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Antiparasitary Potential of Natural and Semi-synthetic Labdane Diterpenes

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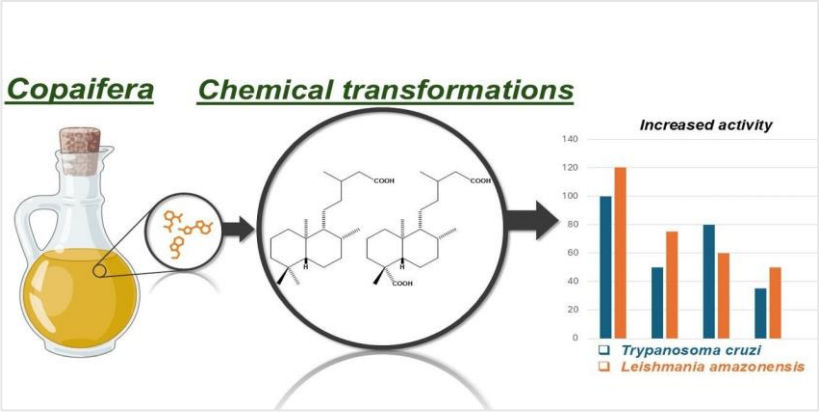
This paper describes the obtention of fourteen derivatives from four natural labdane diterpenes isolated from *Copaifera* oleoresin, named *ent*-copalic acid (**1**), *ent*-3β-acetoxy copalic acid (**2**), *ent*-3β-hydroxy copalic acid (**3**) and *ent*-agathic acid (**4**). All eighteen compounds, derivatives and precursors, were assayed against the promastigote form of *Leishmania amazonensis* and trypomastigote forms of *Trypanosoma cruzi*, revealing two promising compounds with leishmanicidal activity (IC_{50} = 5.94 μM and 5.31 μM) and three promising compounds with trypanocidal activity, two of them (IC_{50} = 13.31 μM and IC_{50} = 15.05 μM) displaying similar activity as the reference drug (IC_{50} = 13.12 μM) and one of them being even more potent with an IC_{50} = 0.425μM.

Graphical abstract



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1. Introduction

American trypanosomiasis, known as Chagas disease, is a neglected tropical disease, caused by the flagellate protozoan *Trypanosoma cruzi* [1]. According to the Drugs for Neglected Diseases Initiative,[2] there are 6 million people infected in 21 countries in Latin America, where Chagas disease is endemic and caused 14,000 deaths, and between 6 and 7 million people infected worldwide. Moreover, about 70 million people are at risk of infection [2].

There are only two drugs, nifurtimox (Nx) and benznidazole (Bz), indicated for the treatment of acute *T. cruzi* infection [3, 4] Nevertheless, none of these drugs are sufficiently efficient in the treatment of the disease, as their efficacy does not surpass 70%. Besides that, the effectiveness of these drugs is even worse in patients within the chronic phase of the disease [5].

These drugs commonly also trigger several side effects in adults, which sometimes need to avoid long-term treatment or abruptly discontinue them [6-8]. The low efficiency of Bz and

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Nx is not attributed to limited tissue penetration, but to low absorption during the first-pass metabolism in the liver. These two processes occur before tissue biodistribution, especially during the chronic phase of the disease, when the parasites are confined mainly to the deep tissues, in which replication occurs [9-11]. Moreover, the high cost, toxicity and drug resistance developed by *T. cruzi* strains are further cons [11-13].

Cutaneous leishmaniasis, in turn, is a vector-borned disease caused by protozoan parasites and is also considered a neglected disease. This is the most common form of leishmaniasis caused by *Leishmania amazonensis*, an infirmity that affects 0.6 to 1 million people each year in 87 countries worldwide [14]. It is known for developing skin lesions, often on the face, bringing severe social stigma, particularly in women and children [15].

After antimonial treatment failure in the 1950's, pentamidine, amphotericin B, paromomycin and miltefosine were used as treatment drugs for leishmaniasis. Despite some positive aspects, all of these treatments are considered unsatisfactory in at least one category: efficacy, cost, safety, and/or treatment failure [16]. Treatment of leishmaniasis is challenging and no vaccine or prophylactic drugs to prevent infection are currently available [17].

