and 215; $[M - 100]^+$ 173 and 187). The peaks presenting *m/z* $[M]^+$ 275, 301, 303 and 329 (RRt = 0.95, 1.08-1.09, 1.04 and 1.21, respectively) (Table 1) might correspond to piperamides with methylenedioxy group in the aromatic ring (*m/z* 135). Two of them, that showed ions at *m/z* $[M]^+$ 303, $[M - 100]^+$ 203 and *m/z* $[M]^+$ 301, $[M - 100]^+$ 201, seemed to contain carbon chains with six carbons, differing by two mass units, thus suggesting that one has minus one double bond (Figure 1). The amide related to the peak with RRt = 1.21, *m/z* $[M]^+$ 329, $[M - 100]^+$ 229, found just in the roots of the species, showed 28 mass units more than that related to the peak with *m/z* $[M]^+$ 301, which might suggest a longer carbon chain. The search for matching compounds at the NIST05 MS database indicated that the peak with *m/z* M^+ 273 (RRt = 1.00) would possibly be piperovatine (**1**). These MS data gave strong structural indications of the piperamides present in the species but were not enough for an unequivocal identification of the compounds, specially taking into account the configuration of the double bonds.

NMR analyses in mixture of the piperamides

NMR analyses of root piperamides

Further study of root fraction by 1D and 2D NMR techniques revealed by the ^1H NMR spectrum data and the ^1H-^1H correlations in the COSY spectrum (Figure 1) the coupling between protons at δ 7.08 (d, *J* 8.2 Hz) and 6.84 (d, *J* 8.2 Hz) characteristic of a *p*-disubstituted aromatic ring

Table 1. GC-EIMS profiles and distribution of the piperamides from the different parts of *Piper ottonoides* - *m/z* (rel.ab.%)

Part of the plant	Compound	RRt	$[M]^+$	$[M + 1]^+$	$[M - 72]^+$	$[M - 100]^+$	Other ions
Root	**1**	1.00	273 (18.3)	274 (3.8)	201 (8.5)	173 (100)	121 (40.3)
	2	1.03	287 (2.3)	–	–	–	121 (100)
	5	1.04	303 (17.0)	–	–	203 (18.7)	135 (100)
	3	1.09	301 (3.5)	302 (0.8)	229 (0.8)	201 (1.2)	135 (100)
	4	1.21	329 (18.4)	330 (3.2)	257 (8.6)	229 (29.9)	135 (100)
Stem	**1**	1.00	273 (18.4)	–	–	173 (100)	121 (44.7)
	2	1.03	287 (2.1)	–	–	–	121 (100)
	3	1.08	301 (3.3)	302 (0.7)	229 (0.7)	201 (1.1)	135 (100)
Fruit	**6**	0.95	275 (4.0)	276 (0.8)	203 (2.1)	175 (5.0)	135 (100)
	1	1.00	273 (20.4)	274 (4.1)	201 (8.3)	173 (100)	121 (53.9)
	2	1.03	287 (2.0)	288 (0.4)	215 (0.3)	187 (0.5)	121 (100)
	5	1.04	303 (10.1)	–	–	203 (8.7)	135 (100)
	3	1.08	301 (3.3)	302 (0.6)	229 (0.6)	201 (1.0)	135 (100)
Leaf	**6**	0.95	275 (3.9)	276 (0.8)	203 (2.1)	175 (4.7)	135 (100)
	1	1.00	273 (14.7)	–	–	173 (44.5)	121 (42.6)
	2	1.03	287 (1.9)	–	–	–	121 (100)
	3	1.08	301 (3.2)	–	–	–	135 (100)

RRt: relative retention time to peak with *m/z* M^+ 273.

