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The toxic effects of Winter Cherry on the physiology of Cotton Bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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Abstract. The cotton bollworm, *Helicoverpa armigera* (Hübner) is considered as one of the most important pests of vegetables worldwide. Nowadays, alternative safe control measures are usually recommended against various insect pests. We investigated the ethanolic extracts of a medicinal plant fruit called winter cherry *Withania somnifera* L. (Solanaceae) against third instar larvae of cotton bollworm. The LC_{50}, LC_{30}, and LC_{10} values were estimated by feeding through larval diet at 1.33, 0.53, and 0.14 (% w/v). Later, the LC_{30} value (0.53 % w/v) obtained was again treated on third instar larvae to find out its sublethal effects on some important molecules and enzymes. The results showed elevated amount of uric acid and also increased activity in acid phosphatase, aspartate aminotransferase, lactate dehydrogenase, glutathione S-transferase in comparison with the controls (fed artificial diet with ethanol alone). However, decreased activity of alanine aminotransferase, alkaline phosphatase, catalase, acetylcholinesterase, superoxide dismutase, and peroxidase were observed. The midgut histology showed disruption in main midgut epithelial cells as well as other cell types in treated vs controls. Chemical components of the ethanolic extract of winter cherry fruits were also identified using GC-MS where the main components included 5-Hydroxymethylfurfural (51.18 %) and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (21.86%). Our results are indicative of a promising natural product that can be regarded as a new source for insect pest control.

Keywords: Extract, Growth and development, Histology, Toxicity, *Whithania somnifera*

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Introduction

The overpopulated world demands more food and agricultural products (Thakur *et al*., 2020). There are many destructive factors that damage agricultural products, including pests, pathogens, and weeds (Samada & Tambunan, 2020). One of the key pests in most agricultural products is the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), which causes several billion dollars of damage every year (Riaz *et al*., 2021).

The cotton bollworm is considered a successful insect pest enjoying global distribution for several reasons such as being polyphagous, facultative diapause, high reproductive rate, ability to migrate (more than 2000 km), and a high motility rate (Silva *et al*., 2018a). Currently, *H. armigera* is observed in at least 128 countries of the world including Pakistan, India, China, Thailand, Greece, Portugal, Spain, Turkey, Australia, and New Zealand (Gonçalves *et al*., 2019; Riaz *et al*., 2021). This pest has a wide host range such as cotton, tomato, soybean, bean, maize and sorghum, peas, tobacco, and other legumes (Dhir, 2017). Larvae are the most destructive life stage that can feed on all parts of the cotton plant and the young larvae can damage the vegetative parts of the plant (Shahid *et al*., 2021). Most of the damages to cotton fields are related to second and third generations of this pest (Mojeni, 2019). It is a serious threat to tomatoes during the growing season, resulting in losses between 20-60% (Toffa *et al*., 2021).

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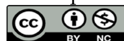

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In recent decades, chemical pesticides are the most common method practiced in controlling cotton bollworm worldwide (Ali *et al*., 2021), however, their disadvantages can be an important risk factor for environmental health (Nicolopoulou-Stamati *et al*., 2016). So, due to the problems caused by using pesticides, the need for alternative and safe methods of control including extracts from plant sources has increased (Kaur *et al*., 2021). These extracts inflict their activities in several ways including anti-nutritional, toxicity, repellency, and insect growth regulatory (IGRs) forms (Silva *et al*., 2018b).

Withania somnifera (L.) Dunal (Solanales: Solanaceae) commonly called Ashwagandha, is a plant in Ayurvedic medicine (traditional Indian medicine) (Shinde *et al*., 2021). It is widely distributed in drier parts of tropical and subtropical regions (Akanksha *et al*., 2022). *W. somnifera* disrupts the endocrine glands of insects and causes abnormalities in molting and metamorphosis, thus hindering their growth (Gaur & Kumar, 2019).

W. somnifera is well-known for its many pharmacological properties including anti-inflammatory, anti-depressant (Dar *et al*., 2015), antibacterial, antifungal (Khanchandani *et al*., 2019), anticancer (Rai *et al*., 2016), antioxidant, antiparkinsonian, hypolipidemic and immunomodulatory activity (John, 2014). Several reports have showed insecticidal activity of *W. somnifera* in some insects such as *Chrysomya megacephala* (Fabricius 1794) (Diptera: Calliphoridae) (Gaur & Kumar, 2021), tobacco caterpillar, *Spodoptera litura* Fab. (Guar & Kumar, 2020), toxicity and insect growth regulatory against red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Guar & Kumar, 2020).

Although there are several reports on *W. somnifera* in various countries as a medicinal herb (Shanmugaratnam *et al*., 2013; Singh & Sharma, 2018), it seems feasible to study its effect on an insect of economic importance. The previous studies on the effect of this plant from Indian sub-continent have resulted in promising potential for pest control. The literature survey clearly shows that none of these studies have taken a comprehensive approach in irreversible damages caused upon application of this plant product (Gaur & Kumar, 2020; Akanksha *et al*., 2022), on such compounds including detoxifying and antioxidant system. No doubt these enzymes play a major role in reducing or removing endogenous and exogenous substances that are harmful to insects, so to enable the insects to circumvent adverse conditions. Hence, in the current study apart from toxicity we have worked on the damages caused to enzymatic and non-enzymatic compounds, detoxifying enzymes, antioxidant system and even the histology of the mid-gut in order to have a deep insight into the effect of this precious medicinal herb.

