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EVALUATION OF RESISTANCE IN TEN GENERATIONS OF *CULEX QUINQUEFASCIATUS* (SAY, 1823) EXPOSED TO METHANOLIC LEAF EXTRACT OF *CALOTROPIS PROCERA*

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ABSTRACT

Mosquitoes constitute a serious Public Health menace, resulting in millions of death worldwide each year. Emergence of insecticide resistant strains of the mosquitoes poses a serious threat and hence calls for alternative control measures. This study assessed the larvicidal efficacy of the methanolic leaf extract of *Calotropis procera* against the 3rd instar larvae of *Culex quinquefasciatus,* after ten generations for 24 hours of exposure. Larvicidal activities of the leaf of the plant were studied on laboratory reared larvae of *Culex quinquefasciatus* at concentration ranges of 15 mg/L to 19 mg/L. The LC50 values were obtained from Probit analysis at 95% confidence limit (CL). Results of the study indicated that the LC50 values obtained from the parent generation (F_0) to the tenth generation (F_{10}) were; 15.79 mg/L, 16.58 mg/L, 17.29 mg/L, 18.07 mg/L, 18.95 mg/L, 19.99 mg/L, 20.65 mg/L, 21.18 mg/L, 22.80 mg/L, 25.16 mg/L and 32.98 mg/L respectively at 24hours. A higher efficacy of activity was exhibited by the extract at the parent generation (F_0) with lowest LC50 of 15.97 mg/L at 24h. The results of this research therefore underscore the efficacy of the plant and further suggest the use of the leaf extracts of *Calotropis procera* in the control of mosquito vectors and indirectly to the diseases they vectored.

Keywords: *Calotropis procera*, *Culex quinquefasciatus*, Generations, Lethal Concentration, Resistance ratio

INTRODUCTION

Culex quinquefasciatus Say is an important vector of a wide variety of pathogens and parasites of medical and veterinary diseases worldwide and is a potential vector of the filarial worm *Wuchereria bancrofti*, the agent of bancroftian filariasis (Jitpakdi *et al.*, 1998; Triteeraprapab *et al.*, 2000; Pumidonming *et al*., 2005). It also helps in transmitting malaria, dengue, chikungunya, filariasis Japanese encephalitis etc. which lead to thousands of deaths yearly [World Health Organization (WHO), 1995].

Culex quinquefasciatus is one of the most widespread mosquitoes in the world. It is found throughout most of pan and subtropical Americas (Andreadis *et al*., 2010), the Neotropics, Afrotropics (Diaz-Badillo *et al*., 2011), Indomalayan, Australian (David *et al*., 2012) and Eastern Asian regions of the world (Rios-Ibarra *et al*., 2010). It is also present in the United Kingdom and parts of the Middle East such as Pakistan, Iran among others. It is an important vector of periodic filariasis in parts of the world (Rios-Ibarra *et al*., 2010) and is known to transmit *Wuchereria bancrofti* to some degree in many regions of the globe. Of the estimated global 128 million lymphatic filarial cases, 91% are caused by *Wuchereria bancrofti* (Andreadis *et al*., 2010). Currently, the main tool for mosquito control is the use of diverse synthetic chemicals as larvicides and adulticides (Govindarajan and Rajeswary, 2014). The drastic effects of synthetic insecticides in the environment have received wide public apprehension (Morin and Comrie, 2010). Indeed, misuse of synthetic insecticides in agriculture and public health programmes has caused many problems like insecticide resistance, resurgence of pest species, environmental pollution, toxic hazards to humans and other non-target organisms (Sarwar *et al*., 2009). Chemical insecticides have the advantage of speedy action and easy application. However, their continuous use causes the development of resistance in insects in many parts of the world such as South America, Sudan, Sri Lanka, Nigeria, Burkina Faso, Egypt, Guatemala, USA, Turkey, and Syria (Malcolm, 1988; WHO, 1992). Consequently, several insecticides have been withdrawn. To overcome the problem of development of resistance in insects, attention is being given to natural products because of their biodegradable nature, non-toxic to other organisms, safer to use, easily available and cost effective, and have been used as alternative methods to control vectors of biological importance (WHO, 2002). A botanical phytochemical, with mosquitocidal potential are now recognized as potent alternative insecticides to replace synthetic insecticides in mosquito control programs due to their excellent larvicidal, ovicidal, adulticidal and repellent properties (Govindarajan *et al*., 2008). However, it is not known if these vectors will develop resistance to botanicals with continual usage. Therefore, this research aims at evaluating the resistance development on exposures of *Culex quinquefasciatus* larvae to methanolic extract of *Calotropis procera*. The result of this study may further strengthen the acceptance of using botanical as good substitute to synthetic insecticides.

