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RESEARCH ARTICLE

LARVICIDAL EFFICACY OF FRACTIONATED ESSENTIAL OILS OF *TAGETES MINUTA*, *OCIMUM KILIMANDSCHARICUM*, AND *ARTEMISIA AFRA* AGAINST *ANOPHELES GAMBIAE* S.S.

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Abstract

The growing concern over environmental ills caused by synthetic insecticides employed to control malaria vectors has renewed interest in the search for ecofriendly alternatives. This study evaluated the larvicidal efficacy of fractions obtained from essential oils (EOs) of *Tagetes minuta*, *Ocimum kilimandscharicum* and *Artemisia afra* against *Anopheles gambiae* s.s. A total of 23, 14 and 7 fractions were isolated from EOs of *T. minuta* (coded 1 to 23), *O. kilimandscharicum* (coded A to N) and *A. afra* (coded X-1 to X-7), respectively. Preliminary tests were done to eliminate fractions with less potent compounds from which one fraction (2), two fractions (A and F), and two fractions (X-1 and X-2) were selected for subsequent bioassays. Chemical analysis of the five fractions with potent compounds was done using Gas Chromatography-Mass Spectrometry (GC-MS) with the major constituents being β-ocimene (56.29%) in fraction 2, α-cubebene (29.39%) in fraction A, β-caryophyllene (31.48%) in fraction F, and β-thujone (37.41%) in fraction X-1, and ethyl-2-octynoate (40.80%) in fraction X-2, respectively. Data obtained were subjected to probit analysis using STATISCA 6.0 statistical software to model the relationship between the response variables and factors. Fraction F was the most efficacious under laboratory conditions, followed by X-1, A, X-2, and 2, respectively. A similar trend of larval mortalities was observed under simulated field conditions (SFC) with significantly higher ($P \leq 0.05$) mortalities recorded under SFC than under laboratory conditions. The outcome in all the tests performed were both dose and time depended. Two fractions, Fraction F- a slightly polar eluate from *O. kilimandscharicum*, and Fraction X-1- a nonpolar eluate from *A. afra*, exhibited high potency and should be explored further with the aim of formulating commercially viable larvicides.

Introduction:-

Malaria is a major global public health problem and a leading cause of morbidity and mortality in many countries (WHO, 2013). Many approaches have been developed to control the mosquito vectors (Sanei-Dehkordi, *et al*., 2016) but despite intensive efforts, the disease incidence continues to proliferate and cause enormous mortality and

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debilitation rates (Cuthbert, *et al*., 2019) with huge economic losses (Afolabi, *et al*., 2018) in the resource-limited settings of Africa, Asia, Latin America, and beyond (Karunamoorthi, *et al*., 2014). Chemical control using long lasting insecticide treated nets (LLINs) remains the most effective tool for intervention, with pyrethroids being the only class, out of the four conventional insecticides, recommended for use in LLINs by WHO (Kisinza, *et al*., 2017; WHO, 2018; Zoh, *et al*., 2018).

The ever-changing climatic conditions and complex social structures (Cuthbert, *et al*., 2019), and the development of multiple resistances by mosquito vector species to synthetic insecticides of different classes currently in use (Uragaya, *et al*., 2015; WHO, 2018), pose a bigger challenge in combating the disease (Pavela, *et al*., 2019). Prevention and control efforts of human malaria, according to WHO (2018), are threatened by the resistance of the malaria vectors to pyrethroids that are used in all insecticide treated mosquito nets (ITNs), and to the four insecticide classes used for indoor residual spraying (IRS).

Anopheles gambiae s.s., the major Afrotropical malaria vector, is predominantly associated with the most virulent malaria parasite, *Plasmodium falciparum* (Zoh, *et al*., 2018), whose multi-drug resistance to artemisinin combined therapy (ACT), the current drugs of choice, has been reported from many parts of the world (WHO, 2018; Hamilton, *et al*., 2019). Camara, *et al*. (2018), reported that *An. gambiae* mosquitoes had developed widespread and strong resistance to organochlorines and pyrethroids, and moderate level resistance to organophosphates. Lynd, *et al*. (2018), and Okia *et al*. (2018), detected a high frequency of phenotypic resistance to two pyrethroids, deltamethrin and permethrin, and an organochlorine, dichlorodiphenyltrichroethane (DDT) in *An. gambiae* s.s. This rapid evolution of insecticide resistance phenotypes was attributed to a selective pressure with multiple origins, the most significant being exposure to synthetic insecticides used in vector control (Camara, *et al*., 2018), which according to Lynd *et al*. (2018), and Okia *et al*. (2018), provides an enormous challenge for LLIN-based vector control programmes.

It has also been reported that during the period 2010-2017, insecticide resistance existed in 68 of the 80 endemic countries to at least one insecticide in one malaria vector from one collection site, with three countries reporting resistance for the first time, and the detected resistance to each class being; 82% for organochlorines, 82% for pyrethroids, 63% for carbamates, and 50% for organophosphates (WHO, 2018). According to Uragaya *et al* (2015), there are limited options for management of insecticide resistance prompted by conventional insecticides, necessitating the search for ecofriendly alternatives for effective control of the malaria vectors.

