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Article

# Isolation and Structural Characterization of Two New Furanoditerpenes from *Pterodon emarginatus* (Fabaceae)

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A furanoditerpene-enriched fraction was obtained from the fruits of *Pterodon emarginatus* and submitted to semipreparative high performance liquid chromatography (HPLC). Two new furanoditerpenes, 6α,19β-diacetoxy-7β,14β-dihydroxyvouacapan and 6α-acetoxy-7β,14β-dihydroxyvouacapan, in addition to the known compound methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate were obtained. Compound structures were determined by 1D and 2D nuclear magnetic resonance (NMR) experiments and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS). The major compound methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate was evaluated against promastigote forms of *Leishmania amazonensis* and *L. braziliensis*, presenting the concentration which causes lysis on 50% of parasites $IC_{50}$ < 30 μg $mL^{-1}$.

**Keywords:** *Pterodon*, furanoditerpenes, vouacapan, *Leishmania*

## Introduction

According to the website The Plant List,[1] *Pterodon* (Fabaceae) comprises two native Brazilian species, popularly known as "sucupira-branca" or "faveiro": *Pterodon abruptus* (Moric.) Benth. (synonym: *P. apparicioi*) and *P. emarginatus* Vogel (synonyms: *P. polygalaeflorus*, *P. polygaliflorus* and *P. pubescens*). These species are found in the Brazilian Cerrado and their fruits have often been used in popular medicine for their anti-rheumatic, analgesic and anti-inflammatory properties.[2] Vouacapan furanoditerpenes are constituents of these fruits, and present some pharmacological properties, such as anti-inflammatory,[3] antiproliferative against human cancer cells,[4] antiedematogenic[5] and antinociceptive activities.[6]

In the present study, two new furanoditerpenes, as well as one that has been previously described in the literature, were obtained from *P. emarginatus* through semi-preparative high performance liquid chromatography (HPLC). Their structures were determined via one and two-dimensional nuclear magnetic resonance (1D and 2D NMR) experiments, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS), and comparison with data from literature. In addition to the chemical study, compound methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate was evaluated against promastigote forms of *Leishmania amazonensis* and *L. braziliensis*.

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## Experimental

### General procedures

NMR experiments were performed on a Bruker Avance III 11.75 Tesla spectrometer at 25 °C, using a 5 mm broadband probehead with a z-gradient. Spectra were obtained at 500.13 MHz for $^{1}H$, using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard. Complete signal assignment was also obtained by heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum correlation (HSQC) experiments, and eventually by $^{13}C$ analysis. A Model 9.4 T Solarix mass spectrometer (Bruker Daltonics, Bremen, Germany) was set to operate over a mass range of *m/z* 150-2000. The ESI source conditions were as follows: nebulizer gas pressure: 3 bar; capillary voltage: 3.5 kV; transfer capillary temperature: 250 °C. Ions accumulated in the hexapole collision cell in $5 \times 10^{-3}$ s were then transported to the analyzer cell (ICR) through the multipole ion guide system (another hexapole). The time-of-flight in the hexapole was 0.5 ms. Each spectrum was acquired by accumulating 200 scans of time-domain transient signals in 4 mega-point time-domain data sets. All mass spectra were externally calibrated using arginine (for ESI+) and sodium salt of trifluoroacetic acid (NaTFA) (for ESI–) solutions (*m/z* 200-2000). A resolving power, $m/\Delta m_{50\%}$ ca. 730 000, in which $\Delta m_{50\%}$ is the full width at half maximum height, with *m/z* ca. 400 and mass accuracy of < 1 ppm, provided the unambiguous molecular formula assignments for singly charged molecular ions. Mass spectra were acquired and processed using data analysis software (Bruker Daltonics, Bremen, Germany). The MS data was processed and compounds' elemental compositions were determined by measuring *m/z* values. Semi-preparative HPLC was performed using a Shimadzu LC-8A model (Kyoto, Japan), equipped with an SPD-20A UV detector and a Shimadzu C18 column (250 × 20 mm, 5 μm). Data processing was performed by LC Solution. All solvents used in chromatographic separations were of HPLC grade (Merck KGaA, Darmstadt, Germany), and analytical grade solvents were used for extraction (Qhemis, Brazil).

### Plant materials

Fruits of *P. emarginatus* were collected in the Cerrado region of Bela Vista, Goiás State, Brazil. A voucher specimen was authenticated by Prof José Realino de Paula and deposited in the herbarium of Universidade Federal de Goiás (UFG), Brazil, under No. 27,155.

