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Research Article

BIOCHEMICAL STATUS AND HISTOLOGICAL CHANGES OF *CATHARANTHUS ROSEUS* ETHANOLIC LEAVES EXTRACT AGAINST MOSQUITO LARVAE *AEDES AEGYPTI, ANOPHELES STEPHENSI AND CULEX QUINQUIFASCIATUS* (DIPTERA:CULICIDAE)

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ABSTRACT

The mortality was observed in larvae after 24 h of exposure. The LC_{50} values were 157.8, 173.1 and 192.3 ppm, respectively. Larvae that were alive after 24 h of extract treatment at LC_{50} were collected for biochemical and histological examination. The quantitative estimations of glycogen, protein and AchE (brain) was carried out for the *Culex quinquifasciatus* body tissues of normal and ethanol extract treated larvae of *Aedes aegypti*, *Anopheles stephensi* and *Cx. quinquifasciatus*. The extracts produced significant alterations in the biochemical profiles of Aedes, Anopheles and Culex larvae. Further, the impacting factors of extracts on carbohydrate, protein contents and AchE activity of larvae are species specific. The results indicate altered metabolic activity of the larvae. Histological changes in the mid-gut region of third instar larvae of *Cx. quinquifasciatus* exposed to LC_{50} of *Catharanthus roseus* ethanolic extract presented an irregularly structured brush border after 24 h. The cells began to swell via a slight vacuolization, with disorganized, shortened and confluent microvilli membranes. The structural disorganization of the mid-gut epithelium was evident; cells did not show the characteristic morphology and had destroyed features when compared to control. Based on the results of biochemical status and histological changes of ethanol extracts of *C. roseus*, it can be concluded that the extract could be a new novel source of various larvicidal active compounds for controlling mosquito vectors.

Keywords: *Catharanthus roseus, Aedes aegypti, Anopheles stephensi, Culex quinquifasciatus,* Biochemical.

INTRODUCTION

Mosquito vectors play a very predominant role in the transmission of malaria, dengue fever, yellow fever, filariasis and several diseases which are today among the greatest health problems in the world. Mosquitoes are one of the major medically significant vectors, and they transmit parasites and pathogens, which are continuing to have a devastating effect on human beings and other animal's eco-friendly approaches of agriculture pest control. Elumalai *et al.* (2015); Lakshmanan *et al*. (2017). Several mosquito species belonging to genera *Anopheles*, *Aedes* and *Culex*, are the vectors for the pathogens of various diseases and contribute significantly to poverty and social debility in tropical countries (Jiang *et al*., 2009).

One of the approaches for controlling mosquitoes borne diseases is the interruption of disease transmission either by killing, preventing mosquito bite by using repellents or by causing larval mortality in a large scale at the breeding centers of the vector. The control of mosquito larvae worldwide depends on the continued application of organophosphates and insect growth regulators (Rahuman *et al.*, 2009). These problems have accentuated the need for newer strategies for mosquito larvae control. Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and has led to resurgences in the mosquito population. It has also lead to the development of resistance, ecological imbalance, harm to human and animals and undesirable effects on non-target organisms (Kamaraj *et al.*, 2008). One such possibility is the use of botanicals which are readily biodegradable, non-toxic and show broad-spectrum target specific activity (Sharma *et al.*, 2005). The mosquito control at the larval stage of development with phytochemicals that occur in the

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oils, leaves, and roots of plants is one of the techniques which afford a cheaper and environment friendly method of mosquito larval control (Shyamapada, 2011).

