

Antileishmanial and Cytotoxic Activity of Synthetic Aromatic Monoterpenes

Edison OSORIO ^{1,2}, Gabriel ARANGO ^{1*}, Sara ROBLEDO ²,
Diana MUÑOZ ², Luz JARAMILLO ^{1,2} & Iván VÉLEZ ²

¹ Grupo de Investigación en Sustancias Bioactivas GISB, Sede de Investigación Universitaria SIU,
Universidad de Antioquia, Calle 62 N° 52-59, Torre II, Lab 22,9. Medellín, Colombia.

² Programa de Estudio y Control de Enfermedades Tropicales PECET,
Universidad de Antioquia. Medellín, Colombia.

SUMMARY. The *in vitro* activity of synthetic monoterpenes were evaluated against promastigotes and amastigotes of *L. (V.) panamensis* and compared to Glucantime®. Cytotoxic activity was determined against U-937 cells. Seven compounds showed low toxicity to U-937 cells (LC₅₀ >100.0 µg/ml) and four of them were active against amastigotes form (EC₅₀ <60.0 µg/ml). No correlation was found between promastigote and amastigote activities, suggesting again that the chemotherapeutic potential of anti-leishmanial drugs depend on their action against amastigotes. The 3-isopropyl-2,5-dimethoxy-6-methyl-phenylamine (T6) compound was the most active showing activity at concentrations similar to those reached in serum by pentavalent antimonials (13.6 µg/ml) but the 5-isopropyl-4-methoxy-2-methyl-3-nitro-phenol (T4) compound was the most selective for *Leishmania* rather than mammal cells (SI = 8.7). This study concludes that monoterpen derivatives show promising antileishmanial potential and could be considered as new lead structures in the search for novel antileishmanial drugs.

RESUMEN. "Actividad Antileishmania y Citotóxica de Monoterpenos Aromáticos Sintéticos". Fue evaluada la actividad antileishmania *in vitro* de monoterpenos sintéticos contra promastigotes y amastigotes de *L. (V.) panamensis* y comparada con la del Glucantime®. La actividad citotóxica fue determinada contra células U-937. Siete compuestos mostraron una baja citotóxicidad en las células U-937 (CL₅₀ >100.0 µg/ml) y cuatro de ellos fueron activos contra la forma amastigote (CE₅₀ <60.0 µg/ml). No se halló correlación entre las actividades contra los promastigotes y amastigotes, sugiriendo una vez más que el potencial quimioterapéutico de fármacos anti-leishmania depende de su acción contra los amastigotes. El más activo de los compuestos fue el derivado 3-isopropil-2,5-dimetoxi-6-metil-fenilamina (T6) mostrando actividad a concentraciones similares a las alcanzadas por los antimoniales pentavalentes a nivel sanguíneo (13.6 (g/ml), sin embargo el compuesto 5-isopropil-4-metoxi-2-metil-3-nitrofenol (T4) presento la mayor selectividad hacia *Leishmania* que hacia las células mamíferas (IS = 8,7). Este estudio concluye que los derivados monoterpénicos muestran una potencial actividad antileishmania y podrían ser considerados en la investigación de nuevos agentes leishmanicidas.

INTRODUCTION

The leishmaniasis comprise a group of clinically complex diseases that can be divided into cutaneous (CL), mucosal (MCL) and visceral (VL) forms, all caused by different species of protozoan parasites of the genus *Leishmania*, transmitted by phlebotomine sand flies of the genera *Phlebotomus* and *Lutzomyia*. These diseases are endemic to at least 88 tropical and subtropical countries, with approximately 350 million people at risk of suffering the infection, a global prevalence of 12 million and an incidence of 1.0-1.5 million cases per year for the disfiguring forms of the disease (simple or diffuse CL and MCL) and 0.5 million cases per year for the potentially fatal visceral form. Each one of these clinical forms is associated with differ-

ent *Leishmania* species. All clinical forms of the disease are considered to be public health problems because they are potentially fatal (VL), may cause mutilations (MCL) or may compromise working capacity because of secondary effects of chemotherapy or stigmatization caused by deforming lesions. According to the World Health Organization, incidence of the diseases increased 42-fold from 1985-1998 and Leishmaniasis is now considered to be the second most important cause of death among the parasitic infections ¹.

L. (V.) panamensis is widely distributed in several tropical countries including Colombia where is responsible for more than 90% of the CL and most of the MCL cases ². Control of the leishmaniasis by elimination of the insect vec-

KEY WORDS: Antileishmanial activity, Cytotoxic activity, Intracellular amastigotes and monoterpen.

PALABRAS CLAVE: Actividad antileishmania, Actividad citotóxica, Amastigotes intracelulares y monoterpenos.

* Author to whom correspondence should be addressed. E-mail: gjarango@quimbaya.udea.edu.co

tors or mammalian reservoirs is costly and often impractical. Since no effective vaccine is available, treatment of the disease presently constitutes an important alternative to control. The treatment available currently has been used for more than 50 years and consists of the intramuscular administration of meglumine antimoniate (Glucantime®). This drug is effective in most cases but its potential cardio- and nephrotoxicity requires medical supervision during its administration, which makes treatment very expensive. Although effective, other drugs such as amphotericin B and pentamidine are even more toxic than the antimonials, require closer supervision and are even more expensive³. On the other hand, cheaper drugs such as allopurinol and mefloquin that can be administered orally have proved to be ineffective against CL caused by *L. (V) panamensis*^{4,5}. Further complication the situation is the appearance of parasite strains that are resistant to these drugs⁶. Therefore, there is an urgent need for new leishmanicidal drugs of low toxicity and cost. One of the sources of potential anti-leishmanial drugs are the so called "natural remedies" that have been used in traditional medicine to treat. The antiprotozoan activity of plant extracts and compounds isolated from them is attributed to intermediate metabolites such as alkaloids, terpenoids, essential oils, phenolic and polyphenolic compounds, lectins and polypeptides⁷.

