In vitro Evaluation of Antileishmanial Activity and Toxicity of Essential Oils of Artemisia absinthium and Echinops kebericho

ARTICLE *in* CHEMISTRY & BIODIVERSITY · APRIL 2011

Impact Factor: 1.52 · DOI: 10.1002/cbdv.201000331 · Source: PubMed

4 AUTHORS:

Yinebeb Tariku
Jimma University
12 PUBLICATIONS **85** CITATIONS
SEE PROFILE

Ariaya Hymete
Addis Ababa University
45 PUBLICATIONS **270** CITATIONS
SEE PROFILE

Asrat Hailu
Addis Ababa University
159 PUBLICATIONS **2,588** CITATIONS
SEE PROFILE

Jens Rohloff
Norwegian University of Science and Techn…
85 PUBLICATIONS **958** CITATIONS
SEE PROFILE

In vitro Evaluation of Antileishmanial Activity and Toxicity of Essential Oils of *Artemisia absinthium* and *Echinops kebericho*

by **Yinebeb Tariku**[a]), **Ariaya Hymete**[b]), **Asrat Hailu**[c]), and **Jens Rohloff***[d])

[a]) Department of Chemistry, College of Natural Science, Jimma University, P.O. Box 378, Jimma, Ethiopia

[b]) Department of Pharmaceutical Chemistry, School of Pharmacy, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

[c]) Department of Immunology, Microbiology and Parasitology, Faculty of Medicine, Addis Ababa University, P.O. Box 9086, Addis Ababa, Ethiopia

[d]) The Plant Biocentre, Department of Biology, Norwegian University of Science and Technology (NTNU), N-7491 Trondheim (phone: +4773590174; fax: +4773590177; e-mail: jens.rohloff@bio.ntnu.no)

Potential toxicity, costs, and drug-resistant pathogens necessitate the development of new antileishmanial agents. Medicinal and aromatic plants constitute a major source of natural organic compounds. In this study, essential oils of *Artemisia absinthium* L. and *Echinops kebericho* MESFIN were investigated by GC and GC/MS analyses. Isolated oils were screened for antileishmanial activity against two *Leishmania* strains (*L. aethiopica* and *L. donovani*), and toxicity on the human monocytic leukemia (THP-1) cell line and red blood cells *in vitro*. GC/MS Analysis revealed 65 compounds (93.74%) for *Artemisia absinthium* and 43 compounds (92.85%) for *Echinops kebericho* oil. The oils contained the oxygenated monoterpene camphor (27.40%) and the sesquiterpene lactone dehydrocostus lactone (41.83%) as major constituents, respectively. Both oils showed activity against promastigote (*MIC* 0.0097–0.1565 μl/ml) and axenic amastigote forms (EC_{50} 0.24–42.00 nl/ml) of both leishmania species. Weak hemolytic effect was observed for both oils, showing a slightly decreased selectivity index (*SI* 0.8–19.2) against the THP-1 cell line. Among the two oils tested, *E. kebericho* exerted strong antileishmanial activity that was even higher than that of amphotericin B with significant cytotoxicity. This study, therefore, demonstrated the potential use of both oils as source of novel agents for the treatment of leishmaniasis.

Introduction. – Leishmaniasis is a vector-borne parasitic disease caused by several species of *Leishmania* that can infect humans and other mammals. The disease prevails in 88 tropical and subtropical countries on five continents where *ca.* 350 million people live [1]. Ethiopia, being located in a vector and intermediate host-breeding area, is affected by the widely distributed cutaneous leishmaniasis (caused dominantly by endemic *L. aethiopica*) and visceral leishmaniasis distributed in lowlands and semi-desert areas (caused by *L. donovani*) [2]. Several drugs such as pentavalent antimonials, paromomycin, and amphotericin B are available for treatment of the disease, but they are too expensive in most endemic areas. Moreover, increased drug resistance and associated treatment failure, high costs, and significant toxicity complicate appropriate treatment [3]. Therefore, the development of new antileishmanial compounds is imperative. In this regard, plants are indispensable sources of diverse types of bioactive organic compounds, also including the complex group of essential oil-related compounds.

Essential oils consist of lipophilic volatile and semi-volatile constituents isolated from various plant morphological parts either by pressing, extraction, or various distillation techniques. Chemically, they consist of complex mixtures comprising terpenes and/or aromatic compounds. Essential-oil constituents contribute to beneficial or adverse effects based on their characteristic biological activity. Their medicinal potential has attracted the attention of many scientists and encouraged them to study essential-oil composition, and carry out chemical and pharmacological investigations. Many studies also indicate potential leishmanicidal effect of essential oils from various plants including *Croton cajucara* [4], *Ocimum gratissimum* [5], *Chenopodium ambrosioide* [6], *Echinacea purpurea* [7], *Annona foetida* [8], *Artemisia herba-alba* [9], and *Piper auritum* [10]. Essential oils obtained from *Achillea millefolium*, *Syzygium aromaticum*, and *Ocimum basilicum* [11], and *Artemisia* spp. [12] exhibited toxic effects against trypanosomes, which are protozoal species closely related to *Leishmania*.

