ISSN 2278- 4136
ZDB-Number: 2668735-5
IC Journal No: 8192
Volume 1 Issue 4
Online Available at www.phytojournal.com

Journal of Pharmacognosy and Phytochemistry

Isolation of Crotepoxide From Berries of *Croton macrostachyus* and Evaluation of Its Anti-Leishmanial Activity

Habtamu Gelaw[1], Legesse Adane[1]*, Yinebeb Tariku[1], Asrat Hailu[2]

1. Department of Chemistry, Jimma University, Jimma, Ethiopia.
   [E-mail: adanelegesse@gmail.com]
2. Department of Microbiology Immunology and Parasitology, Faculty of Medicine, Addis Ababa University, P.O. Box 9086, Addis Ababa, Ethiopia

The chloroform extract of the berries of *Croton macrostachyus* was subjected to column chromatography with chloroform: ethyl acetate mixture as eluting solvent that led to isolation of Crotepoxide. Evaluation of antileishmanial activity of crotepoxide against promastigotes and amastigotes form of *L. aethiopica* showed that the compound is less active as compared to the reference antileishmanial drugs (amphotericin B and miltefosine). Regardless of the observed activity, further *in vivo* tests are recommended before drawing any conclusion about the potential of the compound as a new antileishmanial drug candidate.

Keyword: Mimusops elengi, Ethnobotany, Phytochemistry, Triterpenoids, Saponins

1. Introduction

Use of natural products for curing human diseases is as ancient and universal as medicine itself. Most of the currently available drugs for treatment of different human and animal diseases obtained from natural products especially medicinal plants[1-4]. Such drugs have been discovered after observing the medicinal use of a particular plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by herbalists, and subsequent isolation of bioactive compounds from the plant or part of the plant that was used traditionally for treatment of different human illnesses.

Moreover, some compounds obtained from natural sources have also been used as leads or precursors that can be modified synthetically to improve their therapeutic activities[1,2]. Introduction and development of several new and highly specific *in vitro* bioassay techniques, chromatographic methods, spectroscopic techniques and other standardized pharmacological methods have also made it much easier to screen, isolate and identify potential drug compounds quickly and precisely from natural sources to alleviate human illnesses[5-8].

Though natural products (e.g., medicinal plants) have many medicinal uses, there are several reasons that necessitate isolation and characterization of bioactive compounds from them. Some of the reasons are (i) distribution of medicinal plants is not uniform throughout the world to be used by people everywhere; (ii) most of the medicinal plants are under threat of extinction due to climate changes and population pressure[9,10]; (iii) isolation and purification of compounds from natural sources is tedious,

expensive and time consuming process; and (iv) the need to identify the chemical compounds that are responsible for the observed medicinal value of the plant. Once, such compounds are known and their properties and structures determined they can be prepared synthetically without relying on plants. Moreover, the amounts of natural products (bioactive compounds) obtained from medicinal plants are very small. Thus, many plants should be destroyed to harvest significant quantities of such compounds for disease treatment[11,12]. All these facts necessitated isolation and characterization of bioactive compounds from medicinal plants in order to characterize those compounds, and subsequently synthesize them in large scales in laboratories or industries in reasonable costs[7,12].

One of the plant species that is known for its medicinal use is *Croton macrostachyus* (Euphorbiaceae). The plant is native to some Eastern African countries such as Eritrea, Ethiopia, Kenya, Tanzania, Uganda and Nigeria. It is one of the eight *Croton* species found in Ethiopia[13]. In areas where it is native, the plant (or its parts) is used for treatment of several human health problems that include symptoms of diabetes[14], malaria[15-17], dysentery[18], stomachache[18], ascariasis and taeniasis[18,19], abdominal pain[20], gonorrhoeae , wounds, ringworm infestation, hemorrhoids [21,22], venereal diseases[23], cough[23], rheumatism[24] and as a purgative in cases of ascariasis[22-24]. It is also used to stop bleeding in child birth[18]. The seeds are eaten to induce abortion[19] while the seed oil is used as a purgative[23]. In Ethiopia, the plant also has folk medicinal uses as purgative, various skin diseases, management of helminthes and venereal diseases, and to induce abortion[25-27]. These wide range of medicinal uses of *Croton macrostachyus* led scientists to isolate compounds from its different parts. Some of the compounds isolated from this plant include cyclohexane diepoxides such as crotepoxide (**1**)[28-31], lupeol (**2**) and betulin (**3**)[30,32-34], *cis*-clerodane (**4**), crotomacrine (**5**)[31], 3β-Acetoxy tetraxer-14-en-28-oic acid (**6**), trachylina-19-oic acid (**7**), trachylina-18-oic acid (**8**), neoclerodan-5,10-en-19,6β; 20,12-diolie (**9**), 3α,19-dihydroxy trachylina (**10**), 3α,18,19-triydroxy trachylina (**11**)[32-34] (Figure 1).

Some of the compounds isolated from different parts of the plant have been tested for their biological activities[35], and scientific investigations are still being carried out by different research teams to examine biological activities of crude extracts or pure compounds isolated from *Croton macrostachyus*, and most of them showed promising activities. *In vitro* anthelmintic activities of crude aqueous and hydro-alcoholic extracts of the seeds of *Croton macrostachyus* were found to show significant activity on the egg and adult stage of *H. contortus*[36].

