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In vitro cytotoxic and leishmanicidal activity of isolated and semisynthetic ent-pimaranes from Aldama arenaria

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Conflict of Interest: The authors declare that they have no conflict of interest.

Abstract:
Two pimaranes ent-pimara -8(14),15-dien-19-oic acid (1) and ent-8(14),15-pimaradien-3β-ol (2), isolated from Aldama arenaria, and six semi-synthetic derivatives methyl ester of the ent-pimara-8(14),15-dien-19-oic acid (3), ent-pimara-8(14),15-dien-19-ol (4), acetate of ent-pimara-8(14),15-dien-19-ol (5), ent-pimara-8(14),15-dien-19-ol succinic acid (6), acetate of ent-8(14),15-pimaradien-3β-ol (7), ent-8(14),15-pimaradien-3β-ol succinic acid (8) were evaluated in vitro for their cytotoxic activities to childhood leukemia cell lines and leishmanicidal activity against the parasite Leishmania amazonensis. Among these compounds, 1 to 6 presented moderate cytotoxic activity, with compound 4 being the most active (GI₅₀ of 2.6 µM for the HL60 line) and the derivatives 7 and 8 inactive. Against the parasite Leishmania amazonensis, the most promising derivative was acetate of ent-pimara-8(14),15-dien-19-ol (5), with EC₅₀ of 20.1 µM, selectivity index of 14.3, and significant reduction in the parasite load. Pimarane analogues 1, ent-pimara-8(14),15-dien-19-oic acid, and 2, ent-8(14),15-pimaradien-3β-ol, presented different activities, corroborating the application of such molecules as prototypes for the design of other derivatives that have greater cytotoxic or leishmanicidal potential.

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Multidisciplinary Center of Chemical, Biological and Agricultural Researches (CPQBA)

Paulínia, August 17th 2021

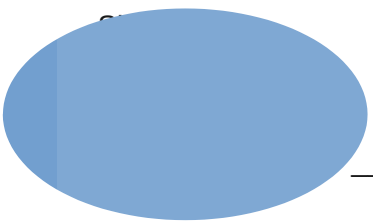
Dear Editor Oliver Kayser,

I am writing to present our manuscript entitled "*In vitro* cytotoxic and leishmanicidal activity of isolated and semi-synthetic ent-pimaranes from *Aldama arenaria*", by the authors Adriana da Silva Santos de Oliveira, Gabrielly Galdino Conrado, Natalia Grazzia, Danilo Ciccone Miguel, Gilberto Carlos Franchi Júnior and Vera Lúcia Garcia. We would be very grateful if this manuscript were considered for publication at Planta Medica.

This manuscript describes an original work and it is not under consideration by any other simultaneous journal and has not been previously published in any language elsewhere. All authors approved the manuscript as well as this submission.

In this work, we demonstrate unpublished results regarding the in vitro potential activities cytotoxic to childhood leukemia cell lines and leishmanicidal against the parasite Leishmania amazonensis. Two pimaranes [ent-pimara-8(14),15-dien-19-oic acid (1) and ent-8(14),15-pimaradien-3β-ol (2)], isolated from *Aldama arenaria*, and six semi-synthetic derivatives were evaluated. The results showed promising in vitro action of the pimaranes, corroborating the application of such molecules as prototypes for the design of other derivatives that have greater cytotoxic or leishmanicidal potential. It is likely to be of great interest to researchers, scientists, visionary companies and all others concerned with Planta Medica.

Thank you for your consideration of this manuscript.



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In vitro cytotoxic and leishmanicidal activity of isolated and semisynthetic *ent*-pimaranes from *Aldama arenaria*

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ABSTRACT

Two pimaranes *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*-8(14),15-pimaradien-3β-ol (**2**), isolated from *Aldama arenaria*, and six semi-synthetic derivatives methyl ester of the *ent*-pimara-8(14),15-dien-19-oic acid (**3**), *ent*-pimara-8(14),15-dien-19-ol (**4**), acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**), *ent*-pimara-8(14),15-dien-19-ol succinic acid (**6**), acetate of *ent*-8(14),15-pimaradien-3β-ol (**7**), *ent*-8(14),15-pimaradien-3β-ol succinic acid (**8**) were evaluated *in vitro* for their cytotoxic activities to childhood leukemia cell lines and leishmanicidal activity against the parasite *Leishmania amazonensis.* Among these compounds, **1** to **6** presented moderate cytotoxic activity, with compound **4** being the most active (GI_{50} of 2.6 µM for the HL60 line) and the derivatives **7** and **8** being inactive. Against the parasite *Leishmania amazonensis*, the most promising derivative was the acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**), with EC_{50} of 20.1 µM, selectivity index of 14.5, and significant reduction in the parasite load. Pimarane analogues **1**, *ent*-pimara-8(14),15-dien-19-oic acid, and **2**, *ent*-8(14),15-pimaradien-3β-ol, presented different activities, corroborating the application of such molecules as prototypes for the design of other derivatives that have greater cytotoxic or leishmanicidal potential.

Keywords: leishmanicidal activity, cytotoxic activity, *Leishmania amazonensis*, pimaranes, *Aldama arenaria* (syn. *Viguiera arenaria*), Asteraceae

Abbreviations:

Childhood leukemia cell lines: myeloid (K562), promyelocytic (HL60), acute T-cell (JURKAT, MOLT-4), Burkitt lymphoma (RAJI, RAMOS), B lymphoid (NALM-6), and acute lymphocytic leukemia (REH).

DE: dichloromethane extract of *A. arenaria* roots

GI_{50}: concentration for 50% of maximal inhibition of cell proliferation

EC_{50}: effective concentration needed to inhibit 50% of cell growth

CC_{50}: cytotoxic concentration capable of reducing 50% of the host cell population

MOI: multiplicity of infection

SI: selectivity index

Introduction

Aldama arenaria (Baker) E. E. Schill. & Panero, classified previously as *Viguiera arenaria* (Baker), Asteraceae, is a species found in the center-east of the state of São Paulo [1,2]. It has aromatic underground organs and can be chemically characterized by the presence of diterpene compounds of the pimarane type, class of secondary metabolites with numerous described biological activities, and sesquiterpene lactones [3-5]. Studies with isolated and semi-synthetic compounds of this species showed inhibitory action of the contraction of the vascular smooth muscle, *in vitro* activity against the protozoan *Trypanosoma cruzi*, capacity to reduce the influx of extracellular Ca^{2+} and consequent induction of hypotension in normotensive rats, vasorelaxant action, antibacterial, schistosomicidal and antiproliferative activity [6-13]. As a result, and continuing the research project of our working group, we evaluated pimaranes 1, 2 and six semi-synthetic derivatives.

Among the different types of cancers, leukemias comprise about 2.4% of incidents in the world, and statistics show the emergence of 437,033 new cases in 2018. It is one of the most common cancers, involving clonal neoplastic proliferation of immature cells or blasts of the hematopoietic system. It has two main subtypes identified on the basis of malignancy: lymphoid cells (B cells and T cells) or myeloid cells (granulocytic, erythroid, and megakaryocytic cells); and whether the disease is initially chronic or acute. In 2018 alone, when added together, all types of leukemia caused around 309,006 deaths worldwide. In Brazil, as in developed countries, cancer among children and adolescents aged 1 to 19 years represents the leading cause of death from diseases (8% of the total). Currently, mainly due to the significant progress in the treatment of cancer in childhood, around 80% of children and adolescents can be cured, if diagnosed early and treated in specialized centers [14].

They are usually more aggressive cancers, with few treatment options and, thus, poor prognosis. The main therapy for childhood leukemia is chemotherapy, but other treatments include transplantation, specific drugs, immunotherapy, surgery, and radiotherapy. Side effects such as hair loss, mouth sores, loss of appetite, diarrhea, nausea, and vomiting are commonly observed [15]. Some drugs widely used in the treatment of these patients come

from a plant species, such as: vincristine, etoposide, and teniposide [16-18]. These worrying data show that we must always look for new, innovative, more efficient, and less aggressive drugs, to ensure an ever greater chance of cure and survival for patients.

Leishmaniasis is a disease typical of poor countries, which affects more than 12 million people worldwide. It is a Neglected Tropical Disease (NTD) transmitted by different species of infected sandflies (genus *Phlebotomus*) and is defined as a set of parasitic diseases caused by protozoa of the genus *Leishmania*. It is known that more than 90 species of sandflies transmit these parasites, and it is estimated that, annually, there are 0.7 to 1 million new cases, of which 20 to 30 thousand result in deaths. Medications available for the treatment of leishmaniasis are few and have serious side effects and resistance, such as pentavalent antimonials. In the case of visceral Leishmania, other drugs can be used, such as liposomal amphotericin B and oral miltefosine, but these also present problems, such as high cost and teratogenic effect [19-20]. In Brazil, this disease is directly related to geographic regions, and the most common species are *Leishmania (Vianna) braziliensis* and *Leishmania amazonensis* [21].

In its biological cycle, the parasite has two morphological forms: promastigote, a flagellate form found in the female of the insect vector (invertebrate host), and amastigote, present in mammalian hosts. The amastigote forms are intracellular parasites commonly found in macrophages and are located in the phagolysosome or parasitophorous vacuole, where they multiply [22].

Knowing the high impact that these diseases have on society and the importance of studying substances isolated from plant species, this study aimed to evaluate *in vitro* isolated and semi-synthetic pimaranes of *A. arenaria* (Figure 1) against the leishmanicidal [*Leishmania amazonensis* parasite] and cytotoxic activities to childhood leukemia cell lines: myeloid (K562), promyelocytic (HL60), acute T-cell (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH).

[FIGURE 1]

Results and discussion

The yield obtained for the dichloromethane extract of the roots (DE) of *A. arenaria* in the turbo extraction was 14.1% (m/m, 42,36 g), a yield higher than that observed by Ambrosio et al. (2004) in their ultrasound extraction [3]. This extract presented the compounds **1** and **2** as major compounds by gas chromatography coupled to a mass spectrometry detector (GC-MS). Isolation of pimaranes **1** and **2** from DE by flash chromatography was efficient, with yields of 15.99 and 7.54 % (m/m, 3,52 g and 1,66 g, respectively) and purity greater than 95%, respectively, proving to be a fast, appropriate, and economic technique.

The characterization of pimaranes **1** and **2** was performed by comparing the spectroscopic data obtained with those described in the literature, and served as a reference to confirm the structures of semi-synthetic derivatives **3** to **8** (Tables 1, 2, and 3) [10,11,25-29].

The fragmentation pattern obtained in the mass spectra of these compounds is characteristic of pimaranes, presenting as base peak the fragments of *m/z* 121.10, 135.10 and 257.2 [30].

Regarding the synthesis of pimarane derivatives, the proposed synthetic routes were considered easy to perform and satisfactory, as their yields ranged from 80 to 100% (Figures 2 and 3).

[FIGURES 2 AND 3]

The pimaranes **1** (*ent*-pimara-8(14),15-dien-19-oic acid), **2** (*ent*-8(14),15-pimaradien-3-β-ol alcohol), **4** (*ent*-pimara-8(14),15-dien-19-ol alcohol), and **7** (derived in C-3 *ent*-8(14),15-pimaradien-3β-acetoxy) were described in the species *A. arenaria* and *Gnaphalium gaudichaudianum* and biologically explored for antibacterial, vasorelaxant, trypanocidal, and cytotoxic activities [6-13,23].

Derivatives **3** and **4**, obtained by microbial transformation, were evaluated for their capacity to inhibit vascular smooth muscle contraction, its spasmolytic effects, and its anticariogenic activities [24,25]. No activities were described for derivatives **5**, **6**, and **8**.

The cytotoxic activity of pimaranes **1** to **8** was evaluated *in vitro* for cell lines myeloid leukemia (K562), promyelocytic leukemia (HL60), acute T-cell leukemia (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH). Table 4 shows the GI_{50} values (concentration necessary to inhibit 50% of cell growth) obtained for the different samples and different lines. Vincristine was used as a reference chemotherapeutic drug.

Pimaranes with different functional groups at C19 (**1**, **3**, **4**, **5**, and **6**) had similar cytotoxicity values, with GI_{50} ranging from 2.6 ± 0.5 to 11.5 ± 0.1 µM. Derivative **4**, *ent*-pimara-8(14),15-dien-19-ol, was the most promising and presented GI_{50} of 2.6 ± 0.5 µM for the HL60 cell line.

Among the molecules with different substituents in C3 (**2**, **7**, and **8**), the compound **2** was the most active of all, with GI_{50} ranging from 4.2 ± 1.0 to 7.8 ± 0.7 µM.

Among the evaluated pimaranes, derivatives **7** and **8** were the least active, with EC_{50} > 15 µM; in this case, the addition of an acetate function or a succinate radical in C3 significantly reduced the cytotoxic activity for all lines tested, compared to **2**.

