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Bio-guided isolation of leishmanicidal and trypanocidal constituents from *Pituranthos battandieri* aerial parts

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ABSTRACT

Protozoan pathogens that cause neglected tropical diseases are a major public health concern in tropical and developing countries. In the course of our ongoing search for new lead compounds as potential antiprotozoal agents, this study aims to perform a bio-guided fractionation of *Pituranthos battandieri*, using an *in vitro* assay against *Leishmania amazonensis* and *Trypanosoma cruzi*. Two known polyacetylenes, (−)-panaxydiol (**1**) and (−)-falcarindiol (**2**) were identified from the ethanolic extract of the aerial parts of *P. battandieri* as the main bioactive constituents. Compounds **1** and **2** showed similar potency (IC_{50} values of 5.76 and 5.68 μM, respectively) against L. *amazonensis* to miltefosine (IC_{50} value of 6.48 μM), the reference drug, and low toxicity on macrophage cell lines J774. Moreover, compound **1** exhibited moderate activity (IC_{50} 23.24 μM) against *T. cruzi*. In addition, three known furanocoumarins, 8-geranyloxypsoralen (**3**), 8-geranyloxy-5-methoxypsoralen (**4**), and phellopterin (**5**) were isolated. Their structures were elucidated by NMR and MS analysis. Compounds **1** and **2** are described for the first time in the *Pituranthos* genus, and this is the first report on their antiprotozoal activity. These results highlight this type of polyacetylenes as an interesting scaffold for the development of novel antiparasitic drugs.

1. Introduction

Tropical parasitic diseases such as leishmaniasis and trypanosomiasis are some of the main public health issues around the world, which affect hundreds of millions of people and domestic animals. Therefore, there is an urgent need to discover the next generation of antiparasitic drugs against *Leishmania* and *Trypanosoma* to improve the current therapeutic options, which suffer from serious drawbacks such as resistance, inefficacy, toxicity, long courses of administration, and high costs [1,2].

Despite recent *advances in modern medicine* in the field of infectious diseases, medicinal plants are still recognized as the preferred primary health care system in most countries, mainly due to the inaccessibility of therapeutic treatments [3]. In fact, natural products play an important role in the development of drug candidates for leishmaniasis and trypanosomiasis [4–6]. In this regard, the bio-guided fractionation, isolation, and identification of natural compounds from plants constitute a versatile methodology for discovering new drugs [7].

The genus *Pituranthos* (Apiaceae or Umbelliferae family) has more than 20 species, some endemic to North Africa and often found in arid desert areas [8,9]. Several species of this genus have been used in traditional folk medicine to treat fever, hepatitis, asthma, rheumatism, diabetes, and digestive problems [9–11]. *Pituranthos* species are characterized by the presence of bioactive natural products such as coumarins [12], furanocoumarins [13], and terpenoids [14]. In Algeria, there are four species, *P. battandieri, P. scoparius*, *P. chloranthus,* and *P. reboudii*. *P. battandieri* Maire, synonymous with *Deverra battandieri* (Maire) Chrtek [15], is an endemic species to the Algerian and Moroccan Sahara [16,17]. A previous study reported the chemical analysis of the

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essential oil of the aerial parts of *Pituranthos battandieri* by steam distillation and analyzed by GC and GC–MS [18].

As a part of our research aiming to identify new antiparasitic lead compounds, the current study reports the bio-guided fractionation of the ethanolic extract of the aerial parts from *P. battandieri* against promastigotes of *Leishmania amazonensis* and epimastigotes of *Trypanosoma cruzi*.

