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In vitro* antileishmanial effects of antibacterial diterpenes from two Ethiopian *Premna* species: *P. schimperi* and *P. oligotricha Solomon Habtemariam*

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Published: 6 June 2003

Received: 3 April 2003

BMC Pharmacology 2003, 3:6

Accepted: 6 June 2003

This article is available from: <http://www.biomedcentral.com/1471-2210/3/6>

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Abstract

Background: Three antibacterial diterpenes: (5R,8R,9S,10R)-12-oxo-ent-3,13(16)-clerodien-15-oic acid (**1**), 16-hydroxy-clerod-3,13(14)-diene-15,16-olide (**2**) and ent-12-oxolabda-8,13(16)-dien-15-oic acid (**3**) were previously isolated from *Premna schimperi* and *P. oligotricha*. Since andrographolide and other structurally related diterpenes were shown to have antileishmanial activity, the aim of the present study was to assess the *in vitro* effect of premna diterpenes against *Leishmania aethiopica*; the causative agent of cutaneous leishmaniasis in Ethiopia.

Results: The diterpenes showed potent concentration-dependant suppressive effect on the viability of axenically cultured amastigotes of *L. aethiopica*. The clerodane diterpenes **1** and **2** were most active (LD₅₀ values 1.08 and 4.12 µg/ml respectively) followed by andrographolide and **3**. Compounds **1** and **2** appear to be over 20 and 10-times respectively more selective to leishmania amastigotes than the permissive host cell line, THP-1 cells or the promastigotes stage of the parasites.

Conclusion: The clerodane diterpenes (**1**, **2**) which were more potent and selective than labdanes (andrographolide and **3**) are promising for further studies and/or development.

Background

Leishmaniasis is a vector-borne disease that is transmitted by sandflies and caused by obligate intracellular protozoa of the genus *Leishmania*. Current estimates indicate that leishmaniasis affects people in 88 countries, with 350 million at risk of contracting the disease and approximately 2 million new cases reported each year [1,2]. During the last decade, the geographic region of leishmania as well as incidence of infection significantly increased mainly due to increased urban development and immunosuppressive illnesses such as AIDs [1,3].

For many decades, the first choice of drug treatment for leishmaniasis has been the pentavalent antimonials,

sodium stibogluconate (Pentostam) and meglumine antimonate [4]. Both drugs require parenteral administration and apart from being expensive, shown to have variable efficacies and produce some severe toxic side effects, the developments of resistance by the parasites have been reported [5–7]. In such cases, the use of other parenteral and rather toxic alternative drugs such as amphotericin B (AMB) and pentamidine are inevitable [4]. Although recent developments in the search of new as well as improved formulations of old drugs have been promising [4,8], there is still an urgent need for new anti-leishmanial drugs. As part of a systematic search programme for antileishmanial agents of natural products-origin, the present study assesses the potential of two clerodane (**1**,

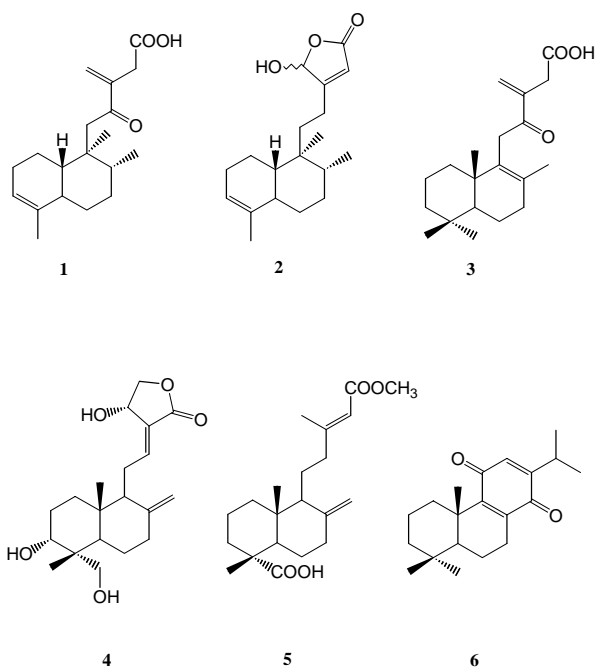


Figure 1
Structures of premna diterpenes (1–3) and other related antileishmanial compounds (4–6).

