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# Terpenes from *Copaifera* Demonstrated in Vitro Antiparasitic and Synergic Activity

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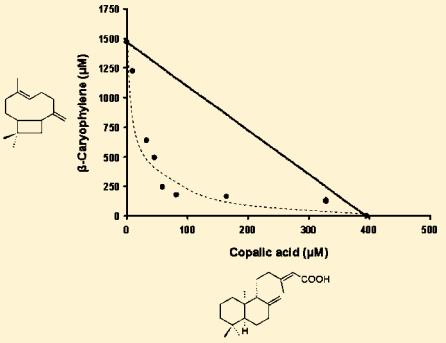
**ABSTRACT:** To discover new possible therapies for Chagas' disease, we evaluated against all *Trypanosoma cruzi* life stages the in vitro trypanocidal and synergistic activity of terpenes isolated from *Copaifera* oleoresins collected in the Amazon and investigated their possible mechanism of action. Seven acid diterpenes and one sesquiterpene were tested. Terpenes promoted changes in oxidative metabolism followed by autophagic processes in the parasite cell leading to selective death. Furthermore, they were more effective against replicative forms, in particular amastigotes. A synergistic effect occurred. Cytotoxicity to erythrocytes and nucleated cells was moderate. This is the first study showing synergic activity between two terpenes against *T. cruzi*. Combinations of natural compounds can show high activity and may lead to new alternative treatments in the future.




## INTRODUCTION

Chagas' disease is an endemic disease in Latin America, affecting over 15 million people.[1] It is caused by *Trypanosoma cruzi*, a kinetoplastid protozoan that is transmitted by triatomine bugs. Although Chagas' disease is vector-borne, in the Amazon region, oral infections have occurred due to food contamination with vector excreta containing the parasite.[2] Although the disease is endemic to Latin America, migration of infected people has spread it outside the region.[3]

Current drugs used to treat parasitic diseases are highly toxic or have become less effective against resistant strains. In recent decades, research on the development of new drugs based on natural products for the treatment of neglected diseases has intensified. Naturally occurring molecules are continually being found and evaluated for their biological activity, including metabolites isolated from endophytic microorganisms and animals.[4,5] However, plants are still the major source for bioprospecting, since they have traditionally been used to cure diseases since ancient times.

Natural product research is a growing field, partly due to its potential as a source for new medicines and treatments for neglected diseases, especially those for which there is no cure or the available treatment has severe side effects or low effectiveness.[6] Studies with natural products usually involve an extraction process followed by isolation and identification of compounds. Bioguided fractionation is a useful and preferred method, although most of these studies still do not result in isolated compounds, as inferred by a recent review for Chagas' disease.[7] This review found that the causative parasite was the target for studies investigating the biological activity of almost 400 plant species in the last 15 years, although only slightly more than 100 compounds were actually isolated and evaluated.

It is difficult to find published studies that compare compounds obtained from the same plant. Usually, the compounds are evaluated separately or are compared to a synthetic drug. Recently, it was demonstrated that amiodarone, an antiarrythmic compound also prescribed to chagasic patients, acts synergistically against *T. cruzi*, when associated with posaconazole, an antifungal compound.[8] Despite the fact that synthetic available drugs are still preferred for the investigation of synergic activity, natural compounds can become a more viable alternative to treatments than the use of drug combinations.

The *Fabaceae* is one of the most important plant families in ethnopharmacology. Members of the fabaceaean genus *Copaifera* occur in many countries in the Americas and Africa. In Brazil, they are popularly known as copaiba trees and are present in several regions; however, they are most abundant in the Amazon region and in the western parts of the country. *Copaifera* sp. trees produce an oleoresin known as copaiba oil, which is used by natives for its medicinal properties.[9]

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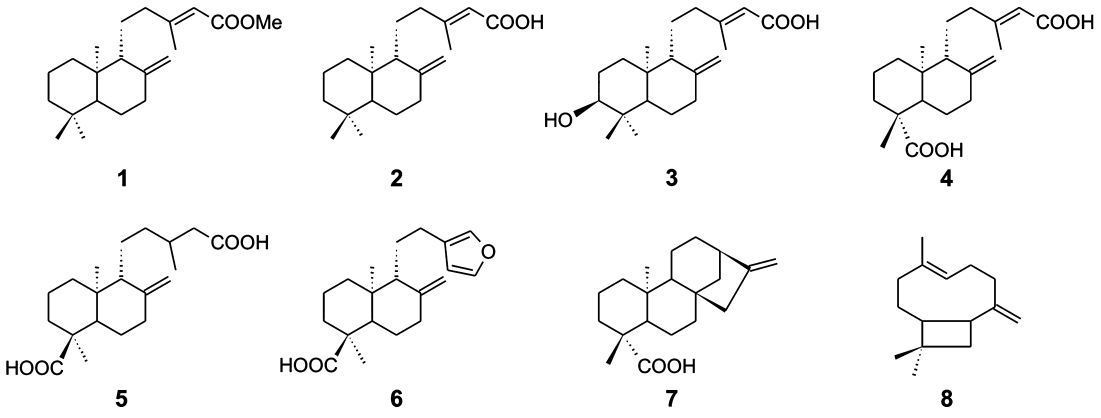


**Figure 1.** Terpenes evaluated for antitrypanosomal activity. Methyl copalate (**1**), copalic acid (**2**), 3$\beta$-hydroxycopalic acid (**3**), agathic acid (**4**), pinifolic acid (**5**), polyaltic acid (**6**), kaurenoic acid (**7**), and $\beta$-caryophylene (**8**).

