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Effect of *Calendula officinalis* extracts on the nutrient components of different tissues of tobacco cutworm, *Spodoptera litura* Fabricius

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ABSTRACT

The impact of leaf and flower extracts of *Calendula officinalis* on total protein and total carbohydrate contents of the hemolymph, midgut and midgut content of *Spodoptera litura* larvae were studied. Among the leaf and flower extracts tested in the hemolymph, the highest reduction in protein content of the larvae was caused by chloroform leaf and benzene flower extracts whereas in the midgut, chloroform leaf and petroleum ether flower extracts reduced the protein to the maximum. Aqueous leaf and flower extracts had least activity in both hemolymph and midgut. However, in the midgut content, methanol leaf and chloroform flower showed maximum reduction in protein content and petroleum ether leaf and methanol flower showed least reduction. Total carbohydrate content was reduced by chloroform leaf, and petroleum ether flower extracts in hemolymph with least reduction in benzene leaf and flower extracts treated larvae. In the midgut, chloroform leaf and flower extract caused maximum reduction whereas in the midgut content, methanol leaf and petroleum ether flower extracts showed reduction in carbohydrate content, while aqueous leaf and flower extracts showed an increase in carbohydrate content in midgut content contrary to midgut. However, when compared to control, the protein and carbohydrate content were reduced in all the tissues of treated larvae irrespective of the extracts tested. These results suggest that *Calendula* extracts affect the nutritional status of *Spodoptera litura* larvae.

Key words: Botanicals, biochemistry, haemolymph, tobacco cutworm

INTRODUCTION

Botanical insecticides are currently studied more and more because of the possibility of their use in plant protection as an alternative method to the broad use of conventional pesticides. Several plant based insecticides are said to influence various biochemical components such as proteins, carbohydrates and lipids within the body of insects, thereby altering the internal metabolism of the insect, causing their reduced activity or mortality. Roya Khosravi and Sendi (2010) have reported reduction in the total protein, lipid and carbohydrate contents in the fifth instar larvae of lesser mulberry pyralid *G. pyloalis* after treatment with methanolic extract of *Artemesia annua* L meanwhile, Vijayaraghavan *et al.* (2010) reported the same in the leaf webber larva, *Crocodolomia binotalis* due to the influence of the extracts of *Strychnos nuxvomica* Linn, *Vitex negundu* Linn., *Lippia nodiflora* Burm. Reduction in protein content in *Spodoptera litura* larvae due to the activity of neem and annona seed extracts (Vijayaraghavan and Chitra, 2002), azadirachtin (Zhiwei Huang *et al.,*2004), *Ageratum conyzoides*, *and Ageratum vulgaris* extracts (Brisca Renuga and Sahayaraj, 2009) have been previously reported. Effect of methanol extracts of *Lantana wightiana* on total proteins, total carbohydrates of *S. litura* have been previously studied (Martin Rathi and Gopalakrishnan, 2010).

Calendula officinalis (Asteraceae) is a widely cultivated plant in Europe and North America for ornamental and medicinal purposes. In India, it is a commonly grown garden plant especially in the colder regions. The extracts from the aerial parts of *C. officinalis* showed antifeedant activity against the cabbage butterfly and potato beetle (Wawrzyniak, 2002). The chemical composition of *C.officinalis* includes sesquiterpenes, flavonol glycosides, carotenoids, flavonoids, triterpenoid saponins, triterpene alcohols, phenolic acids, tannins, sterols, mucilage, tocopherols etc. (Ahmed *et al.,* 1993). The triterpenoid, saponins, sesquiterpenes and phenolic acids are involved in the plant defense system against herbivore attack.

Tobacco cutworm, *Spodoptera litura* Fabricius (Noctuidae), is a polyphagous lepidopteran pest on tobacco. It also has been recorded on several other crops like cauliflower, castor, cotton, banana and mulberry. The fully grown caterpillars are the most voracious feeders and cause extensive damage by defoliation. This pest may become serious during the seedling stage. Majority of the strains of *S. litura* from South India exhibited high resistant levels of 61- to 148- fold to various organic pesticides and conventional synthetic pyrethroids (Kranthi *et al*., 2002; Chitra and Rao, 1996). Henceforth, the use of insecticides for controlling this pest is on the rise and has the ability to develop resistance to many more insecticides (Murugesan and Dhingra, 1995). Although several plants have been tested for their insecticidal properties on *S. litura*, the use of Calendula in control of this particular pest is quite novel. The phytoconstituents present in Calendula showed antifeedant effect on the pest. Hence, the present investigation was undertaken to study the impact of the extracts of *C. officinalis* on the digestive physiology of the tobacco cutworm larvae in the light of exploring the possibility of its use as biocontrol agent.