2) and a labdane (3; see Fig. 1) diterpenes from two Ethiopian *Premna* sp.: *P. shimperi* Engl. and *P. oligotricha* Baker (Verbenaceae). The diterpenes (1–3; Fig. 1) were more toxic to the axenically-cultured amastigotes of *L. aethiops* parasites than the insect vector-stage, promastigotes, and the clerodane diterpenes (1, 2) appears to be much more selective to axenic amastigotes than the permissive host cell line, THP-1 cells.

Results

Effects of test compounds on promastigotes viability

A variety of *in vitro* methods are now available to assess the susceptibility of leishmania parasites to experimental agents [9] and references therein. An initial attempt to use the MTT (data not shown) and the alamar blue-based cell viability measurements (Fig. 2) in the present study was unsuccessful as the reductions of the dyes by *L. aethiops* promastigotes was very slow. Despite it being time consuming, the gold standard in leishmania promastigotes susceptibility assays has been microscopic counting and was used here effectively for routine assessment of leishmanicidal activity.

As shown in Fig. 3a, premna diterpenes (1–3) as well as andrographolide (4) displayed a weak activity against the

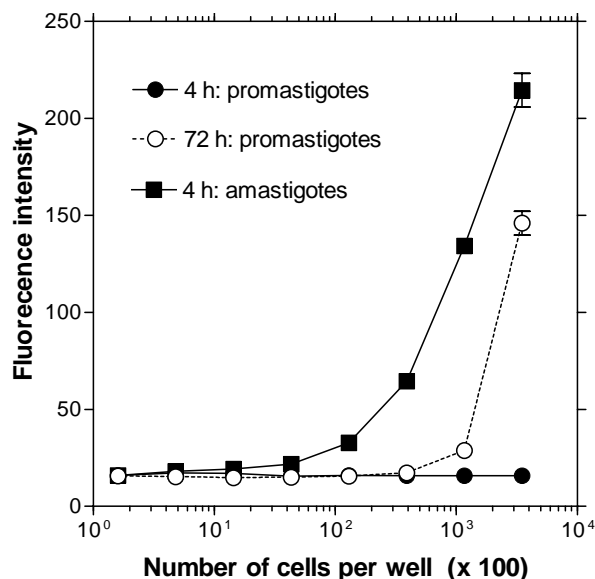


Figure 2
Differential ability of promastigotes and axenically cultured amastigotes of *L. aethiops* in reducing alamar blue. Alamar blue was added to promastigotes or axenically-cultured amastigotes and cells incubated for indicated period of time. Fluorescence associated with alamar blue reduction was quantified as described in the methods section.

promastigotes stage of leishmania parasites. LD₅₀ values for compounds 1 and 4, which were by order of magnitude more potent than 2 and 3, were also shown in Table 1. For comparison purposes, the effect of the standard antileishmanial agent, AMB was also assessed. The observed effect for AMB (Fig. 3a and Table 1) was in close agreement with those reported before [10] and appears to be much stronger than those of the diterpenes (1–4) studied.

Effects of test compounds on axenically cultured amastigotes viability

Typical morphological features of the axenically cultured *L. aethiops* parasites (two weeks old following initiation of differentiation) exhibiting a rounded cellular appearance together with loss or flagella and the expected clump formation is shown in Fig. 4. Since this axenically cultured parasites could readily reduce alamar blue (Fig. 2), the bioassay of drugs against this stage of the parasite was relatively easy. In comparison to the promastigotes, axenically cultured amastigotes of *L. aethiops* were more susceptible to diterpenes 1–4 than their promastigotes counterpart (Fig. 3, Table 1). The order of potency was: AMB > 2 > 1 > 4 > 3.

