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Posted Date: 24 July 2024

doi: 10.20944/preprints202407.1954.v1

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Article

Antiparasitary Potential of Natural and Semi-Synthetic Labdane Diterpenes

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

Keywords: leishmaniasis; chagas disease; copalic acid; structural modification; trypanocidal and leishmanicidal activities

1. Introduction

American trypanosomiasis, known as Chagas disease, is a neglected tropical disease, which is 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 between 6 and 7 million people infected worldwide. Moreover, there are about 70 million people at risk of infection, and, in addition, the disease is responsible for 14,000 deaths in the endemic region [2].

There are only two drugs, Nifurtimox (Nx) and Benznidazole (Bz), indicated for the treatment of acute *T. cruzi* infection [3,4]. Nevertheless, both drugs could be considered not good enough to treat the disease because their efficacy is not higher than 70%. Besides that, when the patient is in the chronic phase of the disease, the performance of those drugs is even worse [5].

Furthermore, those drugs commonly 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 Nx could not be attributed to limited tissue penetration, but to low absorption during 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 the medicine resistance developed by the strains of *T. cruzi* are further cons [11–13].

Cutaneous leishmaniasis, in turn, is a vector-borned disease caused by protozoan parasites and is also considered as a neglected disease. This is the most common form of leishmaniasis caused by *L. amazonensis*, an infirmity that affects 0.6 to 1 million people each year at 87 countries worldwide

 doi:10.20944/preprints202407.1954.v1

[14]. It is known to develop skin lesions often on the face, which brings severe social stigma, particularly for 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 them were considered unsatisfactory at least in one item such as efficacy, cost, safety, or treatment failure [16]. Treatment of leishmaniasis in 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 therapeutics options in mandatory.

Given this context, the need of 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 has been the specialized metabolites from natural sources [19]. Among the diversity of natural substances to be explored, there are the diterpenes. They constitute a numerous class of compounds that has 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. So, 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 our research group's interests in producing diterpene derivatives to reach 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**) (Figure 1). This allowed the evaluation of the biological profile against *Trypanosoma cruzi* of eighteen substances, in the search for more active and less toxic compounds, as described.

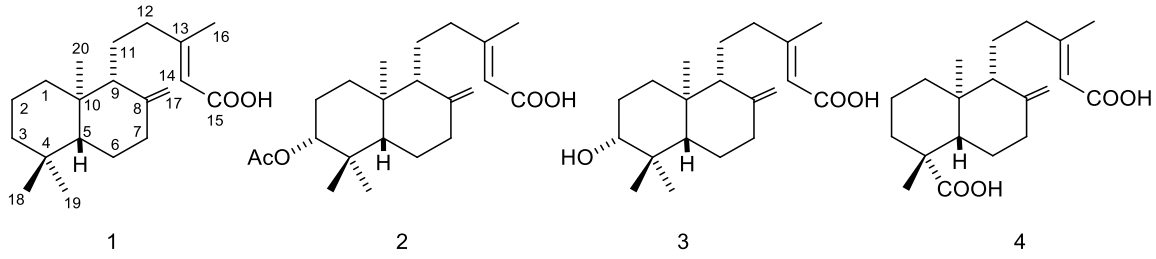

Figure 1. Structures of the natural diterpenes.

2. Materials and Methods

2.1. Chemistry

2.1.1. 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 09/2010.

2.1.2. Extraction and Isolation

The major constituents present in the oleoresin sample, the natural diterpenes entcopalic acid (**1**), *ent*-3β-acetoxycopalic acid (**2**), *ent*-3β-hydroxy-copalic acid (**3**) and *ent*-agathic acid (**4**) were

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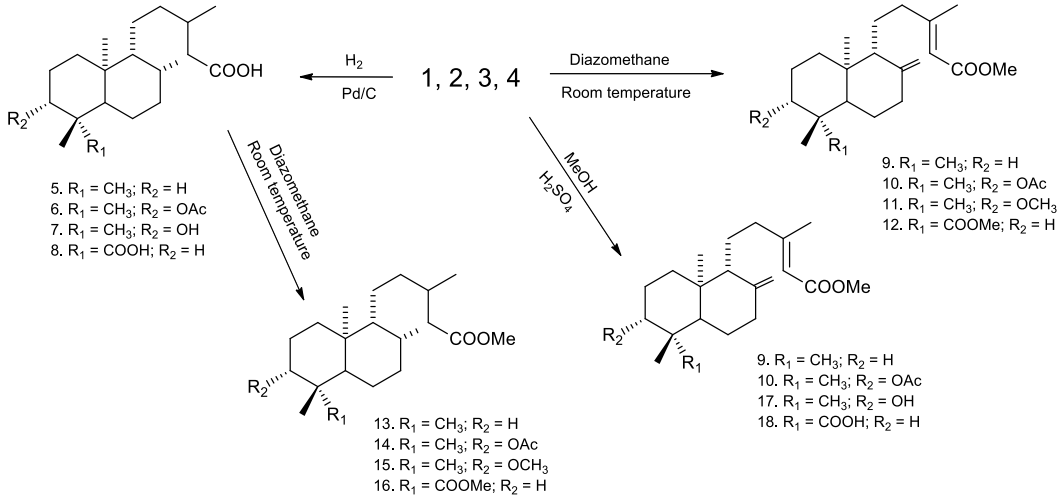
isolated according to the methodology previously described by our research group [27]. The identification of the 4 isolated diterpenes was performed, by comparative analysis, from their NMR data with previously published data in literature [28,29], as performed before [25]. Especial attention was given to compound **1**, to which all structures comparisons are made. NMR spectra and data are in Figures S1–S8 and Tables SI and SII, in supplementary material. Purities of the isolated compounds were estimated, by NMR, to be around 95%.