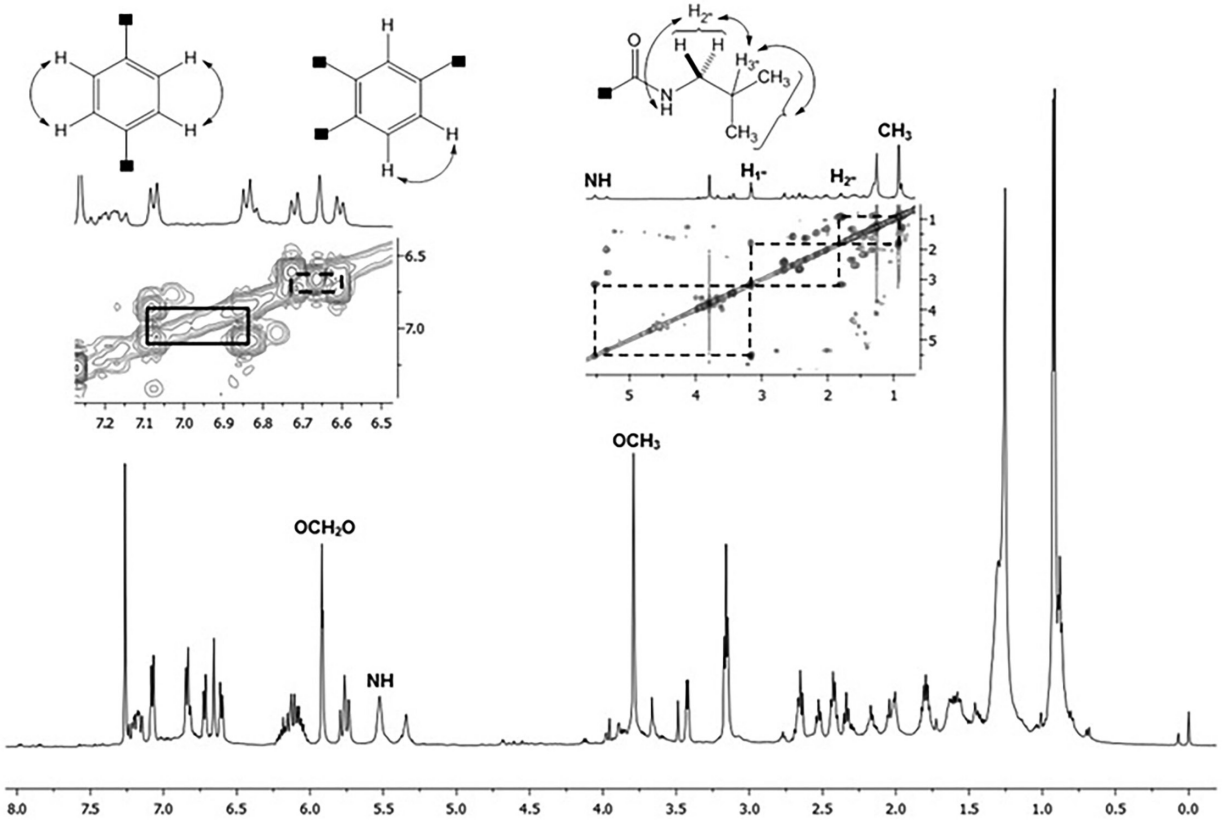


Figure 1. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) and selected correlations in the ^{1}H-^{1}H COSY spectrum of *Piper ottonoides* root fraction for characterization of the piperamide isobutyl group and the aromatic substituent patterns.

and a set of signals at δ 6.72 (d, *J* 7.8 Hz), 6.60 (d, *J* 7.8 Hz) and 6.66 (bs) characteristic of a trisubstituted aromatic ring. These data together with the singlets at δ 3.79 and 5.92, characteristic of methoxy and methylenedioxy groups, respectively, in combination with the described MS data confirmed the presence of *p*-methoxy and methylenedioxy aromatic substituted piperamide in this fraction. The amidic protons (δ 5.53, bs) were also revealed. The COSY

spectrum also confirmed the presence of an isobutyl group bearing to the amidic nitrogen through the sequential correlation among the signal at δ 5.53 with those at δ 3.16 (t, *J* 6.4 Hz), 1.80 (m) and 0.92 (d, *J* 6.5 Hz) (Figure 1).

The long range ^{1}H-^{13}C correlation in the HMBC spectrum among the signals at δ 3.16 (t, *J* 6.4 Hz) and 5.72-5.80 (bt) with the signal at δ_C 166.4 (Figure 2a) revealed an amidic α,β-unsaturated carboxyl.

The *J*-RES spectrum (Figure 3) allowed discriminating the signal at δ 5.72-5.80 in at least three doublets at δ ca. 5.73, 5.74 and 5.78 with the same coupling constant (*J* 15.8 Hz) thus showing α-carboxyl double bonds with *trans* stereochemistry. These signals (H-2) were taken as key-signals in the assignment of the carbon chains that connect the amidic carboxyl to the corresponding aromatic system. The COSY spectrum showed strong correlations between the signal at δ ca. 5.76 (d, *J* 15.8 Hz) (H-2) with those at δ 7.19 (dd, *J* 15.6 and 10.9 Hz) (H-3) and between this last one with the signal at δ ca. 6.12 (m) (Figure 3). The *J*-RES spectrum (Figure 3) revealed that the signal at δ ca. 6.12

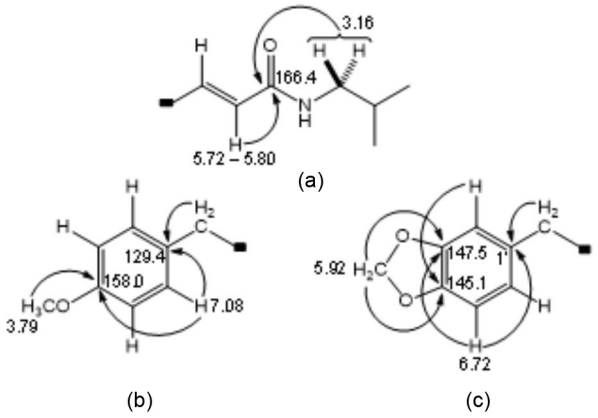


Figure 2. Key HMBC correlations of the *Piper ottonoides* root fraction for: (a) characterization of the α,β-unsaturated amidic carboxyl system; (b) and (c) assignment of the quaternary aromatic carbons and the connections between benzylic protons with their corresponding aromatic systems.