2. Materials and methods

2.1. General procedures

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter in $CHCl_3$ at 25 °C and the $[\alpha]_D$ values are given in 10^{-1} deg. cm^2/g. IR (film) spectra were measured on a Bruker IFS 55 spectrophotometer. ^1H, ^{13}C NMR, DEPT, COSY, ROESY HSQC, and HMBC spectra were carried out at 300 K on a Bruker Avance 600 spectrometer, with the pulse sequences given by Bruker, the chemical shifts are given in δ (ppm) with residual $CDCl_3$ (δ_H 7.26, δ_C 77.0) as an internal reference, and coupling constants in Hz. The EIMS and HREIMS data were obtained on a Micromass Autospec spectrometer. HRESIMS (positive mode) were measured on an LCT Premier XE Micromass Electrospray spectrometer. Silica gel 60 (particle size 15–40 and 63–200 μm, Macherey-Nagel) was used for column chromatography (CC), while silica gel 60 F_{254} was used for analytical thin layer chromatography (TLC). Sephadex LH-20 was purchased from Pharmacia Biotech. The developed TLC plates were visualized by UV light and then spraying with HOAc-H_2SO_4-H_2O (80:16:4) system, followed by heating at 100 °C for 3 min. All the used solvents were analytical grade from Panreac. Reagents and deuterated solvents were from Sigma-Aldrich. For bioassays, Schneider's medium (Sigma-Aldrich), RPMI 1640 and LIT media (Gibco®), Alamar Blue® reagent (Invitrogen, Life Technologies), EnSpire® Multimode Plate Reader (Perkin Elmer), and Leika DMIL inverted microscope (Leika, Wetzlar, Germany) were used. Miltefosine, used as a reference drug, was provided by Æterna Zentaris.

