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Bioactive Chemical Constituents from the Leaf of *Oreosyce africana* Hook.f (Cucurbitaceae) with Mosquitocidal Activities against Adult *Anopheles arabiensis*, the Principal Malaria Vector in Ethiopia

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Abstract

The use of botanical insecticides can serve as an alternative to the environmentally undesirable inorganic insecticides as a means of malaria vector control. The objective of the study was to identify the biologically active compounds of *Oreosyce africana* (Cucurbitaceae) leaf extracts by using a bioassay-guided approach on adult mosquitoes. The dichloromethane fraction of *O. africana* crude methanol extract was subjected to bioassay-guided fractionation by flash column chromatography, preparative thin-layer chromatography and high performance liquid chromatography to obtain purified fractions. The purified fractions were assayed against adult *Anopheles arabiensis* Patton. Gas chromatography coupled to mass spectrometry was used for identification of the active chemical compounds against adult mosquitoes. The LC_{50} and LC_{90} values were determined based on probit analysis. Two purified fractions of *O. africana,* fraction IV or B2'O and B2''O, had the most potent adulticidal activities with an LC_{50} of 2.206 and LC_{90} of 7.811 ppm and with an LC_{50} of 2.62 and LC_{90} of 11.779 ppm at 24 hrs post-exposure, respectively. Among the purified fractions of *O. africana* LC_{50} ratio of B2'O and B2''O showed significantly different values at $P<0.05$. The structural elucidation of the active ingredients using GC-MS analysis showed the presence of 9,12-Octadecadienoic acid (Z,Z) as the major chemical constituent (98.35%) in B2'O fraction and dibutyl phthalate as the major chemical constituent (97.75%) in B2''O fraction with their molecular peaks at *m/z* 280 and 278, respectively. These results suggest that the purified fractions of *O. africana* possess a very high adulticidal effect against the principal malaria vector, *An. arabiensis*, in Ethiopia and have a potential for development into an affordable botanical mosquitocide as an alternative to the existing inorganic insecticides with environmental toxicity.

Keywords: *Oreosyce africana*; *Anopheles arabiensis;* Adulticidal activity; Octadecadienoic acid; Dibutyl phthalate

Introduction

In spite of available effective preventive, treatment and case management measures for a very long time, malaria remains a global public health problem and a leading cause of morbidity and mortality in many tropical countries. WHO [1] estimates for the year 2010 showed that there were 219 (154–289) million malaria cases and 660,000 (490,000–836,000) deaths due to malaria. Out of these about 80% of the cases and 90% of the deaths occurred in Africa while the remaining cases and deaths occurred mainly in South-East Asian and Eastern Mediterranean regions. In Ethiopia, *Anopheles arabiensis* Patton is the primary vector responsible for malaria transmission [2,3] whereas *An. pharoensis* Theobald, *An. funestus* Giles and *An. nili* (Theobald) are regarded as secondary vectors [4].

The initial malaria eradication campaigns against the mosquito vectors were very successful in the developed parts of the world, particularly with the coupled application of dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides [5]. However, many countries in Africa, Asia and Latin America lacked the basic health infrastructure, and hence these campaigns could not yield the expected results [6]. In Ethiopia, as in several other countries, vector control through application of chemical insecticides was frustrated with the development of strong resistance of mosquitoes to the synthetic insecticides [7-9]. The discovery of DDT insecticidal properties in 1939 and the subsequent development of organochlorine and organophosphate insecticides limited plant product research since the answers to insect control were thought to have been found [10]. On the other hand, some inherent features and use patterns of the conventional synthetic insecticides lead to toxicity to humans, livestock, fish, birds, and other beneficial organisms as a result of adverse effects on the environment, resulting from contamination of soil, water, and air. Resurgence of insect vector populations because of the emergence and spread of physiological and behavioral resistance to a wide range of insecticides has been a serious impediment in malaria control. On top of this is the higher costs of spraying operations, and high degree of refusal by the public to permit indoor spraying have remained challenges to the malaria control programs in developing countries. Thus, the drawbacks characteristic of the synthetic insecticides, has necessitated the search for an environment friendly and less costly plant products as an alternative vector control tools for use in the integrated vector management programs. In additions, mosquito control using plant materials can be obtained from local sources and are likely to generate local employment, reduce dependence on

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expensive imported products, and stimulate efforts to enhance public health [11]. The value of local focus on medicinal pant research was also emphasized by Kuppusamy and Murugan [12] who showed that the selection of insecticidal plants could be optimized through initial ethnobotanical screening of plants that indigenous local communities use in their traditional medical systems. It is known that some plant derivatives have been in use as insecticides for quite some time. Among these, pyrethrum produced from *Chrysanthemum cinerariifolium* (Asteraceae), rotenone from *Derris elliptica* (Fabaceae), azadirachtin from *Azadirachta indica* (Meliaceae), and nicotine from *Nicotiana tabacum* (Solanaceae) were widely used [13]. While these botanical bioactive products have the advantage of a relatively low mammalian toxicity and a fairly broad spectrum of activity compared with many synthetic insecticides, their short half-life of activity is the disadvantage [14]. The low persistency of the existing botanical insecticides has been shown to be due to their susceptibility to the effects of light and air as demonstrated for pyrethrum [15]. The present study was designed with the objective of evaluating the adulticidal activities of purified fractions of an indigenous plant *Oreosyce africana* Hook.f. against adult *An. arabiensis* Patton, the primary vector of malaria in Ethiopia.

