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Research Article
Susceptibility of *Culex quinquefasciatus* (Say) Larvae to Methanolic Extracts of *Annona reticulata*

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Abstract

Background and Objective: Environment pollution and resistance of many pests to the most frequently used chemical insecticides gave the rationale of altering to replace them with natural herbal extracts for pests and vector control. This study investigated the effect of methanolic extract of *Annona reticulata* on the mortality and development of larvae of *Culex quinquefasciatus*. **Materials and Methods:** Methanolic herbal extracts were analyzed using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) to identify the phytochemical compounds in them. Ten mosquito larvae were used as replicates and exposed to each of the five concentrations of the plant extract (30, 100, 150, 200 and 250 mg mL^{-1}) and ten larvae were exposed to double distilled water and considered as control. **Results:** Phytochemical analysis revealed the presence of phenols, steroids, quinones, tannins and saponins. Statistical analysis showed a significant strong correlation and regression between exposure to the different concentrations of the extract and mortality of *Culex quinquefasciatus* larvae where $R^2 = 0.982$, the Correlation value is 0.991099 ($p<0.05$). Results also showed that the extracts affect the development of larvae more than mortality. The effect of the extracts on the early larval stages was significantly high compared to the late stages of larvae. **Conclusion:** According to our knowledge results of this study has been reported for the first time in Saudi Arabia where *Annona reticulata* is neglected regionally and no study approved its efficacy as a botanical herbal extract against mosquitoes.

Key words: *Culex quinquefasciatus*, herbal extract, *Annona reticulata*, larvae, mortality, insecticides, quinones

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Numerous species of mosquitoes cause a real threat to humans and or animals to their vectorial capacity to transmit serious pathogens like filarial warms, malaria parasites, yellow fever and dengue fever viruses[1]. Many parts of the world were identified as endemic areas for diseases caused by these pathogens[2].

Many vector control campaigns focus on reducing densities of larval stages in their breeding habitats because they assume that targeting adults may reduce the density of adults for the short time[3]. Besides, dispersal of larval stages of mosquitoes is limited compared to the adult stages thus the eradication of a larval population in a breeding site is easier and more applicable[4].

Chemical insecticides belonging to various families are regularly used for the control of numerous families of pests and/or vectors to reduce their densities. Many studies recorded that using of these insecticides exponentially correlated with the appearance of insecticide resistance[5]. To save the environment from the pollution caused by the chemical insecticides as well as to prevent the insecticide resistance problem it's recommended to use natural products for insects control such as herbal extracts[6]. The relatively high cost of chemical control compared to herbal extracts as well as the opportunity of affecting non-targeted organisms by chemical insecticides are additional advantages encouraged altering from the chemical vector control to the natural product's control[5].

Products of various plants have been used to control either (or both) larval or (and) adult stages of mosquitoes[4]. Some herbal products are also used as repellents to prevent mosquitoes-human contact[7]. In Saudi Arabia, few studies reported the efficacy of some plant extracts on mosquitoes larvae such as *Solenostemma argel*[8]. Another study approved the efficacy of three herbal extracts on the control of *C. quinquefasciatus* larvae[9].

According to our knowledge, no published study investigated the efficacy of *Annona reticulata* on the control of Saudi strains of mosquitoes. This gave the rationale to conduct this research.

MATERIALS AND METHODS

Study area: Plants and wild mosquitoes were collected from different localities proximal to Hfouf in Al Ahsa (Eastern Region around 328 km from Riyadh Capital of Saudi Arabia).

Study design and study settings: A prospective study has been conducted in Saudi Arabia between January-June, 2017. Wild populations of *C. quinquefasciatus* larvae, laboratory-reared larvae of the same species and leaves of wild *Annona reticulata* trees were used in this study.

Collection and rearing of mosquito larvae: Larvae of *C. quinquefasciatus* larvae were collected from stagnant water bodies in variant localities in Saudi Arabia distributed in the Eastern, Southern and Central regions. Plastic pipettes and white dishes were used for the manual collection of the larvae. Larvae transferred to the Entomology Research Lab in the Faculty of Science at Princess Nourah Bint Abdurrahman University for morphological identification and rearing. Mosquito larvae anaesthetized using Triethyleamine solution, identified under dissecting microscopes using the classical keys of Knols[10] then placed into labelled white dishes containing powder of yeast and biscuit mixture with (1:3) ratio. Pupae were collected via pipettes periodically and transferred to containers containing double distilled water placed inside adult mosquito cages. Each cage was 30×30 cm in size and 30 cm in height.

