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Bioassay-Guided Isolation of Dehydrocostus Lactone from *Echinops kebericho* as a Leishmanicidal Drug

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Bioassay-Guided Isolation of Dehydrocostus Lactone from *Echinops kebericho* as *a* Leishmanicidal Drug

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Abstract: Several bio-actors are involved in the occurrence of leishmaniasis, which is made too complex for prevention and control options. Currently, all forms of leishmaniasis are being treated with chemical drugs, which have limitations and adverse effects on patients. Searching the bioactive molecules from plant sources allows the development of a novel drug. The essential oil and solvent extract of the roots of *E. kebericho* showed antileishmanial activity. But looking for biological activities of the isolated active ingredient from tubers of *E. kebericho* was scant. Thus, the isolation of the leishmanicidal compound from the roots of *E. kebericho* through a bioassay-guided technique was conducted in this study. Antileishmanial activity test of the present finding showed that the essential oil and hexane fraction from roots of *E. kebericho* had significantly active against *L. major* and *L. tropica*. Dehydrocostus lactone, one of the major constituents of the essential oil and hexane fraction, showed twice potency of the standard drug miltefosine on leishmanicidal activities against *L. major* and *L. tropica*. Besides, the broad-spectrum biological activities of dehydrocostus lactone could be a potential drug candidate for co-infected patients caused by an association of *leishmania* and other pathogens.

Keywords: Bioassay; Dehydrocostus lactone; *Echinops kebericho*; Leishmaniasis

1. Introduction

Leishmaniasis is a major health problem in the tropical region, which is caused by more than 20 species of the genus of protozoa parasite known as *leishmania*. Over 90 species of sandflies are the transmitting vectors and more than 70 animal species including humans are used as reservoir hosts [1]. The involvement of several bio-actors for leishmaniasis, made it too complex for prevention and control of the health problem associated with it. Worldwide, 50,000 to 90,000 and 600,000 to 1 million cases have been reported annually for visceral and cutaneous forms of leishmaniasis, respectively [1]. Cutaneous leishmaniasis is the most common form in Ethiopia, with an estimated 20,000 to 50,000 cases annually and *Leishmania aethiopica*, *Leishmania tropica*, and *Leishmania major* are the causative agents [2].

Affordable, safe, and short-course treatment is one of the strategies among leishmaniasis prevention tools by the World Health Organization [3]. So far, some leishmanicidal drugs such as pentavalent antimonial, paromomycin, amphotericin B, liposomal amphotericin, pentamidine, and miltefosine have been availed, but they have limitations like drug resistance, high cost, least availability, toxicity, and painful route of administration [4][5]. Therefore, it is vital to search for leishmanicidal molecules that have efficacy and tolerable safety. Medicinal plants are a good source of secondary metabolites that have diverse biological activities [6]. *Echinops kebericho* Mesfin is an endemic and endangered Ethiopian medicinal plant belonging to the genus *Echinops* and family Asteraceae. Its root is a potential medicinal part and has been used for treating various ailments such as dispelling nightmares in children, constipation, headache, heart pain, stomachache, typhus, as fumigant after childbirth, intestinal pains, lung TB, leprosy, syphilis, cough, ward off evil eye,

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toothache, and vomiting [7][8][9][10][11][12][13]. Besides, it is used as Snake and Mosquito repellant [14][15].

The essential oil extracted from roots of *E. kebericho* has major constituent of eudesm-7 (11) -en-4-ol, caryophyllene oxide, τ-cadinol, β-cubebene, β-patchoulene, longifolene, cyperene, dehydrocostus lactone, β-phellandrene, germacrene B, α-selinene, isoshyobunone, modephene, α-pinene, and β-pinene [16][17]. Moreover, β-sitosterol, stigmasterol, campesterol, β-amyrene, lupeol, and ursolic acid, together with a series of fatty acids were identified from the solvent extract of *E. kebericho* roots [18][19].