The main objective of antineoplastic chemotherapy is to exterminate cancer cells; however, this cannot always be done in a directional and selective way, almost always damaging healthy cells. This is because these drugs act in a non-specific way, harming both normal and malignant cells. The combination of several chemotherapeutic agents and their efficiency has been shown to be a beneficial tool for the treatment of malignant tumors, with cure rates of 75 to 90% in different types of cancer [31].

Additionally, compound **4** was evaluated for B lymphoid leukemia (NALM-6), Burkitt lymphoma (RAMOS), acute T-cell leukemia (MOLT-4), myeloid leukemia (K562), promyelocytic leukemia (HL60), and acute lymphocytic leukemia (REH) lines to verify whether this molecule had selectivity (Table 5).

The results obtained showed that compound **4** does not present any selectivity when evaluated for these other lines, with EC_{50} > 19 µM.

These data indicate moderate action of pimarane derivatives **1** to **6** isolated from *A. arenaria* and semi-synthetic ones, with a concentration-dependent profile and little selectivity among the evaluated lines.

Leishmanicidal activity was evaluated *in vitro* against the promastigote forms of *L. amazonensis* (MHOM/BR/73/M2269). About 20 described species can be associated with clinical manifestations in humans [32].

The initial screening was performed with pimaranes (**1** to **8**) at concentrations of 50 and 100 µg/mL (Figure 4).

[FIGURE 4]

This assay revealed that only pimaranes **1**, **2**, **3**, **5**, and **7** were able to decrease the cell viability of the parasites at rates lower than 30%, showing mortality of promastigote parasites in more than 82% for some samples. These samples were evaluated against parasites at different concentrations (0.15 to 660 µM), resulting in sigmoidal concentration curves versus parasite cell viability (Figure 5).

[FIGURE 5]

Through sigmoidal regression analysis of these data, it was possible to calculate the effective concentration needed to inhibit 50% of cell growth (EC_{50}) for pimaranes **1**, **2**, **3**, **5**, and **7** (Table 6).

Among the most active samples, compound **5** was the most promising derivative, with an EC_{50} from 16.02 to 24.82 µM. When we compare its activity with its precursor, pimarane **1**, we see an increase in action due to the addition of an acetate in C19. In the case of derivative **7,** we can see a less pronounced action when this substituint is positioned in C3.

Comparing the mean EC_{50} values obtained, we conclude that only derivative **5** has a potential leishmanicidal action *in vitro* (Figure 6).

[FIGURE 6]

The cytotoxicity of these molecules was evaluated in fibroblast cells (L929) and in macrophages and compared by the selectivity index, determined as the ratio between the

CC_{50} for macrophages and the EC_{50} for *L. amazonensis*. A selectivity greater than 100 times is expected for pathogens, however, such higher rates are quite uncommon [32] (Table 8).

Among the pimaranes evaluated, only acetate **5** showed potential action against parasite *L. amazonensis*, with a selectivity index greater than 10, compared to the macrophage and low cytotoxicity regarding the L929 line.

In vitro infection was performed with compound **5** in macrophages extracted from the femur and tibia of Black mice in a 24-well plate, at four concentrations from 4.73 to 37.82 µM. The micrographs obtained from the infection (Figure 7) showed that the coverslips containing only the infected macrophages (control) had a high degree of infection, represented by the circumferences with a diameter of less than 5 µM (arrowhead in Figure A). In Figure B, one can observe healthy macrophages and the absence of intracellular parasites. The photomicrographs of the coverslips can be seen in Figure 7.

[FIGURE 7]

By counting the numbers of macrophages, infected macrophages, and amastigote parasites, it was possible to plot the graphs that are shown in Figures 8 to 10.

Assessing the number of amastigotes per 100 macrophages, we verified a small reduction in the number of infected macrophages at the highest concentrations (18.91 and 37.82 µM), compared to the untreated control group. In this case, there was no significant difference in infection between the two highest concentrations. This profile is also observed when we verify the percentage of infection (Figure 8).

[FIGURES 8 AND 9]

Regarding the infectivity index, we can infer that pimarane 5 contributed to the reduction of the parasite load when evaluated at the highest concentrations (Figure 10), reducing macrophage infection by more than 50% compared to the untreated control. This inhibition is quite significant for a patient, because macrophage cells are the main cells infected by parasites of the genus *Leishmania*, playing a crucial role in the initial immune response to infections.

[FIGURE 10]

The results of the *in vitro* evaluation of macrophage infection allow us to conclude that pimarane **5** was active against *L. amazonensis* amastigotes at concentrations 18.91 and 37.82 µM.

Conclusion

This study has shown that pimaranes isolated from *A. arenaria* and their semi-synthetics derivatives have *in vitro* cytotoxic action in childhood leukemia lines and leishmanicidal activity when evaluated for the *L. amazonensis* line.

Among the pimaranes evaluated, the alcohol **4**, *ent*-pimara-8(14),15-dien-19-ol, presented greater cytotoxicity, with an GI_{50} of 2.6 for the promyelocytic leukemia (HL60) cell line, without showing any specific selectivity. The most promising pimarane against *L. amazonensis* parasites was the acetate from *ent*-pimara-8(14),15-dien-19-ol (**5**), with an $EC_{50}$of 20.1 µM and selectivity index of 14.5.

Pimarane analogues *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*- 8(14),15-pimaradien-3β-ol (**2**) presented different activities, indicating that these precursor molecules can be considered prototypes for the planning of other derivatives that have greater cytotoxic or leishmanicidal potential. Additionally, other *in vitro* and *in vivo* studies are needed to unravel their mechanisms of action and toxicities.

Material and methods

Plant material

Plant material was collected from Ecological Station of Itirapina, São Paulo, Brazil (22°14'S, 47°51'W) in its natural state. The identification was carried out by the botanist Mara Angelina Galvão Magenta, and the exsiccate was deposited in the herbarium of the "Luiz de Queiroz" School of Agriculture – ESALQ-USP, under number 111847 and study permits from CGEN, process no. 010216/2012-0 and SisGen A7C6E5C. This matrix plant provided seeds used

for the production of 20 seedlings that were cultivated for 2 years in the experimental field of CPQBA/UNICAMP and processed for the production of extracts.

Extraction and isolation of *ent*-pimara-8(14),15-dien-19-oic acid (1) and *ent*- 8(14),15-pimaradien-3β-ol (2) from the roots of *A. arenaria*

Fresh roots of *A. arenaria* were ground and extracted in an Ultra Turrax (IKA) disperser at room temperature with dichloromethane (1g/10mL) for 5 min, followed by filtration and re-extraction of the plant residue with two more portions of dichloromethane. The pooled extracts were evaporated under reduced pressure, providing the crude dichloromethane extract (DE).

DE (22 g) was fractionated in a medium pressure liquid chromatograph (CombiFlash Rf+ /Teledyne ISCO) in a chromatographic column containing 220 g of silica gel 60 (High Performance Gold, spherical particle 20-40 µm and pore size of 60Å), gradient of hexane and ethyl acetate with mobile phase, flow rate of 50 mL/min, and wavelength of 254 nm. 1020 fractions of 18 mL each were collected. The resulting fractions were analyzed by thin layer chromatography (TLC), pooled according to similarity and evaporated under reduced pressure. The pooled fraction enriched in pimarane **1** was crystallized in dichloromethane in a freezer, filtered, and vacuum dried. CG-MS ions (*m/z*) [relative intensity, %]: 302.20 [34]; 123.10 [34]; 121.10 [100]; 91.10 [40]; 79.10 [33]. ^{1}H NMR data: δ= 0.66 (s, 3H, 10-CH_3), 1.01 (s, 3H, 13-CH_3), 1.27 (s, 3H, 4-CH_3), 4.91 (dd, 1H), 4.96 (dd, 1H), 5.16 (s, 1H), 5.72 (dd, 1H). ^{13}C NMR: δ = 184.3 (C-19), 147.2 (C-15), 137.9 (C-8), 128.0 (C-14), 112.9 (C-16), 56.1 (C-5), 50.5 (C-9), 44.0 (C-4), 39.2 (C-1), 39.2 (C-10), 38.5 (C-13), 37.9 (C-3), 35.8 (C-7), 36.4 (C-12), 29.3 (C-17), 29.2 (C-18), 24.1 (C-6), 19.6 (C-11), 19.2 (C-2), 13.8 (C-20).

To obtain pimarane **2**, fractions from the DE flash chromatography containing large amounts of pimarane alcohol were pooled and methylated with the reagent TMSD (trimethylsilyl diazomethane). Because of the similar Rf values, this reaction is necessary for the conversion of the acid **1** to the correspondent ester ensuring the isolation of alcohol. The reaction remained under stirring for 2h. After completion of the reaction, confirmed by TLC,

the sample was vacuum dried. This sample enriched in pimarane **2** (4.9g) was fractionated again in a medium pressure liquid chromatograph in a chromatographic column containing 80 g of silica gel 60 (High Performance Gold, spherical particle 20-40 µm and pore size of 60Å), gradient of hexane and ethyl acetate with mobile phase, flow rate of 10 mL/min, and wavelength of 254 nm. 322 fractions of 15 mL each were collected. The resulting fractions were analyzed by TLC, pooled according to similarity and vacuum evaporated. The pooled fraction enriched in compound **2** was solubilized at heat (45 ºC) and then crystallized in methanol at room temperature. The crystallisate from pimarane **2** was centrifuged and washed with cold methanol. After separating the supernatant, the precipitate was vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 288.20 [8]; 270.20 [11]; 255.20 [17]; 135.10 [100]; 91.10 [22]. ^{1}H NMR: δ = 0.83 (s, 3H, 4-CH_3), 1.02 (s, 3H, 4- CH_3), 1.07 (s, 3H, 10-CH_3), 1.17 (s, 3H, 13-CH_3), 3.3 (dd, 1H), 4.89 (dd, 1H), 4.96 (dd, 1H), 5.16 (d, 1H), 5.74 (dd, 1H). ^{13}C NMR: δ = 147.3(C-15), 137.9 (C-8), 128.1 (C-14), 112.8 (C-16), 79.2 (C-3), 54.1 (C-5), 51.2 (C-9), 39.0 (C-4), 38.6 (C-13), 38.1 (C-10), 37.1 (C-1), 35.7 (C-7), 35.7 (C-12), 29.4 (C-17), 28.4 (C-18), 27.5 (C-2), 22.1 (C-6), 19.1 (C-11), 15.7 (C-19), 14.7 (C-20).

Obtaining the derivatives of *ent*-pimara-8(14),15-dien-19-oic acid (1) and *ent*-8(14),15-pimaradien-3β-ol (2)

Methyl ester of *ent*-pimara-8(14),15-dien-19-oic acid (**3**): 502.9 mg of pimarane **1** was weighed into a 50 mL flask and 10 mL of a mixture of dichloromethane and methanol (9:1) was added. 2.50 mL of the TMSD reagent (trimethylsilyl diazomethane) was added to the reaction medium and the system was left under stirring for 2h. After completion of the reaction, confirmed by TLC, the sample was vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 316.20 [15]; 257.20 [14]; 180.10 [26]; 121.10 [100]; 91.10 [18].

***ent*-pimara-8(14),15-dien-19-ol** (**4**): In an inert atmosphere, 50 mL of anhydrous ethyl ether and 200.6 mg of lithium and aluminum hydride were added to a 250 mL flask. In another

container, 503.0 mg of derivative **3** were solubilized in 5 mL of anhydrous ethyl ether. The solubilized derivative **3** was then added slowly to the reaction medium. The system remained under heating at 40 ºC and stirring for 1h. After completion of the reaction, confirmed by TLC, 10 mL of a mixture of ethyl ether and distilled water (1:1) were added, followed by 2 mL of distilled water. The reaction medium was filtered under cotton, and anhydrous sodium sulfate was added to the ether phase. The organic phase was filtered again and vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 288.30 [6]; 257.20 [100]; 135.10 [20]; 121.10 [21]; 91.10 [26].

Acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**): In an inert atmosphere, 150.5 mg of derivative **4** and 40 mL of dichloromethane were added to a 125 mL flask. A further 2 mL of pyridine, 103.7 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 1.3 mL of acetic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 1h. After completion of the reaction, confirmed by TLC, 25 mL of distilled water and 1 M hydrochloric acid solution were added until pH 3. The reaction medium was partitioned with 50 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 330.30 [18]; 257.20 [91]; 135.10 [100]; 93.10 [38]; 43.10 [41].

