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EFFECT OF AZADIRACHTIN ON GROWTH AND THE ACTIVITY OF MIDGUT ENZYMES OF THE COCKROACH *PERIPLANETA AMERICANA*

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Abstract: Azadirachtin, a potent antifeedant was isolated from Sri Lankan neem seeds. Effects of azadirachtin on body weight of adult male and female cockroaches was examined for 7 days. Body weight and faecal production of treated insects were compared with that of the control group. Generally, the body weight of all the treated cockroaches decreased while that of the control insects increased continuously. All treated cockroaches produced fewer faeces when compared with untreated, fed control group insects. The reduction of body weight and faecal production were prominent at the doses >1μg/g body weight of cockroaches. Both *in vivo* and *in vitro* experiments were carried out in order to examine the effect of azadirachtin on midgut enzymes of adult female cockroaches. The activity of protease, invertase and amylase were measured separately in both azadirachtin treated cockroaches (1μg/g body weight) and the control cockroaches using the methods previously described. The *in vivo* results indicate that the azadirachtin treated cockroaches showed 50% reduction of the activity of all three enzymes and azadirachtin had no inhibitory effect on the activity of midgut enzymes *in vitro*. The same experiment was carried out with the ligatured cockroaches in order to study the effect of neurohormones on the secretion of midgut enzymes. In contrast, similar results were obtained from the azadirachtin treated insects and the control insects suggesting that the toxic effect of azadirachtin is associated with a disruption of endocrine events in cockroaches.

Keywords: azadirachtin, invertase, Neem, *Periplaneta americana*, protease.

INTRODUCTION

The Neem tree, *Azadirachta indica* is emerging as an important source of insecticides. All parts of the tree are biologically active, but the most effective one is neem seed kernel due to their high concentration of azadirachtin,[1] one of the many complex terpenoids present in the seeds of the neem tree. It was originally identified as the major component in the antifeedant activity of the plant. Since then, the compound has been shown to have a range of biological activities on the metamorphosis and development of certain insects.[1,2,3]

Azadirachtin has subtle effects, which may provide clues as to its cellular mode of action. For example, adult locusts treated with azadirachtin become sluggish and show reduced locomotor and flight activity.[1] Insect's muscle structure has also been affected by azadirachtin. Histological studies of midgut muscle of *Schistocerca*

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gregaria and *Locusta migratoria* show that the muscle becomes swollen and disrupted in a dose-dependant and time dependant manner after azadirachtin treatment.[2] Protein synthesis in brain, corpus cardiacum, haemolymph and suboesophageal ganglion (SOG) in *S. gregaria* has recently been shown to be affected by azadirachtin treatment. Two-dimensional electrophoresis revealed polypeptide profiles showing mainly disappearance but also induction of proteins within 3 days of treatment with 2.5 μg/g body weight of azadirachtin.[3]

A great deal of work throughout the world over the last 30 years has indicated clearly that various extracts of the neem tree have great potential as biological insecticides. Almost all the research work has been concentrated on agricultural pests and stored product pests and limited attempts have been made on the use of biological pesticides to control household pests. The purpose of this study was to understand a few physiological activities related to azadirachtin towards one common household pest in Sri Lanka, namely *Periplaneta americana*.

The American cockroach, *Periplaneta americana* is an economically important household pest, which is controlled primarily by the use of synthetic insecticides. Repeated application of insecticide has resulted in the development of resistance to chlorinated hydrocarbons, organophosphate, carbamate and pyrethroid insecticides.[4]

The work reported here was focused to understand the effect of azadirachtin on the following.

1 The effect of azadirachtin on the development and faecal production of American cockroaches and to determine whether azadirachtin could be used to control American cockroaches.

2 The physiological effects of azadirachtin on various types of insects has already been reported. But very little is known about effect of azadirachtin on the digestive enzymes of the cockroach, *Periplaneta americana*. Hence the second objective of this investigation was to determine the effect of azadirachtin on the activity of midgut enzymes of cockroaches *in vivo* and *in vitro* and the effect of neurohormones on the secretion of midgut enzymes.