Materials and Methods

Plant materials

The leaves of *Oreosyce africana* Hook.f. (Cucurbitaceae) were collected from Oromia Region, Ethiopia, in October 2011. The leaves were stored dry at room temperature and were extracted by 80% aqueous methanol (SCP, England) within two months. Ethnobotanical information on the medicinal uses of the species were collected by interviewing local traditional herbalists and knowledgeable elders after explaining the purpose of the research and obtaining consent through blessings that constituted the traditional ethical clearance. The dried plant material was stored at room temperature until the extraction process started. Along with the collection of plant materials for extraction and analysis, voucher specimens were collected, pressed, dried and authenticity confirmed by taxonomic experts at the Department of Plant Biology and Biodiversity Management and deposited at the National Herbarium of Ethiopia, Addis Ababa University for further reference.

Extraction and solvent fractionation

The powdered leaves of *O. africana* was extracted with 80% aqueous methanol (SCP, England) at room temperature for 72 hr in a rotator shaker (VWR, USA) within two months of collecting. The residue was filtered and the extraction procedure repeated three times under the same condition. The filtrates were combined and evaporated to dryness under reduced pressure at 45°C by using a rotary evaporator (Stuart®RE300, UK). The extract was fractionated by solvent partitioning with dichloromethane (Carlo Erba, France) and deionized water (water deionizer, EASYpureII, USA) and partitioned three times. After drying over anhydrous sodium sulfate, the dichloromethane extract was evaporated by using a rotary evaporator to give the dichloromethane fraction.

Flash column chromatography

The dichloromethane fraction obtained by solvent partitioning was purified based on the procedure Alkofahi et al. [16] as shown in Figure 1. The residue of dichloromethane fraction was chromatographed in a column of silica gel (Merck 25-100 mesh size, Germany) and a glass column (40×5 cm) successively eluted with hexane, hexane-chloroform (95:5, v/v), hexane-chloroform (90:10), hexane-chloroform (80:20),

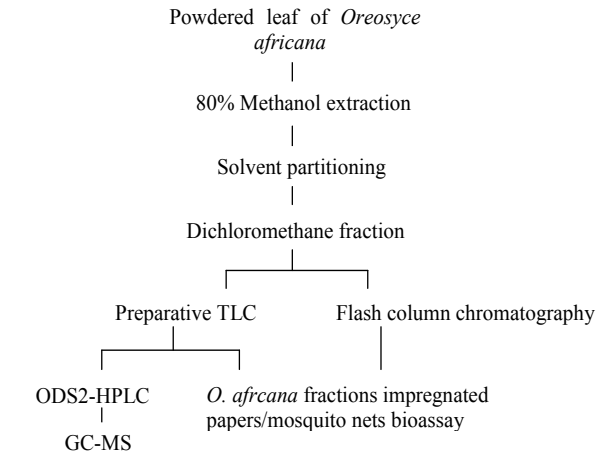

Figure 1: Purification procedure for the extracts from *Oreosyce africana* leaf.

hexane-chloroform (70:30), hexane-chloroform (60:40), and hexane-chloroform (50:50). In each case, 100 ml of solvent mixture was added and 20 ml fractions were collected using 50 ml conical flasks placed under the stopcock.

Thin-layer chromatography

Isolation of the metabolite of *O. africana* was monitored during the separation stages with thin-layer chromatography (TLC) on Kieselgel gel 60 F_{254} plates (Merck) which has a 0.5 mm thickness of silica sorbent (Merck no. 64271, Darmstadt, Germany) using chloroform-methanol (9:1, v/v) following the method of Okeke et al. [17]. The components of TLC plate were visualized by spraying with 1% sulfuric vanillin solution, then heating on a hot plate at 110°C for 5 min. The retention factor (R_f) value of the fraction was determined using the formula described by Ettre [18].

$$R_f \text{ value} = \frac{\text{Distance travelled by the spot (cm)}}{\text{Distance travelled by the solvent (cm)}}$$

Preparative thin-layer chromatography

The concentrate obtained by solvent evaporation of dichloromethane fraction of *O. africana* leaf extract was dissolved with chloroform and then applied to 16 sheets of Kieselgel 60 F_{254} TLC plates (20 × 20 cm, 0.25 mm thickness; Merck, Germany) following the method described by Jiao et al. [19] and Sasidharan et al. [20]. These were developed in chloroform-methanol (5:1) mixed solvent. 1.5 cm zone of the distinct bands of the purified fractions was scraped off from its plate and extracted eight times with 100 ml of chloroform-methanol (9:1) solvent. The extraction solvent was evaporated under rotary vacuum evaporator and extracted fractions were coded as B2'O and B2"O. These two fractions were stored at -20°C until used in the mosquito bioassay tests.

