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ETHANOLIC AND ESSENTIAL OIL EXTRACTS OF *CYMBOPOGON CITRATUS* (DC.) STAPF ARE TOXIC TO ADULT *ANOPHELES GAMBIAE* S.S

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Anopheles gambiae s.s, *C. citratus*, mosquitocidal agents, ethanolic extract, essential oil, toxicity, malaria

ABSTRACT

This study sought to determine the toxicity of the ethanolic and essential oil extracts of *Cymbopogon citratus* against adult stages of *Anopheles gambiae* s.s and to compare mortality rates resulting from the application of the two extracts. Five different concentrations of each extract were derived by serial dilution and these were applied to adult stages of the test subject respectively. A total of 720 two to five-day old laboratory reared adult *Anopheles gambiae* were exposed for a period of 24 hours to the varying concentrations of the plant extracts as well as positive and negative controls with the aid of the WHO kit method. Deltamethrin at 0.5% and Ethanol at 0.2% were employed as positive and negative control. The application of increasing concentrations of the essential oil extracts resulted to increasing mortality among test subjects with the highest mean mortality of 100% recorded against the two highest concentrations of 8 and 10 µg/ml and LC_{50} of 1ug/ml within 24 hours. Ethanolic extracts concentrations of the plants however demonstrated lower toxicity than the essential oil extracts with the highest mean mortality recorded as 86.50% at a concentration of 1 mg/ml and an LC_{50} of 1 mg/ml. Chi-square probability value of P = 0.222 recorded from probit analysis proved that mortality resulting from essential oil extract concentration is dose-dependent however, mortality resulting from the application of ethanolic extract of the plant is not dose-dependent as a probability value of P = 0.897 was obtained. This study proves that the ethanolic and essential oil *C. citratus* plant extracts are toxic to adult Anopheles gambiae s.s. It is recommended that further studies be carried out to determine the effect of the extracts on non-target organisms in the environment and to also investigate the susceptibility of other mosquito species to these extracts.

MAIN PAPER STARTS HERE…

INTRODUCTION

The application of residual insecticide sprays is the most common chemical method of controlling mosquito species. Nevertheless, insecticide resistance is a major threat to existing vector control methods because of a great deal of reliance on one class of insecticides, the Pyrethroids (Permethrin and Deltamethrin). Pyrethroids are not very effective, seeing that resistance has been reported in 27 countries in sub-Saharan Africa including Nigeria (Ranson *et al.*, 2011; WHO 2011; WHO, 2014).

Prior to the advent of insecticide resistance, Dichloro, diphenyl, trichloroethane (DDT) of the Organochlorine class was used for vector control. The World Health organization in 2014, stated that DDT has the longest efficacy of all insecticides currently used for in-

door residual spray as it can last 6 – 12 months compared to 3 - 6 months for pyrethroid based- insecticide. Nonetheless DDT has been banned in most countries due to its adverse toxic effects on man, animal, agriculture, earth's resources and biodiversity (WHO, 2000; Yayock *et al*., 2015).

The exploration for new compounds toward vector control has focused on their lethal effects traditionally (Achee *et al*., 2012). Given that personal protection against mosquito bites is the first line of action against Mosquito-borne disease, other effects such as repellence or irritancy can be used to reduce vector–host contact (Achee *et al*., 2009; Deletre *et al*., 2013). Some plants are known to produce volatiles (essential oils) from their leaves when damaged in order to dissuade herbivores (Gatehouse, 2002). Essential oils are natural products or secondary metabolites that plants produce for their own needs other than nutrition such as protection or attraction. In general, they are complex mixtures of organic compounds that give characteristic odour and flavour to the plants. They are mainly made up of monoterpenes and sesquiterpenes whose main metabolic pathway is through mevalonate leading to sesquiterpenes and from methyl-erythritol leading to monoterpenes. They are located in different parts of the plant. They can be found in the root such as that of the vetiver grass (*Vetiveri azizanioides*), in stems like that of Peteribi wood (*Cordia trichotoma*) and incense, in leaves like in eucalyptus trees (*Eucalyptus citriodora*), citronella (*Cymbopogon nardus*), chinchilla (*Tagetes minuta*) and lemon grass (*Cymbopogon citratus*) (Adlard, 2010).