The essential oil, petroleum ether, and chloroform extracts of roots of *E. kebericho* have anti-leishmanial activity against *Leishmania* species [20]. Assay for biological activities of the isolated active ingredients from roots of *E. kebericho* were obscure. Indeed, this work had the aim to identify the leishmanicidal crude fraction next to the isolated compound against the promastigote stage of *L. major* and *L. tropica* strains. The in vitro method of screening leishmanicidal compound and crude fraction was determined by 96 well plates assay with microscope detection method [21].

2. Results and Discussion

Hydro distillation of dried roots of *E. kebericho* obtained a 0.16% yield of essential oil. The in vitro antileishmanial activity test of the essential oil showed leishmanicidal activity with an IC50 value of 38.3μg/mL for *L. major* and 55.16μg/mL for *L. tropica*. A supportive result has been reported as the essential oils of *E. kebericho* showed concentration-dependent growth inhibitory effects against promastigote forms of *L. donovani* (MIC = 0.0765 μl/ml) and *L. aethiopica* (MIC = 0.0097 μl/ml) [17].

Furthermore, the essential oil profile was characterized, and a total of 43 compounds were identified by GC-MS analysis (Table 2). Dehydrocostus lactone (24.95%), β-guaiene (11.13%), cis lanceol (5.92%), 1,4-methanoazulen-9-one, decahydro-1,5,5,8a-tetramethyl-, [1R-(1α,3aβ,4α,8aβ)] (4.98%), (±)-cadinene (3.94%) δ-neoclovene (3.68%), aromadendrene oxide (3.29%), and caryophyllene (3.05%) were the major constituents of the essential oil. A comparable result of the major constituents of *E. kebericho* essential oil was reported by Tariku et. al., (2011) [16] which 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%).

Dichloromethane-methanol maceration of the dried roots of *E. kebericho* was achieved with a crude extract yield of 10.38% (92.63g). Different yields of fractions were recovered from liquid-liquid partition, such as hexane (55.61g), dichloromethane (8.53g), ethyl acetate (4 g), n-butanol (11.70g) and water fraction (12.79g). From the in vitro test of fractions, the hexane fraction had an antileishmanial activity with an IC_{50} value of 33.3 and 36.6 μg/mL for *L. major* and *L. tropica*, respectively. All other crude fractions were not active against the tested *leishmania* strain. The dichloromethane fraction was not tested due to partial solubility by DMSO.

The major compound from the hexane fraction was isolated by column chromatography, which collected 30 fractions (F1-F30) (Figure 1 b). Amongst the fractions, F8 was the major pure compound and coded as Ek-cpd1 with having Rf value of 0.57 (Figure 1 c) through the solvent system of hexane: ethyl acetate (86:14) ratio, respectively.

The compound Ek-cpd1 was a colorless crystalline solid and its structure was identified using EI-Mass that gave a molecular mass ion peak ($[M]^+$) at m/z 230.2 having a molecular formula $C_{15}H_{18}O_2$ (Figure 2). The ^1H NMR (500 MHz, $CDCl_3$) showed a spectra of δ^1H: 6.19 (1H, d, *J* = 3.5 Hz, H-13a), 5.46 (1H, d, J = 3.2 Hz, H-13b), 5.24 (1H, brs, J = 2.4, 1.5 Hz, H-15a), 5.04 (1H, brs, H-15b), 4.87 (1H, brs, H-14a), 4.79 (1H, brs, H-14b), 3.94 (1H, t, J = 9.3 Hz, H-6), 2.93 (1H , m, H-5), 2.89 – 2.79 (2H, m, H-4, 7), 2.59 – 2.41 (3H, m, H-3a, 9a, 3b), 2.22 (1H, m, H-9b), 2.13 (1H, m, H-2a), 1.97 – 1.79 (2H, m, H-2b,8a), 1.39 (1H, m, H-8b) (Figure 3). From the spectral analysis and comparisons with the literature [22][23], the structure of Ek-cpd1 was characterized as dehydrocostus lactone, which is a sesquiterpene lactone with guaianolides class (Figure 4).

