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Assessment of *Sauromatum guttatum* lectin toxicity against *Bactrocera cucurbitae*

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Abstract

Lectins are proteins that bind specifically to foreign glycans. Due to this binding property, these molecules have potential application as bioinsecticidal tools replacing conventional chemical insecticides. The present study involved purification of phytolectin from the tubers of *Sauromatum guttatum* by affinity chromatography on asialofetuin-linked silica matrix. The purity of the sample was checked by SDS-PAGE at pH 8.3. Purified lectin was incorporated in the artificial diet of a Dipteran model, *Bactrocera cucurbitae* at different concentrations (10, 20, 40, 60 and 80 μgml^{-1}). The lectin significantly affected various developmental parameters that were studied. Percentage pupation and percentage emergence was reduced to 44 % and 7.9%, respectively, at 80 μgml^{-1} concentration as compared to control (100 %). LC_{50} of *Sauromatum guttatum* lectin was calculated to be 19.42 μgml^{-1}. Treatment of insect larvae with LC_{50} of *Sauromatum guttatum* lectin suppressed the activity of hydrolytic enzymes (esterases and acid phosphatases) and oxidative enzymes (superoxide dismutase and glutathione-S-transferase). Thus, with low LC_{50} and high mortality (approximately 92 % at 80 μgml^{-1}) of the insect larvae, *Sauromatum guttatum* lectin offers a possibility to engineer crop plants for improved and safer agriculture.

Key words:

Anti-oxidative enzymes, Araceae, Artificial diet bioassay, Hydrolytic enzymes, Tephritidae.

Introduction

Tephritids (Dipterans) that constitute over 4000 spp., cause enormous threat to fruit and vegetable production throughout the world (Benelli *et al.,* 2014). *Bactrocera cucurbitae* (Coquillett), melon fruit fly, belonging to this family is a polyphagous insect infesting around 81 host plants with damage reaching 30 %-100 % depending upon the season causing both qualitative and quantitative loss (Dhillon *et al.,* 2005). Direct damage is caused by adult females laying eggs under the skin of fruits and vegetables. The eggs hatch into larvae that feed in the decaying flesh of the crop. Infested fruits and vegetables are rendered inedible or drop on the ground (Ekesi and Mohamed, 2011). This fly has repeatedly defied the conventional control measures (Singh *et al.,* 2009). Moreover, the conventional method of controlling these insect pests involve the use of broad spectrum chemical pesticides has proved to be harmful for human health, environment and beneficial to insects as a non-specific target (Beard, 2006; Desneux *et al.,* 2007; Heimpel *et al.,* 2013). This has lead to a shift towards biopesticides that have overall more sustainable ecotoxicological profile as compared to conventional synthetic pesticides (Copping and Menn, 2000).

The use of plant derived pesticides and plant defence proteins has recently been reported as alternative control measure for insects (Ali *et al.,* 2012; Benelli *et al.*, 2013; Campolo *et al.,* 2013). Amongst the plant defence proteins, the most important ones are lectins, protease inhibitors, amylase inhibitors, acrelin and chitinases that are particularly abundant in plant storage organs such as tubers and seeds (Coelho *et al.* 2007; Babu *et al.,* 2012). Of all these proteins, plant lectins have raised special interest because of their specific carbohydrate binding properties to foreign glycans. This feature demonstrates the potential of using plant lectins as naturally occurring

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insecticidal agents against various insect pests that diminish crop production (Macedo *et al.,* 2007). *Galanthus nivalis* L. agglutinin (GNA), which is one of the most intensively studied lectins, has effectively been expressed in a range of crop plants including potato, rice, maize, tobacco, wheat, tomato and sugarcane (Oliveira *et al.,* 2011). Concanavalin A (ConA), peanut agglutinin (PNA), *Morniga*-G, wheat germ agglutinin (WGA) and phytohemaglutinin (PHA) are examples of other lectins that have shown insecticidal activity towards insects (Vasconcelos and Oliveira 2004; Sprawka and Golawska, 2010; War *et al.,* 2013). Studies have indicated little or no harmful effect of expressed lectins on second or third trophic levels, further ensuring relative safety of transgenic plants expressing lectins (Down *et al.,* 2000; Carilini and Grossi-de-sa, 2002; Vasconcelos and Oliveira, 2004).