Temperature and Relative Humidity (RH%) were adjusted to 21±1°C and 77±3% RH during the whole period of the laboratory work in the insectary. Emerged adult male mosquitoes offered 20% Glucose solutions while female adult mosquitoes offered rabbits blood via artificial feeding membranes. Egg rafts were collected daily and replaced with labelled larvae-rearing dishes. Third instars' larvae were specifically pipetted for bioassay tests[11].

Description of *Annona reticulata*: *Annona reticulata* is a plant also called Sarifa or sugar apple natively grown in Western Indis but also commonly found in tropical areas. The leaves and branches are used as natural dies due to the blue pigments they contain. *Annona reticulata* approved significant larvicidal efficacy against mosquito larvae and antimicrobial material against some pathogens infect humans[12,13].

Preparation of herbal extracts: Fresh leaves were collected separately from various locations in Saudi Arabia distributed in the Eastern, Central and Southern localities. The trees were carefully identified and specimens were transferred to the Department of Biology, Faculty of Science, Research laboratory, King Faisal University, Saudi Arabia. Collected leaves were washed using distilled water then let to dry in the laboratory for two weeks (Mean Temperature 21±2°C). Dried leaves were ground using a clean electric grinder. Volumes of

methanol and dried powdered were determined according to the method of Mohankumar *et al.*[14] who recommended adding 15 g of the powder to 250 mL of methanol (100% concentration). The crude extract was then filtered using filter papers and concentrated by evaporation until dried in a water bath. Specific concentrations were prepared as follows: 0.6, 0.3 and 0.1% by vortexing the stock of crude extract and diluting volumes of it by the adequate volume of distilled water. Prepared concentrations were eventually then transferred to labelled dark sterile bottles and preserved at a 4 refrigerator. All steps of herbal extracts preparations were conducted according to the method of Awosolu *et al.*[15]. Phytochemical analysis was performed using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) to identify the chemical compounds in the extracts.

Larvicidal assay: Replicates of larvae (10 larvae/concentration) were exposed to the five concentrations of the plant extract. About 10 larvae were exposed to double distilled water and considered as control. Proportion mortality of larval mortality due to the exposure to the herbal extract was calculated according to the following Eq.[16]:

$$\text{Mortality } (\%) = \frac{\text{Number of dead larvae}}{\text{Total number of larvae}} \times 100$$

RESULTS

Phytochemical analysis showed that the methanolic extract of *Annona reticulata* is positive for phenols, steroids, quinones, tannins and saponins. Results of phytochemical analysis also showed variations in the levels of these elements in the extracts. Phenols and Tannins were existing at high levels while Steroids were present at moderate levels. In contrast to this, Quinones and Saponins were present in low levels (Table 1).

Five concentrations 30, 100, 150, 200 and 250 μg mL^{-1} of the methanolic plant extracts were used for bioassay tests. Statistical analysis showed a significant strong correlation and regression between exposing to all prepared concentrations of *Annona reticulata* methanolic extract and mortality of *Culex quinquefasciatus* larvae where R^2 = 0.982, the Correlation value is 0.991099 ($p<0.05$) (Table 2, Fig. 1).

Results of the cumulative mortality of larvae after exposure to different concentrations of the methanolic extract showed the median concentration of 100 μg mL^{-1} induced high mortality to approximately near half of the population of mosquito larvae (40% cumulative mortality) where the lethal concentration (LC_{90}) was 160.03 μg mL^{-1} while the maximum concentration 250 μg mL^{-1} caused 96% cumulative mean mortality of the population of mosquito larvae and the (LC_{90}) was 262.64 μg mL^{-1}) (Table 3, Fig. 2).

Statistical analysis also showed that the effect of exposure to double LC_{50} concentration of *Annona reticulata* methanolic extract for 24 hrs significantly affected the development to the next developmental stage compared to the effect on mortality where Chi^2 values were 5.5515 and 1.76986 ($p<0.05$), respectively. Besides, the extract significantly affected the earliest larval stages compared to the latest stages of larvae in their mortality and development.

