International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2019; 7(6): 01-04
Received: 01-09-2019
Accepted: 03-10-2019

Baraka Samwel
Department of Natural Products Development and Formulation, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar es Salaam, Tanzania

Ester Innocent
Department of Biological and Pre-clinical Studies, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar es Salaam, Tanzania

Francis Machumi
Department of Natural Products Development and Formulation, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar es Salaam, Tanzania

William N Kisinza
Amani Research Centre, National Institute for Medical Research, National Institute for Medical Research, P. O. Box 9653, Tanzania

Matthias Heydenreich
Institute of Chemistry, University of Potsdam, Baumhaselring 110A, 14469 Potsdam, Germany

Corresponding Author:
Correspondence
Baraka Samwel
Department of Natural Products Development and Formulation, Muhimbili University of Health and Allied Sciences, P. O. Box 65001, Dar es Salaam, Tanzania

Isolation and characterization of mosquito larvicidal compounds from leaves of *Kotschya uguenensis* (Taub) F. Whote

Baraka Samwel, Ester Innocent, Francis Machumi, William N Kisinza and Matthias Heydenreich

Abstract
At 72h of exposure, the crude extract showed larvicidal activity with LC_{50} values of 94.01 µg/mL against *An. gambiae*. Based on bioactivity-guided fractionation, a new *ent*-halimane type diterpene, named *ent*-halim-1(10)-ene-15-oic acid (1) together with a known 3-O-methyl-D-*chiro*-inositol (3) were isolated and identified as the active constituents. *Ent*-halim-1(10)-ene-15-oic acid (1) and 3-O-methyl-D-*chiro*-inositol (3) exhibited strong larvicidal activity with LC_{50} value of 30.05 and 80.73 µg/mL µg/mL, respectively (>95% CI) after 72 h post exposure. The extract and the two compounds (1 and 3) had no significant activity at 24 and 48 h. However the *ent*-halim-1(10)-ene-15-oic acid (1) demonstrated strong activity against *An. gambiae* with significant different being observed against the extract and compound 3. The results indicated that 80% aqueous ethanol leaves extract of *K. uguenensis* and the two isolated constituents have potential for development in the control of *An. gambiae* larvae.

Keywords: *Kotschya uguenensis*, mosquito larvicidal, *Anopheles gambiae*, active compound

1. Introduction
Plants have been providing man with most of his needs like shelter, food, clothing, flavors, medicine and pesticides throughout the world since the ancient times [1]. Plants remain a crucial source of medicines for a large percentage of the world's population, mostly in the developing countries [2]. Terrestrial plants especially higher plants are well known for their chemical diversity. They produce various compounds, many of which show medicinal and pesticidal properties. These compounds serve as defensive mechanism of the plants to resist continuous selection pressure from herbivore predators and other environmental factors [1]. Previously several compounds from the family Fabaceae have been reported to have insecticidal activity [3, 4]
The genus *Kotschya* belongs to the family Fabaceae, it consists of about 30 species which are widely distributed in tropical Africa and Madagascar [5]. Some species of the genus *Kotschya* have been used in traditional medicine to treat headache, delay or absence of menstruation, stomachache and skin diseases [6]. The genus has been indicated various biological activities such as antioxidant [7] and mosquito larvicidal activity [8]. Phytochemical studies of the genus *Kotschya* reported various classes of natural products including flavanoids, terpenoids and steroids [7, 9, 10].
The aerial part of *K. uguenensis* has been used traditionaly to repel the chicken mite, *Dermanyssus gallinae* DeGeer (Acarina: Dermanyssidae) infection [11]. Biological studies showed that the root and stem bark of *K. uguenensis* exhibited growth inhibitory activities against *An. gambiae* with cycloaretenone being mild active and main constituent, that was isolated from ethanolic extract [12]. In the present study, the leaves of *K. uguenensis* were evaluated for mosquito larvicidal activity against *An. gambiae*. Bioactivity-guided fractionation led to isolation of a new *ent*-halimane type diterpene, named *ent*-halim-1(10)-ene-15-oic acid (1) and a known 3-O-methyl-D-*chiro*-inositol (3) which exhibited mosquito larvicidal activity.

