Trypanocidal Activity of Oleoresin and Terpenoids Isolated from *Pinus oocarpa*[§]

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Fractionation with *n*-hexane/ethyl acetate (1:1 v/v) by open column chromatography of the oleoresin from *Pinus oocarpa* Schiede yielded two diterpenes, pimaric acid (**1**) and dehydroabietic acid (**5**), the sesquiterpene longifolene (**3**) and a diterpenic mixture containing pimaric acid (**1**), isopimaric acid (**4**) and dehydroabietic acid (**5**). Subsequently, the isolated compounds, the mixture of **1**, **4** and **5**, the oleoresin and the dehydroabietic acid methyl ester (**2**), were tested *in vitro* against epimastigotes of *Trypanosoma cruzi*, the causative agent of Chagas disease. The most active compounds were **1**, **3** and the oleoresin, being as active as nifurtimox, a drug effective in the treatment of acute infection by American trypanosomiasis and used in this work as positive control.

Key words: Diterpenes, Trypanocidal Activity, *Pinus oocarpa*

Introduction

The flagellate *Trypanosoma cruzi*, the etiological agent of Chagas disease or American trypanosomiasis, affects more than l8 million people in Latin America, leading to approx. 400,000 deaths per year. This parasite is transmitted to humans by insects that belong to the Reduviidae family, specially by blood-sucking bugs (Schvartzapel *et al.*, 1995), and also by transfusion of infected blood, or ingestion of infected meat. Since 1909 when this disease was initially characterized (Chagas, 1909) several efforts were carried out in order to eradicate it, but in spite of the 75% reduction in the incidence of human cases observed in the South American countries (*i.e.* Argentina, Brazil, Chile, Paraguay, Uruguay and Venezuela) as a consequence of the successful vector control programs (Moncayo, 1993; TDR news), Chagas disease still remains a regional health problem. The drugs nifurtimox, benznidazole and ketoconazole ameliorate acute *T. cruzi* infection (Brener *et al.*, 1993; Jernigan and Pearson, 1993; Castro and Diaz de Toranzo, 1988; Cerecetto *et al.*, 1999, 2000; Cerecetto and Gonzalez, 2002) but exert serious side effects and little or no effect on the chronic phase of the disease (Gorla *et al.*, 1988, 1989; Rodríguez *et al.*, 1995), probably due to the inherent characteristics of the host and the virulence and resistance of the parasite. Development of safer and more efficient therapeutic anti-*Trypanosoma cruzi* compounds continues to be a mayor goal in trypanocidal therapy.

Recently, the trypanocidal activity of diterpenes of clerodane, kaurane and icetexane types were reported (Freiburghaus *et al.*, 1998; Alves *et al.*, 1995; Batista *et al.*, 1999; Da Costa *et al.*, 1996; Scio *et al.*, 2003; Uchiyama *et al.*, 2003). This information prompted the authors to test *in vitro* some pimmarane derivatives, resin and longifolene from *Pinus oocarpa* against epimastigotes of *Trypanosoma cruzi.*

We have previously demonstrated that diverse secondary metabolites have different sites of action and different molecular targets when they interact with enzymes and perturb biological processes (Céspedes *et al.*, 2000, 2001, 2004; Calderón

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et al., 2001; Kubo *et al.*, 2003a, b; Torres *et al.*, 2003).

Pinus oocarpa Schiede, Syn., *Pinus oocarpoides* Lindley ex Laudon, belongs to the Pinaceae family and Abietineae subfamily. It is known in Mexico under several common names as "ocote", "pino resinoso", "ixtaji", "ichtaj", "juncia" etc. (Frei *et al.*, 2000) and growing on the humid lowlands to the east of pacific coast (Leopold, 1950; Miranda and Hernández, 1963; Rzedowski, 1993), together with *Liquidambar* sp., *Podocarpus* sp., *Hymenaea courbaril* and *Quercus* spp., as well as an (sub)-tropical evergreen, partly submontane (broad-leaved) seasonal forest types [*Manilkara zapota* (L.) van Ryen, *Coccoloba barbadensis* Jacq., *Enterolobium cyclocarpum* (Jacq.) Griseb.] (Lorence and Mendoza, 1989).

The local indigenous population used for medicinal purposes different parts of the plant *P. oocarpa*: leaves, resin and stalk, administered as infusion, which have been reported in traditional folk medicine as efficient against inflammation, pimples, hoarseness and dysentery (Aguilar, 1994). With the aim to identify lead compounds for dissect new chemotherapeutic agents, the study of the oleoresin of *Pinus oocarpa* was undertaken. Herein, the bioassay-guided fractionation of "ocoteresin", the isolation of three known diterpenes, one sesquiterpene and their trypanocidal activities are reported.