METHODS AND MATERIALS

Insects: Adult male and female *P.americana* of the same age were used for all the experiments and bioassays. They were provided with a balanced laboratory cockroach diet containing protein, carbohydrate, fat, multi-vitamins and tap water.[4] The cockroaches were maintained in plastic containers. The temperature in all experiments was maintained at 30 ^{0}C ± 2 ^{0}C with a photoperiod of 12:12 (L:D) h.

Chemicals: Azadirachtin (> 95% purity) was isolated from Sri Lankan neem seeds using flash chromatography and Medium Pressure Liquid Chromatography. 3,5 dinitrosalicylic acid, glucose, tyrosine and Coomassie Brilliant Blue (CBB) was purchased from Sigma Chemical Company Ltd.

Bioassay: Four groups of cockroaches, each group containing 7 cockroaches were used for the bioassays. Azadirachtin isolated from neem seed and neem cake was used for the bioassays. Azadirachtin was dissolved in acetone to obtain a series of 0.5, 1, 2, 3 ml/g concentrations. The cockroaches were anesthetized by freezing for 10 to 15 minutes in the refrigerator. Each cockroach was injected 1µl/g body weight of azadirachtin and the cockroaches in controls were injected with 1µl/g body weight of acetone. Injection was made through the arthrodial membrane between coxa and the first thoracic segment. After injection, the cockroaches were placed in separate plastic containers with food and water. Weight loss and faeces produced were determined every 2nd day. The data were statistically analysed using ANOVA.

The in vivo effect of azadirachtin on the activity of midgut enzymes: Treated insects were killed, 5 min and 30 min after injection of 1µg/g body weight azadirachtin in order to isolate the midgut enzymes. The alimentary system of the cockroach was removed from the body, and carefully rinsed with insect saline (NaCl (7.5g/l),$CaCl_2$ (0.2 g/l),KCl(0.35 g/l)and $NaHCO_3$(0.2g/l), before isolating the midgut. The isolated midgut tissues were frozen immediately in the freezer. A group of eight cockroaches were in each treatment. Isolation of midgut of control cockroaches was carried out as the same way as azadirachtin treated cockroaches.

Isolation of the midgut enzymes: The midgut tissues of *P. americana* were homogenized with an ultrasonic homogeniser in 1 ml of 0.01 M phosphate buffer (pH 8.0) to extract the midgut enzymes. The suspensions were centrifuged for 15min at 10,000g and samples of supernatants were taken for estimation of the activity of midgut enzymes.[5]

The in vitro effect of azadirachtin on the activity of midget enzymes: The cockroaches were anaesthetized under ether and dissected in the insect saline. The midgut was removed from the insects and washed with insect saline. The midgut was incubated in 0.80ml of insect saline at 37°C for 30min. and then 0.02ml of azadirachtin was added to the medium. Isolation of midgut enzymes was carried out after 5min and 30min as described in the *in vivo* analysis. 0.02ml of acetone into 0.80 ml insect saline only were added to the controls.

Determination of activity of protease: For the determination of protease activity, casein was used as the substrate. 0.04ml of 1.5% casein solution was prepared in a reaction mixture of 0.01 M phosphate buffer (pH 8.0) and in 0.2 M glycine -sodium hydroxide buffer (pH 11.0). A 200µl supernatant from the midgut homogenate was added to the reaction mixture. Enzyme activity was terminated after 30 min of

incubation at 37 °C by adding 1.28 ml of 5% trichloroacetic acid. The reaction mixture was then filtered through Whatman No:1 filter paper and the filtrate was taken for enzyme activity evaluation. Proteolytic activity was measured at an absorbance of 280nm.[6] Data were analysed statistically using ANOVA.

Determination of activity of Invertase and Amylase: Amylase and invertase activities were determined under optimal experimental conditions.[5,6] 3,5 dinitrosalicylic acid reagent was used to measure free aldehyde groups formed after starch digestion. This reaction was based on the reduction of the dinitrosalicylic acid by the aldehyde groups of glucose units in the starch. The reduced dinitrosalicylic acid can be measured spectrophotometrically at an absorbance of 550nm.