2. Materials and Methods
2.1 General analyses
Silica gel (Mesh 70–230) was used in column chromatography as the stationery phases. Thin layer chromatography (TLC Silica gel 60 F_{254}) was done using aluminum pre-coated silica gel plates. The TLC plates were visualized under UV light at 254 or 366 nm, followed by exposing the plate in iodine vapor or spraying with reagent.

The ^{1}H and ^{13}C NMR spectra of the isolated compounds were obtained using Bruker–Avance 500 and 600 MHz spectrometers. The 2D (COSY, HSQC and HMBC) spectra of the compounds were obtained using standard Bruker software. Chemical shifts were measured in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). CDCl3 and D_2O were used as NMR solvents. Mass spectrometry was done by ESI-Q-TOFmicro (Quadrupole-Time of Flight) and GC-MS.

2.2 Collection of plant material

The leaves of *K. uguenensis* were collected at Ngwazi dam in Mufindi district, Tanzania. The plant was authenticated by the Botanist from the Department of Botany, University of Dar es Salaam and the voucher specimens (No. FMM 3624) are preserved at herbarium of the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences.

2.3 Preparation of extract, fractions and Isolation of compounds

A powdered air-dried leaf of *K. uguenensis* (2.5 Kg) was extracted with 80% ethanol in water three times after every 72 h. The filtrate was concentrated under reduced pressure and kept at 4° C until when required. The crude extract (20 g) was chromatographed over a column of silica gel (200 g) with petroleum ether/ethyl acetate (100:0, 95:5, 80:20, 50:50, 0:100) followed by ethyl acetate/methanol (95:5) as eluents. Basing on TLC analysis, fractions (3-6) and (8-10) obtained by eluting petroleum ether/ethyl acetate and (11-12) obtained by eluting ethyl acetate/methanol (95:5) were concentrated separately and named Fr1, Fr2 and Fr3 respectively. The fraction Fr1 (12 g) was subjected to small silica column with petroleum ether/dichloromethane (10:90) to yield subtraction Fr1A, which further subjected to silica and eluted with solvent system of petroleum ether/dichloromethane/ethyl acetate (49:49:2) to yield 45 mg of colorless oil (compound 1). Fraction Fr3 was subjected to silica gel column chromatography and eluted with ethyl acetate/methanol (97:3) to obtain Fr3A. The Fr3A was further purified in Sephadex LH-20 to yield 68.3 mg of white amorphous powder (compounds 3).

2.4 Mosquito larvicidal activity assay

The larvicidal test was performed according to World Health Organisation (WHO) protocol with minor modification [13]. The stock solutions (50 mg/mL) of stem bark extract were prepared by first dissolving them in DMSO followed by dilution of stock solutions was made with distilled water to make 100, 50, 25, 10 µg/mL solutions for crude extract bioassay and 40, 20, 10, 5 and 2.5 µg/mL solution of isolated compound bioassay. Ten late third instars laboratory reared *An. gambiae* larvae were then introduced in the test solutions and mortality was observed after 24 h, 48 h and 72 h. Control larvae were exposed in 50 mL of 0.5% (DMSO/H_2O). All tests were carried out in triplicate under controlled temperature (26 ± 2 °C) and relative humidity of 75-85%. The number of dead larvae was recorded at 24 h, 48 h, and 72 h post exposure.

2.5 Statistical analysis

The mean percentage mortalities were calculated for each concentration was used in statistical analysis of the experimental data. Lethal concentrations were determined using the Fig P computer program. The regression equations were used to determine LC16, LC50, LC84 and 95% C.I values [14].

3. Results and discussion

3.1 Structure elucidation

The 80% aqueous ethanol of *K. uguenensis* was subjected to repeated normal phase column chromatography and Sephadex LH-20 chromatography, to afford the new a new *ent*-halimane type diterpene, named *ent*-halim-1(10)-ene-15-oic acid (1) along with a known compound, 3-O-methyl-D-*chiro*-inositol (3) [15]. The structure of the known compound was identified by comparison of spectroscopic data obtained with those reported in the literature.