Materials and methods

Insect Rearing

The larvae of *H. armigera* were collected from tobacco farms in Rasht city (37°20'N 104°09'E) in Guilan, north of Iran. The larvae were reared on an artificial diet (powdered cowpea, yeast, wheat germ powder, ascorbic acid, sorbic acid, sunflower oil, formaldehyde, and distilled water) according to Shorey & Hale (1965). The insects were bred in transparent plastic containers (10×5×5 cm) at 26±1°C, 65±10% relative humidity with a photoperiod of 16L:8D. After rearing the insect for three generations to get adapted to the rearing conditions, the third instar larvae of fourth generation were used for the experiments.

Extract Preparation

The *W. somnifera* fruits were obtained from Saravan (27°22′15″N 62°20′03″E) in Sistan and Baluchestan province of Iran. The fruits were dried in an oven at 45°C for 48 hours. Briefly, 50 gr of powdered plant fruits were added to 500 ml of 85% ethanol (Merck, Germany) (Warthen *et al*., 1984). The resulting suspension was placed on a stirrer for one hour and then kept at room temperature for 48 hours. After that, the suspension was again mixed for one hour as above and finally passed through sterile Whatman filter paper No. 4. To remove the ethanol and obtain the concentrated extract, a rotary evaporator apparatus (at 45°C for 7 minutes) was used. The extract was further dried at 45°C in an oven for 2-3 days. Finally, a 50% stock solution was prepared from the dried extract with 85% ethanol as the solvent, and the desired concentrations for subsequent experiments were prepared from this stock.

Bioassay

Initially, from stock solution (50%) concentrations of 0.5, 1, 2, 3, 4, and 5% (w/v) were made in ethanol (Merck, Germany). For each bioasaay, 10 mL of each concentration was incorporated to 100 g of artificial diet (already mentioned in insect rearing section) made into pills and then left for thirty minutes under laboratory conditions in order to evaporate the ethanol. The food (10 gr pills) was provided to the newly-ecdysed third instar larvae of *H. armigera* for 3 days and then let them feed on untreated diet until pupation. Four replicates of 10 larvae for each replicate were used for every concentration (a total no. of 240 larvae) and similarly 4 replicates of 10 larvae (No. 40 larvae) with 85% ethanol in the diet were used as controls. The mortality was recorded after 48 h.

Growth and development

In order to study the effect of sublethal concentration (LC_{30}) on growth and development, the LC_{30} value was used on newly-ecdysed third instar larvae in artificial diet with 4 replications of 10 larvae in each replicate (No. 40) and the controls were run simultaneously with 85% ethanol treated diet alone. The remaining larvae in treatment and controls were fed on untreated diet until the first batch of control adults emerged.

Histological studies

Treated (No. 3) and control larvae (No. 3) were dissected under a stereomicroscope (Olympus, Japan) in insect ringer. The midguts were separated and fixed in aqueous Buin's fluid for 24 hours. The tissues were washed under running water to remove the yellow color of the fixative until becoming white. Then, they were dehydrated in graded alcoholic series, cleared in xylol, and embedded in molten wax. The tissues were cut by a rotary microtome at 5 µm thickness (Reichert-Jung Bio cut 2030 Germany). They were then routinely stained in hematoxylin-eosin (Merck) and observed and photographed under a light microscope (M1000 Leica-light microscope) equipped with a camera (Canon EOS 600D).

Preparation of samples for biochemical analysis

The whole larval body 48 h post treatment was homogenized using a glass pestle. Then the samples were diluted with phosphate buffer (1:1 W/V at pH 7). Three replicates of three insects for each replicate were used. The sample was centrifuged at 13000 g for 30 min at 4°C. The supernatant was used as the source of enzymatic and non-enzymatic. The controls were simultaneously run for comparisons.

Enzymes assays

The amount of protein was measured using the total protein kit (Parsazmun Co., Iran) according to the method of Lowry (1951). The absorption read at 545 nm with an ELISA reader device (EPOCH 2 BioTek).

Uric acid was measured by using Parsazmun (Co., Iran) kit. According to Pachla *et al* (1987), at first 50 µL of R1 reagent was incubated with 20 µL of each sample for 10 minutes. Then, 20 µL of R2 reagent was added and incubated again for 10 minutes. The absorption read at 405 nm.

Urea was measured according to Williams-Boyce and Jungreis (1980), 39.6 µL of R1 reagent and 4 µL of R2 reagent were incubated with 20 µL of each sample for 10 minutes. Then the absorption was recorded at 560 nm.

Based on Thomas (1998) method the Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were measured using Parsazmun (Co., Iran) kit. Briefly, 15 µL of each substrate (10 mM) was separately added to 50 µL of fast blue RR (1 mM) before adding 20 µL of enzyme solution and incubated for 5 min. The absorption was read at 340 nm.

The Acid phosphatase (ACP) and Alkaline phosphatase (ALP) enzymes were measured using para-nitrophenol phosphate substrate according to Otto *et al.* (1946). For this purpose, 40 µL of Tris buffer (Tris-HCl 20 mM, pH 5 for ACP, and pH 8 for ALP) and 20 µL of each sample were incubated for 5 minutes. The absorption was read at 545 nm.