**Table 1. Inhibition of *T. cruzi* Life Stages and Cytotoxicity on Mammalian Cells[a]**

| | epimastigotes | trypomastigotes | amastigotes | $LLCMK_2$ | erythrocytes | SI |
|---|---|---|---|---|---|---|
| compd | $IC_{50}$/96 h | $EC_{50}$/24 h | $IC_{50}$/96 h | $CC_{50}$/96 h | $HC_{50}$/3 h | $CC_{50}/IC_{50}$ ama |
| **1** | 83.3 ± 2.2 | 377.3 ± 88 | 2.5 ± 0.06 | 69.1 ± 4.4 | 1597 ± 9.4 | 27.6 |
| **2** | 42.7 ± 6.5 | 444 ± 161.1 | 1.3 ± 0.06 | 39.4 ± 4.6 | 65.7 ± 0.2 | 30.3 |
| **3** | 41.2 ± 0.9 | 453.1 ± 109.3 | 1.8 ± 0.06 | 31.2 ± 6.5 | 125 ± 7.5 | 17.3 |
| **4** | 86.8 ± 14.9 | 823.3 ± 104.7 | 14.9 ± 2.9 | 56.8 ± 6.2 | 1047 ± 10.4 | 3.8 |
| **5** | 854 ± 108.6 | 1630 ± 108.6 | 18.6 ± 3.1 | 251.5 ± 13 | >1552 | 13.5 |
| **6** | 167.7 ± 6.3 | 965.1 ± 66.4 | 28.4 ± 6.3 | 60.1 ± 8.8 | >1582 | 2.1 |
| **7** | 167.2 ± 2.3 | 596 ± 92.7 | 16.5 ± 3.3 | 76.1 ± 4.6 | 463.5 ± 56.5 | 4.6 |
| **8** | 78.4 ± 4.9 | 1593 ± 171.5 | 63.7 ± 9.8 | 1,715 ± 171.5 | >2451 | 26.9 |
| BZ | 6.5 ± 0.7 | 34.5 ± 7.6 | 19.2 ± 3.0 | 614.7 ± 115.2 | >1,921 | 32.0 |

[a]Values of concentration are represented in $\mu$M. $IC_{50}$, inhibitory concentration of 50%; $EC_{50}$, effective concentration of 50%; $CC_{50}$, cytotoxic concentration of 50%; $HC_{50}$, hemolytic concentration of 50%; SI, selectivity index between host cell $LLCMK_2$ and amastigote forms; ama, amastigotes; ±, standard deviation; >, above; and BZ, standard drug benznidazole. Significant differences between compound's activity and control cell growth, ANOVA ($p < 0.0001$).

Copaiba oil exudes from the trunk of these trees and is essentially comprised of sesquiterpenes and acidic diterpenes; the sesquiterpenes are more abundant and are responsible for the oil's aroma. The chemical composition of the oil differs between species and also within the same species, depending on the environmental conditions. Generally, the sesquiterpenes most often mentioned in the literature are $\beta$-caryophyllene, $\alpha$-copaene, and $\beta$-humulene, and the most cited diterpenes are copalic acid, hardwickiic acid, and clorechinic acid.[9,10]

Several studies have investigated copaiba oils and their medicinal properties, including the antinociceptive, anti-inflammatory, antimicrobial, antileishmanial, and bioinsecticidal activities.[11−15] Among compounds that have been isolated from these oils, kaurenoic acid is described as genotoxic, causing DNA fragmentation and micronucleus formation.[16] Two diterpenoids from *Copaifera*, 3-$\beta$-acetoxylabdan-8(17)-13-dien-15-oic acid and alepterolic acid, were recently found to have bioinsecticidal properties.[17]

In the present study, we describe the antiprotozoal and synergistic activities of terpenes commonly found in copaiba oleoresins (Figure 1). The diterpenes methyl copalate (**1**) and copalic (**2**), 3$\beta$-hydroxycopalic (**3**), agathic (**4**), pinifolic (**5**), and polyaltic (**6**) and kaurenoic (**7**) acids are found in the acid fraction of oleoresins from different *Copaifera* species, and the sesquiterpene $\beta$-caryophylene (**8**) is one of the most abundant in the majority of oleoresins. These terpenes were studied for their possible antiparasitic acitivity and cytotoxicity against mammalian cells. Ultrastructural alterations as well as the integrity of mitochondria and cell membranes were also evaluated, as well as parasite oxidative stress.