Although chemotherapy is the most practical and effective treatment applied to all three major forms of leishmaniasis, some unfavourable features of chemotherapy include toxicity, high cost and long-term treatment [18]. Thus, the search for new therapeutic options is mandatory.

Given this context, the need for new trypanocidal and leishmanicidal drugs that could be safer and more efficient for the treatment of Chagas' disease and leishmaniasis is evident and urgent.

One of the main sources of new substances with interesting biological activities are specialized metabolites from natural sources.[19] Among the diversity of natural substances to be explored, diterpenes constitute a numerous class of compounds that have gained prominence, justified by their promising profile that comprise several biological activities such as antimicrobial [20], anticancer [21], anti-HIV [22], anti-inflammatory and antitumoral [23], fungicide [24], antitubercular [25], antitrypanosomal [26], among others.

A considerable number of different structures to be assayed can be obtained by structural modification of those isolated metabolites. The more structural variability of semisynthetic derivatives to be assayed, the higher the chance of success. Thus, the major components of some natural sources can be isolated and submitted to chemical reactions, with the perspective of obtaining more active substances.

Due to interests in producing diterpene derivatives to obtain more active compounds [20, 24, 25] the present work describes the production of 14 semisynthetic derivatives from 4 labdane-type diterpenes, major constituents of *Copaifera langsdorffii* oleoresin, named *ent*-copalic acid (**1**), *ent*-3b-acetoxy copalic acid (**2**), *ent*-3b-hydroxy copalic acid (**3**) and *ent*-agatic acid (**4**) (Fig. 1). The studied reactions – esterification and hydrogenation – were chosen based on their simplicity and were planned to be used in combination, i.e., the goal was to apply simple reactions to different reaction targets, such as the double bonds and carboxyl group. This allowed the evaluation of the biological profile against *L. amazonensis* and *T. cruzi* of all eighteen substances, in the search for more active and less toxic compounds, as described.

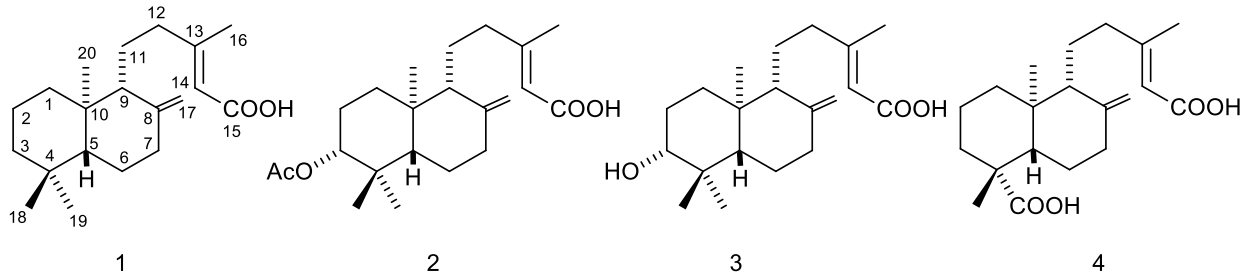


Fig. 1. Structures of the natural diterpenes.

2. Material and Methods

Chemistry

Plant material

The *Copaifera langsdorffii* Desf. oleoresin was purchased from 'Apis-Flora Comercio e Industria', a Brazilian herbal company located in the city of Ribeirão Preto, state of São Paulo under the register: lot 0790310, manufactured in 09/2010.