In vitro, a leishmanicidal test result of Ek-cpd1 (Dehydrocostus lactone) showed an activity with an IC_{50} value of 15.3 μM/mL for *L. major* and 14.2 μM/mL for *L. tropica*. The standard drugs had an

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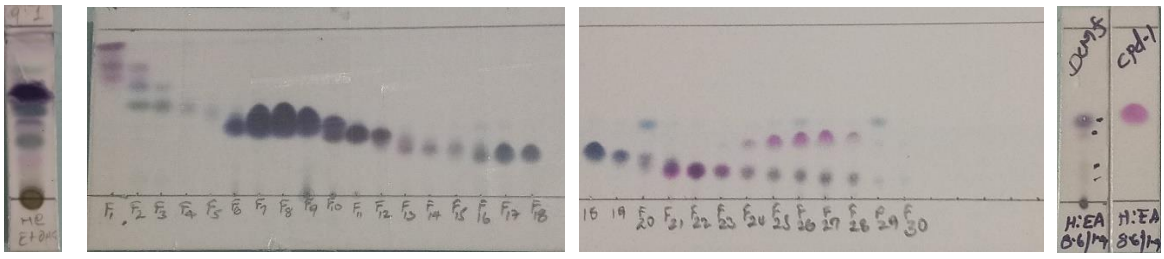
IC_{50} value of 3.39 and 3.41 µM/mL (Amphotericin B), 4.56 µM/mL (Pentamidine), 31.8 and 27.2 µM/mL (Miltefosine) for *L. major* and *L. tropica*, respectively.

Antileishmanial activity test of the present finding showed that the essential oil and hexane fraction from roots of *E. kebericho* had significantly active effects against *L. major* and *L. tropica*. Dehydrocostus lactone is the most abundant constituent of the essential oil and hexane fraction from roots of *E. kebericho*, which showed a potent antileishmanial activity. Specifically, the leishmanicidal test revealed that dehydrocostus lactone had doubled potency of the standard drug miltefosine against the tested *leishmania* species.

No toxicity test has been done in this study. However, previous studies reported that the essential oil of *E. kebericho* showed no mortality in acute oral dose toxicity up to 2000mg/kg body weight and was well tolerated in 200mg/ kg sub-acute toxicity and repeated dose exposure [17]. Additionally, the decoction of *E. kebericho* tuber had LD_{50} greater than 5000 mg/kg in acute toxicity and was well tolerated up to the dose of 600mg/kg in sub-acute toxicity [24]. Moreover, an in vivo cytotoxicity test of dehydrocostus lactone against laryngeal carcinoma showed that inhibition of the growth of the Hep-2 nude mouse xenograft model and had no significant signs of toxicity in the organs of nude mice [25].

Several researchers reported that dehydrocostus lactone has a range of biological activities such as anticancer activity against different cancer cells [26][27][28][29], anti-inflammatory activity [30][31], anti-allergy activity [32], and anti-depressant property [33]. Overall, dehydrocostus lactone could be a potential drug for co-infected patients caused by an association of *leishmania* and other pathogens.



(**a**) (**b**) (**c**)

Figure 1. TLC profiles of (a) crude extract (b) column fractions and (c) isolated pure compound.