(m) contained, among others, at least two double triplets at δ ca. 6.06 (*J* 14.8 and 6.6 Hz) and δ ca. 6.2 (*J* 14.2 and 6.8 Hz) which correspond to double bond protons coupled to other double bond protons and to aliphatic protons. This set

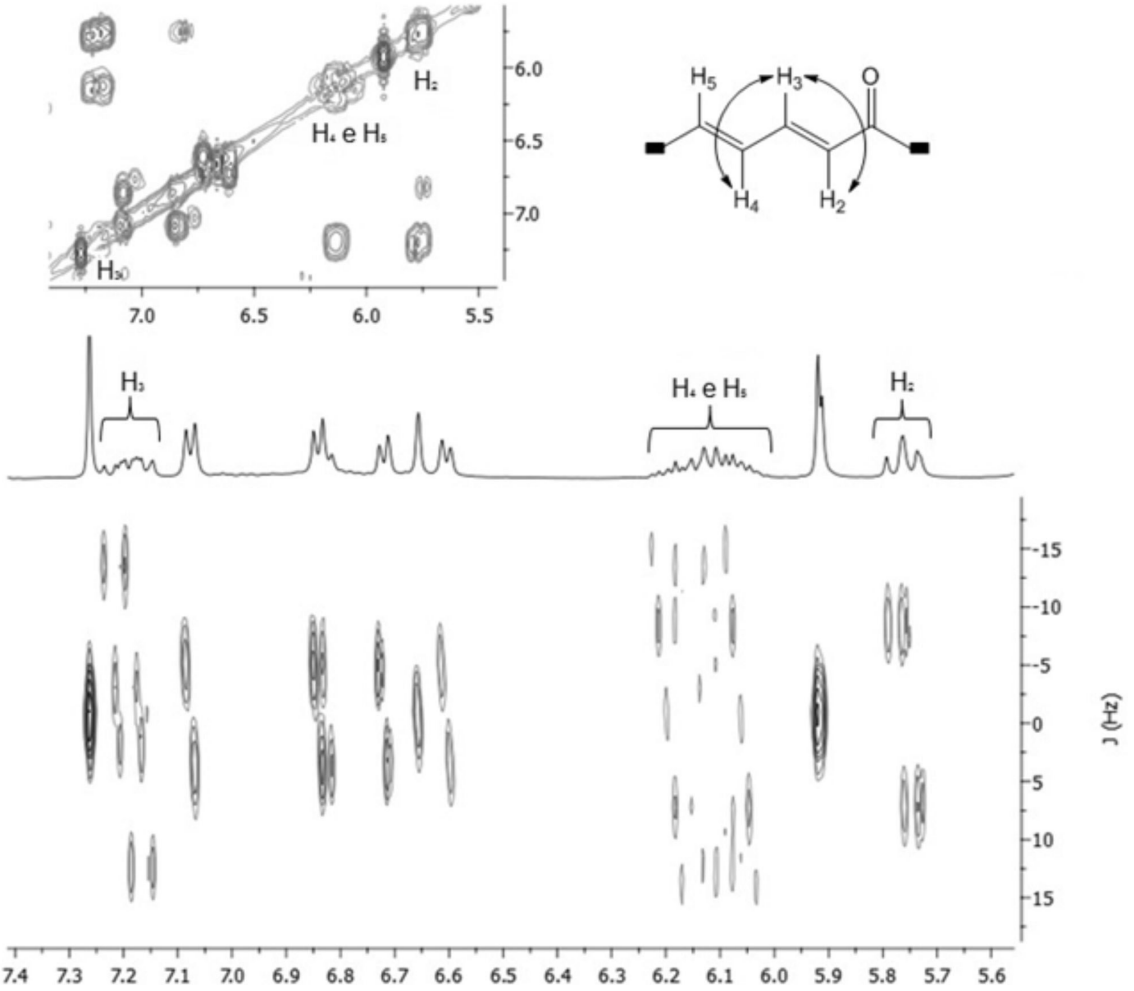


Figure 3. COSY and *J*-RES spectra from δ 5.5-7.4 of *Piper ottonoides* root fraction for characterization of the *E*,*E*-α,β,γ,δ-unsaturated amidic carboxyl system.

of data characterized the presence of an α,β,γ,δ-unsaturated carboxyl system with all double bonds with *E* configuration.

