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Effects of β-sitosterol on growth, development and midgut enzymes of *Helicoverpa armigera* Hübner

Monika Mishra, Aarti Sharma, Vinay Singh Dagar and Sarita Kumar*

Department of Zoology, Acharya Narendra Dev College (University of Delhi), Kalkaji, New Delhi 110019, India

***Corresponding author**: sarita.sanjay90@gmail.com; saritakumar@andc.du.ac.in

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Abstract: *Helicoverpa armigera* is a global agricultural pest of serious concern. Continued use of chemical insecticides as control measures has raised grave health and environment concerns, necessitating a search for botanicals as safe alternatives. The current study investigates the effects of β-sitosterol, a bioactive phytocomponent in *Thevetia neriifolia,* on the growth and development, as well as on midgut enzymes of *H. armigera*. Dietary β-sitosterol produced dose-dependent systemic toxicity and growth inhibitory effects in *H. armigera*; the most significant effects were obtained with 10 μg/mL dietary β-sitosterol. Higher prepupal and pupal mortality in comparison to larval mortality and a comparatively greater reduction in average weight gained by later instars point to cumulative effects of β-sitosterol. The delayed effects were ascertained by the 82.05%-57.89% reduction in adult emergence in comparison to 95.02% emergence in controls. Dose-dependent effects of β-sitosterol were observed as significantly decreased enzyme activities of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) in the larval midgut. Suppression of enzyme activity was obtained in the order ALT>AST>ALP. Impaired activity of gut enzymes possibly lowered the energy reserves and affected nutrient transport through the gut epithelium, affecting the growth and development of *H. armigera.* Our study points to a promising use of β-sitosterol against *H. armigera*, although further examination and field studies are needed to ascertain its possible use in control programs.

Keywords: *Helicoverpa armigera*; β-sitosterol; alanine aminotransaminase; aspartate aminotransaminase; alkaline phosphatase

INTRODUCTION

Helicoverpa armigera is a widely spread destructive pest of many important agricultural crops in Asia, Africa, Australia and Europe [1]. Commonly known as cotton bollworm, the pest feeds on a wide geographical range of host plants, possesses the ability to adapt to new ecosystem and causes great economic losses to cultivated crops [2]. In India alone, this highly polyphagous insect feeds on about 181 plant species spread across 68 botanical families [3]. So far, the major tool applied to keep this pest under check was spraying of crop fields with chemical insecticides. However, this has resulted in the persistent occurrence of residual insecticides in foodstuff and drinking water, raising grave health concerns. The adverse effects of insecticides in the environment has resulted in ecological imbalance, emergence of more aggressive and resistant pests and the elimination of valuable non-target organisms [4]. Several studies have reported the development of resistance in *H. armigera* against almost every group of chemical insecticides [5-10]. All this has raised the necessity to explore and develop novel and eco-safe strategies for *H. armigera* control.

Plants contain a reservoir of endogenous secondary metabolites that provide them with defense against insects and increase their resistance to pest attack. Plant-herbivore interaction thus forms the basis of exploration of plants for pesticidal active natural products as alternates to synthetic counterparts [11]. Many phytocompounds like essential oils, flavonoids, alkaloids, glycosides, esters and fatty acids, are reported to possess anti-insect effects and can be used as alternatives to synthetic insecticides in different ways, such as repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants and attractants [12]. The identification of these chemical factors is crucial in determining the evolutionary and ecological processes of insect-plant interactions

in order to devise novel eco-friendly molecules with practical application in the field.

Our previous studies have shown the significant effects of the dietary stem extract of *Thevetia neriifolia* on larval growth, development and midgut enzymes of *H. armigera* [4,13-15]. The extract of *T. neriifolia* could diminish the levels of phosphatases and transaminases in *H. armigera*. These enzymes play several functions in the insect body and display an important growth diagnostic potential [16]. Alkaline phosphatase (ALP), a brush border membrane marker enzyme [17], is accountable for tissue cytolysis during insect development [18] while transaminases are key enzymes in the formation of non-essential amino acids, gluconeogenesis, and the metabolism of nitrogenous compounds and protein [19]. Involvement of midgut transaminases in the Krebs cycle and energy production affects insect growth and development considerably [20]. Thus, any deviation in these enzymes indicates the adverse effects on gut physiology and growth and development of the target insect.