Among the terpenoids, the monoterpenics have been shown to possess broad antimicro-

bial and antiprotozoan activity. Several monoterpenic with proven leishmanicidal activity include some structural derivatives of *p*-cimene (1-methyl-4-isopropyl benzene) (1) such as Piquerol A (5-isopropenyl-6-methylene-cyclohex-2-ene-1,4-diol) (2) isolated from *Piqueria trinervia* Cav. (Asteraceae)⁸, terpinen-4-ol (4-isopropenyl-1-methyl-cyclohexanol) (3) obtained from *Melaleuca alternifolia* (Myrtaceae)⁹ and espintanol (3-isopropyl-2,4-dimethoxy-6-methyl-phenol) (4) isolated from *Oxandra es-pintana* (Annonaceae)¹⁰ (Fig. 1). Despite their good antiprotozoan activity, the study of these natural products has not progressed to preclinical trials due to their low availability in nature, poor solubility and high toxicity¹¹; nevertheless, they continue to be important as basic structures which can be modified to improve its biological activity, solubility and reduce its toxicity.

The thymol monoterpen (2-isopropyl-5-methylphenol) (5) (Fig. 1) is a phenolic compound also derived from *p*-cimene widely used in the medicinal practice, agriculture, cosmetics and food industry¹²⁻¹⁴. It has potent antimicrobial^{15,16}, antiseptic and disinfectant activities¹² and, improves wound healing in a variety of cutaneous afflictions¹⁷. Therefore, in this paper, the thymol structure was chemically modified at specific regions to evaluate the *in vitro* anti-leishmanial activity of thymol and its derivatives, in comparison to Glucantime®. This knowledge may facilitate the development of novel antiparasitic compounds.

MATERIALS AND METHODS

Maintenance of the parasite

Promastigotes of *L. (V) panamensis* (strain MHOM/CO/87/UA/UA140) were kept in NNN medium at 25 °C. The parasites were periodically passed by way of experimental infection of golden hamsters (*Messocricetus aureatus*) to ensure good levels of infectivity¹⁸. Hamster lesions were aspirated periodically and the material cultivated in NNN medium to obtain promastigotes that were cultivated *en masse* until they reached their stationary growth phase (5 days) and could be used for the *in vitro* infections.

Antileishmanial assay in vitro

Activity was evaluated against promastigotes and intracellular amastigotes of *L. (V) panamensis* (MHOM/CO/87/UA/UA140).

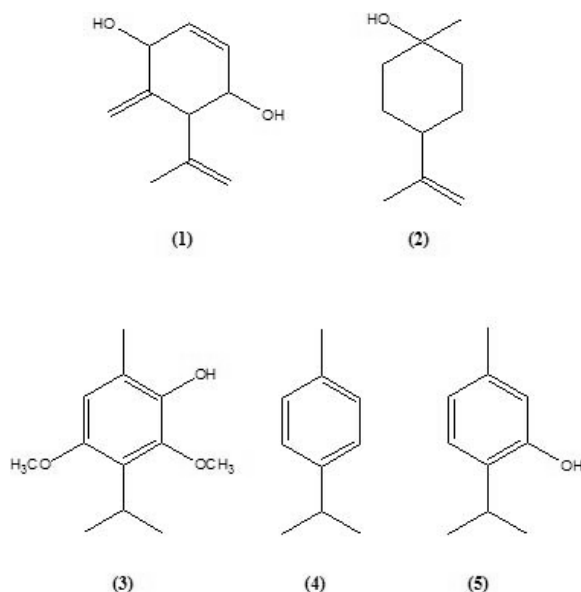


Figure 1. Chemical structures of monoterpenic isoprenoids with antibacterial and antiprotozoal activity: piquerol A (1), terpinen-4-ol (2), espintanol (3), *p*-cimene (4) and thymol (5).

Activity against promastigotes

The capacity of the compounds to kill promastigotes of *Leishmania* spp. was determined based on the viability of the parasites evaluated by the MTT method following a previously reported method¹⁹. In short, the parasites were cultivated in Schneider's medium supplemented with 10% heat-inactivated bovine foetal serum (HIBFS) (Irwing Scientific) for 3 days at 26 °C. Afterwards they were harvested, washed and re-suspended to a concentration of 1×10^6 promastigotes/ml of Schneider's medium with 10% HIBFS. Each well of a 96-well plate was seeded with 100 μ l of every parasite suspension (in triplicate) and 100 μ l of each concentration of the compound to be evaluated was added. The parasites were then incubated at 26 °C, the medium was changed once 48h later and the parasite harvested after 96 h of incubation to measure their viability by measuring the activity of the mitochondrial dehydrogenase by adding 10 μ l/well of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubating at 37 °C for 3 h. Afterwards 100 μ l/well of a 50% isopropanol solution and 10% SDS was added at 30 min to stop the reaction. Parasite viability was determined based on the quantity of formazan produced, this being detected at 570 nm in a Bio-Rad ELISA reader¹⁹. Parasites cultivated in the absence of the compound but maintained under the same conditions were used as controls for growth and viability. Since the drug was solubilized in 0.5% DMSO a control was included in which DMSO was added in the absence of the drug.

Activity against intracellular amastigotes

The *Leishmania* amastigotes are grown in macrophage-like cell lines so before testing the effect of monoterpenes on the parasite we assessed its effect on the non-infected cell line.

The cytotoxic activity of thymol and its derivatives was evaluated on promonocytic human cells of the line U-937 using the MTT enzymatic micromethod. U-937 cells were cultivated in suspension in RPMI 1640 medium (Gibco BRL, Grand Island, NY) enriched with 10% HIBFS and incubated at 37 °C and 5% CO₂, the medium being changed every two days. After 48 h of cultivation the cells were washed, counted and adjusted to a final concentration of 1×10^6 cells/ml in RPMI 1640 medium with 10% HIBFS. The cells were incubated in 96-Flat bottom well plates with decreasing concentrations of the compounds at 37 °C with 5% CO₂ for 96 h, changing the medium after 48h. The toxicity of the compounds was determined by MTT as de-

scribed previously. Cells cultivated in the absence of the compound but maintained under the same conditions were used as controls for growth and viability. Additionally the effect of the compounds was compared with the standard drug (Glucantime®). All the compounds including Glucantime® were evaluated in three independent experiments, each in triplicate. The results are expressed as LC₅₀, calculated by the probit probabilistic method²⁰.