Subsequently, the COSY spectrum showed that the protons at δ ca. 6.12 were coupled to three different aliphatic protons: at δ 3.42 (d, *J* 6.2 Hz), 2.42 (q, *J* 6.9 Hz) and 2.17 (m) (Figure 4). The proton at δ 3.42 (d, *J* 6.2 Hz) had chemical shift and coupling constant compatible to a neighboring benzylic proton to a double bond (Figure 4, highlighted in solid line). The protons at δ 2.42 (q, *J* 6.9 Hz) showed correlations to the protons at δ ca. 6.12 (m) and to those at δ 2.65 (t, *J* 7.2 Hz) (Figure 4, highlighted in dotted line). On the other hand the protons at δ 2.17 (m) correlated to the protons at δ ca. 6.12 (m) and δ 1.45 (m) which in turn were coupled to those at δ 2.50 (t, *J* 7.4 Hz) (Figure 4, highlighted in dashed line). Thereby, these data allowed to deduce the presence of piperamides with three different carbon chain sizes (six, seven and eight carbons) (Figure 5a-c). The connections among these carbon chains and their corresponding aromatic systems were obtained by analyzing some key long range ^{1}H-^{13}C correlations in the HMBC spectrum. The signals at δ 3.42 (d, *J* 6.2 Hz) and at δ 2.65 (q, *J* 6.9 Hz) (benzylic protons of the carbon chain type A and B respectively, Figure 5) were coupled to the quaternary carbon at δ_C 129.4 (Figure 2b). In addition, the correlation among the protons at δ 3.79 (s) (methoxy group) and δ 7.08 (d, *J* 8.2 Hz) with the carbon at δ_C 158.0 allowed confirming the assignment of the aromatic carbon bearing the methoxy group in the *p*-disubstituted aromatic system. In addition, the aromatic proton at δ 7.08 (d, *J* 8.2 Hz) also showed a long range correlation with the carbon at δ_C 129.4. These data revealed in the roots of *P. ottonoides* two different piperamides with a *p*-methoxy substituent in the aromatic system. The data set allowed confirming the presence of piperovatine (**1**) and revealed a new piperamide: *N*-isobutyl-7-(4'-methoxyphenyl)-2*E*, 4*E*-heptadienamide which we named ottonoidenamide (**2**) (Table 2). These structures are in accordance to those presenting MS profiles of the compounds with RRt = 1.00 and 1.03 in the GC-MS analysis (Table 1). Further analyses of the HMBC spectrum revealed, among others, the long range correlations of the signal at δ 5.92 (s) (methylenedioxy protons) with the quaternary carbons at δ_C 147.6 and 145.1 (Figure 2c), the correlation between the benzylic proton at δ 2.65 (t, *J* 7.2 Hz) (carbon chain type B, Figure 5) with