The current study was thus designed to assess the insecticidal potential of *Sauromatum guttatum* Schott lectin against *Bactrocera cucurbitae* as a model insect for Tephritids. The insecticidal effect was evaluated on second instar larvae of *B. cucurbitae* by incorporating *Sauromatum guttatum* lectin in an artificial diet bioassay. Various developmental parameters studied include larval period, pupal period, total developmental period, percentage pupation and percentage emergence. This effect was further evaluated by assessment of activity of some hydrolytic and anti-oxidative enzymes including esterases, acid and alkaline phosphatases, superoxide dismutases, catalases and glutathione-S-transferase.

Materials and Methods

Purification of lectin: Tubers of *Sauromatum guttatum* plant were collected during August. They were washed, crushed and soaked in phosphate buffered saline (PBS, 0.01 M, pH 7.2) overnight at 4°C. The crude extract was filtered with surgical gauze and centrifuged at 11000 rpm for 20 min at 4°C (Centrifuge 5804 R, Eppendorf). Supernatant (crude extract) was stored in aliquots at -20°C with sodium azide (0.02 % w/v).

Sauromatum guttatum lectin was purified from crude extract by affinity chromatography using asialofetuin linked amino-activated silica bead matrix (Cliffmar, UK) as described previously (Shangary *et al.,* 1995). The bound lectin was eluted using Glycine-HCl buffer (100 mM, pH 2.5). Eluted fractions were immediately neutralized with Tris-HCl buffer (2 mM, pH 8.8). Presence of lectin in crude extract and purified fractions was detected by hemagglutination assay (Sandhu *et al.,* 1990). Lowry's method (1951) was used to determine the protein content using bovine serum albumin as standard. SDS-PAGE at pH 8.3, was performed to check the purity of lectin preparation (Laemmli, 1970).

Evaluation of insecticidal potential of *Sauromatum guttatum* lectin:

Maintenance of insect culture: Wild culture of *B. cucurbitae* was obtained from infested *Cucurbita moschata* Duchesne, procured from local market and identified by the Department of Zoology, Guru Nanak Dev University, Amritsar. Flies were reared on protinex (dietary protein supplement by pfizer) and 20 % sugar solution in fruit fly cages (45L × 45B × 50H cm). For oviposition, ripe pumpkin pieces were placed and gravid females were released in cages. For larval development, pumpkin pieces were transferred to battery jars (having autoclaved sand) covered with muslin cloth. Charged pumpkin pieces were removed and dissected in saline water for harvesting the second instar larvae (64 - 72 hr old) that were employed for all studies. Culture conditions were maintained by standard protocol at 25°C±2°C, relative humidity 70 %-80 % and were illuminated under L10:D14 photoperiod regime.

Effect of *Sauromatum guttatum* lectin on developmental parameters: The second instar larvae from pumpkin pieces were harvested in saline water. The experiment was set up in the culture vials in BOD incubator (Calton, Narang Scientific). Affinity purified lectin obtained after dialysis against PBS was incorporated in artificial diet prepared in water medium. The lectin concentrations used were 10, 20, 40, 60 and 80 μgml^{-1}. For control, lectin was replaced with PBS. There were six replicates for each concentration and second instar larvae (n=15) were used in each replicate set up in culture vials (25 mm diameter × 100 mm length). Development of larvae into adult flies was closely monitored and observations were recorded on daily basis. These observations were used to calculate various developmental parameters that were larval period, pupal period, percentage pupation and percentage emergence. Data collection was performed until there was no emergence continuously for 4-5 days in any of the vials. These data were further analysed to calculate lethal concentration of *Sauromatum guttatum* lectin for 50 % mortality (LC_{50}).