Artemisia absinthium L. (Asteraceae/Compositae) is an erect, medium-sized (30–60 cm) plant with greenish silvery leaves and white twigs with strong aroma. The plant is native to Europe, North America, and Asia, and commonly cultivated in temperate climatic zones [13]. In previous works on plant samples from different geographic regions, bioactive compounds including terpenoids (*e.g.*, the sesquiterpenelactone absinthin), flavonoids, coumarins, phenolics, caffeoylquinic acids, sterols, tannins, carotenoids, lignans, glucosides, acetylenes, and bitter principles [14][15] were reported. *A. absinthium* extracts and essential oils were assessed for various biological activities, including antibacterial [16], antitrypanosomal [12], antiplasmodial [17], analgesic and anti-inflammatory [18], antipyretic [19], antidepressant and antioxidant [20], and antifungal effects [14]. *A. absinthium* is known as '*Ariti*' in Ethiopia and used for its aroma in rituals called '*Atete*', and as a remedy for treatment of infectious and non-infectious diseases such malaria, helminths (vermifuge), and animal wounds [14][21]. In several countries and regions, *A. absinthium* is traditionally used in food industry for the preparation of aperitifs, bitters, and spirits [22].

Echinops kebericho Mesfin, locally called '*kebericho*', is an erect massive root stock-bearing perennial herb or shrub that grows up to a height of 1.2 m. The plant belongs to the genus *Echinops* that comprises 125–130 species in the family Asteraceae/Compositae distributed in semi-humid zones of tropical and North Africa, Mediterranean basin, and temperate regions up to Central Asia [13][23]. Twelve species occur in Ethiopia confined to the highlands of the country [24]. *Echinops* species were reported to contain a wide range of chemical constituents comprising alkaloids, saponins, phytosterols, polyphenols, carotenoids, sesquiterpene lactones/alcohols, lignans, acetylenic and thiophene compounds, and essential oil [25–28]. Reports and ethnobotanical surveys also evidence long traditional use of the plant for preparation of medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, billharzia, syphilis, and amoebic dysentery [29][30]. Extracts and essential oils of the roots of *E. kebericho* were also assessed for their antimicrobial, antihelminthic, and molluscicidal activities [27][31].

Based on the reported biological activities and medicinal properties, and the plants' significance in traditional medicine in Ethiopia, the present study was aimed at investigating the *in vitro* leishmanicidal potential and toxicity profile of essential oils

obtained from *Artemisia absinthium* L. and *Echinops kebericho* MESFIN using two isolated *Leishmania* parasites and control cell types.

Results and Discussion. – *Yield of Essential Oils.* The yield of essential oils was 1.39% for *A. absinthium* and 0.13% for *E. kebericho*. The color of the oils of *A. absinthium* and that of *E. kebericho* were blue and light yellow, respectively. The yield of *Artemisia* oil was higher than that previously reported for samples from Morocco with 0.57% [32], while the yield of *Echinops* from fresh tubers was much higher than that reported in [28] using dried Ethiopia samples (0.05%).

GC/MS Analysis. A total of 65 compounds representing 93.74% were identified in the essential oils of *A. absinthium*. In the oil of *E. kebericho,* 43 compounds representing 92.85% of the total essential oil constituents were identified. Essential oil composition of the studied plants are presented in *Table 1*. The oil of *A. absinthium* contained mainly oxygenated monoterpenes (41.20%), followed by oxygenated sesquiterpenes (27.06%), aromatic hydrocarbons (9.39%), monoterpene hydrocarbons (8.03%), and sesquiterpene hydrocarbons (4.99%). Major constituents of this oil were camphor (27.40%), davanone (16.43%), ethyl (*E*)-cinnamate (5.81%), (*E*)-nerolidol (4.63%), and chamazulene (4.00%). Similar investigations carried on volatile constituents of a closely related species from Ethiopia (*A. rehan*) revealed camphor (26.4%) and davanone (41.2%) as the two major constituents [33]. The main components and dominant classes of terpenoids were found to differ compared to chemotypes previously reported of the same species, such as α-thujone, β-thujone, sabinene, myrcene, (*E*)-sabinol, (*E*)-sabinyl acetate, linalyl acetate and geranyl propionate [32][34], caryophyllene oxide, *p*-cymene, and 1,8-cineole [22], and (*Z*)-chrysanthenol in [35]. Some reports indicate possibility of essential oil composition variations among the same plant species growing in different countries and geographic locations [22][34–36], plant habitat and season of collection [37], and the extraction technique employed [38].

Sesquiterpene lactones, monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygenated monoterpene structures represented 41.83, 23.97, 14.90, and 2.80%, respectively, of the total essential oil of *E. kebericho*. The main constituents of the oil were dehydrocostus lactone (41.83%), β-phellandrene (10.84%), germacrene B (5.38%), α-selinene (4.13%), α-pinene (3.63%), and β-pinene (3.62%). Qualitatively, these results do not agree with those in previous reports on essential oil of the same species using a similar extraction technique, where eudesm-7(11)-en-4-ol (14.3%), caryophyllene oxide (9.7%), and τ-cadinol (8.3%) were reported as main constituents [28]. The difference in % oil yield and chemical composition of *E. kebericho* could be due to the fact that hydrodistilled dried tubers were studied in the previous work, while the current study employed fresh samples.