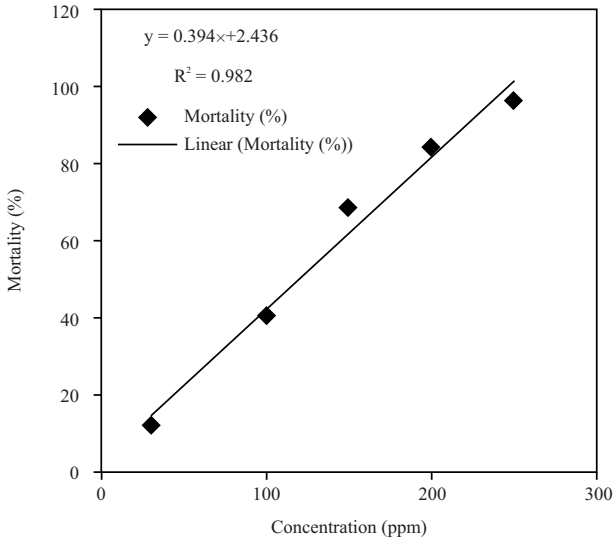

Fig. 1: Linear regression and correlation between mortality of larvae and concentrations of the herbal extract

Table 1: Phytochemical analysis of *Annona reticulata* methanolic extract

Components	Results
Amino acids	-
Proteins	-
Carbohydrates	-
Phenols	+++
Steroids	++
Tannins	-
Quinones	+
Acidis	-
Resins	-
Oils and fats	-
Tannins	+++
Saponins	+
Resins	-

-: Negative, +: Positive with low level, ++: Positive with a moderate level and +++: Positive with high level

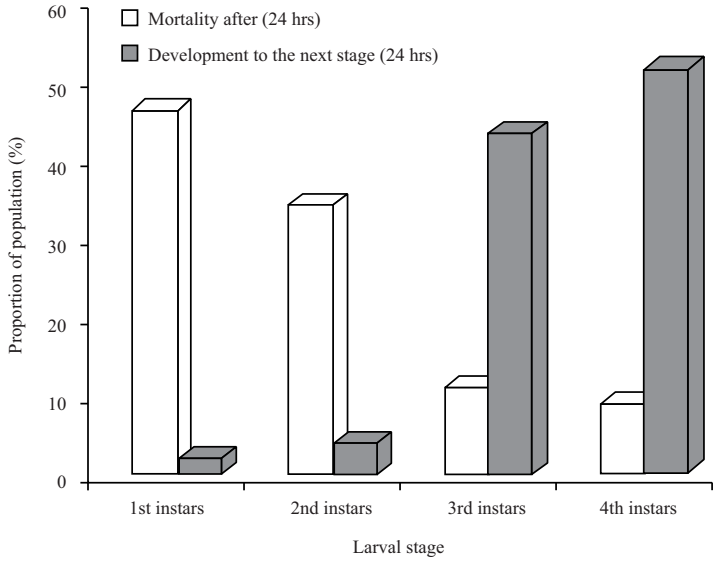

Fig. 2: Mortality and development of larvae after 24 hrs of exposure to double LC_{50} concentration of the extract
*Chi^2 values ($p<0.05$) = 1.76986 and 5.5515 for mortality and larval development, respectively

Table 2: Effect of exposure periode to *Annona reticulata* methanolic extracts on the average mortality of *C. quinquefasciatus* larvae

Exposure periode (hrs)	Mortality (%) in control	Average mortality (%)/concentration 30 (µg mL^{-1})	p-value	100 (µg mL^{-1})	p-value	150 (µg mL^{-1})	p-value	200 (µg mL^{-1})	p-value	250 (µg mL^{-1})	p-value
6	0	1	8.46	3.6	4.1	15.4	0.02	19.6	0.4	23	0.9
12	0	2.1		6.3		16.7		20.1		23.9	
24	0	3.1		10.4		17.5		21.9		24.4	
48	0	5.8		19.7		18.4		22.4		24.7	

Table 3: Cumulative mortality of *Culex quinquefasciatus* larvae after exposure to different concentrations of *Annona reticulata* methanolic extract

*Conc. (µg mL^{-1})	Mortality (%)	LC_{50} (µg mL^{-1})	LC_{90} (µg mL^{-1})	95% FL	Slope±SE
30	12	31.2	139.4	84.9-179.1	2.49±33.94
100	40	52.3	160.03	101.48-298.2	2.49±14.14
150	68	71.5	199.7	128.7-496.3	2.49±5.66
200	84	53.17	236.8	151.48-638.5	2.49±16.97
250	96	95.24	262.64	172.23-931.30	2.49±25.46

*Conc: Concentration (µg mL^{-1}), *LC_{50}: Median lethal concentration, LC_{90}: Lethal concentration, FL: Fiducial limits and DF: Degree of freedom

