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Bioassay Guided Fractionation of N-hexane Extract of *Salvia verbenaca* (L.) Briq. ssp verbenaca Maire (*S. clandestina* Batt. non L) whole Plant from Morocco and Synthetic Molecules as Antileishmanial Agents

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Abstract: We have previously reported on the antileishmanial potential of *S. clandestina* from different solvents and reported that n-hexane extract exerted the strongest antileishmanial activity against *Leishmania infantum*. In this paper, we now report on the bioassay-guided fractionation of n-hexane extract, its effect on *L. infantum*, and their GC-MS analysis. Furthermore, four synthetic molecules derived from 6-nitro-1Hindazoles were synthesized and assessed for their antileishmanial activities. Six fractions resulting from the chromatographic separation of the n-hexane extract showed antileishmanial activity, with the highest activity reported for F3 (170 µg/mL). GC-MS analysis leads to the identification of several phytocompounds such as dihydro-ar-tumerone, and β-Tumerone in F1, 1,2-Benezenedicarboxylic acid, diethyl ester, and 1-Docosene, in F2, 2-Pentadecanone, 6, 10, 14-trimethyl-, 9, 12-Octadecadienoic acid (Z, Z)-, and 24(Z)-Methyl-25-homo-cholesterol in F3. M1 and M4 exhibited strong antileishmanial activity against *L. infantum*, *L. tropica* (IC50 = 5.53 µg/mL, IC50 = 248.72 µg/mL, IC50 = 102.93 µg/mL, and IC50 = 200 µg/mL, respectively). This indicates that *S. clandestina* is a promising source of antileishmanial effect for developing drugs with promising results and fewer side effects. Thus, further research is being done to isolate and identify the pure bioactive antileishmanial compounds.

Keywords: antileishmanial activity; *Salvia clandestina*; bioassay-guided; GC-MS; phytocompounds.

1. Introduction

Leishmaniasis is a cosmopolitan anthropozoonosis observed in humans and some animals, including rodents and dogs, and is caused by an obligate intracellular parasite belonging to the *Leishmania* genus [1]. In hosts, the final parasitic forms found are amastigotes, after promastigotes forms [2]. Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (CML) are the different clinical manifestations of this disease in

humans [3]. The infection is transmitted by the bite of infected female phlebotomine sand flies from the *Phlebotomus* genus, which can lead to deadly mucocutaneous and visceral forms [4,5]. The *Leishmania* subgenus is distributed in the Old and New Worlds, while the *Viannia* subgenus is found only in the New World. In the Western Hemisphere, several species regularly infect people: *Leishmania (Leishmania) amazonensis, L. (Viannia) braziliensis, L. (V.) peruviana, L. (V.) colombiensis, L. (L.) donovani, L. (L.) garnhami, L. (V.) guyanensis, L. (L.) infantum chagasi, L. (V.) lainsoni, L. (V.) lindenbergi, L. (L.) mexicana, L. (V.) naiffi, L. (V.) panamensis, L. (L.) pifanoi, L. (V.) shawi,* and *L. (L.) venezualensis*. In the eastern hemisphere, there are far fewer species that infect humans: *L. (L.) donovani, L. (L.) infantum, L. (L.) aethiopica, L. (L.) major,* and *L. (L.) tropica* [6,7].

Leishmaniasis infects over 1 million new cases annually worldwide, with up to 65,000 annual deaths, and threatens about 350 million people [8,9]. The incidence rates are nearly 0.2-0.4 and 0.7-1.2 million for VL and CL each year, respectively [10]. Leishmaniasis occurs in 88 countries located in southern Europe, Africa, Asia, southern Asia, Central America, and the Mediterranean [11]. In Morocco, since the identification of CL due to *L. tropica* in 1987 and *L. major* [12,13] and VL caused by *L. infantum* in 1921 [3], the disease continues to develop gradually in the form of epidemic outbreaks in peri-urban and urban areas rural and geographical extension, which are previously free from it. For this reason, leishmaniasis continues to be seen as a public health problem and became a notifiable disease by Ministerial Decree N° 683-95 of March 31, 1995 [14]. This disease presents several syndromes depending on the variety of parasites, reservoirs, vectors, and environmental dynamics: Zoonotic CL caused by *L. major* zymodeme MON 1 is manifested in an endemo-epidemic manner in pre-Saharan areas; Anthroponotic CL caused by *L. tropica* is localized in endemo-epidemic foci in the center of the country, and VL caused by *L. infantum* is occurred in the north of the country in a hypo-endemic mode in the rural household [15].

Parasite drug treatments are mainly pentavalent antimonial compounds, including sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) [16]. Amphotericin B, miltefosine, paromomycin, and pentamidine are the second-line drugs used to treat this disease in cases of the ineffectiveness of pentavalent antimonials [17]. All these drugs have several limitations, such as toxicity, hypoxia, nausea, vomiting, nephrotoxicity, drug resistance, and high cost [18]. In addition, investigators revealed no response to the treatment used in several case reports [19,20]. Thus, the identification of new drugs should receive attention. In this context, pharmacological research on parasite disease is oriented toward screening and identifying phytomolecules with efficacy and tolerable safety.

In Morocco, ethnobotanical studies carried out in several regions, including Tafilalt and Sefrou, reported the traditional use of various medicinal plants against leishmaniasis pathology, including *Salvia clandestina* (*S. clandestina*) [21,22]. This has an extensive application in traditional medicine and has been used especially against Leishmanial strains [23]. Our last study tested the leishmanial cytotoxicity of n-hexane, dichloromethane, and methanol extracts from Salvia verbenaca against *L. major, L. tropica,* and *L. infantum* using MTT assays [24]. From this research, the n-hexane extracts demonstrated an important inhibition rate and effectively inhibited the growth rate against *L. infantum* promastigotes (IC_{50} = 14.11 µg/mL). The antileishmanial study on Moroccan *S. clandestina* showed that n-hexane extract exhibited promising therapeutic capability as an antileishmanial drug.

In this study, based on the efficacy of n-hexane extract, we investigated the *in vitro* effect of n-hexane fractionation on promastigotes forms of *L. infantum*. Furthermore, we

identified phytocompounds in each fraction. Four molecules synthesized from a new series of 6-nitro-1Hindazoles were also tested against these leishmanial species (*L. major*, *L. tropica*, and *L. infantum*).

2. Materials and Methods

2.1. Chemicals and solvents.

Growth media used for culturing Leishmanial strains (Novy, McNeal, and Nicolle (NNN), boiling hot brain of cattle (CC medium), fetal calf serum (SVF), and RPMI 1640), phosphate-buffered saline (PBS), streptomycin, penicillin were obtained from Biowest and Biotechnics Solution Society.

