



ORIGINAL ARTICLE

Biologically-guided isolation of leishmanicidal secondary metabolites from *Euphorbia peplus* L.



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Abstract Leishmaniasis is a worldwide health problem, highly endemic in developing countries. Moreover, the severe side effects and the reported drug resistance make it an urgent need to search for effective drugs that can replace or supplement those currently used. In a research program designed to investigate the antileishmanial activity of plants collected from the Egyptian flora, twenty extracts from fifteen plants growing in Egypt have been investigated for *in vitro* leishmanicidal activity against *Leishmania donovani* promastigotes. Among the tested extracts, the methanol extract of *Euphorbia peplus* aerial parts exhibited a significant antileishmanial activity as it produced 100% inhibition of growth with activity similar to amphotericin B. The total extract was subjected to liquid-liquid fractionation using solvents of different polarities, followed by testing the antileishmanial activity of the successive fractions. Phytochemical exploration of the active *n*-hexane fraction (which produced 75% inhibition of growth) led to isolation of four compounds: simiarenol (1), 1-hexacosanol (2), β -sitosterol (3), and β -sitosterol-3-*O*-glucoside (4) from the biologically active sub-fractions. Structure elucidation was aided by 1D and 2D NMR techniques. In conclusion, *E. peplus* plant has many non-polar secondary metabolites that can be used as drug leads for treatment of leishmaniasis.

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1. Introduction

Leishmaniasis is a vector-borne disease which is transmitted by sandflies. It is caused by about 20 different species of the genus *Leishmania* (Habtariam, 2003). This disease has a wide

range of clinical symptoms that ranges from self-healing ulcers, which is called cutaneous leishmaniasis, to progressive nasopharyngeal infections (mucocutaneous leishmaniasis) and fatal disseminating visceral leishmaniasis (Ahuja et al., 2007). Due to the occurrence of visceral leishmaniasis as an opportunistic infection in HIV-infected patients, the expansion of the AIDS pandemic makes the emergence of leishmaniasis/HIV co-infection a serious problem (Rocha et al., 2005). Since long time attention was paid toward the promising potential of medicinal plants for treatment of prevailing ailments. Moreover, the high cost and limited availability of effective pharmaceutical products suggest the use of native plants for symptomatic treatment of leishmaniasis in areas where it is

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endemic. In traditional medicine, the treatment of this disease usually consists of oral administration of crude plant extracts for visceral leishmaniasis, and topical preparations for the treatment of skin infections (Chan-Bacab and Peña-Rodríguez, 2001).

The genus *Euphorbia* comprises the largest among genera of the family Euphorbiaceae. It includes about 1600 known species (Ali et al., 2013), ranging from annuals to trees; all contain latex and have unique flower structure. Some of the reported folk medicinal uses of *Euphorbia* include treatment of skin diseases, gonorrhoea, migraines, intestinal parasites, and warts (Jassbi, 2006). In Iran some species are used as purgative (Upadhyay et al., 1976). In addition, different species of *Euphorbia* contain macrocyclic diterpenoids with antibacterial, anticancer, PGE2-inhibitory, anti-HIV, and analgesic activity (Jassbi, 2006). *Euphorbia peplus* L. is originally native to Europe and North Africa (Zhi-Qin et al., 2010). The plant has a milky sap that is used in traditional medicine for treatment of non-melanoma skin cancer; the active compounds have been determined to be diterpene esters (Ramsay et al., 2011). In our search for leishmanicidal secondary metabolites, twenty extracts from fifteen plants growing in Egypt have been investigated for *in vitro* leishmanicidal activity against *Leishmania donovani* promastigotes. Among the tested extracts, the methanol extract of *E. peplus* aerial part exhibited a powerful leishmanicidal activity. Moreover, biologically-guided isolation of four compounds from the aerial parts of *E. peplus* is presented.