Extraction and isolation

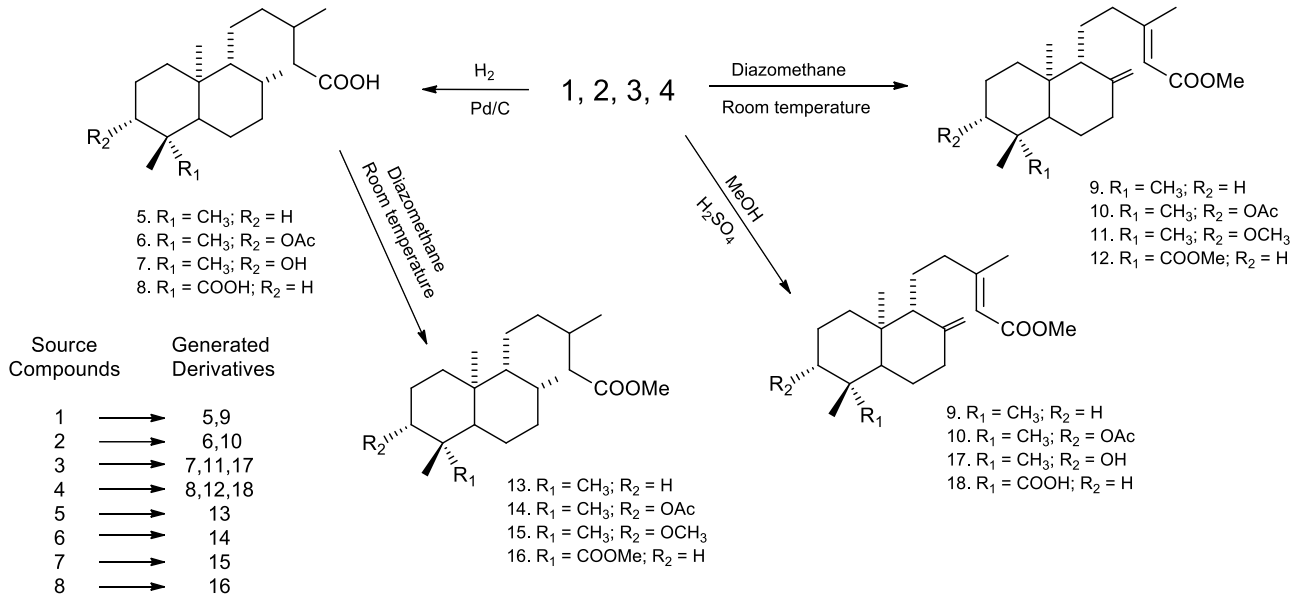
The major constituents present in the oleoresin sample, natural diterpenes ent-copalic acid (**1**), *ent*-3β-acetoxycopalic acid (**2**), *ent*-3β-hydroxy-copalic acid (**3**) and *ent*-agathic acid (**4**), were isolated according to the methodology previously described [27], carefully adapted to the isolation of only those four major diterpenes. For this, about 30.0g of oleoresin were chromatographed over silica gel 60 H (Merck, art. 7736) using vacuum liquid chromatography (VLC) with increasing amounts of EtOAc in *n*-hexane as eluent. This procedure furnished six fractions (500 mL each), named F1 (6.9 g; pure *n*-hexane), F2 (4.3 g; 80% *n*-hexane:20% EtOAc), F3 (5.1 g; 60% *n*-hexane:40% EtOAc), F4 (3.1 g; 40% *n*-hexane:60% EtOAc), F5 (3.8 g; 20% *n*-hexane:80% EtOAc) and F6 (2.9 g; pure EtOAc); masses measured after solvent evaporation. After an initial analysis by thin-layer chromatography (TLC), fraction F3 (1.0 g) was fractionated over silica gel 60 (Merck, art. 7734) using classic chromatography (isocratic, *n*-hexane: EtOAc 7:3), to obtain 650.0 mg of compound **1** (ent-copalic acid). Both F4 and F5 were initially chromatographed by VLC over silica gel 60 H (Merck, art. 7736) as described above, to result in additional fractions (F4.1–F4.5 and F5.1–F5.5). Compound **2** (*ent*-3β-acetoxycopalic acid) was obtained (330.0 mg) from F4.3 (1.2 g) through medium pressure chromatography (*flash*

chromatography) using silica gel 60 (Merck, art. 9385), isocratic *n*-hexane:EtOAc:$CHCl_3$ (5:2:3) as mobile phase, and a flow rate of 5 mL/min. Subfraction 5.4 (390.0 mg) was also chromatographed by flash chromatography as described above. This procedure led to the isolation of 220.0 mg of compound **4** (*ent*-agathic acid). Thin layer chromatography (TLC) analysis of F5.2.5 showed a main spot, which was later purified by preparative thin layer chromatography using silica gel PF254 (Merck art. 9385; 1 mm thickness) and isocratic *n*-hexane: EtOAc 1:1 as mobile phase. This procedure yielded 190.0 mg of compound **3**.