Calotropis procera (Sodom apple), also called "babambi" in Fulfulde, belongs to the family Asclepiadaceae and is a soft wooded, evergreen perennial shrub. It is a xerophytic erect shrub, bearing purple spotted pink scented flowers (Shrivastava *et al*., 2013). The latex of *C. procera* is used as purgative, while the flower and dried leaves are considered as digestive aids, useful in cough, asthma and anorexia. The root bark is useful in treating skin diseases and intestinal worms; it also possesses analgesic, anticonvulsant, and sedative effect. It is highly recommended in the treatment of leprosy and hepatitis. Oil extracted from leaves of *C. procera* is very efficacious in treating cases of paralysis. Fresh leaves are utilized to relieve rheumatic pains and inflammation in joints (Verma *et al*., 2011). *Calotropis procera* possesses alkaloids, cardiac glycosides, anthraquinone, tannins, saponins, flavonoids, steroids, terpenoids, reducing sugars, and resins which are supposed to have significant antibacterial activity (Shaalan *et al*., 2005). The extracts of *C. procera* possess good larvicidal activity against mosquitoes and more studies shows that the extract contained more active compounds for future use against other stages in mosquito control (Shahia *et al*., 2010). The phytochemicals present in *C. procera* extracts has been found to act as antioxidants by scavenging free radicals and thus possessing therapeutic potentials (Patel *et al*., 2014).

MATERIALS AND METHOD

Collection and processing of plant materials: Fresh healthy leaves of *Calotropis procera* was collected from the botanical garden, Ahmadu Bello University, Zaria main Campus. Authentication of plant samples was done at the herbarium unit, Department of Botany, Ahmadu Bello University, Zaria and was assigned a Voucher no of 009219.

The leaves were thoroughly washed with tap water to avoid dusts and other unwanted materials accumulated on the leaves from their natural environment. The dust free leaves were then allowed to dry under shade at room temperature for about 20 days. The dried *Calotropis procera* was powdered by using mortar and pestle, sieved with a mesh size of 0.002 mm to collect fine powder.

Extraction and preparation of plant material: The dried *C. procera* leaves was pulverized and the material, thus obtained was cold macerated in organic solvent (methanol). Stock solution of the extract was prepared from 100mg of the solid residue dissolved in one litre (1L) of distilled water to obtain 100 mgL^{-1} concentration. Serial dilution of this was made with distilled water to obtain test concentrations of 10 mgL^{-1}, 20 mgL^{-1}, 30 mgL^{-1} and 40 mgL^{-1}. Control concentration, devoid of the plant extract, consisted of 99 ml of distilled water and 1 ml methanol.

Collection and Rearing of Mosquitoes: Blood fed Female of *Culex quinquefasciatus* were collected from within Ahmadu Bello University, Zaria main campus using mechanical aspirators and brought to the laboratory. They were placed in entomological cage containing dechlorinated water for egg laying. The larval stages were fed with mouse pellet and the third instar larvae (L3) were exposed to various concentrations of methanolic leaf extract of *Calotropis procera*. The concentration that killed 50% of the larvae was determined. The survived larvae were reared to adult stage. A quail was introduced into the cage for the emerged female adult to have source of blood meal for about 6 hours, and allowed to lay eggs. The third instar larvae were exposed to determined LC_{50}.This process was repeated until 10 generations were tested and resistance ratio was determined in each case.

Larval bioassay**:** Bioassay was performed according to WHO guidelines (2005). After making test concentrations, batches of 25 third instars larvae were transferred by means of droppers to small disposable test cups, each containing the test concentrations of 10, 20, 30 and 40 mg/L while the control devoid of the extract contained 99 ml of water and 1% methanol.

Median Lethal Concentration (LC_{50}) of Leaves Extract: LC_{50} was determined as described by (Finney, 1971; Raymond, 1985) with little modification.

Resistance ratio (RR) in *Culex quinquefasciatus*: Resistance ratio (RR) was determined using the method of (Brown and Pal, 1971) as follows:

$$\text{Resistance ratio (RR)} = \frac{LC_{50} \text{ of } F_0 \text{ generation}}{LC_{50} \text{ of Tested generation}}$$

Calculated RR values 10 are indicative of high resistance, 5-10 are indicative of medium resistance and < 5 are indicative of low resistance (Mazarri and Georghiou 1995).