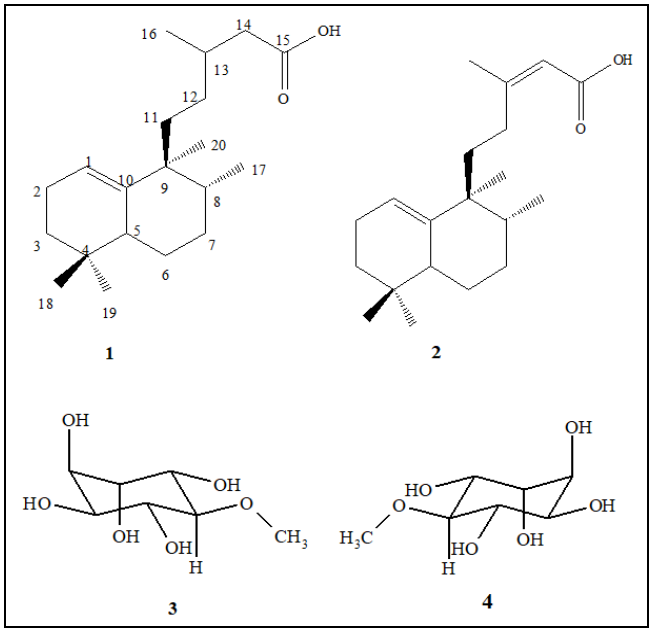

Fig 1: Structure of compounds 1 and 2 isolated from leaves extract of *K. uguenensis* and compounds 3 and 4 reported in literatures

Compound 1 was isolated as colorless oil with a molecular composition of $C_{20}H_{34}O_2$ as inferred from HREIMS *m/z* 306.2575, $[M]^+$. The ^{1}H-NMR spectrum showed the presence of three methyl singlets at δ_H [(0.76, 0.79 and 0.8)], two methyl doublets δ_H [0.73(3H, d, J=7.0) and 0.88 (3H, d, J=6.7)]. Seven methylene protons; six at δ_H (0.88-1.93) and one diastereomeric atoms resonated at δ_H 2.3 and 2.04 (H-14). Also observed were three methine δ_H [1.46 (1H, m), 1.57 (1H, m), 1.78 (1H, m)] and at δ_H 5.19 (1H, t, J=3.3 Hz) due to an olefinic proton (H-1). The 13 C- NMR exhibited 20 carbons signals (Table 1), which were determined by the aid of analysis of HSQC spectrum to five methyls, seven methylene, three quaternary carbons resonating at δ_C 43.85(C-20), 39.69 (C-8) and 31.37 (C-15). Also observed were two olefinic carbons C-1 δ_C (120.04), and C-10 δ_C (142.20) and a carboxyl at δ_C 180.36. These data above suggested that the structure of compound 1 was very similar to *ent*-halima-1(10), 13E-dien-15-oic acid (3) previously isolated from *Polyalthia longifolia* [16]. The only difference was the observation of ethylene group at δ_H (2.3 and 2.04, 2H, m, H-14) instead of olefinic proton δ_H 5.67 (1H, s, H-14) for *ent*-halima-1(10), 13E-dien-15-oic acid (3). This was evident from the shift to high field of C-13 and C-14 from δ_C 114.8 and 115.1 to δ_C 31.37 and 42.22 in the two structures respectively. The H/H COSY coupling between (H-13) δ_H 1.78 and (H-14) δ_H 2.3, 2.04 together with HMBC coupling of (H-13) to carbon signal resonating at δ_C 42.22 (C-14), (H-14) δ_H 2.3, 2.04 to δ_C 31.37

(H-13) and 180.36 (C-15) established the assignment of H-13 and H-14. Based on the NMR spectral data analysis, the compound 1 was identified to be a new ent-halim-1(10)-ene-15-oic acid.