Albert *et al*[37] reported analgesic and anti-inflammatory properties of the aqueous and methylene chloride/methanol extracts of the stem bark of *Croton macrostachyus*. A recent report by Karunamoorthi and Ilango[38] showed larvicidal activity of *Croton macrostachyus* against *Anopheles arabiensis* Patton (a potent malaria vector). Its crude extract was found to demonstrate high activity against reference strain of *N. gonorrhoeae* (ATCC 49226) and mitogenic activity on human lymphocytes and mice spleen lymphocyts[21,39]. A reports by Desta *et al*.[40] and Taniguchi and Kubo[41] indicated antimicrobial and antifungal activities of methanol and dichloromethane extracts of the leaves and stem of *Croton macrostachyus*. There are reports describing molluscicidal activities of crude extracts of the plant[42-44]. There are also reports that show antimicrobial[45], and anticonvulsant[46] activities of crude extracts of *Croton macrostachyus*.

Recent reports also indicated that essential oils from *Croton macrostachyus* possess antibacterial activities[47] and antileishmanial activities[48]. The oils that were obtained from berries of the plant were tested for theirs antileishmanial activities (against *L. donovani* and *L. aethiopica* promastigotes and axenic amastigote stages) and were found to have high efficacy. Based on the observed high efficacy and moderate toxicity of the volatile oil from *Croton macrostachyus*, the authors suggested the potential of this plant species

as a promising source of new lead compounds in the search for safe and effective antileishmanial drugs to replace the currently available commercial drugs such as sodium stibogluconate, sodium antimony gluconate, meglumine antimoniate, pentamidine, isethionate, amphotericin B, miltefosine, paromomycin, sitamaquine and imiquimod. There are serious limitations associated with these drugs, which (i) unpleasant side effects that cause pain at the site of injection, gastrointestinal problems, stiff joints, cardio-toxicity and in some cases, hepatic and renal insufficiency[49,50]; (ii) most of them are expensive and are not affordable by the majority of the patients[51]; and (iii) most of these drugs also have lost their effectiveness due to drug resistance[52,53].

The report by Tariku *et al*.[48] and the aforementioned limitations of the currently available antileishmanial drugs[49-53] led us to carry out isolation of compounds from berries of *Croton macrostachyus*. Moreover, our literature survey did not reveal any information regarding antileishmanial activity test of compounds from this plant species. Therefore, it is important to assess antileishmanial activities of compounds isolated from the berries of this plant in order to find out compounds that are responsible for its observed antileishmanial activities[48]. In this paper, we report isolation of crotepoxide (**1**) from berries of *Croton macrostachyus*, and evaluation of its antileishmanial activities.

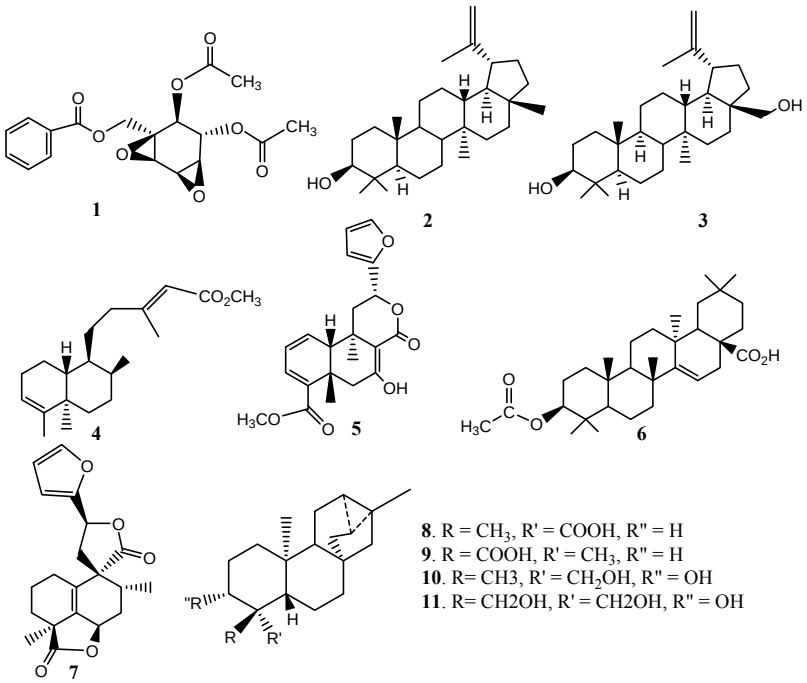
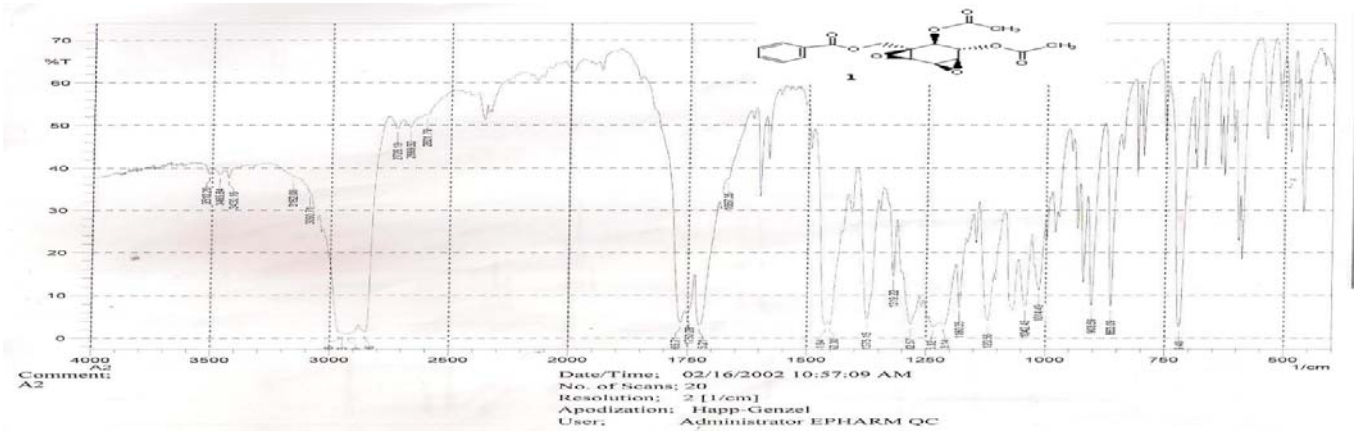