The identification of the 4 isolated diterpenes was performed, by comparative analysis, from their NMR data from previously published data in the literature,[28], [29] as realized in a previous work.[25] Special attention was given to compound **1**, to which all structure comparisons were made. NMR spectra and data are shown in Figures S1-S8 and Tables SI and SII, in the supplementary material. Purities of the isolated compounds were around 95%, as estimated by NMR.

Semi-synthetic derivatives

The series of semi-synthetic derivatives was prepared from the four isolated labdane diterpenes, **1-4**, by a previously described procedure,[25] as shown in Scheme 1.



Scheme 1. Preparation of semi-synthetic diterpenes.

Hydrogenation

In a special bottle (glass tube), 70 mg of the compound to be hydrogenated, 20 mL of absolute ethanol and catalytic quantity of Pd/C were introduced. The atmosphere in the reactor was changed for Hydrogen and the hydrogen pressure was adjusted to 2 atm. The reaction mixture was then stirred at room temperature for 2 hours. Following, the reaction mixture was filtered in Celite® and the solvent was removed by rotary evaporation, obtaining the hydrogenated compounds, **5-8**, with a yield of between 95 to 98%.

Esterification with diazomethane

Initially, diazomethane was prepared with 2.4 g of diazogen (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) placed in a round bottom 125 mL flask and dissolved in 30 mL of anhydrous ethylic ether. The flask was cooled to 0 ºC and a solution of 0.4 g of KOH in 10 mL of anhydrous ethylic ether was added dropwise to the mixture. Then, a distilling system was prepared with this flask, that was heated to 100 ºC, and the diazomethane was distilled and collected in an Erlenmeyer placed in an ice bath (0 ºC) as a solution in anhydrous ethylic ether. Following, the prepared diazomethane solution was added to the solution containing the starting material, 50 mg of substrate in 5 mL of ethylic ether. After each addition, the mixture was stirred for a few seconds until gas evolution (N_2) ceased. The process was repeated until there was no further release of gas with new addition of diazomethane. At the end, the solvent was removed by rotary evaporation and the products, **9-16**, were obtained with a yield of around 95%.

Esterification with methanol

In a round bottom 25 mL flask, a solution of the diterpene (70 mg) in methanol (10 mL) was prepared. Then, 10 drops of concentrated sulfuric acid were slowly added to this solution, which remained stirring at room temperature for 18 hours. 30 mL of distilled water were added to the reaction mixture and then extracted with 3 portions (20 mL each) of ethyl acetate. The organic phase was dried under $MgSO_4$, and the solvent was removed by rotary evaporation. After purification, the analogues **9**, **10**, **17** and **18**, showed approximately 95% of yield.

Purity of all semi-synthetic compounds were estimated by NMR to be around 95%, before performing the biological assays.

NMR structural assignments were made by comparison with previously published data [25, 28] as described before [25]. Spectra are shown in figures S9 to S22.

In vitro trypanocidal evaluation

In the present work, clone B5 of the Tulahuen strain of *T. cruzi*, which expresses β-galactosidase, giving this strain a special characteristic for quantification of the number of parasites, regardless if they are or not within the host cell, was

used.

