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Toxicity, Antifeedant and Sub-Lethal Effects of *Citrullus colocynthis* Extracts on Cotton Bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Authors' Contribution
AG, M Ahmed and RQ conceived the idea. AM and MT conducted the experiment. AG and M Ali analyze the data. AG and MT wrote the paper.

Key words
Citrullus colocynthis, Helicoverpa armigera, IPM, Cotton bollworm, Insecticide resistance.

ABSTRACT

Cotton bollworm, *Helicoverpa armigera* (Hubner) is very important polyphagous insect pest with wide host range known for its economic losses worldwide. Its ability to develop resistance to insecticides faster to identify alternates like biocontrol agents, plant extracts etc. In the present study the extract of *Citrullus colocynthis* in different solvents were evaluated on second instar larvae of *H. armigera* under laboratory studies for toxicity, sublethal and antifeedant effect. The result showed that ethanol based extract was the most effective to control the *H. armigera* followed by the ethyl acetate. The sublethal concentration of ethanol based extract of *C. colocynthis* increased larval and pupal duration as compared with control. The percent pupation, adult emergence and pupal weight decreased in treated populations as compared with control populations. The results of this study can be used in integrated pest management program for the management of *H. armigera.*

INTRODUCTION

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae), commonly known as bollworm, earworm, fruitworm or pod borer is very important insect pest of many important crops in Asia, Africa, Australia and Europe (Fakrudin *et al*., 2004; Teese *et al*., 2013; Hemati *et al*., 2013; Ali *et al*., 2016). It damages approximately 181 plant species including cotton, chickpea, okra and tomato (Manjunath *et al*., 1989; Razmjou *et al*., 2014). The larvae feed mainly on reproductive plant parts resulting severe crop losses and protects themselves because of their cryptic feeding habits (Kamaraj *et al*., 2008; Razmjou *et al*., 2014). During the 1990s, it appeared as a major insect pest of important crops in Pakistan (Ahmad *et al*., 2001) and needed application of more effective methods for its management.

Farmers mostly rely on chemicals as insecticides for the control of insect and mite pests but their frequent use has resulted in development of resistance in number of key pest species (Palumbi, 2008; Matthew, 2008; Xu *et al*., 2010). Insecticide resistance is now a major problem for the chemical control of a wide range of insect pests (Bisset *et al*., 1997; Liu and Yue, 2000). Approximately 500 agricultural pest insect species have developed resistance to various pesticides (Whalon *et al*., 2008). The field populations of *H. armigera* (F.) have developed insecticide resistance to all the insecticides (McCaffery, 1998; Avilla and Gonzales-Zamora, 2010; Namin *et al*., 2014; Abedi *et al*., 2014). In Pakistan, this developed insecticide resistance to almost all the conventional insecticide such as organochlorine (OCs, pyrethroids, organophosphates (OPs) and carbamates (Ahmad *et al*., 1995, 1997, 1998a, b, 1999, 2001).

Environmental and toxicological hazards of insecticides widespread use have spurred the search for alternatives (Munoza *et al*., 2013). Conventional and synthetic compounds of plants for development of more integrated methods of insect pest management have possibilities for future use (Tabashnik, 1994). Plant extracts have certain chemicals which can effectively substitute synthetic pesticides without phytotoxic properties (Schmutterer, 1990; Georges *et al*., 2008; Munoza *et al*., 2013). These plant based pesticides

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have been used in agriculture since two millennia for their efficient pest management and safety to mankind (Bernays, 1983; Thacker, 2002). *Citrullus colocynthis*, an annual herb, is found in warm regions of Pakistan, India, and Africa (Tallamy *et al*., 1997; Hussain *et al*., 2014). This plant shows insecticidal, antifeedant, larvicidal and antioviposition effects against insect pests. Petroleum ether, ethyl acetate, benzene and methanol extracts of *C. colocynthis* seeds showed negative impact on adult emergence of spider mites and pulse beetle (Mansoor *et al*., 2004; Seenivasan *et al*., 2004) and *Culex quinquefasiatus* (Mullai and Jebanesan, 2007; Rahuman *et al*., 2008). The aqueous extract of different parts of *C. colocynthis* significantly reduced the *Rhopalosiphum padi* population (Khalid, 2015).