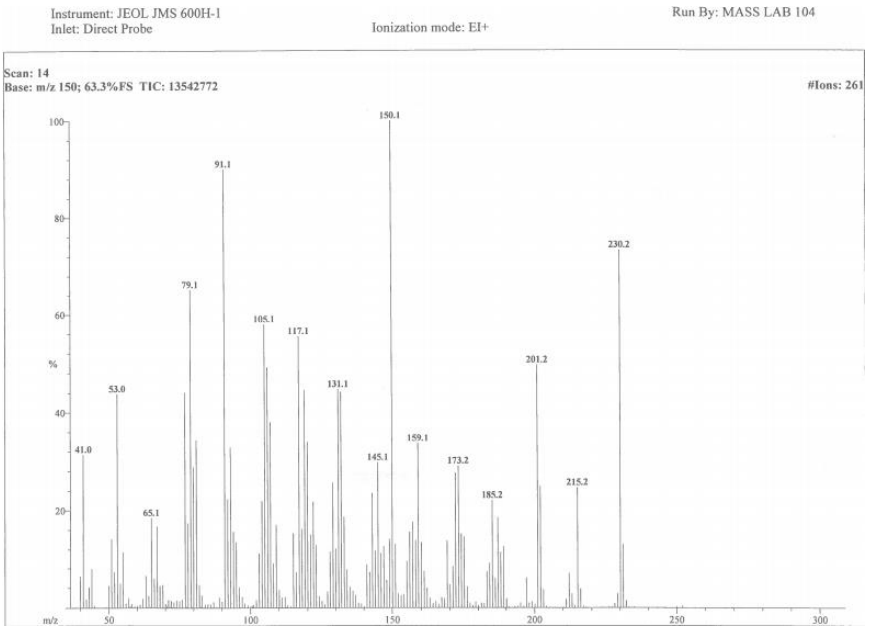


Figure 2. EI-Mass fragmentation of isolated compound (Ek-cpd1).

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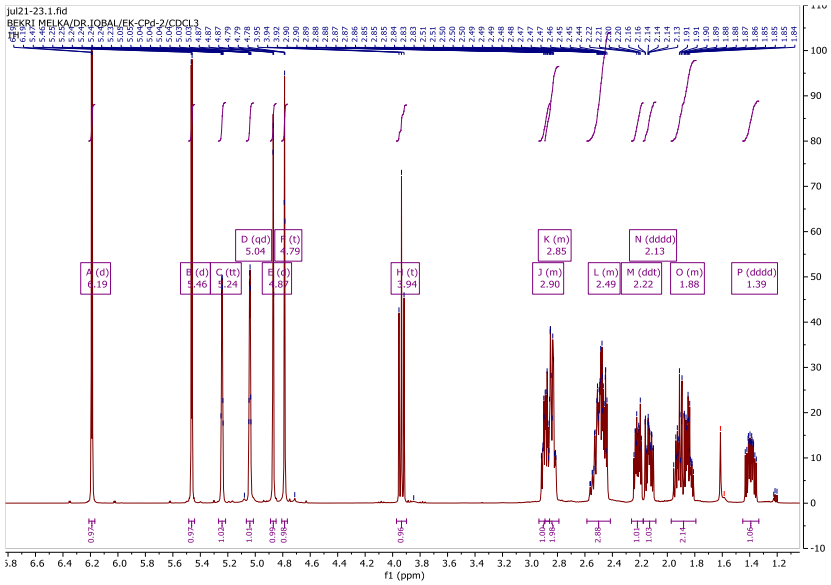

Figure 3. [1]HNMR spectra of isolated compound (Ek-cpd1).

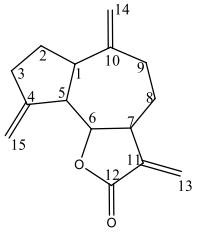

Figure 4. Structure of dehydrocostus lactone.

Table 1. In vitro antileishmanial activity test of crude extract, essential oil, and isolated compounds from roots of E. kebericho.

Tested samples	**Tested Parasites**	
	L. major	*L. tropica*
Crude extract	IC_{50} (µg/mL)±SD	IC_{50} (µg/mL)
Hexane fraction	33.3±0.7	36.6±0.5
Dichloromethane fraction	*NT*	*NT*
Ethyl acetate fraction	>100	>100
N-Butanol fraction	>100	>100
Water fraction	>100	>100
Essential oil	38.3±0.8	55.16±0.9
Compounds	IC_{50} (µM/mL)	IC_{50} (µM/mL)
E.k-cpd1	15.3±0.03	14.2±0.2
Amphotericin B	3.39±0.03	3.41±0.02
Pentamidine	4.56±0.01	4.56±0.01
Miltefosine	31.8±0.2	27.2±0.6

NT: not tested.