2. Materials and methods

2.1. General experimental

1D and 2D NMR spectra were recorded on a Bruker Avance III 400 MHz with BBFO Smart Probe and Bruker 400 MHz AEON Nitrogen-Free Magnet (Bruker AG, Switzerland) using the chemical shift of CDCl₃ solvent peak at 7.24 (s) ppm in ¹H and 77.2 (t) ppm in ¹³C NMR as an internal reference standard. Data were analyzed using Topspin 3.1 Software. LC-ESIMS was obtained using a Bruker Bio Apex FT-MS in ESI mode.

2.2. Material for chromatography

Thin layer chromatography (TLC), pre-coated silica gel 60 F₂₅₄ plates (Fisher Scientific, Suwanee, GA) for TLC; developing system: *n*-hexane-EtOAc (8:2 and 7:3) and visualization using 10% H₂SO₄ in MeOH. Column chromatography (CC) was performed with silica gel (230–400 mesh) and Sephadex LH-20 (Pharmacia Biotech, Uppsala).

2.3. Plant materials

All plant materials were collected in Egypt and were identified by Dr. M. Elgebaly, Faculty of Science, Cairo University. Botanical names and plant parts are listed in Table 1. Voucher samples were deposited in the Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, with voucher numbers listed in Table 1. *E. peplus* L. was collected on March, 2013, from El-Nil public garden, Beni-Suef, Egypt.

Table 1 Plant list and their primary antileishmanial screening result.

Family and plant name (voucher no.)	Part used	% of inhibition
<i>Acanthaceae</i>		
<i>Adhatoda vasica</i> (BUPD-45)	Leaf	45
	Stem	14
<i>Aizoaceae</i>		
<i>Mesembryanthemum crystallinum</i> (BUPD-22)	Aerial part	0
<i>Mesembryanthemum forsskaolii</i> (BUPD-21)	Aerial part	14
<i>Aizoon canariensis</i> (BUPD-46)	Aerial part	17
<i>Trianthemum portulacastrum</i> (BUPD-47)	Aerial part	20
<i>Asteraceae</i>		
<i>Tagetes patula</i> (BUPD-48)	Leaf	12
	Stem	10
	Flower	15
<i>Brassicaceae</i>		
<i>Brassica rapa rapa</i> (BUPD-49)	Aerial part	8
<i>Chenopodiaceae</i>		
<i>Anabasis setifera</i> (BUPD-50)	Aerial part	5
<i>Atriplex lindleyi</i> (BUPD-51)	Aerial part	6
<i>Euphorbiaceae</i>		
<i>Euphorbia helioscopia</i> (BUPD-52)	Aerial part	48
<i>Euphorbia peplus</i> (BUPD-53)	Aerial part	100
<i>Gramineae</i>		
<i>Sorghum bicolor</i> (BUPD-54)	Seedlings	19
<i>Solanaceae</i>		
<i>Solanum nigrum</i> (BUPD-55)	Leaf	14
	Stem	14
	Flower	18
<i>Zygophyllaceae</i>		
<i>Zygophyllum coccineum</i> (BUPD-56)	Aerial part	14
<i>Zygophyllum decumbens</i> (BUPD-57)	Aerial part	6

2.4. Extraction

The collected samples (200 g each) were dried under controlled temperature not exceeding 45 °C, pulverized and then extracted with 80% methanol (300 ml × 3) by percolation. The extracts were then dried under reduced pressure at temperature not exceeding 45 °C. Preliminary testing of the prepared extracts used for antileishmanial activity against the protozoan *L. donovani* (National Center for Natural Products Research NCNPR, University of Mississippi, Oxford, MS, USA), revealed significant activity for the alcohol extract of *E. peplus* (Family Euphorbiaceae). The air-dried aerial part of *E. peplus* (1 kg) was pulverized using a laboratory mill and extracted

with 80% methanol (5L × 5). The methanol was removed by vacuum distillation to give (100 g) residue. Successive fractionation using solvents of different polarities such as *n*-hexane, DCM and EtOAc was done, then the fractions were subjected to antileishmanial screening. The *n*-hexane fraction was the most active (Fig. 1).