Amylase activity was determined in the reaction mixture of 0.4 ml 0.05 M glycine -NaOH buffer (pH 9.5) , 0.2ml 1% starch solution and 0.02ml enzyme extract. Invertase activity was determined in the reaction mixture of 0.48 ml 0.05 M phosphate buffer (pH 7.0), 0.2 ml 4% sucrose solution and 0.02 ml of the enzyme extract. After 10 min incubation at 37 °C enzyme activity was terminated by adding 1.6 ml of 3,5, dinitrosalicylic acid reagent. The reaction mixture was heated for 5 min at 100 °C followed by rapid cooling in an ice bath and dilution with 1.6ml of distilled water. Absorbance of reduced nitrosalicylic acid at 550nm was measured. A calibration curve was constructed using D-glucose under the experimental conditions similar to those used for amylase and invertase. Data were analysed statistically using ANOVA.

Determination of total protein content: The enzyme solution (200 µl) was added into 1.48ml distilled water and mixed with 1.5ml of CBB reagent (0.25 g CBB G 250 dissolved in 12.5ml of 95% ethanol and mixed with 25ml of phosphoric acid and diluted up to 250ml). Absorbance was measured at 595nm after 2-4mins. Bovine Serum Albumin was used to prepare the calibration curve and enzyme activities were estimated with reference to the total protein content of the midgut enzyme solution.

Effect of neurohormones on the activity of midgut enzymes: Adult female cockroaches were anaesthetized by chilling at 0 °C for 5mins. and the cockroaches were head-ligatured and 1 µg/g body weight of azadirachtin was injected into each cockroach.

The control cockroaches were injected with an equal volume of acetone along with head ligation. The activity of midgut enzymes was estimated 8 h after injection in both control and azadirachtin treated cockroaches.

RESULTS

Injection of azadirachtin into the adult male and female cockroaches produced dose response curves. Effects of different concentrations of azadirachtin on body weight

of adult male cockroaches are shown in fig. 1 and effects of azadirachtin on faecal production of adult male cockroaches are shown in fig. 2. The faecal production and body weight of female cockroaches with azadirachtin were similar to male cockroaches. Generally, treated cockroaches produced less faeces compared with untreated-fed insects. Azadirachtin inhibited the growth of cockroaches whereas the development was normal in acetone treated insects. The weight reduction of cockroaches varied with the dose of azadirachtin administered. The body weight of all treated cockroaches decreased except the cockroaches treated with 0.5 µg/g body weight of azadirachtin and controls. Cockroaches treated with 1µg, 2µg and 3µg per gram body weight of azadirachtin showed significant weight reduction during the period of observation. The minimum effective dose of azadirachtin was identified as 1 µg/g body weight and this dose was used to determine the physiological effects of azadirachtin on activity of midgut enzymes of cockroaches.

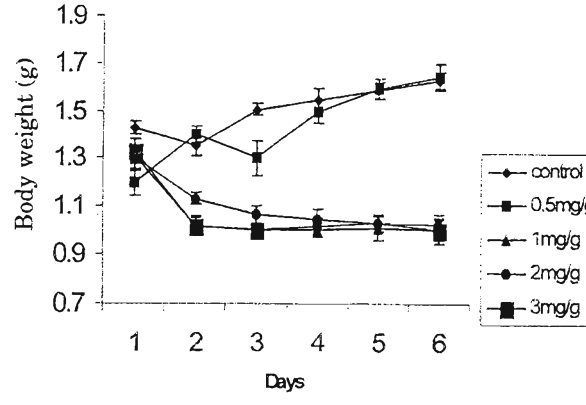

Figure 1: Effect of different concentrations of azadirachtin on body weight of adult male cockroach *Periplaneta americana*. (Each data point represents at least four replicates with a total sample size of 7 individuals).

In order to perform enzyme extractions and enzyme activity determinations in an environment comparable to that found in insect gut, the experiments were carried out in a medium containing insect saline.