Anopheles arabiensis laboratory rearing

Adult female *An. arabiensis* mosquitoes used in the bioassays were reared according to the WHO [21] protocol. The adult colonies were reared and maintained at 25-27°C and 70-80% relative humidity under a photoperiod cycle of 12 hrs light and 12 hr dark in the insectary of the Department of Zoological Sciences, Addis Ababa University. 10% sucrose solution was provided to the adult mosquitoes in soaked cotton pad placed on top of the mosquito nets. Adult female *An. arabiensis*

were periodically blood-fed on restrained rabbits shaved back and belly. The larvae were fed on ground Tetramin® fish food pellets (Tetra holding Inc., Blacksburg, VA, USA); the feed applied on alternate days and the larvae and pupae were kept at 25-27°C and 70-80% relative humidity under a photoperiod cycle of 12 hr light and 12 hr dark.

Evaluation of adulticidal activity

Plastic tube bioassay: The mosquitocidal activities of the purified fractions were determined based on WHO [22] plastic tube bioassay test method. These consisted of holding and exposure cylindrical plastic tubes having a length of 125 mm and a diameter of 44 mm. Concentrations ranging from 4 to 16 ppm of each fraction, which included fractions II,III and IV collected from flash chromatography and B2'O and B2"O collected from preparative TLC were applied to WHO plain papers. Each concentration was prepared in 0.05% DMSO (Carlo Erba, France) in deionized water and shaken vigorously to achieve homogeneity of the contents. The WHO papers treated with the *O. africana* fractions were left to dry at room temperature overnight and then inserted into each exposure tube and the WHO papers treated with 0.05% DMSO in deionized water served as a negative control. The bioassays were performed at 25-27°C and 70-80% relative humidity with non-blood-fed *An. arabiensis* adult mosquitoes of known age (2 to 5 days post-emergence) in batches of 20 in each concentration. The mosquitoes were allowed to acclimatize in the holding tube for 1 hr and then exposed to the fractions on the impregnated paper and the unimpregnated control for 1 hr. At the end of the exposure period, the mosquitoes were transferred back to the holding tubes and kept for 24 hrs recovery period. A pad of cotton soaked with 10% sucrose solution was placed on the mosquito net during the holding period. Each treatment was replicated three times for each concentration.

Cage bioassay: Aerosol bioassays were performed by modifying the method of WHO [21] on adult *An. arabiesnsis* held in laboratory cages covered with mosquito netting (19 × 19 × 19 cm). Twenty five non-blood-fed adult 2 to 5 days old, female *An. arabiensis* were transferred into the test cages with a mouth aspirator. The adult mosquitoes were allowed to acclimatize in the cages for 1 hr before treatment. Each treatment was applied based on the method [23] using a small hand-held compression sprayer that discharged the purified fractions (II, III, and IV) collected from flash chromatography and B2'O and B2"O purified fractions collected from prep. TLC were sprayed into mosquito cages in separate experiments, at a concentration of 8 ppm. The test sample concentrations were prepared in 0.05% DMSO in deionized water and shaken vigorously to achieve homogeneity of the contents. The control cages were sprayed with the same amount of 0.05% DMSO in deionized water. 10% sucrose solution soaked on cotton pad, as a source of nutrition, was placed on top of the mosquito netting, in the test as well as in the control group. The Bioassay tests were carried out at 25-27°C and 70-80% relative humidity in the laboratory and mosquito mortality was recorded 24 hrs post-treatment.

Cone bioassay: Polyester fabric nets were purchased from retail shops in Addis Ababa and were cut into 25 × 25 cm size for use in the experiments. The nets were folded and soaked in disposable plastic petri dishes containing solutions of B2'O or B2"O in 0.05% DMSO at concentrations of 4, 8, and 16 ppm. Nets were treated one day before bioassay and stored at ambient room temperature [24]. The impregnated and the control nets were dried in shade by laying them flat on the table. The impregnated nets were fixed to a cardboard and three standard WHO plastic cones were placed and fixed with tape over the area of the treated net. Ten non-blood-fed, 2 to 5 days old, female *An. arabiensis* each were introduced into the three test cones through a hole on the mosquito nets. The same numbers of non-blood-fed adult female mosquitoes were also introduced into the cone that was fixed over a net impregnated with 0.05% DMSO in deionized water as a negative control. Both test and negative control mosquitoes were exposed for 15 minutes under ambient room temperature. At the end of the exposure period, the mosquitoes were transferred into recovery paper cups covered with untreated nets and were held for 24 hrs with access to 10% sucrose solution. Mortality was determined after 24 hrs recovery period.

Chemical characterization of the mosquitocidal constituents

High performance liquid chromatography analysis: The active fractions of *O. africana* were further purified by high performance liquid chromatography (HPLC) using Waters LC-2000 model equipped with Waters 600 pump controller (Milford, MA, USA). 10 µl of each sample fraction-B2'O and B2"O injected manually into a column of Tracer Extrasil ODS2, TR416059 (particle diameter, 5 µm; pore size, 30 nm; column size, 25 × 0.46 cm) (Teknokroma, Barcelona, Spain) and eluted by use of solvent system: methanol-water (80:20, v/v) with a running time of 0-30 min. Column temperature of 20°C and flow rate of 1.0 ml/min were maintained and the eluate detected at 254 nm using Waters 2487 dual absorbance UV detector. All data were recorded and processed by Millennium 32 software from Waters (Milford, MA, USA).