Antileishmanial Activity and Toxicity Profile. Essential oils of *A. absinthium* and *E. kebericho* showed concentration-dependent growth inhibitory effects (*Table 2*) against promastigote forms of *L. donovani* (*MIC* 0.1565 and 0.0765 µl/ml, resp.) and *L. aethiopica* (*MIC* 0.1565 and 0.0097 µl/ml, resp.). These oils also had effects on axenic amastigote forms of *L. donovani* (EC_{50} 42.00 and 0.50 nl/ml, resp.) and *L. aethiopica* (EC_{50} 7.94 and 0.24 nl/ml, resp.). The 50% cytotoxic concentrations (CC_{50}) on THP-1 cells for *A. absinthium* and *E. kebericho* oil were 152.04 and 0.40 nl/ml, respectively. *A.*

Table 1. *Chemical Composition* [% peak area] *of the Essential Oils of* A. absinthium *and* E. kebericho *Obtained by Hydrodistillation and GC/MS Analysis*

Artemisia absinthium L.			*Echinops kebericho* Mesfin		
Compound	*KI*[a])	Area	Compound	*KI*	Area
Ethyl 2-methylbutanoate	845	1.01	α-Thujene[b])	932	0.14
Propyl 2-methylpropanoate	866	0.23	α-Pinene[b])	936	3.63
Tricyclene	927	0.09	Camphene[b])	950	0.28
α-Thujene[b])	932	0.14	Sabinene[b])	973	0.72
α-Pinene[b])	936	1.61	β-Pinene	978	3.62
Propyl 2-methylbutanoic acid	943	0.65	β-Myrcene[b])	987	0.30
Camphene[b])	950	2.56	α-Phellandrene	1002	0.96
Sabinene[b])	973	0.06	*p*-Cymene[b])	1015	0.14
6-Methylhept-5-en-2-one	978	0.29	β-Phellandrene	1023	10.84
β-Myrcene[b])	987	1.35	Limonene[b])	1025	2.25
α-Terpinene	1013	0.47	(*Z*)-β-Ocimene	1029	0.83
p-Cymene	1015	0.18	(*E*)-β-Ocimene	1041	0.11
1,8-Cineole	1024	0.11	Terpinolene[b])	1082	0.15
Limonene[b])	1025	0.59	Camphor[b])	1123	0.16
(*Z*)-2,6-Dimethylocta-1,5,7-trien-3-ol	1048	0.03	4-Terpineol[b])	1164	0.24
γ-Terpinene	1051	0.76	Bornyl acetate[b])	1270	2.24
(*E*)-Sabinene hydrate	1053	1.75	(*E*)-Sabinyl acetate	1278	0.16
Terpinolene[b])	1082	0.22	α-Isocomene	1388	0.20
(*Z*)-Sabinene hydrate	1083	0.46	β-Elemene	1389	1.87
Linalool	1086	2.82	Longifolene	1411	0.14
(*E*)-Pinan-2-ol	1114	0.07	β-Caryophyllene[b])	1421	0.38
(*Z*)-*p*-Menth-2-en-1-ol	1116	0.13	7,8-Dihydro-β-ionone	1422	0.15
Camphor[b])	1123	27.40	α-Patchoulene	1457	0.21
(*E*)-*p*-Menth-2-en-1-ol	1124	0.06	β-Ionone	1468	1.00
Camphene hydrate	1143	0.16	Selina-4,7-diene	1469	0.32
Borneol	1150	0.55	γ-Muurolene[b])	1474	0.25
4-Terpineol[b])	1164	2.61	β-Selinene[b])	1486	0.93
Isogeranial	1166	0.03	δ-Selinene[b])	1492	0.75
α-Terpineol	1176	0.52	Viridiflorene	1493	0.34
Methyl hydrocinnamate	1248	0.07	α-Selinene	1498	4.13
Bornyl acetate[b])	1270	3.14	Germacrene B	1561	5.38
(*Z*)-Methyl cinnamate	1272	0.10	Caryophyllene oxide[b])	1578	0.40
Dihydrocarveol acetate	1295	0.55	α-Guaiol	1593	0.11
Ethyl dihydrocinnamate	1322	0.83	τ-Cadinol	1633	0.27
Eugenol	1331	0.09	γ-Eudesmol[b])	1641	0.54
Ethyl (*Z*)-cinnamate	1348	1.80	8-Cedren-13-ol	1657	2.84
Methyl (*E*)-cinnamate	1354	0.45	(11*E*,13*Z*)-Octadeca-1,11,13-triene	1665	1.11
Benzyl 3-methylbutanoate	1366	0.05	α-Bisabolol	1673	0.24
(*Z*)-Jasmone	1371	0.15	Pentadecanal	1702	0.28
Methyl eugenol	1407	0.19	Benzyl benzoate	1730	0.59
α-Santalene	1418	0.08	(*Z*)-Lanceol	1740	0.48
β-Caryophyllene[b])	1421	0.30	β-Acoradienol	1764	1.34
endo-Arbozol	1435	0.81	Dehydrocostus lactone	1867	41.83
Ethyl (*E*)-cinnamate	1451	5.81			
α-Humulene	1455	0.03			
γ-Muurolene[b])	1474	0.22			
β-Selinene[b])	1486	0.25			

Table 1 (cont.)