Phytochemicals and their derivatives have been considered potential suitable replacements to synthetic insecticides (Sanei-Dehkordi, *et al*., 2016), with major research efforts directed towards investigating their insecticidal activity against various developmental stages of the mosquito vectors in a bid to reduce the frequent over reliance on conventional insecticides (Pavela, *et al*., 2019). Elsewhere, products from *Tagetes minuta* (Asteraceae), *Ocimum kilimandscharicum* (Lamiaceae) and *Artemisia afra* (Asteraceae) have yielded promising efficacy against a number of organisms including the malaria vectors when used alone (Bekele, 2018). The current study sought to evaluate the efficacy of fractions isolated from essential oils (EOs) of *T. minuta*, *O. kilimandscharicum* and *A. afra*, against third instar larvae of *An. gambiae* s.s. under laboratory and simulated field conditions.

Methodology:-

The study was carried out at the Centre for Global Health Research (CGHR) CDC-KEMRI located about 10 Km from Kisumu City along the Kisumu-Busia road. The station lies on the coordinates 0°4'40"N 34°40'38"E. Kisumu City, in western Kenya, is an area endemic for major infectious diseases including malaria.

Research Design

This study employed an experimental research design and followed the post-test only assessment. Two groups were randomly selected, the experimental and control groups, with the latter comprising of positive and negative controls. The positive controls contained the recommended 1 g/L of Bi-Larv^{TM} 25 WP (a registered trademark product of Bayer Pharmaceutics Company in wettable powder containing 25% diflubenzuron, an insect growth regulator (IGR) as the active ingredient, and is listed under WHO Pre-Qualification (WHO-PQ) scheme) while the negative controls contained 1 mL acetone in 250 mL solution.

Harvesting and Identification

Fresh full-bloom aerial parts of *T. minuta*, *O. kilimandscharicum*, and *A. afra*, were collected from the confines of the University of Eldoret (UoE) located 11 km North of Eldoret town in Uasin Gishu County, Kenya. The town is about 320 km Northwest of Nairobi and lies between 0º15.5'N and 0º20'N latitudes, and on longitude 35º19'E. The altitude of the area is 2080 metres above sea level. Samples of the plant materials were deposited at the UoE's herbarium for identification. The three plants were identified as *Tagetes minuta*, voucher number (VN): MU/0003/87, *Ocimum kilimandscharicum*, VN: MU/0033/89, and *Artemisia afra*, VN: MU/0107/87. The freshly collected plant materials were taken to the laboratory for processing.

Extraction of Essential Oils

The plant materials were chopped into small pieces and stuffed into a round-bottomed flask. EOs of each plant species were extracted separately by steam distillation following the methods described by (Pavia, *et al*., 1982; Baser and Buchbauer, 2010). For each plant species, 500 grams of chopped fresh plant material was stuffed separately into a 1000 mL conical flask which was tightly corked with a two-delivery tubes fitted cork, one connected to 1000 mL round bottomed flask containing boiling water (source of steam), and the other connected to a din and stack connected to a Liebig condenser. Steam from the boiling water in the round bottomed flask, heated using a Bunsen burner, was passed through the plant material in order to vaporize the EOs. During the distillation process, a continuous stream of cold water was run through the condenser to liquefy the vaporized EOs. The EOs obtained were dried using anhydrous calcium chloride and stored in tightly corked glass vials at 4^0C awaiting further use.

Fractionation of Essential Oils by Column Chromatography

50g of EOs of *T. minuta*, *O. kilimandscharicum*, and *A. afra* were separately weighed accurately using an analytical balance of 0.001g sensitivity then each was mixed with 50g accurately weighed silica gel. The mixtures were thoroughly stirred using pestle and mortar and the resulting homogeneous pastes left to stand for 48 hours in a dry place at room temperature and pressure (rtp) for them to dry.

Each of the dry mixtures was packed in a column using slurry method as described by Pavia *et al*, (1982) and Baser and Buchbauer, (2010), with hexane as the eluent. The sample mixtures were carefully added separately to the column then a piece of cotton wool was placed on top of the sample to prevent disturbance of the sample layer during addition of the eluent and to obtain horizontal bands during elution. The polarity of the moving phase (eluent) was increased from non-polar (100% hexane: 0% ethyl acetate) to polar (0% hexane: 100% ethyl acetate) by increasing the polarity at 5% intervals while collecting the eluates in clean dry test tubes. Polarity was further increased using methanol at the same intervals from 100% ethyl acetate to 100% methanol (high polar) with the last concentration added twice to elute high polar compounds.