## RESULTS

**Inhibition of *T. cruzi* Life Stages.** *Epimastigotes.* All compounds showed activity against epimastigotes up to the maximum concentration tested. Compounds **2**, **3**, and **8** were the most active, with $IC_{50}$ values of 42.7, 41.2, and 78.4 $\mu$M, respectively. Compound **4** showed 50% inhibition at 86.8 $\mu$M and **1** at 83.3 $\mu$M. Higher concentrations of **7** and **6** were needed to inhibit parasite proliferation, 167.2 and 167.7 $\mu$M, respectively. The $IC_{50}$ of **5** after 96 h was 854 $\mu$M (Table 1).

*Trypomastigotes.* *T. cruzi* in its nonproliferative form was more resistant to the toxic effects of the compounds. Compounds **1**, **2**, and **3** showed 50% activity at similar concentrations of 377.3, 444, and 453.1 $\mu$M, respectively. Also, **8**, **4**, and **6** acids were effective at concentrations from 823.3 to 1595 $\mu$M. Compound **5** was the least active compound against trypomastigotes, with an $EC_{50}$ of 1630 $\mu$M (Table 1).

*Amastigotes.* Amastigotes were more sensitive to the presence of different compounds from *Copaifera*. After 96 h, **2** and **3** as well as **1** inhibited intracellular multiplication of the parasite with concentrations below 3 $\mu$M: 1.3, 1.8, and 2.5 $\mu$M, respectively. Compounds **4**, **7**, and **5** also inhibited growth, at 14.9−18.6 $\mu$M. Compounds **6** and **8** showed $IC_{50}$ values of 28.4 and 63.7 $\mu$M, respectively (Table 1). The selectivity index (SI)


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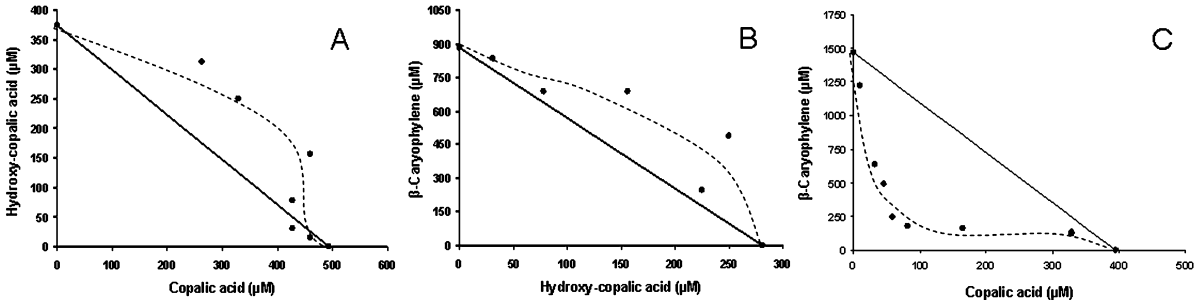


**Figure 2.** Isobolograms of terpene combinations; (A) 3$\beta$-hydroxycopalic acid × copalic acid, (B) $\beta$-caryophyllene × 3$\beta$-hydroxycopalic acid, and (C) $\beta$-caryophyllene × copalic acid. The theoretical line crosses at the $EC_{50}$ concentration of each compound alone. Points represent the combinations of concentrations of both compounds that produced an equal effect (50% of parasite death). Isobolograms of acid diterpenes with benznidazole were not plotted.

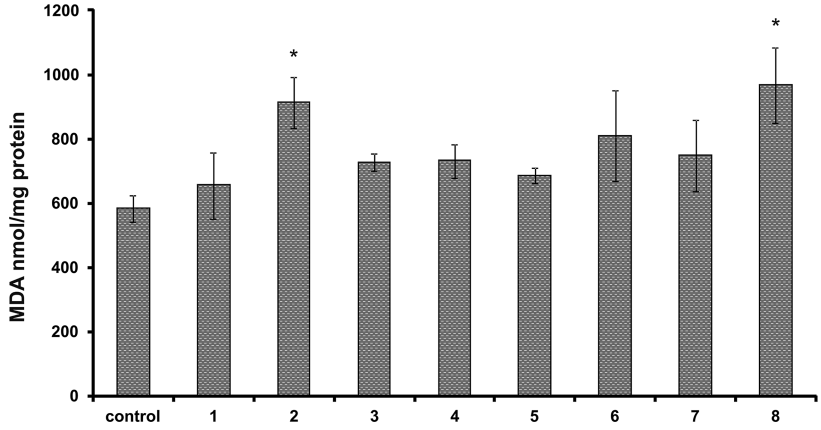


**Figure 3.** Evaluation of lipid peroxidation caused by terpenes on epimastigote forms. Treatment with 400 $\mu$M for 6 h. ANOVA–Dunnett (*$p$ = 0.02).