Gas chromatography-mass spectrometry (GC-MS) analysis of *T. neriifolia* stem extract has revealed the presence of a number of components, with β-sitosterol as one of the important bioactive constituents [15,21]. β-sitosterol is a dominant phytosterol widespread in plants, animals as well as fungi [22]. It is structurally similar to cholesterol and is converted to cholesterol by phytophagous insects for their growth and reproduction [23-25]. It has also been observed as a feeding incitant in silkworms, *Bombyx mori* [26].

Various reports, however, have suggested that a few insects are incapable of converting β-sitosterol to cholesterol, which may affect their growth and development [27-29]. Incorporation of β-sitosterol in the diet of *Dermestes maculatus* inhibited its larval development and transformation into the pupal stage [27]. Treatment of *Pieris rapae* 5th instars with β-sitosterol affected their biochemical profile by reducing the protein and glycogen contents of hemolymph [28]. β-sitosterol, isolated from the petroleum ether extract of *Abutilon indicum,* has been found to be toxic against *Culex quinquefasciatus* [29].

According to available reports, the efficacy and potential use of β-sitosterol as a possible control agent of *H. armigera* has not been investigated. As a continuation of our previous work, in the present study we explored the effects of dietary stress caused by β-sitosterol on the growth and development, as well as on three midgut enzymes, alanine aminotransaminase, aspartate aminotransaminase and alkaline phosphatase, of *H. armigera.* The outcome of the study may help in devising a novel and eco-friendly control strategy.

MATERIALS AND METHODS

Rearing and maintenance of *H. armigera* under controlled conditions

The culture of *H. armigera* was maintained in a Biological Oxygen Demand (BOD) incubator under controlled conditions of 27±1°C temperature and 80±5% relative humidity, with a photoperiod of 12 h daylight/12 h dark [13].

Experimental diet

β-sitosterol was obtained from Sigma Aldrich, US (<99% purity). Dilutions were made in ethanol and stored at 4°C. An artificial diet for *H. armigera* was prepared using chickpea flour [15]. A preliminary toxicity and antifeedant bioassay was conducted with β-sitosterol against *H. armigera* on the basis of which three concentrations of β-sitosterol, 10, 1 and 0.1 µg/mL, which exhibited insignificant toxic and antifeedant activity, were selected to evaluate their impact on the growth parameters of *H. armigera*. The experimental diets possessing final concentrations of 10, 1 and 0.1 µg/mL β-sitosterol were prepared by replacing one part of the 200 mL of dry diets with the required concentration of β-sitosterol. The diet was solidified using agar after evaporation of the solvent. The diet containing only the solvent served as the control.

Examination of the effects of dietary stress of β-sitosterol on *H. armigera* growth parameters

Fifty neonates of *H. armigera*, 5 batches of 10 larvae each, were reared on each experimental diet. The development of each larva was studied and different growth parameters such as larval duration, weight of each larva on the day of successful molting, pupal duration, pupal weight, pupal length and adult

emergence were recorded. Morphological aberrations in the developmental stages and hormone-mimetic effects exerted by dietary stress of β-sitosterol were also noted. Controls were run simultaneously.

Effect of dietary stress of β-sitosterol on gut enzymes of *H. armigera*

The effect of dietary β-sitosterol was investigated on three midgut enzymes of *H. armigera*, alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP). Fourth instars of *H. armigera* were randomly selected from the experimental lines reared on 0.1, 1 and 10 µg/mL of β-sitosterol and killed at -20°C. The midgut was carefully excised, homogenized in ice-cold phosphate buffer (pH 7.0) for 5 min and centrifuged at 13226×g for 30 min at 4°C. The supernatant was used for estimation of enzyme activity. Each assay was conducted in 5 replicates, each containing 10 larvae. Respective control sets were reared simultaneously. The protein content of the samples was quantified using bovine serum albumin (BSA) as standard according to the Bradford protocol [30]. A volume of 800 µL of each standard and sample solution was separately mixed with 200 µL of Bradford reagent followed by incubation in the dark at 37°C. The absorbance was read at 595 nm.

Estimation of ALT activity

The activity of alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) was determined based on the method of Reitman and Frankle [31]. A volume of 500 µL of Reagent A (2.8 M L-alanine and 7 mM α-ketoglutarate in 0.1 M phosphate buffer, pH 7.4) was incubated at 37°C for 3 min in a water bath and added to 100 µL of larval tissue homogenate, incubated again at 37°C for 60 min, followed by the addition of 500 µL of Reagent B (1 mM 2,4 dinitrophenyl hydrazine in 1N HCl). The mixture was kept at 25°C for 20 min after which 5.0 mL of 0.4 N NaOH solution was added to stop the reaction. The mixture was incubated at 25°C for 5 min and absorbance was measured at 505 nm. The pyruvate standard was used to prepare the calibration curve. ALT activity was expressed as moles of pyruvate formed from α-ketoglutarate per mg of total protein per h.