The effect of thymol and its derivatives against intracellular amastigotes of *L. (V) panamensis* was evaluated by microscope examination of infected cells incubated in the presence or absence of the compounds^{4,19}. After 48 h growth the U-937 cells were harvested and washed twice with a Dulbecco phosphate buffered saline (DPBS) solution (Gibco BRL) and adjusted to a concentration of 1×10^6 cells/ml of RPMI 1640 medium (Gibco BRL). In each well of a 24-well cell culture plate, 10⁵ cells were dispensed and exposed to promastigotes of *L. (V) panamensis* (proportion 25:1, parasite/cell). The cells with the parasites were incubated for 2 h with 5% CO₂ at 34 °C. By then, most of the U-937 cells were attached to the glass. The non-attached cells and the free parasites were then washed twice with PBS, fresh medium was added and 24 h later replaced fresh medium containing the compound to be evaluated. The range of concentration for every extract was selected according to the LC₅₀ that was calculated beforehand. The infected cells were exposed to the compounds for 96 h, changing the medium once after 48 h. The cells were washed with PBS and fixed with methanol (JT Baker) for 20 min and stained with Giemsa (Merck). The percentage of infection was calculated by dividing the number of infected cells obtained in presence of each compound by the number of cells obtained in absence of treatment and multiplying by 100. About 200 cells were evaluated at random in every well, recording the number of infected and uninfected cells. Infected cells cultivated in the absence of the compounds served as controls for the infection. The data reported is the average of three independent experiments conducted in triplicate. The results obtained both for promastigotes and amastigotes were expressed as 50% of the effective concentration (EC₅₀) which was calculated by the probit method²⁰. The selectivity index (SI) was calculated by dividing the cytotoxic activity observed in U-937 cells by the antiprotozoan activity in promastigotes or amastigotes (SI = LC₅₀/EC₅₀).

Synthesis of Aromatic Monoterpenes

Thymol, and other reagents were purchased from Merck. The synthesis of thymol derivatives was analyzed by thin layer chromatography (TLC), silica gel chromatography column (CC), combined gas chromatography - mass spectrometry (GC-MS) and UV spectral analysis. TLC was carried out with out with silica gel 60 GF₂₅₄ chromatoplates in aluminium 0.25 mm thick; silica gel 60F with a particle diameter of 0.063-0.2 mm was used for CC analysis. A Mineralight model UVGL-58 (254 and 366 nm) ultraviolet lamp was used to visualise the plates. Analysis by GC- MS technique was performed with Varian 3800 equipment coupled with a Varian Saturn GC/MS 2000 mass selective detector. A variable wavelength (200-700 nm) Spectronic Genesys 2 Spectrophotometer and automatic data recorder were used for ultraviolet spectroscopy analysis. The spectra of RMN ¹H and RMN ¹³C were determined in deuterated chloroform with 0.05% TMS at 300 MHz and 75 MHz respectively in a Bruker apparatus. The pathway for synthesis and the compounds obtained are summarised in Fig. 2.

6-isopropyl-3-methyl-2,4-dinitro-phenol (T1)

To obtain this compound, 0.066 moles of thymol was dissolved in concentrated acetic acid at 5 °C, after which 20 ml of nitric acid was

mixed with 50 ml of acetic acid. The two solutions were mixed at ambient temperature with shaking for 1 h. The reaction was stopped with ice and the mixture refrigerated at 4 °C for 48 h. It was then neutralised with 25% ammoniac before being extracted with dichloromethane and the compound purified by column chromatography. This provided 11.237 g of yellow crystals (reaction yield 70%) [Molecular formula = C₁₀H₁₂N₂O₅, pf 51-52 °C; UV (EtOH) λ max (log ε) 211.0 (4.12), 267.0 (3.68), 399.0 (3.87) nm; (EtOH + NaOH 0.1 M) λ max (log ε) 209.0 (4.11), 262.0 (3.72), 383.0 (4.12) nm; IR (KBr) ν_{max} 3401 (OH), 3037 (Ar-H), 2969 (CH), 2875 (CH₃), 1547 (NO₂), 1333 (NO₃), 1235 (CO, phenol); MS m/z 241 [M+1]⁺(52), 240 [M]⁺(15), 223 [M-OH]⁺(100), 177 [M-OH-NO₂]⁺(19), 149(12), 119(13), 104(7), 91[C₇H₇]⁺(35), 39(13); RMN ¹H (CDCl₃) δ 7.90 (1H, s, H-5), 3.40 (1H, sep, J=7.0 Hz, H-7), 1.30 (6H, d, J=7.0 Hz, H-8,9), 2.60 (3H, s, H-10), 9.80 (1H, br s, OH); RMN ¹³C-JMOD (CDCl₃) δ 154.12 (C-1), 138.18 (C-2), 126.40 (C-3), 138.18 (C-4), 127.44 (C-5), 129.00 (C-6), 27.73 (C-7), 22.24 (C-8), 22.24 (C-9), 16.86 (C-10)].

1-Isopropyl-2-methoxy-4-methyl-3,5-dinitro-benzene (T2)

To obtain this compound 0.025 moles of T1 were dissolved in acetone and 15.6 g of sodium