The compounds in the different eluates were determined using thin layer chromatography (TLC) by carefully spotting them on TLC plates as described by Baser and Buchbauer, (2010). The TLC plates were then immersed in developing solvent mixtures containing ethyl acetate and hexane, at different ratios (2:8, 3:7, and 4:6, respectively) containing two drops of acetic acid to prevent tailing. The TLC plates were removed from the developing solvent when the solvent fronts had moved about ¾ of the distance, and the solvent front marked immediately (Pavia *et al*, 1982). The TLC plates were then immersed in a visualizer, containing 5% sulphuric acid in methanol for 30 seconds, then left to dry before viewing the compounds using an ultra violet (UV) lamp (Pavia *et al*, 1982). The locations of the compounds were marked using a pencil for calculation of retention factor (R_f) values.

The fractions obtained at different polarities were coded differently for each plant with those obtained from *T. minuta* laboratory coded 1 to 23, with 1 comprising of non-polar eluates while 23 comprised of high polar eluates. Fractions obtained from *O. kilimandscharicum* were coded A to N, with A comprising of non-polar eluates while N comprised of high polar eluates. Fractions obtained from *A. afra* were coded X-1 to X-7, with the former comprising of non-polar eluates and the latter comprising of high polar eluates. The contents of test tubes with similar R_f values were mixed together and then concentrated by vacuum distillation using a Rotavapor at 40°C.

Laboratory Rearing of Anopheles gambiae s.s.

100 three-day old females of *An. gambiae* s.s. Kisumu strain were obtained from the insectary at the CGHR CDC-KEMRI, Kisumu and provided with a blood meal from a laboratory rabbit that was sheared on the back. Twenty fully engorged blood fed females were put into rearing cages measuring 30 cm x 30 cm x 30 cm. 30 mL plastic cups

containing oviposition substrate were provided in the cages. Laid eggs were transferred to 500 mL white plastic trays half-filled with dechlorinated water. Freshly hatched larvae were fed on a sprinkle of larval food comprising of a mixture of ground dog biscuits and brewer's yeast, and the ration was gradually increased as larvae increased in size. The rearing water was replenished after every 48 hours to prevent fouling and larval mortalities. Up to 100 pupae were transferred to 100 mL plastic cups containing rearing water and put into the rearing cages for adult emergence.

For colony maintenance, adult males and females were kept together and provided with ad libitum 10% sucrose solution, while females were provided with blood meal from a sheared rabbit following the method used by Amerasan, *et al*., (2012). The larvae used in both laboratory and SFC experiments were obtained from the reared colony, which was maintained at a temperature of 27±2°C, relative humidity (RH) of 75±5%, and a 12L:12D (Light: Dark) photoperiod.

Bioassays under Laboratory Conditions

1% stock solutions of all the 44 fractions obtained from column chromatography were prepared from which serial dilutions were done following WHO (2005) protocol. 200 ppm test solutions of each fraction were prepared in 250 mL volumes contained in 500 mL white plastic trays, unto which thirty third instar larvae of *An. gambiae* s.s. were exposed for 48 hours to eliminate those with less potent compounds. Each setup comprised of triplicate sets of the same fraction and the tests were repeated three times. Larval food was added to each test solution every morning. Fractions, whose solutions caused at least 10% larval mortality within 48 hours, were selected and used in subsequent bioassays.

Fraction 2, obtained from non-polar eluates (100 - 90% n-hexane: 0 - 10% ethyl acetate) of *T. minuta* , fractions A and F obtained from non-polar eluates (100 - 95% n-hexane: 0 - 5% ethyl acetate) and slightly polar eluates (90 - 85% n-hexane: 10 - 15% ethyl acetate) of *O. kilimandscharicum*, and fractions X-1 and X-2 obtained from non-polar eluates (100 – 95% n-hexane: 0 – 5% ethyl acetate) slightly polar eluates (95 - 90% n-hexane: 5 - 10% ethyl acetate) of *A. afra*, respectively, were selected. Eight different ppm concentrations of the five fractions, ranging from 25 to 200 ppm at intervals of 25 ppm, were prepared and their efficacy tested against third instar larvae of *An. gambiae* s.s. alongside positive (1 g/L Bi-Larv^{TM} 25 WP) and negative (1 mL acetone) controls in 250 mL solutions, under laboratory conditions. Thirty larvae were exposed to each treatment.

Bioassays under Simulated Field Conditions

Simulated field conditions (SFC) trials were done, following the WHO protocol on pesticide evaluation (WHO, 2005), using the fractions that had been selected under laboratory conditions. Sixty 5L basins were placed in rows that were one by one metre apart. The basins were smeared with clay soil on the inside to near brim and filled with 1000 mL of dechlorinated water, marked and left overnight. In the day that followed, dechlorinated water was filled to mark and left to settle before a batch of 50 laboratory-reared early third instar larvae of *An. gambiae* s.s. was released into each container and a sprinkle of larval food added. After 3 hours of larval acclimation, the set-ups were treated with different doses of the selected fractions in a completely randomized manner using micro-pipettes.

The containers were covered with a mosquito netting material to prevent other mosquitoes and/or insects from laying eggs and to protect the water from falling debris. Live larvae were counted after every 24 hours to score post-treatment larval mortality. The water level in the containers was maintained to mark for 72 hours. The remaining larvae and/or pupae were destroyed after 72 hours and the smeared mud discarded and replaced with fresh mud before the next trial. The test trials were repeated five times. The positive control contained the recommended 1 g/L concentration of Bi-Larv^{TM} 25 WP while the negative control contained 4 mL of acetone in 1000 mL solution.