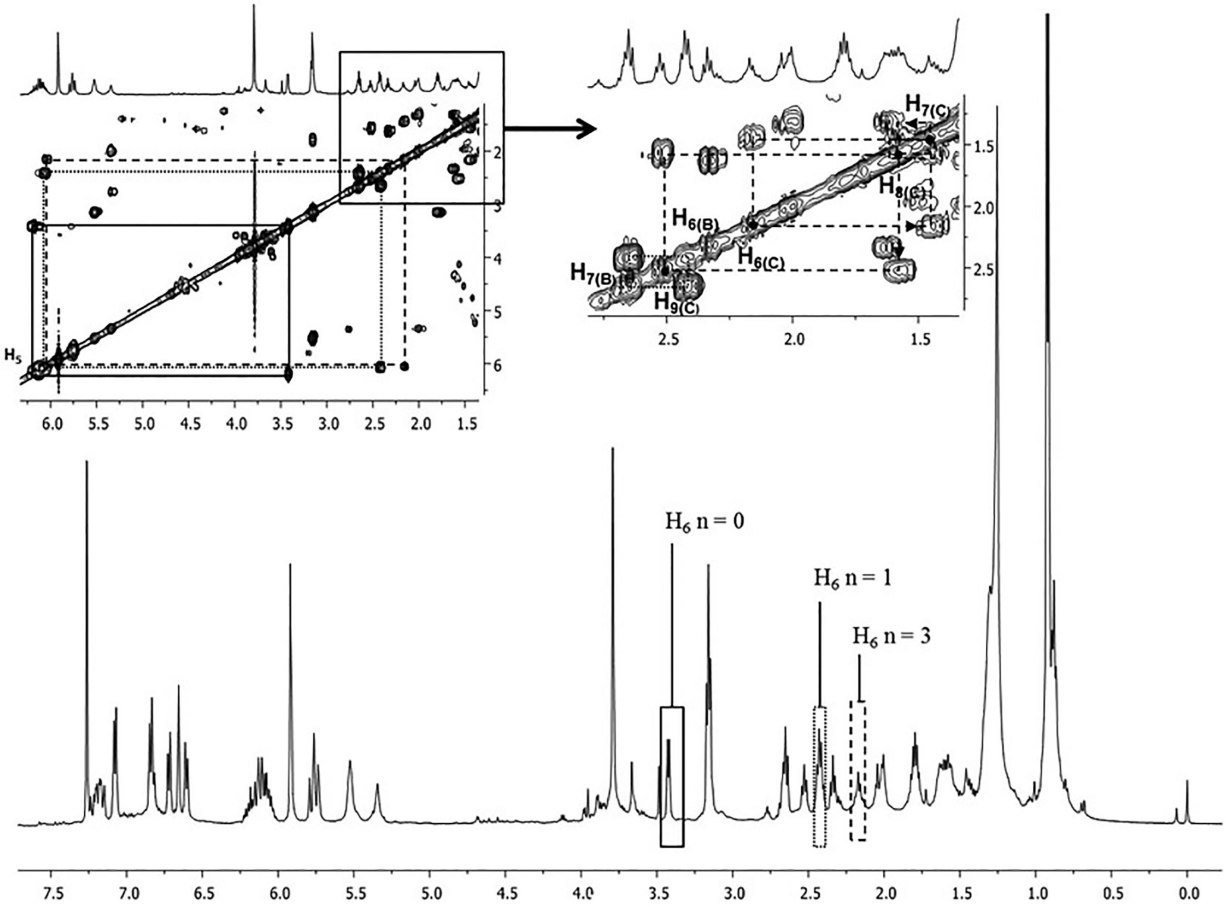


Figure 4. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) and selected correlations in the ^{1}H-^{1}H COSY spectrum of *Piper ottonoides* root fraction for characterization of the piperamide different types of carbon chains.

the quaternary carbon (C-1') at δ_C 134.5 and the benzylic proton at δ 2.50 (t, *J* 7.4 Hz) (carbon chain type C, Figure 5) with the quaternary carbon (C-1') at δ_C 136.2 (Figure 2c), showing at least two different piperamides with this aromatic system. These data indicated the presence of chingchengenamide A (**3**)[25,29,30] and pipercallosine (**4**)[8] which appear in the GC chromatogram with RRt = 1.09 and 1.21 (Table 1), respectively.

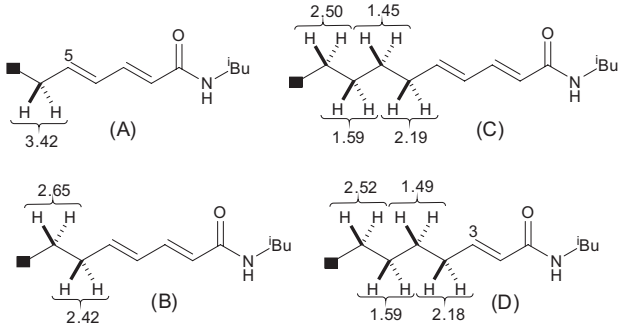


Figure 5. Types of carbon chains characterized in the piperamides found in *Piper ottonoides* root fraction.

Additional analysis of the COSY spectrum showed the ^{1}H-^{1}H correlation between the signals at δ ca. 5.76 (d, *J* 15.8 Hz) (H-2) and 6.82 (m) which in turn sequentially correlated to those at δ 2.18 (m), 1.45 (m), 1.59 (m) and to the benzylic protons at δ 2.52 (t, *J* 7.4 Hz) (Figure 6). The ^{1}H-^{13}C correlation in the HMBC spectrum between the protons at δ 2.52 (t, *J* 7.4 Hz) and the quaternary carbon (C-1') at δ_C 136.2 was similar to those found for the compounds **3** and **4** (Figure 2c). The data set revealed the presence of pipercallosidine (**5**)[20,31] which appears in the GC chromatogram with RRt = 1.04 (Table 1).

Table 2. ^{1}H (500 MHz) and ^{13}C (by HSQC and HMBC) NMR data of compound **2** in $CDCl_3$

Position	δ_H in ppm, mult. (*J* in Hz)	δ_C in ppm
1	–	166.4
2	5.78, d (15.7)	122.2
3	7.19, dd (15.7, 10.9)	141.0
4	6.12, m	128.9
5	6.12, m	141.8
6	2.42, m[a]	34.9
7	2.65, t (7.5)	34.9
1'	–	129.4
2'	7.08, d (8.2)	129.4
3'	6.84, d (8.2)	113.9
4'	–	158.0
5'	6.84, d (8.2)	113.9
6'	7.08, d (8.2)	129.4
1''	3.16, t (6.4)	47.1
2''	1.80, m	28.6
3''	0.92, d (6.5)	20.1
4''	0.92, d (6.5)	20.1
OCH_3	3.79, s	55.3
NH	5.53, bs	–

[a]Overlapped signal.