Figure 1. Examples of compounds isolated from *Croton macrostachyus*[28-34].

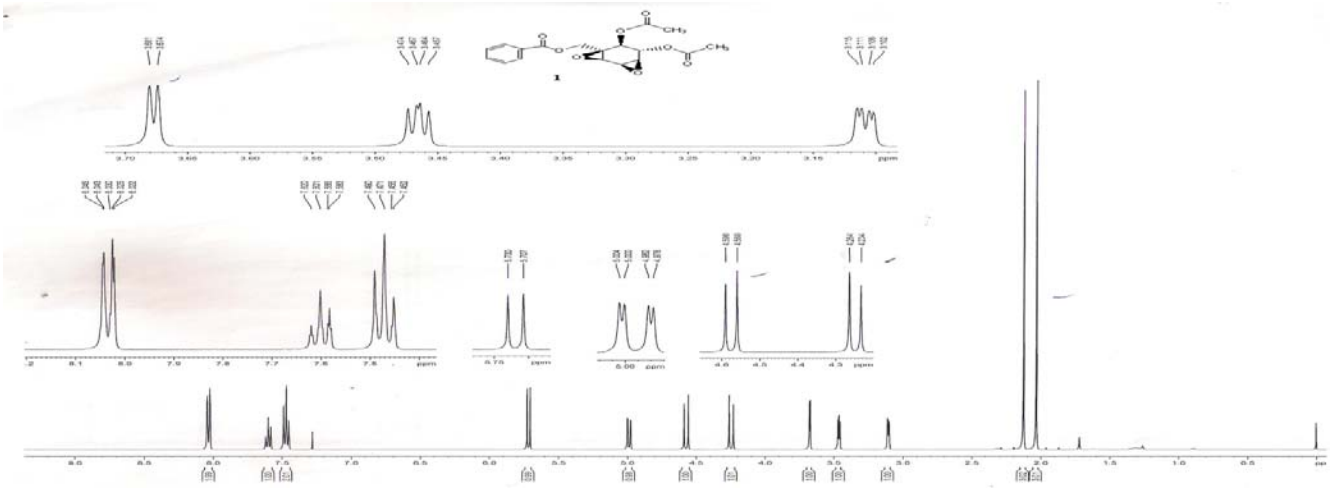
2. Results and Discussions

The compound (**1**) was obtained from chloroform extract of berries of *Croton macrostachyus* (See experimental section). It is a white solid with observed melting point value of 149-150 ^{0}C. In the IR spectrum (Supplementary material 1), the absorption band at 720 cm^{-1} indicated the presence of mono-substituted benzene ring whereas the absorption bands at 863 cm^{-1} and 903 cm^{-1} suggested the presence of epoxy rings. The bands at 1235 cm^{-1} and 1213 cm^{-1} showed the presence of benzoate and acetoxyl C-O stretching vibrations, respectively. The absorption bands at 1725 cm^{-1} and 1765 cm^{-1} suggested the C=O stretch values of benzoate ester and acetoxyl ester group, respectively. The absorption band at 3090 cm^{-1} indicated C-H stretching frequency of aromatic ring whereas a band 2851 cm^{-1} was attributed to

methylene C-H stretching vibration. The ^{1}H-NMRspectrum of compound was found to be consistent spectrum of compound was found to be consistent with reported data for crotepoxide (**1**) (Figure 2) [31].



IR spectrum of compound 1.



^{1}H-NMR spectrum of compound **1**.