LLCMK2 cells were used and distributed in 96-well plates (5×10^4 cells/mL). The cells were infected with T. cruzi (5×10^5 parasites/mL) after 2 hours. The cultures were incubated for 24 h, 37°C, and 5% CO_2 and then washed with phosphate buffered saline (PBS). Serial dilutions (0.5; 2.0; 8,0 and 32μM) of compounds in DMSO: RPMI solution (0,5:100) were added to the cultures and incubated for 72 h, 37°C, and 5% CO_2. After incubation, the media was removed and followed by the reaction with chlorophenol red-β-D-galactopyranoside (CPRG) buffer (200 μM CPRG, 2% Trion X-100, and 50 mM $MgCl_2$ in PBS) for 4 h, 37°C. The plates were read at 570 nm in ELISA reader (Synergy™ H1, Biotek). All assays were performed in triplicate. Wells containing only culture medium and cells of the LLMCK2 lineage were used as positive control and parasitized cells treated only with DMSO: RPMI solution were used as negative control. Bz was used as reference drug at the same concentrations of the tested compounds. All assays were performed in triplicate. Determination of 50% inhibitory concentration values (IC50) was carried out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA).

In vitro leishmanicidal evaluation

All 18 compounds were evaluated against *Leishmania amazonensis* (IFLA/BR/67/PH8) promastigotes (1 x 10^6 parasites per well) according to a previous reference [30]. Compounds were assayed at 6.25, 12.5, 25, 50, and 100 μM. Amphotericin B (0.5 μM) was used as positive control, while RPMI 1640 medium containing 0.1% DMSO was used as negative control. The determination of IC_{50} was carried out by non-linear regression curves of a GraphPad Prism version 5.0 Windows software (GraphPad software, USA).

3. Results and Discussion

After isolation, purification, and structure confirmation of the four major constituents from the *C. langsdorffii* oleoresin (**1** to **4**), these were submitted to structural modification through hydrogenation and esterification methods, as described above. This resulted in fourteen analogue structures (**5** to **18**), as shown in scheme 1. The chemical structures of these products were confirmed according to NMR data, by careful comparison to the precursor's data. The absence of olefinic protons signals in compounds **5** to **8** indicates the expected structure for all cases. Moreover, the presence of a new methyl signal (position 17), the shifting of CH_3-16 to a more shielded position and a new CH_2 signal (position 14) in all four derivatives confirm the structures shown in scheme 1. The identification was even easier for all obtained esters. As the hydrogen signal from the carboxyl group is not detected in the most common spectral width, for products **9**, **10**, **13** – **15**, **17** and **18**, the only difference in the product ^{1}H-NMR spectrum, in relation to the precursor, is one more signal near 3.5 ppm, assigned to the -OCH_3 group of the formed methyl ester. For compounds **11**, **12** and **16**, two methoxy NMR signals were observed.

The natural compounds and their derivatives were assayed against trypomastigote forms of *T. cruzi* and against promastigote forms of *L. amazonensis*. All biological results are presented in Table I.

Table 1. *In vitro* antileishmanial and antitrypanosomal activity of natural labdane diterpenes and their derivatives (IC_{50}±SD).

Compounds	*L. amazonensis* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)	Compounds	*L. amazonensis* IC_{50} (μM)	*T. cruzi* IC_{50} (μM)
1	30.65±7.39	>100	10	14.65±4.65	>100
2	87.49±26.86	>100	11	8.27±1.43	15.05±3.2
3	89.47±26.49	0.425±0.08	12	8.73±1.71	>100
4	>100	>100	13	26.39±4.58	>100
5	>100	>100	14	11.35±1.92	13.31±2.8
6	5.94±1.53	>100	15	8.88±3.51	>100
7	>100	>100	16	5.31±1.03	>100
8	>100	>100	17	61.25±24.11	68.36±19.5
9	17.20±4.59	>100	18	>100	>100
Amphotericin B	0.043±0,015	---	Amphotericin B	0.043±0,015	---
Benznidazole	---	13.12±3.7	Benznidazole	---	13.12±3.7

Antileishmanial: *Leishmania amazonensis;* promastigote; MHOM/BR/PH8. Antitrypansomal: *Trypanosoma cruzi*; trypomastigote; clone B5, Tulahuen strain.