***ent*-pimara-8(14),15-dien-19-ol succinic acid** (**6**): In a 125 mL flask and inert atmosphere, 141.5 mg of derivative **4** and 40 mL of dichloromethane were added. A further 4 mL of pyridine, 205.5 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 3.05 g of succinic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 78h. After completion of the reaction, confirmed by TLC, 1 M hydrochloric acid solution was added until pH 3. The reaction medium was partitioned with 50 mL of dichloromethane. The extraction was repeated three more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered

and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 288.20 [5]; 257.2 [100]; 135.10 [21]; 121.10 [24]; 91.10 [28].

Acetate of *ent*-8(14),15-pimaradien- 3β-ol (**7**): In a 125 mL flask and inert atmosphere, 240.8 mg of pimarane **2** and 75 mL of dichloromethane were added. Additional 3.2 mL of pyridine, 173.4 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 2.10 mL of acetic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 1h. After completion of the reaction, confirmed by TLC, 1 M hydrochloric acid solution was added until pH 2. The reaction medium was partitioned with 100 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 330.30 [8]; 255.20 [29]; 135.10 [100]; 119.10 [23]; 43.10 [31].

***ent*-8(14),15-pimaradien-3β-ol succinic acid** (**8**): 262.0 mg of pimarane **2** was solubilized in 80 mL of dichloromethane in a 250 mL flask. A further 7.5 mL of pyridine, 380.5 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 8.01 g of succinic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 144h. After completion of the reaction, confirmed by TLC, 200 mL of distilled water were added and the medium acidified with 1 M hydrochloric acid until pH 2. The reaction medium was partitioned with 100 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 288.30 [7]; 255.20 [16]; 135.10 [100]; 121.10 [14]; 91.10 [21].

Structural determination of pimaranes

The structural determination of pimaranes **1** to **8** was carried out by the analysis of ^{1}H and ^{13}C NMR spectroscopic data, mass spectra, and data described in the literature [19-23].

Evaluation of *in vitro* cytotoxic activity

The cytotoxicity of pimaranes **1** to **8** was evaluated *in vitro* in childhood leukemia cell lines from ATCC (American Type Culture Collection), namely: myeloid leukemia (K562), promyelocytic leukemia (HL60), acute T-cell leukemia (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH), To assess the cytotoxic potential, samples diluted in dimethyl sulfoxide (100 mg/mL) were used. This first dilution was at the maximum concentration of 0.1% DMSO, and, in each well, the concentration reached a maximum of 0.03%, not representing cytotoxicity. Cell viability was assessed by the MTT method (3-[4,5-dimethyl-triazol-2-yl]-2,5-diphenyltetrazolium bromide) in 96-well plates [33].

Cells were cultured in appropriate plastic bottles with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% inactivated fetal bovine serum (FBS) for one hour at 56°C, 10 mM of 4-(2-acid) hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 $g.L^{-1}$ of sodium bicarbonate, 1% of penicillin G (100 U/mL), 100 mg/mL of streptomycin, and 50 µg/mL of amphotericin B in an oven with 5% CO_2 atmosphere at 37ºC. Before the experiments, the number of viable cells was determined by the trypan blue exclusion method.

The cells were plated using serial dilution and the epMotion 5070 equipment (Eppendorf, Vaudaux, Schonenbuch, Switzerland), which distributed 2×10^4 cells per well (96-well plates) and added the substances in different concentrations (0.01 to 1000 µg/mL) for 48 h at 37ºC and 5% CO_2. The chemotherapy drug vincristine was used as a positive standard control. After the treatment period, the culture medium was removed by centrifugation and discarded, 100 µL of MTT solution (0.5 mg/mL) was added, and the plate was incubated again for 4h. After this period, the medium was removed and the formazan precipitate was dissolved into 100 µl DMSO per well. The plate was then analyzed in a microplate reader at 540 nm (Bio-

Tek Power Wave XS) for optical density reading. The control group of untreated cells, incubated with the growth medium only, was considered as 100% of viable cells for calculation of dose-response curves and, consequently, to calculate the GI_{50} (concentration needed to inhibit 50% of cell growth) using sigmoidal regression in the Graphpad Prism 5.0 software for Windows (GraphPad Software), according to the equation: Relative cell viability = (sample absorbance / control absorbance) x 100. The samples were evaluated in triplicate, and the results were expressed as mean ± standard deviation by Excel 2016 software (Microsoft).

Evaluation of *in vitro* leishmanicidal activity in promastigote forms

Promastigote of *L. amazonensis* (strain MHOM/BR/73/M2269) kindly donated by Prof. Dr Silvia Uliana (University of São Paulo, Brazil) were cultured in 25 mL flask with 5 mL of medium 199 (Sigma-Aldrich) supplemented with 50 units/mL penicillin, 0.1 mM adenine, 50 µg/mL streptomycin, pH 7.4, 40 mM HEPES, 0.0001% biotin, 0.0005% hemin, and 10% fetal bovine serum (FBS-Vitrocell).

The samples were diluted in dimethyl sulfoxide (10 mg/mL, maximum final concentration of 0.07 %) and submitted to the biological activity test according to the MTT method (3-[4,5-dimethyl-triazol-2-yl]-2,5-diphenyltetrazolium bromide) in 96-well plates [19,34,35]. In this procedure, the culture of *L. amazonensis* containing 2.5×10^6 promastigote parasites in logarithmic phase and maintained in culture medium 199 was evaluated against the sample in different concentrations (50 to 100 µg/mL), for 24 hours. After this period, 30 µL of an MTT solution (3-[4,5-dimethyl-triazol-2-yl-2,5-diphenyltetrazolium, 5 mg/mL) was added to each well and the plates were incubated in the oven at 26 °C (2 hours). To lyse the cells, 30 µL of SDS (20% sodium dodecyl sulfate) was added, and the absorbance was read in a microplate reader (595 nm). Results were expressed as percentages of cell viability compared to the group of untreated parasites (control, equivalent to 100%).

Defining the most promising samples, cell mortality above 20%, cell viability curves were drawn with at least 6 concentrations and at least three independent assays. Using sigmoidal

regression in the Origin software, it was possible to calculate the effective concentration for a 50% reduction in cell viability (EC_{50}) for each sample.

In vitro cytotoxicity assessment for fibroblast and macrophage cell lines

The fibroblast culture maintained with 20 mL of complete RPMI medium was transferred to falcon tube, washing with complete RPMI medium. The solution was centrifuged at 1000 xg and 4°C for 5 minutes. The supernatant was discarded and an additional 10 mL of RPMI medium was added. The number of cells was determined in a Neubauer chamber, with 2×10^5 cells per well, and the samples were evaluated according to the MTT method described above.

The macrophages used for cytotoxic evaluation were differentiated from bone marrow precursor cells extracted from the tibia and femur of mice. These cells were cultured in 7.5 cm diameter plates containing 10 mL R2020 medium, modified from RPMI Lonza medium, containing: RPMI, 20% fetal bovine serum, 20% L929 fibroblast supernatant and gentamicin, and kept in an oven at 37°C with 5% CO_2 atmosphere. After 4 days, another 5 mL of R2020 medium was added and, in 7 days, the macrophages had already gone through the differentiation process and were ready for plating. Macrophages adhered to the bottom of the plate were removed with a sterile cell scraper (Corning), collected in RPMI medium, centrifuged at 1000G for 5 minutes at 4°C, and resuspended in R105 medium (RPMI + 10% FBS + 5% of L929 supernatant). The number of cells was determined in a Neubauer chamber, with 2×10^5 cells per well in the cytotoxic analysis and 5×10^5 cells in the *Leishmania* infection process, according to the MTT method.

Evaluation of *in vitro* infection of macrophages with *L. amazonensis*

To assess the viability of intracellular amastigote parasites after exposure to the pimaranes under study, macrophage cells were infected with *L. amazonensis* promastigotes. Macrophages were derived from bone marrow precursor cells obtained from C57BL/6J mice provided by the "Multidisciplinary Center for Biological Investigation on Laboratory Animal

Science" at the University of Campinas (CEMIB-Unicamp). Briefly, 5×10^5 macrophages were cultured on coverslips in 24-well plates and infected for 24h before being exposed to the test sample. Infection schemes followed multiplicities of infection (MOI) equivalent 5 to 10 stationary phase promastigotes per macrophage. An untreated group was used as a negative control. Infections were analyzed under immersion with an optical microscope, considering three parameters: total percentage of infected cells; number of parasites per 100 macrophages; and infectivity index, which combines the two previous parameters (infectivity index = percentage of infected macrophages multiplied by the average number of amastigotes for 100 macrophages).

Supplementary information

^{1}H and ^{13}C NMR spectra were obtained on a Bruker 400 operating at 400 and 100 MHz, respectively, using $CDCl_3$ as solvent and TMS as reference. Data from Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) analyses of compounds **1**-**8**, and dose-response curves from the biological tests can be request as supplementary information.

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Conflict of Interest

The authors declare no conflict of interest regarding the authorship and/or publication of this article.

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Legends for Figures

Figure 1. Isolated pimaranes from *A. arenaria ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*- 8(14),15-pimaradien-3β-ol (**2**) and semi-synthetic ones (**3** to **8**).

Figure 2. Synthesis scheme for obtaining semi-synthetic derivatives **3** to **6**.

Figure 3. Synthesis scheme for obtaining semi-synthetic derivatives **7** and **8**.

Figure 4. Cell viability graph obtained from the initial screening of pimaranes **1** to **8** for *L. amazonensis* promastigote parasites.

Caption – Each bar represents the mean ± standard deviation of cell viability for three independent experiments.

Figure 5. Cell viability curves of *L. amazonensis* promastigote parasites versus concentration log (μM) obtained for pimaranes **1**, **2**, **3**, **5**, and **7**.

Caption – Each bar represents the mean ± standard deviation of cell viability for three independent experiments.

Figure 6. Graph of EC_{50} (μM) obtained for pimaranes **1**, **2**, **3**, **5**, **7** and amphotericin against *L. amazonensis* promastigote parasites.

Caption – EC_{50}: effective concentration needed to inhibit 50% of cell growth; AB: amphotericin B. Each bar represents the mean EC_{50} for three independent experiments. Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 7. Photomicrographs of coverslips obtained from the infection and *in vitro* treatment of macrophages infected with *L. amazonensis* amastigotes and treated with derivative **5**. **A** – Infection control; **B** – Macrophages treated with pimarane **5** (37.82 μM). Bar = 5 micrometers.

Figure 8. Number of amastigotes per 100 macrophages versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 9. Percentage of infected macrophages versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 10. Infectivity index versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Table 1. ^{1}H NMR data of pimaranes **3**-**6** ($CDCl_3$, 400 MHz, δ in ppm).

Position	3	4	5	6
4-CH_3	1.20 (*s*, 3H)	1.05 (*s*, 3H)	0.98 (*s*, 3H)	0.98 (*s*, 3H)
10-CH_3	0,55 (*s*, 3H)	0.75 (*s*, 3H)	0.75 (*s*, 3H)	0.75 (*s*, 3H)
13-CH_3	1.00 (*s*, 3H)	1.00 (*s*, 3H)	0.97 (*s*, 3H)	0.97 (*s*, 3H)
14	5.14 (*s*, 1H)	5.16 (*s*, 1H)	5.18 (*s*, 1H)	5.17 (*s*, 1H)
15	5.71 (*dd*, 1H)	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)
16	4.91 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)
16	4.94 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)
19	-	3.40 and 3.80 (Ha and Hb)	3.90 and 4.30 (Ha and Hb)	3.90 and 4.35 (Ha and Hb)
19-$OCOCH_3$	-	-	2.10 (*s*, 3H)	-
19-$OCO(CH_2)_2CO_2H$	-	-	-	2.70 (*dt*, 2H)
19-$OCO(CH_2)_2CO_2H$	-	-	-	2.62 (*dt*, 2H)
4-$COOCH_3$	3,63(s, 3H)	-	-	-

Table 2. ^{1}H NMR data of pimaranes **7** and **8** ($CDCl_3$, 400 MHz, δ in ppm).

Position	7	8
4-CH_3	0.87 (*s*, 3H)	0.75 (*s*, 3H)
4-CH_3	0.87 (*s*, 3H)	0.87 (*s*, 3H)
10-CH_3	0.75 (*s*, 3H)	0.87 (*s*, 3H)
13-CH_3	0.98 (*s*, 3H)	1.00 (*s*, 3H)
3	4.50 (*dd*, 1H)	4.55 (*dd*, 1H)
16	4.90 (*dd*, 1Ha)	4.96 (*dd*, 1Ha)
16	4.96 (*dd*, 1Ha)	4.96 (*dd*, 1Ha)
14	5.18 (*s*, 1H)	5.20 (*s*, 1H)
15	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)
3-$OCOCH_3$	2.10 (*s*, 3H)	-

3-OCO(CH$_2$)$_2$COOH	-	2.70 (2H)
3-OCO(CH$_2$)$_2$COOH	-	2.68 (2H)

Table 3. ^{13}C NMR data of pimaranes **3**-**8** ($CDCl_3$, 100 MHz, δ in ppm).