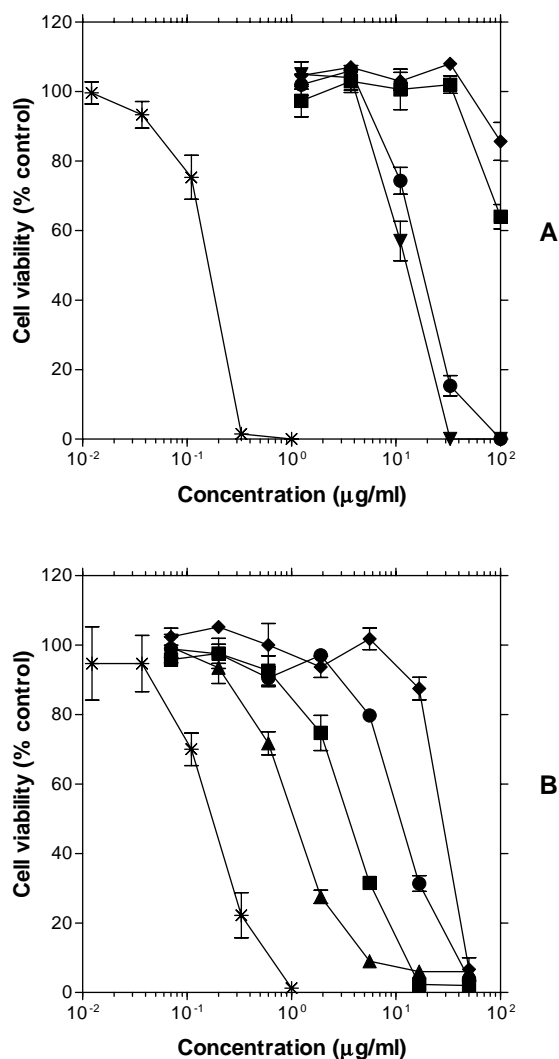


Figure 3
Effects of diterpenes (1–4) and AMB on *L. aethiopica* promastigotes (panel-A) and axenic amastigotes (panel-B) viability. Experiments were carried out as described in the methods section. Data points represent mean and SEM values obtained from three (panel-A) or 5 (panel-B) replicates. Symbols indicate: * AMB, ■ 1, ▲ 2, ◆ 3, and ● 4.

Cytotoxicity of test compounds in THP-1 monocytes

The cytotoxicity of 1–3 and the positive controls, andrographolide (4) and AMB is shown in Fig. 5 and Table 1. The concentrations of test compounds that produced up to 100% lethality in axenic amastigotes did not show toxicity to THP-1 cells. In agreement with previous studies that showed cytotoxicity of 1–3 to mammalian cells, in particular cancer cells [11,12], the compounds

were found toxic to THP-1 cells but only at concentrations higher than those found to be lethal to axenic leishmania amastigotes (Fig. 5a). Assessment of the LD₅₀ values (Table 1) revealed that the clerodane diterpenes (1, 2) were much more selective to axenic leishmania amastigotes than permissive monocyte/macrophage host cells, THP-1 or promastigotes. The standard antileishmanial drug, AMB, did also show some toxicity to THP-1 cells at its higher concentrations (Fig. 5b).

Discussion

In traditional medicine, the leaves extracts of *Premna schimperi* are used to treat external injuries and secondary infection in wounds [13]. Previous systematic biological and phytochemical studies on this plant have identified the clerodane diterpene 1 as the antibacterial principle [13]. Subsequent studies on a related species, *P. oligotricha*, resulted in the isolation of two antibacterial diterpenes, a clerodane (2) and labdane (3) [13,14] together with various other compounds [15–17]. Primarily due to owing the presence of an α,β -unsaturated moiety, compound 1–3 did also show cytotoxicity to various cancer cell lines [11,12]. A related labdane diterpene, andrographolide (4, see Fig. 1), is also shown to possess antiproliferative effects in cancer cells [18] but its non-toxic concentrations are known to possess various other biological activities including antiinflammatory, antiviral and antithrombotic [19] and cytoprotective effects from toxins [20,21]. Recently, andrographolide (4) has also demonstrated to have antileishmanial effects in the *in vivo* model of leishmaniasis [22]. Another antileishmanial diterpenes of close structural similarity with compounds 1–3 are labdane 5 [23] and abietane, 6 (Fig. 1) and its derivatives [24], which have been shown to induce leishmanial cell death at concentrations below that cause toxicity in mammalian host cells. With the established antileishmanial effects of structurally related diterpenes, the aim of the present study was to assess the *in vitro* effect of compounds 1–3 against promastigotes and axenically-cultured amastigotes of leishmania parasites.