The data on enzymatic activity are reported in relation to the total protein content in the enzyme extract. The data obtained for activity of protease, invertase and amylase of azadirachtin treated insects were compared with that of the control cockroaches. The results of the effect of azadirachtin on the activity of midgut enzymes *in vivo* and *in vitro* are shown in Table 1. Fig.3 shows the activity of protease, invertase and amylase in the midgut of the cockroaches 5mins after injection.

In order to determine the neurohormonal effect on the activity of midgut enzymes, the cockroaches were ligatured, subsequently the effect of azadirachtin

was also examined by injecting it into the haemolymph of the ligatured cockroaches. The results show (table 2) that the activity of midgut enzymes in treated cockroaches was similar to that of the control cockroaches.

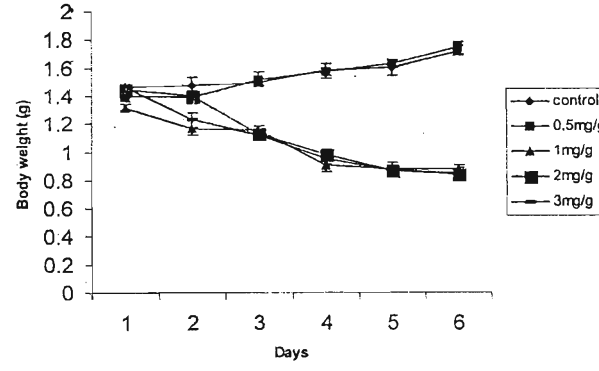

Figure 2: Effect of different concentrations of azadirachtin on body weight of adult female cockroach *Periplaneta americana*. (Each data point represents at least four replicates with a total sample size of 7 individuals).

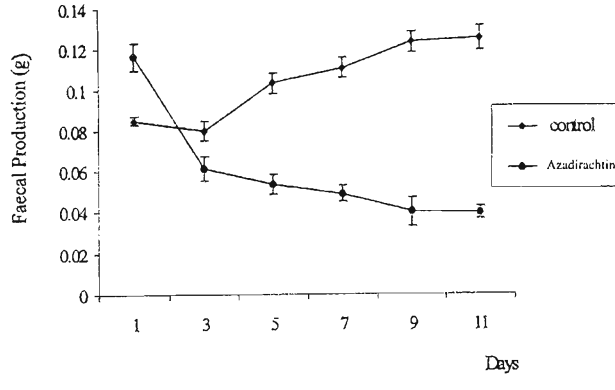

Figure 3: Effect of azadirachtin on faecal production of adult male cockroach *Periplaneta americana*. (Each data point represents at least four replicates with a total sample size of 7 individuals).

Azadirachtin incorporated into the haemolymph of the cockroaches significantly reduced the activity of midgut enzymes. Data obtained 5mins and 30mins after injection of azadirachtin showed similar pattern of results. The control cockroaches showed higher level of enzyme activity than in the azadirachtin treated cockroaches. The results indicate that the activity of midgut enzymes of the treated cockroaches was dramatically reduced to below 50% of that of the control.

Table 1: Effect of azadirachtin on activity of midgut enzymes of cockroach *Periplaneta americana in vivo* and *in vitro*

Enzyme	Incubation time Mins	Enzyme activity µg/total of protein/hr.	
		Treated insects	Control insects
In vivo			
Protease	5	8.42±(0.004)[a]	15.79±(0.02)[b]
Protease	30	8.12±(0.03)[a]	14.09±(0.05)[b]
Amylase	5	8.53±(0.032)[c]	15.79±(0.01)d
Amylase	30	8.34±(0.02)[c]	15.45±(0.05)[d]
Invertase	5	13.32±(0.005)[e]	27.91±(0.03)[f]
Invertase	30	13.05±(0.05)[e]	27.45±(0.05)[f]
In vitro			
Protease	5	16.70±(0.04)[g]	15.45±(0.04)[g]
Protease	30	15.76±(0.034)[g]	14.09±(0.05)[g]
Amylase	5	28.85±(0.05)[h]	28.51±(0.05)[h]
Amylase	30	14.09±(0.05)[i]	15.85±(0.06)[i]
Invertase	5	15.76±(0.034)[j]	14.0±(0.005)[j]
Invertase	30	28.51±(0.01)[k]	30.05±(0.04)[k]