Gas chromatography-mass spectrometry (GC-MS): The GC and GC-MS analyses were performed using the method described by Mohan and Maruthupandian [25]. Gas chromatography measurements were carried out with Hewlett-Packard HP5890 Series II (Agilent Technologies, USA) and GC-MS analysis was conducted with gas chromatograph model 7890B (Agilent Technologies, USA) coupled to Hewlett-Packard MS model 6977A (Agilent Technologies, USA) with mass selective detector. The analysis was carried out on a GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) employing the following conditions: Column Elite-1 fused silica capillary column (30 mm × 0.25 mm 1D × 1 µm df, composed of 100% dimethyl poly siloxane). Helium (99.999%) was used as a carrier gas operating in electron impact mode at a constant flow of 1 ml/min and each sample injected contained a volume of 2 µl was applied manually (split ratio of 10:1) under injector temperature of 250°C and ion-source temperature of 280°C. The oven temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/min until 200°C and then rose at 5°C/min up to 280°C and was held for the last 9 minutes (isothermal at 280°C). The GC-MS instrument was used in the electron impact mode under an ionization voltage of 70 eV. The detection of mass spectra was completed in 38 min. Interpretation of the mass spectra from GC-MS data was conducted using the database of National Institute of Standards and Technology (NIST) library. The mass spectra of the unknown components were compared to the spectra of the known compounds and determined by searching in the NIST library.

Data analysis

The LC_{50} and LC_{90} values of *O. africana* fractions II, III, IV, B2'O, and B2"O were determined by probit regression analysis using the statistical package PoloPlus (version 2.0, LeOra Software, Petaluma, California, USA; 2007). The 95% confidence limits of upper confidence limit (UCL) and lower confidence limit (LCL) for the lethal concentration in ppm (LC_{50} and LC_{90}) were determined using probit regression analysis of the statistical package PoloPlus. Hypothesis testing to compare concentration-mortality probit lines was evaluated by likelihood ratio tests for equality and parallelism (chi-square tests

and P values for comparing the slope and intercept). When the slopes of the concentration-mortality lines were not significantly different, lines were considered parallel, indicating that the two treatments had the same relative potency (i.e., the same variability in response) [26]. PoloPlus analysis was used to determine adulticidal efficacies among purified *O. africana* fractions II, III, B2'O, and B2"O. Statistically significant differences between the compared LC_{50} concentrations, the 95% confidence intervals for lethal concentration ratios were calculated. In this pair wise comparison, lethal concentrations were considered significantly different if the value '1' did not fall within the confidence interval for the ratio [26].

Results

Thin-layer chromatography analysis of *Oreosyce africana* fractions

Extracts of dichloromethane fraction of *O. africana* were applied onto the columns and the fractions with similar R_f values on the TLC plates were combined and designated as fractions II to IV as follows: $O_{6\&7}$ as fraction II eluted with hexane-chloroform (95:5, v/v); $O_{8\text{-}10}$ as fraction III eluted with hexane-chloroform (90:10); and $O_{11\text{-}14}$ as fraction IV eluted with hexane- chloroform (80:20). The dichloromethane fraction of *O. africana* leaf extract was also applied to preparation. TLC for purification Sixteen plates of prep. TLC were used for the purification of the fractions and yielded two major yellow bands on prepared TLC. From the combined fractions two active purified fractions B2'O and B2"O were obtained after separation by prep. TLC on silica gel plates with chloroform-methanol (5:1). In TLC analysis, two yellow spots corresponding to B2'O (R_f=0.5) and B2"O (R_f = 0.46) were observed by spraying with 1% sulfuric vanillin reagent.

Bioassay of chromatographic fractions of *Oreosyce africana* on adult *Anopheles arabiensis*

Comparison of the adulticidal activities of purified potent fractions of *O. africana* collected following flash column chromatography and prep. TLC against the malaria vector, *An. arabiensis* adults are presented in Table 1. The hypothesis test for parallelism was rejected (χ^2=22.90; P=0.001) showing that slopes differed significantly. Among the fractions tested, the highest adulticidal activity was observed with fractions of IV or B2'O with an LC_{50} and LC_{90} values of 2.206 and 7.811 ppm followed by fraction of B2"O with an LC_{50} and LC_{90} values of 2.62 and 11.779 ppm (Table 1).

Effects of purified potent fractions of *O. africana* against adult *An. arabiensis*

The effect of the purified potent fractions, B2'O and B2"O isolated from *O. africana* were compared with WHO papers treated with 0.05% lambdacyhalothrin (positive control) against *An. arabiensis* adults (Figure 2). The adulticidal activities of purified fractions B2'O and B2"O at a concentration of 8 ppm each against *An. arabiensis* showed 89% and 80% adult mortality, respectively while with 0.05% lambdacyhalothrin, the WHO standard, the mortality of adult *An. arabiensis* was 74% (Figure 2). There was no mortality in the untreated controls.

Efficacy of active components of *O. africana* as aerosol sprays on adult *An. Arabiensis*

Five purified fractions of *O. africana* exhibited different levels of adulticidal activity. With regard to treatment by aerosol sprayed inside the cage against adult *An. arabiensis* with *O. africana* purified fractions II, III, IV, B2'O and B2"O at 8 ppm concentration each resulted in 49, 41, 89, 89 and 80% mortality, respectively (Figure 3).