For various reasons including simplicity in *in vitro* culture maintenance, routine screenings of antileishmanial chemotherapeutic agents are often based on promastigotes susceptibility assays. The cellular, molecular and biochemical characteristics of amastigotes, responsible for the clinical manifestation of leishmaniasis in humans, however differ from promastigotes [25]. Hence, the chemotherapeutic potential of drugs for leishmaniasis must further be validated using the intracellular-amastigote forms of the parasites. Since anti-amastigote assays using *ex vivo* cultured macrophages or permissive cell lines has various limitations [25], recent researches in many laboratories have focussed on the development of *in vitro* culture conditions for maintenance and bioassays using

Table 1: Antileishmanial activity of diterpenes (1–4) and AMB. LD₅₀ values in µg/ml obtained from a minimum of three separate experiments are shown.

Drugs	<i>L. aethiopica</i>		THP-1
	promastigotes	amastigotes	
AMB	0.189 ± 0.01 (n = 3)	0.183 ± 0.04 (n = 6)	>10
1	> 100	4.12 ± 0.28 (n = 5)	>100
2	11.67 ± 0.88 (n = 3)	1.08 ± 0.25 (n = 5)	12.13 ± 1.42 (n = 4)
3	> 100	19.2 ± 2.24 (n = 5)	26 ± 3.89 (n = 4)
4	18.33 ± 1.05 (n = 3)	4.52 ± 0.97 (n = 5)	7 ± 1.08 (n = 4)

axenically-cultured amastigotes. For some leishmania species, including the *L. aethiopica* strain (MHOM/ET/72/L100) used in the present study, temperature elevation alone could result in differentiation of promastigotes to amastigotes [26–28]. Lowering the pH to 5.5 together with temperature elevation and 5% CO₂-air mixture have now been widely used not only to differentiate promastigotes to amastigotes but also for their long-term culture [28,29]. Hence, this well established condition and medium 199 [28] were used in the present study to obtain and maintain *L. aethiopica* axenic amastigotes. The resulting axenic amastigotes of *L. aethiopica* could differentiate back to promastigotes by centrifuging and re-suspending them in promastigotes medium (RPMI 1640) followed by incubation at 22 °C. Phase contrast microscopic observations revealed the expected loss of flagella, cell rounding, aggregate formation and the metabolic differences as evidenced by the ability of axenic amastigotes to readily reduce alamar blue. While axenically cultured amastigotes were able to reduce alamar blue in 4 h, promastigotes were only weakly able to do so after long term incubation.

In agreement with previous report on the antileishmanial effect of abietane diterpenes [24], the effect of diterpenes 1–4 was more pronounced in the amastigotes, mammalian-stage of the parasite than the promastigote, insect-stage. On the other hand, the antileishmanial effect of AMB which is known to be mediated through formation of membrane channels permeable to water and ions [30] and references there in) was not developmental stage specific as both promastigotes and amastigotes were equally susceptible to AMB. The order of potency of diterpenes 1–3 was similar with their antibacterial effects in previous studies [13,14]: i.e. 2 > 1 > 3.

It is interesting to note that differences between the toxicity of the compounds studied to axenic amastigotes and the permissive host cells, THP-1 cells were observed. In previous studies, 50–500 µg/ml concentrations of andrographolide (4) were reported as non-toxic to peritoneal macrophages [22] but the study had limitations as cells were exposed to the drug for only one hour. In the

present assay system, the same incubation time period (72 h) as the antileishmanial assay was used so that direct comparison between toxicities to host cell and parasites could be made. Andrographolide (4) and the other labdane diterpene (3) appear to be less selective as amastigotes/THP-1 toxicity ratio (see Table 1) was less than two. On the other hand, the amastigotes/THP-1 toxicity ratio for the clerodane diterpenes 1 and 2 were over 20 and 10 respectively. AMB did also show small but significant toxicity at its higher concentrations. The most toxic side effects that limit the clinical use of AMB, however, are neurotoxicity and nephrotoxicity [31].