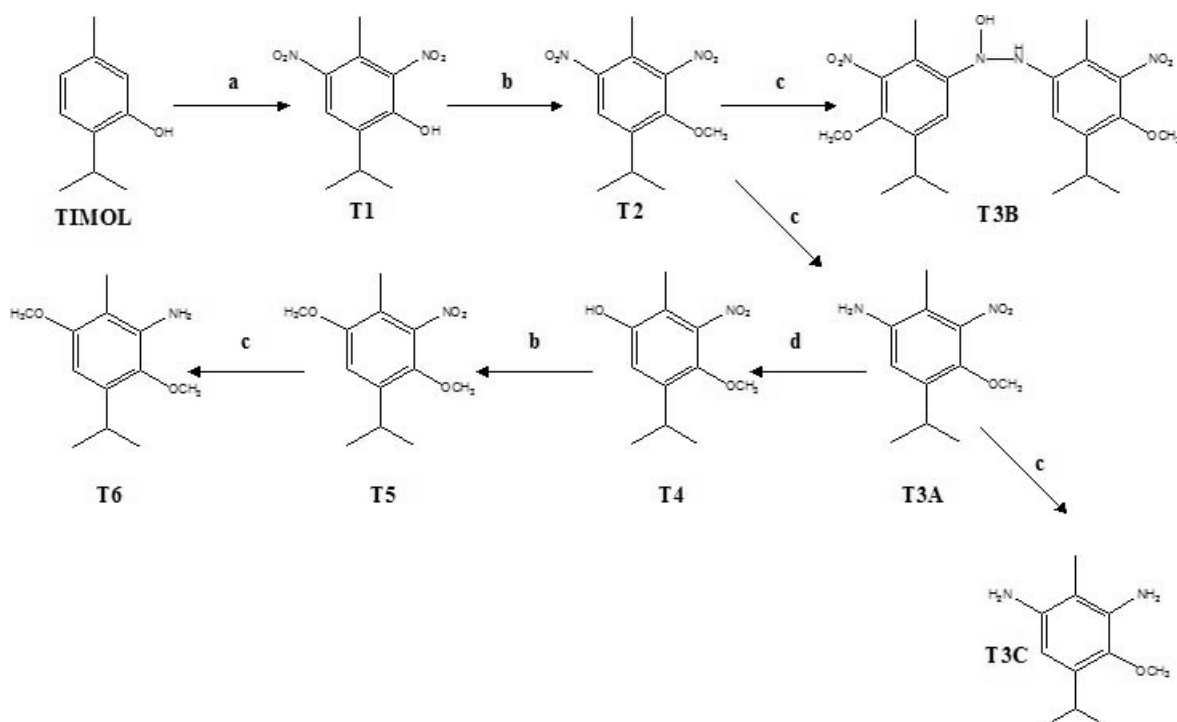


Figure 2. Synthesis reactions of aromatic monoterpenes derivative of thymol. Reaction conditions: (a) HNO₃/CH₃COOH; (b) (CH₃)₂SO₄/Na₂CO₃-Acetone; (c) Fe/HCl-CH₃OH; (d) NaNO₂/H₂SO₄.

carbonate was dissolved in dimethyl sulphate. The two solutions were mixed and shaking for 3 h. The mixture was then filtered and concentrated under reduced pressure. The compound was purified using CC of silica gel, yielding 5.872 g of amorphous yellow crystals (reaction yield 92%) [Molecular formula = $C_{11}H_{14}N_2O_5$. pf 41-42 °C; UV (EtOH) λ max (log ϵ) 213.0 (4.11), 258.0 (3.65) nm; IR (KBr) ν_{max} 3037 (Ar-H), 2970 (CH), 2873 (CH₃), 1540 (NO₂), 1340 (NO₄), 1277 (CO, ether), 1091 (COC, ether) cm⁻¹; MS m/z 254 [M]⁺(21), 237(100), 220(21), 205(22), 162(8), 121(10), 91(24); RMN ¹H (CDCl₃) δ 8.00 (1H, s, H-6), 3.35 (1H, sep, $J=7.0$ Hz, H-7), 1.30 (6H, d, $J=7.0$ Hz, H-8,9), 2.45 (3H, s, H-10), 3.90 (3H, s, OCH₃); RMN ¹³C-JMOD (CDCl₃) δ 131.00 (C-1), 152.50 (C-2), 143.52 (C-3), 124.96 (C-4), 143.52 (C-5), 125.24 (C-6), 27.31 (C-7), 23.59 (C-8), 23.59 (C-9), 14.58 (C-10), 64.25 (OCH₃)].

5-Isopropyl-4-methoxy-2-methyl-3-nitrophenylamine (T3A)

To obtain this compound 0.0125 moles of **T2** and 1.396 g of iron filings were mixed in absolute methanol with shaking and heating. Separately, 0.125 ml of concentrated hydrochloric acid was dissolved in 25 ml of absolute methanol. The two solutions were mixed and shaking for 2 h with constant heating and shaking. Heating was then stopped and the mixture added to a 15% solution of potassium hydroxide in methanol. It was then filtered and washed with methanol. The filtrate was concentrated under reduced pressure and the compound purified by silica gel CC, yielding 1.908 g of needle-like yellow crystals (reaction yield 68%) [Molecular formula = $C_{11}H_{16}N_2O_3$. pf 80-82 °C; UV (EtOH) λ max (log ϵ) 214.0 (4.11), 238.0 (4.04), 291.0 (3.41) nm; IR (KBr) ν_{max} 3473 (NH₂), 3386 (NH₂), 3035 (Ar-H), 2964 (CH), 2870 (CH₃), 1630 (NH₂), 1526 (NO₂), 1377 (NO₂), 1240 (CO, ether), 1109 (COC, ether) cm⁻¹. MS m/z 224 [M]⁺(100), 209(15), 207(17), 175(3), 164(10), 147(11), 91(3), 39(3); RMN ¹H (CDCl₃) δ 7.24 (1H, s, H-6), 7.88 (2H, br s, NH₂), 3.83 (1H, sep, $J=6.9$ Hz, H-7), 1.82 (6H, d, $J=6.9$ Hz, H-8,9), 2.60 (3H, s, H-10), 4.35 (3H, s, OCH₃); RMN ¹³C-JMOD (CDCl₃) δ 143.00(C-1), 140.80 (C-2), 146.60 (C-3), 154.10 (C-4), 141.20 (C-5), 114.53 (C-6), 26.73 (C-7), 24.04 (C-8), 24.04 (C-9), 12.18 (C-10), 64.16 (OCH₃)].

***N,N'*-Bis-(5-isopropyl-4-methoxy-2-methyl-3-nitro-phenyl)-*N*-hydroxy-hydrazine (T3B)**

This was formed as a subproduct of the re-

duction of compound **T2** to **T3A**, appearing as whitish crystals [molecular formula $C_{22}H_{30}N_4O_7$. pf 140-142 °C; UV (EtOH) λ max (log ϵ) 211.0 (4.41), 238.0 sh (4.10), 320.0 (4.08) nm; IR (KBr) ν_{max} 3036 (Ar-H), 2968 (CH), 2872 (CH₃), 1532 (NO₂), 1455 (NOH), 1340 (NO₂), 1266 (CO, ether), 1102 (COC, ether), 1054 (NOH) cm⁻¹. MS m/z 462 [M]⁺(12), 429(86), 355(88), 73(100); RMN ¹H (CDCl₃) δ 8.10 (1H, s, H-6), 7.20 (1H, s, H-6'), 2.90 (2H, sep, H-7,7'), 0.80 (12H, d, H-8,9,8',9'), 1.95 (3H, s, H-10), 1.87 (3H, s, H-10'), 3.53 (3H, s, OCH₃), 3.48 (3H, s, OCH₃), 3.02 (1H, br s, NH); RMN ¹³C-JMOD (CDCl₃) δ 143.28 (C-1), 123.62(C-2), 155.03 (C-3), 148.62 (C-4), 126.90 (C-5), 124.23 (C-6), 27.27 (C-7), 23.88 (C-8), 23.88 (C-9), 13.52 (C-10), 64.24 (OCH₃), 139.00 (C-1'), 123.50 (C-2'), 157.16 (C-3'), 141.78 (C-4'), 126.00 (C-5'), 122.62 (C-6'), 27.27 (C-7'), 23.72 (C-8'), 23.72 (C-9'), 13.14 (C-10'), 64.10 (OCH₃)].