Sub-lethal effects may be defined as behavioural and physiological changes occur in individuals that survive to the exposure to a pesticide at sub-lethal dose/concentration (Desneux *et al*., 2007; Hui *et al*., 2010). Various studies on sub-lethal effects of various insecticides have been conducted to determine the harmful and non-lethal impacts of insecticides on life span, fecundity, fertility and olfactory learning of insects (Shi *et al*., 2011; Biondi *et al*., 2012; Gontijo *et al*., 2013; Guo *et al*., 2013). In this paper the toxicity and antifeedant activity of different extract of *C. colocynthis* were studied. Moreover, the sub-lethal effects of ethanolic extract on different biological parameter of *H. armigera* were also studied.

MATERIALS AND METHODS

Insect collection and rearing

A population of *H. armigera* was collected from the cotton growing areas and cultured at 25 ± 2 C, 60 ± 5% RH with 16:8 (light:dark) cycle. The larvae were provided with artificial diet until pupation. The pupae were placed in a box lined with tissue paper. The adults were transferred to rearing jars and fed with 10% sugar solution. Nappy liner strips were hanged for egg laying. The eggs were collected on daily basis. After hatching, neonates were shifted to the artificial diet and 2nd instar larvae were used for bioassays and biological studies.

Collection of plant material and extract preparation

Citrullus colocynthis fruits were collected from Bhakkar and all the impurities were removed completely by hand picking. *C. colocynthis* plant was dried at room temperature. Dried fruits were pulverized in fine powder with electric grinder and sieved through a muslin cloth. For solvent extract, 50 grams of the powdered fruit was soaked in 100 ml of commercial ethanol, methanol, ethyl acetate and hexane separately for 24 h. The mixtures were stirred for one hour and then kept under 4°C in a refrigerator for 48 h. This was stirred again for one hour. The solution was passed through Whatman filter paper No. 4 twice. The solvent was dissolved in 10 ml of respective solvent to be used as stock solution.

Toxicity bioassays

Bioassay experiments were performed by using diet immersion method. Fresh artificial diet was prepared with different serial concentrations with different solvent extracts of *C. colocynthis*. The prepared artificial diet was cut into small pieces and place in Petri dish. The diet prepared with respective solvent alone was used as control. One larva per Petri dish was released on the treated diet. Four replications were made against each concentration and 10 larvae per replication were used. An organophosphate insecticide, (profenofos) was used as standard to compare the results of plant extracts. Mortality data was noted after 7 days. Larvae that failed to respond to gentle contact with a fine brush were considered as dead.

Sub-lethal effects on biological parameters

Sublethal effect of ethanolic extract of *C. colocynthis* against *H. armigera* larvae were evaluated using a sublethal concentration of LC_{25} calculated from the toxicity experiment.

Development and survival of larval stages of H. armigera *to adults*

Twenty 2nd instar larvae were placed on artificial diet incorporated with the LC_{25} concentration of ethanol extract of *C. colocynthis* in individual Petri dishes and same with ethanol alone as control. Each treatment was replicated five times. Larvae were examined daily and the development time from 2nd instar larva to pupa was noted. The weight of the pupa was noted one day after pupation. The pupae were kept individually in 250 ml plastic cups covered with white netting. Adult emergence was noted daily.

Adult longevity, mating success, fecundity and egg viability of H. armigera

On emergence, adults (< 24 h-old) were paired to estimate the longevity, fecundity and egg viability. The adults were paired as follows: Control (untreated) male x Control (untreated) female (10 pairs) and insecticide treated male x insecticide treated female (10 pairs). Each pair was kept in transparent cup coved with white netting. Honey solution was provided as a food source. When a female started egg laying, the pair was shifted to a new cup daily until they died. Mating success, number of eggs laid per female, proportion of eggs that hatched and adult longevity were recorded.