The method of King (1965) was followed to measure Lactate dehydrogenase (LDH) using Parsazmun (Co., Iran) kit. First, reagents R1 and R2 were incubated for 20 min at laboratory conditions and then the activity of the enzyme was measured by adding 20 µL of samples. The absorption was read at 340 nm.

Glutathione S-transferase (GST) was measured using Parsazmun (Co., Iran) kit with CDNB (1-chloro2,4-dinitrobenzene) and DCNB (1,2-dichloro-4-nitrobenzene) as substrates. The absorption was read at 340 nm (Oppenorth, 1979).

The activity of the superoxide dismutase (SOD) enzyme followed the method of Marklund & Mellerklund (1974). Initially, 10 mg of bovine serum albumin was added to 100 µL of xanthine oxidase and 2 mL of 0.1 M phosphate buffer at pH 7. Then, 100 µL of the solution was added to 500 µL of phosphate buffer containing 70 µmol of NBT and 125 µmol of xanthine. The obtained solution was incubated in the dark for 20 minutes. Then, 100 µL of the solution and 20 µL of each enzyme sample were mixed, and absorption was read at 340 nm.

The method of Reddy *et al.* (1985) was adopted for peroxidase (POX) activity. For this purpose, 50 µL of pyrogallol and 50 µL of 1% H_2O_2, and 20 µL enzyme samples were mixed and incubated for 2 minutes. The absorption was read at 430 nm.

According to the method of Luck (1974), for measuring the activity of Catalase (CAT), 100 µL of 1% hydrogen peroxide and 20 µL of each sample were mixed and incubated for 10 minutes. The absorption was read at 430 nm.

According to the method of Ellman *et al.* (1961), for measuring the activity of Acetylcholin esterase (AChE), 80 µL of 100 mM phosphate buffer at pH 7 and 50 µL of acetylcholine iodide were mixed with 50 µl of 100 mM DTNB and then were incubated for 5 minutes. After adding 20 µL of each sample, they were incubated again for 30 minutes. The absorption was read at 405 nm.

Chemical analysis of ethanolic extract of *W. somnifera*

The analysis of the ethanolic extract of *W. somnifera* was done using gas chromatography (Agilent Technologies 7890B) mass spectrometer (Agilent model 5977A) in a HP-5MS column with a length of 30 cm, an inner diameter of 0.25 mm and a thickness of the stationary phase layer of 0.25 micrometers. The temperature of the injection chamber was 280 °C and helium carrier gas (99.9%). Compounds were identified by comparing the peaks of each sample at different inhibition times with the peaks in the device library (NIST: National Institute of Standards Technology, Wiley 7n.1 mass computer library) (Adams, 2007).

Statistical Analysis

Determination of mortality and lethal concentrations were done by Polo-Plus Robertson *et al.* (2007) software. For toxicity tests, 240 larvae were used in 4 replicates of 10 newly ecdysed third instar larvae for each concentration. A similar assay was run for controls (40 larvae were used). Data from enzymatic and non-enzymatic activities were analyzed by one-way analysis of variance (ANOVA). The differences between means in treatments were compared at 5% level by Tukey's multiple range tests. No transfer of arcsine-square-root equation were required as they appeared normal.

Results

Bioassay of ethanol extract on *H. armigera* larvae

The ethanolic extract of *W. somnifera* caused mortality in third instar larvae of *H. armigera* after 48 hours. The LC_{50} value was estimated 1.33 % (w/v). The mortality rate in treated larvae was dose-dependent. The confidence limits (CL) and the slope of regression are shown in Table 1.

Morphological deformities caused by *Withania somnifera* ethanol extract on *H. armigera* larvae

When third instar larvae of *H. armigera* were treated with ethanolic extract of *W. somnifera* fruits, in addition to mortality, several deformities were also observed including larval-pupal intermediates (Fig. 1). We also observed reduced feeding and lethargy of the larvae and prolongation of larval stages.

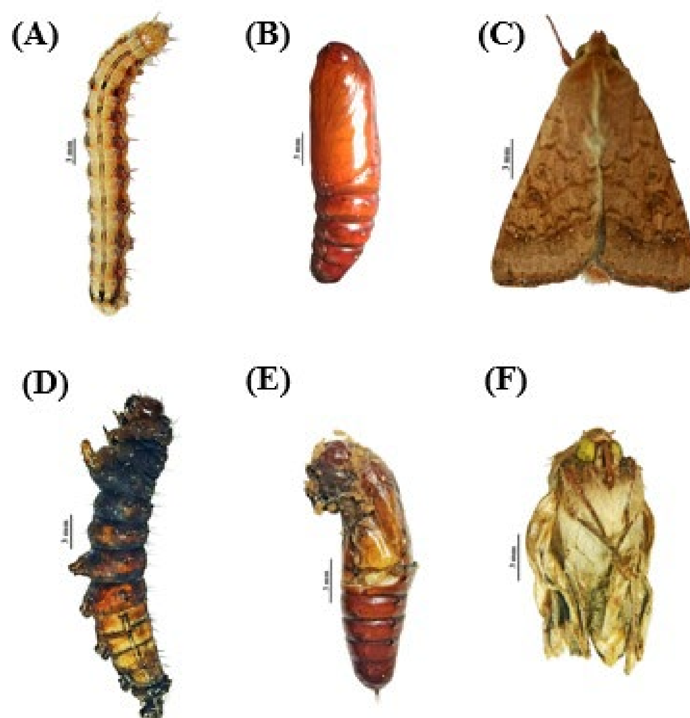

Fig. 1. Types of deformities produced after treatment of *Helicoverpa armigera* larvae with fruit ethanolic extracts of *Withania somnifera*. (A) Normal sixth instar larva, (B) normal puparium, (C) normal adult, (D) deformed sixth instar larva, (E) ventral view of larva-pupal intermediate, (F) adultoid with crumpled wings.