The biochemical changes induced by these chemicals have been reported (Bhagawan *et al*., 1992; Reddy *et al*., 1993). The biochemical parameters are valuable in assessing and predicting the toxicological effect on the insects. Further, the potential effects of botanical insecticides on the biochemical milieu of insects are of great interest in biological control applications (Sak *et al*., 2006). Carbohydrates supply a major portion of energy to the living system the Carbohydrate along with protein and lipid from the principal classes of organic compounds that are also found in an insect. When insects are exposed to any toxic or polluted medium, sudden stress is developed for which the insects should meet more energy demand to overcome the toxic stress (Jebakumar & Jayaraman, 1988). The biochemical responses of some Lepidopteran insects on exposure to different insecticides on certain aspects of carbohydrate metabolism have been well documented (Nath, 2000). In insects, the glycogen is the chief reverse carbohydrates as in other animals (Howden & Kilby, 1961) and formed primarily from glucose and serve as a reverse energy.

Proteins are the known biological compound which regulates and integrate several physiological and metabolic processes in the body through hormones, enzymes and nucleoproteins. An understanding of the metabolism of protein and amino acids are important. Most of them can be synthesized inside the body of animals are called non-essential amino acids which cannot be synthesized by the body of animals are called essential amino acids and must be supplemented only through diet (Kilby & Candy, 1975). Amino acids are utilized for the production of hormones and enzymes, and the composition of total protein in the body may be significantly modified (Ridley, 1988).

Enzymes are attractive as indicators because they are more easily quantified than the other indicators. Metabolic pathway mainly depending on enzyme activities may be affected due to the destruction under stress, reflecting the changes in enzyme activities (Ganguly *et al*., 1997). In the process of transition of impulses between the neurons, acetylcholinesterase (AChE) is a key enzyme, which hydrolyses the neurotransmitter acetylcholine (ACh) in cholinergic synapses of neurons (Colovic *et al*., 2013). Therefore, AChE plays a crucial act for every function of living being including insects. Most of the current insecticides such as organophosphorus (OP) is based on inhibiting acetylcholinesterase (AChE), yet the knowledge of this enzyme and its mechanism of action is limited (Aldridge & Reiner, 1969; Ehrich *et al*., 2011). AChE catalyses the hydrolysis of acetylcholine, a neurotransmitter for cholinergic neurotransmission in insects. The neurotoxic compounds hydrolyse the neurotransmitter acetylcholine (ACh) to terminate neuronal excitement at the postsynaptic membrane (Delfino *et al*., 2009). *Catharanthus roseus* L (Apocynaceae), also known as *Vinca rosea*, is a native plant to the Caribbean Basin and has been historically utilised for treating a wide range of diseases. The European herbalists used this plant for conditions as varied as the treatment for headache, to a folk remedy for diabetes. It is known to have more than 400 known alkaloids, some of which are already approved antineoplastic agents to treat leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilms tumor, and other cancers. Its vasodilating and memory enhancing properties have been shown to alleviate vascular dementia and Alzheimer's disease.

In recent years, much effect has been focused on the exploration of bioactive, chemical compounds from indigenous plants for mosquito control in India. In this study, biochemical status and histological changes of *Catharanthus roseus* ethanolic leaves extract against mosquito larvae *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquifasciatus*. The results of the present study would be useful in promoting research aiming toward the development of new agents for mosquito control programme.

MATERIALS AND METHODS

Test mosquitoes species

All experiments were carried out against laboratory reared vector mosquitoes *viz*., *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* free of exposure to insecticides and pathogens. Cyclic generations of vector mosquitoes were maintained at 25-29 0C and 80-90% R.H. in the insectariums, with a photo period of 14 h light, 10 h dark. Larvae were fed on larval food (powdered dog biscuit and yeast in the ratio 3:1).Third instar larvae were used for the studies.

Plant material collection and identification

C. roseus (pink variety) was taxonomically authenticated by the Department of Botany, Annamalai University, Chidambaram, and the voucher specimen was kept in the herbarium (Bot/Her/751) of our University. Fresh leaves of *C. roseus* (pink variety) were collected during September, from the Thiruchotruthurai, Thanjavur, and the leaves were shade dried and then ground to a fine powder.