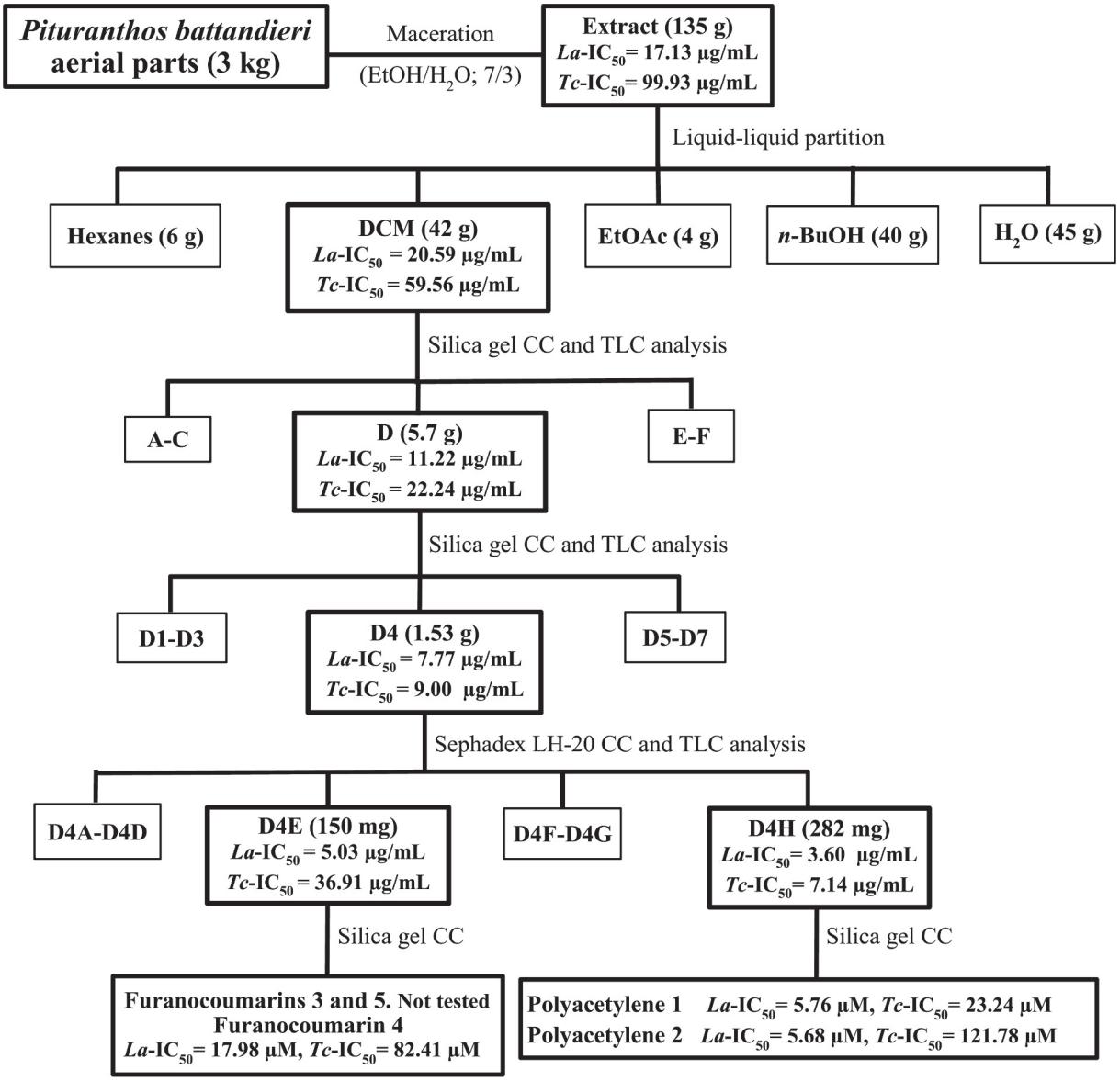

Fig. 1. Flowchart of antiprotozoal bio-guided fractionation of *P.battandieiri* aerial parts against promastigotes of L. *amazonensis* and epimastigotes of *T.cruzi*. *La*: *Leishmania amazonensis*. *Tc*: *Trypanosoma cruzi*. IC_{50}: Inhibitory Concentration that inhibits 50% of the growth of the tested parasite. The IC_{50} values are included only for the fractions/subfractions with the highest activity.

2.2. Plant material

The aerial parts of *Pituranthos battandieri* Maire were collected near the hydraulic Djorf Torba dam in the Wilaya Béchar in Southern Algeria (GPS coordinates: 30°31′0" N and 2°45'0" W) during the flowering stage in April 2017. The plant material was authenticated by M. Mohamed Benabdelhakem, Director of the nature preservation agency, Béchar. A voucher specimen (PBA 54/04/10) was deposited at the Herbarium of the VARENBIOMOL research unit, Université Frères Mentouri Constantine 1, Algérie.

2.3. Extraction

The air-dried powdered leaves of *P. battandieri* (3.0 kg) were extracted by repeated maceration (three times) with EtOH-H_2O (2 L, 70:30) at room temperature for two days. The extract was filtered, and the solvent was removed under reduced pressure at 40 °C on a rotary evaporator affording a green residue (135 g, 4.5%). The hydroalcoholic extract was refrigerated at −8 °C and an aliquot was lyophilized for antiprotozoal assays against L. *amazonensis* promastigote forms and epimastigote stage of *T. cruzi*.

2.4. Bio-guided fractionation and isolation

The extract was initially fractionated by liquid-liquid partition (Fig. 1). Thus, the extract was suspended in water (H_2O, 1.3 L) and successively extracted with hexanes (3 × 1 L), dichloromethane (DCM, 3 × 1 L), ethyl acetate (EtOAc, 3 × 1 L), and *n*-butanol (*n*-BuOH, 3 × 1 L). The organic phases were dried with Na_2SO_4, filtered and concentrated under reduced pressure at 40 °C to afford the corresponding organic fractions: hexanes (6 g), DCM (42 g), EtOAc (4 g), and *n*-BuOH (40 g). The *aqueous* phase was lyophilized to yield 45 g of *aqueous* fraction. All fractions from the liquid-liquid procedure were assayed for their antiprotozoal activity.