Carbon	3	4	5	6	7	8
1	38,2	39.2	39.0	39.0	38.0	37.9
2	19,2	18.6	18.5	18.5	28.4	29.0
3	38,2	35.4	35.7	35.7	81.0	81.7
4	44,1	38.2	38.2	37.1	37.9	38.0
5	56,1	55.7	55.7	55.7	54.2	54.2
6	24,3	22.4	22.5	22.4	21.3	21.9
7	35,8	37.7	36.0	36.0	35.6	35.6
8	138,0	138.0	137.9	137.8	137.8	137.7
9	50,5	51.4	51.5	51.5	51.1	51.1
10	39,2	35.7	38.6	38.2	38.3	38.6
11	19,6	19.2	19.3	19.3	19.1	19.1
12	36,4	36.1	36.2	36.2	35.6	36.7
13	38,5	36.5	36.2	38.5	38.6	38.7
14	127,9	128.1	128.2	128.3	128.3	128.3
15	147,2	147.3	147.2	147.2	147.3	147.3
16	112,8	112.7	112.9	112.9	112.8	112.8
17	29,3	29.4	29.4	29.0	29.4	29.4
18	29,0	27.0	27.5	27.5	22.0	23.9
19	177,9	65.2	67.0	67.4	16.9	16.9
20	13,5	15.8	15.8	15.8	14.8	14.8
-OCO-	-	-	171.4	172.2	171.0	171.8
-OCO-CH$_3$	-	-	-	29.0	24.0	35.5
-CH$_2$CH$_2$-	-	-	-	28.9	-	29.7/29.0
-CH$_2$CH COOH-	-	-	-	177.7	-	177.8
OCH$_3$	51,2	-	-	-	-	-

Table 4. GI_{50} values (μM) of pimaranes **1** to **8** for different leukemia lines.

PIMARANES	CELL LINES / GI_{50} (MM)*			
	K562	**HL60**	**RAJI**	**JURKAT**
1	6.5 ± 2.6	11.5 ± 0.1	10.5 ± 0.4	8.6 ± 1.1
2	6.8 ± 0.9	4.2 ± 1.0	5,7 ± 0.3	7.8 ± 0.7
3	6.5 ± 0.4	4.7 ± 0.9	5.9 ± 0.8	4.5 ± 1.3
4	3.7 ± 1.3	2.6 ± 0.5	3.95 ± 0.5	4.4 ± 0.4
5	7.3 ± 0.3	5.1 ± 0.3	10.8 ± 2.7	7.4 ± 1.2
6	8.6 ± 1.0	8.1 ± 0.4	8.1 ± 0.2	7.9 ± 0.9
7	36.7 ± 4.5	33.7 ± 1.1	41.0 ± 0.8	40.2 ± 3.1
8	> 100	17.5 ± 1.0	> 100	20.1 ± 2.5
VINCRISTINE	47.6 ± 9.8	21.2 ± 0.4	NE	12.7 ± 0.2

Caption – GI_{50}: concentration necessary to inhibit 50% of cell growth; K562: myeloid leukemia; HL60: promyelocytGI leukemia; RAJI: Burkitt lymphoma; JURKAT: acute T-cell leukemia; Standard deviation for n=3; NE: Not evaluated.

Table 5. GI_{50} values (µM) of pimaranes **4** for the different leukemia lines.

	CELL LINES / IC_{50} (MM)*			
	NALM6	**RAMOS**	**MOLT4**	**REH**
4	22.5 ± 4.5	24.6 ± 4.9	19.4 ± 0.7	33.3 ± 1.9
VINCRISTINE	0.001 ± 0.0	0.121 ± 0.073	0.001 ± 0.0	0.001 ± 0.0

Caption – GI_{50}: concentration necessary to inhibit 50% of cell growth; B-lymphoid leukemia (NALM-6), Burkitt lymphoma (RAMOS), acute T-cell leukemia (MOLT-4), promyelocytic leukemia (HL60), and acute lymphocytic leukemia (REH); Standard deviation for n=3.

Table 6. EC_{50} values (µM) of pimaranes **1**, **2**, **3**, **5**, and **7** obtained for *L. amazonensis* promastigote parasites.

Compound	EC_{50} (µM) promastigotes			Mean and CI (µM)
1	126.2	149.9	79.7	114.7[a] (50.9 to 258.1)
2	59.2	66.7	38.0	53.1[b] (25.4 to 111.1)
3	65.2	44.8	70.5	59.1[b] (32.3 to 107.9)
5	18.6	25.5	17.3	20.1[b,c] (12.1 to 33.6)

7	35.8	53.0	56.7	47.5[b] (25.6 to 88.2)
AB	0.09	0.02	0.08	0.05[c] (0.005 to 0.49)

Caption – EC_{50}: effective concentration needed to inhibit 50% of cell growth; CI: 95% Confidence Interval obtained for a minimum of n=3 independent tests; Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Numbers followed by the same letter are statistically similar.

Table 7. Selectivity index (SI) and EC_{50} values (μM) of pimaranes **1** to **3**, **5**, and **7** for promastigote parasites, *L. amazonensis* macrophages, and L929 fibroblast cells.

Pimarane	Promastigotes EC_{50} (μM)	Macrophages CC_{50} (μM)	Selectivity Index (SI)	Fibroblasts L929 CC_{50} (μM)
1	114.7	100.3	0.9	87.6
2	53.1	208.2	3.9	136.2
3	59.1	229.8	3.9	189.7
5	20.1	292.0	14.5	215.5
7	47.5	208.9	4.4	165.2
AB	0.05	1.99	39.80	1.36

Caption – EC_{50}: effective concentration necessary to inhibit 50% of cell growth in the promastigote parasite; CC_{50}: cytotoxic concentration capable of reducing 50% of the host cell population; AB: amphotericin B.

In vitro cytotoxic and leishmanicidal activity of isolated and semisynthetic *ent*-pimaranes from *Aldama arenaria*

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ABSTRACT

Two pimaranes *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*-8(14),15-pimaradien-3β-ol (**2**), isolated from *Aldama arenaria*, and six semi-synthetic derivatives methyl ester of the *ent*-pimara-8(14),15-dien-19-oic acid (**3**), *ent*-pimara-8(14),15-dien-19-ol (**4**), acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**), *ent*-pimara-8(14),15-dien-19-ol succinic acid (**6**), acetate of *ent*-8(14),15-pimaradien-3β-ol (**7**), *ent*-8(14),15-pimaradien-3β-ol succinic acid (**8**) were evaluated *in vitro* for their cytotoxic activities to childhood leukemia cell lines and leishmanicidal activity against the parasite *Leishmania amazonensis.* Among these compounds, **1** to **6** presented moderate cytotoxic activity, with compound **4** being the most active (GI_{50} of 2.6 µM for the HL60 line) and the derivatives **7** and **8** being inactive. Against the parasite *Leishmania amazonensis*, the most promising derivative was the acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**), with EC_{50} of 20.1 µM, selectivity index of 14.5, and significant reduction in the parasite load. Pimarane analogues **1**, *ent*-pimara-8(14),15-dien-19-oic acid, and **2**, *ent*-8(14),15-pimaradien-3β-ol, presented different activities, corroborating the application of such molecules as prototypes for the design of other derivatives that have greater cytotoxic or leishmanicidal potential.

Keywords: leishmanicidal activity, cytotoxic activity, *Leishmania amazonensis*, pimaranes, *Aldama arenaria* (syn. *Viguiera arenaria*), Asteraceae

Abbreviations:

Childhood leukemia cell lines: myeloid (K562), promyelocytic (HL60), acute T-cell (JURKAT, MOLT-4), Burkitt lymphoma (RAJI, RAMOS), B lymphoid (NALM-6), and acute lymphocytic leukemia (REH).

DE: dichloromethane extract of *A. arenaria* roots

GI_{50}: concentration for 50% of maximal inhibition of cell proliferation

EC_{50}: effective concentration needed to inhibit 50% of cell growth

CC_{50}: cytotoxic concentration capable of reducing 50% of the host cell population

MOI: multiplicity of infection

SI: selectivity index

Introduction

Aldama arenaria (Baker) E. E. Schill. & Panero, classified previously as *Viguiera arenaria* (Baker), Asteraceae, is a species found in the center-east of the state of São Paulo [1,2]. It has aromatic underground organs and can be chemically characterized by the presence of diterpene compounds of the pimarane type, class of secondary metabolites with numerous described biological activities, and sesquiterpene lactones [3-5]. Studies with isolated and semi-synthetic compounds of this species showed inhibitory action of the contraction of the vascular smooth muscle, *in vitro* activity against the protozoan *Trypanosoma cruzi*, capacity to reduce the influx of extracellular Ca^{2+} and consequent induction of hypotension in normotensive rats, vasorelaxant action, antibacterial, schistosomicidal and antiproliferative activity [6-13]. As a result, and continuing the research project of our working group, we evaluated pimaranes 1, 2 and six semi-synthetic derivatives.

Among the different types of cancers, leukemias comprise about 2.4% of incidents in the world, and statistics show the emergence of 437,033 new cases in 2018. It is one of the most common cancers, involving clonal neoplastic proliferation of immature cells or blasts of the hematopoietic system. It has two main subtypes identified on the basis of malignancy: lymphoid cells (B cells and T cells) or myeloid cells (granulocytic, erythroid, and megakaryocytic cells); and whether the disease is initially chronic or acute. In 2018 alone, when added together, all types of leukemia caused around 309,006 deaths worldwide. In Brazil, as in developed countries, cancer among children and adolescents aged 1 to 19 years represents the leading cause of death from diseases (8% of the total). Currently, mainly due to the significant progress in the treatment of cancer in childhood, around 80% of children and adolescents can be cured, if diagnosed early and treated in specialized centers [14].

They are usually more aggressive cancers, with few treatment options and, thus, poor prognosis. The main therapy for childhood leukemia is chemotherapy, but other treatments include transplantation, specific drugs, immunotherapy, surgery, and radiotherapy. Side effects such as hair loss, mouth sores, loss of appetite, diarrhea, nausea, and vomiting are commonly observed [15]. Some drugs widely used in the treatment of these patients come

from a plant species, such as: vincristine, etoposide, and teniposide [16-18]. These worrying data show that we must always look for new, innovative, more efficient, and less aggressive drugs, to ensure an ever greater chance of cure and survival for patients.

Leishmaniasis is a disease typical of poor countries, which affects more than 12 million people worldwide. It is a Neglected Tropical Disease (NTD) transmitted by different species of infected sandflies (genus *Phlebotomus*) and is defined as a set of parasitic diseases caused by protozoa of the genus *Leishmania*. It is known that more than 90 species of sandflies transmit these parasites, and it is estimated that, annually, there are 0.7 to 1 million new cases, of which 20 to 30 thousand result in deaths. Medications available for the treatment of leishmaniasis are few and have serious side effects and resistance, such as pentavalent antimonials. In the case of visceral Leishmania, other drugs can be used, such as liposomal amphotericin B and oral miltefosine, but these also present problems, such as high cost and teratogenic effect [19-20]. In Brazil, this disease is directly related to geographic regions, and the most common species are *Leishmania (Vianna) braziliensis* and *Leishmania amazonensis* [21].

In its biological cycle, the parasite has two morphological forms: promastigote, a flagellate form found in the female of the insect vector (invertebrate host), and amastigote, present in mammalian hosts. The amastigote forms are intracellular parasites commonly found in macrophages and are located in the phagolysosome or parasitophorous vacuole, where they multiply [22].

Knowing the high impact that these diseases have on society and the importance of studying substances isolated from plant species, this study aimed to evaluate *in vitro* isolated and semi-synthetic pimaranes of *A. arenaria* (Figure 1) against the leishmanicidal [*Leishmania amazonensis* parasite] and cytotoxic activities to childhood leukemia cell lines: myeloid (K562), promyelocytic (HL60), acute T-cell (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH).

[FIGURE 1]

Results and discussion

The yield obtained for the dichloromethane extract of the roots (DE) of *A. arenaria* in the turbo extraction was 14.1% (m/m, 42,36 g), a yield higher than that observed by Ambrosio et al. (2004) in their ultrasound extraction [3]. This extract presented the compounds **1** and **2** as major compounds by gas chromatography coupled to a mass spectrometry detector (GC-MS). Isolation of pimaranes **1** and **2** from DE by flash chromatography was efficient, with yields of 15.99 and 7.54 % (m/m, 3,52 g and 1,66 g, respectively) and purity greater than 95%, respectively, proving to be a fast, appropriate, and economic technique.