Extraction

The leaves of the plant were first washed with tap water, then shade dried and finely ground into fine powder. The finely ground plant leaf powder (1.0 kg/solvent) was loaded in Soxhlet apparatus and was extracted from ethanol extract individually. The solvents from the extracts were removed using a rotary vacuum evaporator (Buchi Labortechnic AG, Switzerland) to collect the crude extract. The standard stock solutions were prepared at 1% by dissolving the residues in acetone. From this stock solution, different concentrations were prepared, and these solutions were used for larvicidal bioassay.

Biochemical Estimations

Treatment of larvae 100 *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* larvae each were treated with ethanolic extract of *C. roseus* at LC50 values were 157.8, 173.1 and 192.3 ppm, against *Aedes, Anopheles* and *Culex* larvae for 24 hours. A pinch of larval food (yeast powder) was supplied during experimentation. Similar conditions were maintained for control with 1 mL solvent. After the exposure of 24 hours, the live larvae were removed from the beaker and washed with water and the whole body tissue stored in the -20 freezer for further biochemical analysis.

Estimation of Glycogen

The colorimetric method by Kemp & Van Heijningen, (1954) was adopted for the quantitative estimation of glycogen.

Estimation of Protein

The protein content was determined by adopting the procedure of Lowry *et al*. (1951).

Estimation of Acetylcholinestarase

Acetyl cholinesterase (AchE) activity was estimated by the following method adopted by Mushigeri & David (2005).

Specimen Preparation for Light Microscopic Study

Histological changes of the midgut epithelial cells of treated larvae *Cx. quinquefasciatus* were observed. After the exposure of 24 hours, the alive larvae were collected for examination. The larvae were rinsed with distilled water before fixation with the bouins solution, followed by dehydration in graded ethanol and toluene series. Then, the larvae were embedded in paraffin, sectioned and stained with Haematoxylin and Eosin before the examination using compound microscope (Al Mehmadi & Al Khalaf, 2010).

Statistical analysis

Statistical analysis for all the experiments was performed using SPSS 11.5 software. The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) and were expressed as mean ± S.D. The values were considered statistically significant if the *p*-value was less than 0.05.

RESULT AND DISCUSSION

The glycogen level of body tissue of control larvae (*Ae. aegypti, An. stephensi and Cx. quinquifasciatus*) were 14.6 ± 0.8, 14.0 ± 1.1 and 16.3 ± 1.2 mg/g respectively. The amount of glycogen level in the *C. roseus* leaves extracts treated larvae were about 9.3 ± 1.2, 8.6 ± 0.8 and 9.6 ± 0.8 mg/g respectively. The Glycogen level was drastically reduced in extracts treated larvae. This might be due to the reason that more sugars were metabolized to meet the energy expenses during stressful conditions, leading to carbohydrate level depletion in the treated larvae (Table 1). The values of mean glycogen level of the *Ae. aegypti, An. stephensi and Cx. quinquifasciatus* of control and treated larvae significantly differed at 0.05% level**.** The protein level of body tissue of control larvae (*Ae. aegypti, An. stephensi* and *Cx. quinquifasciatus*) were 63.6 ± 2.4, 59.6 ± 2.0 and 61.6 ± 1.4 mg/g respectively. The amount of protein level in the ethanolic extract of *C. roseus* leaves treated larvae of *Ae. aegypti, An. stephensi* and *Cx quinquifasciatus* were about 51.0 ± 1.1, 49.0 ± 1.7 and 50.3 ± 1.4 mg/g, respectively. Protein level appears to be gradually decreased in the treated larvae when compared to control larvae (Table 2). The values of the mean protein level of the body tissue of control and treated larvae significantly differed at 0.05 % level. The quantity of protein in all the body tissues gradually decreased in *C. roseus* leaves extract treated larvae, which may be due to intensive proteolysis to meet the extra energy demand when the larvae are subjected to ethanolic extract stress.