± denotes SE
Means with the same letters do not differ significantly at P=0.05
(P<0.05 was considered as significantly different)

Table 2: Effect of head ligaturing on the activities of midgut enzymes of cockroach *Periplaneta americana*

Enzyme	Azadirachtin treated Cockroaches µg/total protein/hour	Control Cockroaches µg/total protein/hour
Protease	15.40±(0.05)[a]	14.90±(0.03)[a]
Amylase	14.95±(0.04)[b]	16.81±(0.03)[b]
Invertase	29.05±(0.05)[c]	29.73±(0.03)[c]

To determine whether the azadirachtin would retain its inhibitory activity when azadirachtin was mixed with the enzyme solution in an artificial medium using insect saline, the experiment was performed *in vitro*. The addition of azadirachtin to the incubations had no inhibitory effect on the enzyme activitity and the result reveal that the activity of the enzymes in azadirachtin treated medium was similar to that of the controls.

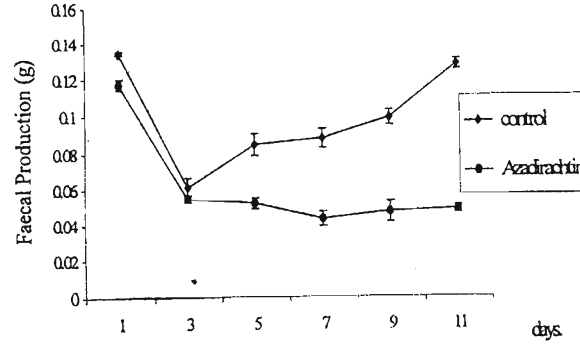

Figure 4: Effect of azadirachtin on faecal production of adult female cockroach *Periplaneta americana*. (Each data point represents at least four replicates with a total sample size of 7 individuals).

DISCUSSION

Previous studies have demonstrated that azadirachtin was effective in disrupting growth and development of various species of insects. Margosan-O (340µg azadirachtin per milliliter) was tested against German cockroaches.[7] The results indicate azadirachtin caused increased mortality and delayed development of the nymphs. The fecundity of Homopteran insects is strongly influenced by neem extracts or azadirachtin. For example, sweet potato whitefly, *Bemisia tabaci*, confined to cotton treated with neem seed extract deposited less than 20% of eggs compared with controls for up to 7days after treatment.[1] The present study demonstrates the potential of azadirachtin as a growth retardant for the cockroach *P.americana*. Effects of different concentrations of azadirachtin on the body weight of cockroaches were compared with that of control. Azadirachtin is well known as a potent growth inhibitor in insects. With azadirachtin at 1µg / g body weight or higher shows a reduction of the weight of the cockroaches suggesting that azadirachtin caused a strong antifeedant and toxic effects in *P. americana*. This decrease in body weight and increase in motility suggests altered feeding behavior or this behavior may be caused by alteration in neural control centers by azadirachtin.[8]

In terms of nutritional physiology, it has been recently shown with several lepidopteran species, that avoidance of primary antifeedant activity can be achieved

quite easily after applying azadirachtin either topically or by injection.[9] Accordingly evidence is available that insect growth inhibitory and antifeedant effects are independent of each other. The effects of azadirachtin on gut physiology are mostly related to efficiency of diet conversion or inhibition of digestive enzymes.[10] Dysfunction of midgut due to necrosis following azadirachtin treatment in locusts has also been demonstrated.[2]

The experiments reported here were performed to examine the effects of azadirachtin on the activity of midgut enzymes of adult female cockroaches. It has been already reported that when a physiologically effective dose of azadirachtin (2-3 μg azadirachtin / g body weight) was injected, it induced an inhibitory effect on the growth of *P. americana*.[4] The activity of all three enzymes, namely protease, invertase and amylase, in the gut contents of azadirachtin treated cockroaches was reduced to 50% of that seen in controls 5mins after injection of azadirachtin. However, it was clearly shown that the inhibitory effects of azadirachtin 5mins and 30mins after injection were similar, suggesting that the inhibitory effect of azadirachtin would be due to secondary antifeedant effect or the toxic effect.