The characterization of pimaranes **1** and **2** was performed by comparing the spectroscopic data obtained with those described in the literature, and served as a reference to confirm the structures of semi-synthetic derivatives **3** to **8** (Tables 1, 2, and 3) [10,11,25-29].

The fragmentation pattern obtained in the mass spectra of these compounds is characteristic of pimaranes, presenting as base peak the fragments of *m/z* 121.10, 135.10 and 257.2 [30].

Regarding the synthesis of pimarane derivatives, the proposed synthetic routes were considered easy to perform and satisfactory, as their yields ranged from 80 to 100% (Figures 2 and 3).

[FIGURES 2 AND 3]

The pimaranes **1** (*ent*-pimara-8(14),15-dien-19-oic acid), **2** (*ent*-8(14),15-pimaradien-3-β-ol alcohol), **4** (*ent*-pimara-8(14),15-dien-19-ol alcohol), and **7** (derived in C-3 *ent*-8(14),15-pimaradien-3β-acetoxy) were described in the species *A. arenaria* and *Gnaphalium gaudichaudianum* and biologically explored for antibacterial, vasorelaxant, trypanocidal, and cytotoxic activities [6-13,23].

Derivatives **3** and **4**, obtained by microbial transformation, were evaluated for their capacity to inhibit vascular smooth muscle contraction, its spasmolytic effects, and its anticariogenic activities [24,25]. No activities were described for derivatives **5**, **6**, and **8**.

The cytotoxic activity of pimaranes **1** to **8** was evaluated *in vitro* for cell lines myeloid leukemia (K562), promyelocytic leukemia (HL60), acute T-cell leukemia (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH). Table 4 shows the GI_{50} values (concentration necessary to inhibit 50% of cell growth) obtained for the different samples and different lines. Vincristine was used as a reference chemotherapeutic drug.

Pimaranes with different functional groups at C19 (**1**, **3**, **4**, **5**, and **6**) had similar cytotoxicity values, with GI_{50} ranging from 2.6 ± 0.5 to 11.5 ± 0.1 µM. Derivative **4**, *ent*-pimara-8(14),15-dien-19-ol, was the most promising and presented GI_{50} of 2.6 ± 0.5 µM for the HL60 cell line.

Among the molecules with different substituents in C3 (**2**, **7**, and **8**), the compound **2** was the most active of all, with GI_{50} ranging from 4.2 ± 1.0 to 7.8 ± 0.7 µM.

Among the evaluated pimaranes, derivatives **7** and **8** were the least active, with EC_{50} > 15 µM; in this case, the addition of an acetate function or a succinate radical in C3 significantly reduced the cytotoxic activity for all lines tested, compared to **2**.

The main objective of antineoplastic chemotherapy is to exterminate cancer cells; however, this cannot always be done in a directional and selective way, almost always damaging healthy cells. This is because these drugs act in a non-specific way, harming both normal and malignant cells. The combination of several chemotherapeutic agents and their efficiency has been shown to be a beneficial tool for the treatment of malignant tumors, with cure rates of 75 to 90% in different types of cancer [31].

Additionally, compound **4** was evaluated for B lymphoid leukemia (NALM-6), Burkitt lymphoma (RAMOS), acute T-cell leukemia (MOLT-4), myeloid leukemia (K562), promyelocytic leukemia (HL60), and acute lymphocytic leukemia (REH) lines to verify whether this molecule had selectivity (Table 5).

The results obtained showed that compound **4** does not present any selectivity when evaluated for these other lines, with EC_{50} > 19 µM.

These data indicate moderate action of pimarane derivatives **1** to **6** isolated from *A. arenaria* and semi-synthetic ones, with a concentration-dependent profile and little selectivity among the evaluated lines.

Leishmanicidal activity was evaluated *in vitro* against the promastigote forms of *L. amazonensis* (MHOM/BR/73/M2269). About 20 described species can be associated with clinical manifestations in humans [32].

The initial screening was performed with pimaranes (**1** to **8**) at concentrations of 50 and 100 µg/mL (Figure 4).

[FIGURE 4]

This assay revealed that only pimaranes **1**, **2**, **3**, **5**, and **7** were able to decrease the cell viability of the parasites at rates lower than 30%, showing mortality of promastigote parasites in more than 82% for some samples. These samples were evaluated against parasites at different concentrations (0.15 to 660 µM), resulting in sigmoidal concentration curves versus parasite cell viability (Figure 5).

[FIGURE 5]

Through sigmoidal regression analysis of these data, it was possible to calculate the effective concentration needed to inhibit 50% of cell growth (EC_{50}) for pimaranes **1**, **2**, **3**, **5**, and **7** (Table 6).

Among the most active samples, compound **5** was the most promising derivative, with an EC_{50} from 16.02 to 24.82 µM. When we compare its activity with its precursor, pimarane **1**, we see an increase in action due to the addition of an acetate in C19. In the case of derivative **7,** we can see a less pronounced action when this substituint is positioned in C3.

Comparing the mean EC_{50} values obtained, we conclude that only derivative **5** has a potential leishmanicidal action *in vitro* (Figure 6).

[FIGURE 6]

The cytotoxicity of these molecules was evaluated in fibroblast cells (L929) and in macrophages and compared by the selectivity index, determined as the ratio between the

CC_{50} for macrophages and the EC_{50} for *L. amazonensis*. A selectivity greater than 100 times is expected for pathogens, however, such higher rates are quite uncommon [32] (Table 8).

Among the pimaranes evaluated, only acetate **5** showed potential action against parasite *L. amazonensis*, with a selectivity index greater than 10, compared to the macrophage and low cytotoxicity regarding the L929 line.

In vitro infection was performed with compound **5** in macrophages extracted from the femur and tibia of Black mice in a 24-well plate, at four concentrations from 4.73 to 37.82 µM. The micrographs obtained from the infection (Figure 7) showed that the coverslips containing only the infected macrophages (control) had a high degree of infection, represented by the circumferences with a diameter of less than 5 µM (arrowhead in Figure A). In Figure B, one can observe healthy macrophages and the absence of intracellular parasites. The photomicrographs of the coverslips can be seen in Figure 7.

[FIGURE 7]

By counting the numbers of macrophages, infected macrophages, and amastigote parasites, it was possible to plot the graphs that are shown in Figures 8 to 10.

Assessing the number of amastigotes per 100 macrophages, we verified a small reduction in the number of infected macrophages at the highest concentrations (18.91 and 37.82 µM), compared to the untreated control group. In this case, there was no significant difference in infection between the two highest concentrations. This profile is also observed when we verify the percentage of infection (Figure 8).

[FIGURES 8 AND 9]

Regarding the infectivity index, we can infer that pimarane 5 contributed to the reduction of the parasite load when evaluated at the highest concentrations (Figure 10), reducing macrophage infection by more than 50% compared to the untreated control. This inhibition is quite significant for a patient, because macrophage cells are the main cells infected by parasites of the genus *Leishmania*, playing a crucial role in the initial immune response to infections.

[FIGURE 10]

The results of the *in vitro* evaluation of macrophage infection allow us to conclude that pimarane **5** was active against *L. amazonensis* amastigotes at concentrations 18.91 and 37.82 µM.

Conclusion

This study has shown that pimaranes isolated from *A. arenaria* and their semi-synthetics derivatives have *in vitro* cytotoxic action in childhood leukemia lines and leishmanicidal activity when evaluated for the *L. amazonensis* line.

Among the pimaranes evaluated, the alcohol **4**, *ent*-pimara-8(14),15-dien-19-ol, presented greater cytotoxicity, with an GI_{50} of 2.6 for the promyelocytic leukemia (HL60) cell line, without showing any specific selectivity. The most promising pimarane against *L. amazonensis* parasites was the acetate from *ent*-pimara-8(14),15-dien-19-ol (**5**), with an $EC_{50}$of 20.1 µM and selectivity index of 14.5.

Pimarane analogues *ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*- 8(14),15-pimaradien-3β-ol (**2**) presented different activities, indicating that these precursor molecules can be considered prototypes for the planning of other derivatives that have greater cytotoxic or leishmanicidal potential. Additionally, other *in vitro* and *in vivo* studies are needed to unravel their mechanisms of action and toxicities.

Material and methods

Plant material

Plant material was collected from Ecological Station of Itirapina, São Paulo, Brazil (22°14'S, 47°51'W) in its natural state. The identification was carried out by the botanist Mara Angelina Galvão Magenta, and the exsiccate was deposited in the herbarium of the "Luiz de Queiroz" School of Agriculture – ESALQ-USP, under number 111847 and study permits from CGEN, process no. 010216/2012-0 and SisGen A7C6E5C. This matrix plant provided seeds used

for the production of 20 seedlings that were cultivated for 2 years in the experimental field of CPQBA/UNICAMP and processed for the production of extracts.

Extraction and isolation of *ent*-pimara-8(14),15-dien-19-oic acid (1) and *ent*- 8(14),15-pimaradien-3β-ol (2) from the roots of *A. arenaria*

Fresh roots of *A. arenaria* were ground and extracted in an Ultra Turrax (IKA) disperser at room temperature with dichloromethane (1g/10mL) for 5 min, followed by filtration and re-extraction of the plant residue with two more portions of dichloromethane. The pooled extracts were evaporated under reduced pressure, providing the crude dichloromethane extract (DE).

DE (22 g) was fractionated in a medium pressure liquid chromatograph (CombiFlash Rf+ /Teledyne ISCO) in a chromatographic column containing 220 g of silica gel 60 (High Performance Gold, spherical particle 20-40 µm and pore size of 60Å), gradient of hexane and ethyl acetate with mobile phase, flow rate of 50 mL/min, and wavelength of 254 nm. 1020 fractions of 18 mL each were collected. The resulting fractions were analyzed by thin layer chromatography (TLC), pooled according to similarity and evaporated under reduced pressure. The pooled fraction enriched in pimarane **1** was crystallized in dichloromethane in a freezer, filtered, and vacuum dried. CG-MS ions (*m/z*) [relative intensity, %]: 302.20 [34]; 123.10 [34]; 121.10 [100]; 91.10 [40]; 79.10 [33]. ^{1}H NMR data: δ= 0.66 (s, 3H, 10-CH_3), 1.01 (s, 3H, 13-CH_3), 1.27 (s, 3H, 4-CH_3), 4.91 (dd, 1H), 4.96 (dd, 1H), 5.16 (s, 1H), 5.72 (dd, 1H). ^{13}C NMR: δ = 184.3 (C-19), 147.2 (C-15), 137.9 (C-8), 128.0 (C-14), 112.9 (C-16), 56.1 (C-5), 50.5 (C-9), 44.0 (C-4), 39.2 (C-1), 39.2 (C-10), 38.5 (C-13), 37.9 (C-3), 35.8 (C-7), 36.4 (C-12), 29.3 (C-17), 29.2 (C-18), 24.1 (C-6), 19.6 (C-11), 19.2 (C-2), 13.8 (C-20).

To obtain pimarane **2**, fractions from the DE flash chromatography containing large amounts of pimarane alcohol were pooled and methylated with the reagent TMSD (trimethylsilyl diazomethane). Because of the similar Rf values, this reaction is necessary for the conversion of the acid **1** to the correspondent ester ensuring the isolation of alcohol. The reaction remained under stirring for 2h. After completion of the reaction, confirmed by TLC,

the sample was vacuum dried. This sample enriched in pimarane **2** (4.9g) was fractionated again in a medium pressure liquid chromatograph in a chromatographic column containing 80 g of silica gel 60 (High Performance Gold, spherical particle 20-40 µm and pore size of 60Å), gradient of hexane and ethyl acetate with mobile phase, flow rate of 10 mL/min, and wavelength of 254 nm. 322 fractions of 15 mL each were collected. The resulting fractions were analyzed by TLC, pooled according to similarity and vacuum evaporated. The pooled fraction enriched in compound **2** was solubilized at heat (45 ºC) and then crystallized in methanol at room temperature. The crystallisate from pimarane **2** was centrifuged and washed with cold methanol. After separating the supernatant, the precipitate was vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 288.20 [8]; 270.20 [11]; 255.20 [17]; 135.10 [100]; 91.10 [22]. ^{1}H NMR: δ = 0.83 (s, 3H, 4-CH_3), 1.02 (s, 3H, 4- CH_3), 1.07 (s, 3H, 10-CH_3), 1.17 (s, 3H, 13-CH_3), 3.3 (dd, 1H), 4.89 (dd, 1H), 4.96 (dd, 1H), 5.16 (d, 1H), 5.74 (dd, 1H). ^{13}C NMR: δ = 147.3(C-15), 137.9 (C-8), 128.1 (C-14), 112.8 (C-16), 79.2 (C-3), 54.1 (C-5), 51.2 (C-9), 39.0 (C-4), 38.6 (C-13), 38.1 (C-10), 37.1 (C-1), 35.7 (C-7), 35.7 (C-12), 29.4 (C-17), 28.4 (C-18), 27.5 (C-2), 22.1 (C-6), 19.1 (C-11), 15.7 (C-19), 14.7 (C-20).