Effect of asialofetuin bound lectin on juvenile development: To further validate the fact that the effect of *Sauromatum guttatum* lectin on developmental parameters was due to lectin-sugar interaction, a similar diet bioassay was set up with lectin pre-incubated with asialofetuin. Affinity purified *Sauromatum guttatum* lectin (LC_{50}: 19.42 μgml^{-1}) was incubated (37°C, 1 h) with asialofetuin (4 $mgml^{-1}$), an inhibitory glycoprotein. A small fraction of bound lectin was used to perform hemaglutination assay to confirm asialofetuin binding to lectin. This bound lectin was incorporated in artificial diet of the larvae along with control.

Analysis of insect enzymes: Lectin at LC_{50} was incorporated in artificial diet bioassay in a similar experimental setup as described earlier. There were six replicates for each concentration and control. Larvae were harvested every 0, 24, 48, 72 and 96 hrs at beginning of the experiment from the control and treatment vials. Appropriately weighed larvae were crushed and enzyme extracts were prepared as mentioned in the respective protocols. For each stage, enzyme activity was measured spectrophotometrically. The method given by Lowry *et al.* (1951)

was used to estimate protein concentration of enzyme extracts followed by calculation of specific activity (unitsmg^{-1}).

Larval homogenates were prepared and activity of esterases, acid phosphatases and alkaline phosphatases was estimated by the method given by Katzenellenbogen and Kafatos (1971) and MacIntyre (1971). Superoxide dismutase activity was calculated according to the protocol given by Kono (1978). Catalase activity was estimated by the method of Bergmeyer (1974). The protocol given by Chein and Dauterman (1991) was followed to estimate the activity of glutathione-S-transferase.

Statistical analysis: Regression analysis was performed on the data obtained from juvenile development observation. Probit analysis was performed to calculate LC_{50} of *Sauromatum guttatum* and the entire enzyme assay data were analysed with student's *t*-test. All the analysis were performed with SPSS 16.0 (SPSS, Inc., Chicago, Illinois, USA). A value of $p<0.05$ was considered significant unless mentioned.

Results and Discussion

In the current study, *Sauromatum guttatum* lectin turned out to have a significant anti-insect activity towards *B. cucurbitae*. The affinity purified lectin had a titre of 2^6. Purity of lectin preparation was indicated by a single band in SDS-PAGE at pH 8.3.

Out of various developmental parameters tested, a significant decrease was observed in percentage pupation and percentage emergence. However, no significant effect was observed on larval period, pupal period and total developmental period. Larvae treated with 80 µgml^{-1} *Sauromatum guttatum* lectin, the percentage pupation and percentage emergence were reduced be to 44 % and 7.9 %, respectively, with respect to control (100 %). There was a significant proportion of variance due to *Sauromatum guttatum* lectin treatment in the percentage pupation, $R^2 = 0.895$, $F(1,5) = 34.011^{(p<0.01)}$ (Fig. 1A). Regression analysis showed that the relationship between percentage pupation and concentration was significantly negative due to *Sauromatum guttatum* lectin treatment, $\beta = 56.218$, $t(1) = 16.506^{(p<0.01)}$. Similarly, a significant proportion of variance in the emergence could be explained due to *Sauromatum guttatum* lectin in diet, $R^2 = 0.718$, $F(1,5) = 10.164^{(p<0.05)}$ (Fig. 1B). A negative relationship between percentage emergence and concentration was also significant, $\beta = 17.996$, $t(1) = 5.650^{(p<0.01)}$. A significantly lower percentage emergence of treatment groups with respect to control can be explained on the basis of decreased percentage pupation, thus affecting the overall development of the insect. LC_{50} was calculated to be 19.42 µgml^{-1} with confidence limits of 15.31 µgml^{-1} to 26.46 µgml^{-1} and $R^2 = 0.911$. LC_{50} of *Sauromatum guttatum* lectin was lower than some previously reported Araceous lectins (*Arisaema jacquemontii, A. intermedium, A wallichianum, Erythrina indica, Caladium bicolor, Colocasia esculenta*; LC_{50} ranging from 29.0 µgml^{-1} to 86 µgml^{-1}) tested towards this insect (Kaur *et al.* 2006, 2009, 2011; Singh *et al.* 2009; Thakur *et al.* 2013). A lower LC_{50} can be interpreted as higher activity of *Sauromatum guttatum* lectin as compared to other molecules mentioned and this might be attributed to its unique sugar specificity which makes it bind to the glycoprotein receptors on the peri-trophic membranes with higher affinity and, hence acquire higher toxicity.

