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Hitherto unobserved inhibition of insect chitinase enzyme by natural terpene

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Abstract
It is more benefit to develop an antimosquito agent to attack the larval development rather than using chemicals directly influenzing chitin metabolism. Gossypol a natural toxic terpene has shown antimicrobial activity. The LC_{50} dose of 195.4 ppm terpenoid aldehyde exposure for 48 hr among third stage larva of *Anopheles stephensi* has exhibited a block on the synthesis of ecdysone by interfering neuro transmitting acetylcholine in our earlier observation. In order to corroborate the above concept and the developmental disorder due to terpene, the terpene exposed experimental mosquito larval heads are homogenized and injected in to the nymph of *Periplanata Americana.* The hameolymph samples have shown significantly reduced chitinase enzyme activity while comparing the corresponding values of cockroach. This would suggested the impairment of chitin metabolism through the inhibition of ecdysone hormonal regulation by the terpene compound. Further the injected nymphs have prolonged larval period, restriction on the number of moulting and never attain adult characters. The above results are discussed with relation to antimosquito principle.

Keywords: Inhibition of chitinase by terpene. gossypol as antimosquito agent. Natural chitinase inhibition and development disorder

1. Introduction
Chitin metabolism plays an important role for the moulting and development of insects. The chemical nature of chitin is a polymer of N-acetyl-β-0 glucosamine. It is usually binds with insect cuticle proteins such as resilin which has high residue of amino acids glycine and proline. Chitin together with resilin gives an elevated elasticity for the cuticle. The synthesis and degradation of chitin are by carbohydrate splitting enzyme β-N acetylglycosaminidases. In insects two types of chitinolytic enzymes are existing. They are endo and exo chitinases. The endo β-N acetylglycosaminidase involves for the chitin degradation while poly-N – acetylglycosaminidase is engaging for the polymerization and synthesis of chitin during moulting. The mixed functions of these two enzymes are under the control of ecdysone hormone secreted by the corpora allata (Kingan and Adams, 2000; Chang *et al.* 2003) [5].

Chitin metabolism is highly significant bio event during insect development. Therefore inhibition or interruption of the chitin metabolism leads to insect developmental defects. This subject becomes an important objective for the control of insect pests and disease causing vector insects. Two different ways have been practiced to disturb the chitin metabolism. Enzyme inhibitors such as peptidyl nucleosides and acyl urease are used to block the chitin synthesis. Likewise substances which can interfere the ecdysone hormone of chitin metabolism during insect moulting (Hans and Lars, 2003; Proespraiwong *et al.* 2010) [3]. In our earlier studies in this line we found that a terpenoid aldehyde which is known for the high content of phenols and terpenes derived from cotton seed oil has developed ecdysone hormonal block by inhibiting acetylcholine of the oesophagel ganglion (so called 'brain') in *Anopheles stephensi* mosquito species. It is difficult to isolate or elute chitinase enzyme from the mosquito larval haemolymph. Hence the present study is an attempt of an extension to the above research and test the phenomenon by using cockroach as insect model.

Materials and Methods
The dissected out heads of *Anopheles stephensi* mosquito species and the nymphs of cockroach were served as materials. The phenolic rich terpenoid aldehyde was derived from cotton seed oil. The methonolic extract and the method of exposure studies are explained in details. The toxic dose of LC_{50} was found out by probit mortality calculation. It was known as 48 hr exposure of 195.4 ppm for the III stage larva of *Anopheles stephensi*. (Agatha Christy and Jayaprakash, 2017) [1]. The dissected heads of 100 mosquito third stage larva prior to the exposure of terpenoid aldehyde were used as controls while the exposed mosquito larval heads are served as experimental. In each case 100 heads are homogenized in mechanical stirrer in aliquot medium. Then it was centrifuged at 1000 rpm for 20 minutes. The supernatant was used to determine the effect of head extract on the chitinase enzyme activity in cockroach. The extract was injected in to the pleural region of nymph of cockroach. An amount of 5 points of extract using DB diabetic syringe was injected. Both the control nymphs and nymphs which were given experimental head extracted were observed for their development as well as estimation of chitinase enzyme specific activity in their haemolymph samples.