As it can be seen, compound **3** was the only natural compound that showed expressive anti-trypanosome activity (IC_{50} = 0.425 μM). Moreover, most of the active compounds obtained (**11** and **17**) are derived from this compound. It can be stated that all active compounds presented in this work (**3**, **11**, **14** and **17**) feature a substituent in position 3, which seems to be a requisite for this kind of structure to be active. Another kind of requirement to display some activity seems to be the presence of two oxygenated functional groups, as all active compounds in this work. A careful look at compound **1** and its derivatives (**5**, **9** and **13**) shows the inactivity of only one functional group. Moreover, even with two functional groups, it seems that one of them is required in position 3, as compound **4** and all its derivatives (**8**, **12**, **16** and **18**) are completely inactive. In addition, it can also be stated that the hydrogenation is not one of the best transformations to perform in these diterpenes in the search for trypanocide substances, as from four active compounds in this group, only one presents single bonds between 8,17 and 13,14 carbons. Furthermore, the ester function also seems important for activity in this type of skeleton, since it constitutes four of the functional groups of a total of eight in the active compounds (**3**, **11**, **14** and **17**). These latter results agree with a previous published work from Chavez and his co-workers [31], which states that all prepared esters where more potent than the precursor acid diterpene.

Regarding trypanocidal activity, the present results are promising, since two obtained derivatives (**11** and **14**) present activity at the same magnitude than the reference drug, Benznidazole. Moreover, one of the assayed natural precursors (**3**) was even more potent, in a greatness of ten, than the positive control. These can be considered very

promising results in the search for active compounds against *T. cruzi*. When compared to the results in the literature for diterpenes versus *T. cruzi* [31-34] or more specifically, labdanes against *T. cruzi* [35-37] it can be reaffirmed that the results obtained in the present work are very promising. In one work [33] that prepared 32 derivatives, the most active compound displayed activity only with a concentration three-fold the Bz concentration in μg/mL. Ullah and co-workers [34] show the most active compound needing 3.7 times the concentration of Bz in μM to be active. Besides that, another published work dealing with trypanocidal activity of diterpenes [31] concluded that only a moderate activity was reached. Only one of work [32] presented promising results of the same magnitude of ours; nevertheless, the structures assayed were completely different from all compounds here presented. Regarding labdane diterpenes against *T. cruzi*, the three cited references showed only assayed seven substances of this class. Four of them [36] re somewhat different than the copalic analogues, and involved two potentially active, displaying a high value for lysis in one unique concentration (125 μg/mL). Nevertheless, IC_{50} was not calculated neither was there a positive control, turning results not comparable. In the other work [37], two labdanes, more like copalic analogues, were assayed and one of them displayed also promising activity against *T. cruzi*. They were compared to Nx (IC_{50} = 7.7 μM) as positive control and one of the assayed compounds demonstrated to be as active as it (IC_{50} = 9.8 μM). The only reference found showing the same kind of structure, was the work by Sartorelli and his co-workers [35]. In this work, *ent*-copalic acid is assayed and does not show significant activity against the amastigote form of *T. cruzi*.

Thus, relatively to the literature, the results are interesting and from our knowledge, our work presents the most promising evidence of trypanocidal activity of copalic acid analogues labdane diterpenes.

Regarding the comparison between natural (**3**) and semi-synthetic (**11**, **14** and **17**) active products. The most active compound is the natural diterpene **3**, precursor of **11** and **17,** showing that in both cases the structural modification turned the activity worse. Nevertheless, from the perspective of compound **14**, derived from **2** and with potentially the same activity as Bz, the structural modification seemed to be extremely important. However, results should not be evaluated in such an isolated manner. The obtention of more than one promising substance in *in vitro* assays enhances the chance to get one or two active compounds in future *in vivo* experiments. Toxicity evaluation can also bring even more important information for the constant search for new trypanocide agents.

With respect to leishmanicidal activity, a greater number of natural compounds expressed some measurable activity. Nevertheless, none of them displayed good activity, being the best with IC_{50} = 30.65 μM (compound **1**). On the other hand, the activity improvement by chemical transformations is more expressive in this case. From the fourteen transformations performed in this work, nine of them improved leismanicidal activity, two of them caused no change and only three transformations decreased activity.