Chemical Analysis of Fractions with Potent Compounds

Sample Preparation

A stock solution of 1mg/ml of each sample was prepared and to it 1µl (100pg/µl) of internal standard, 1-heptene was added followed by 50 mg of Na_2SO_4 (drying agent). The samples were vortexed for 1 min, extracted by ultrasonication in sonication bath (Branson 2510, Danbury, CT, USA) for 10 min, centrifuged at 13,000 rpm for 5 min at 5°C and the supernatant filtered by passing through glass wool each before analysis by GC-MS. The analysis was replicated 3-times.

Gas Chromatography Coupled Mass Spectrometry (GC-MS) Analysis

GC-MS in full scan mode was used to detect and profile all the compounds present in the extracts. The extracts were analyzed by GC-MS on a 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) linked to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) by using the following conditions: inlet temperature 270°C, transfer line temperature of 280°C, and column oven temperature programmed from 40°C to 285°C with the initial temperature maintained for 5 min then 10°C/min to 280°C held at this temperature for 10.5 min and finally 50°C/min to 285°C and held at this temperature for 31.9 min. The GC was fitted with a HP-5 MS low bleed capillary column (30 m× 0.25 mm i.d., 0.25 µm) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 ml/min served as the carrier gas. The mass selective detector was maintained at ion source temperature of 230°C and a quadruple temperature of 180°C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. A 1.0 µl aliquot of extract was injected in the split/ splitless mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40–550 m/z mass range in the full scan mode. The filament delay time was set at 5 min.

The compounds were identified by comparison of gas chromatographic retention time and fragmentation pattern with that of the authentic standards. When there was lack of corresponding reference compounds, the structures were proposed on the basis of their general fragmentation and using reference spectra published by library–MS databases: National Institute of Standards and Technology (NIST). The peak area of the internal standard, 1-heptene was used for quantification.

Data analysis:

The bioassays performed against *An. gambiae* s.s. during this study consisted of mortality and survival, all considered as response variables against varying concentrations of the fractions. The response variables were binary in nature (died/survived) and naturally followed a binomial distribution (Agresti, 1990). Therefore, the relationship between the response variables and factors were modeled using Probit analysis using STASTICA 6.0 statistical software. Maximum likelihood was used to estimate the regression coefficient (R square) because it gives more precise estimation of necessary parameters for correct evaluation of the results (Finney, 1952). During Probit analysis, all data were transformed to $\log_{10}$ to linearize the relationship between the response variables and factors. The response frequency was observed as response variables from a total observation of 30 larvae of *An. gambiae* s.s., while the concentrations of the fractions were covariate.

The resulting probability outcomes were multiplied by 100 to determine the expected percentage of the response frequency. To test for the significance of the Probit plots, Z statistics were calculated; the larger the Z statistics the larger the differences from the smaller sized Z. Nevertheless, the differences were verified using P-value of ≤ 0.05. The modeled fit was confirmed using chi-square goodness of fit test between the observed response values and predicted probability of response values. The resultant graph plotted consisted of Probits of response variables in Y-axis and $\log_{10}$ concentration in X-axis.

The LC_{50} and LC_{90} were determined by projecting the Y axis for a probit of 5.00 and 9.00 and taking the inverse $\log_{10}(X)$ of the concentration of the fractions. The LC_{50} and LC_{90} were then compared using One Way Analysis of variance (One-Way ANOVA) to test the efficacy of different fractions. Where significant differences existed following ANOVA, means were separated using Duncan's Multiple Range Test (DMRT). In all analyses, significant differences were declared at $P \leq 0.05$.

Results:-

Based on the laboratory bioassays using different fractions, it was observed that larval mortalities followed a dose-response pattern, with lower mortality recorded at lower exposure doses and higher mortality at higher exposure doses (Figure 1). Among the five fractions tested, larvicidal efficacy was highest in treatment solutions containing Fraction F, LC_{50} 58.75 ppm and 37.40 ppm while the least efficacy was observed in Fraction 2 with LC_{50} 81.50 ppm and 66.25 ppm after 24- and 48- hours of exposure, respectively.

During the experiment it was established that the differences in the LC_{50} and LC_{90} values of the mosquito larvae subjected to the different fractions from *T. minuta*, *O. kilimandscharicum*, and *A. afra* were significant ($P \leq 0.05$). Fraction F had the lowest concentration required to kill 50% and 90% of the *An. gambiae* s.s. larvae, followed by fractions X-1, A, X-2 and 2, respectively. Fractions X-2 and 2 showed lower efficacy and were unable to kill up to

90% of the mosquito larvae after 24- and 48-hours of exposure in concentrations of up to 200 ppm (Table 1). There was a significant difference ($P \leq 0.05$) between the efficacy of fractions and the positive control. The trend in larvicidal activity observed when the various fractions 2, A, F, X-1 and X-2 were tested against *An. gambiae* s.s., larvae alongside positive control was: Positive control > Fraction F > Fraction X-1 > Fraction A > Fraction X-2 > Fraction 2.