indicates the toxicity for the parasite when compared to the host. The higher the selectivity is the more specific is the toxicity of the compound. For amastigotes, when compared to LLCMK2 cells, it was shown that **8**, **1**, and **2** were 26–30 times more toxic to the parasite than to host cells. Compounds **5** and **3** had SIs of 13.5 and 17.3, respectively. Compounds **6**, **4**, and **7** were less selective, with SIs of 2.1, 3.8, and 4.6, respectively (Table 1). The percentage of infected cells after the treatment with the $IC_{50}$ of the terpenes did not vary significantly, although the number of amastigotes per cell was reduced in 48% for compound **2**, 40% for **3**, 38% reduction after the treatment with **1**, **4**, **6**, and **7**, and 25% reduction of amastigote growth for compounds **5** and **8**.

*Synergic Effect.* Compound **8** combined with **2** showed strong synergy, FICI = 0.12, resulting in a reduction of 25 and 11 times the $EC_{50}$, respectively (Figure 2C). No interaction was detected between **8** and **3**, FICI = 0.86 (Figure 2B) and between **2** and **3**, FICI = 0.6 (Figure 2A). Indifferent index values were also obtained with combinations of benznidazole with **3** (FICI = 1.01) and with **2** (FICI = 0.95).

*Cytotoxicity.* The hemolytic assay showed different toxicities of the compounds on human red blood cells, and the percentage of hemolysis depended on the concentration. The majority of compounds showed low toxic effects, resulting in 50% hemolysis in concentrations above 400 $\mu$M. Compound **5** was the least toxic drug, causing 8% hemolysis at 1552 $\mu$M. Compounds **2** and **3** were hemolytic for 50% of the erythrocytes in concentrations below 200 $\mu$M. Amphotericin B is a commercial drug with activity against fungi and some protozoan parasites but is also known for its hemolytic activity and presented hemolysis of 50% at 50.8 $\mu$M (Table 1). Terpenes had moderate toxicity to nucleated cells. Compound **8** showed the lowest toxic effect, with $CC_{50}$ at 1715 $\mu$M and **5** at 251.5 $\mu$M. Compounds **1**, **4**, **6**, and **7** caused 50% host cell death at concentrations ranging from 56.8 to 76.1 $\mu$M. $CC_{50}$ values of **2** and **3** were determined as 39.4 and 31.2 $\mu$M, respectively (Table 1).

*Lipoperoxidation.* Quantification of lipid peroxidation showed that only **8** and **2** were able to cause alterations. Both compounds were responsible for an increase of oxidative stress in the parasite of approximately 30–40%. Compounds **1** and **3**–**7** caused no lipoperoxidation after 6 h of treatment (Figure 3).

*Flow Cytometry.* Only epimastigotes were susceptible to drug interference up to the maximum concentrations tested for both evaluations. The cell membrane integrity was affected by the majority of the compounds (Figure 4, column I), except for **1** and **5**, for which the percentage of gated cells was similar to that in the negative control cells (Figure 4I-1,I-5). Loss of mitochondrion potential (Figure 4, column II) could not be observed for the same compounds as for cell membrane permeability. Compound **8** caused a strong mitochondrial potential alteration (Figure 4II-8), and a partial effect was detected for **2** and **3** (Figure 4II-2,II-3).

*Ultrastructural Alterations.* Epimastigotes treated with 50% of the lethal dose of the terpenes showed cell alterations (Figure 5). The sesquiterpene **8** produced total disorganization of the kinetoplast and an initial formation of concentric


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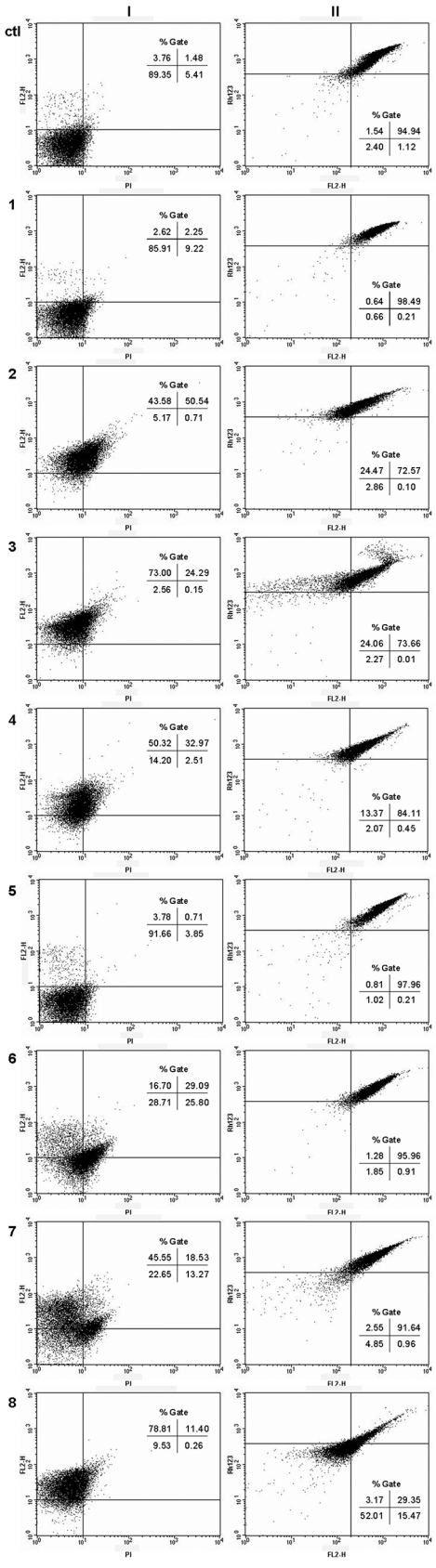