The adulticidal potential of (*Cymbopogon citratus*) have been explored by some researchers. In a study titled "Evaluation of adulticidal activities of some selected plant species against *Anopheles gambiae*" (Bukar and Lawan, 2021) show cased the potential adulticidal efficacy of *C. citratus* against *An. gambiae*. Here, *C. citratus* extract demonstrated a mortality of 66% at the highest concentration of 20mg/ml and at 10mg/ml, 42% mortality was recorded.

This work is being carried out to compare the adulticidal (toxic) activity of ethanolic and essential oil extracts of *Cymbopogon citratus* (DC.) Stapf (Lemon grass) using Laboratory reared adult *Anopheles gambiae*.

MATERIALS AND METHOD

Plant Material: Large portions of the leaves of *C. citratus* were harvested washed and air dried until a constant weight was achieved. Some of the resulting dry plant materials were taken to the Herbarium and Ethnobotany unit of the National Institute for Pharmaceutical Research and Development, Idu (NIPRID) Abuja where they were identified by Mr Lateef, A.A. Following this, the dried plant parts were respectively ground into powder to attain homogeneity and poured into air-tight containers to prevent deterioration.

Extraction: Ethanolic extracts of *C. citratus* and *O. gratissimum* were prepared using Ethanol (70%) as solvent for extraction. 500g of dry plant powder of each study plant was poured into white plastic bottles respectively. This was done with the aid of a funnel. 1000mls of ethanol was then added to the individual plants contained in the bottles and the mixture was shaken together to achieve homogeneity. Maceration was allowed to occur over 48 hours with intermittent shaking at room temperature. The resulting mixtures were then filtered twice into beakers using a 12.5 mm Whatman filter paper. Subsequently, the collected filtrates of each plant were concentrated at 60^0C in a water bath (XTME-7000) with the aid of a Rotary evaporator (rpm 120) under reduced pressure and dried to obtain a constant weight. The residue was then poured into small glass bottles, labelled and weighed, and finally stored in the laboratory refrigerator at 4^0C until use. Essential Oil was extracted from plant material by Hydro distillation Using Clevenger Apparatus. Dry plant material was loaded unto the round-bottom flask in the Clevenger machine and water was poured into the same flask. The flask was then placed upon a steam jacket at 100^0c to begin the extraction process. The water in the flask begins to boil and steam rises from the flask into a condenser. The condensates which is a mixture of oil and water falls into a burette. Following this, the oil separates from the water as it floats above the water. The water below was transferred back into the round-bottom flask through a diagonal conduit. After 2 hours of carrying out this procedure, the left-over oil in the burette was collected and measured.

Serial Dilutions: A 1% stock solution was made by weighing 200 mg (0.2 g) of ethanolic extract of plant into an empty test-tube. 20 ml of ethanol was added to the test-tube, the mixture was shaken together vigorously to ensure even dissolution and labelled appropriately. This made a stock solution of 20 ml, with a concentration of 10 mg/ml. Serial dilutions of 0.1% (1 mg/ml), 0.01% (0.1 mg/ml), 0.001% (0.01 mg/ml), and 0.0001% (0.001 mg/ml) concentrations were then made from the stock (W.H.O., 2005). As a result, five different ethanolic extract concentrations were obtained namely 10 mg/ml, 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml. Varying concentrations of the essential oil extract was also achieved by making a 1 % stock solution adding 0.5 ml of essential oil to 0.5 ml of ethanol in a test-tube. After homogeneity was achieved, 49 ml of distilled water was added to the test-tube and this was shaken vigorously to achieve a homogenous 50 ml stock solution. This gave a final concentration of 10ug/ml. Other concentrations of 8ug/ml, 5ug/ml, 2.5ug/ml, and 1.25ug/ml were then made from the stock by dilution with distilled water.

Laboratory maintenance of test organisms: At emergence, adults were transferred into improvised plastic cages of diameter (22cm x 30cm x 18cm) and maintained with a cotton pad soaked with 10% glucose solution, at a temperature of 30^0C ± 2.00 and a relative humidity of 78% ± 2.00 for a period of 2 – 5 days during which they were used for Bioassay.