MATERIALS AND METHODS

Culturing of test insects

Spodoptera litura egg masses were obtained from the National Bureau of Agriculturally Important Insects (NBAII), Bangalore and reared in the laboratory on castor leaves (*Ricinus comunis* L. Euphorbiaceae) until pupation and adult emergence. Neonate larvae were transferred with the help of fine camel hair brush to tender castor leaves. The petioles of the leaves were inserted into glass vials containing water to facilitate the leaves to retain moisture and stay fresh for long hours. The later instar larvae were transferred to individual plastic containers, with fresh castor leaves. The leaves were changed once in two days for early instars and daily for late instars. The pupae were sexed and transferred to adult emergence chamber. Upon emergence, 10% sugar solution compounded with a few drops of vitamin E was provided to the adult moths that were allowed to mate. The females were offered fresh castor leaves to deposit eggs on. Eggs with leaf were kept in Petri plates for hatching and the above procedure was continued for mass culture of insects (Pratibha *et al.,* 2010). The mass culture and the experiments were carried out at 25 ±2°C, 70±5% relative humidity (RH), with 12:12 L:D cycle.

Extraction and formulation of botanicals

The shade dried leaves and flowers of *C. officinalis* were ground into powder. 500g of powder was then extracted with petroleum ether, benzene, chloroform, methanol and water, in succession in the increasing order of the polarity, in a Soxhlet apparatus to get respective crude extracts (Martin Rathi and Gopalakrishnan, 2005). The crude extract obtained was dissolved in minimal quantity of the solvent that served as a stock. The required dilution from the stock were prepared in acetone and emulsified with a few drops of sandovit.

Bioassay and treatments

Based on the previous trials with different concentrations (0.1%-10%), bioassays were performed with fourth instar larvae of *S. litura* using 10% concentration of different extracts of *C. officinalis* leaves and flowers. Positive control leaves were treated with acetone and the feed of untreated leaves served as negative control. A minimum of 30 larvae per concentration were used for all the experiments and the experiments were replicated thrice. Fresh castor bean leaves were sprayed with 10% of different extracts of *C. officinalis* and allowed to air dry for 20 min. The freshly molted fourth instar larvae (10-day old; 0.07±0.03g) were starved for 4 hours and then fed with treated and untreated leaves till it reaches its next instar. The uneaten leaves were removed every 24 hrs, and the larvae were fed with fresh treated leaves. At the end of the 3 days, the larvae were sacrificed to estimate total protein content (Lowry *et al.,* 1951) and total carbohydrate content (Dubois *et al.,* 1956) in hemolymph, midgut and midgut content of both treated and untreated larvae.

Preparation of tissue extracts for biochemical analysis

Hemolymph(HL)

Hemolymph samples of treated fourth instar larvae of *S. litura* were collected by puncturing the proleg using a pair of sterile scissors and drawing the exudate into 10 mL insect Ringer's solution (Miyazawa and Arakawa, 1999) containing a few crystals of sodium thio-sulphate to prevent melanization. The hemolymph sample was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was used as the source for estimations.

Midgut tissue (MG)

The biochemical source for estimations was prepared as per the methodology of Applebaum and Applebaum *et al.* (1961). The larvae were frozen at -21°C and the entire digestive tract dissected out in ice-cold insect Ringer's solution. The malphigian tubules, adhering tissues and gut contents were removed. The gut was homogenized for 3min at 4°C in ice-cold citrate-phosphate buffer (pH 6.8) using a tissue grinder (IKA®. T/18 Dispenser, Japan). Homogenized gut was suspended in ice cold buffer. The homogenate was centrifuged at 500 rpm for 15min and the supernatant was used for estimations.

Midgut content (MCN)

The midgut content was separated from the midgut tissue and homogenized with ice-cold citrate-phosphate buffer (pH 6.8) using a tissue grinder (IKA®. T/18 Dispenser, Japan). Homogenized sample was suspended in ice cold buffer. The homogenate was centrifuged at 500 rpm for 15 min and the supernatant served as the source for estimation of both proteins and carbohydrates.