2.5. Chromatographic isolation

Hexane fraction (25 g) was subjected to CC over silica gel for column (200 g, 4 cm × 65 cm) using gradient elution starting with pet. ether-DCM till 100% DCM then adding EtOAc in 5% increments. Seven collective fractions were obtained after TLC monitoring. Column subfractions were subjected to primary antileishmanial screening, and three fractions were found active as shown in Fig. 1. Fraction 3 (eluted with 50% DCM in pet. ether) was rechromatographed over silica gel eluted with pet. ether-EtOAc in 5% increments to get compound **1** (25 mg) and another sub-fraction that was rechromatographed over Sephadex-LH-20 eluted with DCM-MeOH (80:20) to get compound **2** (63 mg). Fraction 4 (eluted with 75% DCM in pet. ether) was rechromatographed using silica gel column and isocratic elution with 10% EtOAc in pet. ether to get compound **3** (12 mg). Fraction 7 (eluted with 100% EtOAc) was rechromatographed on silica gel column eluted with DCM-MeOH in a gradient fashion to give compound **4** (18 mg) (see Fig. 2).

2.6. Characterization of the isolated compounds

Compound (1): Simiarenol: ESIMS showed $[M+1]^+$ at m/z 427.1743 indicating a molecular formula of $C_{30}H_{50}O$. 1H NMR (400 MHz, $CDCl_3$): δ 5.64 (d, $J = 5.0$ Hz, 1H), 3.50 (m, 1H), 2.18–1.18 (m, 24H), 1.16 (s, 3H), 1.07 (s, 3H), 1.03 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H), 0.91 (overlapped d, 3H), 0.85 (overlapped d, 3H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.0, 122.0, 76.4, 60.1, 51.8, 50.9, 50.3, 44.3, 42.8, 40.8, 39.3, 38.6, 35.4, 34.8, 34.2, 30.8, 29.1, 29.0, 28.3, 27.8, 25.5, 24.1, 22.9, 21.9, 19.9, 18.1, 17.9, 16.1, 15.8, 15.0. NMR data were consistent with the literature (Duarte, 2008).

Compound (2): 1-Hexacosanol: ESIMS showed $[M+1]^+$ at m/z 383.2057 indicating a molecular formula of $C_{26}H_{54}O$. ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 63.1 (CH_2-OH), 32.7 (CH_2), 31.9 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 25.4 (CH_2), 22.7 (CH_2), 14.2 (CH_3) indicating unsaturated primary fatty alcohol. ^{13}C NMR data were consistent with fatty alcohols (Osborne and Stevens, 1996).

Compound (3): β -sitosterol: ESIMS showed $[M+1]^+$ at m/z 415.2953 indicating a molecular formula $C_{29}H_{50}O$. ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.77, 121.72, 71.81, 56.78, 56.06, 50.14, 45.84, 42.33, 42.31, 39.78, 37.26, 36.51, 36.15, 33.95, 31.91, 31.67, 29.16, 28.25, 26.08, 24.31, 23.07, 21.09, 19.82, 19.40, 19.04, 18.79, 11.99, 11.86. Data were consistent with previously published data (Kongduang et al., 2008).

Compound (4): β -sitosterol-3-O-glucoside: ESIMS spectrum showed an M^+ at m/z 576.4 and for the aglycon β -sitosterol at m/z 414.4. CO-TLC with authentic sample using $CHCl_3$ -