Two broad singlets at δ 2.03 and 2.13 were attributed to methyl groups of the two acetoxyl groups of crotepoxide. The peaks at δ 3.11, 3.46 and 3.67 were attributed to methine protons attached to C-4, C-5 and C-6 of cyclohexane ring, respectively. The AB type peaks at δ 4.26 and 4.59 can be attributed to protons at C-7. The peaks at δ5.00 and 5.71 were attributed to protons of C-3 and C-2, respectively. The peaks at δ 7.49, 7.62 and 8.04 were attributed to the protons attached to the aromatic carbon atoms (C-3', C-5'), (C-4') and (C-2', C-6'), respectively. The observed and reported ^{1}H-NMR data of crotepoxide (**1**) (Figure 2) are given in Table 1 and its ^{1}H-NMR spectrum is given as supplementary material (Supplementary material 2).

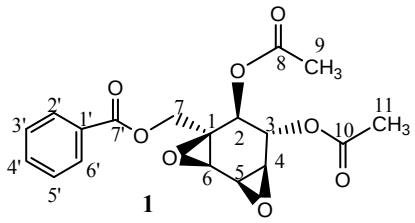
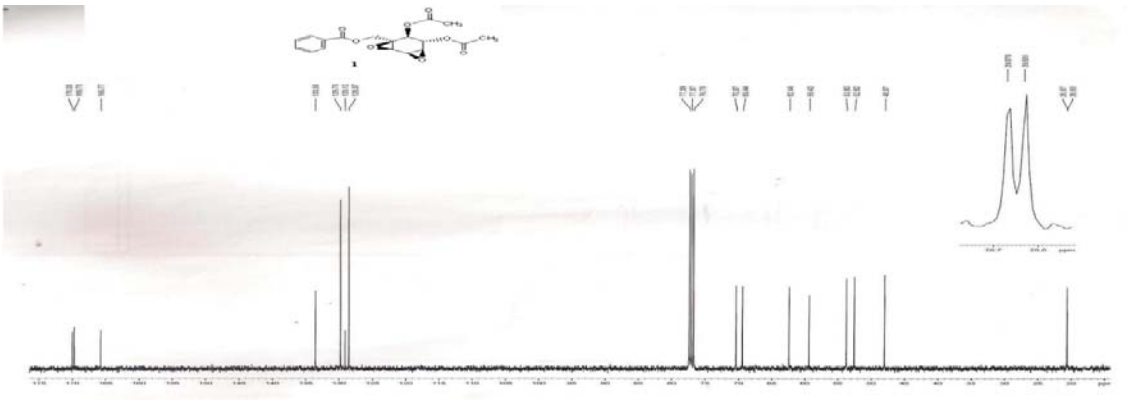


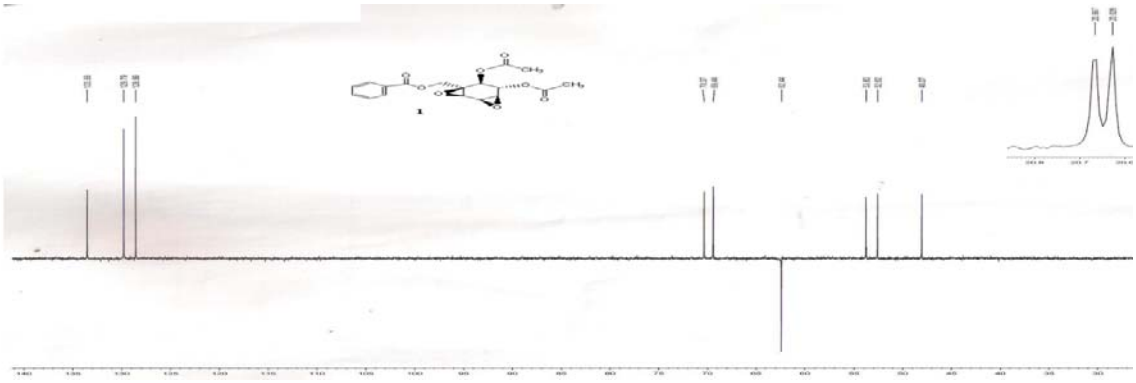
Figure 2. The chemical structure of crotepoxide (**1**) along the numbers given to distinguish its carbon atoms

Analyses of the ^{13}C-NMR and DEPT spectra of the compound also showed the presence of two

acetoxyl groups at or near 20.67, one methylene carbon at δ 62.44 (C-7), five methine carbons at δ 70.37 (C-3), 69.44 (C-2), 53.80 (C-6), 52.62 (C-4) and 48.07 (C-5), a methylene carbon at 62.44 (C-7), five quaternary carbons at δ 59.42 (C-1), 129.12 (C-1'), 165.75 (C-7'), 169.75 (C-8) and 170.05 (C-10) and five aromatic carbons at δ 128.57 (C'-2 and C-6'), 129.75 (C-3' and C-5') and at 133.55 (C-4'). The observed ^{13}C-NMR and DEPT spectra (Table 2) were also consistent with the reported data for crotepoxide (**1**). ^{13}C-NMR and DEPT-135 spectra of the compound are given as supplementary materials (Supplementary material 3 and 4).



^{13}C-NMR spectrum of compound 1.



DEPT-135 data of compound 1.

Table 1: Observed ^{1}H- NMR (400 MHz, $CDCl_3$) spectral data along with reported ^{1}H-NMR data of Crotepoxide (**1**).