The AchE activity of the brain of control larvae were 7.96 ± 0.18, 7.0 ± 0.15 and 7.53 ± 0.26 µmoles formazone formed/mg wet wt of tissues/hour respectively. The ethanolic extract of *C. roseus* leaves extract treated larvae of (*Ae. aegypti, An. stephensi* and *Cx. quinquifasciatus*) AchE activity of brain were about 3.3 ± 0.2, 2.93 ± 0.14 and 3.06 ± 0.14 µmoles formazone formed/mg wet wt of the tissues/hour respectively. The AchE activity gradually reduced in the treated larvae than compared to control insects (Table 3). The AchE activity in the brain of control and treated larvae differ significantly at 0.05% level. The enzymatic measurements performed in LC_{50} treated larvae revealed a neurotoxic activity and a stimulation of the detoxification system as evidenced by an inhibition of AchE.

The cross and longitudinal section of control third instar larvae of *Cx. quinquefasciatus* mid gut region consists of epithelial cells resting upon a basement membrane. This membrane is surrounded externally by circular and longitudinal muscle fibers, respectively. The epithelium consists of columnar cells with clusters of small regenerative cells, each of which contains a relatively large nucleus and strongly basophilic cytoplasm. The epithelium is also protected from food particles by a detached sheath-a peritrophic membrane-surrounding a lumen. The epithelial cells revealed long and regularly placed microvilli borders toward the lumen (Figure A and C). Histopathological changes in the mid-gut region of third instar larvae exposed to LC_{50} of *C. roseus* ethanolic extract presented an irregularly structured brush border after 24 h. The epithelial cells began to swell via a slight vacuolization, with disorganized, shortened and degraded microvilli membranes. The structural disorganization of the mid-gut epithelium was evident; cells did not show the characteristic morphology, had become more destroyed. Degenerating nuclei, some time distinct elongations protruding into the lumen. Rupture of peritropic membrane, the epithelial cells appeared to have expanded into the gut

lumen (Figure B and D). Thus, the results from the present study revealed that the ethanolic extract of this leaf has substantial property for a larvicidal natural product.

In the present study, the diminished glycogen content in body tissues of *Aedes*, *Anopheles* and *Culex* about 9.3 ± 1.2, 8.6 ± 0.8 and 9.6 ± 0.8 mg/g larvae indicates its rapid utilization for energy generation; a demand caused by rutin extracted from *C. roseus* leaves to extract as a consequence toxic stress during the experiment. Finally, glycogenolysis seems to be the result of increased secretion of catecholamine due to the stress of plant extracts treatment (Tiwari & Singh, 2005). Larvae also secrete catecholamine in excess amount, during stress, which depletes glycogen reserves (Nakano & Tomlinson, 1967). Anaerobic and aerobic segments are two important components of carbohydrate metabolism. In the first case, the breakdown of glucose or glycogen through Embden-Meyerhof pathway (glycolysis) takes place while the next one consists oxidation of pyruvate to acetyl co-A to be utilized through citric acid cycle (Nelson & Cox, 2005).

Table 1. Amount of glycogen content of control and treated 3rd instar larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*

Body Tissues	Control mg/g	Treated (24 hours) mg/g
Ae. aegypti	14.6 ± 0.8[a]	9.3 ± 1.2 [b]
An. stephensi	14.0 ± 1.1 [a]	8.6 ± 0.8b[b]
Cu. quinquefasciatus	16.3 ± 1.2 [a]	9. 6 ± 0.8 [b]

Values are mean ± Standard deviation of six individual observations. Values are significantly different at $p < 0.05$.

Table 2. Amount of protein content in the controland treated 3rd instar larvae of *Ae. aegypti An. stephensi* and *Cx. quinquefasciatus.*

Body Tissues	Control mg/g	Treated (24 hours) mg/g
Ae. aegypti	63.6 ±2.4[b]	51.0 ± 1.1 [a]
An. stephensi	59.6 ± 2.0[b]	49.0 ± 1.7[a]
Cu. quinquefasciatus	61.6 ± 1.4 [b]	50.3 ± 1.4[a]

Values are mean ± Standard deviation of six individual observations. Values are significantly different at $p < 0.05$.