The active fraction (DCM, 42 g) was subjected to column chromatography (CC) over silica gel (63–200 μm, 9 × 40 cm) eluting with mixtures of hexanes-EtOAc of increasing polarity (100:0 to 0:100, 1 L each one) to afford ten fractions, which were combined based on their TLC profile into six fractions (A to F). According to their antiprotozoal activity, the active sub-fraction (D, 5.7 g) was subjected to further silica gel flash chromatography (15–40 μm, 6.5 × 30 cm) using mixtures of hexanes-diethyl ether of increasing polarity (100:0 to 0:100) to afford seven sub-fractions (D1 to D7) based on their TLC profiles. Thus, the sub-fraction D4 (1.55 g) was then chromatographed over Sephadex LH-20 (4 × 60 cm) by isocratic mixture of hexanes-$CHCl_3$-MeOH (2:1:1) to give eight sub-fractions (D4A to D4H) after their TLC analysis. The active sub-fraction D4E (150 mg) was purified by silica gel CC (15–40 μm, 1 × 30 cm) using hexanes-DCM mixtures of increasing polarity (0–100%) to yield seven sub-fractions according to TLC analysis (D4E1 to D4E7). NMR analysis of sub-fractions D4E3, D4E4, and D4E6, allowed us to identify furanocoumarins **3** (16 mg), **4** (9.4 mg), and **5** (15 mg), respectively. The sub-fraction D4H (282 mg) was purified by silica gel CC (15–40 μm, 2 × 30 cm) using hexanes-EtOAc mixtures of increasing polarity (0–40%) affording nine sub-fractions grouped based on their TLC profile (D4H1 to D4H9). Sub-fractions D4H5 and D4H7 were identified as the polyacetylenes **1** (7.5 mg) and **2** (15 mg), respectively.

2.5. Leishmanicidal activity

In vitro leishmanicidal activity of the crude extract, fractions, sub-fractions, and compounds was determined on the promastigote forms of L. *amazonensis* (MHOM/BR/77/LTB0016) by the modified Alamar Blue® [19]. All samples were dissolved in dimethyl sulfoxide (DMSO) and further dilutions were made in RPMI 1640 medium. Promastigotes were maintained in RPMI 1640 liquid medium (Gibco) supplemented with 20% heat inactivated fetal bovine serum, vitamins, and amino acids at 26 °C. Logarithmic phase cultures of promastigotes were used for experimental purposes, and the *in vitro* assay was performed in sterilized 96-well microtiter plates (CorningTM). Parasites were added to the plates at a concentration of 1×10^6 cells/mL. The final volume was 200 μL in each well. Following addition of 10% of Alamar Blue Assay Reagent®, the plates were incubated at 26 °C. After 72 h, the plates were examined with an Enspire microplate reader at excitation wavelengths of 570 nm and emission wavelengths of 585 nm.

2.6. Trypanocidal activity

In vitro trypanocidal activity of the crude extract, fractions, sub-fractions, and compounds was evaluated on the epimastigote stage of *T. cruzi* (Y strain) using the Alamar Blue® assay [19]. All samples were dissolved in DMSO and further dilutions were made in Liver Infusion Broth (LIT) medium. Epimastigotes were maintained in LIT liquid medium at 26 °C. Logarithmic phase cultures of epimastigotes were used for experimental purposes, and the *in vitro* assay was performed in sterilized 96-well microtiter plates (CorningTM). Parasites were added to the plates at a concentration of 1×10^5 cells/mL. The final volume was 200 μL in each well after the addition of 10% of Alamar Blue Assay Reagent®. Following 72 h of incubation at 26 °C, the plates were examined with an Enspire microplate reader at excitation and emission wavelengths of 570/585 nm.

2.7. Cytotoxicity activity

Cytotoxicity was evaluated against murine macrophage J774. A1 cell line. Cells were incubated with different concentration of the tested compounds at 37 °C in a 5% CO_2 humidified incubator for 24 h. Briefly, macrophages were seeded in duplicate on a 96-well microtiter plate with 50 μL from a stock solution of 2*10 cells/ mL. Cells were allowed to adhere for 15 min and 50 μL of serial dilution of the tested drugs were added. The viability of the macrophages was determined with the Alamar Blue assay as previously described [20]. Dose response curves were plotted and the LC_{50} were obtained. The analyses were performed in triplicate.

2.8. Statistical analysis

The percentage of inhibition and 50% inhibitory concentration (IC_{50}) was determined by linear regression analysis with 95% confidence limits. All experiments were performed three times in duplicate and the mean values were also calculated. A paired two-tailed *t*-test was used for analysis of the data. Values of $p < 0.05$ were considered significant. The inhibition curves statistical analysis was undertaken using the Sigma Plot 12.0 software program (Systat Software Inc).