The LC_{30} (0.53 % w/v) treated larvae prolonged larval period (20.0 ± 1.03 compared to controls 12.75 ± 0.79) showing significant changes (F = 4.71, df = 1, 39, p = 0.0362) (Table 2).

Histology of midgut

Figure 2A shows the structure of larval midgut in control with three distinct cell types including the principal epithelial cells (EPC), the goblet cells (GC) and the regenerative cells (RC). The EPC are columnar with distinct nucleus, similarly the GC and RC are normal in structure in control larvae. However, in the LC_{30} treated larvae the hyperplasia in nucleus of EPC is evident at the same time disintegration of the cell boundaries particularly toward lumen of mid gut has made a double layer and unusually thick area. Similar disintegration in other cell types has made them difficult to be differentiated in their respective forms (i.e., GC and RC).

Effect of *W. somnifera* fruit ethanol extract on the activity of biochemical and enzymatic components

The protein content (0.866 ± 0.011) (F = 38.81, df = 1, 5, p = 0.003) reduced significantly after treatment of *H. armigera* larvae by LC_{30} concentration of plant extract. The amount of uric acid (0.361 ± 0.019) and urea (0.042 ± 0.005) increased in LC_{30} treated third instar larvae of *H. armigera* compared to the controls (0.175±0.058) (F = 9.17, df = 1, 5, p = 0.039), (0.003 ± 0.0003) (F = 52.65, df = 1, 5, p = 0.002), respectively. The activity of ALT (0.308 ± 0.035) decreased significantly in LC_{30} concentration (F = 22.85, df = 1, 5, p = 0.009), conversely, the activity level of AST (0.308 ± 0.002) (F = 360.67, df = 1, 5, p = 0.000) and ACP (0.130 ± 0.006) enzyme (F = 117.38, df = 1, 5, p = 0.000) increased significantly compared to the control. Reduced ALP (0.043 ± 0.003) (F = 2.56, df = 1, 5, p = 0.018) and enhanced LDH (0.188 ± 0.021) (F = 8.41, df = 1, 5, p = 0.044) are seen compared to the control, however, not significant. The activity of GST with two substrates of DCNB (0.368 ± 0.020) and CDNB (0.432 ± 0.015) increased in LC_{30} concentration compared to the control (F = 89.94, df = 1, 5, p = 0.001) and (F = 104.97, df = 1, 5, p = 0.001) acetylcholinesterase (0.123 ± 0.012) (F = 17.43, df = 1, 5, p = 0.014) activity slightly decreased compared to the control.

In LC_{30} concentration treated insects, the activity of some antioxidant systems including superoxide dismutase (0.341 ± 0.037) (F = 2.02, df = 1, 5, p = 0.000), peroxidase (1.493 ± 0.071) (F = 3.61, df = 1, 5, p = 0.013), catalase (0.164 ± 0.001) (F = 387.82, df = 1, 5, p = 0.000) decreased compared to the controls.

Table1. The LC values (% w/v), from *Withania somnifera* by ethanolic extract on third instar larvae of *Helicoverpa armigera*

Ethanolic Extract	Time	LC_{10}	LC_{20}	LC_{30}	LC_{50}	LC_{90}	Slope ± SE	X^2 (df=4)	P - value
W. somnifera	48*	0.14 (0.02 ± 0.32)	0.31 (0.07 ± 0.57)	0.53 (0.18 ± 0.86)	1.33 (0.80 ± 1.87)	12.64 (6.72 ± 54.84)	4.59 ± 0.29	1.71	0.43

*48 h after treatments; LC: lethal concentration (% w/v for oral toxicity); X^2: chi-square value; df: degrees of freedom