DISCUSSION

A piece of scientific evidence for the effectiveness of *Annona reticulata* in the control of *C. quinquefasciatus* larvae have been provided from this study. This evidence is valuable because searching for alternatives to control pests and vectors became an essential issue during the last decades due to the devastating hazard of chemical control to both environment and non-targeting organisms[17]. Some herbal extracts are found to be potentially effective to replace synthetic chemical insecticides in the control of numerous pests (and vectors) due to their high pests (or vectors) specificity and their relative minimum negative impact on the environment and the vertebrate and invertebrate organisms living there[18]. In this study, concentration was given to *C. quinquefasciatus* mosquitoes because it's the most dominant domestic species of mosquitoes in Saudi Arabia which makes it necessary to select a friendly insecticide to control the larvae of this species in their breeding habitats which are mostly proximal to human dwellings[19]. International studies approved the potentiality of the neglected herbal extracts of *Annona reticulata* on the control of *C. quinquefasciatus* larvae in some countries[20,21].

Results of phytochemical analysis exhibited the presence of some bioactive compounds like Phenols, Tannins, Steroids, Quinones and Saponins. These results coincide with the results

of previous similar published studies that recorded the same compounds in the extracts of *A. reticulate* leaves[22,23]. The presence of these components indicates the possibility of utilizing this herbal extract as a potential insecticide[24]. Alkaloid compounds such as Steroids have been found to reduce the combining site at the acetylcholine enzyme in more than one living organism[25] which also adds to the assumption that *A. reticulate* herbal extracts can be recommended as a potential insecticide.

The findings of this study indicated a significant effect of the methanolic extract of *A. reticulate* on the mortality and development of the native Saudi strands of *C. quinquefasciatus* larvae. Larvicidal tests specifically revealed that the herbal extracts of *A. reticulate* are highly toxic against *C. quinquefasciatus* larvae especially the first two instars. Many studies are in agreement with these findings[26,27] thus, using these herbal extracts against the early developmental stages of *C. quinquefasciatus* mosquitoes would be more valuable rather than the late stages. Toxicity of *A. reticulate* indicated in this study by the ability of the extract to induce a high mortality rate and to reduce the number of larvae succeeded to complete their development to the next stage in the life cycle. More than one study has already reported significant variations in the susceptibility of mosquitoes during their different stages to the phytochemical compounds found in the herbal extracts[28]. The key result of this study is the determination of the recommended lethal dose (100 μg mL^{-1}) that induced high mortality to approximately 40% of mosquito larvae. A previous study also recommended this concentration as the lethal dose to control mosquito larvae[29]. Another golden outcome of this study is the finding that *A. reticulate* methanolic extract retard the development of the mosquito larvae more than killing them. This indicates the relatively slow but less toxic effect of this herbal extract on invertebrate organisms in the environment. Coinciding to this result, a study reported that the best herbal extracts recommended to be used as pesticides should induce acute toxicity to the targeted pests as well as less residual capacity[28]. Finally, despite the advantages already found and discussed in this study of using *A. reticulate* as effective pesticides still some disadvantages of using herbal extracts in general as alternatives to synthetic chemical insecticides like unknown properties of active ingredients of many herbal pesticides were reported in some studies[21]. Further studies are essential to evaluate the sustainability of *A. reticulate* in the environment and to ensure its safety to non-targeting organisms before recommending it as a botanical pesticide for *C. quinquefasciatus* larvae. The effect of this herbal extract on other species of mosquitoes as well as other developmental stages such as pupae and adults should be investigated.

CONCLUSION

Methanolic extracts of *A. reticulate* retarded the development of *C. quinquefasciatus* larval development and induced significant mortality especially in the early stages of larvae and didn't induce adverse effects at LC_{50} and LC_{90} values. Further studies should be conducted to ensure the safety of these extracts on the non-targeted organisms before they can be recommended as potential pesticides against *C. quinquefasciatus* larvae. According to our knowledge results of this study are the first records in Saudi Arabia.

SIGNIFICANCE STATEMENT

This study discovered the potential efficacy of *A. reticulate* in the control of the domestic mosquito species *C. quinquefasciatus* that can be beneficial for using natural products as insecticides replacing the chemical control which has disadvantages such as environmental pollution and the resistance of insects to chemical insecticides. This study will help the researchers to uncover the critical areas of the suitability of using *A. reticulate* to control mosquito larvae that many researchers were not able to explore in the Middle East. Thus, a new theory on these herbal extracts as potential natural insecticides against *C. quinquefasciatus* mosquitoes may be arrived at.

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