Hydrogen No.	Observed data	Reported data[31]
H-9	2.03	2.04
H-11	2.13	2.14
H-4	3.11	3.11
H-5	3.46	3.46
H-6	3.67	3.67
H-7A	4.26	4.25
H-7B	4.59	4.58
H-3	5.00	5.00
H-2	5.71	5.71
H-3', H-5'	7.49	7.48
H-4'	7.62	7.61
H-2', H-6'	8.04	8.03

As discussed above, all the observed spectral data (IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135) of isolated compound were consistent with that of crotepoxide (**1**) reported in literature [31] suggesting that it is identical to crotepoxide (**1**). Moreover, the observed melting point (149-150 ^{o}C) was comparable to reported melting point value (i.e., 150-151 ^{o}C) of crotepoxide (**1**) [31].

The *in vitro* antileishmanial activity of the isolated compound was evaluated by its ability to inhibit promastigotes and amastigotes forms of *L. aethiopica.* amphotericin B and miltefosine were used as reference drugs in the experiment. The result of the study revealed that observed IC_{50} values of crotepoxide (**1**) to be 219.7 and 229.70 microgram/ml against promastigotes and amastigotes, respectively. The corresponding values the reference drugs were 0.003 microgram/ml (for amphotericin B) and 0.1 microgram/ml (for miltefosine) against promastigotes whereas the IC_{50} values amphotericin B and miltefosine against amastigotes were found to be 0.03 and 0.12 microgram/ml, respectively. The results indicated that the isolated compound **1** is less active against both promastigotes and amastigotes as compared to that of the reference drugs namely amphotericin B and miltefosine. Previous studies also revealed immunomodulatory [55], anti-PAF [56], antimicrobial [57], cytotoxic and antitumor [35,58,59] and weak antioxidant [59] activities of crotepoxide (**1**).

Table 2: The observed ^{13}C-NMR and DEPT-135 (400 MHz, $CDCl_3$) spectral data.

Carbon No.	^{13}C-NMR δ (in ppm)	DEPT-135 δ (in ppm)	Remark
9, 11	20.67	20.67	CH_3 of acetoxyl group
5	48.07	48.07	CH
4	52.62	52.62	CH
6	53.80	53.80	CH
1	59.42	-	Quat. C*
7	62.44	62.44	CH_2
2	69.44	69.44	CH
3	70.37	70.37	CH
2', 6'	128.57	128.56	Aromatic C
1'	129.12	-	Quat. C*
3', 5'	129.79	129.79	Aromatic C
4'	133.55	133.55	Aromatic C
7'	165.77	-	Quat. C*
8	169.75	-	Quat. C*
10	170.05	-	Quat. C*

*Quat.C: Quaternary carbon; ***carbonyl carbons of ester functional groups*

3. Conclusions

The result of our study suggests further *in vivo* tests to decide the potential of compound **1** as candidate antileishmanial agent. Moreover, due to its reported immunomodulatory activity, the authors would like to recommend further *in vitro* test to be conducted on compound **1** in combination with the currently existing antileishmanial drugs.

Materials and Methods

Collection of Plant Material: The berries of *Croton macrostachyus* were collected from Addis Ababa (Yeka subcity) on November 28, 2010. The berries were air-dried and crushed into small pieces to facilitate homogenous drying. The dried plant material was ground to suitable size to facilitate the extraction process.

3.1 Extraction and Isolation: Soxhlet extraction was performed by employing a gradient

extraction principle [54]. The solvents used for the extraction were petroleum ether and chloroform. 150 gram of berries of the plant material was subjected for extraction. The extracts of the two solvents were collected separately, and were subjected to vacuum drying using rotavapor in order to remove the solvents. The process gave 11.18 gm and 3.5 gm crude extracts of petroleum extract and chloroform extract, respectively. A portion (3.5 gm) of petroleum extract was adsorbed onto 4 gm of silica gel and loaded into a chromatographic column packed with 70 gm of silica gel. The column was then eluted with chloroform: ethyl acetate mixture gradually increasing the polarity (from 100:0% to 0:100%). Several fractions of 40 ml each were collected. The 5th fraction of the 50:50% ethyl acetate: chloroform solvent system gave a pure compound identified to be crotepoxide (**1**).

3.2 Characterization: The isolated compound was characterized using different spectroscopic techniques. Nuclear Magnetic Resonances (^{1}H-NMR, ^{13}C-NMR and DEPT-135) were recorded using 400 MHz and deuterated solvents (at the Department of Chemistry, Addis Ababa University). Infra-red (IR) spectra were recorded on IR perestinge-21 FTIR instrument (at Ethiopian Pharmaceutical Industry, Addis Ababa). Melting point values of the compounds were determined using Melting point was recorded using Thomas HOOVER capillary melting point apparatus.

3.3 Test strains and source of macrophages: The standard *L. aethiopica* strain (MHOM/ET/82/117-82) (obtained from Leishmaniasis Diagnostic and Research Laboratory, Faculty of Medicine, Addis Ababa University) and murine peritoneal macrophage isolated from *BALB/c* mice were used in the *in vitro* assay.

3.4 Reference drugs: Reference drugs used were amphotericin B (Fungizone®, Bristol-Myers Squibb, Rueil-Malmaison, France) and miltefosine (Impavido®, Zantaris GmBH, Germany).