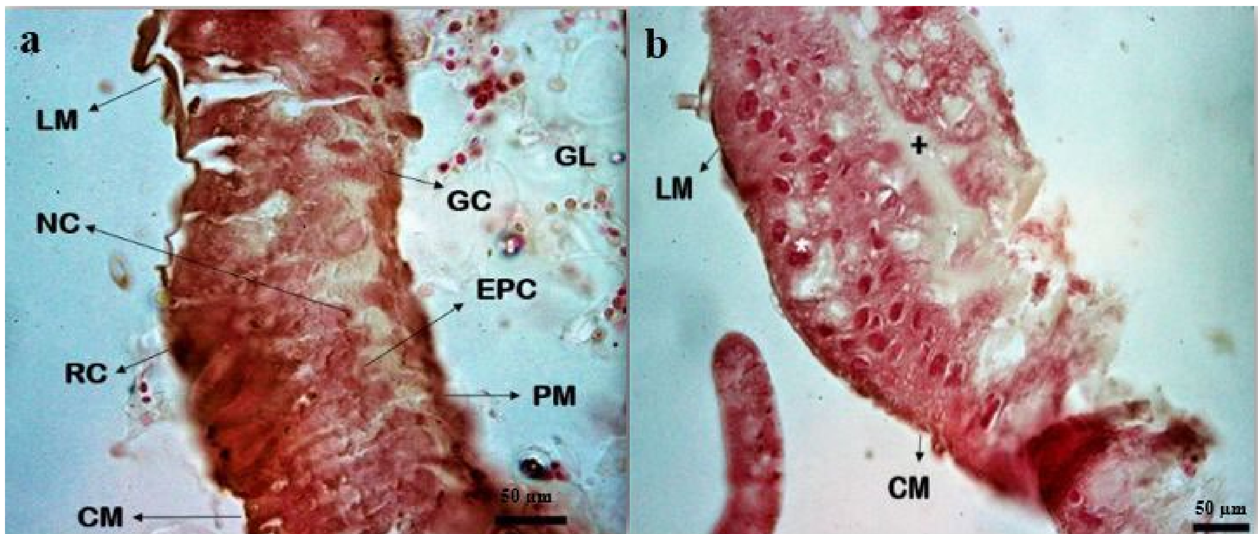

Fig. 2. The effect of *Withania somnifera* fruit ethanolic extract on the histology cross-section of midgut of cotton bollworm *Helicoverpa armigera* stained with hematoxylin and eosin, covering cell, goblet cells, regenerative cell and peritrophic membrane are clearly visible in the control (A). In LC_{30}, the double-layered of the inner part of tissue is due to the disintegration of the cells (+) and the enlargement of the nucleus is also seen (B) (3000x). Regenerative cell (RC), goblet cell (GC), longitudinal muscle (LM), circular muscle (CM), gut lumen (GL), peritrophic membrane (PM), epithelium cell (EPC), nucleus (NC).

Chemical composition of ethanolic extract of *W. somnifera*

The results of the analysis of the chemical components of ethanolic extracts of *W. somnifera* fruits by gas chromatography-mass spectrometry (GC-MS) with their retention time (RT), Molecular Formula, and concentration (%) are presented in Table 4. In this GC-MS, 15 active compounds were identified, the main compounds of which are: 5-Hydroxymethylfurfural (51.182%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (21.863%), 9-Octadecenoic acid (Z)-, methyl ester (6.238%).

Discussion

The use of artificial/chemical pesticides, despite overcoming pests, in excess has affected many beneficial and nonpathogenic nontarget organisms and microorganisms, further reducing soil and water quality and ultimately destroying resources. Biodegradable green synthesis of nanopesticides based on plant extract systems are satisfactory alternative solutions (Isman, 2023).

Table 2. Effect of *Withania somnifera* ethanolic extract on developmental stages (days) of *Helicoverpa armigera*

Treatment	3rd instar larva	4th instar larva	5th instar larva	6th instar larva	Total
Control	2.45 ± 0.13b	2.8 ± 0.24b	3.95 ± 0.23a	3.55 ± 0.19b	12.75 ± 0.79b
LC_{30}	4.3 ± 0.3a	4.9 ± 0.14a	5.75 ± 0.32a	5.25 ± 0.27a	20.2 ± 1.03a

Means in a column followed by the same letters are not significantly different ($p < 0.05$ Tukey's test).

Table 3. Effect of *Withania somnifera* ethanolic seed extract on some biochemical compounds of *Helicoverpa armigera* third instar larvae (48 h after treatment) (U/mg protein)

Enzymes	Control	LC_{30}	df	F-value	p
Protein	1.038 ± 0.025a	0.866 ± 0.011b	1, 5	38.81	0.003
Uric acid	0.175 ± 0.058b	0.361 ± 0.019a	1, 5	9.17	0.039

Urea	0.003 ± 0.0003b	0.042 ± 0.005a	1, 5	52.65	0.002
ALP	0.049 ± 0.000a	0.043 ± 0.003a	1, 5	2.56	0.018
ACP	0.055 ± 0.003b	0.130 ± 0.006a	1, 5	117.38	0.000
ALT	0.495 ± 0.016a	0.308 ± 0.035b	1, 5	22.85	0.009
AST	0.259 ± 0.000b	0.308 ± 0.002a	1, 5	360.67	0.000
LDH	0.107 ± 0.017b	0.188 ± 0.021a	1, 5	8.41	0.044
CAT	0.237 ± 0.003a	0.164 ± 0.001b	1, 5	387.82	0.000
POX	1.715 ± 0.091a	1.493 ± 0.071a	1, 5	3.61	0.013
SOD	0.460 ± 0.074a	0.341 ± 0.037a	1, 5	2.02	0.000
AChE	0.167 ± 0.012b	0.123 ± 0.012a	1, 5	17.43	0.014
GST (DCNB)	0.107 ± 0.017b	0.368 ± 0.020a	1, 5	89.94	0.001
GST (CDNB)	0.236 ± 0.011b	0.432 ± 0.015a	1, 5	104.97	0.001

Means followed by different letters in each row are significantly different according to the Tukey's test ($p < 0.05$) (Mean ± SE). Data related any row were analyzed separately.

The present study revealed that the ethanolic extract of *W. somnifera* was effective against *H. armigera* by increasing larval duration and mortality rate. The secondary metabolites present in this plant in various parts including root, stem, leaf, seed, and fruit have shown their negative impact on some insect pests. The reports by Gaur & Kumar (2020) indicate a detrimental effect of acetone extract of *W. somnifera* on larval *Sarcophaga ruficornis* when applied topically. They assessed the LC_{50} of 28.19, 43.49, 47.48 and 48.16 g/μL against 0, 1, 2 and 3 days of third instar larvae. In the present study, the ethanolic extract showed LC_{50} value of 1.33 % (mL/g diet) depicting a lower LC value compared to the mentioned authors. Insect growth and metamorphosis are dependent on orderly released endocrine hormones (Bian *et al.*, 2022). Any changes in this order retard or eventually disrupt the process of growth, development, and metamorphosis, especially if they are used during the sensitive period of insect growth.