5-Isopropyl-4-methoxy-2-methyl-benzene-1,3-diamine (T3C)

To obtain this compound 0.00156 moles of **T3A** and 0.175 g of iron filings were mixed in 100 ml of absolute methanol with shaking and heating. Compound **T3C** was prepared under the same conditions described for compound **T3A**, producing 170.12 mg of brown needle-like crystals (reaction yield 55%) [Molecular formula = $C_{11}H_{18}N_2O$. pf 94-95 °C; UV (EtOH) λ max (log ϵ) 212.0 (4.08), 290.0 (3.52) nm; IR (KBr) ν_{max} 3467 (NH₂), 3376 (NH₂), 3246 (NH₂), 3035 (Ar-H), 2961 (CH), 2870 (CH₃), 1610 (NH₂), 1225 (CO, ether), 1151 (COC, ether) cm⁻¹; MS m/z 195 [M+1]⁺(60), 194 [M]⁺(100), 179(95), 162(21), 136(10), 91(7), 65(7), 39(5); RMN ¹H (CDCl₃) δ 5.80 (1H, s, H-6), 3.40 (1H, sep, $J=6.9$ Hz, H-7), 1.20 (6H, d, $J=6.9$ Hz, H-8,9), 2.20 (3H, s, H-10), 3.90 (3H, s, OCH₃), 3.09 (4H, br s, NH₂); RMN ¹³C-JMOD (CDCl₃) δ 142.08 (C-1), 112.35 (C-2), 140.79 (C-3), 142.21 (C-4), 131.20 (C-5), 114.55 (C-6), 26.73 (C-7), 24.00 (C-8), 24.00 (C-9), 12.12 (C-10), 64.13 (OCH₃)].

5-Isopropyl-4-methoxy-2-methyl-3-nitrophenol (T4)

To obtain this compound 0.003 moles of **T3A** was dissolved in 20% sulphuric acid. A second solution was prepared with 213.4 mg sodium nitrate dissolved in water. Solutions 1 and 2 were then mixed with continuous shaking. The mixture was left to decant, filtered and washed with 60% sulphuric acid. It was then heated with shaking at 150-160 °C for 10 min, then cooled

and taken to pH 6-7 with ammoniac. The compound was then was extracted with dichloromethane and purified by silica gel CC, producing 307.35 mg of needle-like dark brown crystals (reaction yield 45%) [Molecular formula = $C_{11}H_{15}NO_4$. pf 149-151 °C; UV (EtOH) λ max (log ϵ) 214.0 (3.98), 278.0 (3.40) nm; (EtOH + NaOH 0.1 M) λ max (log ϵ) 213.0 (4.10), 241.0 (3.93), 297.0 (3.50) nm; IR (KBr) ν_{max} 3403 (OH), 3036 (Ar-H), 2967 (CH), 2872 (CH₃), 1530 (NO₂), 1374 (NO₂), 1197 (CO, phenol) cm^{-1} ; MS m/z 226 [M+1]⁺(58), 225 [M]⁺(100), 208(58), 193(2), 176(4), 164(8), 150(10), 121(18), 105(8), 91(18); RMN ¹H (CDCl₃) δ 6.75 (1H, s, H-6), 3.25 (1H, sep, $J=6.8$ Hz, H-7), 1.21 (6H, d, $J=6.8$ Hz, H-8,9), 2.12 (3H, s, H-10), 3.79 (3H, s, OCH₃), 5.40 (1H, br s, OH); RMN ¹³C-JMOD (CDCl₃) δ 151.02 (C-1), 115.21 (C-2), 142.70 (C-3), 142.70 (C-4), 129.15 (C-5), 115.50 (C-6), 27.11 (C-7), 24.26 (C-8), 24.26 (C-9), 14.60 (C-10), 64.49 (OCH₃)].

1-Isopropyl-2,5-dimethoxy-4-methyl-3-nitrobenzene (T5)

To obtain this compound 0.001 moles of **T4** were dissolved in acetone. Solution 2 was prepared mixing 686.4 mg of sodium carbonate with dimethyl sulphate. Solutions 1 and 2 were mixed with continuous shaking for 3 h. The mixture was then filtered and the filtrate concentrated under reduced pressure. The compound was purified by silica gel CC, producing 231.38 mg of amorphous whitish crystals (reaction yield 87%). [Molecular formula = $C_{12}H_{17}NO_4$. pf 54-56 °C; UV (EtOH) λ max (log ϵ) 209.0 (4.01), 276.0 (3.33) nm; IR (KBr) ν_{max} 3036 (Ar-H), 2966 (CH), 2871 (CH₃), 1534 (NO₂), 1373 (NO₂), 1264 (CO, ether), 1091 (COC, ether) cm^{-1} ; MS m/z 240 [M+1]⁺(58), 239 [M]⁺(100), 224(12), 190(3), 178(12), 162(17), 147(4), 105(7), 91(6), 79(2), 39(3); RMN ¹H (CDCl₃) δ 6.80 (1H, s, H-6), 3.35 (1H, sep, $J=6.9$ Hz, H-7), 1.20 (6H, d, $J=6.9$ Hz, H-8,9), 2.10 (3H, s, H-10), 3.80 (3H, s, OCH₃), 3.86 (3H, s, OCH₃); RMN ¹³C-JMOD (CDCl₃) δ 130.01 (C-1), 142.00 (C-2), 142.00 (C-3), 118.15 (C-4), 154.52 (C-5), 110.15 (C-6), 27.28 (C-7), 24.30 (C-8), 24.30 (C-9), 15.01 (C-10), 64.41 (OCH₃), 56.81 (OCH₃)].