Bioassay: The W.H.O kit method was employed for this bioassay. The experiment was carried out three times and a total of 28 kits

were used per round. This comprised of 14 holding tubes and 14 exposure tubes. Whatman No.1 filter papers (12 x 15 cm) were place on a wooden rack in six (11) replicates. Ten of the papers were labelled respectively, each with a concentration of ethanolic extract (10 mg/ml, 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml) and essential oil extract (10ug/ml, 8ug/ml, 5ug/ml, 2.5ug/ml, and 1.25ug/ml) prepared and they were subsequently impregnated with 2 ml of the labelled ethanolic extract concentration and allowed to stand for 5 minutes. The last filter paper was used as negative control as it was impregnated with 2 ml of the solvent for extraction – ethanol - while positive control was a Permethrin impregnated paper of 0.5% concentration. Twenty (20) laboratory raised adult mosquito which were blood starved and of 2 -5 day old were then introduced into each of the 8 holding tubes and allowed to acclimatize for a period of 1 hour. Following this, the holding tubes were clamped unto the exposure tubes and through the aid of the opening between the two tubes the mosquitoes were transferred into the exposure tubes by gentle blowing. They were allowed to sit in the exposure tubes for a period of 1 hour. After the exposure period, the test organisms were transferred back into the holding tubes to recover. The mosquitoes were maintained in the holding tubes with a cotton pad soaked with 10% glucose solution for 24 hours, at a temperature of 30^0C ± 2.00 and a relative humidity of 78% ± 2.00. Mortality per concentration was observed and recorded at 24 hours. Mean and Percentage mortality was also calculated and recorded. For both plant extracts, the assay was conducted in triplicates. Dead mosquitoes were preserved with silica gel in Eppendorf tubes.

Statistical analysis: Probit regression analysis was carried out using SPSS version 23 to determine the minimum lethal concentration of each extract type and the corresponding chi-square values were recorded.

Morphological Identification of Adult Mosquito: A dissecting microscope (Leica) was employed for this procedure. Whole dead mosquito specimens were placed on the stage of the microscope one at a time. The image was then focused using the adjustments on the microscope until a clear image was obtained. Following this the specie of the mosquito specimen was identified by observing the following features: i. Palps; ii. Wings; iii. Thorax; and iv. Legs (Kent, 2006).

Species Specific PCR Identification of Mosquitoes: This was carried out on dead mosquito species identified as *Anopheles gambiae* from the morphological identification procedure. The result of this analysis was used to confirm that which was obtained from the morphological identification of mosquito species. Identification of *Anopheles gambiae* Complex was carried out using the procedure in Scott *et al*., 1993.

RESULTS

The ethanolic and essential oil extracts concentrations of *C. citratus* were applied to test subjects in different experiments. Table 1 below show details of percentage mean mortality obtained from the application of each extract concentration as well as the LC_{50} and the corresponding Chi-square probability values after a 24 hours exposure period. The essential oil extracts demonstrated the highest toxicity against adult *Anopheles* mosquito overall as these extracts on application resulted in 100% mean mortality of adult stages at 8.0 µg/ml and 10 µg/ml respectively. Ethanolic extracts concentrations of the plant however, showed a lower toxicity when compared to the essential oil as the highest mean mortality observed was 86.50% at a concentration of 1 mg/ml. The highest ethanolic extract concentration of the plant (10 mg/ml) demonstrated the lowest mortality of 65.00%. Hence the intermediate ethanolic extract concentrations gave the highest mortality. Positive control (Deltamethrin 0.05%) resulted in a mortality of 86.65 % while negative control (Ethanol 1%) showed zero mortality after 24 hours. LC_{50} values obtained from Probit analysis for both ethanolic and essential oil extract concentration groups were found to be 1.00 mg/ml and 1 µg/ml respectively. The corresponding chi-square value obtained from probit analysis for essential oil extract concentrations was P = 0.222, while that obtained for ethanolic extract concentrations was P = 0.897.

Table 1: Toxic Activity of the Ethanolic and Essential Oil Extracts of *Cymbopogon Citratus* (DC.) Stapf (Lemon Grass) on Adult Stages of *Anopheles gambiae* s.s

Extract / Control (Concentration)	Mean Mortality ± S.E.	Mean Mortality (%)	LC_{50}	X^2 Value
Essential oil (µg/ml)				
1.25	13.33±0.88	66.65	1.00	0.222*
2.5	15.00±0.33	75.00		
5.0	18.60±0.33	93.00		
8.0	20.00±0.00	100.00		
10.0	20.00±0.00	100.00		
Ethanolic (mg/ml)				
0.001	15.30±0.33	76.50	1.00	0.897
0.01	16.00±0.57	80.00		
0.1	17.00±0.00	85.00		
1.0	17.30±0.88	86.50		
10.0	13.00±0.57	65.00		
(+) control (0.5%)	0.00±0.00	0.00		