N-hexane, toluene, ethyl acetate, and formic were used for the extraction of phytochemicals, and Dimethyl sulphoxide (DMSO) was used to resuspend the extracts. DMSO was procured from Genome Biotechnologie (Casablanca, MAR).

2.2. Natural extract and synthetic molecules.

2.2.1. Plant material and preparation of extract.

S. clandestina was collected from Morocco, especially from Skhirat (Northwest of Morocco). Plant identification was carried out by Pr. Fatima Ezzahra EL ALAOUI-FARIS (Faculty of Sciences Rabat, Morocco). The whole plant was air-dried at room temperature in the shade. The powdered materials were then weighed (200 g) and extracted with n-hexane (1.2 L) using the Soxhlet apparatus. The filtrate obtained was concentrated in a rotary evaporator (Heidolph Typ VV 1, Germany) to obtain the n-hexane extract. The extracts were kept at 4°C until further use.

2.2.2. Bioguided fractionation of the n-hexane extract of S. clandestina.

A portion of n-hexane extract (6.257 g) of *S. clandestina* was subject to a column by chromatography over silica gel ($SiO_2$60, Merck: 7734), using Toluene/Ethyl acetate/Formic acid (TAF) solvent (6.5/3/0.5). Fractions having similar Thin Layer Chromatography (TLC) (silica gel 60F 254, Merck) profiles were combined. 10 µL of sub-fraction was spotted at the baseline of the TLC plate at 1.0 cm intervals and then allowed to dry at room temperature. The fraction was separated on TLC using a mobile phase containing Toluene/Ethyl acetate/Formic acid (TAF) solvent (6.5/3/0.5). After spraying with sulfuric vanillin reagent, the TLC plate was dried for 5 min in a hot air oven to highlight several spots and visualized under the UV transilluminator at 254 and 365 nm. In this part, the bio-guided fractionation was carried out in parallel with the antileishmanial test against *L. major*, *L. tropica*, and *L. infantum*.

2.2.3. GC-MS analyses of n-hexane fraction of S. clandestina.

The fractions, diluted in chloroform, underwent GC-MS analysis following the procedure outlined by Talbaoui et al. [25]. Analysis was conducted using a TRACE GC ULTRA apparatus coupled with a Polar Polaris Q mass spectrometer. A nonpolar VB5 capillary column (30 m × 0.25 mm, film thickness 0.25 µm) was employed. Injector and detector temperatures were maintained at 250°C and 300°C, respectively. The oven temperature was programmed to increase at a rate of 4°C/min from 40 to 180°C, and then at 20°C/min from 180 to 300°C. Helium served as the carrier gas at a flow rate of 1 mL/min, with samples (0.5 µL) injected in

splitless mode. Identification of individual components within the fractions was achieved by comparing their relative retention times with authentic samples or by referencing relative retention indices (RRI) against those of a homologous series documented in the literature.. Every compound underwent confirmation through a comparison of its mass spectra with both the NIST02 library data of the GC/MS system and Adams libraries spectra [26]. Abundances for individual components were determined by normalizing the GC peak areas of each compound without applying any correction factors.

2.2.4. Synthesis of synthetic molecules.

Four molecules, namely 5-((3-bromo-6-nitro-1H-indazol-1-yl) methyl)-3-phenyl-4, 5-dihydroisoxazole (M1), 3-bromo-6-nitro-1- (oxirane-2-yl methyl) -1H-indazole (M2), 3-bromo-6-nitro-1- (oxirane-2-yl methyl) -1H-indazole (M3), and 3-bromo-6-nitro-1 - ((1-phenyl-1H-1,2,3-triazol-4-yl) methyl) -1H-indazole (M4), were synthesized from of 6-nitro-1Hindazoles in the chemistry laboratory at the Faculty of Sciences of Rabat and tested against *Leishmanial* strains.

M1: A solution containing 0.01 mole of 3-bromo-6-nitro-1-vinyl-1H-indazole and 0.02 mole of Benzaldehydeoxime was added to 40 mL of dichloromethane with 20 mL of NaCl solution. After stirring for 5 hours to 10 hours, the organic phases were decanted, washed twice with water, and dried over magnesium sulfate. The phases were then filtered, and the solvent was removed under reduced pressure. The pure products were obtained by chromatography on a silica column, using a mixture of hexane/ethyl acetate as eluent in the respective proportions (8/2). The compound was obtained as a yellow powder.

M2: 0.01 mol (0.5 g) of 6-nitroindazole and 0.01 mol (1.2 mL) of 2- (chloromethyl) oxirane reacted in 40 mL of tetrahydrofuran (THF) with 0.01 mol of sodium bicarbonate. Potassium (1.38 g) and 0.16 g of Tetra-n-butylammonium bromide (BTBA). The mixture was stirred for 48 h after removing THF with vacuum. The final product was purified by chromatography on silica gel with hexane and ethyl acetate (8:2) and recrystallized from ethanol.

M3: In a 250 mL round bottom flask (0.06 mol) of ethyl 2- (3-bromo-6-nitro-1H-indazol-1-yl) acetate was added to 25 mL of ethanol and (0.12 mol) of hydrazine hydrate was added slowly. The reaction mixture was heated at reflux for 3 to 5 hours. TLC continuously monitored the reaction. After completion of the reaction, the resulting mixture was concentrated and cooled. The crude was filtered and recrystallized from methanol.

M4: 0.08 mol of 3-bromo-6-nitro-1-(prop-2-yn-1-yl)-1H-indazole and 0.02 mol of benzyl azide were placed in a reactor containing 60 mL of absolute ethanol. The mixture was heated at reflux for 4 hours and checked using TLC. After completion of the reaction, the solvent was evaporated under reduced pressure, and the obtained residue was purified using column chromatography on silica gel.

2.2.5. NMR-TMS spectrometry analysis

The melting points were determined using a Büchi-Tottoli apparatus and were not corrected. ^{1}H and ^{13}C NMR spectra were recorded in CDCl3 and DMSO d6 with TMS as internal reference using Bruker AC 300 (^{1}H) or 75 MHz (^{13}C) instruments; chemical changes were given in ppm downstream of TMS. The multiplicities of the ^{13}C NMR resources were

attributed to lower distortion reduction by polarization transfer experiments (DEPT). Column chromatography was performed on SiO_2 (Merck 60 silica gel 0.063-0.200 mm).

2.3. Leishmanias culture and conservation.

2.3.1. Identification of Leishmania culture.

Leishmania strain cultures were identified according to a molecular biological protocol by PCR-ITS1 method that targets the internal transcribed ribosomal 1 part located at the level of the operon ribosome of the different *Leishmania* species as described previously [3]. The leishmaniasis species were identified as *Leishmania infantum* (MHOM/MA/1998/LVTA), *Leishmania tropica* (MHOM/MA/2010/LCTIOK-4), and *Leishmania major* (MHOM/MA/2009/LCER19-09). The sequence data have been submitted to the National Reference Laboratory of Leishmaniasis, National Institute of Health (Rabat). A voucher strain was kept in the Biochemistry-Immunology laboratory at the Faculty of Sciences of Rabat, the Department of Biology.