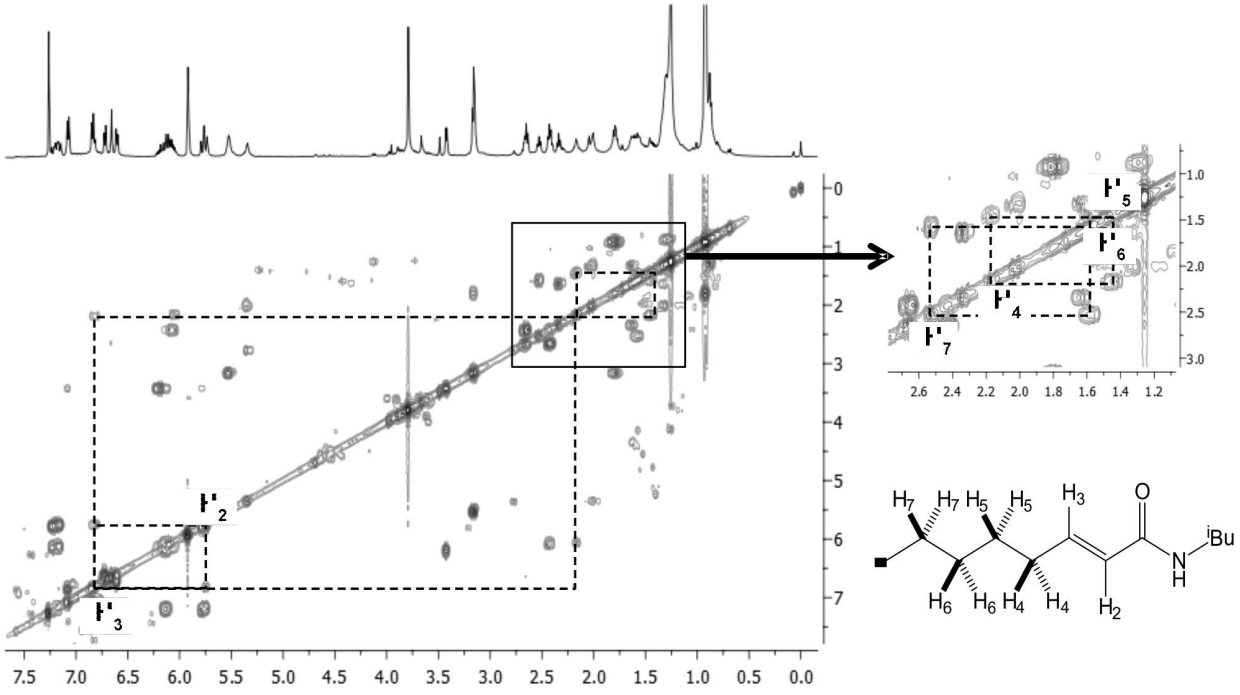


Figure 6. ^{1}H-^{1}H COSY spectrum correlations of the *Piper ottonoides* root fraction for characterization of the seven-carbon chain containing α,β-unsaturated amidic carboxyl system.

The TOCSY spectrum confirmed the proton sequence in the carbon chains. The additional positive HRESITOFMS analysis of the root fraction exhibited $[M + Na]^+$ ions compatible with the characterized compounds. The ion at *m/z* 310.1645 (calculated 287.1879 for $C_{18}H_{25}NO_2$) allowed confirming the structure of the new piperamide **2** (Figure 7 and Figures S3 and S24 in the Supplementary Information (SI) section).

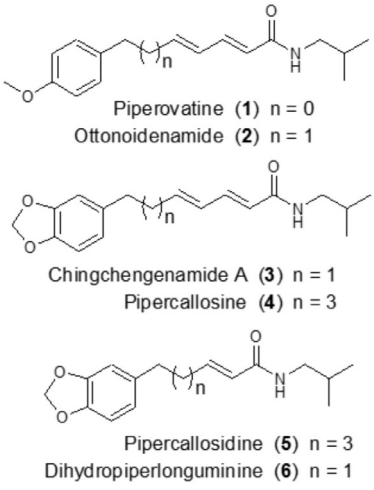


Figure 7. Structures of compounds **1**-**6**.