The most significant transformation was obtained through esterification, as in almost all cases an improvement of activity was observed. However, hydrogenation, which decreased activity in most cases, led to one of the most active compounds – compound **6** – with IC_{50} = 5.94 μM. Moreover, the most active compound obtained in this work, with an IC_{50} = 5.3 μM, was produced by both transformations in sequence (compound **16**).

These results seem to be promising but are not so easy to compare to other results from the literature, mostly because there are several different species of *Leishmania*. This work evaluated the activity of compounds against promastigote forms of *L. amazonensis*. Using the Web of Science search tool, we were unable to find articles combining the terms "labdane" and "*Leishmania amazonensis*". Nevertheless, we could find some leishmanicidal results for labdane diterpenes against *L. donovani*, [38-40], most with promastigote forms, but also most results in μg/mL, not μM.

Therefore, the only way to make a slight comparison would be considering the positive control activity compared to the assayed substances' activities. The results obtained by Fokialakis and co-workers are expressed in μg/mL for 21 results labdane [40]. A total of 6 results present IC_{50} values that are considerably higher (IC_{50} above 30.0 μg/mL) than for amphotericin B (IC_{50} = 0.17 μg/mL), the positive control, and 11 results could be considered intermediary (30.0 μg/mL > IC_{50} > 10.0 μg/mL). On the other hand, there are four results that could be considered promising with IC_{50} lower than 10 μg/mL (between 3.5 and 8.0 μg/mL). For this present work, similar results were achieved, despite being for *L. amazonensis* and with IC_{50} values expressed in μM. Compounds **1**-**5**, **7**, **8**, **17** and **18** are not active with IC_{50} above 30.0 μM, while amphotericin presents IC_{50} = 0.043 μM. With intermediary activity, compounds **9**, **10**, **13** and **14** displayed IC_{50} values between 10.0 and 30.0 μM. The best activities were obtained for compounds **6**, **11**, **12**, **15** and **16**, which presented IC_{50} below 10 μM, highlighting compounds **6** (IC_{50} = 5.94 μM) and **16** (IC_{50} = 5.31 μM).

Another partial comparison can be done with the work of Afolayan and co-workers,[39] in which three labdanes, very similar to the structures here presented, were assayed against *L. donovani* with results of IC_{50} expressed in μM, obtaining very similar results, with the best activity reaching IC_{50} = 7.82 μM. The results obtained by Ghorbani and collaborators [38] can be considered the best ones of the discussed set. Despite assaying only two labdane diterpenes, the obtained results can be considered promising (both IC_{50} between 0.06 and 0.09 μM). These substances were new structures at the occasion and presented different organic functions (aldehyde and epoxide) than the compounds in this present work and although the authors did not present IC_{50} for amphotericin B in that work, we are clearly facing good results.

4. Conclusions

A group of four natural substances were evaluated, from which a promising trypanocide was identified. Moreover, three other trypanocidal compounds were obtained through simple structural modifications, two of them also very promising. In addition, those transformations allowed the obtention of two considerably promising anti-leishmanial agents. As an overall result, compound **11** can be considered interestingly antiparasitary, displaying activity against both parasites.

It can be concluded that labdane-type diterpenes are promising in the search for antiparasitary compounds (against *T. cruzi* and *L. amazonensis*) and that structural modification is certainly a profitable route to accomplish this goal.

Supporting Information

Supplementary material is available at the journal's website as PDF file, with free access.

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Author Contributions

Sérgio de Albuquerque – investigation, methodology, project administration, and resources; Lizandra G. Magalhães – investigation, methodology, project administration, and resources; Daiane A. dos Santos: investigation, methodology, and validation; Vinícius J. M. Rodrigues: investigation, and writing-original draft; Analuz S. Machado: investigation, visualization, and writing-original draft; Julian C. S. Pavan – investigation, methodology, supervision, and writing-original draft; Ana C. F. S. Rocha – visualization, and writing-original draft; Aline N. S. Parra – investigation, and visualization; Vladimir C. G. Heleno – conceptualization, methodology, project administration, resources, supervision, visualization, writing-original draft, writing-review and editing.

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