2.3.2. Culture of Leishmania species.

The cultivation of the species followed the protocol outlined by Et-Touys et al. [24]. In brief, parasite cultures of each *Leishmania* species were washed with phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 10 minutes. Cells were then resuspended in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Streptomycin mixture. Cultures were maintained at 23°C.

2.3.3. Antileishmanial activity.

Before assessing the antileishmanial activity, the cellular density of each species was determined using light microscopy. Once the cellular density reached a threshold concentration of 10^6 cells/mL, *L. infantum*, *L. tropica*, and *L. major* promastigotes were subjected to two washes with phosphate-buffered saline (PBS) and centrifuged at 2500 rpm for 10 minutes. To assess the anti-promastigote activity, 100 µL of parasite cultures were reconstituted in a 96-well tissue culture plate in a fresh culture medium, following the protocol of Et-Touys and coworkers [24]. In brief, parasites were incubated at $2.5x10^6$ cells/well for 72 hours at 23°C in the presence of various concentrations (µg/mL) dissolved in 1% DMSO. The final concentration of DMSO did not exceed 1%, ensuring it remained non-toxic to the parasites [27-28]. Negative controls consisted of sterile PBS and a 1% DMSO solution (vehicle), while Glucantime ® served as a positive control.

2.3.4. Cell viability assay.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was used to assess the viability of leishmania species as described by Essid et al. [27] (Sigma-Aldrich, USA). In brief, 10 µL of MTT (10 mg/mL) were added to each micro-well and incubated for 3 hours at 23°C. After, to stop the reaction, 100 µL of 50% (v/v) isopropanol-10% (w/v) sodium dodecyl sulfate (SDS) mixture was added to each well in order to dissolve insoluble formazen formed after tetrazolium dye reduction. Absorbance was measured at 560 nm using an ELISA plate reader (Statfax 2100) after 30 minutes of incubation at room temperature.

All assays were conducted in triplicate and compared to the negative control (parasites) and reference drug (Glucantime). Cell viability was also evaluated by determining which inhibited half of the cell population (IC_{50}), obtained by modeling the percentage of inhibition versus concentration of extract using the Original Program. The following formula was used to calculate the inhibition percentage (I) [27]:

$$\boldsymbol{I}(\%) = \mathbf{100} \times \frac{(\boldsymbol{Absorbance\ of\ untreated\ cells - Absorbance\ of\ treated\ cells})}{\boldsymbol{Absorbance\ of\ untreated\ cells}} \quad (1)$$

2.3.5. Data analysis.

A one-way ANOVA analysis of variance was performed in the statistical analysis. We considered that the difference is significant for $P \leq 0.05$. The experiments were conducted in six replicates, and the results were expressed as mean ± SD.

3. Results and Discussion

3.1. Bioguided fractionation of the n-hexane extract of S. clandestina.

Previously, we reported that n-hexane extract from *S. clandestina* whole plant exhibited an important inhibition effect against *L. major* and *L. tropica* IC_{50} values (155.43 µg/mL and 24.56 µg/mL, respectively). Furthermore, this extract showed a high inhibition effect on the *L. infantum* promastigotes growth with an IC_{50} value of 14.11 µg/mL [24]. To identify the phytocompounds responsible for antileishmanial activity, the n-hexane extract was subjected to chromatography separation. This last was carried out using chromatography on a silica gel column ($SiO_2$60, Merck: 7734), and the fractions having similar TLC profiles were combined. After bioassay-guided fractionation of the n-hexane extract of *S. clandestina*, we obtained six fractions (F1, F2, F3, F4, F5, and F6) (Figure 1). Since n-hexane showed the highest effect on the *L. infantum* promastigotes growth (IC_{50} = 14.11 µg/mL), the fractions obtained from n-hexane fractionation were tested against *L. infantum* promastigotes.

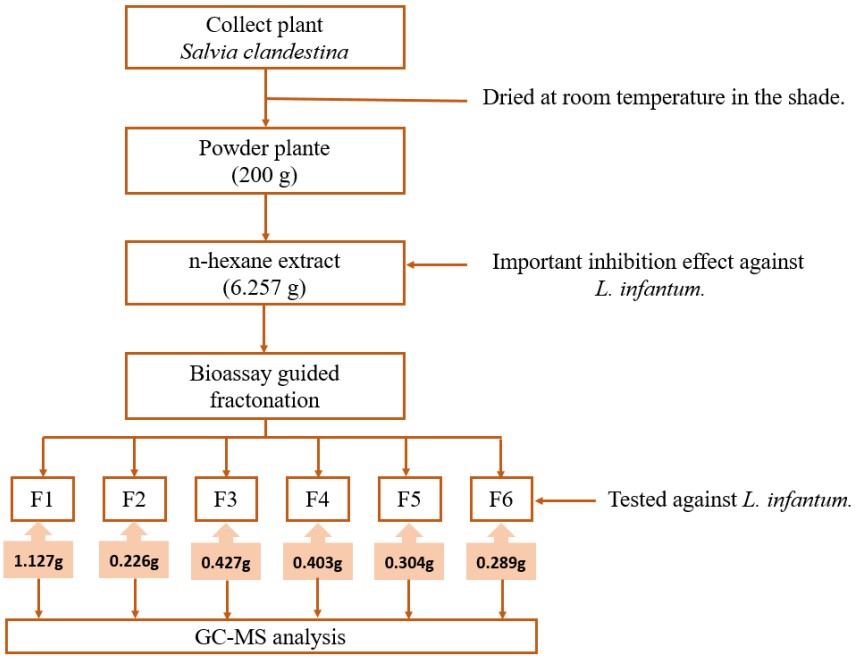


Figure 1. Fractionation schema of n-hexane extract of *S. clandestina.*

3.2. Analysis of antileishmanial property of fractions of n-hexane extract of S. clandestina.

Therefore, the cytotoxic potential of each fraction obtained from S. clandestina n-hexane fractionation was investigated on the *L. infantum* promastigotes growth. Promastigotes were exposed to increasing concentrations ranging from 1 µg/mL to 200 µg/mL. The MTT assay, as described in the section cell viability assay, indicated that each fraction (F1 to F6) revealed different cytotoxic activities towards *L. infantum* promastigotes. As shown in Figure 2, the F3 fraction of *S. clandestina* n-hexane fractionation presented an important inhibiting effect against *L. infantum* with IC_{50} values of 170 µg/mL. At the same time, the other fractions of *S. clandestina* n-hexane fractionation presented less important inhibition effects on the promastigotes growth with IC_{50} > 200 µg/mL, for F1, F2, F4, F5, and F6, respectively (Figure 2). In this bioassay, Glucantime ® (IC_{50} > 250 µg/mL) was used as a positive control drug to compare the parasite inhibition with a fraction of the n-hexane extract of *S. clandestina*. Although there are no studies on the fractionation of *S. clandestina* n-hexane extract, several investigators have shown the antileishmanial activity of several crude solvent extracts.