Estimation of AST activity

AST activity was estimated following a similar procedure to that described for ALT, but with minor modifications. Reagent A was replaced by a mixture consisting of 75 mM L-aspartic acid and 17 mM α-ketoglutarate in 0.1 M phosphate buffer (pH 7.4). AST activity was expressed as moles of oxaloacetate formed from α-ketoglutarate per mg of total protein per h.

Estimation of ALP activity

The activity of alkaline phosphatase (ALP) was determined based on the method described in [32] but with modifications. One mL of bicarbonate and carbonate buffer (0.1 M, pH 10.4) each was mixed with 1.0 mL of 0.02 M disodium phenyl phosphate and incubated at 37°C for 3 min. One hundred µL of tissue homogenate was added to the mixture which was incubated for 15 min at 37°C. The volumes of 800 µL of 0.5 M NaOH, 1.2 mL of 0.5 M $NaHCO_3$, 1.0 mL of 4-aminoantipyrine and 1.0 mL of potassium ferricyanide were added, mixed and the absorbance was recorded at 510 nm. ALP activity was expressed as moles of phenol formed from phenyl phosphate per mg of total protein per h.

Statistical analysis

Data obtained from growth and development bioassays and estimation of enzyme activities were subjected to analysis of variance (ANOVA). The means were separated using Tukey's post hoc honestly significant difference (HSD) test for statistical significance considered for $P \leq 0.05$.

RESULTS

Effect of β-sitosterol on the growth parameters of *H. armigera*

A pronounced lethal and dose-dependent effect of 0.1, 1.0 and 10.0 µg/mL dietary β-sitosterol was recorded on the 2nd larval instars and pupal stages of *H. armigera* in comparison to the controls ($P<0.05$). Doses of 1.0 µg/mL and 10 µg/mL dietary β-sitosterol also induced significant toxic effects on the prepupal (37.83-54.76%) stages ($P<0.05$) (Table 1). However, 10 µg/mL dietary

Table 1. Mortality (%) at different developmental stages of *Helicoverpa armigera* when reared on a β-sitosterol-containing diet.

Dietary concentration of β-sitosterol (µg/mL)	I Instar	II Instar	III Instar	IV Instar	V Instar	VI Instar	Pre-Pupal	Pupal
	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM	Mortality (%)±SEM
Control	4.08±0.77 a	2.08±0.45 a	4.25±0.89 a	2.22±0.54a	2.27±0.28 a	0.0±0.0	2.32±0.35 a	4.76±0.64 a
0.1	6.0±0.81 a	8.38±0.63 b	0.0±0.0	2.38±0.32 a	0.0±0.0	2.43±0.56 a	2.50±0.23 a	17.94±2.18 b
1	6.0±0.86 a	9.09±0.52 b	0.0±0.0	5.0±0.69 b	2.68±0.73 a	2.63±0.49 a	37.83±2.93 b	34.78±1.85 c
10	12.0±1.19 b	10.63±1.54 b	4.54±0.73 a	0.0±0.0	4.32±0.91 a	5.87±0.74 b	54.76±2.84 c	42.10±2.56 d

Mean±SEM, calculated for five replicates, each replicate contains 10 larvae; means in each column followed by the same letters are not significantly different (P<0.05, one-way ANOVA followed by Tukey's all pair wise multiple comparison test)

Table 2: Effect of dietary β-sitosterol on *Helicoverpa armigera* larval and prepupal weight.

Dietary concentration of β-sitosterol (µg/mL)	Mean weight of III instars (g) ± SEM	Mean weight of IV instars (g) ± SEM	% Change in weight	Mean weight of V instars (g) ± SEM	% Change in weight	Mean weight of VI instars (g) ± SEM	% Change in weight	Mean weight of pre-pupae (g) ± SEM	% Change in weight
Control	0.0391 ± 0.0013 a	0.1282 ± 0.0062 a	227.87 (+)	0.3014 ± 0.0199 a	135.10 (+)	0.4926 ± 0.0141 a	63.43 (+)	0.4641 ± 0.0131 a	5.80 (-)
0.1	0.0447 ± 0.0034 a	0.1333 ± 0.0076 a	198.21 (+)	0.2995 ± 0.0142 a	124.68 (+)	0.4231 ± 0.0173 b	41.26 (+)	0.3916 ± 0.0165 b	7.44 (-)
1	0.0392 ± 0.0062 a	0.1026 ± 0.0162 a	161.73 (+)	0.2168 ± 0.0152 b	111.3 (+)	0.2968 ± 0.0281 c	36.91 (+)	0.2763 ± 0.0130 c	6.90 (-)
10	0.0442 ± 0.0014 a	0.0911 ± 0.0081 b	106.81 (+)	0.1721 ± 0.0103 b	89.12 (+)	0.2165 ± 0.0101 c	25.79 (+)	0.1906 ± 0.0056 d	11.96(-)