JH analogs, ecdysone agonists (EAs), and chitin synthesis inhibitors (CSIs) are in this category (Saha & Joy, 2014; Subramanian & Shankarganesh, 2016). The plant compounds can also act as antagonists of growth-regulating hormones and interfere with the growth and development of insects (Khare *et al.*, 2019). The extract of various parts of *W. somnifera* also showed prolongation of larval instars in studied insects by few days (Gaur & Kumar, 2017, 2019 and 2020). Similarly, we found prolongation of juvenile stages of *H. armigera* by 8 days compared with control. The change in larval duration is an indication of hormonal disbalance inserted by metabolites present in *W. somnifera* extracts. Usually, the arrest in ecdysis occurs in larvae when the rate of juvenile hormone increases in hemolymh (Lapcharoen *et al.*, 2005).

Deformed larva, pupa and adults in the current study are an indication of changes in hormone both quantitively and qualitatively, so that the larva or pupa are unable to extrude and become deformed. Similar results on deformation by *W. somnifera* extracts are available in the literature (Afraze *et al.*, 2020; Gaur & Kumar, 2010). Such an effect on larval or pupal metamorphosis has been reported in *Azadirachta indica* extracts and *Artemisia annua* extracts (Shekari *et al.*, 2008; Mojarab-Mahboubkar & Jalali Sendi, 2016; Afraze & Sendi, 2021).

The midgut has an important role in insect growth and development (Wu *et al.*, 2020). This part contains various digestive enzymes that digest and absorb food compounds (Terra *et al.*, 2019), Also, the midgut is the main target of some factors such as pathogens, insecticides, extracts and essential oils, viruses and bacteria (Chapman, 2013). There are various reports that show the destructive effect of plant-based compounds such as azadirachtin and the disruption of protein digestion (Shu *et al.*, 2018). Zhao *et al* (2019) reported that in *Bactrocera dorsalis* Hendel larvae (Dip.: Tephritidae), severe damages to the cells of midgut and a decrease in the activity of digestive enzymes were observed, followed by a disturbance in process of digestion and absorption of food under the influence of azadirachtin treatment. The enlargement of the nuclei, disintegration of the cell boundaries was observed in the current study by ethanolic extract of *W. somnifera* on midgut tissue of *H. armigera.* Such disruption in midgut epithelial cells will eventually lead to trouble in digestion and absorption which are well documented in literature (Dinesh-Kumar *et al.*, 2018; Murfadunnisa *et al.*, 2019; Afraze *et al.*, 2020).

Table 4. Chemical compounds of ethanolic extract of *Withania somnifera*

Peak No.	RT (min)	Name	%
1	6.033	Furfural	4.027
2	7.140	2-Furanmethanol	1.136
3	9.547	2-Furancarboxaldehyde, 5-methyl-	2.949
4	10.153	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	2.615
5	15.904	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	21.863
6	18.934	5-Hydroxymethylfurfural	51.182
7	19.855	2-Methoxy-4-vinylphenol	1.654

8	30.897	Tetradecanoic acid, ethyl ester	0.142
9	33.653	Hexadecanoic acid, methyl ester	2.190
10	35.015	Hexadecanoic acid, ethyl ester	0.660
11	37.080	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	2.041
12	37.259	9-Octadecenoic acid (Z)-, methyl ester	6.238
13	37.740	Methyl stearate	1.159
14	38.471	Linoleic acid ethyl ester	1.192
15	38.611	n-Hexadecanoic acid	0.951

RT: Retention Time

Proteins are key substances in the production of enzymes, integument, and various hormones (Nation, 2022). Any exogenous materials including plant extracts may disturb their activity by various means (Kalaivani *et al*., 2011). A decreasing amount of protein by sublethal dose LC_{30} (0.53 % W/V) is indicative of exertion caused by winter cherry fruit extract. Similarly, in *Spodoptera exigua* the extracts of Zygophyllacae and Euphorbiaceae at and in *Xanthogaleruca luteola* by the leaf extracts of *Artemisia annua* (Rizwan-ul-Haq *et al*., 2010; Shekari *et al*., 2008). It is clear that the reduction of protein greatly impairs growth performance (Wilson *et al*., 2019).

Uric acid and urea are the end product of the metabolic breakdown of purine nucleotides. In insects, there exists an unfavorable interaction between the level of protein and uric acid, meaning that the larvae with decreasing protein (as in our study) show enhanced uric acid. Since protein is incorporated and recovers the destroyed tissues, hence uric acid production is increased as a side product of protein catabolism (Nathan, 2006; Chaitra *et al*., 2020).