**Figure 4.** Flow cytometry analysis of epimastigotes of *T. cruzi*. Columns: (I) cell membrane integrity indicated with the fluorescence of propidium iodide (PI) and (II) mitochondrial membrane potential evaluated with the fluorescence of rhodamine 123 (Rh123). Lines: ctl, control cells without treatment; 1, methyl copalate; 2, copalic acid; 3, 3$\beta$-hydroxycopalic acid; 4, agathic acid; 5, pinifolic acid; 6, polyaltic acid; 7, kaurenoic acid; and 8, $\beta$-caryophyllene. Treatment with 400 $\mu$M for 6 h. Analysis of 10000 events with the percentage of gated cells indicated in the quadrants.

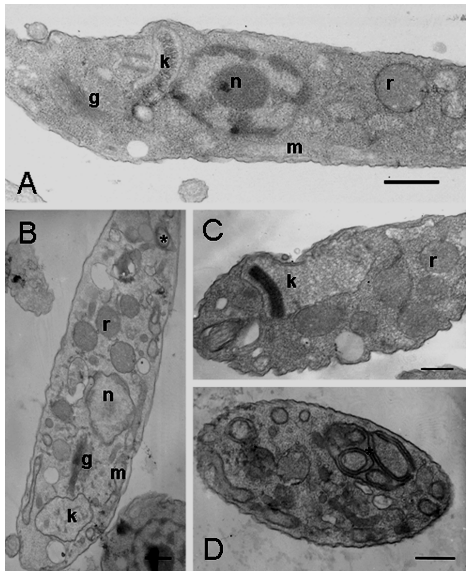


**Figure 5.** Transmission electron microscopy of epimastigotes of *T. cruzi*. (A) Control, (B) $\beta$-caryophyllene, (C) copalic acid, and (D) 3$\beta$-hydroxycopalic acid. Key: n, nucleus; k, kinetoplast; r, reservosomes; g, Golgi apparatus; m, mitochondrion; and asterisk, membranous vacuoles formation. Bars = 1 $\mu$m.

membranous vacuoles (Figure 5B). Diterpenes caused changes in the parasite ultrastructure, although in different ways. Compound **2** treatment resulted in pronounced swelling of the mitochondrion in almost all of the treated cells (Figure 5C), while **3** caused organelle disorganization and membranous vacuole formation in all parasite body parts (Figure 5D).

## DISCUSSION

In this study, we attempted to show that combinations of compounds obtained from the same plant could give better results than the standard drug evaluation method. Compounds **2** and **3** were the most active compounds of those that we tested individually. In the case of **2**, the absence of a hydroxyl changed the "indifferent activity" (combined with **3**) to "synergic activity" when combined with **8**. The sesquiterpene **8** showed good activity against replicative forms, but its combination with **2** revealed a synergic activity, increasing the activity against trypomastigotes 40-fold.

In a recent study, Pelizzaro-Rocha et al.[18] evaluated the activity of parthenolide, a sesquiterpene lactone isolated from *T. parthenium*, and parthenolide showed a strong synergistic effect with benznidazole against epimastigote forms. Another sesquiterpene, psilostachyin isolated from *A. tenuifolia*, inhibited growth irreversibly in low concentrations and caused alterations in cellular ultrastructure resembling those that we observed.[19]

Plant extracts from Kenya have shown suppressive synergic activity against *Plasmodium* parasites. Gathirwa et al.[20] combined root extracts of *U. acuminata* and *F. virosa*, resulting in a FIC of 0.42 in vitro. Strong in vitro and in vivo synergy was observed for leaf extracts from *L. schweinfurthii* and *R.*


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*natalensis*, with a FIC of 0.44 and 87% of parasitaemia reduction. In our study, an index of 0.12 was obtained by the combination of compounds **2** and **8** for in vitro evaluation of antitrypanosomal activity. Similarly, it was demonstrated by Fieck et al.[21] that the combination of antimicrobial peptides apidaecin and magainin acts synergistically against *T. cruzi* with a FIC of 0.12, while magainin and melitin presented an index of 0.41.