To further validate the fact that the effect of *Sauromatum guttatum* lectin on developmental parameters was due to lectin-sugar interaction, a similar diet bioassay was set up with lectin pre-incubated with asialofetuin. A comparison of the results of bioassay with bound lectin, unbound lectin and control showed that diet containing lectin bound to asialofetuin had no effect on the *Bactrocera* larvae development and the results were comparable to control. These findings provided evidence that *Sauromatum guttatum* lectin interacts with the surface glycoproteins present on the cells lining digestive tract of the insect. As the binding domains of the lectin were blocked, shown by negative results in hemagglutination assay, there was no interaction of lectin molecules with the surface receptors of the

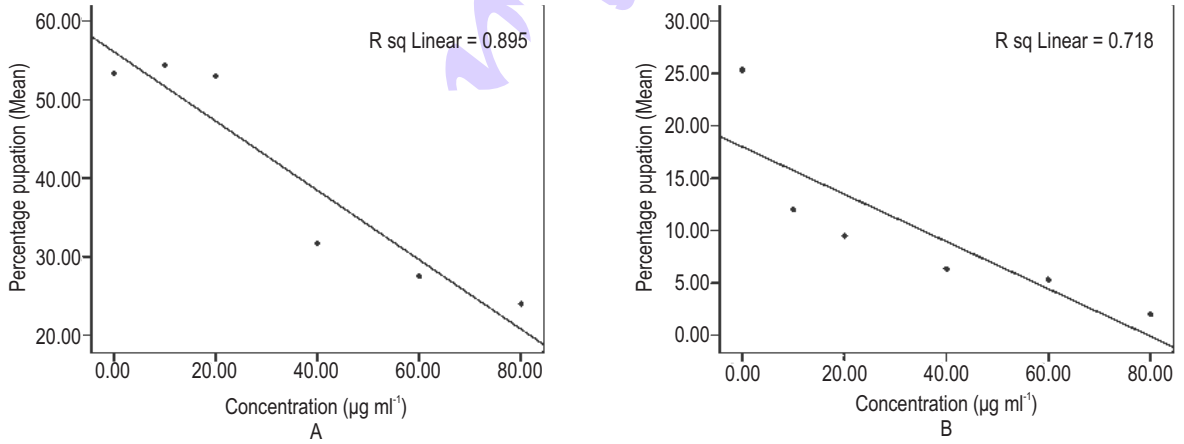

Fig.1.: Effect of dietary SGA on (A) percent pupation (B) percentage emergance of *B. cucurbite.*