### Extraction and isolation

Oleoresin extraction was performed by mechanical pressing using a continuous mini-press (MPE-40 ECIRTEC, Brazil). The extraction yield was 30% in weight. Regarding prior purification, 10.06 g of *P. emarginatus* fruit oil were extracted with 100 mL hexane. The insoluble fraction was solubilized in 100 mL methanol:water (9:1, v/v) and submitted to partitioning with 105 mL of a dichloromethane:hexane (6.3:3.7, v/v) mixture. Following agitation, distilled water was added until phase separation was complete (45 mL). The dichloromethane:hexane (DHF) fraction was submitted to rotaevaporation at 40 °C and used to isolate furanoditerpenes.

Approximately 170 mg of DHF was purified by semi-preparative reversed phase (RP)-HPLC under the following column chromatography conditions: Shimadzu C18 column (250 × 20 mm, 5 μm), detection wavelength of 190 and 220 nm, injection volume of 1.5 mL, mobile phase of acetonitrile:water (65:35, v/v) mixture acidified with 0.05% acetic acid (v/v), at a flow rate of 8 mL $min^{-1}$. Three samples were injected and fractions related to the three main chromatographic peaks (retention time ca. 17, 26 and 30 min) were collected, concentrated in a rotary evaporator at 40 °C, and freeze-dried (MicroModulyo115 freeze-dryer-ThermoFisher Scientific). The major constituents of the three collected fractions had their structures established by NMR and corroborated by mass spectrometry as 6α,19β-diacetoxy-7β,14β-dihydroxyvouacapan (**1**) ($[M-H]^-$ of *m/z* 433.22309), 6α-acetoxy-7β,14β-dihydroxyvouacapan (**2**) ($[M + Na]^+$ of *m/z* 399.21415), 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**) ($[M - H]^-$ of *m/z* 403.21248).

### Antileishmanial assay

Promastigote forms of *L. amazonensis* and *L. braziliensis* were cultivated in M199 (Sigma-Aldrich) supplemented with 10% bovine fetal serum (Gibco), 0.22% $NaHCO_3$, and 0.5% penicillin-streptomycin solution (Sigma-Aldrich) at 23 °C for 6 days. Promastigote forms of *L. amazonensis* and *L. braziliensis* ($10^6$ parasites $mL^{-1}$) were deposited in 96-well microplates and treated with different concentrations of compound **3** (8, 32 and 128 μg $mL^{-1}$) for 24 and 72 h.

After treatments, 50 μL of tetrazolium dye MTT (2.5 mg $mL^{-1}$) were added to each well in the plate, which was incubated for 4 h at 23 °C. Formazan crystals produced by viable cells were solubilized with 50 μL *per* well of SDS (10% m/v) and plates were incubated at 37 °C for 60 min. Following incubation, absorbance was measured at 570 nm using a microplate reader (BioTek, Synergy H1 Hybrid

Reader). Bioassays were performed in triplicate, with dimethylsulfoxide (DMSO) solution being used in the same concentration that was applied for sample solubilization, as negative control and amphotericin B as reference drug.

The leishmanicidal activity of compound **3** was expressed as $IC_{50}$ values, corresponding to the concentration which causes lysis on 50% of parasites. These values were calculated by the statistical method of sigmoid concentration-response curve, via GraphPad Prism 5.0.

## Results and Discussion

Vouacapan furanoditerpenes were obtained from an enriched sample (DHF) by semipreparative RP-HPLC. Fractions amounting to shaded peaks 1, 2, and 3-4 (Figure 1) were collected from three injections, which produced white solids following concentration and freeze-drying steps. Fractions from shaded areas 3 and 4 (Figure 1) were grouped, hence yielding 30.0 mg of a pure compound. Fractions 1 and 2 yielded 7.5 mg each. As may be observed in the chromatogram and confirmed by $^1$H NMR spectra, fractions 1 and 2 contain impurities due to the coelution of substances whose retention times are close to those of the major compounds (Figure 1, particularly at 190 nm).