NMR analyses of fruit piperamides

The study of the ^{1}H, COSY, HMBC and *J*-RES NMR spectra of the fruit fraction revealed some similar data to those previously described for the root piperamides, showing the presence of piperovatine (**1**), ottonoidenamide (**2**) and chingchengenamide A (**3**). In addition, the analysis of the COSY spectrum showed the correlation between the signal at δ 5.70 (d, *J* 15.0 Hz) (H-2) and that at δ 6.78 (m) which is compatible to an α,β-unsaturated carboxyl system similar to those found in the compound (**5**) (Figures 6 and 8). The signal at δ 6.78 (m) presented ^{1}H-^{1}H correlation to the signal at δ 2.36 (m) which in turn correlated to that at δ 2.61 (t, *J* 7.8 Hz) (benzylic protons). The ^{1}H-^{13}C correlation in the HMBC spectrum between the protons at δ 2.61 (t, *J* 7.8 Hz) and the quaternary carbon (C-1') at δ_C 135.0 was similar to those found for the compounds **3** and **4** (Figure 2c) thereby showing an aromatic system bearing a methylenedioxy group. This data set is compatible to that of dihydropiperlonguminine (**6**)[32] which appears in the GC chromatogram with RRt = 0.95 (Table 1).

NMR analyses of leaf and stem piperamides

Similar NMR spectrum analyses of the leaf and stem fractions led to the characterization of piperovatine (**1**), ottonoidenamide (**2**), chingchengenamide A (**3**) and pipercallosidine (**5**) in both fractions and the additional presence of dihydropiperlonguminine (**6**) in leaves of *P. ottonoides.*

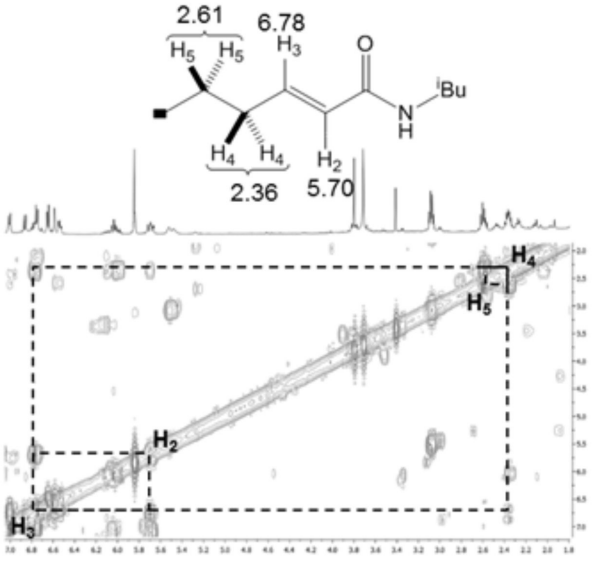


Figure 8. ^{1}H-^{1}H COSY spectrum correlations of the *Piper ottonoides* fruit fraction for characterization of the five-carbon chain containing α,β-unsaturated amidic carboxyl system.

The ^{1}H NMR spectrum profiles of the studied parts of *Piper ottonoides* and the suggested fragments of the main peaks observed in the GC-EIMS of the piperamides are shown in Figures 9 and 10 respectively.

Conclusions

This study unambiguously established by a combination of MS fragmentation and extensive 1D and 2D NMR spectroscopic analyses, without prior isolation, the structure of the new piperamide ottonoidenamide (**2**), as well as the amides piperovatine (**1**) and chingchengenamide A (**3**) in fruits, leaves, stems and roots of *P. ottonoides*; the additional presence of pipercallosine (**4**) and pipercallosidine (**5**) in roots and, of dihydropiperlonguminine (**6**) in fruits and leaves. The presence of piperovatine (**1**) that has been reported to have anesthetic, tongue-numbing and piscicidal activities and of pipercallosine (**4**) that showed anesthetic activity,[19,20,22,33] should be associated to the described properties of the species.

Supplementary Information

Supplementary Information (NMR spectra, GC-MS profiles, HRMS data from root fraction and ^{1}H and ^{13}C NMR data of compounds **1**, **3**-**6**) is available free of charge at http://jbcs.sbq.org.br as PDF file.