3.5 Culture conditions*; Promastigote culture:* *L. aethiopica* was grown 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 and 100 µg/mL streptomycin solution (Sigma Chemical Co., St. Louis, USA) at 22 ^{0}C following previously described methods.

3.6 Murine Peritoneal Exudate Macrophages (MPEM): The isolation and differentiation of MPEM was carried using a standard method reported in literature[55]. First, the MPMs were induced by injecting a 2 ml of 2% starch solution into a mouse peritoneum. 24 hrs, after injection, the test mice was killed by CO_2 suffocation, degloved and injected with cold RPMI. Then peritoneal exudates were removed using a sterile 10 ml syringe with 21G 5/8 needle and collected in sterile plastic tubes placed in ice bath.

3.7 Intracellular amastigote culture: The intracellular amastigotes forms of *L. aethiopica* were obtained using a standard procedure [60]. Briefly, after harvesting the exudates, the cell suspension was determine by counting using a Neubauer hemocytometer, and the cell density was adjusted to 4000. Finally, 100 µl of the cell suspension was seeded into 16-chamber tissue plate and incubated at 37 ^{0}C for 24 hrs to allow differentiation of MPEMs. Promastigotes in stationary phase were used to infect differentiated MPMs in a 16-well culture plates in the infection ratio of 7:1 (parasites to MPEM).

3.8 Biological assays

3.8.1 Promastigote-based assay: In a 96-well microlitre plate, the test compound was serially diluted to twice the final test concentrations (1.00 mg, 0.33 mg, 0.11 mg, 0.037 mg, 0.012 mg, 0.004 mg and 0.0013 mg) in 100 µL culture medium with each test concentration in duplicate. Then, 100 µL of suspensions containing 3.5× 10^6 promastigotes/mL in a logarithmic phase were added to each well.

Contents of the plates were then maintained at 22°C 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 activity was expressed as the MIC values after 72 hrs of incubation. The reference drugs and medium with 1% DMSO were included as controls.

3.8.2 Antiamastigote-based assay: The intracellular amastigotes forms of *L. aethiopica* were obtained using a standard method [41]. *L. aethiopica* promastigotes in stationary phase were used to infect differentiated MPEMs in a 16-well culture plates in the infection ratio of 7:1. Then contents of the plates were incubated at 37 °C in humidified 5% CO_2 incubator for 24 hrs. The amastigote growth inhibitory activity of the test compound was carried by dispensing different concentrations (1.00 mg, 0.33 mg, 0.11 mg, 0.037 mg, 0.012 mg, 0.004 mg and 0.0013 mg) into duplicate wells of 16-well culture plates on which intracellular amastigotes were attached. Then the contents were incubated at 37 ^{0}C for five days in a 5% CO_2 incubator. After 5 days of exposure of the infected cells to the test compound and the reference drugs, the overlay from all of the wells was removed. After 30 seconds of fixing with 100% methanol, the slides were stained with 10 % Giemsa for 10 minutes. After rinsing and air-drying the slides were ready for examination under oil immersion (x100). During examination, infected and uninfected macrophages were counted in each well in a total of 100 macrophages. The % inhibition values were derived by comparison with the % infection of macrophages in control wells. The same procedure was applied for the reference drugs-amphotericin B and miltefosine.

3.8.3 Supplementary materials: The IR and NMR spectroscopic data of compound **1** are also available.

4. Acknowledgements

The authors thank Department of Chemistry, Jimma University, for financial and material supports, and Department of Microbiology Immunology and Parasitology, Faculty of Medicine, Addis Ababa University, for providing lab facilities and test strains to carry out the antileishmanial activity tests.

5. Reference

1. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. Metabolites 2012; 2:303-336.
2. Mishra BB, Tiwari VK. Natural products in drug discovery: Clinical evaluations and investigations. Opport. Challenge. Scope. Natural Prod. Med Chem 2011; 1-62.
3. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007; 70:461-477.
4. Rates SMK. Plants as source of drugs. Toxicon 2001; 39:603-613.
5. Keifer PA. NMR spectroscopy in drug discovery: Tools for combinatorial chemistry, natural products, and metabolism research. Prog Drug Res 2000; 55:137-211.
6. Satyajit D, Zahid L, Alexander I. Natural product isolation Method. Biotechnol J 2001; 20:1-7.
7. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha Y. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 2011; 8:1-10.
8. Nassar AEF, Talaat RE. Strategies for dealing with metabolite elucidation in drug discovery and development. Drug Discov Today 2004; 9:317-327.
9. Medicinal plants in danger thousands of medicinal species worldwide face extinction. http:/www.suite101.com/article/medicinal-plants-under-threat-a88180. 14 September, 2012.
10. Vicki B. Back to Nature: Extinction of medicinal plants threatens drug discovery. JNCI 2008; 100:838-839.
11. The American society of Pharmacognosy: Story of taxol.http:/www.phcog.org/Taxus/Taxus_Web.html. 14 September, 2012.
12. Li JWH, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier?. *Science* 2009; 325:161-165.
13. Gilbert MG. Euphorbiaceae. In: Flora of Ethiopia and Eritrea. Canellaceae to Euphorbiaceae. (Edwards S, Tadesse M, Hedberg I, Eds.), Vol. 2, part 2, Addis Ababa, Ethiopia, Uppsala, Sweden 1995.
14. Moshi MJ, Uiso FC, Mahunnah RLA, Mbwambo ZH, Kapingu MC. A survey of plants used by traditional healers in the management of non-insulin dependent diabetes mellitus. East Centr Afri J Pharmarceut Sci 2000; 3:30-39.