The structures of the new compounds 6α,19β-diacetoxy-7β,14β-dihydroxyvouacapan (**1**) and 6α-acetoxy-7β,14β-dihydroxyvouacapan (**2**) were elucidated by NMR experiments and confirmed by MS and data from literature (Figure 2).[7,8] These experiments were also used to identify compound **3** as methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate, previously identified in *P. apparicioi* and *P. emarginatus*.[9-11]

The $^1$H NMR spectrum for compound **1** showed hydrogen furans at $\delta_H$ 6.42 (1H, d, *J* 1.9 Hz, $H_{15}$) and $\delta_H$ 7.26 (1H, d, *J* 1.9 Hz, $H_{16}$). Two acetyl groups were assigned by the signals at $\delta_H$ 5.36 (1H, dd, *J* 11.8 and 9.2 Hz, $H_6$),

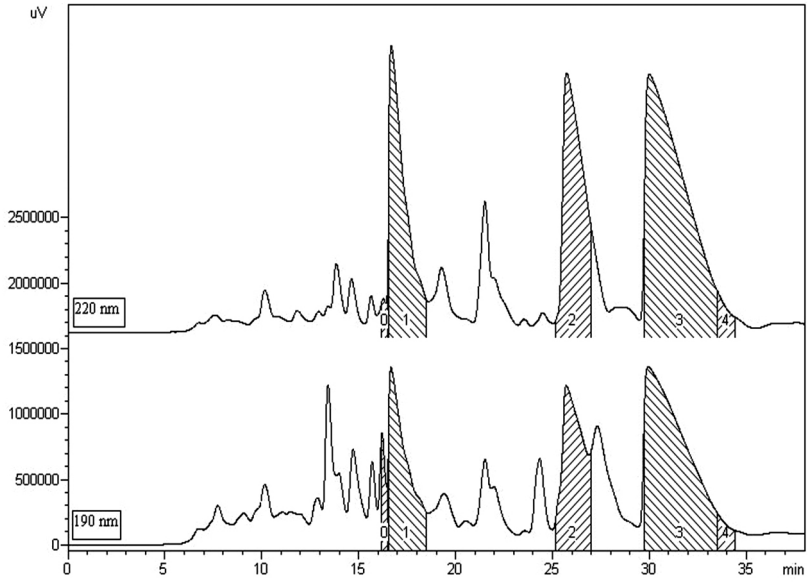


**Figure 1.** Chromatographic profile of the enriched fraction of the *P. emarginatus* oleoresin, obtained via semi-preparative HPLC at 190 and 220 nm. Shaded areas 1, 2, and 3-4 correspond to the collected fractions. The chromatogram at 220 nm is displaced on the y-axis for a clearer presentation.

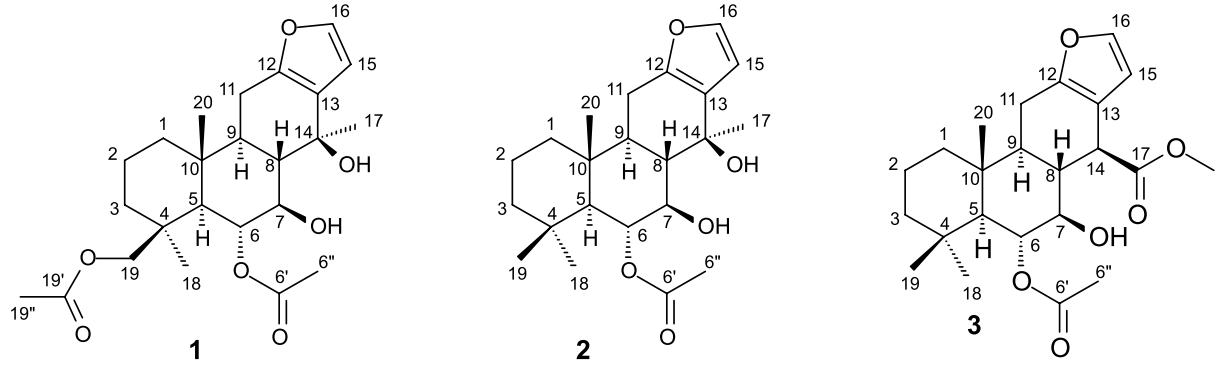


**Figure 2**. Structure of vouacapan diterpenes **1**, **2** and **3**.

$\delta_H$ 4.12 (1H, d, *J* 11.3 Hz, H-19a), $\delta_H$ 4.22 (1H, d, *J* 11.3 Hz, H-19b), $\delta_H$ 2.19 (3H, s, $H_{6''}$), and $\delta_H$ 2.05 (3H, s, $H_{19''}$). Three methyl groups were observed at $\delta_H$ 1.46 (3H, s, $H_{17}$), 1.14 (3H, s, $H_{18}$) and 1.05 (3H, s, $H_{20}$).