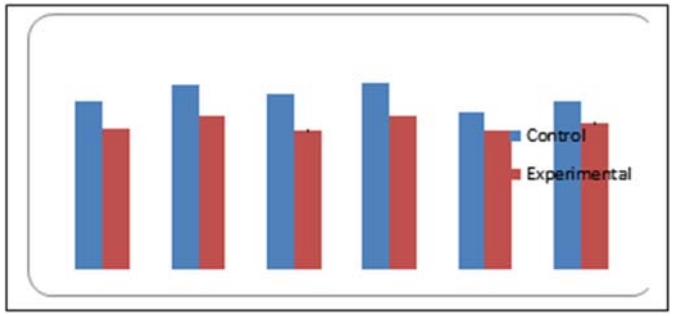
Method for chitinase enzyme activity
The chitinase enzyme activity was measured by the release of N-acetylglucosamine by hydrolysis. Standard graph of straight line was constructed by the known concentration of N-acetylglucosamine by UV spectrophotometer, the known quantity of N-acetylglucosamine was added to make up a mixture of 250 µl acetic acid at pH 5.0. The absorbance was measured at 585 nm. The same procedure was followed for the haemolymph samples of cockroach test insect. One unit of chitinase specific activity was expressed as µml of N-acetylglucosamine for 1 hr.

Results and Discussion

The results of chitinase enzyme activity in haemolymph samples of cockroach are furnished in table 1. From the data it is evident that the control insect has a mean value of 1142.28 µml N-acetylglucosamine per one hour. On the other hand the experimental batch has a mean value of 949.29 µml N-acetylglucosamine per hour. This would suggested that injection of mosquito head extract after the treatment of LC_{50} dose of 195.4 ppm of cotton plant derived phenolic rich terpenoid aldehyde has reduced the activity of chitinase enzyme of cockroach. It is recalled here from our earlier results in *Anopheles stephensi* mosquito larva observation that terpenoid aldehyde has significant functional aspects of oesophagel ganglion by blocking the neuro transmitting molecules namely acetylcholine and interfering moulting. The present study is also corroborated that the terpenoid aldehyde treated brain cells of *Anopheles stephensi* injection in nymphs of cockroach inhibits the synthesis of chitinase enzyme.

Table 1: Quantitative estimation of chitinase specific activity in haemolymph samples of Periplanata americana.

Control	Experimental
1109.13 ± 0.11@	932.18 ± 0.15
1216.09 ± 0.16	1011.11 ± 0.61
1153.16 ± 0.54	914.15 ± 0.13
1231.07 ± 0.13	1009.12 ± 0.33
1039.13 ± 0.42	917.09 ± 0.12
1105.11 ± 0.27	962.07 ± 0.16
Mean - 1142.28	Mean - 949.29



@ µml of N-acetylglucosamine /1 hr.

This could also be reflected on the development of cockroach. The development of cockroach is said to be incomplete metamorphosis. The egg case called ootheca gets mature, bursts and releases several small young nymphs. The nymphs after periodic moulting attain adult stage. There is no pupal stage in between nymph and adult. There was an extended pupal stage in experimental cockroach when comparatively observed with that of control nymphs. The gradual moulting and assumption of nymph attaining all adult characters by periodic ecdysis under normal hormonal action is described as paurometabolic metamorphosis. Normally *Periplanata americana* tooks eight to twelve moulting and the development lasts for 9 to 13 months. To our surprise the experimental batch of cockroach observed in this present study has shown an extraordinary extended nymphal stage. There was no ecdysis after 4 or 5 moulting. They do not developed adult characters such as wings and gonads even beyond sixteen months of survival period. The above foregoing observation and the analysis of chitinase enzyme specific activity would unambiguously disclose that terpenoid aldehyde of cotton plant have the ability to impair insect moulting through the inhibition of chitinase enzyme activity.

Conclusion

Chitin polymers do not exist in vertebrates. It is confined to arthropods and certain fungal species. Therefore development of antimosquito larval agents which interfere chitin metabolism is advantageous. Instead of directly inhibiting chitin synthesis by chemicals, blocking hormonal regulation of chitin through anti ecdysone steroid hormone is interest of recent innovation (Retnakaran, 2001) [8]. The ecdysone antagonist can disturb the development of mosquito. Gossypol a terpenoid aldehyde of cotton origin has rich terpenes and phenols. It has been shown as potential toxin against many microbial types. It is desirable to undertake much more clear studies in future to apply this terpene as antimosquito agent.

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