Artemisia absinthium L.			*Echinops kebericho* MESFIN		
Compound	*KI*[a])	Area	Compound	*KI*	Area
δ-Selinene[b])	1492	0.05			
Davana ether	1495	0.13			
(*E*,*E*)-α-Farnesene	1498	0.06			
(*E*)-Isodavanone	1535	0.95			
(*Z*)-Isodavanone	1543	0.99			
allo-Davanone	1549	0.59			
(*E*)-Nerolidol	1553	4.63			
Caryophyllene oxide[b])	1578	0.07			
Davanone	1586	16.43			
Isoaromadendrene epoxide	1590	0.10			
Methyl jasmonate	1611	0.54			
Methyl *epi*-jasmonate	1637	0.10			
β-Eudesmol	1641	0.81			
3-*Oxo*-β-ionone	1648	0.06			
Geranyl tiglate	1675	0.22			
Longifolol	1707	2.14			
Chamazulene	1719	4.00			
(*E*,*E*)-3,7,11,15-Tetramethylhexadeca-1,6,10,14-tetraen-3-ol	2046	0.04			
TOTAL DETECTED		93.74	TOTAL DETECTED		92.85
Monoterpenes total		49.23	Monoterpenes total		26.77
Monoterpene hydrocarbons		8.03	Monoterpene hydrocarbons		23.97
Monoterpenes oxygenated		41.20	Monoterpenes oxygenated		2.80
Sesquiterpenes total		32.05	Sesquiterpenes total		62.95
Sesquiterpene hydrocarbons		4.99	Sesquiterpene hydrocarbons		14.90
Sesquiterpenes oxygenated		27.06	Sesquiterpenes oxygenated		48.05
Diterpenes		0.04	Sesquiterpene lactones		41.83
Aliphatic hydrocarbons total		2.68	Aliphatic hydrocarbons total		1.39
Aromatic hydrocarbons total		9.39	Aromatic hydrocarbons total		0.59
Carotenoid-derived hydrocarbons		0.35	Carotenoid-derived hydrocarbons		1.15
Oxygenated structures total		80.72	Oxygenated structures total		57.58

[a]) *KI* = *Kovats* index. [b]) Compounds which are common to both species.

absinthium and *E. kebericho* oils revealed selectivity-index (*SI*) values of 3.6 and 0.8 for *L. donovani* and 19.2 and 1.7 for *L. aethiopica*, respectively. The 50% lytic-concentration levels (LC_{50}) of the oils determined in blood hemolysis test were 1.52 and 2.62 µl/ml, respectively. In general, *E. kebericho* oil exerted an extremely strong leishmanicidal effect, which was even higher than that of amphotericin B showing significant cytotoxicity.

Several studies have also revealed the potential leishmanicidal effects of various plant essential oils [4–10]. Moreover, various other volatile oil constituents such as monoterpenes [4][39], sesquiterpenes [40], sesquiterpene lactones [41][42], diterpenoids [43][44], and triterpenoids [45] also possessed antileishmanial effects. Crude essential oils, and isolated monoterpenes and sesquiterpenes including α-cadinol, β-

Table 2. *The* in vitro *Antileishmanial Activity of Essential Oils of* A. absinthium *and* E. kebericho *on Promastigote and Axenic Amastigote Forms of* L. donovani (*L. d.*) *and* L. aethiopica (*L. a.*), *and Toxicity Profile on the Human Monocytic Leukemia Cell Line* (THP-1) *and Sheep Erythrocytes*

Test Substances	*MIC* [μg/ml] Promastigote (mean ± SD)		EC_{50} [μg/ml] Axenic Amastigote (mean ± SD)		Cytotoxicity CC_{50} [μg/ml] (mean ± SD)	Selectivity index $CC_{50,THP\text{-}1}$/ $EC_{50,Leishmania}$		Blood hemolysis LC_{50} [μg/ml] (mean ± SD)
	L. d.	*L. a.*	*L. d.*	*L. a.*		*L. d.*	*L. a.*	
Essential Oils								
A. absinthium	0.1565[a])	0.1565[a])	42.00 ± 9.80[b])	7.94 ± 5.58[b])	152.04 ± 8.40[b])	3.6	19.2	1.52 ± 0.69[a])
E. kebericho	0.0765[a])	0.0097[a])	0.50 ± 0.7[b])	0.24 ± 0.01[b])	0.40 ± 0.01[b])	0.8	1.7	2.62 ± 1.00[a])
Reference Drugs								
Amphotericin B	0.0244	0.0244	0.018 ± 0.006	0.047 ± 0.008	13.27 ± 1.80	282	737	47.71 ± 2.06
Negative Controls								
Complete RPMI medium (with 1% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a]) μl/ml. [b]) nl/ml.

elemene, α-humulene [46][47], and isoprenoids including geraniol and farnesol [48] were reported to be active against tumor cell lines. Varying levels of one or several of these compounds were identified in our essential oils tested (*Table 1*). Although major components of essential oils generally represent specific biological features, it has also been shown that various minor components may contribute to biological activities, possibly by synergistic effects between the components [49].