Natural compounds can also have their chemical structures modified to improve parasitic activity and reduce toxicity to the host.[22] Compound **7** showed moderate activity against *T. cruzi* but also partial cytotoxicity. Recently, it was reported that several benzaldehyde-thiosemicarbazone derivatives from kaurenoic acid displayed more trypanocidal activity and a less toxic effect against $LLCMK_2$ than the natural diterpene.[23]

Against intracellular amastigotes, **1**−**3** showed strong activity, and they proved to be approximately 30 times more toxic to the parasite than to the host cells. After 96 h of treatment, the percentage of infected cells remained similar to control cells, although the average number of amastigotes was significantly lower in cells treated with the $IC_{50}$ of the terpenes.

Toxicity results indicated that **1** is one of the less toxic compounds to erythrocytes and **8** to $LLCMK_2$ cells. The hemolytic effect can be associated with the membrane disruption visualized in the transmission electron microscopy. In general, host cell $LLCMK_2$ was more sensitive to the presence of terpenes, but this could be partially explained because of the longer incubation time, its origin from a commercial cell line, and also because the monolayer can be more easily detached from the well bottom in the presence of toxic compounds in the culture medium than can the cells in a living organism.

Campos et al.[24] reported that terpenes from *C. cajucara*, a plant species found in the Amazon Forest, showed more activity against amastigotes of *T. cruzi* than against other life stages, and this parasitic activity differed among the strains of *T. cruzi* tested. This is comparable to the activity of terpenes typically found in *Copaifera*, as we demonstrated here. Compounds that are found to be more active against intracellular forms indicate a promising future for the discovery of new drugs that affect the parasite where it is most difficult to reach, that is, inside the host cells.

The lipid peroxidation reaction occurs in the presence of reactive oxygen species (ROS) and may be associated with mitochondrial damage or inhibition of the detoxification system. Almost none of the compounds caused lipid peroxidation, except for **8** and **2**, which caused oxidative stress during the treatment. These compounds also affected the mitochondrial membrane potential. Compounds **3** and **4** altered the mitochondrial potential but did not produce oxidative stress. A recent study showed that the trypanocidal action of the naphthofuranquinones is associated with mitochondrial dysfunction, leading to increased ROS generation and parasite death.[25]

Rhodamine 123 is a cationic fluorescent dye that is widely used to probe the mitochondrial membrane potential. Positively charged molecules are attracted by mitochondria in response to the highly negative membrane potential generated by the electrochemical gradient across the inner membrane. Rh123 and other amphiphilic cationic dyes accumulate in the matrix, and any disturbance in electron transport can cause loss of the dye, reducing fluorescence.[26] Changes in the mitochondrial membrane potential may be associated with many cellular processes, including oxidative stress, apoptosis, and autophagy.[27]

Compounds **2**, **3**, and **8** affected the cell membrane permeability, lipid peroxidation, and mitochondrial potential. Part of these results may be correlated with ultrastructural alterations observed by electron transmission microscopy, despite the fact that the cell damage produced by both diterpenes were more severe. Mitochondrial and kinetoplast swelling observed after treatment with **8** and **2** may explain the lipoperoxidation and the loss of labeling with Rh123. For compound **3**, the presence of all membranous vacuoles could imply the existence of autophagic processes.

Intense cytoplasmic vacuolization and structural disorganization of the parasite cell, together with swelling of the kinetoplast-mitochondrion system, were also observed after treatment with the terpenes acetyl aleuritolic acid and *trans*-dehydrocrotonin.[24] Both terpenes inhibited the activity of trypanothione reductase, and mitochondrion alterations may be associated with this enzymatic effect. In this study, compound **2** promoted severe ultrastructural alterations when compared to **3** and **8**, confirming its high antiprotozoal activity in low doses.

Propidium iodide is a fluorescent intercalating agent used for DNA labeling, which does not cross the cell membrane of viable cells, and for this reason, it is an efficient molecule to evaluate cell permeability and integrity.[28] In this study, treatment with terpene compounds resulted in a decrease in cell volume. The loss of integrity of the cell membrane disrupts ion transporters that are responsible for osmotic regulation. Several terpenes interfered with cell membrane permeability, due to DNA labeling. A drug can act on cell membranes by insertion into the lipid bilayer, by blocking ion or amino acid transport (which is an important mechanism of osmotic regulation in *T. cruzi*), or by several other mechanisms.[29] This increase in permeability can cause the loss of intracellular contents, and these uncontrolled osmotic changes can also initiate a process of programmed cell death.

Menna-Barreto et al.[30] studied another diterpene, geranylgeraniol, isolated from *P. pubescens* (Fabaceae), which was more effective against amastigotes than against other life stages of the parasite. Geranylgeraniol altered the mitochondrial potential, and the observation of concentric membranous arrangements inside the damaged mitochondria suggested that this compound causes an autophagic process that leads to cell death.