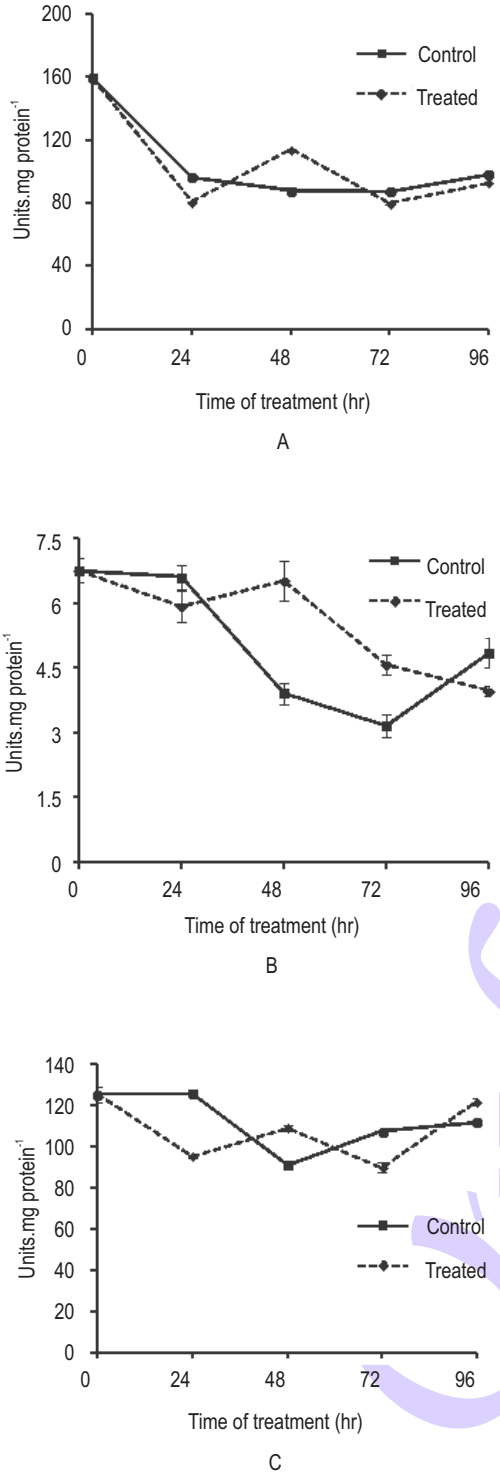

Fig.2.: Activity of various enzymes of *B.cucurbitae* larvae at 0, 24, 48, 72 and 96 hr of treatment of LC_{50} of SGA (A) esterases (B) acid phosphates (C) alkaline phosphatases. Each point represents mean value of the activity. Error bars indicate standard error of mean.

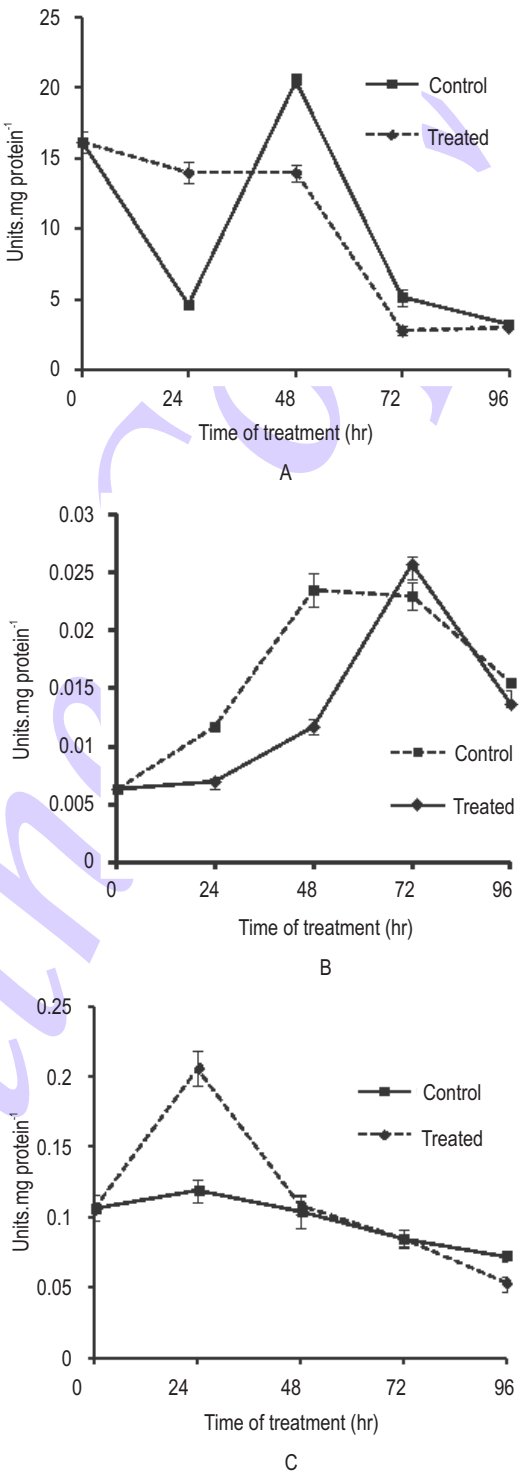

Fig.3.: Activity of various enzymes of *B.cucurbitae* larvae at 0, 24, 48, 72 and 96 hr of treatment of LC_{50} of SGA (A) superoxide dismutase (B) catalase (C) glutathione-S-transferase. Each point represents mean value of the activity. Error bars indicate standard error of mean.

digestive tract membranes and consequently no deleterious effects were observed. Some studies have previously demonstrated insecticidal lectins binding to the surface receptors lining insect midgut (Bandopadhyay *et al.,* 2001; Coelho *et al.,* 2007; Macedo *et al.,* 2007; Oliveira *et al.,* 2011). Furthermore, being resistant to digestion in insect digestive system, lectins might alter the intestinal protein content creating nutritional imbalance leading to detrimental effects on developmental parameters studied (Oliveira *et al.,* 2011).