Statistical Analyses: One-way Analysis of Variance (ANOVA) was used to determine the significant difference on the mortality and Resistance status of the larvae of *Culex quinquefasciatus* between the control and the experimental groups. P-value of less than 0.05 was significantly different. Probit analysis was also used to determine LC_{50} (lethal concentration to cause 50% mortality in the population) by plotting regression line.

RESULTS

The results of the larvae of *Culex quinquefasciatus* exposed to methanol leaf extract of *Calotropis procera* are presented in Table 1. The percentage mortality of the larvae decreased steadily from the F0 generation (56.20%) to the F10 generation (9.40%). The lethal concentration that killed fifty per cent (LC_{50}) of the F0 population was 15.79 mg/L. There was a gradual but steady increase of the LC_{50} values from 16.58 mg/L (F1 generation) to 32.98 mg/L (F10 generation). The percentage mortality and the LC50 across the 10 generations were inversely proportional. Resistance ratio values had a steady increase from the first filial generation (1.050 fold) to the tenth filial generation (2.089 fold).

Table 1: One-Way Analysis of Variance (ANOVA) on the resistance status of the larvae of *C. quinquefasciatus* from F1 generation to the F10 generation after exposure to 24 hours

Generation	Number of Larva Exposed	% Mortality	Mean (±S. E) Mortality	LC_{50} mg/L	Resistance Ratio (Fold)
F0	100	56.2	14.05±1.05	15.79	
F1	100	52.0	13.05±1.01	16.58	1.050
F2	100	48.2	12.05±0.94	17.29	1.095
F3	100	44.4	11.10±0.91	18.07	1.144
F4	100	40.6	10.15±0.87	18.95	1.200
F5	100	36.8	9.20±0.89	19.99	1.266
F6	100	32.2	8.05±0.89	20.65	1.308
F7	100	27.6	6.90±0.94	21.18	1.341
F8	100	21.0	5.25±0.83	22.80	1.444
F9	100	16.4	4.20±0.67	25.16	1.593
F10	100	9.4	2.35±0.80	32.98	2.089

Significant at $p \leq 0.05$

DISCUSSION

Calotropis procera is a promising botanicals for use in the control of vector species. In our studies, we found the methanol leaf extract of this plant effective against the larvae of *Culex quinquefasciatus*. Other researchers (Singh *et al*., 2005; Elimam *et al*., 2009; Kumar *et al*., 2012; Anjum *et al*., 2016) reported similar effects of *Calotropis procera* against *Culex quinquefasciatus* and other mosquito species. Despite the efficacy of *Calotropis procera* on vector species, there seems to be paucity of information on the selection pressure of this plant on vector species. Thus, it is not known whether continual exposure of the extract of *Calotropis procera* on vector species will cause the development of resistance or not.

In this study, we found out that the extract of *Calotropis procera* caused slight decrease of mortality with subsequent exposures. According to the World Health Organization procedure for testing (WHO, 2016), if resistance ratio is less than 5 fold, the resistance is low; if it is between 5 and 10 fold, it is moderate; resistance ratio above 10 fold means that the resistance is high. Thus, exposure of *Culex quinquefasciatus* to the extract of *Calotropis procera* yielded low resistance of 2.089 fold. This makes it interesting because it is most likely going to address the issue of resistance which is prevalent in the use of synthetic insecticides (Hemingway and Ranson, 2004; Rivero *et al*., 2010; Chareonviriyaphap *et al*., 2013). Similar to this report is the use of *Bacillus thuringiensis* var. *israelensis* which yielded only 2.78 fold tolerance after 20 generations (Saleh *et al*., 2003).

On the other hand, selection with synthetic insecticides has always yielded resistance in multiple folds. For instance, El-Sheik *et al*. (2014) selected the populations of *Culex pipiens* with lambda-cyhalothrin and malathion for 11 generations and recorded 57 and 305 fold resistance. Similarly, when the population *Culex quinquefasciatus* was selected with deltamethrin in India, there was approximately 298-fold resistance in the tenth generation (Sarkar *et al*., 2009). This rapid increase may be attributed to the fact that synthetic insecticides are reported to increase the levels of detoxifying enzymes, thus making them effective against the insecticides in question (Zibaee and Bandani, 2010).

CONCLUSION

In conclusion, methanol leaf extract of *Calotropis procera* shows toxic effect on the *C. quinquefasciatus* larvae resulting in high mortality recorded. The leaf of *Calotropis procera* could thus be added to the growing list of botanicals with anti-mosquito properties that could be harnessed for the control of mosquito species and replacement for synthetic pesticides. Slow resistance development on exposure to the extract of this plant is added advantage over synthetic insecticides.

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