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Impact of cow urine extract of *Azadirachta indica* leaves on the digestive enzymes of stored product pest, *Tribolium castaneum*

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Abstract
Tribolium castaneum is a major stored product pest, infesting a number of grains from store houses to other storage places also. Many synthetic pesticides were used for control of stored product pests. Synthetic pesticides minimise these pests as well as create a resistance to synthetic pesticides among those pests. To overcome this problem, the attention had been made for biopesticides to control stored product pests. In this study cow urine extract of *A. indica* leaves were used as a biopesticide. Based on the LC_{50} value sublethal concentrations were fixed as 0.08, 0.16 and 0.24%. Both *T. castaneum* larvae and adult were treated with food grains with sublethal concentrations. The digestive enzymes were analysed before pupation in the case of larvae and after 10 days in adults. A dose dependant reduction was observed in amylase and protease but in the case of invertase at lower doses invertase activity was slightly increased but at higher doses activity was decreased.

Keywords: *T. castaneum*, biopesticide, digestive enzymes

Introduction
Stored grain loss in weight and quality of products due to insects is a serious problem worldwide. It is estimated that stored grain loss of over 10% occur each year due to insect pests among the stored houses throughout the world and tropics, in particular, *T. castaneum* is a major secondary pest of processed or damaged grains (Danahaye *et al*., 2007) [4].
To minimise the use of synthetic pesticides, it is necessary to seek safe, convenient, environmental, and low-cost alternative pest control methods. Considerable efforts have focused on plant derived materials that are potentially useful as commercial insecticides. Plant derivatives are less toxic or nontoxic to mammals, other vertebrates, and invertebrates. Plant products have several uses in insect control.
Throughout the world, the new trend is use of biopesticides for insect pest control in storage of cereals (Rizvi *et al.,* 2001) [13].
Living on a starch-rich diet, many insects depend on the enzymatic activity of amylase, or more precisely, α-amylase, for survival. α-Amylase (α-1,4-glucan-4-glucano hydrolase; EC3.2.1.1) is a hydrolytic enzyme found in microorganisms, plants and animals. This enzyme catalyzes the endohydrolysis of long α-D-(1, 4)-glucan chains, such as starch and related carbohydrates, allowing organisms to use starch as an energy source (Baker, 1991; Strobl *et al.*, 1998).
In the light of above mentioned facts, the present investigation was carried out to investigate the efficacy of cow urine extract of *A. indica* leaves on the digestive enzymes for the stored grain pest, *T. castaneum.*

Materials and methods
Plant collection
Neem leaves were collected from college campus (Queen Mary's College, Chennai, Tamil Nadu). It was ensured that the plant was healthy and uninfected. Leaves were washed under running tap water to remove dirt. Then washed with distilled water, air dried and chop in to small pieces.

Cow urine
The indigenous cattle, scientifically called as *Bos indicus*, mainly inhabitat the Indian subcontinent.

Indigenous cow's early morning fresh urine was collected and kept in an air tight container.

Preparation of bioformulation

250g of chopped *A. indica* leaves were soaked in one litre of cow urine separately in an air tight container for 10 -15 days. Thereafter the leaves were pressed between the palms, remains were thrown out and the extract was filtered (Gupta, 2005) [7].

The filtered solution serves as the stock solution. From the stock solution different concentrations were prepared using distilled water.

Bioassay

Newly moulted third larvae of *T. Castaneum* and newly emerged *T. Castaneum* adult were separated into different containers and fed with fifty grams of rava grains treated with different concentrations of the extract. Control insects were maintained with rava grains without any treatment. Based on the LC_{50} value (0.8%) three sublethal concentrations (0.08, 0.16 and 0.24%) were fixed and insects were treated for the enzyme studies.

Sample preparation for enzyme analysis

The sub lethal concentration of cow urine extract of *A. indica* treated larvae were anaesthetised using ice blocks. The entire larvae and adult were homogenised for 3 min at 0 ºC using a chilled tissue grinder. The homogenate was centrifuged for 15 min at 12000 rpm at 2 ºC in a centrifuge and the supernatant was used as enzyme source. Enzymatic activities of amylase, protease and invertase were estimated.

The protease activity of *T. castaneum* was determined according to Birk *et al*., (1962) [1]. One unit of protease activity was defined as the amount of enzyme that produced 1 Mm of Tyrosine per minute under the above conditions.