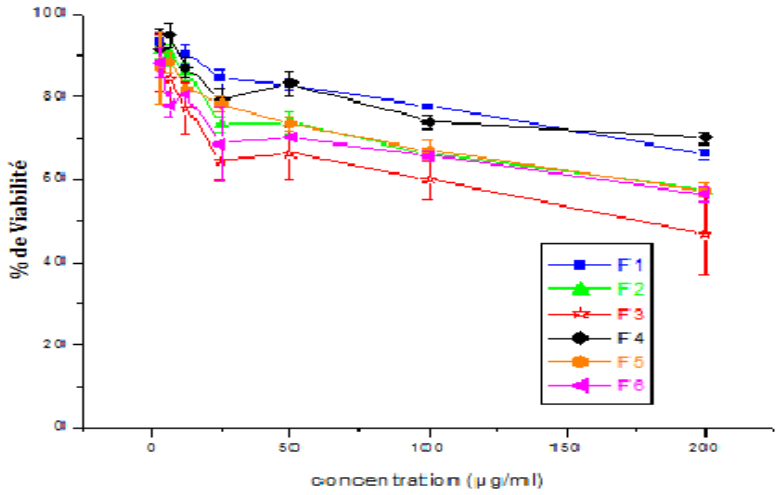


Figure 2. Antileishmanial activity of the six fractions from *S. clandestina* n-hexane extract fractionation against *L. infantum*.

3.3. GC-MS analysis of n-hexane fractions of S. clandestina.

GC-MS analysis was performed to identify the phytocompounds present in n-hexane fractions of *S. clandestina* (F1, F2, F3, F4, F5, and F6). The GC-MS spectra were represented in Figure 3. Since the F3 fraction presented the highest antileishmanial activity, we studied in detail the compounds in this fraction. F3 fraction contained several bioactive compounds such as 9,12-octadecadienoic acid, 2,6,10-Trimethyl (Figure 5),14-ethylene-14-pentadecane, and 2-hydroxy-1-(hydroxymethyl)ethyl ester. No activity were signaled for 2-pentadecanone, 6,10,14-trimethyl-; (Z)6,(Z)9-pentadecadien-1-ol and 1,2-epoxy-1-vinyl cyclododecene (Figure 4) [29,30]. 2,6,10-Trimethyl,14-ethylene-14-pentadecane presented an enzyme inhibitor, anticancer, and antiproliferative activity [31-33], while 9,12-octadecadienoic acid (Z, Z)- showed an anti-inflammatory, antimicrobial, and antityrosinase activities [34-36]. Recently, Tabrez et al. [37] studied, through *in silico* study, the mechanism of action of 9,12-octadecadienoic acid with essential enzymes of *Leishmania* growth, survival, virulence, and transmission inside the host including sterol 24-c-methyltransferase (SMT), trypanothione reductase (TR), pteridine reductase (PTR1) and adenine phosphoribosyltransferase (APRT). From this study, 9,12-octadecadienoic acid possessed a higher binding affinity with SMT, TR,

PTR1, and APRT, which may obstruct the substrate accessibility of these proteins and lead to their subsequent inhibition. Furthermore, the acute toxicity study of 9,12-octadecadienoic acid showed a safe antileishmanial drug candidate. Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, showed an antihemolytic and antioxidant activity [38]. In 2019, Achakzai and coworkers [39] showed that whole-plant hexane fraction of *Achillea wilhelmsii* exhibited antileishmanial activity (58.27 ± 0.52 µg/mL) and presented the same compound (hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester). The antipyretic, antipruritic, and antimutagenic activities of stigmast-5-en-3-ol (3 beta) (Figure 6) have been reported by Devakumar et al. [31]. Rahelivao and coworkers [40] isolated 24(Z)-methyl-25-homo-cholesterol from methanol *Spyridia* sp. (Spyridiaceae) extract, which exhibited no activity in the agar diffusion assay against several bacterial strains. However, no antileishmanial activity has been tested for this compound.

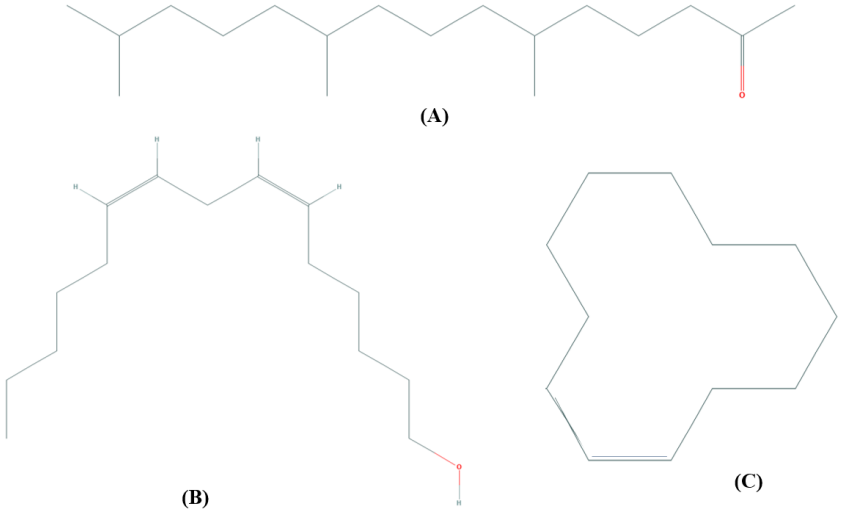


Figure 4. 2-pentadecanone, 6,10,14-trimethyl- (A), (Z)6, (Z)9-pentadecadien-1-ol (B), 1,2-epoxy-1-vinyl cyclododecene (C).