(-) control (0.2%)	17.33±0.00	86.65

Table 2: Morphological Identification of Mosquito Species

Structure	Observation / Characteristic	Inference
Wings	One pale spot in 3rd dark area of vein 1	
Thorax	Creamy yellow scales along the centre back	*Anopheles gambiae*
Legs	Legs are irregularly speckled; Hind legs are white	
Palps	Three pale bands on the palps with a wide pale band at the tip	

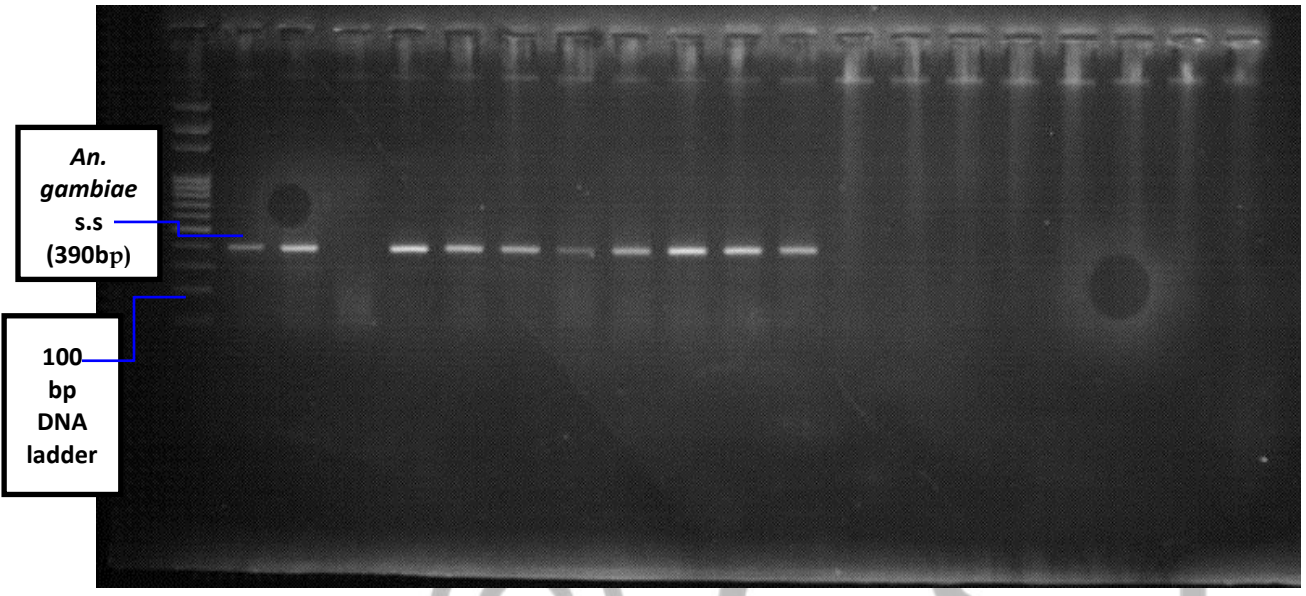

Figure 1: Gel Electrophoresis Result and PCR Identification of *Anopheles* Mosquito Samples