Amylase activity of *T. castaneum* was determined by the method of Ishaaya (1970) [7] using the modified 3, 5 –di nitro-salicylic acid reagent.

Invertase activity was determined as previously described (Ishaaya, 1970 and 1971) [9, 10] using the 3, 5- dinitrosalicyclic acid reagent for determining the free aldehydic groups of glucose formed after sucrose digestion.

Results

Optimum conditions for enzyme reaction i.e. pH, initial velocity enzyme and substrate concentration, were determined in a series of preliminary experiments in which individual factors were varied. The optima for amylase, invertase and protease activities were pH 5.5, 6.0, 6.8 respectively when phosphate buffers were used for the analysis. The results were tabulated (Table 1 and 2).

Cow urine extract of *A. indica* leaves showed moderate inhibitory activity on *T. castaneum* larvae. In this view, at 0.24% of extracts inhibitory activity of amylase was found to be 227.83±7.13 µg maltose / reaction, whereas control showed 878.56±7.11 µg maltose / reaction. A significant negative correlation was found to be -0.9952 and $P<0.01$.

T. castaneum adult insects treated with cow urine extract of *A. indica* leaves were showed a minimal inhibitory activity on amylase when compared to control. A significant negative correlation was observed as -0.9938 and $P<0.01$.

A highly significant negative correlation of -0.999 was obtained between the concentration of cow urine extract of *A. indica* leaves and protease activity of *T. castaneum* final instar larvae. Protease activity was found to be 46.29±0.92 µg tyrosine / reaction in the control and was greatly reduced to 12.08±0.52 µg tyrosine / reaction when treated with 0.24% of *A. indica.*

Discussion

Ishaaya *et al.,* (1971) [10] found that in *S. littoralis* larvae, increase in the protein content of the diet increased the protease activity alone but not the amylase activity. Engelman (1969) [7] concluded that the mechanism of enzyme synthesis is substrate specific. A positive correlation is also obtained by Chockalingam *et al.,* (1987) [2] between the enzyme activity of *E. fraterna* and substrate concentration when treated with carrot seed extract.

A direct inhibition of the enzyme of digestion (Singleston and kratzer, 1973) [14]. Another hypothesis explained that the lowered food consumption would result in the observed decreased activities of digestive enzymes.

This phenomena was already reported in several other species of insects (Engelman, 1969; Gooding, 1975; Muraleedharan and Prabu, 1979; Christopher, 1983) [5, 6, 11, 3]. All these hypothesis seem to be applicable in the present study.

However an increase in invertase activity was also observed in *E. fraterna* treated with plant extracts of *D. metel* flowers and roots. The invertase enzyme is responsible for regulating the toxic substances in the alimentary canal of insects (Noorjahan, 1982). Hence an observed increase in the activity of invertase may be due to the toxic stress created by the certain protein fractions or other active compounds present in food. Those active compounds (Hori, 1969) [8] can stimulate digestive enzymes, probably through a hormonal mechanism. The present study suggests that the invertase can also be used to determine the adaptability of the insects to the toxic stress.

Table 1: Impact of cow urine extract of *A. indica* leaves on the digestive enzymatic activities of larvae of *T. castaneum* (Mean ± SD).

Concentration of extracts (%)	Amylase (µg maltose/reaction)	Invertase (µg glucose /reaction)	Protease (µg tyrosine /reaction)
Control	878.56±7.11	672.52±4.09	46.29±0.92
0.08	755.33±4.21	812.46±3.91	39.21±0.94
0.16	536.47±8.01	797.03±6.24	25.35±0.61
0.24	227.83±7.13	256.42±2.78	12.08±0.52

Table 2: Impact of cow urine extract of *A. indica* leaves on the digestive enzymatic activities of adult of *T. castaneum* (Mean ± SD).

Concentration of extracts (%)	Amylase (µg maltose/reaction)	Invertase (µg glucose /reaction)	Protease (µg tyrosine /reaction)
Control	365.54±11.07	486.12±5.19	27.33±0.49
0.08	351.14±9.10	437.27±3.18	25.71±0.37
0.16	272.23±4.11	284.65±5.07	19.09±0.41
0.24	155.46±1.02	202.40±3.05	14.23±0.27

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