Materials and Methods

Plant material and chemicals

The oleoresin was collected as a hard syrup from resinous exudate of *P. oocarpa*, growing in the tropical rain forest in Chiapas State, Mexico, and stored at – 20 °C. Longifolene, pimaric acid, and the mixture (pimaric, isopimaric and dehydroabietic acids) were isolated from a hexane extract of oleoresin of *P. oocarpa* by an open column chromatography. Dehydroabietic acid methyl ester was prepared by reaction with diazomethane and purified by preparative thin layer chromatography. Dimethyl sulfoxide and other solvents were purchased from Sigma-Mexico, S.A. de C.V. and nifurtimox(4-[(5-nitrofurfurylidene)amino]-3-methylthiomorpholine-1,1-dioxide), the active compound of Lampit™ (BAYER), was isolated from the commercial drug Lampit™ from Bayer Argentina, S.A.

Apparatus

^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra at 75 MHz, respectively, on a Varian VXR-300S spectrometer; chemical shifts (ppm) are related to $(CH_3)_4Si$ as internal reference; $CDCl_3$ and acetone-d_6 from Aldrich Chemical Co. were used as solvents; coupling constants are quoted in Hz. IR spectra were obtained in KBr on a Perkin Elmer 283-B and a FT-IR Nicolet Magna 750 spectrophotometers. UV spectra for pure compounds were determined on a Shimadzu UV-160. Optical rotation was measured on a JASCO DIP-360 spectropolarimeter. Melting points were determined on a Fisher-Johns hotplate apparatus and remain uncorrected. A spectronic 20 D+ model Genesys 5 spectrophotometer was used to determine parasite culture optical density at 580 nm. An inverted microscope and a Neubauer chamber were used to evaluate the parasite number and mobility.

General experimental procedures

The oleoresin material was dried, milled and extracted with hexane. This extract was fractionated by an open chromatography column for bioassays evaluation. Each one of the fractions was tested for trypanocidal activity (Table I) and then re-submitted to column chromatography using SiO_2 (G 60, Merck) as solid phase. Elution carried out with *n*-hexane/ethyl acetate mixtures afforded the active fractions, which were analyzed by thin layer chromatography (TLC) using different solvent systems (*n*-hexane/ethyl acetate and dichloromethane/methanol mixtures). Repeated TLC of these fractions led to the isolation of the secondary metabolites which were purified by preparative TLC and identified, comparing with authentic samples, as pimaric acid (**1**) (70 mg), longifolene (**3**) (250 mg), and a mixture of pimaric (**1**) + isopimaric (**4**) + dehydroabietic acid (**5**) (2:2:6, 3.15 g) from which, 25 mg of dehydroabietic acid methyl ester (**2**) were obtained by methylation with diazomethane (Fig. 1). All compounds were analyzed and characterized by their IR, UV, ^{1}H NMR, ^{13}C NMR spectra, melting point and R_f values compared with authentic samples. Analytical TLC was performed on silica gel 60 F_{254} (Merck) plates and the spots were visualized by spraying with 10% H_2SO_4, followed by heating at 110 °C.

Biological evaluation

Trypanocidal activity was assayed on epimastigote forms of *T. cruzi* Y strain, cultured at 28 °C in liver infusion tryptose medium (LIT), supplemented with 10% inactivated fetal calf serum. Parasites in logarithmic growth phase (from an initial culture with 2 × 106 epimastigotes/ml) were incubated with increasing concentrations of test compounds (50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml) dissolved in dimethylsulfoxide (DMSO) (1% final concentration) for 2, 24, 48, 72 and 96 h. Parasite growth response was determined at 2 h, and then every 24 h in an inverted microscopy, a Spectronic 20 D+ spectrophotometer at 580 nm and a Neubauer chamber. Morphology was analyzed in fixed preparations stained with Giemsa. All assays were carried out in triplicate. The activity was calculated as growth inhibition (in %) and the data express the mean of three different experiment.

Statistical analysis

Data shown in the figures and tables are the means of three replicates of trypanocidal activity and are presented as mean ± standard error. Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures and PROBIT analyses. The results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with a Student-Newman-Keuls (SNK) test. The I_{50} values were calculated by PROBIT analysis on the basis of the percentage of growth inhibition obtained at each concentration of the samples. I_{50} is the concentration producing 50% inhibition at 48 h. Complete statistical analyses were done *via* the MicroCal Origin 6.0 statistical and graphs PC program.

Results and Discussion

In clinical practice, trypanocidal drugs must act upon *T. cuzi* infective stages (trypomastigote and amastigote). Nevertheless, in this study we used the epimastigote noninfective form, since culture and handling in the laboratory are easier and safer. Moreover, replicative, noninfective cells have been used in several other studies for screening natural and synthetic compounds with potencial trypanocidal activity (Pozas *et al.*, 2005). Agents showing biological activity against *T. cruzi* epimastigotes have proved to be also efficient against the infective forms (Maya *et al.*, 2003).