2.1.3. Semi-Synthetic Derivatives

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



Scheme 1. Preparation of semi-synthetic diterpenes.

2.1.4. Hydrogenation

In a special bottle (glass tube), were placed 70 mg of compound to be hydrogenated, 20 mL of absolute ethanol and catalytic quantity of Pd/C. 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. At the end of this time, 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 from 95 to 98%.

2.1.5. 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, and it 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. After, 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 yielding around 95%.

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2.1.6. 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. The reaction mixture was added 30 mL of distilled water 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 around 95%, before sending to biological assays.

NMR structural assignments were made by comparison with previously published data [25,28] and was undertaken exactly as described before [25]. Spectra are in Figures S9 to S22.

2.2. In Vitro Trypanocidal Evaluation

For the present work, clone B5 of the Tulahuen strain of *T. cruzi* was used, which has the property of expressing β-galactosidase, which gives this strain a special characteristic for quantification of the number of parasites, regardless of they are or not within the host cell.

LLCMK2 cells was used and distributed in 96-well plates (5×10^4 cells/mL). After 2 hours the cells were infected with T. cruzi (5×10^5 parasites/mL). The cultures were incubated for 24 h, 37°C, and 5% CO_2 and them 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 were 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 the assays were performed in triplicate. As a positive control we used wells containing only culture medium and cells of the LLMCK2 lineage and as negative control, parasitized cells treated only with DMSO: RPMI solution. Bz was used as reference drug at same concentrations of tested compounds. All assays were performed in triplicate.

2.3. In Vitro Leishmanicidal Evaluation

All 18 compounds were evaluated against *Leishmania amazonensis* (IFLA/BR/67/PH8) promastigotes (1 x 106 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 mM) was used as positive control, while as the negative control, RPMI 1640 medium containing 0.1% DMSO was used. The IC50 (50% *L. amazonensis* promastigote flagellar motility inhibition) was obtained by using GraphPad Prism 6.

3. Results

After the isolation, purification, and structure confirmation of the four major constituents, from the *C. langsdorffii* oleoresin (**1** to **4**), they were submitted to structural modification through hydrogenation, and esterification methods described above. This resulted in fourteen analogue structures (**5** to **18**), as shown scheme 1. These products had their chemical structures confirmed according to NMR data, by careful comparison to the precursor's data. For compounds **5** to **8**, the absence of olefinic protons signals 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 on scheme 1. For all obtained esters, the identification was even easier. 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**, there were two methoxy NMR signals 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 expressed in Table 1.

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Table 1. *In vitro* antileishmanial and antitrypanosomal activity of natural labdane diterpenes and their derivatives.

Compounds	*L. amazonensis* IC_{50} (M)	*T. cruzi* IC_{50} (M)	Compounds	*L. amazonensis* IC_{50} (M)	*T. cruzi* IC_{50} (M)
1	30.65	>100	**10**	14.65	>100
2	87.49	>100	**11**	8.27	15.05
3	89.47	0.425	**12**	8.73	>100
4	>100	>100	**13**	26.39	>100
5	>100	>100	**14**	11.35	13.31
6	5.94	>100	**15**	8.88	>100
7	>100	>100	**16**	5.31	>100
8	>100	>100	**17**	61.25	68.36
9	17.20	>100	**18**	>100	>100
Anfothericin B	0.043	---	**Anfothericin B**	0.043	---
Benznidazole	---	13.12	**Benznidazole**	---	13.12

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

4. Discussion

As can be seen, compound **3** was the only natural compound that showed expressive anti-trypanosome activity (IC_{50} = 0.425mM). Moreover, most of the active compounds obtained (**11** and **17**) are derived from this compound to. One can all state that all active compounds in this work (**3**, **11**, **14** and **17**) features 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 if there are two functional groups, it seems to be required one of them in position 3, as compound **4** and all its derivatives (**8**, **12**, **16** and **18**) are completely inactive. In addition, one can state that the hydrogenation is not one of the best transformations to perform in these diterpenes in the search for trypanocide substances, because from four active compounds in this group, only one presents single bonds between 8,17 and 13,14 carbons. Furthermore, the ester function seems also important to activity in this type of skeleton, since it constitute four of the functional groups in a total of eight in the active compounds (**3**, **11**, **14** and **17**). These latter results agree with previous published work from Chavez and his co-workers [31], which states that all prepared esters where more potent than the precursor acid diterpene.