Cone bioassay of B2'O and B2"O fractions impregnated nets on *Anopheles arabiensis*

Based on high adulticidal potency of fractions B2'O and B2"O of *O. africana*, dose-response bioassay for the adulticidal activities of the two fractions against adult *An. arabiensis* was conducted and mortality was observed after 24 hrs exposure period. The percentage adult mortality against the logarithmic concentration values was plotted based on the mean of three replicates (Figure 4). The results of adulticidal activity tests

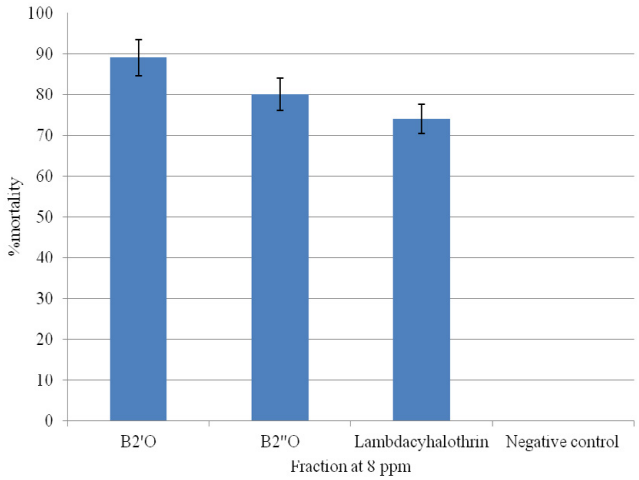

Figure 2: Comparison of activities of potent fractions of *Oreosyce africana* (B2'O and B2"O) at 8 ppm with 0.05% lambdacyhalothrin (positive control) on adult *Anopheles arabiensi* after 24 hrs exposure period on impregnated papers in WHO test tubes (n=45 in each test).
Note: Positive control (0.05% lambdacyhalothrin); negative control (0.05% DMSO in deionized water).

Fractions*	LC_{50} ppm (95% CL)	LC_{90} ppm (95% CL)	Slope ± SE	χ^2 (P)	LC_{50} ratio (95% CL)
II	6.906 (6.047-7.950)	23.731 (18.580-33.420)	2.391 ± 0.229	1.666 (0.56)[a]	1.287(0.961-1.725)
III	7.388 (6.329-8.776)	32.366 (23.443-52.165)	1.997 ± 0.215	1.687 (0.53)[a]	1.203(0.887-1.633)
IV	2.206 (1.734-2.632)	7.811 (6.470-10.173)	2.334 ± 0.273	0.571 (0.53)[a]	4.030(2.898-5.603)[b]
B2'O	2.206 (1.734-2.632)	7.811 (6.470-10.173)	2.334 ± 0.273	0.571 (0.53)[a]	4.030(2.898-5.603)[b]
B2"O	2.620 (1.031-3.952)	11.779 (7.263-47.258)	1.963 ± 0.232	2.480 (0.41)[a]	3.393(2.428-4.742)[b]
Negative control**	0.0	0.0	0.0	0.0	0.0

** DMSO (0.05%) in deionized water.
[a] Good fit of the data to the probit model (*P*>0.05).
[b] LC_{50} ratio significant (*P*<0.05), 95% confidence interval did not comprise the value 1.0.
Table 1: Adulticidal activity of different fractions of *O. africana* (fraction II, III and IV using flash column chromatography; B2'O and B2"O using prep. TLC) against *Anopheles arbiensis* adults after 24 hr exposure period on impregnated papers in WHO test tubes (n=45 in each test).
* The codes used for the fractions.

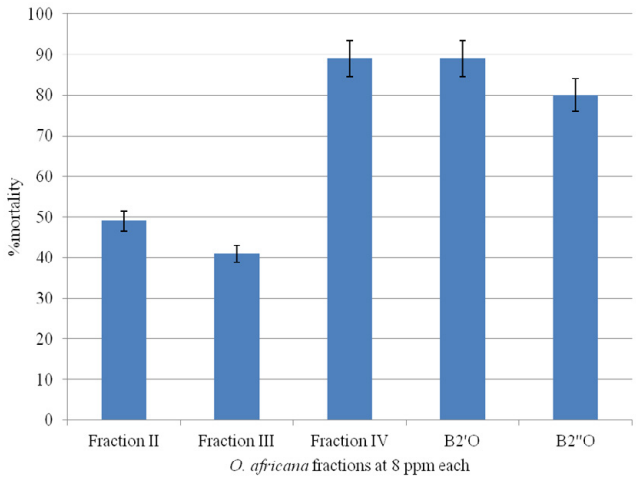

Figure 3: Measure of mortality of *Anopheles arabiensis* adults following aerosol spray treatment with fractions of *Oreosyce africana* after 24 h exposure period (n=75 in each test).