Aminotransferases (AST and ALT) play a very important role in insect physiology in transferring an amino group from an amino acid to a ketoacid in the catabolism of amino acids. These enzymes cause the return of amino acids transferred to adipose tissue during metabolic pathways to the hemolymph (Rivera-Perez *et al*., 2017; Go *et al*., 2022). According to our findings, ethanol fruit extracts of *W. somnifera* increased AST and decreased ALT activities in third instar larvae of *H. armigera*. A comparable finding was also reported in *Pieris rapae* L. (Hasheminia *et al*., 2011) and in *Sitophilus granarius*, *Tribolium confusum* and *Acanthoscelides obtectus* (Kısa *et al*., 2018). The increase of AST may be due to involvement in detoxification processes (Shekari *et al*., 2008) or could be due to possible damages on various tissues (Zibaee *et al*., 2011). However, the reduction of ALT activity in larvae might be due to the effects of plants metabolites on the synthesis or functional levels of this enzyme directly or indirectly by changes inserted on cell cytomorphology or the neurosecretory hormonal pattern (Mageed *et al*., 2018; Saheir, 2018).

Acid phosphatases and alkaline phosphatases (ACP and ALP) play an important role in separating phosphate groups from molecules (nucleic acid, protein, and alkaloids) (Li *et al*., 2021). This process is called dephosphorylation and it is most effective in an alkaline environment (Kalender *et al*., 2005). They are also effective in digesting and absorbing food in the midgut and transferring it to fat bodies (Basiouny, 2021). In our study, while the activity of ACP increased, that of ALP decreased after treatment by *W. somnifera* extract in *H. armigera*. Different activities of ALP and ACP in treated larvae may indicate a change in the physiological balance in nutritional metabolism and intermediary metabolism or endocrine disruption through progressive or retrogressive larval activity inserted by plant extract (Hasheminia *et al*., 2011; Shahriari *et al*., 2017).

Lactate dehydrogenase (LDH) is a tetrameric and glycolytic enzyme that exists in the tissues of all animals and catalyzes the reversible process of converting pyruvate to lactate with NADH (Granchi *et al*., 2017). When oxygen is absent or in shortage, LDH converts pyruvate to lactate, the final product of glycolysis. Carbohydrate metabolism also contains LDH and it has been used as the criterion of exposure to stress (Mojarab-Mahboubkar *et al*., 2022). Goharrostami *et al*. (2022) reported reduced activity of LDH in thymol treated larvae of *Glyphodes pyloalis* Walker. There are several reports on LDH activity inserted by botanicals e.g. a decreased activity as seen in azadirachtin treated larvae of *G. pylolais* which could be due to the allelochemical effects of plants on the peritrophic membrane and damage to the cell surfaces, caused by tissue changes in the midgut (Khosravi & Sendi, 2013).

Glutathione S-transferases (GSTs) are the main phase II detoxification enzymes that are mostly found in the cytosol. In addition to the role of catalyzing the synthesis of electrophilic substrates into glutathione, these enzymes also perform a wide range of other functions. They also have peroxidase and isomerase activities (Krishnan & Kodrík, 2006). They have an active role in the detoxification of endogenous and exogenous substances and toxin metabolites for insects (Fan *et al*., 2022). Here, an increased level of GST with both substrates (CDNB and DCNB) has been observed in *H. armigera* by *W. somnifera* treatment. In another study, extract of *Melia azedarach* L. and *Amaranthus viridis* L. leaves when used against *S. exigua* decreased the GST activity which indicates the ability of these two extracts to inhibit detoxification enzymes (Rachokarn *et al*., 2008). Overall, two main mechanisms are involved in an increase in the activity of GST that is, induction of new isozyme synthesis or

overexpression of major GST isozymes (Mojarab-Mahboubkar *et al*., 2022). This can be related to the defense system of *H. armigera* in confronting stress factors such as allelochemicals induced by the essential oils or extracts of plants (AlJabr *et al*., 2017). SOD by using different processes converts superoxide anion into hydrogen peroxide and oxygen, and then CAT and POX break down the produced hydrogen peroxide and convert it into water and oxygen (Johnson & Giulivi, 2005). CAT is a hemoprotein that catalyzes the decomposition of hydrogen peroxide into water and oxygen and protects cells from oxidative damage by hydrogen peroxide and hydroxyl. POXs are iron-containing glycoproteins that oxidize many organic and inorganic substrates using hydrogen peroxide (Adwas *et al*., 2019). Biological systems are affected by oxidative stress through reactive oxygen species such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxides, which are natural oxidative processes in cells and extracellular fluids (Jena *et al*., 2013). The Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) are important antioxidant enzymes (Aqeel *et al*., 2022).

The changes in these enzyme activities might be related to the suppression of excess production of ROS via SOD, CAT, and GPx. But during the process of detoxification, potentially toxic H_2O_2 is generated, where CAT and GPx act to remove these peroxides, while SOD dismutase superoxide anions directly (McCord & Fridovich, 1969; Yasur & Rani, 2015). Pesticides, plant metabolites, and extracts must be neutralized by the antioxidant systems of insects (Chowanski *et al*., 2016). On the other hand, plants can be the main source of natural antioxidants that could donate hydrogen ions, quench singlet oxygen, and chelate metal in order to reduce the stresses caused by any source (Vujanović *et al*., 2019). The current study clearly depicts the decreasing trend in all studied antioxidant enzymes indicating the reducing power of extract of winter cherry to combat the antioxidant system of *H. armigera*. Contrary to our study, when 1% agglutinin from *Polygonum persicaria* L. (Polygonales: Polygonaceae; PPA) extracts was used against larvae of *H. armigera* increased in SOD, CAT, and POX enzymes oxidative stress. According to this report, absorption of PPA increases the concentration of superoxide and hydrogen peroxide radicals, which will activate these enzymes (Rahimi *et al*., 2018). The increase in antioxidant system after treatment with essential oil or extracts are well documented in literature (Gao *et al*., 1995; Zhang *et al*., 2002; Jun *et al*., 2003). However, all these studies are confined to a particular age post treatment the activities according to a scheduled time was not explored. However, studies after treatment with *Beauveria bassiana* in several insects show an initial increase in antioxidant enzymes including SOD, CAT and POX but decreased even lower than control (Tian *et al*., 2015; Zhang *et al*., 2020; Gao *et al*., 2022). These studies concluded from the results obtained that after a particular time post infection the insect is weakened and unable to produce further SOD, CAT and POX. We did not explore the activities of these enzymes in a time bound manner post treatment which need further research. It seems the antioxidant enzymes must have increased initially (before 48 h) and then decreased as reported by Gao *et al*. (2022) which corresponds to our report.