Mean±SEM, calculated for five replicates, each replicate contains 10 larvae; (+)indicates weight gain, (-)indicates a decrease in the weight of larvae and pupae; means in each column followed by the same letters are not significantly different (P<0.05, one-way ANOVA followed by Tukey's all pair wise multiple comparison test)

β-sitosterol did not result in any mortality in 4th instars although a significant mortality was recorded with 1.0 µg/mL dietary β-sitosterol (P<0.05). Results showed a direct correlation between the dietary concentration of β-sitosterol and its systemic toxicity on *H. armigera* developmental stages (Table 1).

The growth inhibitory effects of dietary β-sitosterol in *H. armigera* were shown by reduced average weight gain in the larvae during development (Table 2). Most significant effects (P<0.05) were revealed by the rearing of *H. armigera* neonates on the 10.0 µg/mL β-sitosterol-containing diet, which reduced the mean weight of 6th instars and prepupae by 56.04% and 59.93%, respectively, in comparison to the weight gained by control larvae (P<0.05). Less pronounced adverse effects were observed with 1.0 µg/mL dietary β-sitosterol, which caused a 39.74% reduction in weight gain in 6th instars (Table 2). The 0.56- and 0.37-fold decreases in pupal weight with dietary 1.0 and 10.0 µg/mL β-sitosterol, respectively (P<0.05), further confirmed its growth inhibitory effects (Table 3). Dietary β-sitosterol also reduced adult emergence in *H. armigera*, resulting in 82.05-57.89% adult emergence as compared to 95.02% emergence observed in controls (P<0.05) (Fig. 1). However, β-sitosterol could not induce any hormone-mimetic effects specified by the absence of larval-pupal or pupal-adult intermediates, and deformed adults during development.

Table 3: Effect of dietary β-sitosterol on *Helicoverpa armigera* pupal weight.

Dietary concentration of β-sitosterol (µg/mL)	Mean weight of pre-pupa (g) ± SEM	Mean weight of pupae (g) ± SEM	% Change in weight (-)
Control	0.4641±0.0131 a	0.4317±0.0112 a	6.98
0.1	0.3916±0.0161 b	0.3534±0.0171 b	9.75
1	0.2763±0.0130 c	0.2452±0.0115 c	11.25
10	0.1906±0.0056 d	0.162 ±0.0056 d	14.53

Larvae reared in five replicates, each replicate contains 10 larvae; (-) indicates decrease in pupal weight; means in each column followed by the same letters are not significantly different (P<0.05, one-way ANOVA followed by Tukey's all pair wise multiple comparison test)

Effect of β-sitosterol on the midgut enzymes of *H. armigera*

Among the three midgut enzymes investigated, the control set of *H. armigera* showed maximum activity for ALP, followed by ALT and AST. A significant decrease in all three midgut enzymes of *H. armigera*

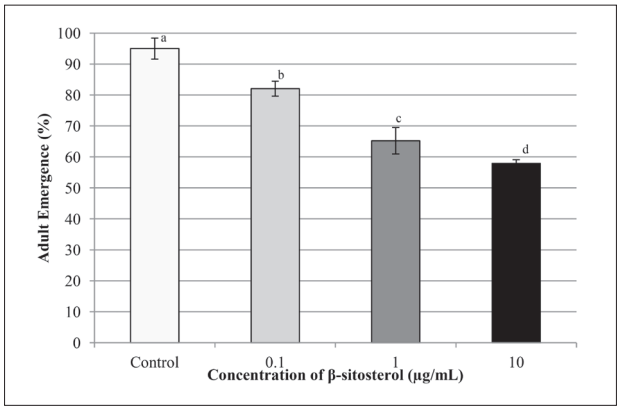

Fig. 1. Percent adult emergence in *Helicoverpa armigera* larvae reared on the control diet and diet containing 0.1, 1 and 10 µg/mL β-sitosterol. Larvae were reared in five replicates, each replicate contained 10 larvae. Bars (±SEM) with different letters differ significantly ($P<0.05$, one-way ANOVA followed by Tukey's all pair wise multiple comparison test).