3-Isopropyl-2,5-dimethoxy-6-methylphenylamine (T6)

To obtain this compound 0.0005 moles of **T5** and 60 mg of iron filings were dissolved in absolute methanol with shaking and heating. Solution 2 was prepared by dissolving 0.125 ml of

concentrated hydrochloric acid in absolute methanol. Compound **T6** was prepared under the same conditions used for compound **T3A**, producing 69.59 mg of brown needle-like crystals (yield reaction 62%). [Molecular formula = $C_{12}H_{19}NO_2$. pf 67-69 °C; UV (EtOH) λ max (log ϵ) 208.0 (4.02), 275.0 (3.30) nm. IR (KBr) 3469 (NH₂), 3379 (NH₂), 3036 (Ar-H), 2966 (CH), 2870 (CH₃), 1534 (NH₂), 1132 (CO, ether), 1091 (COC, ether) cm^{-1} . MS m/z 209 [M]⁺(100), 194(49), 177(7), 162(4), 134(8), 119(5), 91(1), 65(1); RMN ¹H (CDCl₃) δ 6.20 (1H, s, H-6), 3.30 (1H, m, H-7), 1.20 (6H, d, H-8,9), 2.00 (3H, s, H-10), 3.80 (3H, s, OCH₃), 3.76 (3H, s, OCH₂), 3.34 (2H, br s, NH₂); RMN ¹³C-JMOD (CDCl₃) δ 140.01 (C-1), 156.10 (C-2), 139.86 (C-3), 98.38 (C-4), 156.00 (C-5), 109.70 (C-6), 27.15 (C-7), 24.58 (C-8), 24.58 (C-9), 15.01 (C-10), 61.13 (OCH₃), 56.43 (OCH₃)]. The compounds obtained were dissolved in 0.5% DMSO prior to evaluation.

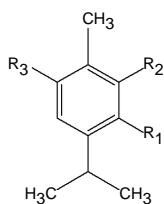
Statistical Analysis

A completely randomised, balanced and unifactorial design was used. The results were expressed as the mean plus or minus the standard deviation ($X \pm SD$) of the data, obtained in triplicate. Tukey's comparison test was also applied at the 5% significance level.

RESULTS

All the products were identified by the ¹H-RMN, ¹³C-RMN and EM spectroscopy techniques. The spectra of ¹H-RMN clearly showed the protons of the isopropyl chain in the form of an A₆X system (a doublet corresponding to the two methyl groups of about 1.25 ppm, coupled to a septuplet of almost 3.35 ppm corresponding to the methan proton, with a binding constant close to 7 Hz). The protons of the aromatic methyl in position *para* resonate as a singlet of about 2.2-2.4 ppm. Mass spectrometry studies showed important peaks for the oxygenated derivatives of *p*-cimene at [M-15]⁺, due to loss of the methyl group and the formation of a substituted hydroxytropilium ion. The presence of the tropilium ion [C₇H₇]⁺ at $m/z = 91$ is also characteristic. Peaks were also observed at [M-43]⁺, these being due to the elimination of the isopropyl chain.

The capacity of monoterpenes to kill mammalian cells was evaluated against promonocytic human cells of the U-937 line using the MTT enzymatic micromethod. The cytotoxic activities for each one of the evaluated compounds are



Compound	R ₁	R ₂	R ₃	LC ₅₀ ^a	EC ₅₀ ^b		SI ^c	
					Pro	Am	Pro	Am
Thymol	OH	H	H	>400.0 ± 0	194.3 ± 3.9	>400.0 ± 0	>2.0	>1.0
T1	OH	NO ₂	NO ₂	101.7 ± 0.7	0.4 ± 0	58.8 ± 7.7	148.1	1.7
T2	OCH ₃	NO ₂	NO ₂	176.3 ± 18.7	0.31 ± 0.03	176.3 ± 0	568.7	1.0
T3A	OCH ₃	NO ₂	NH ₂	410.4 ± 2.4	17.1 ± 1.0	318.0 ± 31.3	24.0	1.3
T3B	OCH ₃	NO ₂	C ₁₁ N ₃ H ₁₆ O ₄	220.2 ± 25.7	10.0 ± 1.0	210.0 ± 0	22.0	1.0
3C	OCH ₃	NH ₂	NH ₂	87.1 ± 6.3	8.2 ± 1.2	26.7 ± 1.0	10.6	3.3
T4	OCH ₃	NO ₂	OH	376.9 ± 74.4	>100.0 ± 0	43.4 ± 1.5	NC	8.7
T5	OCH ₃	NO ₂	OCH ₂	368.3 ± 85.0	>100.0 ± 0	131.5 ± 0.9	NC	2.8
T6	OCH ₃	NH ₂	OCH ₂	23.8 ± 0.4	>100.0 ± 0	13.6 ± 4.0	NC	18
Glucantime				416,4 + 0	400.0 + 1.2	6.7 ± 0.1	1.0	62,1

Table 1. *In vitro* cytotoxicity and anti-leishmanial activity of aromatic monoterpenes. ^a Cytotoxic activity in U-937 human promonocytic cells (µg/ml) ± SD. ^b Leishmanicidal activity (µg/ml) ± SD; Prom: promastigotes; Am: amastigotes. ^c IS, Index of Selectivity = CL₅₀ in U-937 / CE₅₀ in Prom or Am.

summarised in Table 1. Most of them showed a low toxicity (LC₅₀ >100.0 µg/ml); only **T6** and **T3C** showed to be more toxic (LC₅₀ = 23.8 and 87.1 µg/ml, respectively). The least toxic compound was **T3A** showing a toxicity comparable to that of Glucantime® (LC₅₀ = 410.0 and 400.0 µg/ml, respectively).

To determine the capacity of monoterpenes to kill *Leishmania* and compare its effectiveness against the two biological forms of the parasite, promastigotes and intracellular amastigotes obtained from infected U-937 cells were incubated in the presence or absence of each of the compounds. **T6** was the most active compounds against intracellular amastigotes (EC₅₀=13.6 µg/ml). Other compounds showing a potential antileishmanial activity were **T1**, **T3C** and **T4** (Table 1). The anti-*Leishmania* activity of these compounds against promastigotes were substantially different from that detected against amastigotes (Fig. 3). Unlike Glucantime®, which is much more toxic to intracellular amastigotes than against extracellular promastigotes (EC₅₀ = 6.7 µg/ml and 4.0 mg/ml, respectively), most of the monoterpenes presented stronger anti-*Leishmania* activity against the latter forms, with values below 1.0 µg/ml for the compounds **T1** and **T2** in promastigotes versus 58.8 and 176.3 µg/ml in amastigotes (Table 1).