Obtaining the derivatives of *ent*-pimara-8(14),15-dien-19-oic acid (1) and *ent*-8(14),15-pimaradien-3β-ol (2)

Methyl ester of *ent*-pimara-8(14),15-dien-19-oic acid (**3**): 502.9 mg of pimarane **1** was weighed into a 50 mL flask and 10 mL of a mixture of dichloromethane and methanol (9:1) was added. 2.50 mL of the TMSD reagent (trimethylsilyl diazomethane) was added to the reaction medium and the system was left under stirring for 2h. After completion of the reaction, confirmed by TLC, the sample was vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 316.20 [15]; 257.20 [14]; 180.10 [26]; 121.10 [100]; 91.10 [18].

***ent*-pimara-8(14),15-dien-19-ol** (**4**): In an inert atmosphere, 50 mL of anhydrous ethyl ether and 200.6 mg of lithium and aluminum hydride were added to a 250 mL flask. In another

container, 503.0 mg of derivative **3** were solubilized in 5 mL of anhydrous ethyl ether. The solubilized derivative **3** was then added slowly to the reaction medium. The system remained under heating at 40 ºC and stirring for 1h. After completion of the reaction, confirmed by TLC, 10 mL of a mixture of ethyl ether and distilled water (1:1) were added, followed by 2 mL of distilled water. The reaction medium was filtered under cotton, and anhydrous sodium sulfate was added to the ether phase. The organic phase was filtered again and vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 288.30 [6]; 257.20 [100]; 135.10 [20]; 121.10 [21]; 91.10 [26].

Acetate of *ent*-pimara-8(14),15-dien-19-ol (**5**): In an inert atmosphere, 150.5 mg of derivative **4** and 40 mL of dichloromethane were added to a 125 mL flask. A further 2 mL of pyridine, 103.7 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 1.3 mL of acetic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 1h. After completion of the reaction, confirmed by TLC, 25 mL of distilled water and 1 M hydrochloric acid solution were added until pH 3. The reaction medium was partitioned with 50 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried. GC-MS ions (*m/z*) [relative intensity, %]: 330.30 [18]; 257.20 [91]; 135.10 [100]; 93.10 [38]; 43.10 [41].

***ent*-pimara-8(14),15-dien-19-ol succinic acid** (**6**): In a 125 mL flask and inert atmosphere, 141.5 mg of derivative **4** and 40 mL of dichloromethane were added. A further 4 mL of pyridine, 205.5 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 3.05 g of succinic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 78h. After completion of the reaction, confirmed by TLC, 1 M hydrochloric acid solution was added until pH 3. The reaction medium was partitioned with 50 mL of dichloromethane. The extraction was repeated three more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered

and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 288.20 [5]; 257.2 [100]; 135.10 [21]; 121.10 [24]; 91.10 [28].

Acetate of *ent*-8(14),15-pimaradien- 3β-ol (**7**): In a 125 mL flask and inert atmosphere, 240.8 mg of pimarane **2** and 75 mL of dichloromethane were added. Additional 3.2 mL of pyridine, 173.4 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 2.10 mL of acetic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 1h. After completion of the reaction, confirmed by TLC, 1 M hydrochloric acid solution was added until pH 2. The reaction medium was partitioned with 100 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 330.30 [8]; 255.20 [29]; 135.10 [100]; 119.10 [23]; 43.10 [31].

***ent*-8(14),15-pimaradien-3β-ol succinic acid** (**8**): 262.0 mg of pimarane **2** was solubilized in 80 mL of dichloromethane in a 250 mL flask. A further 7.5 mL of pyridine, 380.5 mg of the 4-dimethylaminopyridine (DMAP) catalyst, and 8.01 g of succinic anhydride were added to the reaction medium. The system remained at room temperature and under stirring for 144h. After completion of the reaction, confirmed by TLC, 200 mL of distilled water were added and the medium acidified with 1 M hydrochloric acid until pH 2. The reaction medium was partitioned with 100 mL of dichloromethane. The extraction was repeated two more times, grouping the organic phases. The organic phase was then dried with anhydrous sodium sulfate, filtered and vacuum dried, and recrystallized in dichloromethane at room temperature. GC-MS ions (*m/z*) [relative intensity, %]: 288.30 [7]; 255.20 [16]; 135.10 [100]; 121.10 [14]; 91.10 [21].

Structural determination of pimaranes

The structural determination of pimaranes **1** to **8** was carried out by the analysis of ^{1}H and ^{13}C NMR spectroscopic data, mass spectra, and data described in the literature [19-23].

Evaluation of *in vitro* cytotoxic activity

The cytotoxicity of pimaranes **1** to **8** was evaluated *in vitro* in childhood leukemia cell lines from ATCC (American Type Culture Collection), namely: myeloid leukemia (K562), promyelocytic leukemia (HL60), acute T-cell leukemia (JURKAT, MOLT-4), Burkitt lymphoma (RAJI and RAMOS), B lymphoid leukemia (NALM-6), and acute lymphocytic leukemia (REH), To assess the cytotoxic potential, samples diluted in dimethyl sulfoxide (100 mg/mL) were used. This first dilution was at the maximum concentration of 0.1% DMSO, and, in each well, the concentration reached a maximum of 0.03%, not representing cytotoxicity. Cell viability was assessed by the MTT method (3-[4,5-dimethyl-triazol-2-yl]-2,5-diphenyltetrazolium bromide) in 96-well plates [33].

Cells were cultured in appropriate plastic bottles with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% inactivated fetal bovine serum (FBS) for one hour at 56°C, 10 mM of 4-(2-acid) hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 $g.L^{-1}$ of sodium bicarbonate, 1% of penicillin G (100 U/mL), 100 mg/mL of streptomycin, and 50 µg/mL of amphotericin B in an oven with 5% CO_2 atmosphere at 37ºC. Before the experiments, the number of viable cells was determined by the trypan blue exclusion method.

The cells were plated using serial dilution and the epMotion 5070 equipment (Eppendorf, Vaudaux, Schonenbuch, Switzerland), which distributed 2×10^4 cells per well (96-well plates) and added the substances in different concentrations (0.01 to 1000 µg/mL) for 48 h at 37ºC and 5% CO_2. The chemotherapy drug vincristine was used as a positive standard control. After the treatment period, the culture medium was removed by centrifugation and discarded, 100 µL of MTT solution (0.5 mg/mL) was added, and the plate was incubated again for 4h. After this period, the medium was removed and the formazan precipitate was dissolved into 100 µl DMSO per well. The plate was then analyzed in a microplate reader at 540 nm (Bio-

Tek Power Wave XS) for optical density reading. The control group of untreated cells, incubated with the growth medium only, was considered as 100% of viable cells for calculation of dose-response curves and, consequently, to calculate the GI_{50} (concentration needed to inhibit 50% of cell growth) using sigmoidal regression in the Graphpad Prism 5.0 software for Windows (GraphPad Software), according to the equation: Relative cell viability = (sample absorbance / control absorbance) x 100. The samples were evaluated in triplicate, and the results were expressed as mean ± standard deviation by Excel 2016 software (Microsoft).

Evaluation of *in vitro* leishmanicidal activity in promastigote forms

Promastigote of *L. amazonensis* (strain MHOM/BR/73/M2269) kindly donated by Prof. Dr Silvia Uliana (University of São Paulo, Brazil) were cultured in 25 mL flask with 5 mL of medium 199 (Sigma-Aldrich) supplemented with 50 units/mL penicillin, 0.1 mM adenine, 50 µg/mL streptomycin, pH 7.4, 40 mM HEPES, 0.0001% biotin, 0.0005% hemin, and 10% fetal bovine serum (FBS-Vitrocell).

The samples were diluted in dimethyl sulfoxide (10 mg/mL, maximum final concentration of 0.07 %) and submitted to the biological activity test according to the MTT method (3-[4,5-dimethyl-triazol-2-yl]-2,5-diphenyltetrazolium bromide) in 96-well plates [19,34,35]. In this procedure, the culture of *L. amazonensis* containing 2.5×10^6 promastigote parasites in logarithmic phase and maintained in culture medium 199 was evaluated against the sample in different concentrations (50 to 100 µg/mL), for 24 hours. After this period, 30 µL of an MTT solution (3-[4,5-dimethyl-triazol-2-yl-2,5-diphenyltetrazolium, 5 mg/mL) was added to each well and the plates were incubated in the oven at 26 °C (2 hours). To lyse the cells, 30 µL of SDS (20% sodium dodecyl sulfate) was added, and the absorbance was read in a microplate reader (595 nm). Results were expressed as percentages of cell viability compared to the group of untreated parasites (control, equivalent to 100%).

Defining the most promising samples, cell mortality above 20%, cell viability curves were drawn with at least 6 concentrations and at least three independent assays. Using sigmoidal

regression in the Origin software, it was possible to calculate the effective concentration for a 50% reduction in cell viability (EC_{50}) for each sample.

In vitro cytotoxicity assessment for fibroblast and macrophage cell lines

The fibroblast culture maintained with 20 mL of complete RPMI medium was transferred to falcon tube, washing with complete RPMI medium. The solution was centrifuged at 1000 xg and 4°C for 5 minutes. The supernatant was discarded and an additional 10 mL of RPMI medium was added. The number of cells was determined in a Neubauer chamber, with 2×10^5 cells per well, and the samples were evaluated according to the MTT method described above.

The macrophages used for cytotoxic evaluation were differentiated from bone marrow precursor cells extracted from the tibia and femur of mice. These cells were cultured in 7.5 cm diameter plates containing 10 mL R2020 medium, modified from RPMI Lonza medium, containing: RPMI, 20% fetal bovine serum, 20% L929 fibroblast supernatant and gentamicin, and kept in an oven at 37°C with 5% CO_2 atmosphere. After 4 days, another 5 mL of R2020 medium was added and, in 7 days, the macrophages had already gone through the differentiation process and were ready for plating. Macrophages adhered to the bottom of the plate were removed with a sterile cell scraper (Corning), collected in RPMI medium, centrifuged at 1000G for 5 minutes at 4°C, and resuspended in R105 medium (RPMI + 10% FBS + 5% of L929 supernatant). The number of cells was determined in a Neubauer chamber, with 2×10^5 cells per well in the cytotoxic analysis and 5×10^5 cells in the *Leishmania* infection process, according to the MTT method.

Evaluation of *in vitro* infection of macrophages with *L. amazonensis*

To assess the viability of intracellular amastigote parasites after exposure to the pimaranes under study, macrophage cells were infected with *L. amazonensis* promastigotes. Macrophages were derived from bone marrow precursor cells obtained from C57BL/6J mice provided by the "Multidisciplinary Center for Biological Investigation on Laboratory Animal

Science" at the University of Campinas (CEMIB-Unicamp). Briefly, 5×10^5 macrophages were cultured on coverslips in 24-well plates and infected for 24h before being exposed to the test sample. Infection schemes followed multiplicities of infection (MOI) equivalent 5 to 10 stationary phase promastigotes per macrophage. An untreated group was used as a negative control. Infections were analyzed under immersion with an optical microscope, considering three parameters: total percentage of infected cells; number of parasites per 100 macrophages; and infectivity index, which combines the two previous parameters (infectivity index = percentage of infected macrophages multiplied by the average number of amastigotes for 100 macrophages).

Supplementary information

^{1}H and ^{13}C NMR spectra were obtained on a Bruker 400 operating at 400 and 100 MHz, respectively, using $CDCl_3$ as solvent and TMS as reference. Data from Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) analyses of compounds **1**-**8**, and dose-response curves from the biological tests can be request as supplementary information.

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Conflict of Interest

The authors declare no conflict of interest regarding the authorship and/or publication of this article.

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Legends for Figures

Figure 1. Isolated pimaranes from *A. arenaria ent*-pimara-8(14),15-dien-19-oic acid (**1**) and *ent*- 8(14),15-pimaradien-3β-ol (**2**) and semi-synthetic ones (**3** to **8**).

Figure 2. Synthesis scheme for obtaining semi-synthetic derivatives **3** to **6**.

Figure 3. Synthesis scheme for obtaining semi-synthetic derivatives **7** and **8**.

Figure 4. Cell viability graph obtained from the initial screening of pimaranes **1** to **8** for *L. amazonensis* promastigote parasites.

Caption – Each bar represents the mean ± standard deviation of cell viability for three independent experiments.

Figure 5. Cell viability curves of *L. amazonensis* promastigote parasites versus concentration log (μM) obtained for pimaranes **1**, **2**, **3**, **5**, and **7**.