Table 1: LC_{50} and LC_{90} values of mortalities of *An. gambiae* s.s. larvae caused by the fractions isolated from EOs of *T. minuta*, *O. kilimandscharicum* and *A. afra* after 24- and 48- hours exposure periods

	Exposure time			
	24 h		48 h	
Treatments	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Fraction X-1	64.10^{c}	Na	43.80^{c}	85.80^{c}
Fraction A	68.20^{d}	Na	54.90^{d}	Na
Fraction F	58.75^{b}	91.80^{b}	37.40^{b}	75.80^{b}
Fraction X-2	72.50^{e}	Na	60.80^{e}	Na
Fraction 2	81.50^{f}	Na	66.25^{f}	Na
Positive control	53.75^{a}	84.20^{a}	31.50^{a}	69.40^{a}
F-value	134.9730	886.0000	973.0800	5124.0000
P-value	<0.001	<0.001	0.0005	<0.001

Values with different superscripts along the column are significantly different ($P \leq 0.05$). Na denotes not attained.

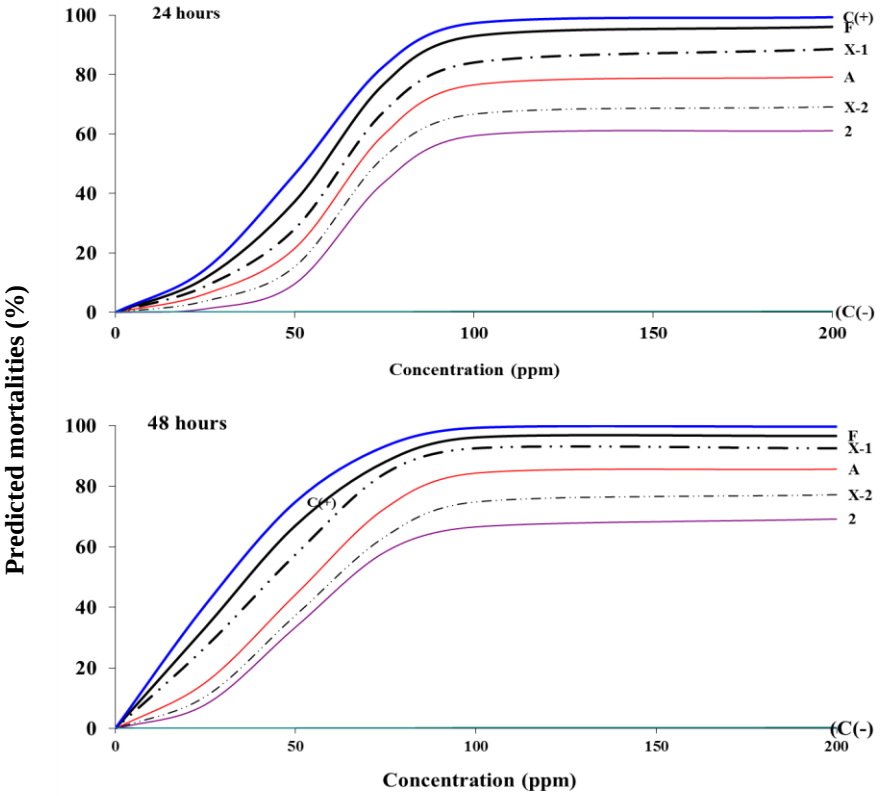

Figure 1: Mortalities of *An. gambiae* s.s. larvae caused by fractions isolated from EOs of *T. minuta*, *O. kilimandscharicum*, and *A. afra* after 24- hours and 48- hours of exposure.

Key:
C (+) – Positive Control (containing1 g/L Bi-Larv™ 25 WP)
C (-) – Negative control (containing 4 mL/L acetone)
F – Fraction F (containing 31.5% β-Caryophyllene)
X-1– Fraction X-1 (containing 37.4% β-thujone)

A – Fraction A (containing 29.3% α-Cubebene)
X- 2 – Fraction X-2 (containing 40.8% Ethyl-2-octynoate)
2– Fraction 2 (containing 56.3% β-ocimene)

Larvicidal activity of Fractions against *An. gambiae* s.s. Larvae under SFCs

The mortality trends observed under SFC experiments were similar to those observed under laboratory conditions and exhibited dose- and time-dependent patterns (Figure 2). However, the mortality rates were significantly higher under SFCs compared to those observed under laboratory conditions. The most effective fraction, with the lowest concentration required to kill 50% and 90% of *An. gambiae* s.s. larvae was Fraction F with LC_{50} values of 40.00 ppm and 29.20 ppm, while the least effective treatment solution contained Fraction 2 which was unable to cause 50% mortality of *An. gambiae* s.s. larvae at concentrations of up to 200 ppm after 24 and 48 hours of exposure, respectively.