DISCUSSION

In this study, the antileishmanial activity of aromatic monoterpenes were evaluated against intracellular and extracellular forms of *L. (V)*

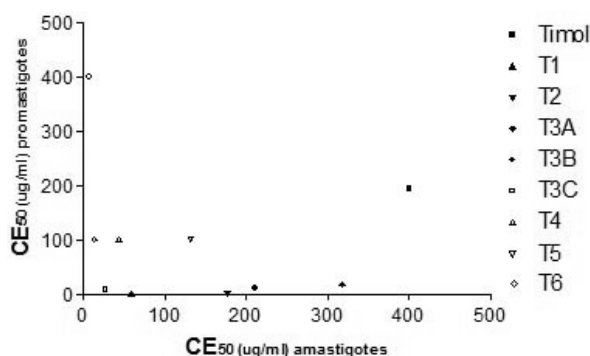


Figure 3. Activity of monoterpenic isoprenoids correlation between intracellular amastigotes and promastigotes of *L. (V) panamensis*. The mean of the results obtained by triplicate both in promastigotes and intracellular amastigotes is expressed as 50% of the effective concentration (CE₅₀) calculated by the Probit method.

panamensis. In addition, its potential toxic effect toward human cells were evaluated as well. Our results show that just one of the evaluated compounds (**T6**) showed leishmanicidal activity *in vitro* against intracellular amastigotes to concentrations similar to the levels obtained in serum for pentavalent antimony (9-12 µg/ml) ²¹ (Table 1). Other three compounds were active at concentrations less than 60 µg/m suggesting that they potential leishmanicidal could be improved. Although, none of the compounds proved to be better than Glucantime® (based on the selectivity indices), the compounds **T1**, **T3C**, **T4** and **T6** have potential for future investigation on anti-*Leishmania* therapy because of

the possibility of reducing toxicity or improving activity by means of structural modifications.

The antimicrobial activity of thymol has been documented before on pathogens such as *Escherichia coli* and *Bacillus subtilis*^{18,22}, *B. cereus*²³ and *Staphylococcus aureus* and *Pseudomonas aeruginosa*²⁴. The antimicrobial effect is attributed to lysis of the microorganisms induced by structural and functional plasma membrane damage²⁴⁻²⁶. Based on capturing confocal scanning laser microscopy, it was determined that thymol disrupt the microbial cell membrane caused an increased permeabilization to the nuclear stain Ethidium bromide²⁴, thymol was also found to desintegrate the outer membrane of *E. coli* and *S. typhimurium* at level close to the minimum Inhibitory Concentration²⁷. Further damage may be related to nutrient uptake, nucleic acid synthesis and ATPase activity²⁸. The detrimental effects of thymol on proton motive force are strongly correlated with leakage of potassium and phosphate ions even at concentrations some what lower than the minimum inhibitory concentration²⁴. This leakage of ions may lead to a decrease in the pH gradient across the cytoplasmic membrane, a collapse of the membrane potential, and the inhibition of ATP synthesis. Finally, these events are followed by cell death^{23,25}. Although the antimicrobial effects of essential oils and their components on the cell membrane are well established, further studies on the mode of action of thymol at various levels against *Leishmania* parasites and the mechanisms which may regulate the selectivity for protect mammalian cells but no parasites cells are needed to expand the knowledge on usage of such natural derivatives compounds in therapeutic of leishmaniasis disease.

The leishmanicidal activity of the compounds against promastigotes was substantially different to that observed with intracellular amastigotes. These results agree with previous observations that there is no correlation between the sensitivity of promastigotes and that of amastigotes^{19,29} and confirm that it is not sufficient to extrapolate the activities obtained for one form of the parasite to the other. The cellular, molecular and biochemical characteristics of promastigotes differ considerably from those of amastigotes (the forms responsible for the clinical manifestations in man) so that the therapeutic value of anti-*Leishmania* drugs should be evaluated and validated in the latter.

Once established inside the mammal host, the parasites must transform into amastigote

forms to resist the potentially toxic microenvironment of the host cell; therefore, it is not suitable evaluate the therapeutic potential of new compounds against the extracellular form of the parasite because drug could be or could not be active against the intracellular form; this is the case of the pentavalent antimonials or amphotericin B which are not effective against promastigotes but they do kill intracellular amastigotes (Table 1), possibly because the drug is actually activated into an active compound by intracellular metabolization mechanisms. It has been hypothesised that pentavalent antimonial acts as a prodrug that is converted to the more toxic trivalent antimony at or near the site of action³⁰. This hypothesis was further supported by the observations that trivalent antimonial is more toxic than pentavalent antimonial against both parasite stages of different *Leishmania* species³¹. Additionally, the evaluation of compounds against intracellular amastigotes is more adequate since this can give an idea of whether the drug acts directly on the parasite or indirectly, for example by activating effector mechanisms of the macrophage. This is the case of Amphotericin B which activate macrophage oxidative burst³².

Although the relationship between structure and activity was not studied, the in vitro activity of the **T3C** and **T6** may be related to the presence of amino groups in positions 1 and 3, respectively, confirming the importance of the nitrogen atom in this position for activity³³. Furthermore, it is important to emphasize that all the compounds that present methoxyl and nitro groups in contiguous positions, *i.e.*, **T2**, **T3A**, **T3B**, **T4** and **T5**, showed weak activity against the intracellular parasites but a protective effect on the host cells, expressed in their high LC₅₀ values. This protective effect could be due to the methoxyl group which need to be evaluated.

In conclusion, our results confirm the fact that organic synthesis based on chemically known compounds of natural origin allows modifications to be made in the basic structure, so that the initial properties of the compounds are improved. An increase in activity, a reduction of toxicity, or improvement of solubility are the results of structural changes and determine the importance of the synthesis in the search for new drugs. As observed in a previous study²⁹ there was no correlation between the sensitivity of the promastigote and amastigote forms of the parasite to chemotherapeutic agents. Given that in leishmaniasis the amastigote is the form of

the parasite responsible for the disease, it should constitute the chemotherapeutic target in the studies of new anti-*Leishmania* agents.