Analysis of chemical compounds, especially secondary metabolites in plants, is an important issue due to their complex structure, chemical diversity, low abundance, and diversity even in one species (Trivedi *et al*., 2017) and using several specific techniques including GC-MS can be performed (Tetali *et al*., 2021). The GC-MS study on ethanolic extract obtained from winter cherry fruits is indicative of 15 compounds. The largest portion belonged to 5-Hydroxymethylfurfural, whose biological activity has not been explored in any individual but it is the main based chemical with alcohol and aldehyde functional groups convertible to 2,5-furandicarboxylic acid, 5-formyl-2-furancarboxylic acid and 2,5-diformylfuran (Tjallinks *et al*., 2023) that have antifungal activities (Del Poeta *et al*., 1998). Reports of chemical composition in fruits of winter cherry in the literature (Bhatia *et al*., 2013) do not correspond to our reports and it seems that these differences must be related to different climatic and soil conditions where the winter cherry grows.

The results of current research clearly show toxicity, histological disruption in the midgut and enzymatic impairments of winter cherry fruits extract on *H. armigera*. The extract also showed abnormalities in growth and development causing larval, pupal and pupoids indicating the effect of this extract on endocrine organs. These activities show different sites of action on pest insect which is winning margin in any candidate biopesticide. Therefore, we conclude that winter cherry can be a potential plant-based pesticide that certainly needs further research. However, a proper formulation that can effectively be used for the control of insect pests of economic importance is prerequisite in integrated pest management which certainly needs further research.

Author Contributions

Yasaman Ohadi: Methodology, Formal analysis, Investigation; **Roya Azizi**: Formal analysis, Investigation, Software, Draft preparation; **Jalal Jalali Sendi**: Conceptualization, Final review and edit, Visualization, Supervision, Project administration and Funding acquisition.

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Data Availability Statement

All data supporting the findings of this study are available within the paper.

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Ethics approval

Insect and plant were used in this study. All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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اثرات سمی پنیرباد بر فیزیولوژی کرم غوزه‌ی پنبه *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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چکیده

کرم غوزه‌ی پنبه *Helicoverpa armigera* (Hübner) یکی از مهم‌ترین آفات سبزیجات در سراسر جهان به شمار می‌رود. امروزه معمولاً اقدامات ایمن جایگزین برای مبارزه با حشرات آفت مختلف توصیه می‌شود. ما عصاره‌ی اتانولی میوه‌ی یک گیاه دارویی به نام پنیرباد *Withania somnifera* L. (Solanaceae) را علیه لارو سن سوم کرم غوزه‌ی پنبه بررسی کردیم. مقادیر LC_{50}، LC_{30} و LC_{10} به صورت گوارشی در رژیم غذایی ۱/۳۳، ۰/۵۳ و ۰/۱۴ (٪ w/v) برآورد شد. بعداً، مقدار LC_{30} (0.53 % w/v) به دست آمده مجدداً بر روی لاروهای تیمار شده به دلیل تأثیر آن بر برخی مولکول‌ها و آنزیم‌های مهم انجام شد. نتایج حاکی از افزایش میزان اسید اوریک و افزایش فعالیت اسید فسفاتاز، آسپارتات آمینوترانسفراز، لاکتات دهیدروژناز، گلوتاتیون اس-ترانسفراز در مقایسه با گروه شاهد (تغذیه با رژیم غذایی مصنوعی با اتانول به تنهایی) بود. با این حال، فعالیت آلانین آمینوترانسفراز، آلکالین فسفاتاز، کاتالاز، استیل کولین استراز، سوپراکسید دیسموتاز و پراکسیداز کاهش یافت. بافت‌شناسی معده‌ی میانی اختلال در سلول‌های اپیتلیال اصلی معده میانی و همچنین سایر انواع سلول‌ها را در گروه تیمار در مقابل شاهد نشان داد. اجزای شیمیایی عصاره‌ی اتانولی میوه‌های پنیرباد نیز با استفاده از GC-MS شناسایی شد که اجزای اصلی آن شامل ۵-Hydroxymethylfurfural (۵۱/۱۸ درصد) و 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (۲۱/۸۶ درصد) بود. نتایج ما حاکی از یک محصول طبیعی امیدوارکننده است که می‌تواند به عنوان منبع جدیدی برای کنترل حشرات آفت در نظر گرفته شود..

کلمات کلیدی: عصاره، رشد و نمو، بافت‌شناسی، سمیت، *Whithania somnifera*

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