Compounds **1** and **5** did not affect the cell and mitochondrion membrane permeability. Compounds **1**−**3** all have a methyl group, which in this case reduced the activity of the compound. Structures **6** and **7** increased the permeability of the parasite membrane, although they did not cause any decrease in the mitochondrial potential. Trypomastigotes did not undergo any modification in the cell or mitochondrion membrane potentials, even in terpene concentrations 3-fold higher than those used for epimastigotes. The mechanism of action of the terpenes studied may be associated with a specific component in the parasite cell that is present only in or is more expressed in epimastigote forms.

## ■ CONCLUSION

Some terpenes found in copaiba oils can cause parasite death by different mechanisms, including oxidative stress, autophagy, and interference with osmotic regulation. Previous studies on specific metabolic pathways of *T. cruzi* have demonstrated that the effect of drugs on the activity of detoxifying enzymes

can cause changes in the mitochondrion. Amastigote forms are more sensitive to terpenes than other life stages of the parasite. Compounds **2** and **8** show synergistic activity against *T. cruzi*, and considering that many species of the genus *Copaifera* contain both of these compounds, this synergism could occur in almost all copaiba oils and may be one of the reasons for their medicinal properties. Diterpenes **1**−**3** were more toxic, and we can observe a significant loss of activity when a hydroxyl or a methyl radical is present. Compounds terpenes from *Copaifera* demonstrated parasitic activity, which could be improved by modifications of their chemical structure. This investigative study of natural compounds isolated from *Copaifera*, tested both individually and in combination, is the first of its kind for *T. cruzi*.

## EXPERIMENTAL SECTION

**Compounds.** Compounds utilized in this study include the sesquiterpene $\beta$-caryophyllene (≥98.5%), purchased from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO) and the diterpenes methyl copalate, copalic acid, 3$\beta$-hydroxycopalic acid, agathic acid, pinifolic acid, polyaltic acid, and kaurenoic acid, isolated from *Copaifera* oleoresins. Isolated compounds were obtained employing column chromatography[31] and flash chromatography. Further purification was performed after successive recrystallization on ethyl acetate or ethanol. All compounds meet the criteria of ≥95% purity. Purity was observed by the two degrees interval of melting point (Mel-Temp II, Lab. Devices Inc., United States) and the absence of other peaks integrating more than 1% on GC-FID (HP 5890) or signals on NMR $^1$H (300 MHz, Brüker AC-300). GC-FID analyses of acid diterpenes were performed after esterification with diazomethane. For biological evaluation, each compound was first dissolved in dimethylsulfoxide and then added to the appropriate medium so that its final concentration did not exceed 1%.

**Activity in Epimastigotes.** Y strain epimastigotes from cultures maintained in log-phase were inoculated at $1 \times 10^6$ parasites/mL in LIT medium supplemented with 10% fetal bovine serum. Cells were placed in 24-well plates with concentrations of the compounds varying from 3 to 500 $\mu$M. Plates were incubated at 28 °C for 96 h, and the parasites were then counted in a Neubauer Chamber. The 50% growth inhibitory concentrations ($IC_{50}$) were determined.

**Activity in Trypomastigotes.** Mammalian $LLCMK_2$ cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen Co., Grand Island, NY) medium with 10% fetal bovine serum (FBS, Gibco Invitrogen Co.). Cells were kept in flasks for 72 h at 37 °C and 5% $CO_2$ atmosphere. *T. cruzi* trypomastigotes (Y strain) were first maintained in vivo in Swiss mice. At the tenth day after infection (parasitemia peak), the blood was collected and the trypomastigotes were used to infected $LLCMK_2$ cell monolayers. After 5 days of incubation, trypomastigotes were released, the supernatant was harvested and centrifuged at 2000*g* for 10 min, and the parasites were utilized. Trypomastigotes were inoculated at $1 \times 10^7$ parasites/mL in DMEM medium with 10% BALB/c mice blood and 20% FBS. The experiment was prepared in 96-well plates that were incubated for 24 h at 37 °C with different concentrations of the compounds. Parasites were counted by Brenner[32] method, by counting mobile parasites in an optic microscope slide, and the index obtained was used to determine the 50% effective concentration ($EC_{50}$) of the drugs on trypomastigote forms.

**Synergic Activity.** Compounds **2**, **3**, and **8** were combined with each other in different concentrations (50−1225 $\mu$M) to evaluate any possible synergic activity. Both acid diterpenes were also combined with benznidazole (3−40 $\mu$M) to evaluate synergy with the standard drug. The experiment was done in 96-well plates in DMEM medium containing $1 \times 10^7$ trypomastigotes/mL, 10% mouse blood, and 20% FBS, followed by incubation for 24 h at 37 °C. The parasites were counted by the Brenner method as described above. The fractional inhibitory concentration index (FICI) was calculated and interpreted as in previous studies,[33] FICI ≤ 0.5 for synergism, FICI > 4.0 for antagonism, and 0.5 < FICI < 4.0 for no interaction.