15. Kasa M.. Antimalarial activities of local medicinal plants. Trad Med Newsletter (Ethiopia) 1991; *2*:1-3.
16. Giday M, Teklehaymanot T, Animut A, Mekonnen Y. Medicinal plants of the Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia. J. Ethnopharmacology 2007; 110:516-525.
17. Mesfin F, Sebsebe D, Tilahun T. An ethno botanical study of medicinal plants in Wonago Woreda, SNNPR, Ethiopia. J Ethnobiol Ethnomed 2009; 5:28.
18. Kloos H, Tekle A, Yohannes LW, Yosef A, Lemma A. Preliminary studies on traditional medicinal plants in nineteen markets in Ethiopia: use patterns and public health aspects. Ethiop Med J 1978; 16:33-43.
19. Wilson RT, Mariam WG. Medicine and magic in central Tigre: A contribution to the ethnobotany of the Ethiopian plateau. Econ Bot 1979; 33:29-34.
20. Yirga G, Teferi M, Kasaye M. Survey of medicinal plants used to treat human ailments in Hawzen district, Northern Ethiopia. Inter. J Biodivers Conserv 2011; 3:709-714.
21. Geyid A. A study of surface proteins, other adhesions and iron acquiring mechanisms in the pathogenesis of *Neisseria gonorrhoeae* strains isolated from STD patients in Ethiopia. J Ethiop Medical Practice 2002; 4:14-20.
22. Abebe WA. Survey of prescriptions used in traditional medicine in Gondar region, Northwestern Ethiopia: general pharmaceutical practice. J Ethnopharmacol 1986; 18:147-165.
23. Mazzanti G, Bolle P, Martinoli L, Piccinelli D, Grgurina I, Animati F *et al*. *Croton macrostachys*, a plant used in traditional medicine: purgative and inflammatory activity. J Ethnopharmacol 1987; 19:213-219.
24. Kloos H. Preliminary studies of medicinal plants and plant products in markets of central Ethiopia. Ethnomed 1977; 4:63-104.
25. Amare G. Some common medicinal and poisonous plants used in Ethiopian folk Medicine. Addis Ababa University 1976; 3-63.
26. Abate G. Ethiopian traditional medicine, Ed. Demissew S, Addis Ababa. University Press, Addis Ababa, 1989, 99-183.
27. Schmelzer GH, Gurib-Fakim A. Plant resources of tropical Africa medicinal plants I. Buckhuys publishers, Wageningen, Netherlands, 2008, 203-206.
28. Kupchan SM, Hemingway RJ, Coggon P, Mc Phail AT, Sim GA. Crotepoxide, A novel cyclohexane diepoxide tumor inhibitor from *Croton macrostachys*. J Am Chem Soc 1968; 90:2982-2983.
29. Kupchan SM, Hemingway RJ, Smith RM. Tumor inhibitors. XLV. Crotepoxide, a novel cyclohexane diepoxide tumor inhibitor from *Croton macrostachys*. J Org Chem 1969; 34:3898-3902.
30. Addae-Mensah I, Muriuki G, Karanja G, Wandera C, Waibel R, Achenbach H. Constituents of the stem bark and twigs of *Croton macrostachys*. Fitoter 1992; 63:81.
31. Tane P, Tatsimo S, Connolly JD. Crotomacrine, a new clerodane diterpene from the fruits of *Croton macrostachyus*. Tetrahedron Lett 2004; 4:6997-6998.
32. Kapingu MC, Dominique G, Mbwambo ZH, Moshi MJ, Uiso FC, Mahunnah RLA. Diterpenoids from roots of *Croton macrostachys*. Phytochem 2000; 54:767-770.
33. Yibralign Z. *M.Sc.* Thesis, Phytochemical investigation on the stem bark of *Croton macrostachyus* (Bisana), Addis Ababa University, Department of Chemistry, Addis Ababa, 2007; 9-10.
34. Menberu D. M.Sc. Thesis, Chemical Composition of *Croton Macrostachyus*, Addis Ababa University, Department of Chemistry, Addis Ababa, 1981; 2:16.
35. Prasad S, Yadav VR, Sundaram C, Reuter S, Hema PS, Nair MS *et al*. Crotepoxide chemosensitizes tumor cells through inhibition of expression of proliferation, invasion, and angiogenic proteins linked to proinflammatory pathway. J Biol Chem 2010; 285:26987-26997.
36. Eguale T, Tilahun G, Gidey M, Mekonnen Y. *In vitro* anthelmintic activities of four Ethiopian medicinal plants against *Haemonchus contortus*. Pharmacologyonline 2006; 3:153-165.
37. Albert K, Marius M, Benoit NT, Gilbert A, Pierre W, Laure NB *et al*. Anti-Nociceptive and Anti-Inflammatory Activities of Extracts from the Stem Bark of *Croton macrostachyus* (Euphorbiaceae) in Mice and Rats. J Complementary Integrat Med 2009; 6:1553-3840.
38. Karunamoorthi K, Ilango K. Larvicidal activity of Cymbopogon citratus (DC) Stapf. and *Croton macrostachyus* against Anopheles arabiensis Patton, a potent malaria vector. Eur Rev Med Pharmacol Sci 2010; 14:57-62.
39. Tachibana Y, Kato A, Nishiyama Y, Kawanishi K, Tobe H, Juma FD, Ogeto JO, Mathenge SG. Mitogenic activities in African traditional herbal medicines. Planta Med, 1993; 59:354-358.