Conclusion

In summary, the present study demonstrate potent antileishmanial activity of premna diterpenes (1–3). The compounds were more potent in inhibiting the axenically-cultured amastigotes than promastigotes of *L. aethiopica*. The clerodane diterpenes (1, 2) with more selectivity to amastigotes than THP-1 cells are promising and warrant further studies and/or development.

Methods

Chemicals and reagents

The isolation and characterisation of diterpenes (1–3) have previously been described [13–15]. Andrographolide (4) and all tissue culture media and supplements were obtained from Sigma-Aldrich Chemical Company (Dorset, UK). The stock solutions of test compounds were prepared in dimethyl sulfoxide (DMSO) which was subsequently diluted in the appropriate medium. All DMSO concentrations used (less than 1%) did not show any activity in cell viability studies (data not shown).

Cell culture

L. aethiopica (WHO designation: MHOM/ET/72/L100) promastigotes as well as the human monocytic THP-1 cells were cultured with RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cultures were maintained at their

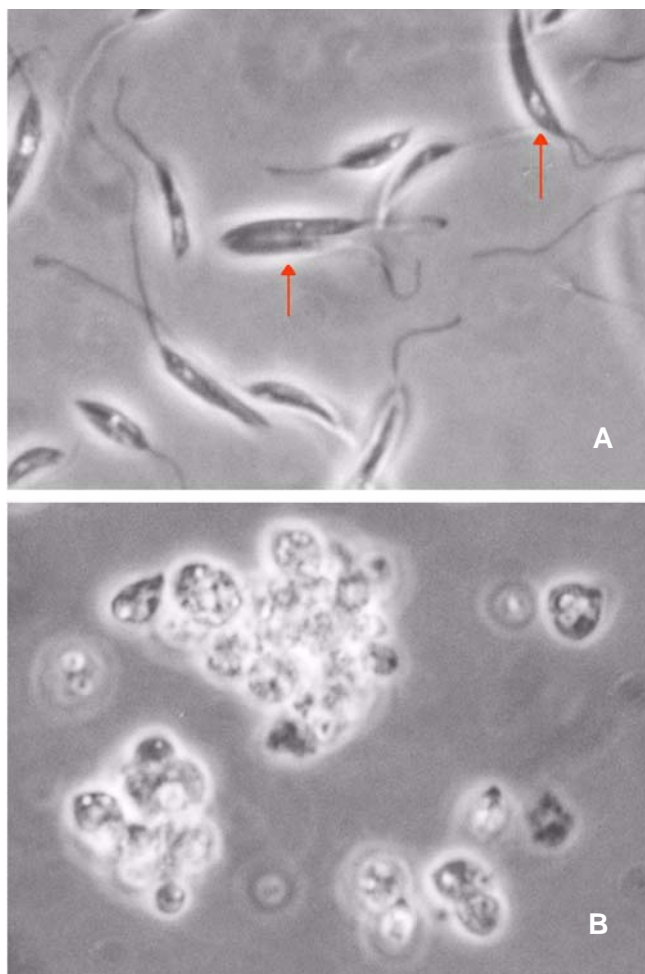


Figure 4
Morphological features of *L. aethiopica* promastigotes and axenically-cultured amastigotes. Mid-log stage promastigotes and amastigote forms were fixed with formaldehyde and viewed under Zeiss Axioskop phase contrast microscope. Photographs were immediately taken using Olympus-2 camera under oil immersion and 1000 × magnification. Arrows indicate promastigotes undergoing cell division.

optimum temperature, i.e. 22 and 37°C (5% CO₂) respectively.