Table 1: ^{13}C and ^{1}H NMR spectroscopic data of compounds 1 in CDCl3

Position	observed δ_C (ppm)	δ_C (ppm) Hara *et al.*, 1995	Observed δ_H (ppm)	δ_H (ppm) Hara *et al.*, 1995	HMBC (H to C)
1	120.04	120.3	5.19 (1H, *t*, *J*= 3.3)	5.33 (H1, *t*, *J*= 3.8)	C-2, 3, 9, 5
2	23.59	23.1	1.93 (2H, m)		C-3, 18
3	33.62	33.2	1.05,1.27 (2H, m)		C-2
4	31.86	31.4			
5	43.85	43.5	1.57 (1H, *m*)		C-10
6	24.24	23.6	1.48 (2H, *m*)		C-17
7	29.68	29.1	1.25,1.91 (2H, *m*)		C-17
8	39.69	39.2	1.46 (1H, m)		
9	43.38	43			
10	142.20	141.1			
11	36.63	37.2	1.86,1.01 (2H, *m*)		C-1, 10
12	30.21	36.3	1.18 (2H, m)		C-20
13	31.37	164.8	1.78 (1H, m)		C-14, 16
14	42.22	115.1	2.3,2.04 (2H, *dd*, *J*=5.7, 8.5)	5.67 (1H, *s*)	C-16
15	180.36	172.4			
16	20.48	19.5	0.88 (3H, *d*, *J*= 6.7)	2.16 (3H, *s*)	C-13
17	16.12	15.6	0.73 (3H, *d*, *J*=7.0)		
18	28.58	28.2	0.76 (3H, *s*)	0.89 (3H, *s*)	C-3, 19
19	26.71	26	0.79 (3H, *s*)	0.84 (3H, *s*)	C-18
20	22.88	22.3	0.8 (3H, *s*)	0.91 (3H, *s*)	C-12

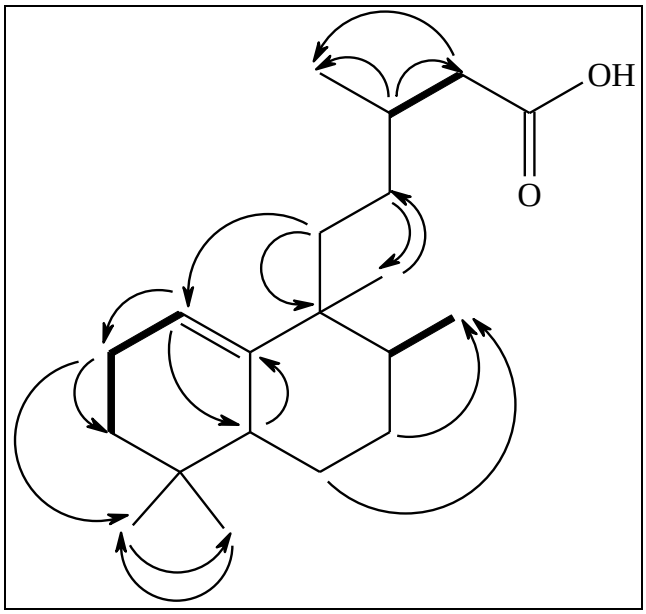

Fig 2: Key HMBC (H-C) and ^{1}H-^{1}H COSY (-)

3.2 Larvicidal bioassay

The 80% aqueous ethanol of *K. uguenensis* leaves showed the larvicidal activity against *An. gambiae* with LC_{50} value of 94.01 µg/mL (Table 2). The isolated constituents, *ent*-halim-1(10)-ene-15-oic acid (1) and 3-O-methyl-D-*chiro*-inositol (3) exhibited strong larvicidal activity with LC_{50} value of 30.05 and 80.73 µg/mL µg/mL, respectively (>95% CI) after 72 h post exposure (Table 2). The extract and the two compounds (1 and 3) showed no significant activity at 24 and 48 h.

However the *ent*-halim-1(10)-ene-15-oic acid (1) demonstrated strong activity against *An. gambiae* with significant different being observed against the extract and compound 3. This may implicate that compound 1 may have a slow prolonged mortality when *An. gambiae* are exposed.