Table 2. Chemical composition analysis of essential oil from roots of E. kebericho.

No.	**RT (minute)**	**MW (g/mole)**	**MF**	**Name of Compounds**	**Relative Concentration (%)**
1	18.1	152	$C_{10}H_{16}O$	(S)-cis-Verbenol	0.21
2	19.1	154	$C_{10}H_{18}O$	Borneol	0.18

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3	23.5	154	$C_{10}H_{18}O$	p-Menth-2-en-7-ol, trans-	0.28
4	24.4	196	$C_{12}H_{20}O_2$	Borneol, acetate	2.08
5	26.7	204	$C_{15}H_{24}$	(±)-Cadinene	3.94
6	27.1	204	$C_{15}H_{24}$	δ-Neoclovene	3.68
7	27.5	204	$C_{15}H_{24}$	β-Elemene	2.67
8	28.4	204	$C_{15}H_{24}$	α-Guaiene	2.36
9	28.7	204	$C_{15}H_{24}$	Caryophyllene	3.05
10	30.5	204	$C_{15}H_{24}$	α-Humulene/α-Caryophyllene	1.36
11	30.8	204	$C_{15}H_{24}$	(-)-Alloaromadendrene	1.07
12	31.7	204	$C_{15}H_{24}$	γ-Muurolene	0.15
13	32.1	204	$C_{15}H_{24}$	β-Eudesmene/β-Selinene	0.11
14	32.4	204	$C_{15}H_{24}$	α-Selinene	0.43
15	33.5	204	$C_{15}H_{24}$	γ-Cadinene	2.19
16	33.7	204	$C_{15}H_{24}$	δ-Cadinene	0.33
17	34.1	204	$C_{15}H_{24}$	β-Guaiene	11.13
18	35.6	222	$C_{15}H_{26}O$	±-trans-Nerolidol	0.48
19	37.7	220	$C_{15}H_{24}O$	Caryophyllene oxide	2.33
20	37.8	220	$C_{15}H_{24}O$	Aromadendrene oxide-(2)	0.74
21	38.2	220	$C_{15}H_{24}O$	1,4-Methanoazulen-9-one, decahydro-1,5,5,8a-tetramethyl-, [1R-(1α,3aβ,4α,8aβ)]-	4.98
22	38.7	220	$C_{15}H_{24}O$	Diepi-α-cedrene epoxide	0.37
23	38.9	222	$C_{15}H_{26}O$	Germacrene D-4-ol	1.84
24	39.1	222	$C_{15}H_{26}O$	Cubenol	0.18
25	39.8	222	$C_{15}H_{26}O$	τ-Cadinol	0.33
26	39.9	222	$C_{15}H_{26}O$	τ-Muurolol	1.32
27	40.1	222	$C_{15}H_{26}O$	δ-Cadinol, (-)-	0.08
28	40.3	222	$C_{15}H_{26}O$	α-Cadinol	1.10
29	40.5	220	$C_{15}H_{24}O$	γ-Gurjunenepoxide-(2)	0.90
30	40.8	204	$C_{15}H_{24}$	Globulol	0.33
31	41.0	220	$C_{15}H_{24}O$	Aromadendrene oxide-(1)	3.29
32	42.0	232	$C_{16}H_{24}O$	9-Methoxycalamenene	1.43
33	42.6	220	$C_{15}H_{24}O$	Cedren-13-ol, 8-	0.09
34	42.8	220	$C_{15}H_{24}O$	Ledene oxide-(II)	0.43
35	44.0	220	$C_{15}H_{24}O$	Lanceol, cis	5.92
36	44.6	220	$C_{15}H_{24}O$	Santalol, cis, α-	0.80
37	45.5	220	$C_{15}H_{24}O$	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol	1.95
38	46.2	236	$C_{15}H_{24}O_2$	Bicyclo[4.4.0]dec-5-ene, 1,5-dimethyl-3-hydroxy-8-(1-methylene-2-hydroxyethyl-1)-	0.39
39	46.6	220	$C_{15}H_{24}O$	α-Copaen-11-ol	0.08
40	47.5	236	$C_{15}H_{24}O_2$	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)-	0.18
41	49.0	230	$C_{16}H_{22}O$	Cycloisolongifolene, 8,9-dehydro-9-formyl-	0.20
42	50.5	202	$C_{15}H_{22}$	Aromadendrene, dehydro-	0.19
43	56.9	230	$C_{15}H_{18}O_2$	Dehydrocostus lactone	24.95

RT: Retention time; MW: Molecular weight; MF: Molecular formula.