Caption – Each bar represents the mean ± standard deviation of cell viability for three independent experiments.

Figure 6. Graph of EC_{50} (μM) obtained for pimaranes **1**, **2**, **3**, **5**, **7** and amphotericin against *L. amazonensis* promastigote parasites.

Caption – EC_{50}: effective concentration needed to inhibit 50% of cell growth; AB: amphotericin B. Each bar represents the mean EC_{50} for three independent experiments. Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 7. Photomicrographs of coverslips obtained from the infection and *in vitro* treatment of macrophages infected with *L. amazonensis* amastigotes and treated with derivative **5**. **A** – Infection control; **B** – Macrophages treated with pimarane **5** (37.82 μM). Bar = 5 micrometers.

Figure 8. Number of amastigotes per 100 macrophages versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 9. Percentage of infected macrophages versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Figure 10. Infectivity index versus concentrations of pimarane **5** (μM).

Caption – Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Equal letters are statistically similar.

Table 1. ^{1}H NMR data of pimaranes **3**-**6** ($CDCl_3$, 400 MHz, δ in ppm).

Position	3	4	5	6
4-CH_3	1.20 (*s*, 3H)	1.05 (*s*, 3H)	0.98 (*s*, 3H)	0.98 (*s*, 3H)
10-CH_3	0,55 (*s*, 3H)	0.75 (*s*, 3H)	0.75 (*s*, 3H)	0.75 (*s*, 3H)
13-CH_3	1.00 (*s*, 3H)	1.00 (*s*, 3H)	0.97 (*s*, 3H)	0.97 (*s*, 3H)
14	5.14 (*s*, 1H)	5.16 (*s*, 1H)	5.18 (*s*, 1H)	5.17 (*s*, 1H)
15	5.71 (*dd*, 1H)	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)
16	4.91 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)	4.90 (*dd*, 1Ha)
16	4.94 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)	4.95 (*dd*, 1Hb)
19	-	3.40 and 3.80 (Ha and Hb)	3.90 and 4.30 (Ha and Hb)	3.90 and 4.35 (Ha and Hb)
19-$OCOCH_3$	-	-	2.10 (*s*, 3H)	-
19-$OCO(CH_2)_2CO_2H$	-	-	-	2.70 (*dt*, 2H)
19-$OCO(CH_2)_2CO_2H$	-	-	-	2.62 (*dt*, 2H)
4-$COOCH_3$	3,63(s, 3H)	-	-	-

Table 2. ^{1}H NMR data of pimaranes **7** and **8** ($CDCl_3$, 400 MHz, δ in ppm).

Position	7	8
4-CH_3	0.87 (*s*, 3H)	0.75 (*s*, 3H)
4-CH_3	0.87 (*s*, 3H)	0.87 (*s*, 3H)
10-CH_3	0.75 (*s*, 3H)	0.87 (*s*, 3H)
13-CH_3	0.98 (*s*, 3H)	1.00 (*s*, 3H)
3	4.50 (*dd*, 1H)	4.55 (*dd*, 1H)
16	4.90 (*dd*, 1Ha)	4.96 (*dd*, 1Ha)
16	4.96 (*dd*, 1Ha)	4.96 (*dd*, 1Ha)
14	5.18 (*s*, 1H)	5.20 (*s*, 1H)
15	5.75 (*dd*, 1H)	5.75 (*dd*, 1H)
3-$OCOCH_3$	2.10 (*s*, 3H)	-

3-OCO(CH$_2$)$_2$COOH	-	2.70 (2H)
3-OCO(CH$_2$)$_2$COOH	-	2.68 (2H)

Table 3. ^{13}C NMR data of pimaranes **3**-**8** ($CDCl_3$, 100 MHz, δ in ppm).

Carbon	3	4	5	6	7	8
1	38,2	39.2	39.0	39.0	38.0	37.9
2	19,2	18.6	18.5	18.5	28.4	29.0
3	38,2	35.4	35.7	35.7	81.0	81.7
4	44,1	38.2	38.2	37.1	37.9	38.0
5	56,1	55.7	55.7	55.7	54.2	54.2
6	24,3	22.4	22.5	22.4	21.3	21.9
7	35,8	37.7	36.0	36.0	35.6	35.6
8	138,0	138.0	137.9	137.8	137.8	137.7
9	50,5	51.4	51.5	51.5	51.1	51.1
10	39,2	35.7	38.6	38.2	38.3	38.6
11	19,6	19.2	19.3	19.3	19.1	19.1
12	36,4	36.1	36.2	36.2	35.6	36.7
13	38,5	36.5	36.2	38.5	38.6	38.7
14	127,9	128.1	128.2	128.3	128.3	128.3
15	147,2	147.3	147.2	147.2	147.3	147.3
16	112,8	112.7	112.9	112.9	112.8	112.8
17	29,3	29.4	29.4	29.0	29.4	29.4
18	29,0	27.0	27.5	27.5	22.0	23.9
19	177,9	65.2	67.0	67.4	16.9	16.9
20	13,5	15.8	15.8	15.8	14.8	14.8
-OCO-	-	-	171.4	172.2	171.0	171.8
-OCO-CH$_3$	-	-	-	29.0	24.0	35.5
-CH$_2$CH$_2$-	-	-	-	28.9	-	29.7/29.0
-CH$_2$CH COOH-	-	-	-	177.7	-	177.8
OCH$_3$	51,2	-	-	-	-	-

Table 4. GI_{50} values (μM) of pimaranes **1** to **8** for different leukemia lines.

PIMARANES	CELL LINES / GI_{50} (MM)*			
	K562	**HL60**	**RAJI**	**JURKAT**
1	6.5 ± 2.6	11.5 ± 0.1	10.5 ± 0.4	8.6 ± 1.1
2	6.8 ± 0.9	4.2 ± 1.0	5,7 ± 0.3	7.8 ± 0.7
3	6.5 ± 0.4	4.7 ± 0.9	5.9 ± 0.8	4.5 ± 1.3
4	3.7 ± 1.3	2.6 ± 0.5	3.95 ± 0.5	4.4 ± 0.4
5	7.3 ± 0.3	5.1 ± 0.3	10.8 ± 2.7	7.4 ± 1.2
6	8.6 ± 1.0	8.1 ± 0.4	8.1 ± 0.2	7.9 ± 0.9
7	36.7 ± 4.5	33.7 ± 1.1	41.0 ± 0.8	40.2 ± 3.1
8	> 100	17.5 ± 1.0	> 100	20.1 ± 2.5
VINCRISTINE	47.6 ± 9.8	21.2 ± 0.4	NE	12.7 ± 0.2

Caption – GI_{50}: concentration necessary to inhibit 50% of cell growth; K562: myeloid leukemia; HL60: promyelocytGI leukemia; RAJI: Burkitt lymphoma; JURKAT: acute T-cell leukemia; Standard deviation for n=3; NE: Not evaluated.

Table 5. GI_{50} values (µM) of pimaranes **4** for the different leukemia lines.

	CELL LINES / IC_{50} (MM)*			
	NALM6	**RAMOS**	**MOLT4**	**REH**
4	22.5 ± 4.5	24.6 ± 4.9	19.4 ± 0.7	33.3 ± 1.9
VINCRISTINE	0.001 ± 0.0	0.121 ± 0.073	0.001 ± 0.0	0.001 ± 0.0

Caption – GI_{50}: concentration necessary to inhibit 50% of cell growth; B-lymphoid leukemia (NALM-6), Burkitt lymphoma (RAMOS), acute T-cell leukemia (MOLT-4), promyelocytic leukemia (HL60), and acute lymphocytic leukemia (REH); Standard deviation for n=3.

Table 6. EC_{50} values (µM) of pimaranes **1**, **2**, **3**, **5**, and **7** obtained for *L. amazonensis* promastigote parasites.

Compound	EC_{50} (µM) promastigotes			Mean and CI (µM)
1	126.2	149.9	79.7	114.7[a] (50.9 to 258.1)
2	59.2	66.7	38.0	53.1[b] (25.4 to 111.1)
3	65.2	44.8	70.5	59.1[b] (32.3 to 107.9)
5	18.6	25.5	17.3	20.1[b,c] (12.1 to 33.6)

7	35.8	53.0	56.7	47.5[b] (25.6 to 88.2)
AB	0.09	0.02	0.08	0.05[c] (0.005 to 0.49)

Caption – EC_{50}: effective concentration needed to inhibit 50% of cell growth; CI: 95% Confidence Interval obtained for a minimum of n=3 independent tests; Statistical analyses of significance by One-way ANOVA with Tukey post-hoc test, $p < 0.05$. Numbers followed by the same letter are statistically similar.

Table 7. Selectivity index (SI) and EC_{50} values (μM) of pimaranes **1** to **3**, **5**, and **7** for promastigote parasites, *L. amazonensis* macrophages, and L929 fibroblast cells.

Pimarane	Promastigotes EC_{50} (μM)	Macrophages CC_{50} (μM)	Selectivity Index (SI)	Fibroblasts L929 CC_{50} (μM)
1	114.7	100.3	0.9	87.6
2	53.1	208.2	3.9	136.2
3	59.1	229.8	3.9	189.7
5	20.1	292.0	14.5	215.5
7	47.5	208.9	4.4	165.2
AB	0.05	1.99	39.80	1.36

Caption – EC_{50}: effective concentration necessary to inhibit 50% of cell growth in the promastigote parasite; CC_{50}: cytotoxic concentration capable of reducing 50% of the host cell population; AB: amphotericin B.

UNIVERSITY OF CAMPINAS

Pluridisciplinary Research Center for Chemical, Biological, and Agricultural Research (CPQBA)

Paulínia, November 17th 2021

Dear Editor Alessandra Braca,

I am writing to submit the corrections of the manuscript entitled, "*In vitro* cytotoxic and leishmanicidal activity of isolated and semisynthetic ent-pimaranes from *Aldama arenaria*."

We would like to thank the reviewers for the valuable comments and suggestions which were useful for the improvement of this paper. We have revised the manuscript accordingly to all comments and issues raised by each reviewer. All suggestions and observations proposed by the referee have been inserted in this new version, highlighted in red. The modifications are:

Editor's Comments

1- The species name *Aldama arenaria* can not be found in the plant list: please check if it exists with a different synonymous (see also reviewer 2 comment).
Reply: *Aldama arenaria* (Baker) E. E. Schill. & Panero, was classified previously as *Viguiera arenaria* (Baker). The data reclassification are in the reference [2] and cited in the manuscript.

2- Title page: Authors names: please double check: Are all authors names spelled correctly? Are they listed in the correct order? Have all contributing authors been mentioned? Note: changes to this part will not be possible after acceptance of the manuscript.
Reply: All information cited in the page is correct.

3- Affiliations: see comment above - please make sure that all affiliations are listed correctly since changes to this part will not be possible later.
Reply: All information about affiliations is correct.

4- Affiliations: Please remove street addresses including ZIP codes from the addresses.
Reply: The street addresses and ZIP codes were excluded.

5- Abstract: please remove the square brackets from the compound names.
Reply: The square brackets were removed from the compound names.

6- Abstract and throughout the manuscript: - For IC50 and EC50 values, confidence intervals should be given. Please do not use ±SDs or SEM (confer http://www.graphpad.com/support/faqid/34/ and http://www.graphpad.com/support/faqid/534/) IC50 values are based on log transformations and therefore the SD is not symmetrical and should not be used.
Reply: We thank the editor for the comment and the references. The SD was removed and CI95% has been added in the text. We modified the term IC50 to GI50, as solicited by the editor.

7- Key words should include the plant's botanical family (as a separate key word).
Reply: The plant's botanical family was included in the key words, as a separate key word.

8- List of abbreviations: “IC50 and EC50: effective concentration needed to inhibit 50% of cell growth”. It is unclear why there should be two different terms used with the same definition. Please note that for Growth inhibition of 50%, the term GI50 is used, for 50% reduction of cell viability, TC50 should be used.
Reply: We modified the term IC50 to GI50, as solicited by editor.

9- There should not be any subtitles in the 'Results' section.
Reply: All subtitles in the Results section were removed.

10- “Fresh roots of A. arenaria were ground and extracted in an Ultra Turrax disperser»: Please indicate whether this was done at room temperature (or else give the temperature).
Reply: The dichloromethane extract (DE) was obtained at room temperature. This information was added to the text.

11- Please remove trademark signs etc. from product names, for instance IKA and CombiFlash.
Reply: The trademarks were removed.

12- Instead of “50 mL.min-1”, please set “50 mL/min»
Reply: We set 50 mL/min.

13- “50 ml of anhydrous ethyl ether” or “10 ml of a mixture”. Please abbreviate litre by ‘L’. Please correct throughout the manuscript.
Reply: We abbreviated liter by L throughout the manuscript.