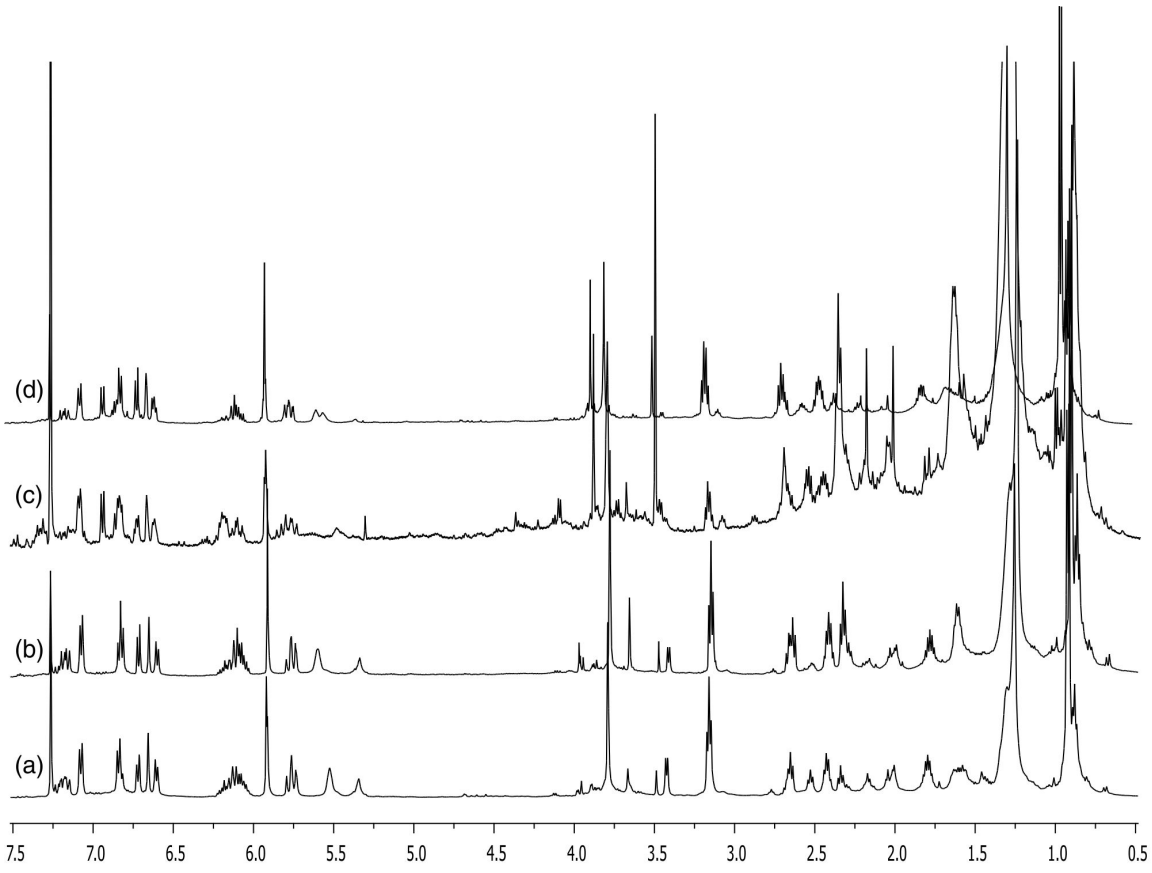
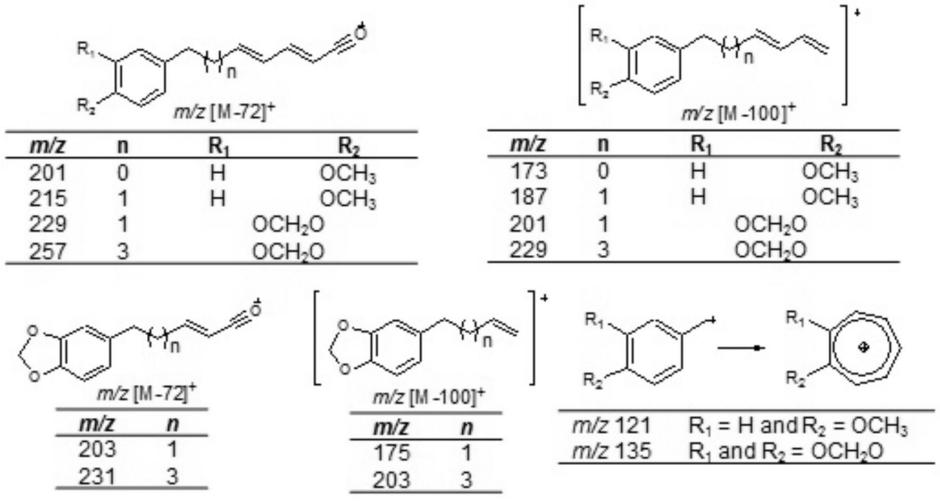


Figure 9. Comparison of the ^{1}H NMR spectrum profiles of the studied parts of *Piper ottonoides*: (a) roots; (b) stems; (c) leaves; (d) fruits.



m/z	n	R_1	R_2
201	0	H	OCH_3
215	1	H	OCH_3
229	1	OCH_2O	
257	3	OCH_2O	

m/z	n	R_1	R_2
173	0	H	OCH_3
187	1	H	OCH_3
201	1	OCH_2O	
229	3	OCH_2O	

m/z	*n*
203	1
231	3

m/z	*n*
175	1
203	3

Figure 10. Suggested fragments attributed to the main peaks observed in the mass spectra of the piperamides present in *Piper ottonoides* (based on Facundo *et al.*[21]).

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