The observed strong antileishmanial activity and toxicity profile of the oils arose possibly due to the fact that most essential oils and a great number of their constituents seem to have no specific cellular targets. As typical for lipophilic structures, they might easily pass through cell walls and cytoplasmic membranes, disrupt the structure of their different layers of polysaccharides, fatty acids, and phospholipids, and make them permeable [49], leading to cell lysis and release of macromolecules [50][51]. Once the oil components have passed the membrane, they may cause coagulation of the cytoplasm, hence damaging lipids and proteins [52][53], interrupt specific metabolic pathways such as biosynthesis of various lipids [45], enhance production of nitric oxide in infected cells [4], or stimulate depolarization in mitochondrial membranes [49][54], which can lead to cell death by necrosis and apoptosis [55].

Conclusions. – Based on the results of this study, it can be concluded that the essential oils from both plants possess strong antileishmanial activity. Many previous studies on these plants also support the observed properties. Therefore, isolation of at least the major constituents of the oils can help to identify chemicals that are responsible for the individual observed activities and cytotoxicities. Additional *in vivo* studies would be needed to justify and further evaluate potential use of these oils as antileishmanial agents.

The authors are thankful to Mrs. *Marga Goris* at the *Royal Tropical Institute*, Amsterdam, for providing THP-1 cell lines and the *Department of Microbiology, Immunology and Parasitology* (DMIP), Addis Ababa University, for access to and use of their laboratory facilities.

Experimental Part

Plant Material. The leaves of *Artemisia absinthium* L. and tubers of *Echinops kebericho* MESFIN were collected from their natural habitats at 'Inchini', located 85 km west of Addis Ababa in December, 2006. The plants were authenticated by Mr. *Melaku Wondafrash*, a plant taxonomist, and specimens were deposited with the National Herbarium, Addis Ababa University under voucher Nos. YT 005/06 and YT 007/06, resp.

Isolation of Essential Oils. Artemisia absinthium and *Echinops kebericho* essential oils were isolated by hydrodistillation of fresh leaves (430 g) and fresh tubers (400 g), resp., using a glass-type *Clevenger* apparatus continuously for 3 h. Oils were carefully separated and stored in an opaque glass vial in a refrigerator ($-20°$) prior to analysis and biological assays.

Analysis of Essential Oils. Volatile oil components were analyzed using a *Varian Star 3400CX* gas chromatograph equipped with automatic injector in splitless mode and a fused silica *Chrompack WCOT CP-Sil-5* cap. column (30 m $\times$ 0.32 mm i.d. and 0.25-μm film thickness) coupled with a *Varian Saturn 3* mass spectrometer. The oven temp. was initially held at 40°, then programmed to 220° at a rate of 3°/min, and finally held at 220° for 3 min. The injector temp. was 220° for all analyses. The carrier gas was He (5 psi) at 50 ml/min through the injector and at 30 cm/s through the column. The MS detector was set at 175°, and an electron ionization (EI) system with ionization energy of 70 eV was used. A mass range of m/z 40–400 was recorded acquiring all mass spectra in EI mode. Components of volatile oils were

identified by comparison of their rel. retention times (t_R) and MS with the standard MS data (IMS Terpene Library, 1989; and NIST MS Database, 2005), retention indices (*RI*s) database search (ESO Database of Essential Oils, 1999), and comparison of MS with reported MS data in literature [56]. Quant. analysis (in %) was performed by peak-area normalization measurements.

Preparation of Stock Solns. Stock solns. of essential oils (100 µl/ml in DMSO) and the reference drug amphotericin B (*Fungizon®*, *Bristol-Myers Squibb*, F-Rueil-Malmaison; 5 mg/ml in PBS) were prepared. Each of these test solns. were frozen at $-20°$ until used.

Test Strains and Cells. The *L. aethiopica* (CL/064/06) and *L. donovani* (VL/15327/06) isolates were obtained from the Leishmania Diagnostic and Research Laboratory collection, Faculty of Medicine, Addis Ababa University. Human monocytic leukemia (THP-1) cell line (ATCC-TIB-202) was donated by Mrs. *Marga Goris* at the Royal Tropical Institute, Amsterdam, and sheep red blood cells were used for the assessment of toxicity potential of the tested essential oils.