Although it is known that the neuroendocrine system is involved in the control of secretion of enzymes from the midgut, the effect of the neurosecretion system is complicated. Following elimination of neurohormone release by head ligation, the data for the activity of midgut enzymes in both azadirachtin-treated and control cockroaches was similar, with only the azadirachtin treated normal cockroaches showed 50% reduction of enzyme activity. This suggests that azadirachtin is having a specific effect on the action of neurohormones which stimulate enzyme activity. These results are comparable with the *in vitro* results, which demonstrate that the enzyme solution incubated with azadirachtin showed the same level of activity as the control experiment.

The effect of neurohormones on the midgut of other insects has also been studied. It is clear that the neurosecretory system can affect the synthesis of digestive enzymes of the midgut. There is evidence that azadirachtin inhibits digestive enzyme secretion. In *Manduca sexta*, azadirachtin inhibits the production of trypsin by the enzyme-secreting cells of the midgut wall and in *Spodoptera litura* azadirachtin significantly affects the digestive enzymes such as protease, invertase and amylase.[11] It is quite evident from the present study that azadirachtin interference with the action of neurohormones *via* digestive enzyme impairment, is apparently one of the modes of action of azadirachtin.

In contrast, azadirachtin has a direct effect on gut muscle contraction and the activity of most digestive enzymes, which are secreted by the midgut of *Manduca sexta* caterpillar.[12] The inhibition of gut contraction by azadirachtin has a marked effect on passage through the gut. This results not only in lower faecal production

but in lower rate of absorption of food. Studies of midgut tissue show that the epithelial cells are much disrupted by the action of azadirachtin.[12]

References

1 Schumutterer H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*, *Annual Review of Entomology* **35**: 271-297.

2 Nasiruddin M. & Mordue A.J. (1993). The protection of barley seedling from attack by *Schistocerca gregaria* using azadirachtin and related analogues, *Entomol., Expet.. Applc.*

3 Annadurai R. S. & Rembold H. (1993). Azadirachtin A modulates the tissue specific 2D polypeptide patterns of the locusts, *S. gregaria, Naturwissenschften*, **80** : 127-130.

4 Prabhakaran S. K. & Kamble S. T. (1996). The effect of azadirachtin on different strains of German cockroaches (Dictyoptera: Blattellidae), *Entomological Society of America*, **25** : 130-134.

5 Ishaaya I., Moore I., & Joseph D. (1971). Protease and amylase activity in larvae of the Egyptian cotton worm, *Spodoptera littoralis.*, *Journal of Insect Physiology*, **17** : 945-953.

6 Ishaaya I, Ascher K.R.S. & Shuval G. (1974). Inhibitory effect of the antifeeding compound AC-24,055 4' (-3,3-dimethyl -1-triazeno) acetanilide on digestive enzymes of *Spodoptera littoralis* larvae, *Pestic. Biochem. Physiol.*, **4**: 19-23.

7 Alder V.E. & Uebel E.C. (1985). Effect of a formulation of neem extract on six species of cockroaches (Orthoptera), *Phytoparasitica* **17**: 3-8.

8 Sieber K.P. & Rembold H. (1987). The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria, Journal Insect Physiology*, **29**: 523-527.

9 Koul O. & Isman M.B. (1991). Effect of azadirachtin on the dietary utilization and development of variegated cutworm *Peridroma saucia, Journal of Insect Physiology*, **37**: 591-598.

10 Peter A.J. & Ananthakrishnan T.N. (1993). Effect of azadrachtin on the food utilization efficiency and digestive enzyme activity in *Cyracanthacris tatarica, Phytophaga*, **5**: 35-43.

11 Koul O., Shankar J.S. & Kapil R.S. (1995). The effect of neem allelochemicals on nutritional physiology of larval *Spodoptera litura*, *Entomologia Experimentalis et Applicata*, **79**: 43-50.

12 Timmins W.A. & Reynolds S.E.(1992). Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* catapillars, reduced growth due to impaired protein digestion, *Entomologia Experimentalis et Applicata*., **63**: 47-54.