Table 3. Acetyl cholinesterase (AchE) activities in the brain tissue of control and treated 3rd instar larvae of *Ae. aegypti*, *An. Stephensi* and *Cx. quinquefasciatus.*

Brain Tissues	Control µmoles formazone	Treated µmoles formazone
Ae. aegypti	7.96 ± 0.18[c]	3.3 ± 0.2 [a]
An. stephensi	7.0 ± 0.15 [b]	2.93 ± 0.14[a]
Cu. quinquefasciatus	7.53 ± 0.26 [bc]	3.06 ± 0.14[a]

Values are mean ± Standard deviation of six individual observations. Values are significantly different at $p < 0.05$

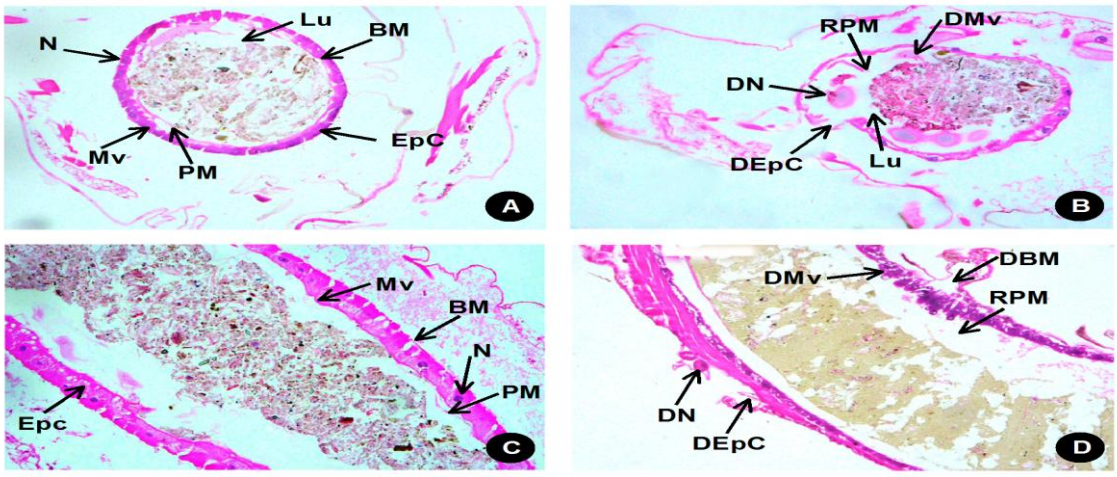

Figure ABCD. Cross (A, B) and longitudinal section (C , D) of midgut region of third instar larvae of *Cx. quinquefaciatus* control (A, C) and treated (B, D) with LC_{50} of *C. roseus* ethanol extract, showing the effects after 24 hrs of exposure.

N	Nucleus	Mv	Microvilli
EpC	Epithelium Cell	Lu	Lumen
PM	Peritrophic Membrane	BM	Basement Membrane
DEpC	Degenerating Epithelial Cell	RPM	Rupture of Peritropic Membrane
DMv	Degraded Microvillai	DN	Degenerating Nuclei
DBM	Degraded Basement Membrane		

Rohani *et al*. (2005) studied the protein synthesized by dengue-infected *Ae. aegypti* and *Ae. albopictus*. They concluded that dengue virus was shown to induce specific protein bands in both *Ae. aegypti* and *Ae. albopictus.* (Rivero & Ferguson, 2003) determined changes in energetic (sugar, glycogen and lipids) and biochemical (protein) levels in *An. stephensi* larvae infected with malaria parasite (Rivero *et al*., 2007) investigated the energy budget of *Ae. aegypti* larvae infected by *Vavraia culicis.* Bakr *et al.* (2010 b) studied the changes in protein content of *Cx. pipiens* mosquito treated with two agricultural waste extracts. Fallatah, (2009) investigated histopathological and biochemical effects of myrrh, pomegranate and black seed on *Cx. quinquefasciatus* larvae and protein analysis showed changes in general protein profile of treated larvae compared to normal larvae Physiological (carbohydrate and lipid) and biochemical (Protein and Amino acid) status of *An. stephensi* larvae treated with various medicinal plants was observed by Senthilkumar *et al.* (2009). Saeed *et al*. (2010) reported that *Cx. pipipens* exposed to *Allium satvium*, *Citrus limon* and *Bti* (*Bacillus thuringiensisvar israelensis*) was observed that the use of plant oil extracts and Bti have a great effect on the total protein content of treated mosquito larvae.