Sauromatum guttatum lectin significantly ($p<0.01$ or $p<0.05$) affected the activity of all the three hydrolytic enzymes studied (Fig. 2). There was decrease in the activity of esterases (EST) in case of treated larvae as compared to control after 96 hr of treatment ($t_{96} = 5.394^{(p<0.01)}$). Similar results have been obtained earlier after treatment of *B. cucurbitae* larvae with pea lectin (Kaur *et al*., 2013). Similarly, reduced activity of EST was observed on the treatment of *Helicoverpa armigera* (Hub.) larvae with groundnut leaf lectin and Con A (War *et al.,* 2013). Phosphatases are another group of hydrolytic enzymes belonging to the class of detoxifying enzymes that display increased activity under metabolic stress induced by insecticides (Luskova *et al.,* 2002; Ahmed *et al.,* 2004). Alkaline phosphatases activity increased ($t_{96} = 4.811^{(p<0.01)}$) in response to *Sauromatum guttatum* lectin feeding insect larvae whereas, acid phosphatases activity was suppressed ($t_{96} = 2.420^{(p<0.05)}$) as compared to control. These findings suggest interference of *Sauromatum guttatum* lectin with insect metabolism. Furthermore, it was proposed that lectins may affect the enzyme activities by binding at sites other than their substrate binding site (Cristofoletti *et al.,* 2006). Lectins can result in increased enzyme activity by modifying their active sites due to physical interactions with them (Sprawka *et al.,* 2012). In addition to this, lectins can also bind to both enzymes and substrates resulting in higher enzyme-substrate affinity leading to altered enzyme activity (Macedo *et al.,* 2007). Alternatively, lectins can bind to glycan receptors at the intestinal surface and block them from enzyme interactions (Matsushita *et al.,* 2002).

Induction of oxidative stress is another mechanism by which various lectins, xenobiotics and insecticides have been reported to interfere with the normal development of insects (Singh *et al.,* 2009; Lozinsky *et al.,* 2012; Kaur *et al*., 2013). The activity of two anti-oxidant enzymes SOD and GST was found to be suppressed ($t_{96} = 0.534^{N.S.}$, $t_{96} = 2.945^{(p<0.05)}$, respectively) (Fig. 3A, C). Catalase activity significantly increased due to treatment ($t_{96} = 4.568^{(p<0.05)}$) (Fig. 3B). These results are in agreement with the previous reports of Thakur *et al.* (2013) where treatment of same insect model with *Colocasia esculenta* agglutinin suppressed SOD activity and increased CAT activity. In the current study, the alternate effect on the activity of two anti-oxidative enzymes indicate that *Sauromatum guttatum* lectin treatment induced reactive oxygen species (ROS) but SOD was not involved in their removal. Decreased activity of GST in treated larvae as compared to control larvae indicated that *Sauromatum guttatum* lectin suppressed the expression of this anti-oxidative enzyme as well. Contrary to these findings, GST activity was found to be raised in case of *Spodoptera* sp., *Drosophila* sp., *Bactrocera* sp. and *Helicoverpa* sp. larvae with various treatments (Rizwan-ul-haq *et al.,* 2010; Lozinsky *et al.,* 2012; Thakur *et al.,* 2013; War *et al.,* 2013).

In conclusion, *S. guttatum* lectin turned out to be an insecticidal molecule for *B. cucurbitae* with an LC_{50} of 19.42 μgml^{-1}. This is indicated by its effect on metamorphosis of insect larvae as reflected by negative effect of *Sauromatum guttatum* lectin on percentage pupation and percentage emergence. In addition, *Sauromatum guttatum* lectin induce metabolic and oxidative stress as indicated by suppression in the activity of various hydrolytic and oxidative enzymes.

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