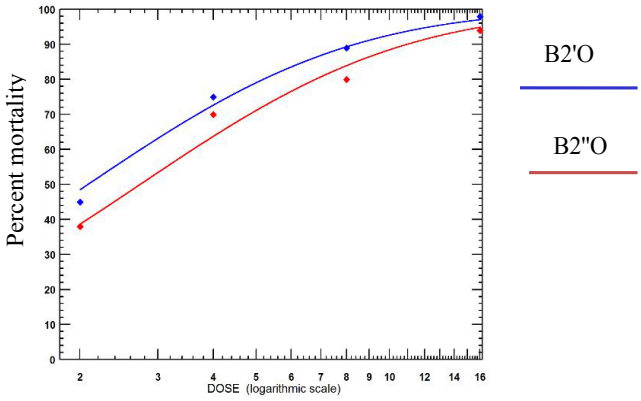

Figure 4: Parallel case regression plot showing percent mortality of adult *Anopheles arabiensis* after 24 hr exposure to different concentrations of purified fractions, B2'O and B2"O, of *Oreosyce africana* using impregnated net in cone bioassay (n=45 in each test).

of B2'O and B2"O clearly showed the percentage of adult *An. arabiensis* mortality to be directly proportional to the concentration of the fractions.

High performance liquid chromatography analysis of O. africana fractions

Purification of B2'O and B2"O of *O. africana* fractions collected from the prep. TLC were determined by using HPLC. This B2'O fraction gave main and minor peaks by HPLC analysis (Figure 5). The retention times (R_t) of the two peaks were 6.398 and 10.471 min with 91% and 5.4% area under the peak, respectively. The HPLC chromatogram showed two peaks of which the first one was the major peak and the second was lesser peak (Figure 6). The first peak appeared at retention time (R_t) 3.565 min with area % of 72.30 and the second peak appeared at R_t 5.175 min with a % of 24.76.

Gas chromatography-mass spectrometry (GC-MS) analysis of B2'O and B2"O

The mass spectrum presented in Figure 7 revealed the structure of 9,12-Octadecadienoic acid (Z,Z), more commonly known as linoleic acid, as the major chemical constituent of the purified isolate, B2'O. The mass spectrometer analysis showed a parent peak at m/z 280, which corresponded to a molecular formula of $C_{18}H_{32}O_2$. There also were other fragmented minor peaks at m/z 264, 110, 95, 81, 67, 55, 41 and 29 (Figure 7). In comparison with the library values, the fragmentation peaks were assigned chemical name, molecular formula, molecular weight, and approximate relative percentages for the compounds. Thus, the GC-MS analysis confirmed the identification of twelve compounds with different retention times from the purified B2'O fraction of *O. africana* leaf extract. The major compounds identified in the purified B2'O fraction were 9,12-Octadecadienoic acid (Z,Z)-, which constituted 98.34847% belonging to the most intense peak, followed by E,E,Z-1,3,12-Nonodecatriene-5,14-diol (0.15081%), Ethanol,2-(9,12-Octadecadienyloxy)-, (Z,Z)- (0.57465%), 10,13-Octadecadienoic acid, methyl ester (0.1329%), 10-Octadecenoic acid, methyl ester (0.11585%), and 9,12-Hexadecadienoic acid, methyl ester (0.11205% (Table 2). The gas chromatographic analysis of purified B2"O fraction revealed seven peaks. The mass spectrometer analysis showed a parent peak at m/z 278 and fragments at m/z 223, 205, 149, 104, 57, and 29 (Figure 8). The major compound with a molecular formula of $C_{16}H_{22}O_{4,}$ appeared as a peak at scan time 25.376 min and this corresponded to the chemical compound, dibutyl phthalate. The characteristics of the seven compounds identified from B2"O purified fraction of the leaf

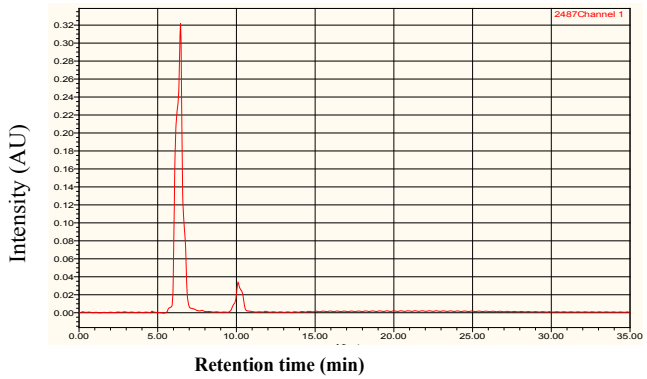

Figure 5: High performance liquid chromatograms for B2'O fraction of *O. africana*, detection at 254 nm.
Note: Chromatographic conditions: column, Tracer Extrasil ODS2, TR416059 (particle diameter, 5 µm; pore size, 30 nm; column size, 25 × 0.46 cm); eluent, methanol: water (80:20, v/v); flow rate, 1 ml/min; temperature, ambient.

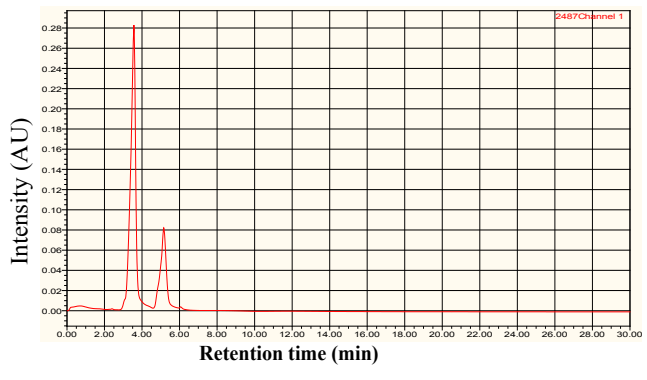

Figure 6: High performance liquid chromatogram for B2"O fraction of *O. africana* extract detected at 254nm.
Note: Chromatographic conditions are the same as in Figure 5.