RESULTS AND DISCUSSION

Plant Extracts on total proteins

The total protein content of hemolymph, midgut and the midgut content was significantly reduced irrespective of the extracts when compared to the positive and negative controls as shown in the table. However, among the extracts tested, significantly decreased levels of proteins was found in hemolymph of the chloroform leaf and benzene flower treated larvae (F=11.69, df=71, p<0.0015). The leaf and flower extracts of petroleum ether, methanol and aqueous though showed drastic reduction in comparison with the control but were not significant when compared to the chloroform and benzene extracts. No mortality was observed during the course of the experiment. The midgut of the larvae treated with chloroform leaf, flower and petroleum ether flower extracts showed significant reduction (F=148.63, df=104, p<0.0001) in total proteins when compared to that of all other extracts and also the controls. There was significant decline in the total proteins of the midgut content of larvae upon treatment with all the plant extracts in comparison with the controls. However, the methanol leaf and chloroform flower extracts showed most significant reduction (F=148.63, df=104, p<0.0001) compared to the remaining extracts. The activity of other extracts was not significantly different when compared with each other.

Earlier studies on *Agrotis ipsilon* (Hufnagel) after treatment with extracts of *Melia azedarach* and *Vinca rosea* (Abo-El-Ghar *et al*., 1996), *Helicoverpa armigera* upon treatment with extracts of *Artemisia annua, Ageratum conyzoides* and *Azadirachta indica* (Padmaja and Rao, 2000), *Dysdercus koenigi* Fab treated with annona seed extracts (Bhagawan *et al.* 1992). Reduction in the total proteins in the midgut tissues of *S. litura* after treatment with *Porterasia coarctata Takeoka* leaf extracts as been reported by Christian Ulrichs *et al.* (2007) and treatment with the leaf extracts of *Lantana wightiana*, *Premna tomentosa* and *Synedrella nodiflora* as observed by Martin Rathi *et al.* (2005) are in agreement with our results. Similar quantitative changes in total proteins in the larvae of *Tribolium confusum* due to the impact of Vetiver oil and ethanol extracts of *Calotropis procera* has been observed by El-Bermawy and Abdel-Fattah (2000). El-Naggar and Abdel-Fattah (1999) showed reduction in the total proteins in the midgut content of *Spodoptera littoralis* larvae when treated with Eucalyptus oil and its combination with gamma radiation. Overall, the chloroform and petroleum ether leaf and flower extracts reduced the proteins in the hemolymph and the midgut whereas the methanol leaf extract caused significant reduction of proteins in the midgut content. The level of protein content is dependant upon rate of synthesis, and breakdown of proteins and even water movement between tissues and hemolymph can also account for changes in protein level.

Table 1. Total protein content and total carbohydrate content (µg/mg) of hemolymph and different tissues of *S.litura* after treatment with *Calendula* extracts

Macromolecules	Treat-ment	Petroleum ether	Benzene	Chloroform	Methanol	Aqueous	Negative Control	Positive Control (Acetone)
Total Proteins in Hemolymph	Leaf	14.5±6.9[b]	15.5±1.9[b]	6.6 ± 0.0[a]	15.5 ±1.9[b]	20.0 ±0.0[b]	153.3±3.3	145.5±3.8
	Flower	12.6±0.0[b]	10.0±0.0[a]	13.3±0.0[b]	13.3±0.0[b]	16.6±0.0[c]		
Total sugars in Hemolymph	Leaf	3.4±1.9[a]	7.8±1.9[c]	3.3 ± 1.2[a]	4.5 ±1.9[b]	4.8 ±1.4[b]	9.4±1.9	8.4±1.9
	Flower	2.2±1.9[a]	6.3±1.9[b]	3.2±1.9[ab]	4.2±1.9[b]	4.3±1.5[b]		
Total Proteins in Midgut	Leaf	51.1±1.9[b]	57.8±1.9[b]	44.4±1.9[a]	78.9±1.9[b]	107.8±1.9[c]	174.4±1.9	151.1±1.9
	Flower	47.8±6.9[a]	77.8±1.9[b]	51.1±1.9[a]	76.7±0.0[b]	95.6±1.9[c]		
Total sugars in Midgut	Leaf	20.0±1.0[a]	30.0±0.7[b]	20.0±1.2[a]	26.7±1.4[b]	40.0±1.3[c]	51.1±1.9	53.3±1.9
	Flower	23.3±0.9[b]	36.7±0.9[c]	16.7±1.4[a]	30.0±1.7[bc]	43.3±1.2[d]		
Total Proteins in Midgut Content	Leaf	83.3±5.8[b]	120.0±0.0[c]	21.1±1.9[a]	192.2±1.9[d]	166.7±3.3[c]	221.1±1.9	214.4±1.9
	Flower	42.2±1.9[b]	167.8±1.9[d]	11.1±1.9[a]	162.2±1.9[d]	128.9±1.9[c]		
Total sugars in Midgut Content	Leaf	26.7±0.6[b]	26.7±0.4[b]	23.3±1.1[b]	13.3±1.2[a]	43.3±1.5[c]	63.3±1.3	64.4±1.9
	Flower	16.7±0.9[a]	23.3±1.1[b]	23.3±1.4[b]	33.3±1.4[bc]	43.3±1.1[c]		