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3. Materials and Methods

3.1. Description of the Experimental Site

The plant sample of *E. kebericho* was collected from the experimental field of Wendo Genet Agricultural Research Center, Ethiopia. All the laboratory experiments, such as extraction, isolation, structure elucidation, and biological activities were conducted at ICCBS, Karachi University, Pakistan.

3.2. Preparation of Plant Sample

The matured *E. kebericho* tubers (12 months old) were harvested from Wendo Genet Agricultural Research Center experimental field (1819 m a.s.l., N 07° 05' 664", E 038° 38' 897"). The tubers were washed with tap water to avoid the mud, then chopped and dried in the shade. The dried tubers were crushed with mortar and pestle. The powdered tuber sample was put in a plastic bag until extraction.

3.3. Essential Oil Extraction

The powdered tubers (200g) were distilled through hydro distillation by using a Clevenger-type apparatus for 4 hours. The distillate was collected using a measuring pipette and stored in cleaned amber vials after drying with anhydrous Na_2SO_4. The essential oil yield was calculated according to the formula described in equation 1. The essential oil was subjected to the antileishmanial activity test and chemical composition analysis.

$$Essential\ oil\ yield\ \left(\frac{v}{w}(\%)\right) = \frac{Amount\ of\ distilled\ oil\ (mL).100}{Amount\ of\ the\ root\ sample\ (g)} \quad (1)$$

3.4. Chemical Composition Analysis of Essential Oil

A GC-MS (Agilent model 7890 A) equipped with GC sampler 120 was used to determine the chemical composition profile of the essential oil. The instrument was conditioned with a split/ split less injector mode, MS detector (7000 Triple Quad), and Zebron ZB-5 capillary column (320 µm internal diameter x 30 m length x 0.25 µm film thickness). The injector was operated in a split ratio of 1:20 with an injection volume of 1.2 µL. Injector and detector temperatures were set at 250 °C. The MSD was operated on scan mode in the 40-700 m/z range and interface temperature was set to 260°C. Helium was used as carrier gas in controlled constant flow mode at a linear velocity of 44.64 cm/sec. The oven was programmed to start at 50 °C, which was held for 5 minutes; then the temperature was ramped at 3 °C/min to 200 °C, which was held for 15 minutes; subsequently the temperature was ramped at 10 °C/ min to 300 °C, which was held for 20 minutes. The solvent delay time was 5 minutes and took 100 minutes for the total run.

3.5. Crude Extraction and Fractionation

The powdered sample of *E. kebericho* tuber (892.1g) was macerated with and divided into two 2000mL volume round bottom flasks using a mixture of dichloromethane and methanol (1:1) solvents (1600mL for each flask) for 48 hours with periodical shaking. The extracted solution was filtered using muslin cloth and the residue was repeated to extract with 800mL maceration solvent. All the extract solutions were collected and filtered with filter paper, and finally concentrated using a Rotary evaporator. The crude extract was added into a 2000mL separatory flask containing 500mL distilled water for further partitioning by liquid-liquid extraction using different organic solvents such as hexane, dichloromethane, ethyl acetate, and n-butanol starting from low to high polarity gradient. The extraction proceeded with adding 1000mL solvent into the separatory flask having crude extract and distilled water. The solutions were Shaking well, and the separatory flask was put on the stand. After completely forming two layers, the lower layer was poured, and the extraction was repeated four times with 1000mL solvent. The upper layers were collected and concentrated for hexane, ethyl