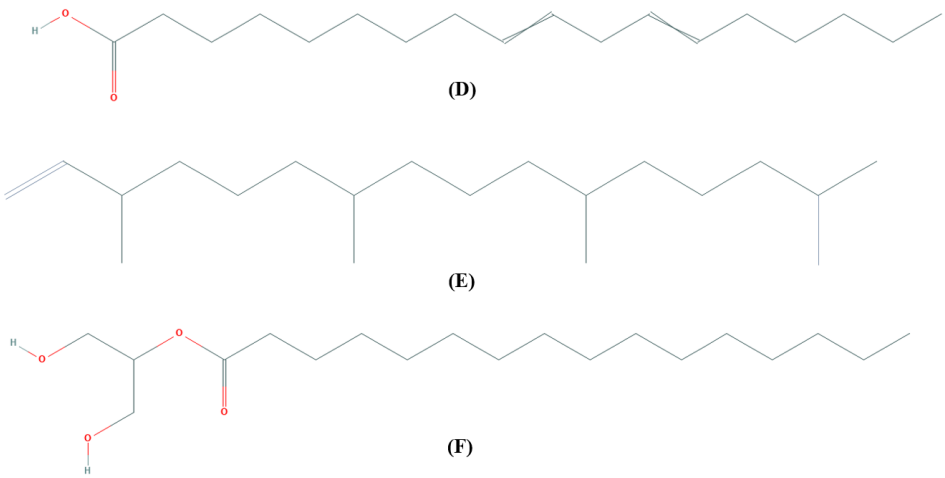
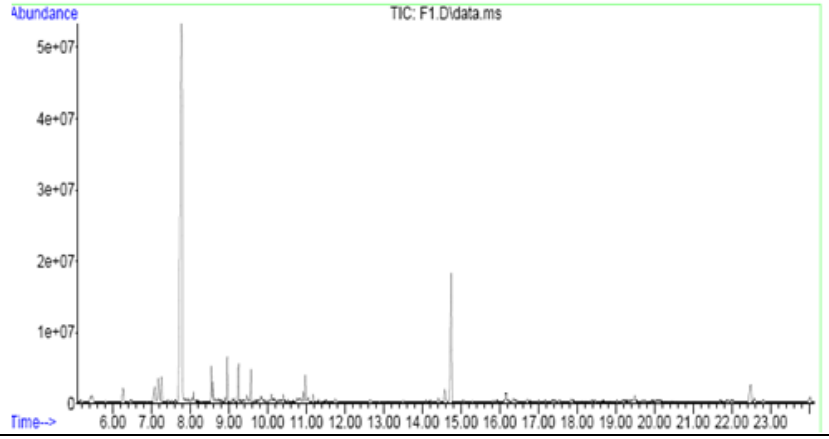


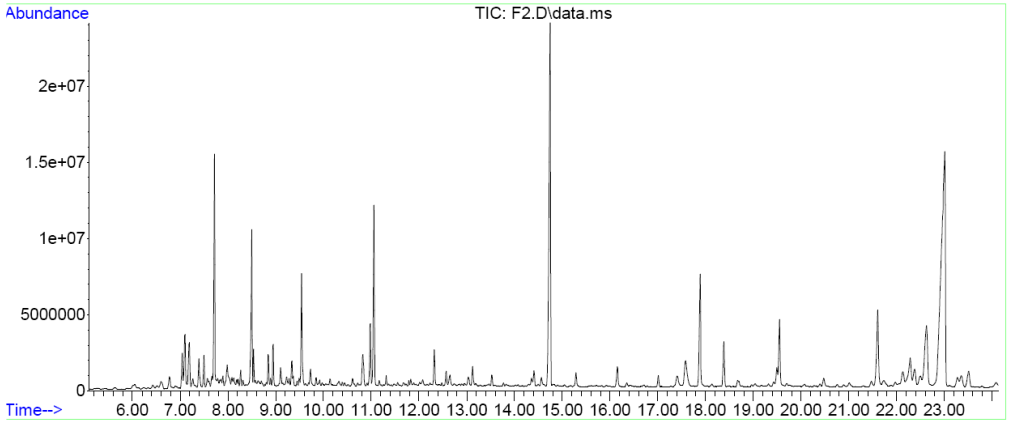
Figure 5. 9,12-octadecadienoic acid (D), 2,6,10-Trimethyl,14-ethylene-14-pentadecane (E), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (F).

F1



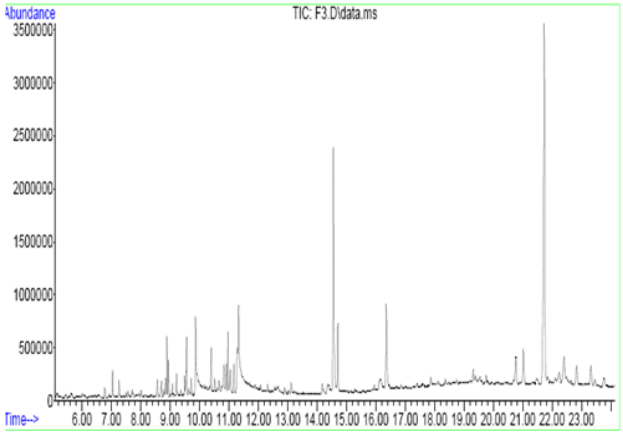
Phytocompounds	Molecular formula	Retention time (min)
Dihydro-ar-tumerone	$C_{15}H_{22}O$	7.17
Béta. Tumérone	$C_{15}H_{22}O$	7.72
(+)-.alpha.-Atlantone	$C_{15}H_{22}O$	8.54
1,2- Benzenedicarboxil acid, bis (2-ethylhexyl) ester	$C_{24}H_{38}O_4$	14.7
Lup-20(29)-en-3-one	$C_{30}H_{48}O$	22.49

F2



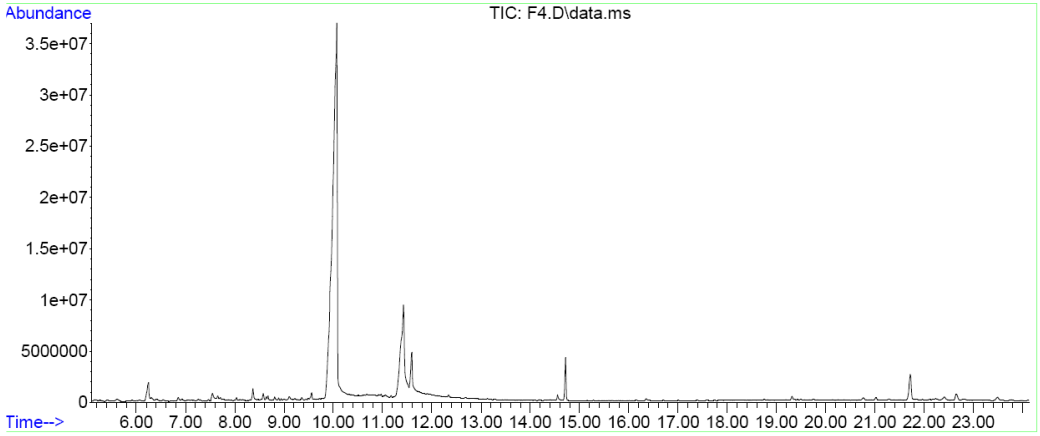
Phytocompounds	Molecular formula	Retention time (min)
1,2-Benezenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	7.09 min
Beta. Tumérone	$C_{15}H_{22}O$	7.71
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]	$C_{20}H_{40}O$	11.05
Acetamide, N-methyl-N-[4-[4-methoxy-1-hexahydropyridyl]-2-butynyl	$C_{13}H_{22}N_2O_2$	12.27
Acetamide, N-methyl-N-[4-[4-methoxy-1-hexahydropyridyl]-2-butynyl]	$C_{13}H_{22}N_2O_2$	14.6
1-Docosene	$C_{22}H_{44}$	16.12
1-Dotriacontanol	$C_{32}H_{66}O$	16.12
1-Nonadecene	$C_{19}H_{38}$	16.12
17-Pentatriacontene	$C_{35}H_{70}$	16.12
Cyclotetracosane	$C_{24}H_{48}$	17.7
Eicosane	$C_{20}H_{42}$	19.47
Nonadecane	$C_{19}H_{40}$	19.47
9-Tricosene, (Z)	$C_{23}H_{46}$	19.56
1-Dotriacontanol	$C_{32}H_{66}O$	19.56
Cyclooctacosane	$C_{28}H_{56}$	21.5
2,2,3,7-Tetramethyltricyclo (5.2.O.O.(1,6))undec-3-ene	$C_{15}H_{24}$	22.63