40. Desta B. Ethiopian traditional herbal drugs. Part II: Antimicrobial activity of 63 medicinal plants. 1993.
41. Taniguchi M, Kubo I. Ethnobotanical drug discovery based on medicine men's trials in the African savanna: Screening of East African Plants for antimicrobial activity II. J Nat Prod 1993; 56:1539-1546.
42. Daffala AA, Amin MA. Laboratory and field evaluation of the molluscicidal properties of habat-el-mollok (Croton spp). East Afri J Med Res 1976; 3:185-195.
43. Khier YM, Salih AM. Investigation of the nature of the molluscicidal factor of Croton macrostachys. J Afri Medicinal Plants 1979; 2:55.
44. Ito Y, Yanase S, Fujita J, Harayama T, Takashima M, Imanaka H. A short- term in vitro assay for promoter substances using human lymphoblastiod cells latently infected with Epstein- Barr virus. Cancer Lett 1981; 13:29-37.
45. Mariita RM, Okemo PO, Orodho JA, Kirimuhuzya C, Otieno JN, Joseph MJ. Efficiency of 13 medicinal plants used by indigenous communities around Lake Victoria, Kenya, against tuberculosis, diarrhoea causing bacteria and Candida albicans. Inter J Pharm Technol 2010; 2:771-791.
46. Ngo BE, Ngah E, Ngo MRM, Minkoulou DMZ, Talla E, Moto FC *et al*. Decoctions of *Bridelia micrantha* and Croton macrostachyus may have anticonvulsant and sedative effects. Epilepsy Behav 2012; 24:319-323.
47. Belay G, Tariku Y, Kebede T, Hymete A, Mekonnen Y. Ethnopharmacological investigations of essential oils isolated from five Ethiopian medicinal plants against eleven pathogenic bacterial strains. Phytopharmacology 2011; 1:33-143.
48. Tariku Y, Hymete A, Hailu A, Rohloff J. Constituents, antileishmanial activity and toxicity profile of volatile oil from berries of *Croton macrostachyus*. Nat Prod Commun 2010; 5:975-980.
49. Trouiller P, Torreele E. Drugs for neglected diseases. Trop Med Intl Health 2001; 6:945-951.
50. Matoussi N, Ameur HB. Treatment of cutaneous leishmaniasis with new aspects of established compounds and on new drugs in patients with *Leishmania major*. Med Mal Infect 2007; 37:257-259.
51. Croft SL, Coombs GH. Leishmaniasis- current chemotherapy and recent advances in the search for novel drugs. Trend Parasitol 2003; 19:502-508.
52. Mishra BB, Singh RK, Srivastava A, Tripathi VJ, Tiwari VK. Fighting against Leishmaniasis: search of alkaloids as future true potential anti-Leishmanial agents. Mini Rev Med Chem 2009; 9:107-123.
53. Kedzierski L, Sakthianandeswaren A, Curtis JM, Andrews PC, Junk PC, Kedzierska K. Leishmaniasis: current treatment and prospects for new drugs and vaccines. Curr Med Chem 2009; 16:599-614.
54. Abdul J. M.Sc. thesis, extraction of antioxidant compounds from red Pitaya using Soxhlet extraction method, Malaysia, Pahang University, 2009.
55. Yuh-Chi K, Lie-Chwen L, Nai-Shian Y, Wei-Jern T, An-Pang L, Ming-Jen L *et al*. Immunomodulatory Principles from *Piper kadsura.* http://www.ejournal.nricm.edu.tw/upload/21614/13/13-3-05.PDF. 23 September, 2012.
56. Jantan SM, Raweh HM, Sirat S, Jamil YH, Mohd Yasin J, Jalil JA. Inhibitory effect of compounds from Zingiberaceae species on human platelet aggregation. Phytomedicine 2008; 15:306-309.
57. kari MA, Neoh BK, Rashid NY, Jalil WNF, Rahmani M, Lajis NH *et al*. Studies on the constituents and medicinal properties of Some Zingiberaceous species. Malaysian J Sci 2005; 24:167-172.
58. Ray-Ling H, Chieh-Fu C, Hui-Yi F, Lie-Chwen L, Cheng-Jen C. Anti-hepatitis b virus of seven compounds isolated from *piper kadsura* (choisy) ohwi. J Chin Med 2001; 12:179-190.
59. Lotulung PDN, Kardono, LBS, Kawanishi, K. Antioxidant Compound from the Rhizomes of *Kaempferia rotunda* L. Pakistan J Biol Sci 2008; 11:2447-2450.
60. Yardley V, Croft AL. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. Intern J Antimicrob 2000; 13:243-248.