**Activity in Amastigotes.** Infected $LLCMK_2$ monolayers were prepared on round coverslips in 24-well plates and treated with concentrations of the compounds ranging from 1.5 to 75 $\mu$M. Plates were incubated at 37 °C with 5% $CO_2$ for 96 h. Coverslips containing the monolayers were washed, fixed with methanol, stained with Giemsa, and permanently prepared in Permount resin. The numbers of infected cells and amastigotes were counted in a total of 200 cells, and the percentage inhibition of amastigotes of 50% was calculated ($IC_{50}$).

**Cytotoxicity on $LLCMK_2$ Cells and RBCs.** The cytotoxicity of the compounds was evaluated against human red blood cells (RBC) and $LLCMK_2$ line cells. Erythrocytes were obtained voluntarily from a healthy human donor with A+ blood type. Blood was collected, defibrinated, and washed in glycosylated saline to remove any free hemoglobin from the defibrinization process. RBCs were inoculated at 3% in glycosylated saline, with different concentrations of the compounds in 96-well plates. The plates were incubated for 3 h at 37 °C, and the supernatant was read at 550 nm. Triton x-100 at 1% was used as the positive control for calculating the percentage of hemolysis, and amphotericin B was used as the hemolytic control for plotting hemolytic curves. $LLCMK_2$ cytotoxicity assays were performed in 96-well plates. Host cells were inoculated and incubated for 24 h, in DMEM medium at 37 °C and 5% $CO_2$, to form monolayers that were treated with different concentrations of the compounds (3−500 $\mu$M) for 96 h. After the incubation period, the monolayers were fixed with 10% trichloroacetic acid (Synth) for 1 h, and then, 0.4% sulforhodamine-B (Sigma-Aldrich) was added to the wells and incubated for 30 min. Excess dye was removed by washing with 1% acetic acid, and an aliquot of 150 $\mu$L of 10 mM Tris buffer (Invitrogen) was added to each well and homogenized for 15 min, and the absorbance was read at 530 nm. The cytotoxic concentration of 50% ($CC_{50}$) was determined by comparing treated monolayers to control cells.

**Lipid Peroxidation.** Epimastigotes were treated with a 400 $\mu$M concentration of the compounds for 6 h at 28 °C. After treatment, cells were washed with phosphate buffer and added to tubes containing 0.37% thiobarbituric acid solution, prepared in 15% trichloroacetic acid and 0.25 N HCl. The tubes were heated at 95 °C for 45 min, followed by cooling and centrifugation. The supernatant was collected, homogenized with butanol (1:1), and centrifuged for phase separation; the upper phase was read at 532 nm. Lipoperoxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA), expressed in MDA nmol protein $mg^{-1}$.

**Flow Cytometry.** The cell and mitochondrion membrane integrity of epimastigotes and trypomastigotes were evaluated after treatment with the compounds. Epimastigotes were treated with a 400 $\mu$M concentration of all terpenes, while trypomastigotes were treated with concentrations from 400 to 1000 $\mu$M copalic acid, 3$\beta$-hydroxycopalic acid, and $\beta$-caryophyllene for 3 h. After incubation, the parasites were washed in PBS and incubated with 300 nM propidium iodide (PI, Sigma-Aldrich) for 10 min and 13 $\mu$M rhodamine 123 (Rh123, Sigma-Aldrich) for 15 min. The cells were washed again and then counted by flow cytometry (FACSCalibur flow cytometer, Becton-Dickinson) with 10000 events for each sample.

**Transmission Electron Microscopy.** Epimastigotes were treated with $IC_{50}$ concentrations of **2**, **3**, and **8** for 96 h at 28 °C. Cells were washed and fixed with 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer and then postfixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide, prepared in a cacodylate buffer. Parasites were washed and then dehydrated in increasing concentrations of acetone (50, 70, 80, 90, 95, and 100%), gradually embedded in Epon resin and polymerized at 60 °C. Ultrathin sections of the samples were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. A Zeiss 900 transmission electron microscope was used to examine the ultrastructure of the parasites.

**Statistical Analysis.** Experiments were performed on different occasions, and the statistical analysis was performed with GraphPad

Prism 5.0 software, one-way analysis of variance, and Dunnett's test. Values of $p < 0.05$ were considered significant.

**Ethics.** Voluntary donations of human blood were obtained according to the Declaration of Helsinki (Ethical principles for medical research involving human subjects). Two donors were chosen for their similar blood type and were instructed about the objectives of the research, and both gave their written consent. Blood (10 mL) was collected by trained professionals and with appropriate medical support. For assays involving mouse blood, male BALB/c mice were obtained from the Central Animal Facility of the University, and the protocol was approved by the Committee on Ethics of Animal Experiments of the State University of Maringá (Acceptance: 058/2010).

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### Notes

The authors declare no competing financial interest.

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