F3



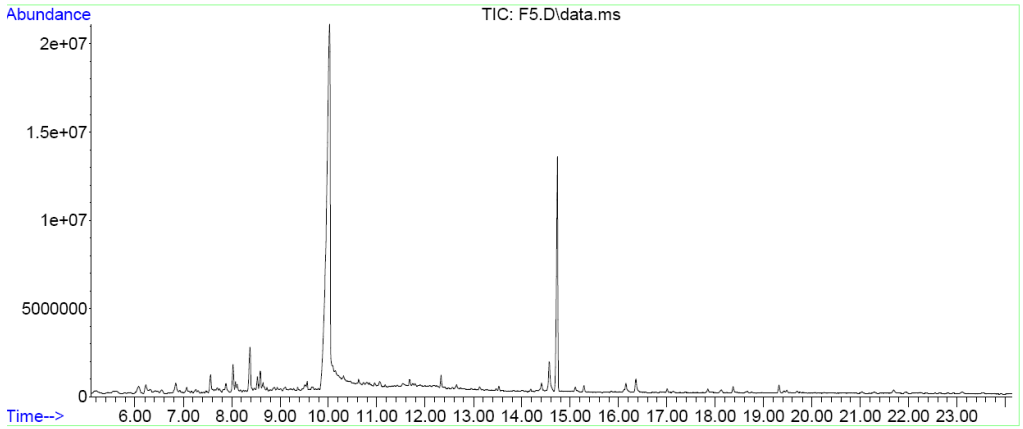
Phytocompounds	Molecular formula	Retention time (min)
2-Pentadecanone, 6,10, 14-trimethyl-	$C_{18}H_{36}O$	78.949
2,6,10-Trimethyl,14-Ethylene-14-Pentadecne	$C_{20}H_{38}$	8.909
9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	11.396
(Z)6,(Z)9-Pentadecadien-1-ol	$C_{15}H_{28}O$	11.396
1,2-Epoxy-1-vinylcyclododecene Cis-cyclododecene	$C_{14}H_{24}O$	13.242
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl exter	$C_{19}H_{38}O_4$	14.55
Stigmast-5-en-3-ol, (3.beta.)	$C_{29}H_{50}O$	21.768
24(Z)-Methyl-25-homocholesterol	$C_{29}H_{50}O$	21.768

F4



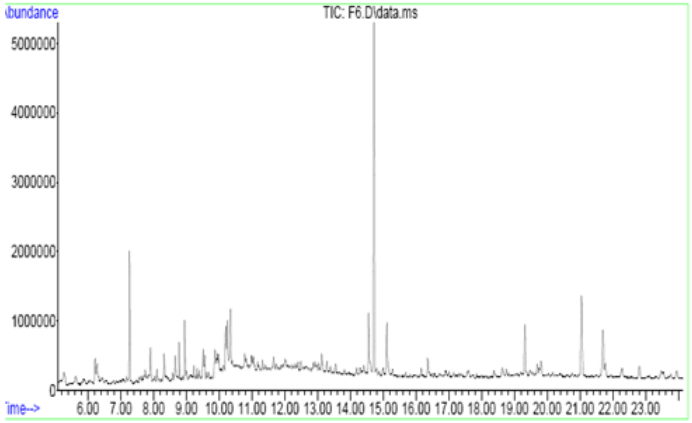
Phytocompounds	Molecular formula	Retention time (min)
Acide 9,12-Octadecadienoic	$C_{18}H_{32}O_2$	11.46
Stigmast-5-en-3-ol,(3.beta.,24S)-	$C_{29}H_{50}O$	21.68

F5



Phytocompounds	Molecular formula	Retention time (min)
Pulegone	$C_{10}H_{16}O$	7.5
Decanoic acid, silver(1+) salt	$C_{10}H_{20}AgO_2$	10.243
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	14.746
1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	14.746
Di-(2-ethylhexyl)phthalate	$C_{24}H_{38}O_4$	14.746

F6



Phytocompounds	Molecular formula	Retention time (min)
13-Oxabicyclo[10.1.0] tridecane. 1,2-Epoxycyclododecane	$C_{12}H_{22}O$	7.3
9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	10.48
Acide 1,2-Benzenedicarboxylic, bis(-ethylhexyl) ester	$C_{24}H_{38}O_4$	14.7
Acetamide, N-methyl-N-[4-[4-methoxy-1-hexahydropyridyl]-2-butynyl]-	$C_{13}H_{22}N_2O_2$	15.398
2,4,6(1H,3H,5H)-Pyrimidinetrione, 5-(1-cyclohexen-1-yl)-5-ethyl-	$C_{12}H_{16}N_2O_3$	22.058
Benzene, 1,4-Bis(trimethylsilyl)-	$C_{12}H_{22}Si_2$	22.37
6 METHYL-2 PHENYLINDOLE	$C_{15}H_{13}N$	22.85

Figure 3. GC-MS analysis of n-hexane fraction (F1 to F6).