The $^{13}C$ NMR associated with cross peaks observed in HSQC and HMBC experiments corroborated typical signals for vouacapan's furan ring at $\delta_C$ 148.2, 141.9, 123.9 and 107.2. Two acetoxy and three carbinolic groups were also observed at $\delta_C$ 172.6, 171.2, 77.5, 75.8 and 72.6.

These data are very similar to that presented for known compound **3** (Table 1), except for the replacement of carbomethoxy and hydrogen groups at $C_{14}$ for hydroxyl and methyl groups. Replacement of hydrogen by one acetoxy group at $C_{19}$ was also observed. The hydroxyl group at $C_{14}$ was confirmed by cross peaks between hydrogens $H_7$, $H_8$ and $H_{15}$ ($\delta_H$ 3.81, 2.02 and 6.42) with $C_{14}$ at $\delta_C$ 72.6 in the HMBC experiment. The doublets at $\delta_H$ 4.12 and 4.22 (*J* 11.3 Hz) as well as the cross peak between $H_{19}$ and carbonyl at $\delta_C$ 171.2 confirmed the position of this new acetoxy group. Cross peaks between these signals and carbons $C_4$, $C_5$ and $C_{18}$ ($\delta_C$ 38.7, 55.0 and 30.6), observed in the HMBC experiment, suggested that the acetoxy group is attached to $C_{19}$. The $C_4$ and $C_{14}$ stereochemistry were evaluated by nuclear Overhauser effect (NOE) experiments (Figures S6 and S7). The observed NOE between $H_5$, $H_7$ and Me-18 corroborate acetoxy group on $C_{19}$. On the other hand, the NOE between Me-17, $H_7$ and $H_{15}$ confirmed hydroxyl group on β position. No NOE was observed between $H_8$ and Me-17. The $C_{14}$ stereochemistry in is agreement with that proposed for two furanoditerpenes voucapanes containing methyl and hydroxyl groups attached on $C_{14}$.[12] The proposed structure is in agreement with 6α,19β-diacetoxy-7β,14β-dihydroxyvouacapan (**1**).

Compounds **1** and **2** show very similar NMR spectra (Table 1). The most significant difference was due to the absence of the acetoxy group attached to $C_{19}$. One methyl group is attached in this position, according to the signal at $\delta_H$ 1.06 (s). Basing on this data, the 6α-acetoxy-7β,14β-dihydroxyvouacapan structure was proposed for compound **2**.

**Table 1.** $^1H$ and $^{13}C$ NMR spectra data for diterpenes **1**, **2** and **3**, isolated from the fruit oil of *P. emarginatus* (500 MHz, $CDCl_3$)