AchE is a significant biomolecule of neuro-signal transmission. For the normal metamorphosis and development of the mosquito larval stages, the signal pathway is essential. AchE influences the moulting of the insect integument by the hormone ecdysone secreted by the neuro endocrinal system. The ethanolic extract of *C. roseus* and its treatment would suggest the functional damage of corpora cardiaca and corpora allata through the inhibition of AchE. It is suggested that extract could be selected as bioactive toxic phytochemical for the control of mosquito population. Previous studies reported that essential oil and plant extracts which are known to have larvicidal activity against mosquitoes and several other compounds had been isolated with AChE inhibition activity (Chaithong *et al*., 2006; De Morais *et al*., 2007; Qin *et al*.,2010). Massoud *et al*. (2001) determined the biochemical changes in *Cx. pipiens* treated with oil and oleo-resin extract of *Myrrh commiphora* molmol which revealed inhibitory action on the protein contents and loss of certain enzymes which affect the metabolic processes. Lerdthusnee & Chareonviriyaphap, (1999) compared isoenzymes patterns of 13 fields collected populations of *Ae. aegypti* by using starch gel electrophoresis.

In this study, histopathological changes in the mid-gut region of third instar larvae exposed to LC_{50} of *C. roseus* ethanolic extract presented an irregularly structured brush border after 24 h. The epithelial cells began to swell via a slight vacuolization, with disorganized, shortened and confluent microvilli membranes. The structural disorganization of the mid-gut epithelium was evident; cells did not show the characteristic morphology, had become more destroyed. Sometimes distinct elongations were protruding into the lumen. The histopathological analyses of the digestive system of the treated larval group were consistent with the results of other previous studies (Arruda *et al*., 2003; Barreto *et al*., 2006). Extracts of *Magonia pubescens* and *Sapindus saponaria* were also reported to cause serious damage to the midgut epithelial cells via processes including cytoplasmic vacuolization (Arruda *et al*., 2003). Sharma *et al*. (2009) reported that extract of *Azadirachta indica* cause Culex larvae damaged body wall and larval tissues. Body wall made of chitin, a protein, and other protenious tissues were destroyed in both the species resulting in an overall decrease in protein levels of the larvae. Extracts of *Copaifera reticulata* cause the partial or complete destruction of midgut epithelial cells via cytoplasmic vacuolization, enlargement of intercellular spaces, and alteration of microvilli, while also affecting the nuclei and nucleoli. The midgut possesses a well-developed brush border in the cell apex because it is the main absorption area in the mosquito gut (Alves *et al*., 2010).

CONCLUSION

It is concluded that the effect of *C. roseus* leaves extracts on carbohydrate and protein contents in treated larvae are species and specific extraction. The lowering of these biochemical components indicates that these extracts can lower the feeding and proper digestion of food. They further interrupt with protein synthesis hormones resulting in its decline. The ethanolic extract of *C. roseus* and its treatment would suggest the functional damage of corpora cardiaca and corpora allata through the inhibition of AchE. Many insecticides that were successful at controlling pest populations in the past have negative, unintentional consequences on the environment. These range from toxicity to non-target animals, persistence in the environment and even the possibility of breaking down into more toxic chemicals. Thus, the current need of the hour is to use naturally procured phytochemicals as mosquito vector control agents. The results of the present study collectively suggested that the extract could be utilized as a bioactive phytochemical concoction (synergic effect) for the control of mosquito population and provides further scope to study the compounds individually or in combination for the better understanding of the mosquito larvicidal activity.

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