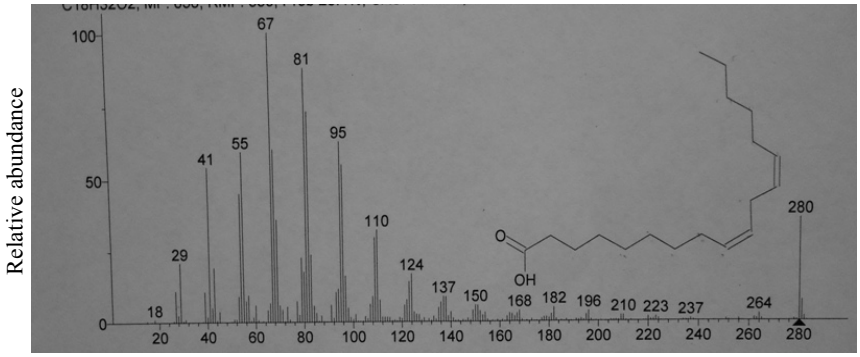

Figure 7: Mass spectrum from GC-MS scan revealing the 9,12-Octadecadienoic acid (Z,Z)- and fragmentation pattern of the peak that eluted at 27.94 min for purified fraction, B2'O from *Oreosyce africana* leaf extract.

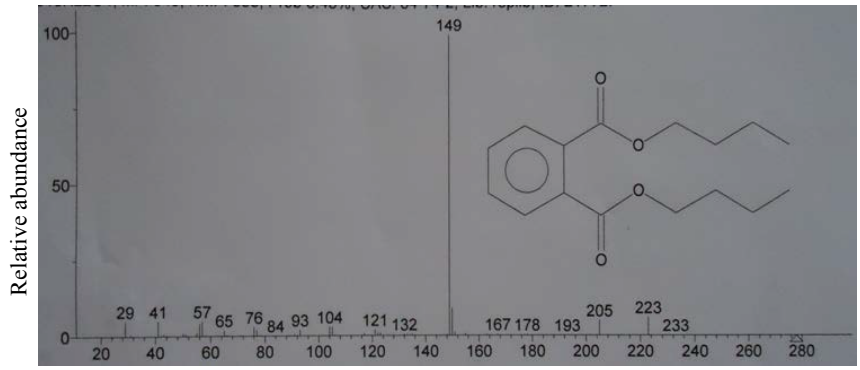

Figure 8: Mass spectra of the dibutyl phthalate and fragmentation patterns of the peak at scan time of 25.376 min for B2''O fraction isolated from *Oreosyce africana* leaf.

sS.No.	Retention time (min)	Name of the compounds	Molecular formula	Molecular weight	Peak area %[a]
1	2.476	9, 12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	98.34847
2	4.377	Isopropyl linoleate	$C_{21}H_{38}O_2$	322	0.06866
3	21.392	9,12-octadecadienoic acid(Z,Z),2-hydroxy-1-[hydroxymethyl)ethyl ester	$C_{21}H_{38}O_2$	354	0.00452
4	24.791	9, 12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308	0.00736
5	27.923	E,E,Z-1,3,12-Nonodecatriene-5,14-diol	$C_{19}H_{34}O_2$	294	0.15081
6	30.382	Ethanol,2-(9,12-Octadecadienyloxy)-, (Z,Z)-	$C_{20}H_{38}O_2$	310	0.57465
7	32.078	10,13-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	0.1329
8	32.681	cis-13,16-Docasadienoic acid, methyl ester	$C_{23}H_{42}O_2$	316	0.03463
9	33.067	9,12-Octadecadienoic acid (Z,Z)-, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	0.07472
10	34.839	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.11585
11	36.663	9,12-Octadecadienoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	0.37539
12	37.265	9,12-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	266	0.11205

[a] Relative percentage of total sample.

Table 2: Compounds identified in the isolated fraction of B2'O of *Oreosyce africana* by GC-MS.

extracts of *O. africana* by GC-MS analysis, including the retention time (R_t), molecular formula, molecular weight and the approximate relative percentages of this B2"O fraction are presented in Table 3. Thus, In GC-MS analysis of the B2"O fraction seven compounds were identified, among which, dibutyl phthalate constituted the largest proportion (97.7535%) followed by Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester (0.38466%), Phthalic acid, butyl tetradecyl ester (0.32408%), and 1,2-Benzenedicarboxylic acid, butyl octyl ester had the least proportion of 0.053% (Table 3).

Discussion

The biological activity of the secondary metabolites from *O. africana* extracts might be due to a variety of compounds in the plant. These compounds may jointly or independently contribute for the adulticidal effects against *An. arabiensis* adults. The enhanced adulticidal activity of purified fraction of *O. africana* compared to their respective crude extracts and semi-purified fractions is evidence that purification of plant extracts would significantly improve the bioefficacy of the active

	Retention time (min)	Name of the compound	Molecular formula	Molecular weight	Peak area %[a]
1	2.506	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	97.7535
2	4.399	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	334	0.053
3	27.891	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	$C_{19}H_{25}NO_5$	347	0.38466
4	29.796	Phthalic acid, butyl dodecyl ester	$C_{24}H_{38}O_4$	390	0.05628
5	30.344	Phthalic acid, butyl tetradecyl ester	$C_{26}H_{42}O_4$	418	0.32408
6	32.795	Phthalic acid, butyl nonyl ester	$C_{21}H_{32}O_4$	348	0.30852
7	34.515	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	0.30972

[a] Relative percentage of total sample.