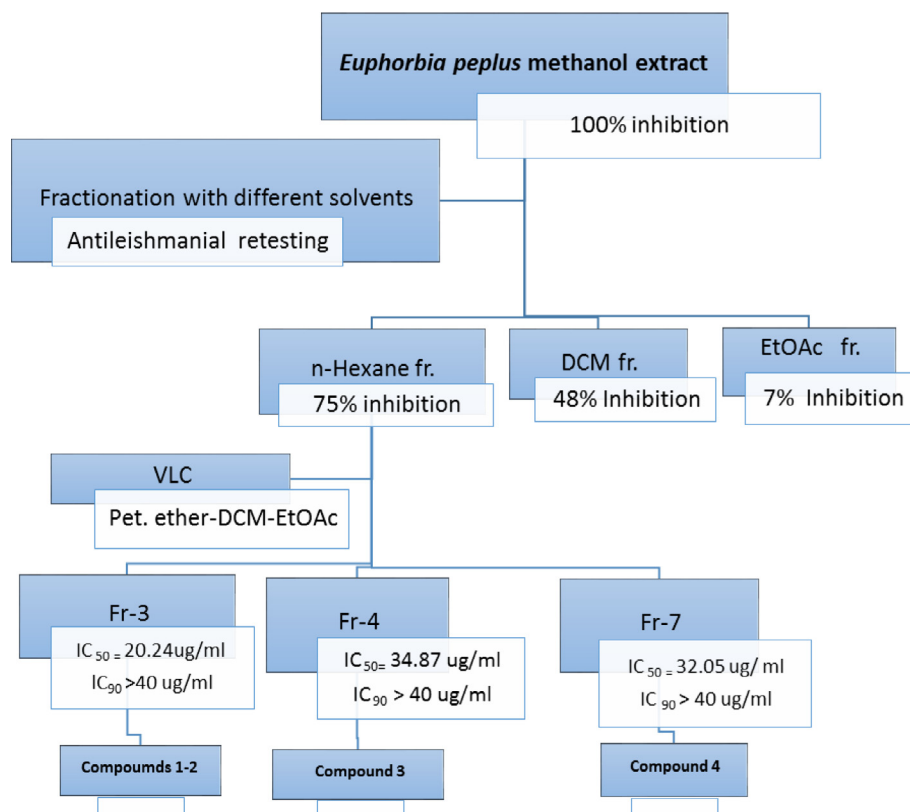


Figure 1 Scheme for biologically-guided isolation of secondary metabolites from *Euphorbia peplus* aerial parts.

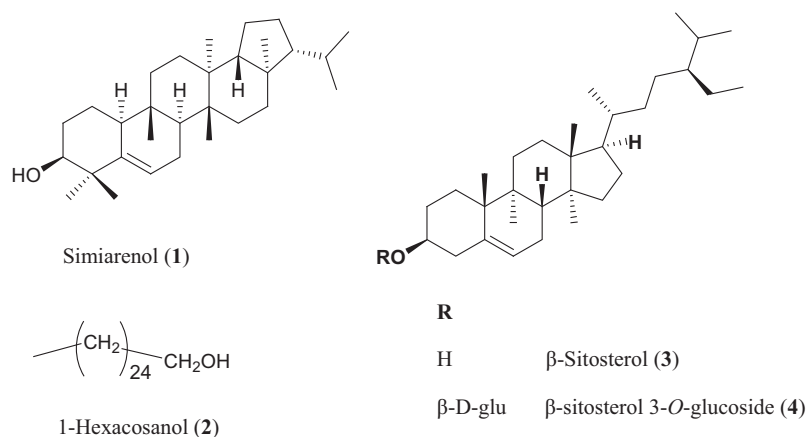


Figure 2 Compounds isolated from *Euphorbia peplus* aerial part.

MeOH (8:2) confirmed the identification of compound **4** as β -sitosterol-3-*O*-glucoside.

2.7. Assay for leishmanicidal activity

Antileishmanial activity was tested *in vitro* on a culture of *L. donovani* promastigotes. In a 96-well micro plate assay appropriately diluted extracts were added to the leishmania promastigotes culture (2×10^6 cells mL^{-1}). The plates were incubated at 26 °C for 72 h and growth of *Leishmania* promastigotes was determined by use of the Alamar blue assay (Moawad et al., 2013). Standard fluorescence was measured by a Fluostar Galaxy plate reader (excitation wavelength: 544 nm; emission wavelength: 590 nm). Pentamidine (IC_{50} 1.01 and IC_{90} 2.03 $\mu\text{g}/\text{mL}$) and amphotericin B (IC_{50} 0.47 and IC_{90} 0.65) were used as the drug controls. Percent growth was calculated and plotted against the tested concentrations to determine the IC_{50} and IC_{90} values.