There were significant differences ($P \leq 0.05$) in the LC_{50} and LC_{90} values obtained when *An. gambiae* s.s. larvae were subjected to different fractions from EOs of *T. minuta*, *O. kilimandscharicum* and *A. afra*, alongside positive and negative controls, under SFCs (Table 2). Of the fractions tested, Fraction F had the least concentration required to cause 50% and 90% mortalities of *An. gambiae* s.s. larvae, respectively. Only two fractions, Fraction F and Fraction X-1, were able to cause up to 90% larval mortalities after both 24- and 48- hours of exposure while fractions A, X-2, and 2 were unable to cause up to 90% mortalities in treatment solutions of up to 200 ppm in both of the exposure durations.

The trend in larvicidal activity observed when the various fractions of EOs extracted from *T. minuta*, *O. kilimandscharicum*, and *A. afra* were tested against larvae of *An. gambiae* s.s., alongside the positive control was: Positive control > Fraction F > Fraction X-1 > Fraction A > Fraction X-2 > Fraction 2.

Table 2: LC_{50} and LC_{90} values of larval mortalities of *An. gambiae* s.s. caused by fractions isolated from EOs of *T. minuta*, *O. kilimandscharicum* and *A. afra* after 24- and 48- hours of exposure under SFCs.

	Exposure time			
	24 h		48 h	
Treatment	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Fraction X-1	46.20^{c}	90.80^{c}	37.70^{c}	83.80^{c}
Fraction A	68.20^{d}	Na	46.20^{d}	Na
Fraction F	40.00^{b}	76.90^{b}	29.20^{b}	60.10^{b}
Fraction X-2	Na	Na	Na	Na
Fraction 2	Na	Na	Na	Na
Positive control	35.40^{a}	69.30^{a}	21.50^{a}	50.80^{a}
F-value	686.9039	269.6925	168.7421	400.5494
P-value	<0.001	<0.001	<0.001	<0.001

Values with different superscripts along the column are significantly different ($P \leq 0.05$). Na denotes not attained.

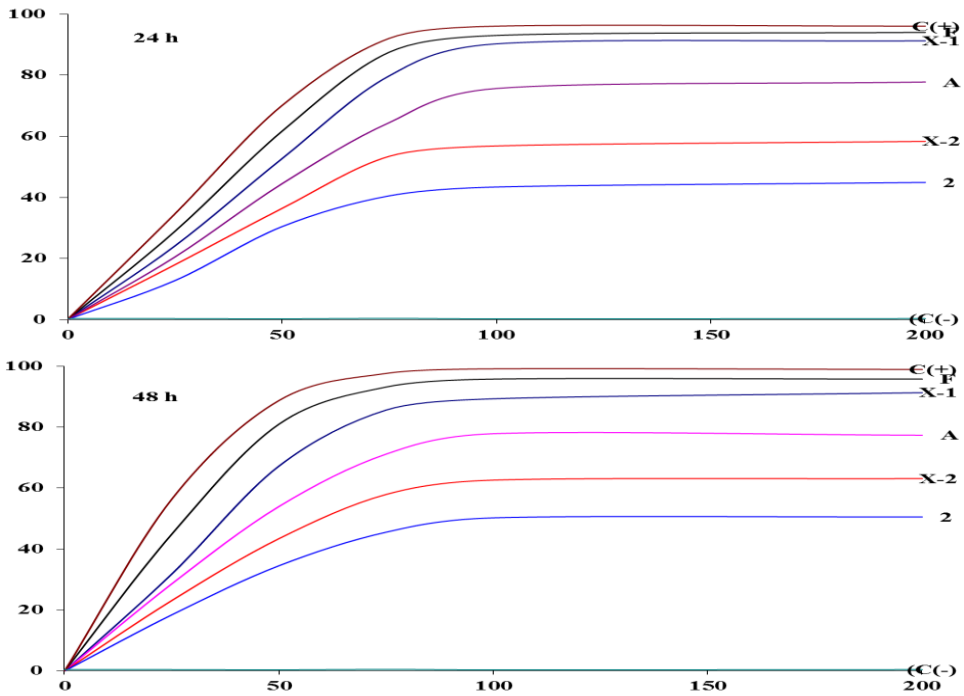

Figure 2: Mortalities of *An. gambiae* s.s. larvae caused by fractions isolated from EOs of *T. minuta*, *O. kilimandscharicum* and *A. afra* after 24- and 48- hours of exposure under SFCs

Key:
C (+) – Positive Control (containing1 g/L Bi-Larv^{TM} 25 WP)
C (-) – Negative control (containing 4 mL/L acetone)
F–Fraction F (containing 31.5% β-Caryophyllene)
X-1– Fraction X-1 (containing 37.4% β-thujone)
A – Fraction A (containing 29.3% α-Cubebene)
X- 2 – Fraction X-2 (containing 40.8% Ethyl-2-octynoate)
2 – Fraction 2 (containing 56.3% β-ocimene)

A total of seven compounds, constituting about 96.62% of the compounds present in Fraction 2, were identified from the GC-MS analysis (Table 3). The major compounds identified in the fraction were β-ocimene (56.29%), 3-methyl-2-hexanol (17.87%), and 4-Octen-3-one (8.39%).