A dose-dependent inhibition of the parasite proliferation was observed. The hexane extract was fractionated and the parasite growth inhibitory activity was assayed with the isolated samples of oleoresin, pimaric acid (**1**), dehydroabietic acid methyl ester (**2**), longifolene (**3**), and a mixture containing pimaric acid (**1**) + isopimaric acid (**4**) + dehydroabietic acid (**5**) (Fig. 1). The trypanocidal activity of these compounds at different concentrations is shown in Tables I and II, and Fig. 2.

At 50 μg/ml, within the first two hours the assayed compounds [**1**, **3**, (**1** + **4** + **5**) and nifurtimox] did not show any effect. At 24, 48 and 72 h the most active compounds were **1**, **3**, oleoresin and nifurtimox, causing 100% of growth inhibition (Table I and Fig. 2).

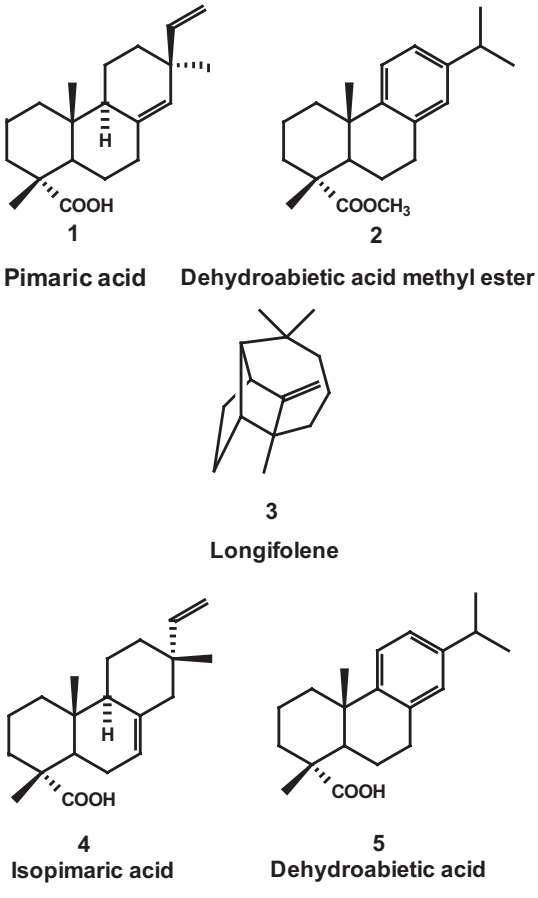

Fig. 1. Chemical structures of diterpenes from *P. oocarpa* assayed against *T. cruzi.*

Table I. Biological activity of compounds reported as % of growth inhibition. Data are the mean of three different experiments.

	2 h				24 h				48 h				72 h				96 h	
[μg/ml] Sample	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200	50	100
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DMSO 1%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nifurtimox	0	0	0	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Mixture (**1** + **4** + **5**)	0	0	0	100	40	90	100	100	19.5	94	100	100	22	93	100	100	17	87
Pimaric acid (**1**)	0	0	0	100	60	90	100	100	46	100	100	100	42	100	100	100	33	100
Dehydroabietic acid methyl ester (**2**)	n.d.	n.d.	n.d.	0	n.d.	n.d.	n.d.	100	n.d.	n.d.	n.d.	100	n.d.	n.d.	n.d.	100	n.d.	100
Longifolene (**3**)	0	0	0	100	100	100	100	100	77	100	80	100	70	100	77	100	46	100
Oleoresin	0	0	0	100	100	100	100	100	89	100	100	100	78	100	100	100	59	100

n.d., concentrations not assayed.

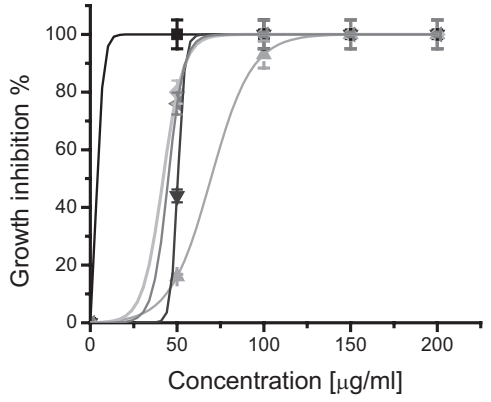

Fig. 2. Inhibition effects at 48 h by assayed compounds on *T. cruzi*: nifurtimox (■), oleoresin (◆), longifolene (**3**) (◀), pimaric acid (**1**) (▼), and mixture (▲).