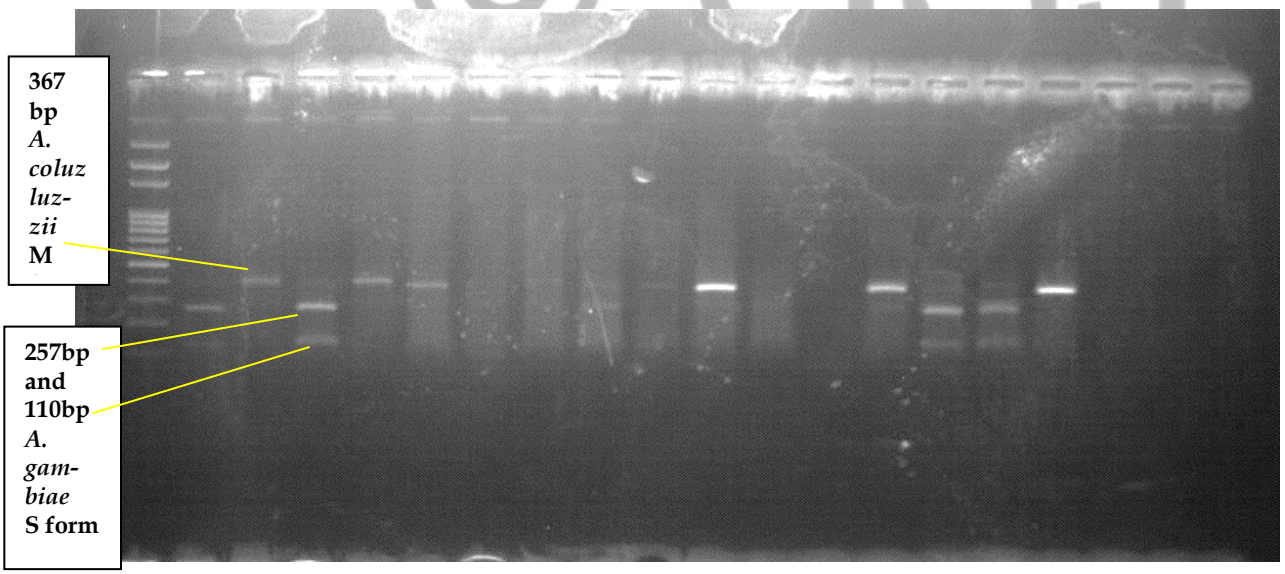

Figure 2: Gel Electrophoresis Result and PCR Identification of M 367 bp (*A. coluzzii*) with 257 and 110bp for S (*A. gambiae*) forms of *Anopheles gambiae* s.s

One species of *Anopheles* mosquito namely *An. gambiae* was identified using the morphological identification method. The result is shown in Table 2 below. The result of polymerase chain reaction (PCR) of the ten randomly selected dead mosquito samples is indicated by bands on Agrose gel electrophoresis are shown in Figure 1 and 2 below. The 390 base pairs band which is seen in ten places in Figure 1 indicate that all ten mosquitoes analyzed are *Anopheles gambiae*. The specific species of the complex were also identified in Figure 2 by the presence of the 367 base pair bands and the 267 base pair bands. The result indicates that six out of the ten are the marsh land form (*A. coluzzii*) while four out of ten are of the savannah form (*A. gambiae*).

Discussion

The result obtained from the application of ethanolic extracts concentrations of *C. citratus* on adult *Anopheles gambiae* s.s in this research is supported by the work of Bukar and Lawan, 2021. Unlike in the latter study mentioned here, where the highest mortality was recorded as 60% at 20 mg/ml, this study has shown for the first time that concentrations as low 0.001, 0.01, 0.1, 1 and 10 mg / ml can result in mortality of the test subject ranging from 65.00 - 86.50 %. This study has also proven that essential oil extracts are most effective in the control of the test subjects as there was increasing mortality as concentration increased with the two highest concentrations of 8 and 10 µg/ml demonstrating 100% mortality. Overall, essential oil extracts were most effective at their highest concentrations while the ethanolic extracts were most effective at their low to intermediate concentrations. The latter could be due to the fact that at low concentrations, penetration of the extract into tissues of the test organism cannot be detected and so they are more effective but at concentrations above 1 mg/ml, the test subjects is able to detect the extract and engage some defense mechanism against its penetration into their internal environment. The results obtained from the application of both extracts and their minimum lethal concentration of 1 mg/ml and 1 µg/ml respectively, indicates that both the ethanolic and essential oil extracts of *C. citratus* are potent deposits of mosquitocidal agents as has been reported in previous research. Chi-square probability value of P = 0.222 recorded from probit analysis proved that there is a significant difference in the mortality resulting from the application of essential oil concentrations of *C. citratus* on test subjects while a P value of 0.897 obtained for ethanolic extract concentrations proved that there is no significant difference in the mortality resulting from the application of the different concentrations on adult stages of *Anopheles gambiae* s.s.

Conclusion

The essential oil and ethanolic extracts of *C. citratus* are toxic to adult stages of *Anopheles gambiae s.s*. These two extracts however act differently as essential oil extracts are most effective at high concentrations while the ethanolic extracts are most effective at lower concentrations. Mortality in essential oil extract is dose-dependent however, it is not dose-dependent in the ethanolic extract. It is recommended that *C. citratus* essential oils be further developed into materials that can be hung in homes or applied topically to human skin to prevent vector-host contact in the prevention of Malaria. Further studies can be carried out to determine the effect of these extracts on human skin. *C. citratus* extracts or whole plant material should be developed into mosquito coils which can serve as a cheap alternative to synthetic insecticide for mosquito control. The effect of the extracts on non-target organisms in the environment should be investigated. More bioassay can also be carried out to determine the susceptibility of other mosquito species to these extracts. (1)

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