Table 2: Mosquito larvicidal activity of *K. uguenensis* crude extract and isolated compound against *An. Gambiae*

Sample	Exposure time (h)	LC_{50} (µg/mL)	95% CI (LCI-UCI)
Crude extract	24	231.73	199.06-269.77
	48	192.51	153.76-241.02
	72	94.01	77.04-114.72
ent-halim-1(10)-ene-15-oic acid (1)	24	179.70	136.44-236.68
	48	82.64	64.79-105.41
	72	30.05	25.60-35.29
3-O-methyl-D-*chiro*-inositol (3)	24	707.95	501.65-999.08
	48	211.34	163.44-273.27
	72	80.73	61.22-106.46

Key: NT= Not tested, NA= Not Applicable, LC LC50 lethal concentration that kills 50% of the exposed larvae, LL lower limit (95% confidence limit), UL upper limit (95% confidence limit)

3-O-methyl-D-*chiro*-inositol (3) is a configurational stereoisomer of quebrachitol [L-(-)-2-Omethyl-chiro-inositol] (4). Quebrachitol (4) has been reported to occur in several plants including *Artabotrys modestus* ssp *macranthus* [17]. Although the two compounds chemically resemble, they have been reported to exhibit different physiological effects [18, 19]. Previously, 3-O-methyl-D-*chiro*-inositol (3) from ethanol extract of *Acacia nilotica*, reported to exhibit larvicidal activity against *Aedes aegypti* (LD_{50}= 267 ppm) and *Culex quinquefasciatus* (LD_{50}= 188 ppm) [20]. However its stereoisomer quebrachitol (4) had no activity against *An. gambiae* [21]. In the current study, 3-O-methyl-D-*chiro*-inositol (3) isolated from the leaves of *K. uguenensis* demonstrated activity (LC_{50}= 707.95 µg/mL) against *An. gambiae*. Furthermore, 3 has been reported to exhibit inhibitory activity against *Helicovarpa armigera* (Noctuidae) and overposition attractant against *Battus philenor* (Papilionidae) [22, 23].

Besides its insecticidal application, 3-O-methyl-D-*chiro*-inositol (3) also has been reported to possess anti-inflammatory, anti-hyperlipidemic, anti-oxidant, cardioprotective and anti-diabetic activities [24, 25].

4. Conclusion

The results of this study demonstrated that ethanol leaves extract of *K. uguenensis* and the isolated compounds; *ent*-halim-1(10)-ene-15-oic acid (1) and 3-O-methyl-D-*chiro*-inositol (3) have potential for development in the control of *An. gambiae* larvae.

5. Acknowledgement

Authors are grateful to the German Academic Exchange Service (DAAD) for funding, Muhimbili University of Health and Allied Sciences (MUHAS) for providing laboratory space for the research work, University of Potsdam through Institute of Chemistry, for research spce and analysis of NMR and MS spectra. We are thankful to Mr. Nicodemus Kihiyo from the Institute of Traditional Medicine, MUHAS for Mosquito larvae rearing, maintenance and supply and Mr. Frank Mbago from Department of Botany, University of Dar es Salaam for identification of the plant.