3. Results and discussion

The ethanolic extract of the aerial parts of *P. battandieri* was evaluated against promastigote forms of L. *amazonensis* and on the epimastigote stage of *T. cruzi* (Table 1). Regarding the reference drugs to treat leishmaniasis and Chagas disease, miltefosine and benznidazole were evaluated for comparative purposes. Miltefosine showed IC_{50} of 2.64 μg/mL against L. *amazonensis*, whereas benznidazole showed an IC_{50} of 1.81 μg/mL against *T.cruzi*. The extract was active against the two tested strains, with a strong effect (IC_{50} = 17.13 μg/mL) against *L. amazonensis* and a moderate activity (IC_{50} = 99.93 μg/mL) against *T. cruzi*. These results prompted us to carry out a phytochemical study by bio-guided fractionation (Fig. 1, Table 1).

DCM fraction showed the highest activity against both parasites, exhibiting IC_{50} values of 20.59 and 59.56 μg/mL against L. *amazonensis* and *T. cruzi,* respectively. Therefore, the DCM fraction was subjected to CC to afford six fractions (A-F), sub-fraction D showed the best biological profile (IC_{50} 11.22 μg/mL on L. *amazonensis* and 22.24 μg/mL on

Table 1
Antiprotozoal activity on promastigote stage of *Leishmania amazonensis* and epimastigote forms of *Trypanosoma cruzi* of the extract, fractions, and sub-fractions from aerial parts of *P. battandieri*.

Samples	*L. amazonensis* IC_{50}[a] ± SD (μg/mL)	*T. cruzi* IC_{50} ± SD (μg/mL)
EtOH extract	17.13 ± 2.42	99.93 ± 7.84
Hexanes Fraction	> 100	> 100
DCM Fraction	20.59 ± 3.71	59.56 ± 2.20
Fr. A	> 50	> 50
Fr. B	> 50	> 50
Fr. C	> 50	> 50
Fr. D	11.22 ± 0.13	22.24 ± 2.14
SubFr. D1	> 50	> 50
SubFr. D2	> 50	> 50
SubFr. D3	> 50	> 50
SubFr. D4	7.77 ± 0.13	9.00 ± 0.86
SubFr. D4A	> 50	> 50
SubFr. D4B	> 50	> 50
SubFr. D4C	> 50	> 50
SubFr. D4D	> 50	> 50
SubFr. D4E	5.03 ± 0.03	36.91 ± 3.79
SubFr. D4F	6.27 ± 0.03	28.68 ± 4.90
SubFr. D4G	n.t.	n.t.
SubFr. D4H	3.60 ± 0.08	7.14 ± 1.12
SubFr. D5	> 50	> 50
SubFr. D6	> 50	> 50
SubFr. D7	> 50	> 50
Fr. E	> 50	> 50
Fr. F	> 50	> 50
EtOAc Fraction	> 100	> 100
n-BuOH Fraction	n.a.	n.a.
H_2O Fraction	n.a.	n.a.
Miltefosine[b]	2.64 ± 0.09	
Benznidazole[c]		1.81 ± 0.50

n.t.: not tested; n.a.: non active

[a] IC_{50}: concentration able to inhibit 50% of the growth of the parasites, and values are expressed as the mean ± standard deviation for three individual experiments.

[b] Miltefosine was usedd as a positive control for leishmanicidal activity.

[c] Benznidazole was usedd as a positive control for trypanocidal activity.

T. cruzi) and was further subjected to CC on silica gel. The sub-fraction D4 was the most active with IC_{50} values less than 10 μg/mL against both parasites, this was a promising indication to continue with the bio-guided fractionation.