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acetate, and n-butanol solvents. For dichloromethane solvent, the lower layers were collected and concentrated. Finally, the water extract was remained and concentrated using a freeze drier. The extract yield was calculated according to the formula described in equation 2.

$$Extract\ yield\ \left(\frac{W}{w}(\%)\right) = \frac{Amount\ of\ extracted\ (g).100}{Amount\ of\ root\ sample\ (g)} \qquad (2)$$

3.6. Isolation of Compounds

The major compounds were isolated using column chromatography with a column diameter of 4cm, a silica gel (70-230 pores size, Merck), and a 40cm pack length. Hexane was used to prepare the slurry of silica gel for column packing. The hexane fraction sample (5g) was prepared as a slurry by mixing 5g silica gel and loaded in the column. Gradient elution of mobile phase was used starting from 200mL hexane and continued with 1000mL hexane: ethyl acetate (95:5); 1000mL hexane: ethyl acetate (90:10) and 1000mL hexane: ethyl acetate (86:14). An eluate of 100mL volume was collected as one fraction and transferred to vials (20 ml) after being concentrated. The purity of the isolated fraction was confirmed by TLC through hexane: ethyl acetate (86:14) solvent system. The spots on the TLC were detected with UV light at 254nm and 366nm wavelength for UV active compounds. The non-UV active compounds were visualized by using vanillin-sulfuric acid reagent. The reagent was prepared by adding 2 mL sulfuric acid to 100 mL acetic acid and cooling it for 10 minutes in the refrigerator. Then 800 mg vanillin was added and mixed well. The reagent was sprayed on the TLC within the Fume hood and dried with a hot gun. Finally, the purified compounds were submitted to spectral analysis such as a 1 mg sample for EI- Mass (JEOL JSM 600H-1) and a 5 mg sample for ^{1}H NMR (AVANCE NEO 500 MHz).

3.7. Antileishmanial Activity Test

An in vitro test with a 96 well plate assay method described by Bouyahya et. al. (2018) [34] was used for antileishmanial activity test against *Leishmania major* 50155 (ATCC) and *Leishmania tropica* 50129 (ATCC). Leishmania promastigote was grown in RPMI-1640 media with 10% fatal bovine serum. The parasite at log. phase was centrifuged at 200 rpm for 10 minutes, and the supernatant was discarded. Fresh media was added to dilute the pallet material till the final density of 10^6 cells/mL. The media (100 µL) was added in all well tissue culture plates except the first column which added 180 µL. The last two rows were used for negative (5% DMSO in media) and positive control (Amphotericin B, Pentamidine, and Miltefosine). The test samples (1 mg) (fractioned extract, essential oil, and isolated compound) were dissolved in 50 µL DMSO and diluted with 950 µL of RPMI-1640 media. The tested solution (20 µL) was added to the first well plate and mixed. A serial dilution of the next well plate was followed. The plates were incubated at dark at 23 ºC for 72 hours. After 72 hours, the activity of the fractioned extract, essential oil, isolated compounds, and drugs were assessed microscopically using an improved Neubauer chamber.

4. Conclusions

Dehydrocostus lactone is the major constituent of essential oil and hexane fraction from the roots of *E. kebericho*. The leishmanicidal activity test confirmed that the antileishmanial property of the roots of *E. kebericho* is due to the presence of sesquiterpene lactone. Particularly dehydrocostus lactone has shown better leishmaniacidal activity than the standard drug miltefosine. However, the toxicity test of dehydrocostus lactone has not been included in this work, previous reports confirmed the presence of toxicity against cancer cells but not the normal cell. Therefore, dehydrocostus lactone is expected to have a high value of selectivity index and is suggested to be safe for use as leishmanicidal drug. Besides, the broad-spectrum biological activities of dehydrocostus lactone could be a potential drug candidate for co-infected patients caused by an association of *leishmania* and other pathogens. The diverse range of biological activities against the different *leishmania* species and forms of leishmaniasis should be tested.

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Conflicts of Interest: All the authors listed in this work have no conflict of interest.

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