3. Results and discussion

Fifteen plants related to the families Acanthaceae, Aizoaceae, Asteraceae, Brassicaceae, Chenopodiaceae, Euphorbiaceae, Gramineae, Solanaceae and Zygophyllaceae (Table 1) were tested for leishmanicidal activity against *L. donovani* promastigotes. Among the tested extracts, the methanolic extract of *E. peplus* showed 100% growth inhibition with activity similar to amphotericin B. The methanolic extract was fractionated using *n*-hexane, DCM, and EtOAc and these fractions were subjected to rescreening as *L. donovani* promastigotes growth inhibitor. The *n*-hexane fraction showed 75% inhibition (Fig. 1). Hexane fraction was subjected to VLC fractionation and the sub-fractions were screened for antileishmanial activity. Three fractions were found active (IC_{50} = 20.24, 34.87, 32.05 $\mu\text{g}/\text{ml}$) and the other fractions showed activity at > 40 $\mu\text{g}/\text{ml}$ and considered inactive. The active fractions were analyzed using HPLC/HRMS in addition to chromatographic isolation of four known compounds elucidated as simiarenol (**1**), 1-hexacosanol (**2**), β -sitosterol (**3**), and β -sitosterol-3-*O*-glucoside (**4**).

The total methanolic extract showed 100% inhibition of growth with activity similar to amphotericin B. Upon fractionation, the *n*-hexane fraction showed 75% inhibition and then

the *n*-hexane subfractions showed activity with IC_{50} = 20.24, 34.87, 32.05 $\mu\text{g}/\text{ml}$ and IC_{90} > 40 $\mu\text{g}/\text{ml}$ compared to pentamidine (IC_{50} = 1.01 and IC_{90} = 2.03 $\mu\text{g}/\text{ml}$) and amphotericin B (IC_{50} = 0.47 and IC_{90} = 0.65 $\mu\text{g}/\text{ml}$) which indicated synergistic effect of secondary metabolites in *E. peplus* total methanolic extract with special concentration in the *n*-hexane fraction.

Although the isolated compounds were previously reported in the title plant, the result of their presence in the active antileishmanial fractions adds to their biologic importance. Compound **1** was assigned to be 5-adianene-3- β -ol which is commonly named as simiarenol. Simiarenol was first isolated from *Rhododendron simiarum* (Aplin et al., 1966). It was previously isolated from *Euphorbia lagascae* and *Euphorbia tuckeyana* (Duarte, 2008) as well as detected in the epicuticular leaf waxes from *Euphorbia characias*, *Euphorbia nicaeensis*, *E. peplus* (Hemmers et al., 1988) and *Euphorbia lathyris* (Hemmers et al., 1989).

Compound **2** was a saturated fatty alcohol (1-hexacosanol). Fatty acids and alcohols exhibit antimicrobial effects as they can prevent the growth of or directly kill bacteria, fungi and other microbes by affecting multiple cellular targets, including the cell membrane (Desbois, 2012).

The inhibiting activity of some triterpenes and sterols for promastigotes and intracellular amastigotes of *Leishmania amanuensis* was previously reported but β -sitosterol was inactive against this species (Torres-Santos et al., 2004), and we reported here the activity of β -sitosterol (**3**) and its 3-*O*-glucoside derivative (**4**) toward *L. donovani* promastigotes.

In conclusion, many of non-polar components in *E. peplus* exhibited synergistic effect to produce leishmanicidal activity against *L. donovani* promastigotes. So far, it is the first report of these compounds to such activity that may be helpful in future to include the *n*-hexane extract in food supplements for treatment of different leishmaniasis caused by such pathogen.

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