This study provides rational bases for the development of a new class of antiparasitic drugs derived from natural products. Further studies of the relationship with structural activity of monoterpenes should be carried out to determine the functional group (or groups) and the best position within the molecule which are responsible for anti-*Leishmania* activity in these compounds.

The results showed here suggest that thymol and its synthetic derivatives may be a lead compounds in the search of new anti-leishmanial drugs.

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REFERENCES

1. Remme, J. H. F., E. Blas, L. Chitsulo, P.M.P. Desjeux, H.D. Engers, T.P. Kanyok, J.F.K. Kayondo, D.W. Kioy, V.Kumaraswami, J.K. Lanzdins, P.P. Nunn, A. Oduola, R.G. Ridley, Y.T. Toure, F.Zicker & C.M. Morel (2002) *Trends Parasitol.* **18**: 421-6.
2. Osorio, L., C. Castillo & M. Ochoa (1998) *Am. J. Trop. Med. Hyg.* **59**: 49-52.
3. Berman, J. (1997) *Clin. Infect. Dis.* **24**: 684-703.
4. Vélez, I., S. Agudelo, E. Hendrickx, J. Puerta, M. Groggl, F. Modabber & J. Berman (1997) *Ann. Intern. Med.* **126**: 232-6.
5. Hirst, S. & L. Stapley (2000) *Parasitol. Today* **16**: 1-3.
6. Sundar, S., D.K. More, M.K. Singh, V.P. Singh, S. Sharma, A. Makharia, P.C. Kumar & H.W. Murray (2000) *Clin. Infect. Dis.* **31**: 1104-6.
7. Cowan, M.M. (1999) *Clin. Microbiol. Rev.* **12**: 564-82.
8. Castro, C., M. Jiménez & M. González (1992) *Planta Med.* **58**: 281-2.
9. Mikus, J., M. Harkenthal, D. Steverding & J. Reichling (2000) *Planta Med.* **66**: 366-8.
10. Hocquemiller, R., D. Cortes, G. Arango, S. Myint, A. Cave, A. Angelo, V. Munoz & A. Fournet (1991) *J. Nat. Prod.* **54**: 445-52.
11. Kayser, O. & A. Kiderlen (2003) *Exp. Opin. Invest. Drugs.* **12**: 197-207.
12. Aeschbach, R., J. Loliger, B.C. Scott, A. Murcia, J. Butler, B. Halliwell & O.I. Aruoma (1994) *Food Chem. Toxicol.* **32**: 316.
13. Lee, S., R. Tsao, C. Peterson & J.R. Coats (1997) *J. Econom. Entom.* **90**: 883-92.
14. Manou, L., L. Bouillard, M.J. Devleeschouwer & A.O. Barel (1998) *J. Appl. Microbiol.* **84**: 36876.
15. Kulevanova, S., A. Kaftandzieva, A. Dimitrovska, G. Stefkov, T. Grdanoska & N. Panovski (2000) *Boll. Chim. Farm.* **139**: 276-80.
16. Shapiro, S. & B. Guggenheim (1995) *Oral Microbiol. Immunol.* **10**: 241-6.
17. Cenas, N., A. Nemeikaite, E. Sergediene, H. Nivinskas, Z. Anusevicius & J. Sarlauskas (2001) *Biochim. Biophys. Acta.* **1528**: 31-8.
18. Rhayour, K., T. Bouchikhi, A. Tantaoui Elaraki, K. Sendide & A. Remmal (2003) *J. Essent. Oil Res.* **15**: 286-92.
19. Weniger, B., S. Robledo, G. Arango, E. Deharo, R. Aragon, V. Munoz, J. Callapa, A. Lobstein & R. Antón (2001) *J. Ethnopharmacol.* **78**: 193-200.
20. Finney, J.D. (1971) *"Probit Analysis: Statistical Treatment of the Sigmoid Response Curve"*. 3er edición, Ed. Cambridge Univ. Press, Cambridge.
21. Chulay, J., L. Fleckenstein & D. Smith (1988) *Trans. Roy. Soc. Trop. Med. Hyg.* **82**: 69-72.
22. Bennis, S., F. Chami, N. Chami, T. Bouchikhi & A. Remmal (2004) *Lett. Appl. Microbiol.* **38**: 454-8.
23. Ultee, A., M.H.J. Bennik & R. Moezelaar (2002) *Appl. Environ. Microb.* **68**: 1561-8.
24. Lambert, R.J., P.N. Skandamis, P.J. Coote & G.J. Nychas (2001) *J. Appl. Microbiol.* **91**: 453-62.
25. Ultee, A., E.P.W. Kets & E.J. Smid (1999) *Appl. Environ. Microb.* **65**: 4606-10.
26. Walsh, S.E., J.Y. Maillard, A.D. Russel, C.E. Catrenich, D.L. Charbonneau & R.G. Bartolo (2003) *J. Appl. Microbiol.* **94**: 240-7.
27. Helander, I.K., H.L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E.J. Smid & A. von Wrigth (1998) *J. Agricult. Chem.* **46**: 3590-5.
28. Denyer, S.P. & W.B. Hugo (1991) *"Biocide induced damage to the bacterial cytoplasmic membrane"*, en "Mechanisms of action of chemical biocides; their study and exploitation the society of for applied bacteriology" (S.P. Denyer & W.B. Hugo, ed.), Oxford Blackwell Scientific Publications, Oxford, Technical Series N° 27, págs. 171-88.
29. Robledo, S., A. Valencia & N. Saravia (1999) *J. Parasitol.* **85**: 360-6.
30. Petit, Y., M. Gallignani, M. Burguera, J.L. Burguera, N. Anez & Y. Lugo (1990) *J. Braz. Chem. Soc.* **1**: 72-5.
31. Roberts, W.L., J.D. Berman & P.M. Rainey (1995) *Antimicrob. Agents Chemother.* **39**: 1234-9.
32. Wolf, J.E. & S.E. Massof (1990) *Infect. Immun.* **58**: 1296-300.
33. Khan, K.M., M. Rasheed, Z. Ullah, S. Hayat, F. Kaukab, M.I. Choudhary, A. Rahman & S. Perveen (2003) *Bioorganic Med. Chem.* **11**: 1381-7.