In addition, the most active sub-fractions of D4 were D4E (IC_{50} 5.03 μg/mL and 36.91 μg/mL against L. *amazonensis* and *T. cruzi*, respectively) and D4H (IC_{50} 3.60 and 7.14 μg/mL against *L. amazonensis* and *T. cruzi,* respectively). Consequently, sub-fractions D4E and D4H were submitted to purification affording the known compounds (**1**–**5**), which were identified as panaxydiol (**1**) [21], falcarindiol (**2**) [22], 8-geranyloxypsoralen (**3**) [23], 8-geranyloxy-5-methoxypsoralen (**4**) [24], and phellopterin (**5**) [25] (Fig. 2) based on their NMR and MS spectroscopic data (Supplementary Materials Figs. S1-S20) and comparison with data reported in the literature. Moreover, compounds **1** and **2** showed a specific rotation of $[\alpha]^{20}_D$ -10.9 (c 0.11, $CHCl_3$) and $[\alpha]^{20}_D$ -25.4 (c 0.13, $CHCl_3$), respectively, establishing their structures as (−)-panaxydiol and (−)-falcarindiol. Compounds **1** and **2** are reported for the first time in *Pituranthos* genus.

Compounds isolated from bio-guided fractionation, except compounds **3** and **5** which were excluded due to low solubility in DMSO, were also assayed for their effect on promastigote forms of L. *amazonensis* and epimastigote forms of *T. cruzi.* (Table 2). The results showed that polyacetylenes **1** and **2** exhibited a potent activity against L. *amazonensis* exhibiting IC_{50} values of 5.76 and 5.68 μM, respectively. Significantly, their activity was similar to the reference drug miltefosine (IC_{50} = 6.48 μM). Furthermore, 8-geranyloxy-5-methoxypsoralen (**4**) showed a moderate leishmanicidal activity (IC_{50} 17.98 μM). In general, crude extract, fractions, sub-fractions, and pure compounds showed higher efficacy against promastigotes of L. *amazonensis* than against epimastigotes of *T. cruzi*. In fact, only compound **1** showed moderate trypanocidal activity (IC_{50} 23.24 μM) (Table 2). Moreover, compounds **1**, **2,** and **4** were tested against macrophage J774 cell line to determine their cytotoxicity (Table 2). These compounds did not affect significantly cell development and showed selectivity index value >2, which is considered selective against promastigotes of L. *amazonensis* [26]. To the best of our knowledge, this study provides the first report of panaxydiol (**1**) and falcarindiol (**2**) as antiprotozoal agents. Thus, overall, we consider that the bio-guided fractionation approach has been a useful tool due to its ability to isolate and concentrate active compounds in a