At 24 h, with 100 μg/ml, the oleoresin and (**3**) caused 100% of growth inhibition and total and partial lysis, respectively. Nifurtimox also provoked 100% of growth inhibition, but total lysis was reached until 48 h. With pimaric acid (**1**) and mixture (**1** + **4** + **5**) growth inhibition was of 90%, without morphological or movement changes (Table I and Fig. 2).

The oleoresin, pimaric acid (**1**), longifolene (**3**), the mixture (**1** + **4** + **5**) and nifurtimox produced 100% of growth inhibition with total lysis at 24 h with 150 μg/ml. Table I and Fig. 2.

With 200 μg/ml the oleoresin extract and longifolene (**3**) showed a significant activity on the parasite within 1 h, producing total and partial lysis, respectively. Pimaric acid and the mixture provoked total parasites lysis at 2 h. With dehydroabietic acid methyl ester (**2**) parasites showed 100% of growth inhibition and lysis within 48 h; and nifurtimox, the positive control, caused 100% of growth inhibition and cell lysis at 24 h (Table I and Fig. 2).

According to the obtained results it is possible to consider that oleoresin, pimaric acid (**1**), longifolene (**3**) and the mixture (**1** + **4** + **5**) have more potent trypanocidal activity than nifurtimox, because the complete inhibitory effect and total lysis of cells was of 100% with 200 μg/ml, within the two first hours of culture with the test compounds, while cultures with nifurtimox showed the same effects only after 48 h (Table I). Oleoresin, pimaric acid (**1**), longifolene (**3**) and the mixture (**1** + **4** + **5**) have also the same biological activity at the same culture time.

At 24 h, with 50 μg/ml, it is easier to visualize the differentiated effects produced by each tested compound: pimaric acid (**1**) and the mixture (**1** + **4** + **5**) provoked 50 and 25% of growth inhibition, respectively. On the other hand, longifolene (**3**) and oleoresin induced 100% of growth inhibition, showing the same activity as nifurtimox, but only the oleoresin produced a total cell lysis (Table I and Fig. 2).

The oleoresin showed the higher activity, probably due to the synergistic effect of the diterpenes. Lysis could be due to the presence of diterpenes; similar effects were reported for some diterpenes from *Alomia myriadenia* (Asteraceae), *ent*-16-hydroxylabda-7,13-dien-15,16-olide and *ent*-12*R*-hydroxylabda-7,13-dien-15,16-olide (Scio *et al.*, 2003), where these compounds produced 100% of lysis at 250 μg/ml. However, in the same work is

reported that *ent*-12*R*,16-dihydroxylabda-7,13-dien-15,16-olide and *ent*-8*S*,12*S*-epoxy-7*R*,16-dihydroxyhalima-5(10),13-dien-15,16-olide did not show biological activity. On the other hand, the known diterpene kauranes [(−)-*ent*-kaur-16-en-19-oic acid, (−)-trachyloban-19-oic acid and (−)-kauran-16-α-ol] from *Viguiera aspillioides* showed significant activity with 50% of growth inhibition with 500 μg/ml. Another natural compound, kolavenol isolated from *Entada abyssinica* (Freiburghaus *et al.*, 1998), has been reported as one of the most potent diterpenes with an IC_{50} value of 2.5 μg/ml, determined at 66 h. It is important to note that the IC_{50} values for pimaric acid (**1**), longifolene (**3**) and oleoresin are 51.1, 44.0 and 42.7 μg/ml, respectively, determined at 48 h (Table II). These values show the potency of the compounds reported herein (Fig. 2) compared to the compounds assayed by Scio *et al.* (2003), tested at 250 μg/ml and 24 h culture, at 4 °C, causing 100 and 99% cell lysis, a potency comparable to gentian violet, but greater than kauranes reported by Batista *et al.* (1999) and Alves *et al.* (1995).

Table II. I_{50} values of compounds observed after 48 h of contact.

Treatment	I_{50} [(μg/ml (μM)]	
Nifurtimox	4.3	(0.015)
Mixture	69.1	–
Pimaric acid (**1**)	51.1	(0.17)
Dehydroabietic acid methyl ester (**2**)	n.d.	–
Longifolene (**3**)	44.0	(0.21)
Oleoresin	42.7	–

n.d., not determined.

Terpenes are well-known to be active against protozoan parasites (Phillipson and Wright, 1991; Phillipson *et al.*, 1995). It is worth to mention that these compounds could be cytotoxic (Scio *et al.*, 2003) and the studies about this and their sites and mode of action are under way. Natural products studies from this species and from others used in traditional medicine in Mexico, as well as new biological activities of the isolated compounds, are in progress.

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