Antipromastigote Assay. The *L. aethiopica* and *L. donovani* isolates were first cultured in tissue-culture flasks containing *RPMI 1640* medium (*Gibco*, *Invitrogen Co.*, UK), supplemented with 10% heat-inactivated fetal calf serum (HIFCS; *Gibco*, *Invitrogen Co.*, UK), and 100 IU penicillin/ml–100 µg/ml streptomycin soln. (*Sigma Chemicals Co.*, St. Louis, USA) at 22° for *L. aethiopica* and at 26° for *L. donovani* according to the methods described in [57][58], resp. The assay was carried out in a 96-well microtitre plate where both oils were serially diluted to twice final test concentrations (0.00244–5.0 µl/ml) in 100 µl of culture medium with each test concentration in duplicate. Then, 100 µl of suspensions containing 3.5×10^6 promastigotes/ml (logarithmic phase) were added to each well. Contents of the plates were further maintained at 22° (for *L. aethiopica*) and 26° (for *L. donovani*) in a 5% CO_2 incubator. The cell density, motility, and morphology for each treatment were determined daily with an inverted microscope, and the antileishmanial activities were expressed as the *MIC* values after a 72 h incubation. The reference drug and medium with 1% DMSO were included as controls.

Antiamastigote Assay. The axenic culture was raised according to the method described in [57] for *L. aethiopica* and in [59] for *L. donovani* with minor modifications. Briefly, promastigotes in late stationary phase were centrifuged and resuspended in medium *199* containing *Hank*'s balanced salts, supplemented with 20% HIFCS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin, and the pH was adjusted to 5.5 (for both strains) with 0.1N HCl. The cells were then incubated at 31° (*L. aethiopica*) or 37° (*L. donovani*) in a humidified 5% CO_2 incubator for 7 d. The antiamastigote assay for the oils tested was performed as described in [60]. In a 96-well microtitre plate, test substances were serially diluted to final test concentrations of 0.000228–0.50 µl/ml in 50 µl of culture medium, and 50-µl suspensions, containing 2×10^7 cells/ml axenic amastigotes, were added to each well. Contents of the plates were then incubated in humidified atmosphere at 31° (*L. aethiopica*) or 37° (*L. donovani*) under 5% CO_2 for 72 h. After incubation for 68 h, 10 µl of fluorochrome resazurin (*Sigma Chemicals Co.*, St. Louis, USA) soln. (12.5 µg dissolved in 100 ml of PBS; pH 7.2) was added into each well, and the fluorescence intensity was measured after a total incubation time of 72 h using a *Victor³ Multilabel Counter* (*Perkin-Elmer*) at excitation wavelength of 530 nm and emission wavelength of 590 nm. Assays with standard antileishmanial drug and medium with 1% DMSO were additionally performed as reference value controls. The background fluorescence intensity of each oil and reference drug was also measured.

Cytotoxicity Assays. The cytotoxic effect of essential oils against human leukemia monocyte THP-1 cells was determined using the resazurin-based fluorescence assay. The human leukemia monocyte THP-1 cells were cultured in *RPMI-1640* supplemented with 10% HIFCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B in a humidified 5% CO_2 incubator at 37° as described in [57]. Serial dilutions and the assay were performed as indicated for the antiamastigote assay, except that 50-µl suspensions containing 1×10^6 THP-1 cells were added to each well and incubated in 5% CO_2/air mixture at 37° for 72 h.

Hemolysis Test. Assay for hemolytic properties of the oils was carried out as described in [41]. Briefly, to 200 µl of 4% red blood cell suspension (in sterile 5% glucose soln.), serial dilutions of the oils (0.00114–2.5 µl/ml) and reference drugs (50–1000 µg/ml) were added, and the mixture was incubated at 37°. After 2 h of exposure, suspensions were centrifuged at 1,000*g* for 10 min, and 100 µl of the supernatants was transferred to a 96-well plate, and absorbance was measured at 540 nm using a *Victor³*

Multilabel Counter (*Perkin-Elmer*). Hemolytic effects were expressed as percentage of the absorbance of the pos. control (*Triton X-114*, 5 µl/ml).

Statistical Analysis. The median effective concentration (EC_{50}) for anti-amastigote assay, median cytotoxic concentration (CC_{50}) for cytotoxicity assay on the THP-1 cells, and 50% lytic concentrations (LC_{50}) for hemolysis assay were all evaluated from sigmoidal dose–response curves using nonlinear regression (sigmoidal dose–response, *GraphPad Prism*®; *GraphPad Software, Inc.*, San Diego, CA), each expressed as means ± SD from triplicate experiments with each test concentration in duplicate.