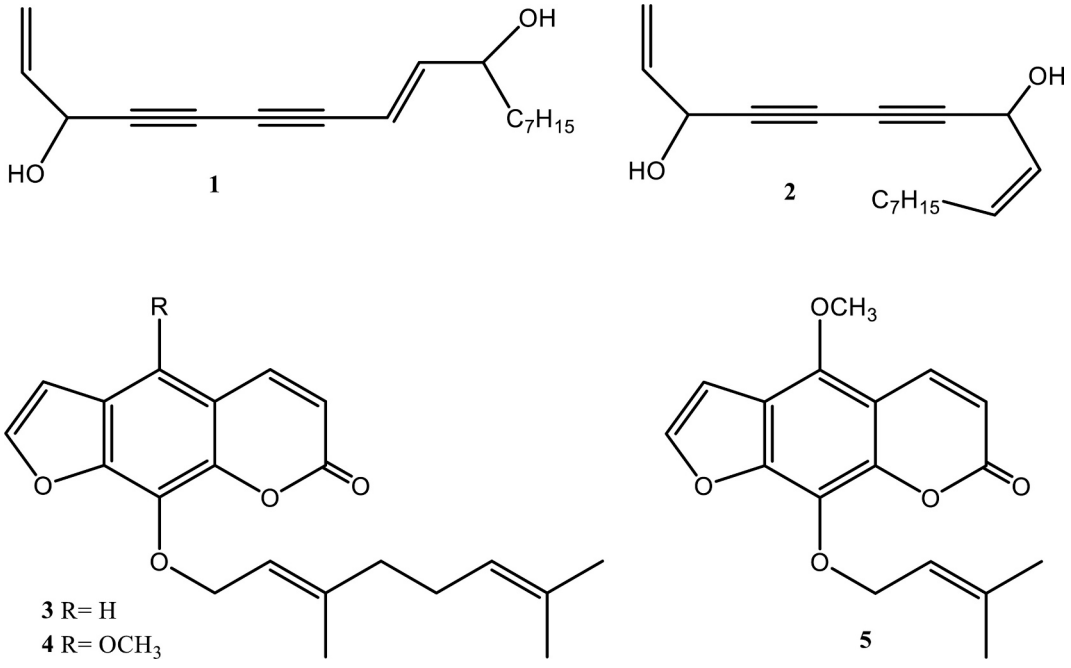

Fig. 2. Chemical structures of isolated polyacetylenes (**1**–**2**) and furanocoumarins (**3**–**5**) from aerial parts of *P. battandieri*.

Table 2
Antiprotozoal activity on promastigote stage of L. *amazonensis* and epimastigote forms of *T. cruzi*, and cytotoxicity activity on macrophage cell line of the isolated compounds[a] from aerial parts of *P. battandieri*.

Compounds	*L. amazonensis* IC_{50}[b] ± SD (μM)	*T. cruzi* IC_{50} ± SD (μM)	J774.A1 LC_{50}[c] ± SD (μM)	SI-L[d]	SI-T[e]
Compound **1**	5.76 ± 0.15	23.24 ± 2.34	44.09 ± 1.77	7.65	1.90
Compound **2**	5.68 ± 0.42	121.78 ± 6.76	40.71 ± 2.19	7.17	0.33
Compound **4**	17.98 ± 0.41	82.41 ± 6.69	68.66 ± 0.16	3.82	0.83
Miltefosine[f]	6.48 ± 0.22		72.18 ± 3.04	11.14	
Benznidazole[g]		6.95 ± 1.92	400.0 ± 4.0		57.55

[a] Compounds **3** and **5** were excluded due to their low solubility in DMSO.
[b] IC_{50}: Inhibitory concentration that inhibits 50% of the growth of the tested parasites, and values are expressed as the mean ± standard deviation for three individual experiments.
[c] LC_{50}: Lethal concentration that reduces 50% of the J774.A1 cell viability, and values are expressed as the mean ± standard deviation for three individual experiments.
[d] SI-L: Selectivity index (LC_{50} /IC_{50}) for L. *amazonensis*.
[e] SI-T: Selectivity Index (LC_{50} /IC_{50}) for *T. cruzi*.
[f] Miltefosine was used as a positive control for leishmanicidal activity.
[g] Benznidazole was used as a positive control for trypanocidal activity.

logical and rational way. The isolated compounds are expected to display higher antiprotozoal activity than the crude extract, as they would be more concentrated. Indeed, panaxydiol (**1**) showed a 11.4 and 16.5-fold increase in activity as compared with EtOH extract against L. *amazonensis* (IC_{50} 1.48 μg/mL *versus* 17.13 μg/mL) and *T. cruzi* (IC_{50} 6.05 μg/mL *versus* 99.93 μg/mL), respectively.

Our findings agree with previous studies revealing that the polyacetylenes show clinically significant antiprotozoal activities against species of *Leishmania* and *Trypanosoma* genus [27–29]. The results for compounds **1** and **2** provide new insights into this type of natural product and may aid the design of antiparasitic agents. As far as we know, there is only one study on the leishmanicidal properties of furanocoumarins [30].

4. Conclusion

The bioguided fractionation of the ethanol extract from the aerial parts of *P. battandieri* led to the isolation of the polyacetylenes (−)-panaxydiol (**1**) and (−)-falcarindiol (**2**) as the main bioactive compounds. These compounds showed similar potency against L. *amazonensis* to miltefosine, the reference drug, and a low toxicity on macrophage cell line J774. Furthermore, the good correlation observed between our results and those of previous works highlights the polyacetylenes as promising antiprotozoal agents. This further supports *P. battandieri* as a source for potential leishmanicidal and trypanocidal agents.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102300.

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