Antifeedant activity

Diet having LC_{50} value of extracts of *C. colocynthis* was weighed in the shape of a small cube and placed in Petri dishes for larval consumption. The diet prepared without extract was used as control. The 2nd instar larvae were starved for almost 8 h before the experiment. Starved larvae were released on treated and control diet in Petri dishes. Data was noted after 24 h. The percent diet consumed was calculated by using feeding deterrence index (DI) (Koul *et al*., 2003).

Deterrence index

$$CAA = (C\text{-}T / C+T) \times 100$$

Where, CAA is corrected antifeedant activity; T is diet consumed in treatment and C is diet consumed in control.

Statistical analysis

LC_{50} and LC_{25} values were determined with Polo-PC software (LeOra Software, 1987). Data for developmental time (larval and pupal duration) was subjected to one–way analysis of variance (ANOVA) and the significant differences between treatments were determined by using Tukey's HSD test ($P < 0.05$) with Satistix 8.1 (2005).

RESULTS

Toxicity bioassay

The $LC_{25,}$ LC_{50} and LC_{90} values of different extract of *C. colocynthis* to *H. armigera* after 7 days were summarized in Table I. The result showed that the ethanol based extract was more toxic as compared with the other extract of *C. colosynthis*. The water based extract showed least toxicity. There was no significant difference ($P > 0.05$) between the toxicity of all the treatments (Table I) but there was significant difference ($P< 0.05$) between all the extract and the positive control.

Development and survival of Helicoverpa armigera

The larval developmental time (2nd instar to pupation) of *H. armigera* treated with sub- lethal concentration of ethanol fruit extract of *C. colocynthis* was significantly longer compare with untreated control larvae (Table II, $F =8.17$, df =5, ^{p} <0.046). The mean pupal developmental time of treated larvae were significantly longer compare with untreated larvae (Table II, $F= 70.8$, DF= 5, $P< 0.001$). The mean developmental time from 2nd instar to adult of treated larvae was also significantly longer than control (Table II, $F= 60.72$, dF= 5, $P< 0.001$). The mean pupal weight of treated *H. armigera* was significantly lower than control treatment (Table II, $F= 161.04$, df =5, $P < 0.0001$). The percent pupation was not significantly different (Table II, $F= 0.34$, df= 5, $P> 0.736$) in the treated insects as comparison with the control treatment. The percent adults emergence was significantly lower in treated population (Table II, $F= 15.76$, df= 5, $P < 0.016$) as compare with the control. The mean adult longevity of treated population was significantly longer than control population (Table II, $F=20.16$, df= 5, $P < 0.01$).

Table I.- Toxicity of *Citrullus colocynthis* fruit extracts in different solvents to 2nd instar larvae of *Helicoverpa armigera* observed after seven days of exposure.

Solvent	LC_{25} (95% CI) %	LC_{50} (95% CI) %	LC_{90} (95% CI) %	Slope ± SE
Hexane	2.63 (0.92-4.16)^{a}	7.57 (6.17-10.28)^{a}	32.18 (19.28-98.19)^{a}	(0.476±0.375)
Ethyl acetate	1.26 (0.35-2.09)^{a}	3.55 (2.21- 4.66)^{b}	25.25(14.41-111.6)^{a}	(0.433±0.323)
Ethanol	0.79 (0.09-1.58)^{a}	2.54 (1.04-3.61)^{b}	23.45(12.91-150.13)^{a}	(0.434±0.321)
Methanol	2.17(0.33-3.96)^{a}	7.18(3.95-9.76)^{ac}	69.31(33.86-692.14)^{a}	(0.428±0.445)
Water	3.53 (2.41-4.42)^{a}	9.92 (7.54-12.76)^{c}	123.01(57.56-768.31)^{a}	(0.301±0.316)
Profenofos	0.02 (0.006-0.044)^{b}	0.079(0.039-0.118)^{d}	0.84(0.514-2.245)^{b}	(0.230±0.285)

Table II.- Mean developmental time (day's ± SE) and Pupal weight of *H. armigera* treated with sub lethal concentration of ethanol fruit extract compared with control.