6. References

1. Gurib-Fakim A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol Aspects Med. 2006; 27(1):1-93.
2. WHO. Global tuberculosis control report, 2002, 286-288.
3. Wink M. Evolution of secondary metabolites in legumes (Fabaceae). South African J Bot, 2013, 1-12.
4. Krishna PM, Knv R, Sandhya S. A review on phytochemical, ethnomedical and pharmacological studies on genus. Brazilian J Pharmacogn. 2012; 22(5):1145-1154.
5. Gillett J, Polhill R, Verdcourt B. Leguminosae (Parts 3and 4), in Flora of Tropical East Africa, Eds. E. Milne - Redhead, RM. Polhill, AA. Balkema, Rotterdam, Netherlands, 1971.
6. Chhabra S, Uiso F, Mshiu E. Phytochemical screening of Tanzanian medicinal plants. J Ethnopharmacol. 1984; 11:157-179.
7. Awouafack MD, Tchuenguem RT, Ito T, Dzoyem JP, Tane P, Morita H. A New Isoflavanol from the Fruits of *Kotschya strigosa* (Fabaceae). Helv Chim Acta. 2016; 99:321-324.
8. Innocent E, Nkunya MH, Hassanali A. Larvicidal activity of *Kotschya uguenensis* plant powders and methanol extracts against *Anopheles gambiae* s.s. larvae in the laboratory and in simulated ponds. J Appl Pharm Sci. 2013; 3(2):122-126.
9. Angyal SJ. The effect of O-methylation on chemical and C-N.M.R. spectra of cyclic polyols. Carbohydr Res. 1983; 123:23-29.
10. Samwel B, Innocent E, Masimba P. Larvicidal activity of *Metarhizium anisopliae* and *Annona squamosa* leaf extract against *Culex quinquefasciatus* and *Anopheles gambiae* larvae. J Appl Life Sci Int. 2015; 3(4):169-176.
11. Innocent E, Joseph C, Gikonyo N, Nkunya M, Hassanali A. Growth disruption activities of polar extracts from *Kotschya uguenesis* (Fabaceae) against *Anopheles gambiae* s.s. (Diptera: Culicidae) larvae. Int J Trop Insect Sci. 2008; 20:220-224.
12. Innocent E, Sempombe J, Masimba PJ, Daniel I, Heydenreich M. Comparison of cycloartenone from four insecticidal *Kotschya species* (Fabaceae) harvested during dry and wet seasons. Pharmacogn Phytochem. 2015; 4(4):97-102.
13. WHO. Report of the WHO informal consultation on the evaluation and testing of insecticides, 1996, 76.
14. Litchfield Jr J, Wilcoxon F. A simple method of evaluating dose-effect experimets. J Pharmacol Exp Ther, 1949, 99-113.
15. Sridhar C, Krishnaraju A, Subbaraju V. Anti-inflammatory Constituents of *Teramnus labialis*. Indian J Pharm Sci, 2006, 111-114.
16. Hara N, Asaki H, Fujimoto Y, Gupta YK, Singh AK, Sahai M. Clerodane and ent-halimane diterpenes from *Polyalthia longifolia*. Phytochemistry. 1995; 38(1):189-194.
17. Nyandoro SS. Some rare Tanzanian plant species as sources of less common metabolites: biomedical potential and conservation status. J Pharmacogn Phytochem. 2014; 3(2):147-157.
18. Ostlund R, Sherman R, Nordin P, Examiner P, Jordan K. Pinitol and derivatives thereof for the treatment of metabolic disoders. United States Patent (19), 1998, (19).
19. Mccance RA, Lawrence RD. An investigation of quebrachitol as a sweetening agent for diabetics, 1933, 986-989.
20. Chaubal R, Pawar PV, Hebbalkar GD *et al*. Larvicidal activity of *Acacia nilotica* extracts and isolation of d - pinitol; a bioactive Carbohydrate. Chem Biodivers. 2005; 2:684-688.
21. Nyandoro SS, Joseph CC, Nkunya MHH. Natural Product Research: Formerly Natural Product Letters New antimicrobial, mosquito larvicidal and other metabolites from two *Artabotrys* species. Nat Prod Res. 2013; 27:37-41.
22. Dreyer D, Binder R, Chan B, Waiss A, Hartwig E, Beland G. Pinitol, a larval growth inhibitor for *Heliothis zea* in soybeans. Experimentia. 1979; 35(9):1182-1183.
23. Papaj D, Feeny P, Sachdev-Gupta K, Lorraine R. D- (+) - Pinitol, an oviposition stimulant for the *Pipevine swallowtail* butterfly, *Battus philenor*. J Chem Ecol, 1992, 18(5).
24. Geethan PKMA, Prince PSM. Antihyperlipidemic Effect of D -Pinitol on Streptozotocin-induced diabetic wistar rats. J Biochem Molecular Toxicology. 2008; 22(4):220-224.
25. Numata A, Takahashi C, Fujiki R, Kitano E, Kitajima A, Takemura T. Plant constituents biologically active to insects. VI. Antifeedants for larvae of the yellow butterfly, *Eurema hecabe mandarina*, in *Osmunda japonica*. Chem Pharm Bull. 1990; 38(10):2862-2865.