The reduction may be due to increased breakdown of proteins to detoxify the active principles present in the plant extracts. It is likely that the insect degrades proteins to resultant amino acids in order to let them enter into the TCA cycle as a keto acid for compensation for the lower energy caused by stress (Nath *et al*., 1997). In addition to this, the antifeedant properties of botanicals also contribute for the reduction of nutrients in the insects. In our earlier studies, it has been observed that *Calendula* leaf and flower extracts showed an antifeedant effect on *Spodoptera litura* and henceforth the consumption and utilization indices of the larvae were significantly decreased (Medhini *et al.,* 2009).

Plant extracts on total carbohydrate

The total carbohydrate content of hemolymph, midgut and the midgut content significantly decreased irrespective of the extracts when compared to the positive and negative controls as represented in the table. In the hemolymph, among all the leaf and flower extracts tested, the amount of carbohydrates was drastically reduced in chloroform and petroleum ether leaf and flower extract ($F=16.99$, $df=71$, $p<0.0001$) treated larvae when compared to benzene, methanol and aqueous extracts. The midgut tissue showed significant reduction in the total carbohydrate content in all the extracts-treated larvae compared to the controls. The most significant reduction was observed in the larvae treated with chloroform leaf, flower and petroleum ether flower extracts ($F=145.14$, $df=71$, $p<0.0001$). However, though the benzene, methanol and aqueous leaf and flower extracts showed considerable reduction but were not significant when compared to each other. The methanol leaf and petroleum ether flower extracts showed significant reduction ($F=148.16$, $df=71$, $p<0.0001$) of total carbohydrates in the midgut content compared to all other extracts whose activity was not significantly different.

Our results are in concurrence with that of Vijayaraghavan *et al.* (2010) who demonstrated reduction in carbohydrate content of the hemolymph of larvae of *Crocidolomia binotalis* Zeller treated with extracts from *Lippia nodiflora* Burm*, Vitex negundo* Linn, and *Strychnos nuxvomica* Linn. Abdul Razak and Sivasubramanian (2007) who observed reduced carbohydrate content in *Chelomenus sexmaculata* Fabricius and *Chrysoperla carnea* Stephens due to the impact of neem, pungam and madhuca. The present study is in concordance with that of Shekari *et al*. (2008) who reported lower glucose in the midgut of the third instar larva of *X. luteola* when treated with *Artemesia annua*. Similar reduction in total carbohydrates of the midgut content was observed in *Spodoptera littoralis* upon treatment with extracts of *Azadirachta indica* and *Citrullus colocynthis* (Sayed Rawi *et al.,* 2011). Overall, the chloroform leaf and petroleum ether flower extracts reduced the proteins in the hemolymph and chloroform leaf and flower extracts in the midgut whereas the methanol leaf extract caused significant reduction of carbohydrates in the midgut content. Phytoconstituents like triterpenoid saponins, flavonol glycosides and phenolic acids lead to antifeedancy in insects thereby reducing the feeding efficiency inducing stress in the insects which in turn reduces some of the vital components in the body. Under such stress conditions, the nutrients get catabolized to meet the high energy demand (Seyoum *et al.,* 2002). Ultimately, insects die due to reduced energy me-tabolism (Etebari *et al*., 2006). In most of the herbivore insects the digestion of leaf material is mediated by several symbiotic microorganisms that reside in the hindgut. Any compound that kills off a reasonable amount of these supporting bacteria could reduce the insect's digestive capabilities is another possibility for the reduction in the nutrient level. When saponins are eaten by an insect, the hydrolytic conditions in the gut will activate the degradation of saponin from an inactive to an active form which could influence the microflora and/or various digestion and absorption processes (Waterman, 1993). Chakraborthy (2008) have reported the antimicrobial activity of *Calendula* extracts. The bioactive factors present in these extracts may show antimicrobial activity against the gut microflora of the larvae thereby contributing for the reduced digestive capacity. Similar observations are made by Adel *et al.* (2000) in their study of activity of a number of saponins and sapogenins on the cotton leafworm *S. littoralis*.

In the present investigation it has been observed that among all the extracts tested the chloroform and petroleum ether extracts of leaves and flowers of *C. officinalis* showed significant impact on the nutrients of interest and caused their reduction irrespective of the tissues tested in the pest. This shows the potency of *Calendula* to be used as a natural pesticide. Further investigations may be focused on investigating the detailed mode of action of each of the phytoconstituents present in this plant for a better understanding of their structure-activity relationship and specificity.

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