Treatment	Larval development	Pupal development	Development time (2nd instar to adult)	Adult longevity	Pupal weight	% pupae	Adult Emergence
Control	16.7± 0.48^{a}	7.76± 0.44^{a}	24.46± 0.04^{a}	10.6 ± 0.61^{a}	213.36± 2.12^{a}	84.4% ± 1.34^{a}	77% ± 0.04^{a}
Treated	18.08± 1.04^{b}	10.11± 0.10b	28.2± 0.94^{b}	12.26± 0.53^{b}	199.43± 0.04^{b}	79% ± 0.027^{b}	68% ± 0.014^{b}

Values within columns with a common letter are not significantly different (P>0.05).

Table III.- Larval weight (mg ± SE) of *Helicoverpa armigera* treated with sub-lethal (LC_{25}) concentration of ethanol fruit extract compared with control.

Treatment	2nd	3rd	4th	5th	6th
Control	3.90±0.08[a]	20.5±0.93[a]	60.51±0.63[a]	282.84±0.67[a]	492.23±1.42[a]
Treated	3.22±0.13[b]	18.5±0.07[b]	59.61±2.51[b]	273.75±0.06[b]	478.06±0.97[b]

Values within columns with a common letter are not significantly different (P>0.05).

The mean larval weight of 2nd. 3rd, 4th and 5th instars was not significantly different as compare with the control in comparison with the control (Table III, F = 32.7, df = 5, P> 0.05). But There was significant difference in larval weight of 6th instar when compared with the control population (Table III, F= 29.8, df= 5, P < 0.001).

Matting success, fecundity and egg viability of Helicoverpa armigera

There was no significant difference between treated and control treatments for the proportion of pairs that produced eggs (Table IV, df= 5, P > 0.05). The number of eggs laid by the treated *H. armigera* was not significantly different as compare with the control (Table IV, df= 5, P> 0.05). Similarly, the egg viability of treated population was not significantly different compared with the control (Table IV, df= 5, P> 0.05)

Antifeedant activity

Decrease in feeding was observed with significant variation of different extracts of 74.5% with ethyl acetate, 72 % with ethanol, 52.5 % with methanol, 36.5% with hexane and 24% with water based (Table V).

Table IV.- Mean reproductive parameter of *Helicoverpa armigera* treated with sub-lethal (LC_{25}) concentration of ethanol fruit extract compared with control.

Treatment	Pairs[a]	MPS[b] %	Egg/female ± SE	% viability of eggs ±SE
Control	15	80	360± 16[a]	69±2.89[a]
Treated	15	65	376± 13[a]	63±3.2[a]

a, total number of pairs; b, mating pair success; Values within columns with a common letter are not significantly different (P>0.05).

Table V.- Deterrence index of different solvent extracts of *C. colocynthis* against 2nd instar of *Helicoverpa armigera* in diet incorporate method at LC_{50} value.

Solvents	Deterrence index
Hexane	36.5%
Ethanol	72%
Methanol	52.5%
Ethyl acetate	74.5%
Water	24%