In the view of trypanocidal activity, our results are promising, since there are two obtained derivatives (**11** and **14**) with 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. Those can be considered very promising results in the search for active compounds against *T. cruzi*. When compared to the results in 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], even the preparation of 32 derivatives, the most active compound displayed activity only with a concentration threefold the Bz concentration in mg/mL. Ullah and co-workers [34] show the most active compound needing 3.7 times the concentration of Bz in mM to be active. Besides that, another published work dealing with trypanocidal activity of diterpenes [31] concluded that there was reached only a moderate activity. Only one of the searched works [32] had promising results of the same magnitude of this present one and, nevertheless, the structures assayed were completely different from all compounds here presented. Regarding labdane diterpenes against *T. cruzi*, the three cited references showed only

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seven substances of this class assayed. Four of them [36] are somewhat different than the copalic analogues, and involved two potentially active, displaying a high value for lysis in one unique concentration (125 mg/mL). Nevertheless, IC_{50} was not calculated neither there was a positive control, turning results not comparable. From the other reference [37], they were two labdanes more like copalic analogues assayed and one of them displayed also promising activity against *T. cruzi*. They were compared to Nx (IC_{50} = 7.7 mM) as positive control and one assayed compound demonstrated to be as active as it (IC_{50} = 9.8 mM). 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 present results are promising, and, from our knowledge, this is the work that most evidence promising trypanocidal activity of copalic acid analogues labdane diterpenes.

One last issue that deserves to be discussed is concerning 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. Moreover, results should not be evaluated in such an isolated manner. The obtention of more than one promising substances at *in vitro* assays, enhances the chance to get one or two active compounds at future *in vivo* experiments. Toxicity evaluation also can bring even more important information for the constant search for new trypanocide agents.

For the leishmanicidal activity case, a greater number of the natural compounds expressed some measurable activity. Nevertheless, none of them displayed good activity, being the best with IC_{50} = 30.65 mM (compound **1**). On the other hand, the improve of activity 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 the esterification, once in almost all cases there was an improvement of activity. However, the hydrogenation, which decreased activity in most cases, led to one of the most active compounds – compound **6** – with IC_{50} = 5.94 mM. Moreover, the most active compound obtained in this work, with an IC_{50} = 5.3 mM, was produced by both transformations in sequence (compound **16**).

These results seem to be promising, but they are not so easy to compare to other results in 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 Web of Science search tool, we were unable to found 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 mg/mL, not mM.

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

 doi:10.20944/preprints202407.1954.v1

Another partial comparison can be done with Afolayan and co-workers article [39], where three labdanes very similar to our structures were assayed against *L. donovani* with results as IC_{50} expressed in mM. For this case, similar results were obtained, with the best activity reaching IC_{50} = 7.82 mM. The results obtained by Ghorbani and collaborators [38] can be considered the best ones of this discussed set. Despite assaying only two labdane diterpenes, the obtained results can be considered promising (both IC_{50} between 0.06 and 0.09 mM). These substances were new structures at the occasion and presented different organic functions (aldehyde and epoxide) then 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.

5. Conclusions

The search for potential trypanocidal and leishmanicidal diterpenes can be considered successful in this work. A group of four natural substances were evaluated, from which it was identified a promising trypanocide. 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.

One can conclude that labdane-type diterpenes can be stated as 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.

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

Author Contributions: Conceptualization, V.C.G.H. and L.G.M.; Methodology, S.A. and V.C.G.H.; Investigation, J.C.S.P., A.N.S.P. and D.A.S.; Resources, V.C.G.H., L.G.M. and A.N.S.P.; Writing - Original Draft Preparation, A.N.S.P., A.S.M. and V.J.M.R.; Writing – Review & Editing, A.C.F.S.R. and L.G.M.; Project Administration, V.C.G.H.; Funding Acquisition, S.A., L.G.M. and V.C.G.H.

Funding:

Acknowledgments: This work was supported by the São Paulo Research Foundation (FAPESP) under grant number 2009/0949-1; the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) under grant (finance code) number 001. Authors are grateful also to CNPq (308348/2018-4). The authors declare no conflict of interests.

Conflicts of Interest: The authors declare no conflict of interest.

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