| H/C | **1** | | **2** | | **3** | |
|---|---|---|---|---|---|---|
| | $\delta$ $^1H$ / ppm (mult., *J* / Hz) | $\delta$ $^{13}C$ / ppm | $\delta$ $^1H$ / ppm (mult., *J* / Hz) | $\delta$ $^{13}C$ / ppm | $\delta$ $^1H$ / ppm (mult., *J* / Hz) | $\delta$ $^{13}C$ / ppm |
| 1 | 1.7-1.6 (m); 1.2-1.1 (m) | 38.4 | 1.7-1.6 (m); 1.0-0.9 (m) | 39.4 | 1.7-1.6 (m); 1.0-0.9 (m) | 39.5 |
| 2 | 1.6-1.4 (m) | 18.0 | 1.6-1.4 (m) | 18.1 | 1.6-1.4 (m) | 18.5 |
| 3 | 1.8-1.7 (m); 1.1-1.0 (m) | 39.5 | 1.2-1.1 (m); 1.4-1.3 (m) | 43.3 | 1.2-1.1 (m); 1.4-1.3 (m) | 43.7 |
| 4 | – | 38.7 | – | 32.9 | – | 33.2 |
| 5 | 1.46 (d, 11.8) | 55.0 | 1.29 (d, 11.6) | 54.3 | 1.28 (d, 11.5) | 55.0 |
| 6 | 5.36 (dd, 11.8, 9.2) | 75.8 | 5.33 (dd, 11.6, 9.2) | 75.8 | 5.21 (dd, 11.5, 9.4) | 75.7 |
| 7 | 3.81 (dd, 10.6, 9.2) | 77.5 | 3.85 (dd, 10.6, 9.2) | 76.7 | 3.31 (dd, 10.6, 9.4) | 81.4 |
| 8 | 2.02 (dd, 12.5, 10.6) | 49.5 | 2.04 (dd, 12.5, 10.6) | 49.6 | 2.37 (ddd, 11.7, 10.6, 9.1) | 42.7 |
| 9 | 1.5-1.4 (m) | 46.9 | 1.5-1.4 (m) | 46.7 | 1.45 (ddd, 11.7, 5.6, 4.9) | 48.2 |
| 10 | – | 37.0 | – | 38.6 | – | 38.6 |
| 11 | 2.62 (dd, 16.6, 6.6)<br>2.45 (dd, 16.6, 10.5) | 22.5 | 2.61 (dd, 16.6, 6.5)<br>2.44 (dd, 16.6, 10.7) | 22.2 | 2.64 (dd, 16.1, 5.6)<br>2.41 (dd, 16.1, 4.9) | 22.2 |
| 12 | – | 148.2 | – | 148.4 | – | 150.5 |
| 13 | – | 123.9 | – | 123.7 | – | 113.6 |
| 14 | – | 72.6 | – | 72.6 | 3.38 (d, 9.1) | 46.6 |
| 15 | 6.42 (d, 1.9) | 107.2 | 6.42 (d, 1.9) | 107.0 | 6.14 (d, 1.7) | 108.6 |
| 16 | 7.26 (d, 1.9) | 141.9 | 7.26 (d, 1.9) | 141.6 | 7.21 (d, 1.7) | 141.6 |
| 17 | 1.46 (s) | 25.9 | 1.47 (s) | 25.6 | – | 175.6 |
| 18 | 1.14 (s) | 30.6 | 1.06 (s) | 36.1 | 1.00 (s) | 36.5 |
| 19 | 4.22 (d, 11.3)<br>4.12 (d, 11.3) | 67.0 | 0.96 (s) | 22.4 | 0.92 (s) | 22.7 |
| 20 | 1.05 (s) | 16.3 | 1.07 (s) | 15.6 | 1.05 (s) | 15.7 |
| 6' | – | 172.6 | – | 172.0 | – | 172.1 |
| 6" | 2.19 (s) | 21.9 | 2.16 (s) | 21.7 | 2.09 (s) | 22.6 |
| 19' | – | 171.2 | – | – | – | – |
| 19" | 2.05 (s) | 20.9 | – | – | – | – |
| OMe-17 | – | – | – | – | 3.70 (s) | 52.5 |

$^1H$ and $^{13}C$ NMR assignments are based on $^1H$ and $^{13}C$ spectra and on $^1H$-$^{13}C$ HSQC and HMBC contour maps; s: singlet; m: multiplet; d: doublet; dd: double doublets; ddd: double double doublets.

Figure 3 shows the ESI(−)-FTICR mass spectrum of these three diterpenes (**1**, **2** and **3**). Diterpene **1** was detected in deprotonated form $[M - H]^-$ of *m/z* 433.22309 with an error of 0.20 ppm. Diterpene **2** was detected as sodium adduct $[M + Na]^+$ of *m/z* 399.21415 with an error of 0.11 ppm. Diterpene **3** was detected in the negative ion mode in deprotonated form $[M - H]^-$ of *m/z* 403.21248 with an error of 0.13 ppm. In all cases, the ultra-high resolution mass spectrum provides the exact mass with an error lower than 1 ppm.

The antiparasitic activity of furanoditerpene **3** was assessed *in vitro* against promastigotes of *L. amazonensis* and *L. braziliensis*, resulting in significant leishmanicidal activity (Table 2).[13]

Diterpenes rank among the classes of secondary metabolites with promising leishmanicidal activity.[14] Leishmaniasis is regarded as a neglected disease, and this is the first study to report on assays carried out with furanoditerpenes vouacapan isolated from fruits of the *Pterodon* genus. Further studies on the pharmacological potential of *Pterodon* furanoditerpenes should be encouraged, as they contribute to the development of drugs for neglected diseases and favor the preservation of Cerrado.