Table 3: Compounds identified in Fraction 2, obtained from EO of *T. minuta*

RT (min)	Compound name	Concentration (ng/mg)	Percentage composition
4.74	Methyl Isobutyl Ketone	2.76±0.01	1.71749
8.24	3-methyl-2-hexanol	29.85±1.02	17.87425
10.07	4-Octen-3-one	13.49±0.01	8.39452
12.52	β-ocimene	90.46±1.01	56.29123
16.50	1,2-dihydro-1,4,6-trimethyl-naphthalene	6.44±0.52	4.00747
20.94	α-Bisabolol	6.59±0.27	4.10081

Ten compounds, constituting about 95.29% of the compounds present in Fraction A, were identified from the GC-MS analysis (Table 4). The major compounds identified in the fraction were α-cubebene (29.40%), 5,8-dimethyl-quinoline (27.02%), and longifolene (10.04%).

Table 4: Compounds identified in Fraction A, obtained from EO of *O. kilimanscharicum*

RT (min)	Compound name	Concentration	Percentage

		(ng/mg)	composition
11.49	α-Terpinene	13.44±0.21	2.64566
12.29	γ-Terpinene	1.67±0.03	0.32874
16.77	Longifolene	50.99±1.03	10.03740
17.15	α-Cubebene	149.24±2.30	29.39961
17.58	8,9-dehydro-cycloisolongifolene	36.46±0.56	7.17717
19.32	5,8-dimethyl-quinoline	137.35±1.27	27.01575
20.13	Humulene II epoxide	39.59±1.87	7.79331
20.83	Isoaromadendrene epoxide	42.51±2.31	8.36811
21.23	Isolongifolene, 9,10-dehydro-	11.65±0.21	2.29331
23.63	Guaiol	1.00±0.01	0.19685

A total of eight compounds, constituting about 96.54% of the compounds present in Fraction F, were identified from the GC-MS analysis (Table 5). Major compounds identified in Fraction F were β-caryophyllene (31.45%), β-thujone (18.37%), caryophyllene oxide (11.73%), α-terpineol (11.52%), and Terpinen-4-ol (11.41%).

Table 5: Compounds identified in Fraction F, obtained from EO of *O. kilimandscharicum*

RT (min)	Compound name	Concentration (ng/mg)	Percentage composition
11.75	Eucalyptol	46.92±.14	3.60923
12.78	α-terpineol	149.74±3.47	11.51846
12.99	Linalool	102.32±2.68	7.87077
13.28	β-thujone	238.76±2.31	18.36615
14.29	Terpinen-4-ol	148.35±2.01	11.41154
16.91	3-Allyl-6-methoxyphenol	7.96±0.21	0.61230
17.76	β-Caryophyllene	409.36±1.57	31.48923
19.84	Caryophyllene oxide	152.46±0.87	11.72769

A total of seven compounds, constituting about 97.72% of the compounds present in Fraction X-1, were identified from the GC-MS analysis (Table 6). The major compounds identified in the fraction were β-thujone (37.41%), Terpinen-4-ol (21.92%), Thujol (13.34%), and α-terpineol (11.55%).

Table 6: Compounds identified in Fraction X-1, obtained from EO of *A. afra*

RT (min)	Compound Name	Concentration (ng/mg)	Percentage composition
11.75	Eucalyptol	59.27±3.21	2.24508
12.43	(Z)-β-terpineol	92.98±3.57	3.52197
13.10	Terpinen-4-ol	578.74±4.68	21.92197
14.20	α-terpineol	305.28±2.37	11.55303
14.31	β-thujone	987.67±3.78	37.41174
14.40	Thujol	352.68±1.34	13.33909
14.53	Cis-p-menth-2-en-7-ol	201.29±5.63	7.62462

Eight compounds, constituting about 96.80% of the compounds present in Fraction X-2, were identified from the GC-MS analysis (Table 7). The major compounds identified in the fraction were Ethyl-2-octynoate (40.81%), 4-propyl-1,6-heptadien-4-ol (26.17%), and 2-Allyl-4-methylphenol (14.62%).

Table 7: Compounds identified in Fraction X-2, obtained from EO of *A. afra*

RT (Min)	Compound Name	Concentration (ng/mg)	Percentage composition
9.08	2-ethyl-hexanal	6.34±0.52	1.19397
10.07	2-Pyrazoline, 1-isobutyl-3-methyl-	3.46±2.15	0.65160
14.10	(E)-2,3-Epoxydecane	28.84±3.28	5.43126
14.66	2,3,4-trimethyl- 2-Cyclopenten-1-one	34.81±3.58	6.55556
17.46	2-Allyl-4-methylphenol	75.73±0.54	14.61770
18.33	4-propyl-1,6-heptadien-4-ol	138.96±3.85	26.16949
18.47	Ethyl-2-octynoate	216.68±3.57	40.80603
20.96	Spiro[5.6]dodecane	9.14±3.52	1.72128