Promastigotes and drug susceptibility assay

For drug susceptibility assays, 10⁶ cells/ml promastigotes were seeded in 96-well flat-bottom plates in final volume of 200 µl. Various concentrations of experimental drugs made in three-fold dilutions were then added to triplicate wells and plates incubated for 72 h at 22°C. At the end of incubation, cell viability was measured by counting the number of motile cells using a haemocytometer. For each

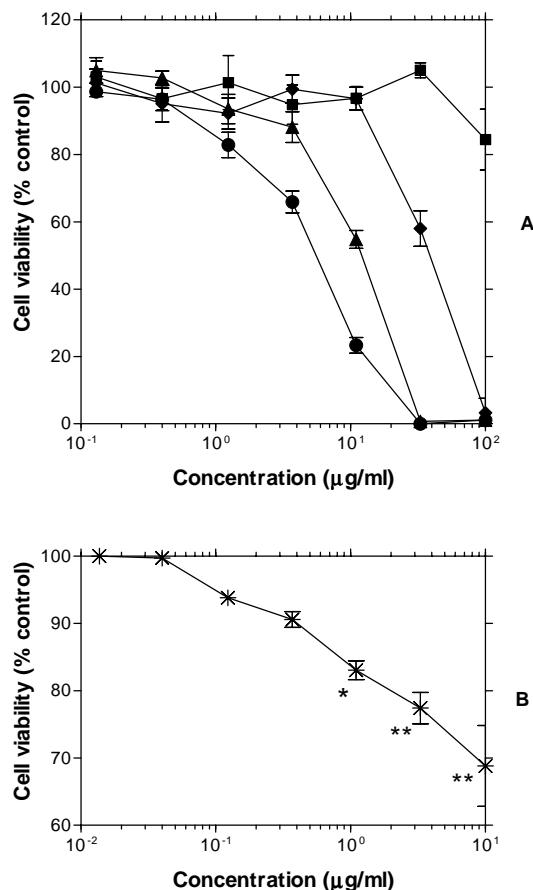


Figure 5
Toxicity of diterpenes (I-4) (panel-A) and AMB (panel-B) in THP-1 monocytes. Cell viability was measured using almar blue as described in the methods section. Data points are mean and SEM values obtained from six replicates. For symbols: see Fig. 3. In the AMB treatment group (panel-B), * and ** represent significant differences (unpaired t-test) compared to untreated control group at the level of $p = 0.05$ and $p = 0.01$ respectively.

drug, the concentration-response curve was plotted from which LD₅₀ values (the concentration of the agent that reduce cell viability by 50% compared to controls) were determined. All experiments were repeated at least three times.

Axenically-cultured amastigotes and drug susceptibility assay

Late stationary phase (3 × 10⁷ cells/ml) promastigotes were centrifuged and re-suspended in medium 199 with Hank's salts supplemented with 20% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and the pH titrated to 5.5 using 1 N HCl. Following incu-

bation of cells at 31 °C, 5% CO₂, amastigote-like rounded morphology together with loss of flagella and cell clumping started appearing within 24 h but these changes were not synchronous for a week as some motile parasites with intermediate forms and short flagella were detected. Drug treatment of the amastigotes was essentially similar with the promastigotes culture described above. Briefly, amastigotes (4 week old following initiation of differentiation) were incubated for 72 h at 31 °C, 5% CO₂ in the presence or absence of drugs. Alamar Blue™ (20 µl; Serotec, UK) was added during the last 4 h of incubation and viability measured fluorometrically by using Labsystem's Fluoroskan Ascent flourimeter at excitation wavelength of 544 nm and emission at 590 nm.

Assessment of drugs toxicity in THP-1 monocytes

THP-1 monocytes were plated onto 96 well plates at a density of 4 × 10⁴ cells per well (in 200 µl volume) in the presence or absence of experimental agents and plates incubated at 37 °C, 5% CO₂ for 72 h. After adding Alamar Blue™ during the last 3 h of incubation, cell viability was measured fluorometrically as described above.

Acknowledgements

The author wishes to thank Dr David Humber of the School of Bioscience (University of East London) for his gift of THP-1 cells and *L. aethiops* promastigotes and Mr Geoff Cooper (University of Greenwich) for his assistance in obtaining the photographs of leishmania parasites.

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