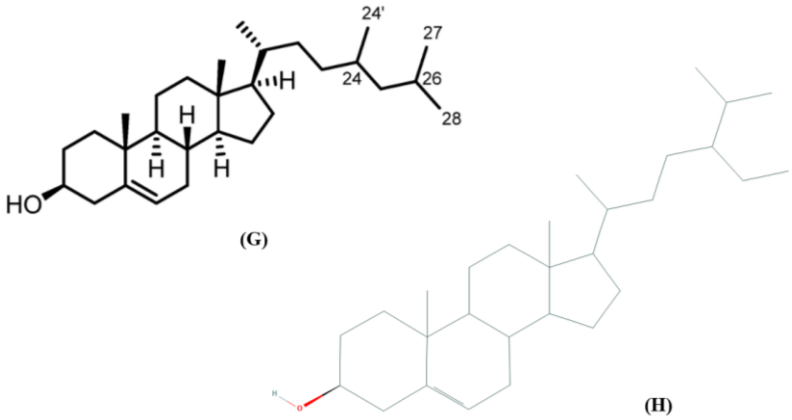


Figure 6. 24(Z)-methyl-25-homocholesterol (G), stigmast-5-en-3-ol, (3.beta) (H).

3.4. Analysis of the antileishmanial activity of synthetic molecules

Four molecules, namely M1, M2, M3, and M4 (Figure 7), synthesized from 6-nitro-1Hindazoles, were carried out on a primary screening for *in vitro* activity against three promastigotes Leishmanial species (*L. tropica*, *L. major*, and *L. infantum*) to investigate the relevance of the aromatic rings. The results are shown in Figures 8, 9, 10, and Table 1, and the IC_{50} was calculated to compare these results. The indazole compound M1, with two groups of oxazole and two groups of pyrazole, exerted a strong antileishmanial activity against *L. infantum*, *L. tropica,* and *L. major* (IC_{50} = 5.53 µg/mL, IC_{50} = 248.72 µg/mL, and IC_{50} > 250 µg/mL, respectively). Several researchers reported the important role of oxazole rings as an anticancer [41], antimicrobial, antidiabetic, and antiobesity [42-45]. Furthermore, pyrazole, an aromatic azole heterocycle with two adjacent nitrogen atoms [46], exhibited several biological activities, and recent research reported a potent *in vitro* antileishmanial activity of dioxolane–pyrazole and tetraoxane–pyrazole against promastigotes of *L. tropica* and *L. infantum* [47]. These two rings explain the effect of M1 against promastigotes of *L. tropica*, *L. major,* and especially *L. infantum.* Compounds M2, synthesized from 6-nitro-1Hindazoles, exerted a low antileishmanial activity against *L. infantum*, *L. tropica,* and *L. major* (IC_{50}> 250 µg/mL). This compound has one ring of oxirane and pyrazole. Several *in vitro* works on the effect of compounds possessing oxirane groups significantly inhibited the growth of Leishmania promastigotes. The antileishmanial effect on promastigotes parasites after treatment with epoxy methoxy flavone revealed an IC_{50} value of 45.45 µM [28]. In addition, a recent study evaluated the effect of epoxy-α-lapachone (oxirane group) against *L. (L.) amazonensis* and reported an IC_{50} value of 37.0 ± 0.4 µM during 24 h [48]. Compound M3 showed low activity against two species, *L. tropica* and *L. major* (IC_{50}> 250 µg/mL), whereas high activity has been reported against *L. infantum* with an IC_{50} = 205 µg/mL. The indazole compound M4 showed strong activity against *L. infantum* in particular (IC_{50} = 102.93 µg/mL). Moderate activity has been recorded against *L. tropica* and *L. major* with an IC_{50} = 200 µg/mL and IC_{50} = 242 µg/mL, respectively. M4 possesses two groups: 1,2,3-triazole and pyrazole. Drugs containing 1,2,3-triazole exhibited promising opportunities in the management of *Leishmaniasis* strains and gave promising results. This is the case of the investigation conducted by Costa and coworkers [49], who testes the effect of compound derived from 1,4-diaryl-1,2,3-triazole against *L. amazonensis* amastigotes and reported a strong activity with IC_{50} value of 4.4 µM. Recently,

1-decyl-3-methyl-4-((oxiran-2-ylmethoxy)methyl)-1H-1,2,3-triazol-3-ium iodide derived from 1,2,3-triazolium salts exhibited promising activity against *L. amazonensis* promastigotes and amastigotes forms with an IC_{50} = 3.61 µM, and IC_{50} = 7.61 µM, respectively [50]. In 2023, Santos and coworkers (2023) [51] assessed the effect of compounds containing 1,2,3-triazole fragments against the *Leishmania braziliensis* and reported that N-((1-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazole-4-yl) methyl)-3,4-dimethoxy cinnamide demonstrated relevant antileishmanial activity with low toxicity in murine cells. The mechanism insights of these compounds involve several targets, including mitochondrial dysfunction through an increase in mitochondrial-ROS, depolarization of mitochondrial membrane potential of *L. amazonensis* promastigotes, nitric oxide production by the host macrophage cells, and by the inhibiting microbial cell wall synthesis through the blockage of lipid biosynthesis [50, 52-53]. M1 and M4 exhibited strong antileishmanial activity against *L. infantum*, *L. tropica*, and *L. major* due to 1,2,3-triazole, pyrazole, and oxazole rings.

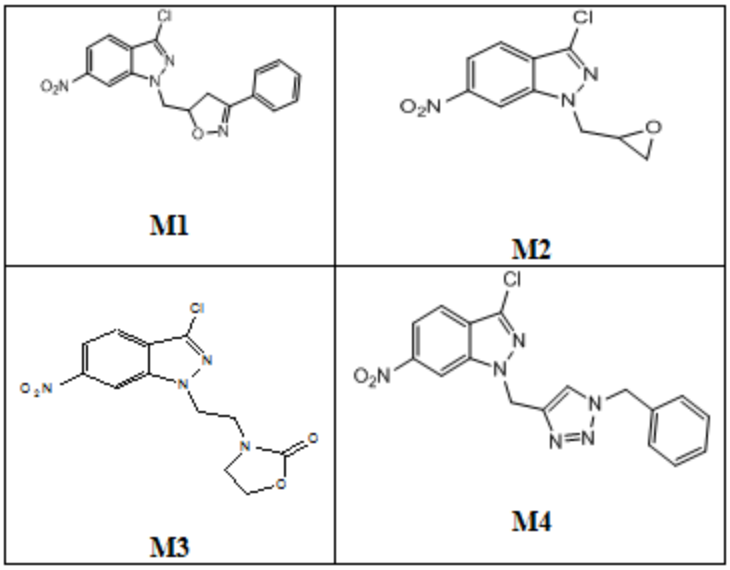


Figure 7. Schematic representation of the four molecules synthesized.