REFERENCES

[1] 'Report of the first meeting of WHO Strategic and Technical Advisory Group on Neglected Tropical Diseases', World Health Organization, WHO/CDS/NTD/2007.2, Geneva, 2007, 26 p.
[2] A. Hailu, T. Gebere-Michael, N. Berhe, B. Meshesha, 'Leishmanisasis', in 'The Epidemiology and Ecology of Health and Diseases in Ethiopia', 1st edn., Eds. N. Berhane, D. Hailemariam, H. Kloos, Sharma Books, Addis Ababa, Ethiopia, 2006, pp. 615–634.
[3] S. L. Croft, S. Sundar, A. H. Fairlamb, *Clin. Microbiol. Rev.* **2006**, *19*, 111.
[4] M. D. S. S. Rosa, R. R. Mendonça-Filho, H. R. Bizzo, I. A. Rodrigues, R. M. A. Soares, T. Souto-Padron, C. S. Alviano, A. H. C. S. Lopes, *Antimicrob. Agents Chemother.* **2003**, *47*, 1895.
[5] T. Ueda-Nakamura, R. R. Mendonça-Filho, J. A. Morgado-Díaz, M. P. Korehisa, P. Filho, A. G. D. Cortez, D. S. Alviano, M. D. S. S. Rosa, C. S. Alviano, C. V. Nakamura, *Parasitol. Int.* **2006**, *55*, 99.
[6] L. Monzote, M. García, A. M. Montalvo, R. Scull, M. Miranda, J. Abreu, *Phytother. Res.* **2007**, *21*, 1055.
[7] S. Soudi, S. H. Mahmoud, A. H. Zavaran, A. Ghaemi, M. J. Asghari, *Iran. J. Pharm. Res.* **2007**, *6*, 147.
[8] E. V. Costa, M. L. B. Pinheiro, J. R. A. Silva, B. H. L. N. S. Maia, M. C. T. Duarte, A. C. F. Amaral, G. M. C. Machado, L. L. Leon, *Quim. Nova* **2009**, *2*, 78.
[9] A. H. H. Mohamed, M. A. El-Sayed, M. E. Hegazy, S. E. Helaly, A. M. Esmail, N. S. Mohamed, *Rec. Nat. Prod*. **2010**, *4*, 1.
[10] L. Monzote, M. García, A. M. Montalvo, R. Scull, M. Miranda, *Mem. Inst. Oswaldo Cruz* **2010**, *105*, 168.
[11] G. F. Santoro, M. G. Cardoso, L. G. L. Guimaraes, L. Z. Mendonça, M. J. Soares, *Exper. Parasitol.* **2007**, *116*, 283.
[12] E. Nibret, M. Wink, *Phytomedicine* **2010**, *17*, 369.
[13] 'Flora of Ethiopia and Eritrea. Vol. 4, Part 2', Eds. I. Hedberg, I. Friis, S. Edwards, Addis Ababa University, Addis Ababa, Ethiopia, 2004, pp. 221–223.
[14] K. S. Bora, A. Sharma, *J. Pharm. Res.* **2010**, *3*, 325.
[15] J. A. S. Teixeira, *Afr. J. Biotechnol.* **2004**, *3*, 706.
[16] C. L. Chopra, M. C. Bhatia, I. C. Chopra, *J. Am. Pharm. Assoc.* **1960**, *49*, 780.
[17] M. M. Zafar, M. E. Hamdard, A. Hameed, *J. Ethnopharmacol*. **1990**, *30*, 223.
[18] F. Ahmad, R. A. Khan, S. Rasheed, *J. Islamic Acad. Sci.* **1992**, *5*, 111.
[19] S. G. Khattak, S. N. Gilani, M. Ikram, *J. Ethnopharmacol.* **1985**, *14*, 45.
[20] M. Mahmoudi, M. A. Ebrahimzadeh, F. Ansaroudi, S. F. Nabavi, S. M. Nabavi, *Afr. J. Biotechnol.* **2009**, *8*, 7170.
[21] H. Yineger, E. Kelbessa, T. Bekele, E. Lulekal, *J. Ethnopharmacol*. **2007**, *112*, 55.
[22] A. Basta, O. Tzakou, M. Couladis, M. Pavlovic, *J. Essent. Oil Res.* **2007**, *19*, 316.
[23] G. Teresa, A. Susanna, N. Garcia-Jacas, R. Vilatersana, J. Vallès, *Folia Geobot.* **2005**, *40*, 407.
[24] M. Tadesse, B. Abegaz, 'A Review of the Genus *Echinops* (Composite, Craude) in Ethiopia with Notes on Phytogeography and Chemistry', Proceedings of the 12th Plenary Meeting of AETFAT, 1990, pp. 605–629.
[25] B. M. Abegaz, M. Tadesse, R. Majinda, *Biochem. Syst. Ecol.* **1991**, *19*, 323.
[26] M. Tene, P. Tane, B. L. Sondengam, J. D. Connolly, *Phytochemistry* **2004**, *65*, 2101.
[27] A. Hymete, T.-H. Iversen, J. Rohloff, B. Erko, *Phytomedicine* **2005**, *12*, 675.
[28] A. Hymete, J. Rohloff, T.-H. Iversen, H. Kjøsen, *Flavour Fragrance J.* **2006**, *22*, 35.