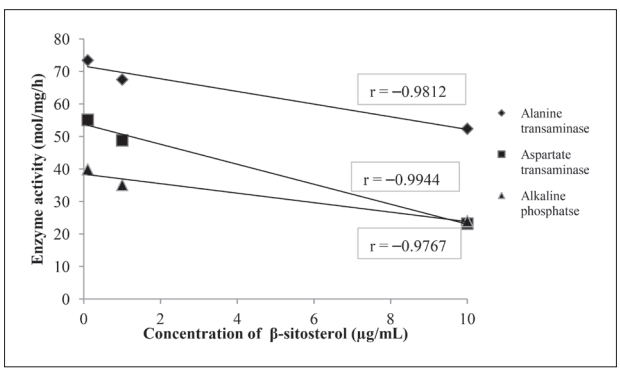

Fig. 2. Correlation between the dietary concentration of β-sitosterol and activity of alanine aminotransaminase, aspartate aminotransaminase and alkaline phosphatase in the midgut of *Helicoverpa armigera* IV instars.

Table 4. Effect of dietary β-sitosterol on the activity of midgut enzymes (mol/mg/h) in *Helicoverpa armigera* early IV instars.

Dietary concentration of β-sitosterol (µg/mL)	**ALT activity±SEM** (mol/mg/h)	**AST activity±SEM** (mol/mg/h)	**ALP activity±SEM** (mol/mg/h)
Control	56.81±0.003 a	41.68±0.006 a	74.07±0.008 a
0.1	55.12±0.005 b	39.88±0.005 b	73.42±0.005 b
1	48.81±0.008 c	35.08±0.009 c	67.51±0.003 c
10	23.14±0.005 d	23.96±0.004 d	52.36±0.008 d

ALT – alanine transaminase, AST – aspartate transaminase, ALP – alkaline phosphatase; five replicates, each contains 10 larvae maintained for the assay; means in each column followed by the same letters are not significantly different ($P<0.05$, one-way ANOVA followed by Tukey's all pair wise multiple comparison test)

larvae was observed with dietary β-sitosterol ($P<0.05$). The suppression was dose-dependent, increasing with the rise in dietary concentration (Table 4, Fig. 2). The decrease in enzyme activity was recorded in the order of ALT>AST>ALP, indicating more pronounced effects of β-sitosterol on the transaminases ($P<0.05$) in comparison to the phosphatase. In comparison to the control diet, 10.0 µg/mL dietary β-sitosterol reduced ALT activity by 59.17% (r=-0.9944), AST activity by 42.51% (r=-0.9767), and ALP activity by 29.31% (r=-0.9812) (Table 4, Fig. 2).

DISCUSSION

Plants possess a pool of secondary metabolites, which have a defensive role against herbivores, such as repellency, feeding deterrence, insecticidal activity, growth regulation, and other effects. These bioactive molecules are continuously explored by researchers owing to their non-detrimental effects on non-target hosts and the environment [33]. Our earlier studies described the effective use of *T. neriifolia* extracts against *H. armigera* [13,14]. Among various bioactive constituents identified in the *T. neriifolia* extract, β-sitosterol was reported to possess insect growth regulatory activity [4]. Likewise, the leaf extract of *Melochia corchorifolia*, which was found to be an effective antifeedant against *H. armigera*, *Spodoptera litura*, *Earias vittella* and *Leucinodes orbonalis*, contained sterols as one of the bioactive constituents [34].

Development of *H armigera* larvae on the β-sitosterol-containing diet adversely impacted their growth and development. Substantial mortality was observed in prepupal and pupal stages as compared to the larval instars, which could be due to the accumulated β-sitosterol in the body leading to various physiological changes. The decline in the weights of later instars and pupal stages due to dietary β-sitosterol pointed to delayed growth inhibitory effects. These results are in agreement with the work carried out on other insect pests with β-sitosterol, where growth regulatory activities of sitosterol were reported against *Spodoptera littoralis* [35]. The sitosterol isolated from *Anemone pavonina* has been found to effectively control *Pheidole pallidula* ants [36]. Although not much study has been carried out on β-sitosterol, other components isolated from different plants have been investigated for their possible effects

on insect pests. β-amyrin isolated from the methanol extracts of *Sarcostemma acidum* possessed antifeedant and growth regulatory activity against *S. litura* [37]. β-sitosterol displayed higher efficacy than α-amyrin acetate isolated from *Catharanthus roseus* leaves that reduced larval growth in *H. armigera* by 35.71% [38]. Similarly, the α-tocopherol isolated from *Ailanthus altissima* inhibited 76% of larval growth in *S. littoralis* when reared on 25 μg/cm^2 dietary α-tocopherol with an ED_{50} value of 222 μg/cm^2 [39]. Citronellol displayed cidal potential against lepidopterans [40].