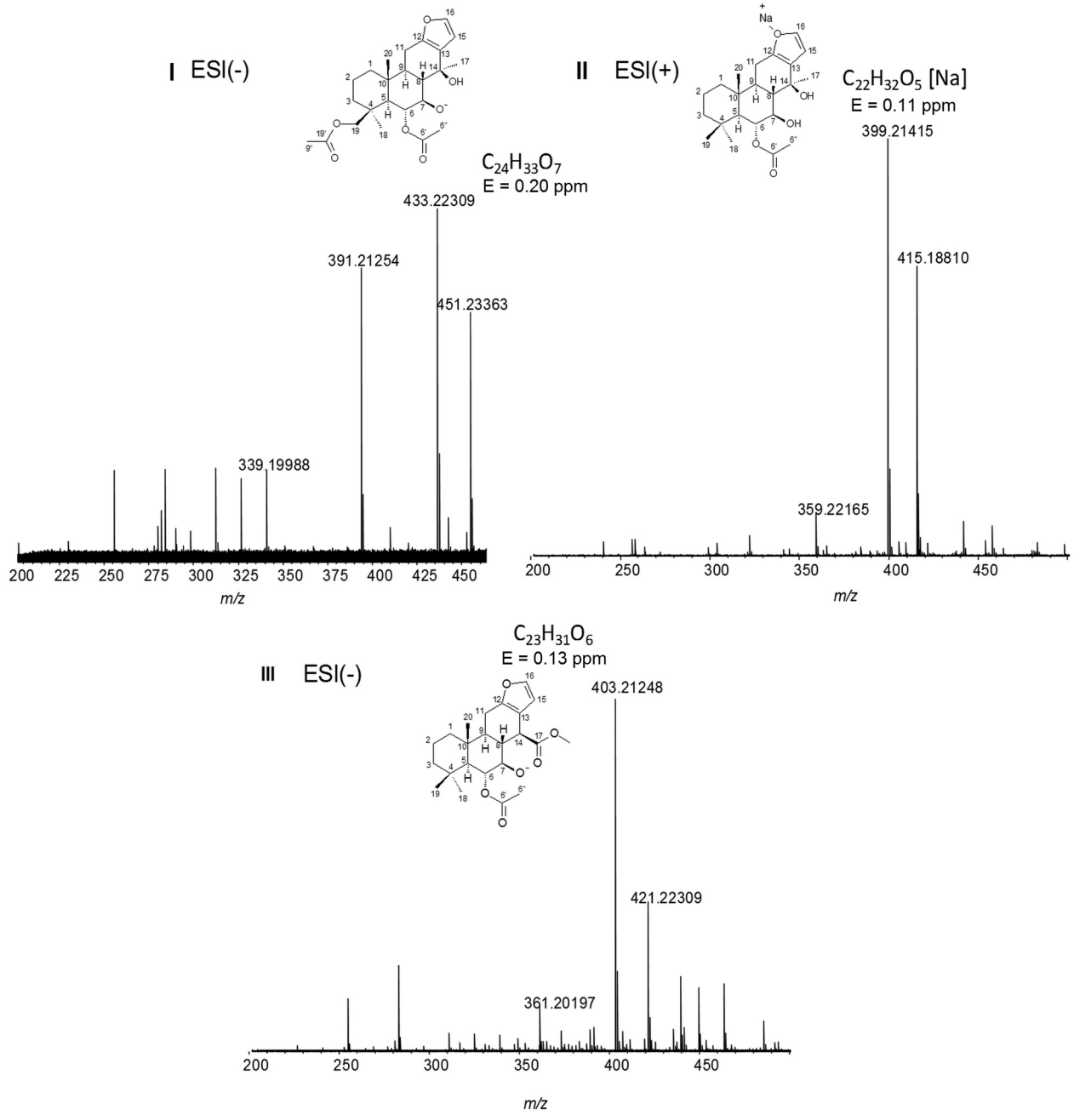


**Figure 3.** ESI-FTICR mass spectrum of vouacapan diterpenes **1**, **2** and **3**.

**Table 2.** Antiparasitic activities of compound **3** and amphotericin B

| Compound | $IC_{50}$ / (μg $mL^{-1}$) | | | |
|---|---|---|---|---|
| | *Leishmania amazonensis* | | *Leishmania braziliensis* | |
| | 24 h | 72 h | 24 h | 72 h |
| **3** | 25.47 ± 1.01 | 20.12 ± 0.76 | 26.12 ± 0.18 | 17.94 ± 1.94 |
| Amphotericin B | 3.09 ± 0.08 | 3.22 ± 0.03 | 2.68 ± 0.23 | 4.84 ± 0.12 |

$IC_{50}$: half-maximal inhibitory concentration; data expressed as mean of triplicates ± standard deviation.

## Conclusions

The phytochemical study of *P. emarginatus* fruits has led to the isolation and elucidation of two new furanoditerpenes, 6α,19β-diacetoxy-7β,14β-dihydroxyvouacapan (**1**) and 6α-acetoxy-7β,14β-dihydroxyvouacapan (**2**), in addition to the known compound methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**). Compound methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**) was found as a promising leishmanicidal agent.

## Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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