[29] D. Abebe, A. Ayahu, 'Medicinal Plants and Enigmatic Health Practices of Northern Ethiopia', Birhanna Selam Printing Press, Addis Ababa, Ethiopia, 1993, pp. 37–44, 83–89.
[30] T. Teklehaymanot, M. Giday, *J. Ethnobiol. Ethnomed.* **2007**, *3*, 12.
[31] M. Ashebir, M. Ashenafi, *Ethiopian J. Health Sci.* **1999**, *9*, 33.
[32] E. Derwich, Z. Benziane, A. Boukir, *Electr. J. Environ. Agric. Food Chem.* **2009**, *8*, 1202.
[33] B. M. Abegaz, P. G. Yohannes, *Phytochemistry* **1982**, *21*, 1791.
[34] A. Judžentienė, F. Tomi, J. Casanova, *Nat. Prod. Commun.* **2009**, *4*, 1113.
[35] A. P. Carnat, M. Madesclaire, O. Chavignon, *J. Essent. Oil Res.* **1992**, *4*, 487.
[36] A. Orav, A. Raal, E. Arak, M. Müürisepp, T. Kailas, *Proc. Estonian Acad. Sci. Chem.* **2006**, *55*, 155.
[37] A. Judžentienė, D. Mockutė, *Chemija* **2004**, *15*, 64.
[38] A. Arino, I. Arberas, G. Renobales, J. B. Domínguez, *Eur. Food Res. Technol.* **1999**, *209*, 126.
[39] S. Robledo, E. Osorio, D. Muñoz, L. M. Jaramillo, A. Restrepo, G. Arango, I. Vélez, *Antimicrob. Agents Chemother.* **2005**, *49*, 1652.
[40] J. Mikus, M. Harkenthal, D. Steverding, J. Reichling, *Planta Med*. **2000**, *66*, 366.
[41] S. T. Tiuman, T. Ueda-Nakamura, B. P. Dias-Filho, D. A. G. Cortez, C. V. Nakamura, *Acta Protozool.* **2005**, *44*, 245.
[42] V. P. Sülsen, F. M. Frank, S. I. Cazorla, C. A. Anesini, E. L. Malchiodi, B. Freixa, R. Vila, L. V. Muschietti, V. S. Martino, *Antimicrob. Agents Chemother.* **2008**, *52*, 2415.
[43] M. J. Chan-Bacab, L. M. Peña-Rodríguez, *Nat. Prod. Rep.* **2001**, *18*, 674.
[44] O. Kayser, A. F. Kiderlen, S. L. Croft, *Stud. Nat. Prod. Chem.* **2002**, *26*, 779.
[45] D. C. Arruda, F. L. D'Alexandri, A. M. Katzin, S. R. B. Uliana, *Antimicrob. Agents Chemother.* **2005**, *49*, 1679.
[46] M. Sylvestre, J. Legault, D. Dufour, A. Pichette, *Phytomedicine* **2005**, *12*, 299.
[47] J. Hou, T. Sun, J. Hu, S. Chen, X. Cai, G. Zou, *Food Chem.* **2007**, *103*, 355.
[48] J. Legault, W. Dahl, E. Debition, A. Pichette, J. C. Maldemont, *Planta Med*. **2003**, *69*, 402.
[49] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, *Food Chem. Toxicol*. **2008**, *46*, 446.
[50] S. D. Cox, C. M. Mann, J. L. Markham, H. C. Bell, J. E. Gustafson, J. R. Warmington, S. G. Wyllie, *J. Appl. Microbiol*. **2000**, *88*, 170.
[51] M. Oussalah, S. Caillet, M. Lacroix, *J. Food Prot.* **2006**, *69*, 1046.
[52] J. E. Gustafson, Y. C. Liew, S. Chew, J. L. Markham, H. C. Bell, S. G. Wyllie, J. R. Warmington, *Lett. Appl. Microbiol*. **1998**, *26*, 194.
[53] A. Ultee, E. P. W. Kets, M. Alberda, F. A. Hoekstra, E. J. Smid, *Arch. Microbiol.* **2000**, *174*, 233.
[54] J. S. Armstrong, *Bioessays* **2006**, *28*, 253.
[55] H. S. Yoon, S. C. Moon, N. D. Kim, B. S. Park, M. H. Jeong, Y. H. Yoo, *Biochem. Biophys. Res. Commun*. **2000**, *276*, 151.
[56] R. P. Adams, 'Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy', Allured Publishing, Illinois, 2001.
[57] S. Habtemariam, *BMC Pharmacol*. **2003**, *3*, 1.
[58] M. Ephros, A. Bitnun, P. Shaked, E. Waldman, D. Zilberstein, *Antimicrob. Agents Chemother.* **1999**, *43*, 278.
[59] Y. Saar, A. Ransford, E. Waldman, S. Mazareb, S. Amin-Spector, J. Plumblee, J. Turco, D. Zilberstein, *Mol. Biochem. Parasitol.* **1998**, *95*, 9.
[60] S. Hoet, F. Opperdoes, R. Brun, V. Adjakidje, J. Quetin-Leclercq, *J. Ethnopharmacol.* **2004**, *91*, 37.

Received November 20, 2010