The dietary β-sitosterol also adversely affected percent adult emergence in *H. armigera;* the total emergence varying from 82.05% to 57.89% as compared to 95.02% emergence in the controls. Phytophagous insects convert β-sitosterol to cholesterol, using it for growth and reproduction [23-25]. In the present study, the adverse effects of β-sitosterol on growth and development of *H. armigera* may possibly be due to the non-conversion of β-sitosterol into cholesterol, leading to its accumulation in the body and causing adverse effects. In a comparable study, flavonoids were reported to negatively impact the growth and development of *H. armigera,* and also exerted dose-dependent effects on adult growth and emergence [41]. In contrast to our study, emergence of deformed adults was reported with flavonoids, with the number directly proportional to the concentration of the extract [41]. This suggests that β-sitosterol does not exert any negative effects on the endocrine system of *H. armigera*.

The effect of β-sitosterol on *H. armigera* estimated on AST, ALT and ALP, resulted in a significant reduction of their activities in a dose-dependent manner. Higher dietary concentrations of β-sitosterol caused more suppression of the enzymatic activity, resulting in negative correlation coefficients. As transaminases are involved in energy production in the Krebs cycle, [20], and ALP plays an active role in membrane transport in intestinal epithelial cells [42, 43], Malpighian tubules [44] and hemolymph [45], any impairment in their activities will have a direct impact on the growth and development of insects. A significant 59.17% reduction in ALT activity in β-sitosterol-fed *H. armigera* larvae indicates reduced energy levels, which may have hampered larval growth and development. Similar reduced midgut enzymatic activities of transaminases and phosphatases were reported in *Cnaphalocrocis medinalis*, the rice leaf folder, fed on a diet containing *Azadirachta indica* and *Vitex negundo* extracts [46]. The effects of different categories of toxic compounds (pyriproxyfen, neemazal and spinetoram) were investigated on the activities of acid phosphatase, alkaline phosphatase, glutamic oxaloacetic transaminase and glutamine pyruvic transaminase in both hemolymph and fat body of the red palm weevil, *Rhynchophorus ferrugineus* 5th instars [16]. Among all of the compounds, spinetoram was found to be the most potent compound, inducing maximum reduction in transaminase and phosphatase activities [16]. In contrast to the present result showing reduced ALT and ASP activities in 4th instars of *H. armigera* after feeding on 10 μg/mL β-sitosterol, increased activity of ALT and AST was demonstrated in 4th instar larvae of *S. littoralis* after feeding with lufenuron [47].

The decreased activity in ALP was also induced in *Bombyx mori* and crickets when subjected to different stress conditions, diseases and toxic chemicals [48-49]. Six essential oils extracted from garlic (*Allium sativum*), mint (*Mentha piperita*), basil (*Ocimum basilicum*), thyme (*Thymus vulgaris*), sesame (*Sesamum indicum*) and chamomile (*Chamaemelum nobile*) decreased ALP activity in *Tribolium confusum* adults and last larval instar, with a maximum reduction of 18% observed with chamomile [50]. Our results are in accordance with the inhibition of ALP activity in *Euprepocnemis plorans* obtained after feeding on limonoids [51].

The presented results suggest that β-sitosterol has the potential to disrupt physiological process and activities that are essential for the survival, acceptable growth and development of *H. armigera*. Reduced larval growth and adult emergence may be attributed to inadequate metabolism of β-sitosterol resulting in its accumulation in the body. The diminished transaminases in the midgut caused by β-sitosterol probably led to a reduction in energy reserves because of an inefficient Krebs cycle. On the other hand, reduced phosphatases likely affected nutrient transport through the gut. However, these assumptions need further study in order to ascertain the effects at the cellular level.

Since β-sitosterol is a plant steroid, it can be utilized as a promising and eco-friendly constituent in insect control formulation. Further investigations and field testing of β-sitosterol to prepare an effective formulation

could help in devising a novel and eco-friendly strategy for *Helicoverpa* management.

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