DISCUSSION

The frequent and indiscriminate use of synthetic insecticides causes environmental and insect pest resistance problems (Tabashnik and Roush, 1990). Another source of natural insecticides used by man for centuries is chemicals derived from the plants which are safe for environment, less toxic to natural enemies and do not persist in nature for long time (Liu *et al*., 2000). In the present study organic and water solvent extracts of *C. colosynthis* fruit were tested against 2nd insatar larvae of *H. armigera*. The results showed that ethanol extract was more toxic to 2nd instar larvae of *H. armigera* followed by ethyl acetate extract when used with diet immersion method.) The petroleum ether, methanol and ethyl acetate leaf extracts of *C. colocynthis* showed toxicity against *C. quinquefasciatus* larvae (Mullai and Jebanesan, 2007). Seed extract of *C. colosynthis* in ethanol has antifeedant and toxic properties against mites (Mansour *et al*., 2004). Water extract of *C. colocynthis* showed toxic effect against *Rhopalosiphum padi* (Khalid, 2015). Petroleum and methanol extracts have already been identified as toxic to many mosquito species with larvicidal and anti-oviposition activities (Rahuman *et al*., 2008). This mortality in mosquitoes was due to the presence of oleic acid and linolic acid (Abdul and Venkatesan, 2008). Fruits extract in four different solvents *i.e.,* n-hexane, methylene chloride, Chloroform and ethanol against have also revealed them effective against *Aphis craccivora* with the highest mortality recorded in ethanol extracts. This mortality in *A. craccivora* was due to the presence of E-Glycosides (Torkey *et al*., 2009). Extract of *C. colocynthis, Azadirachta indica, Ammi majus* and *Mentha microphylla* have toxin effect on *S. litura* and also decreased the total lipid, total fat and total glucose contents of the larvae. These extracts also showed high level of disturbance in the cell wall and midgut of the insect. Presence of organic chemicals including oleic acid, linolic acid, Cucurbiticin B compound and E-Glycoside compounds may be responsible for toxicity and antifeedant effects against tested *H. armigera* larvae.

The ethanol extract of *C. colosynthis* showed significant effect on larval development, pupal duration, total development time, percent pupation and pupal weight. However, there existed non-significant effect

on the adult longevity, larval weights, fecundity and egg viability. Many scientists have documented similar differences in development after exposure of sublethal concentrations of plant extracts (Wondafrash *et al*., 2012; Khani *et al*., 2013; D'Inaco *et al*., 2014). Ma *et al*. (2000) studied the effect of neem extract on 2nd instar *H. armigera* and reported that the neem extract have significant effect on insect development. Arti and Purohit (2009) reported abnormalities in 4th instar larvae of *H. armigera* when treated with *Lantana camara*. The water extract of different parts of neem delayed the larval development as compare with control treatment in *H. armigera* (Wondafrash *et al*., 2012). The methanolic extract of *S. alba* showed 54% pupicidal activity at 2% concentration against *H. armigera* (Sivaraman *et al*., 2014). The extracts of *S. alba* and *C. viscosa* caused 33.93% and 32.86% deformities at adult stage of *H. armigera* respectively (Sivaraman *et al*., 2014). These effects of plant extract on insect development could be due to the effects on neurosecretory cells and on endocrine system that control development in insects (Mordue *et al*., 2005).

In the present study *C. colocynthis* fruits extract in five different solvents *i.e.,* hexane, methanol, ethanol, ethyl acetate and water were used for investigating the antifeedant activity and the results showed that the ethyl acetate extract reduced the feeding activity 74.5 % in comparison with the control population, ethanol extract was the 2nd effective antifeedant extract with 72% reduction in feeding of the test insect, and water was least effective with 24% decrease in food consumption. Dong *et al*. (2005) reported that Lantana leaves showed antifeedant activity even at very low concentration when used against two lepidopterous pests, *Plutella xylostella* and *Spodoptera litura.* Ramya *et al*. (2008) investigated that *Cathranthus roseus* leaf extracts of petroleum ether, methanol and ethyl acetate have antifeedant and larvicidal activity against *H. armigera.* The methanolic extract of *S. alba* showed 71.42% antifeedant activity at 2% concentration against *H. armigera* (Sivaraman *et al*., 2014)

In Pakistan, farmers mostly apply insecticide to control this pest. This situation leads the development of insecticide resistance in *H. arnmigera*. This study showed that extracts of *C. colosynthis* possess insecticidal and developmental inhibiting effect on *H. armigera*. The use of *C. colosynthis* along with the insecticide in the IPM programs delays the development of resistance in this pest. Moreover, the application of *C. colosynthis* also minimizes adverse effect on the human due to the use of synthetic insecticides. However, further detailed studies are suggested after identification of chemicals present in these extracts and their biological and toxicological activities against important insect pests like *H. armigera*.

Statement of conflict of interest

Authors have declared no conflict of interest.

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