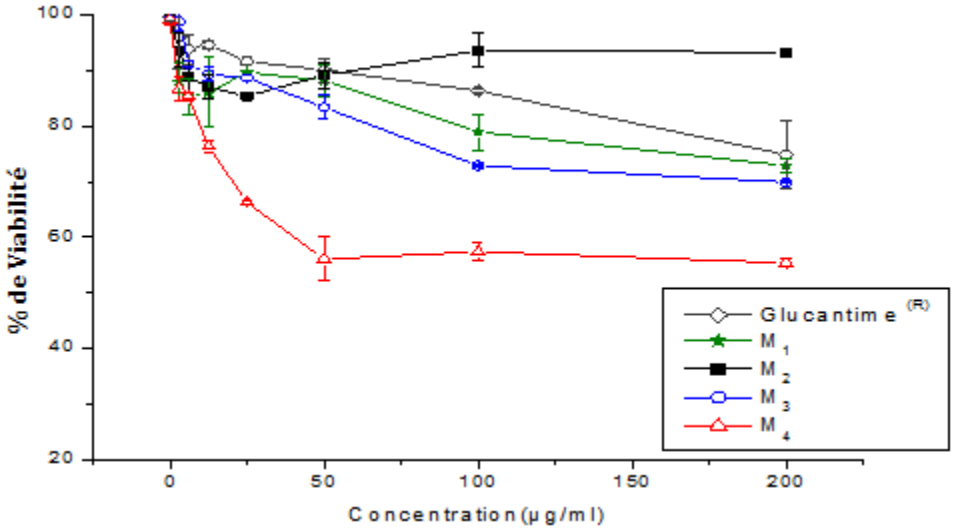


Figure 8. Antileishmanial activity of synthetic molecules against *L. major* promastigotes. Glucantime® was used as a positive control. Data expressed as the mean ± SD of six tests.

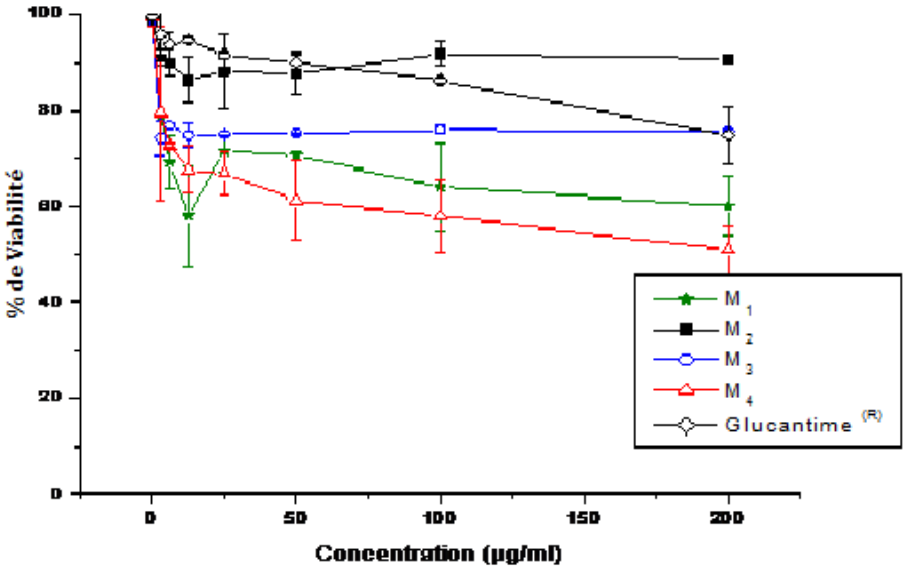


Figure 9. Antileishmanial activity of synthetic molecules against *L. tropica* promastigotes. Glucantime® was used as a positive control. Data expressed as the mean ± SD of six tests.

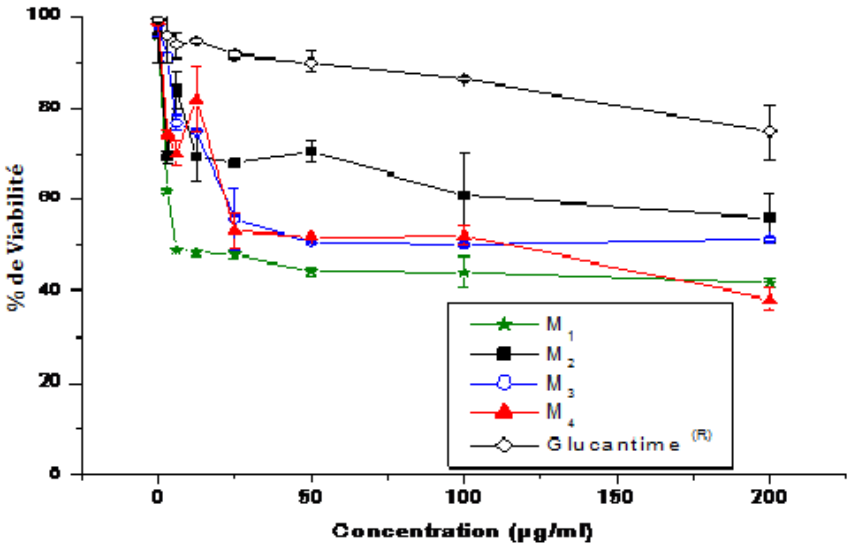


Figure 10. Antileishmanial activity of synthetic molecules against *L. infantum* promastigotes. Glucantime® was used as a positive control. Data expressed as the mean ± SD of six tests.

Table 1. The inhibitory concentration (IC50) values in µg/ml from synthetic molecules towards *L. major*, *L. tropica*, and *L. infantum* promastigotes using the MTT assay.

Molecules	***L. major***	***L. tropica***	***L. infantum***
M1	>250	248.72	5.53
M2	>250	>250	>250
M3	>250	>250	205.84
M4	242.94	200.40	102.93
Glucantime ®	>250	>250	>250

4. Conclusions

The present study showed, on the one hand, that the n-hexane fractionation of *S. clandestina* contained six fractions, and the F3 fraction exhibited the highest antileishmanial activity against *L. infantum*. Furthermore, this fraction presented several compounds, such as 9,12-octadecadienoic acid, hexadecanoic acid, and 2-hydroxy-1-(hydroxymethyl)ethyl ester. These results justify the Moroccan population's use of this plant (S. clandestina) as a beneficial folk plant in treating leishmaniasis as a source of natural compounds in the pharmaceutical

industry. Thus, further research is necessary to separate these compounds in each fraction, elucidate their structure, and identify their antileishmanial activity. These researches are necessary to understand the antileishmanial mechanism of S. clandestina fully. On the other hand, M1 and M4 compounds synthesized from 6-nitro-1Hindazoles M1 exhibited strong antileishmanial activity against *L. infantum*, *L. tropica,* and *L. major* due to 1,2,3-triazole, pyrazole, and oxazole rings. Therefore, these two synthetic compounds could be considered potential antileishmanial activity drugs. However, further investigations are required to assess the toxicity effect of these compounds and ensure their safe application in modern medicines.

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Conflicts of Interest

The authors declare no conflict of interest.

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