14- Please give the sources for the cell lines.

Reply: The sources of childhood leukemia cell lines are from ATCC (American Type Culture Collection) and they were quoted in the text.

15- “which distributed 2×104 cells per well». Please mention the size of the wells (i.e. were 96-well plates used or another format?).

Reply: It was 96-well plates.

16- “After the treatment period, the medium was removed by centrifugation». This might be unclear.

Reply: This phrase was modified to *“After the treatment period, the culture medium was removed by centrifugation and discarded, 100 µL of MTT solution (0.5 mg/mL) was added, and the plate was incubated again for 4h.”*

17- “to calculate the IC50 (concentration needed to inhibit 50% of cell growth), according to the equation: Relative cell viability = (sample absorbance / control absorbance) x 100.». Please give the software used and which model was used to fit the data.

Reply: We used sigmoidal regression in the Graphpad Prisma and Excel softwares.

18- “Evaluation of *in vitro* leishmanicidal activity in promastigote forms”. Please give the final DMSO concentration.

Reply: The final concentration of DMSO was 0.07 % (concentration add to the manuscript).

19- Please give the source for the L. amazonensis

Reply: The source for promastigote parasites of *L. amazonensis* was MHOM/BR/73/M2269, according to what was cited in the text.

20- “The solution was centrifuged at 1000G», Please set 1000 xg

Reply: We set 1000 xg.

21- “Macrophages derived from C57BL/6J mouse bone marrow precursors” Please give the source for the mice or the macrophages.

Reply: The text has been corrected and it reads: “Macrophages derived from C57BL/6J mouse bone marrow precursor cells”.

22- Acknowledgement: Please confirm that you have mentioned all organizations that funded your research in the Acknowledgements section of your submission, including grant numbers where

appropriate. Note: changes to this paragraph will not be possible after acceptance of the manuscript.

Reply: The thanks information is correct.

23- List of references: Please give all authors and do not abbreviate with “et al.”. For instance [31].

Reply: The list or reference has been corrected and three new references were cited to complement the paper.

[2] Schilling EE, Panero JL, A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae) II. Derived lineages. Bot J Linn Soc 2011; 167: 311-331

[5] Reveglia P, Cimmino A, Masi M, Nocera P, Berova N, Ellestad G, Evidente A. Pimarane diterpenes: Natural source, stereochemical configuration, and biological activity. Chirality 2018; 30(10): 1115-1134

[13] Oliveira ASS, Imamura PM, Ruiz ALTG, Appezzato-da-Glória B, de Oliveira T, Garcia VL. Antiproliferative activity from *Aldama arenaria* (Baker) E.E. Schill. & Panero. Bol Latinoam Caribe Plant Med Aromat 2021; 20(1): 51-60

24- Table 1-3: please report NMR data only for new natural and semisynthetic compounds and refer to the known ones in Materials and methods, extraction and isolation section (see also reviewer 2 comments).

Reply: In the Table 1-3 we report NMR data only for new natural and semisynthetic compounds. NMR data for the known ones were referred to in Materials and Methods.

25- Table 4 should be deleted and MS data added in the material and methods, extraction and isolation section.

Reply: Table 4 was deleted and MS data added in the Material and Methods.

26- Tables 5-8: Please check for exaggerated precision. For instance, giving an EC60 value of 118.59 µM means a precision of 1 in 11’859 which is not achievable with the methods used.

Reply: The decimals place value of EC50 has been reduced.

27- Figure 4 and throughout the manuscript. Please do not set “µg.ml-1” but “µg/mL”- Please correct all similar instance.

Reply: In Figure 4 and throughout the manuscript we set µg/mL.

28- Figures 4 and 5, legend: Please give the number of repetitions and the meaning of the error bars.

Reply: Each bar represents the mean ± standard deviation of cell viability for three independent experiments. Phrase added to manuscript.

29- Figure 6, legend: Please give the number of repetitions.

Reply: Graph of EC_{50} obtained for three independent experiments. Information add to caption.

30- Figure 7: This figure has not been uploaded.

Reply: We corrected the figure 7 and we will upload it again.

Reviewer: 1

1) Page 5 of 36, line number 24, 45: Why was the plant A. arenaria chosen? Or why were authors interested in pimarane diterpenes? Some explanation may be helpful for general readers.

Reply: Explanation and references were added to the text to answer this comment.

"It has aromatic underground organs and can be chemically characterized by the presence of diterpene compounds of the pimarane type, class of secondary metabolites with numerous described biological activities, and sesquiterpene lactones [3-5].

Studies with isolated and semi-synthetic compounds of this species showed inhibitory action of the contraction of the vascular smooth muscle, in vitro activity against the protozoan Trypanosoma cruzi, capacity to reduce the influx of extracellular Ca^{2+} and consequent induction of hypotension in normotensive rats, vasorelaxant action, antibacterial, schistosomicidal and antiproliferative activity [6-13]. As a result, and continuing the research project of our working group, we evaluated pimaranes 1, 2 and six semi-synthetic derivatives."

2) Page 7 of 36, line number 34, "different radicals in C3": What is "radicals"? It may be "different substituent" (OH, acetate, succinate).

Reply: The term "radicals" was modified by "substituents"

3) Page 28 of 36, Figure 2 legend: "derivative (3 to 8)" may be "derivative (3 to 6)"

Reply: We corrected the legend to "semi-synthetic derivatives **3** to **6**".

4) Page 31 of 36, Figure 5, legend: against what cells?

Reply: The legend was modified to "Cell viability curves of *L. amazonensis* promastigote parasites versus concentration log (µM) obtained for pimaranes **1**, **2**, **3**, **5**, and **7**".

5) Page 32 of 36, Figure 6, legend: against what cells?

Reply: This legend was modified to "Graph of EC_{50} (µM) obtained for pimaranes **1**, **2**, **3**, **5**, **7** and amphotericin against *L. amazonensis* promastigote parasites".

Reviewer: 2

1- The introduction provides information on both leukemia and leishmaniosis as well as on the studied compounds. Here the only point to be clarified is the correct taxon. The authors used the species name *Aldama arenaria* (Baker) E. E. Schill. & Panero and give *Viguiera arenaria* Baker as a synonym. As neither The Plant List nor The International Composite Alliance list the taxon *Aldama arenaria* it would be helpful to explain that the species was before named *Viguiera arenaria* and placed into the genus Aldama due after phylogenetic analysis. Please also cite the respective reference (https://doi.org/10.1111/j.1095-8339.2011.01172.x). Please, also be aware that you used the name *V. arenaria* at page 5, line 16.

Reply: We thank the reviewer for this comment. The data reclassification are in the reference [2] and it was cited in the manuscript.

2- In the Results and discussion part please also give the absolute amounts obtained for the dichloromethane extract and the two pimaranes. It is also not quite clear of some of the derivatives are previously undescribed compounds.

Reply: The absolute amounts obtained for the DE and the pimaranos **1** and **2** were cited in the text.

3- If so, then the structure elucidation of the new compounds must be added if not so, please indicated more precisely. E.g. do write "No activities were described for compounds 5, 6, and 8" instead of "Derivatives 5, 6, and 8 were not described."

Reply: The phrase "Derivatives 5, 6, and 8 were not described." was modified by "No activities were described for compounds 5, 6, and 8".

4- Furthermore, lines 28 to 42 at page 5 should be moved upwards and appear after the last paragraph of page 4.

Reply: As suggested by the referee, we moved the lines 28 to 42 at page 5 upwards and now it appears after the last paragraph of page 4.

5- Moreover, in the Results and discussion part several text passages are too general and therefore should be removed or moved to the introduction. These passages are from page 5, line 47 to page 6 line 10 as well as from page 6, line 45 to page 6, line 57.

Reply: These passages cited were moved to the introduction.

6- In the Material and methods section, the isolation of compound 2 is a bit confusing. The authors write “To obtain pimarane 2, fractions from the DE chromatography containing large amounts of pimarane alcohol were pooled and methylated with the reagent TMSD for methylation of the acid.” However, compound 2 does not show an acidic function. Does this mean that the authors wanted to get rid of the additional components with similar polarity, e.g. compound 1? Please clarify in the text.
Reply: To better explain this passage we added to the manuscript the phrase “Because of the similar Rf values, this reaction is necessary to the convert the acid **1** in correspondent ester and ensure the isolation of alcohol”.

7- Please write “being inactive” instead of “inactive” at page 2, line 22 and “the acetate” instead of “acetate” in line 24.
Reply: In the lines 22 and 24 we wrote “being inactive” instead of “inactive”, and “the acetate” instead of “acetate”.

8- Please write “syn.” instead of “sin.” In at page 3, line 5.
Reply: On page 3, line 5 we wrote “syn.” instead of “sin.”

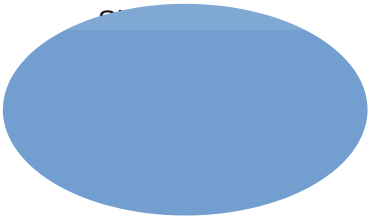
9- Please use “substituent” instead of “radical” as done on page 6, line 35 and page 8, line 5.
Reply: On page 6, line 35 and page 8, line 5, we used “substituent” instead of “radical”.

10- Please write “…and their semi-synthetic derivatives…” instead of “…and semisynthetics…” at page 9, line 28.
Reply: The phrase was modified to “and their semi-synthetics” at page 9, line 28.

11- Please write “evaporated under reduced pressure” instead of “vacuum evaporated” at page 10,
Reply: At page 10, line 33, we wrote “evaporated under reduced pressure”.

12- Please check for the writing of liters. Either l or L. Both versions appear in the text.
Reply: All writing of liters was standard to L.

Aware that we have made all the changes as you requested, we thank you for your consideration of this manuscript.



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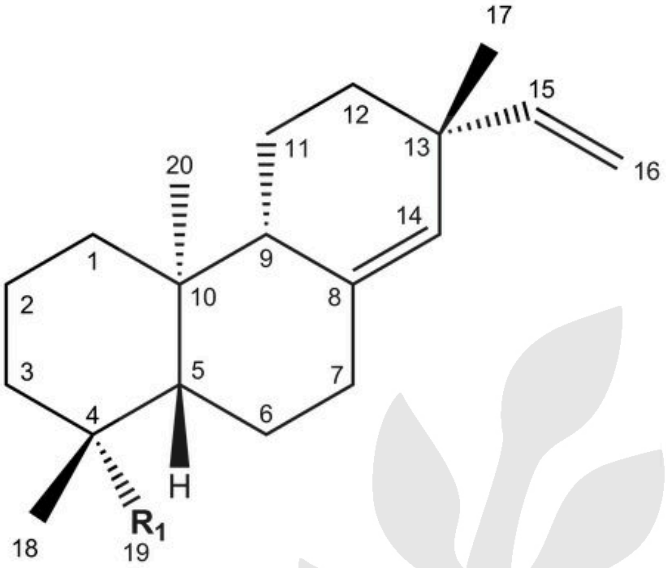
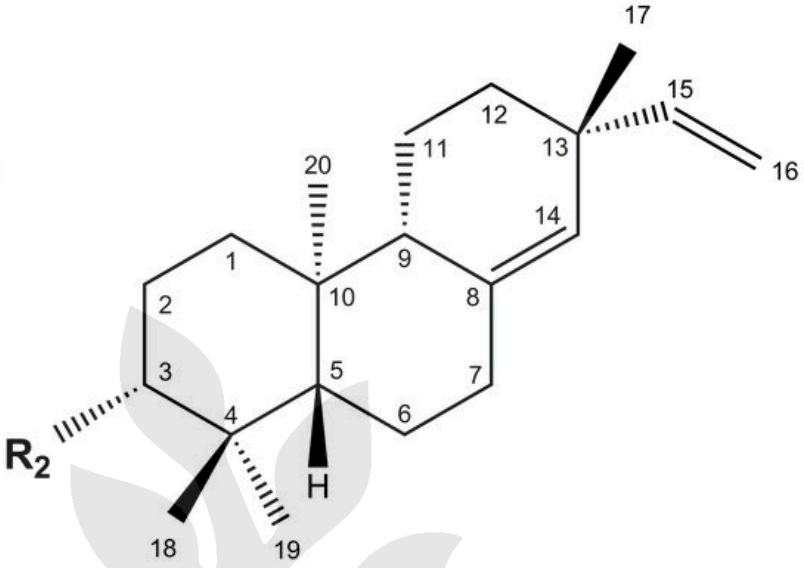


1: R_1= COOH
3: R_1= $COOCH_3$
4: R_1= CH_2OH
5: R_1= $OCOCH_3$
6: R_1= $OCO(CH_2)_2COOH$



2: R_2= OH
7: R_2= $OCOCH_3$
8: R_2= $OCO(CH_2)_2COOH$