Discussion:-

The fractions with remarkably high larvicidal activity against third instar larvae of *An. gambiae* s.s. were obtained from the non-polar and slightly polar eluates of the respective EOs used in this study. This was in agreement with Pineda-Cortel, *et al*., (2019), who reported that the extraction of active biochemical compounds from plants depended on the polarity of the solvents used in extraction. Ghosh *et al*. (2012), also reported that different solvent types significantly affect the potency of extracted phytochemicals, and that moderately polar solvents such as ethyl acetate provided good bioassay results. Furthermore, AhbiRami *et al*. (2014), stated that insecticidal compounds are contained in various proportions in different polarity fractions, being higher in lower polarities. The findings of this study, therefore, were in conformity with those reported by Ghosh, *et al*., (2012), AhbiRami, *et al*., (2014), and Pineda-Cortel, *et al*., (2019), because the five most potent fractions in this study were isolated in the non-polar and slightly polar eluates during column chromatography of *T. minuta* , *O. kilimandscharicum* and *A. afra* EOs, respectively. However, their activities varied with plant species with the two most efficacious fractions being fractions F and X-1 isolated from EOs of *O. kilimandscharicum* and *A. afra*, respectively.

While the most potent fractions from *T. minuta* (Fraction 2) and *A. afra* (Fraction X-1) were obtained from the non-polar eluates, that of *O. kilimandscharicum* (Fraction F) was obtained from the slightly polar eluates. This implies that the most active compounds in the three plants had different molecular weights and solubilities, hence their elution at different polarities. In a previous study and while testing the biological activity of crude EOs of *O. kilimandscharicum* and *A. afra* against *Culex quinquefasciatus*, Runyoro, *et al*., (2010) reported that the former had higher bioactivity than the latter with 323.6 ppm and 457.1 ppm of the EOs required to induce 50% mortality of *Cx. quinquefasciatus* larvae. In the same study, it was also reported that 1000 µg of crude EO of *O. kilimandscharicum* exhibited an inhibition zone of 21.0 mm against *Candida albicans* while a similar quantity of *T. minuta* had a 12.5 mm inhibition zone.

Even though the LC_{50} values reported in current study show great disparity when compared with those reported by Runyoro, *et al*., (2010), the findings of both studies are in agreement that *O. kilimandscharicum* is a better source of bioactive compounds than *A. afra* and *T. minuta*. According to Pineda-Cortel, *et al*. (2019), variations in insecticidal activities of plant extracts could be attributed to several factors such as plant species, part of the plant used, age of the plant, and the targeted vector species. The great disparity is indicative of a higher concentration of active compounds in the fractions used in this study than in the crude EOs in which they could have been masked by the less active or completely inactive constituents.

The mortality trends observed in the semi-field experiments were similar to those in laboratory assays, being dosage- and exposure time-dependent. However, actual mortality rates were comparatively higher under semi-field conditions than those recorded under laboratory conditions in similar treatment concentrations of some fractions. This conformed to the findings of Kweka, *et al*., (2011) and Nyamoita, *et al*., (2013) with both groups attributing the higher larval mortalities under SFCs to contributions of other variables in the surroundings, a normal adaptive biodiversity output. According to Kweka, *et al*. (2011), the exposure of plant extracts to sunlight triggers degradation of phytochemicals to their secondary metabolites, to which higher larval mortalities under SFCs than laboratory conditions had been attributed. Besides, since the larvae used in this study were laboratory bred as opposed to wild forms, their mere introduction into a new set of habitat conditions could on its own make them more susceptible to the treatments to which they were subjected compared to their usual laboratory conditions.

The results of the current study, however, differed from those reported by Karunamoorthi, *et al*., (2014), who while testing the larvicidal efficacy of EOs from *Juniperus procera* against *An. arabiensis*, found larval mortality under laboratory conditions to have been significantly higher with LC_{50} and LC_{90} values of 14.42 and 24.65 mg/L, which were 10 mg/L lower than concentrations recorded under SFCs (LC_{50} and LC_{90} values 24.518 and 34.212 mg/L), respectively. However, while laboratory reared larvae were used under both laboratory and SFCs in this study, Karunamoorthi, *et al*., (2014) had used laboratory reared and wild larvae for laboratory and semi-field experiments, respectively. This could be the reason for disagreement in the findings between their study and the current one. Nevertheless, the authors had still found laboratory reared anopheline larvae to have been more susceptible than wild collected ones. On the other hand, the findings of this study were in line with those of Nyamoita, *et al*., (2013), who while testing the activity of *Vitex payos* against laboratory reared *An. gambiae* s.s larvae, found those under SFCs to have been more susceptible than the ones under laboratory conditions. In conclusion, the development of suitable formulations of biocides from EOs of *T. minuta, O. kilimascharicum*, and *A. afra* is feasible with fractions F from *O. kilimadscharicum* and X-1 from *A. afra* being more promising.

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Conflict of interest

The authors declare no conflict of interest.

Author's contribution

The 1st author participated in data collection and processing, and writing the manuscript, while the 2nd and 3rd authors participated in development of the research question and design, and correcting the manuscript.

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