Table 3: Compounds identified in the isolated fraction of B2"O from *O. africana* by GC-MS.

constituents in the extract. Accordingly, the HPLC method enabled assessment of the levels of the active components of *O. africana* extract purified fractions, B2'O and B2"O, obtained from prep. TLC. The linoleic acid, which was determined by the GC-MS analysis in the B2'O fraction has previously been shown to possess antibacterial potency. Evidence for this comes from investigations on the extracts of the plant *Schotia brachypetal,* in which linoleic acid was the major antibacterial constituent [27]. In addition, the bioactivities of long chain unsaturated fatty acids, such as linoleic acid, have been reported from bioassays against bacteria [28]. Furthermore, the broad spectrum bioactivity of linoleic acid is also evident from its inhibition of parasitemia in mice infected with *Plasmodium vinckei* and *Plasmodium yoelii* in a 4-day suppressive test [29].

The broad bioactivity of the leaf extracts of *O. africana* reported by several workers [25,30,31], characteristics such as their antiinflammatory, hypocholesterolemic, anticancer, hepatoprotective, nematicidal, insectifuge, antihistaminic, antieczemic, antiacne, 5-α reductase inhibitor, antiandrogenic, antiarthritic and immune enhancing may be explained by the presence of various low quantity compounds, which were identified using the GC-MS in the present work. Additional evidence for the insecticidal effects of linoleic acid also comes from *Citrullus colocynthis* (L.) Schrad (Cucurbitaceae) extract that had potent larvicidal activity against fourth instar larvae of *An. stephensi* Liston with LC_{50} value of 9.79 ppm and LC_{90} value of 37.42 ppm [32]. Sayed et al. [33] and Basalah et al. [34] had isolated and identified linoleic acids from *Citrullus colocynthis* spectroscopically. Another study by Edriss et al. [35] had also shown the presence of linoleic acid in the seed extracts of *Jatropha curcas*, a plant known to have larvicidal effects against *An. arabiensis* [36]. Likewise, evidence for dibutyl phthalate, the major B2"O chemical component, detected by GC-MS as a mosquitocidal compound, exists whereby the substance isolated from *Ipomoea carnea* stem extracts that possessed larvicidal activities [37] and from *Urtica dioica* leaf extract that showed antimicrobial activity [38]. The identification of six additional minor compounds from *O. africana* leaf extract would most likely be responsible for the broad spectrum of bioactivity reported by several workers such as antimicrobial and antifouling activities [39]. The fact that percentage mortality of *An. arabiensis* adults increased significantly with exposure time in the cone bioassay test of purified fractions of (B2'O and B2"O), indicates the exposure-time dependence of their lethal effect. Maharaj et al. [40] in a cone bioassay test with dichloromethane extracts of *Ptaeroxylon obliquum* (Ptaeroxylaceae) and *Pittosporum viridiflorum* (Pittosporaceae) against *An. arabiensis* had also found that the more than 50% adult mortality could be achieved in an exposure time dependent manner. In the use of mosquito nets for protection against malaria, physical barrier provided by the nets, usually needs to be supplemented by a chemical barrier consisting of a long-lasting deposit of insecticides on the netting. However, as the most commonly used insecticides are inorganic chemicals that cause concern as environmental hazards, the need for their replacement with organic insecticides, such as B2'O and B2"O, isolated in the present study from *O. africana*, may have to be considered. Similar findings from botanical sources, whereby the phenylpropenoid compounds from *Uvariodendron pycnophyllum* with long term mortality effects to adult *An. gambiae* on impregnated mosquito nets had been suggested as an alternative material for net impregnation [41]. Ramos-López et al. [42] reported that the linoleic acid isolated from *Ricinus communis* exhibited insecticidal activity against *Spodoptera frugiperda*. Furthermore, linoleic acid obtained from extract of *Dirca palustris* had insecticidal activity against *Aedes aegypti* [43]. Treating the nets with active ingredients of *O. africana* is relatively cheap and they are appropriate for an integrated, sustainable community based primary health care program. Therefore, the use of the products of *O. africana* an indigenous plant to Ethiopia for the impregnation of mosquito nets must be seriously considered in malaria control and elimination effort currently in place. This is particularly relevant because of the ever increasing failure of the conventional residual sprays with DDT or other insecticides for malaria vector control in Ethiopia [4,7].

Conclusions

The results of this study indicated that the plant-based compounds such as octadecadienoic acid and dibutyl phthalate have a potential to be developed as an effective alternative to conventional synthetic insecticides for the control of mosquitoes. The use of active compounds isolated from *O. africana* can be used for mosquito net impregnation for adulticidal activity against mosquitoes and can reduce man-vector contact leading to transmission of malaria. The fact that the purified fractions of *O. africana* (B2'O and B2"O) were more potent than the standard WHO recommended 0.05% lambdacyhalothrin, in their toxicity to adult *An. arabiensis* mosquitoes, is a good indication of their potential for use as mosquito control agents.

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