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Research Article

Agrochemistry

Phytochemical and Synergism of Plant and Microbial Insecticides to Eliminate Earias Vittella Gut Epithelial Cells

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Abstract: The spotted bollwom, *Earias vittella* is a very serious and polyphagous pest that attacks many plants of Malvaceae family. Such as Lady's finger (*Abelmoschus esculentus Moench*), which attacks growing points of the plant. The intensive use of highly toxic and broad-spectrum pesticides threatens the sustainability of vegetable production, mainly due to the development of pest resistance. Consequently, it becomes more difficult to manage key pests and secondary pests, which assume major status due to the elimination of natural enemies from the eco system. In the present study, evaluation of the larvicidal, quantitative food utilization and histological activity of *Manihot esculenta*, Azadirachtin (AZA), *Bacillus thuringiensis var. kurstaki* (Btk), and synergistic effects, was carried out against *Earias vittella*. The GC-MS analysis of *M. esculenta* was estimated and seventeen different phytoactive compounds were identified. Larvicidal activity of *M. esculenta*, AZA, and *Btk* provided significant mortality against *E. vittella*. The lowest LC_{50} of individual treatment was from AZA, the LC_{50} being as follows: 0.122%, 0.131%, 0.150%, 0.157%, and 0.168%, and the LC_{50} of synergistic treatment was 1.434ppm, 1.469ppm, 1.531ppm, 1.701ppm, and 1.908ppm for I, II, III, IV, and V instars, respectively. The food utilization result showed that, the food intake of *E. vittella was* significantly reduced after the treatment. Here, synergistic effects controls the food intake, which is highly reduced, and fecal pallet egestion considerably reduces the amount of food consumed. Histological studies state that, after the treatment, the gut of *E. vittella is* drastically damaged by the biopesticides. *M. esculenta*, AZA and *Btk* and their synergism are very effective against the immature stage of *E. vittella*. Gut region of the insect finely eradicated by the biopesticide. Overall, this study concludes that the taken botanical insecticides are eco-friendly, harmless to mammals and other non-target animals.

Keywords: food utilization*; Earias vittella*; Btk; histological; phyto-chemistry

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INTRODUCTION

Agriculture is regarded as one of the most essential components in a country's growth, influencing major economic sectors in both the developing and industrialized worlds. Sustaining quality agriculture production is a necessity, particularly for the under-developed, the developing, and those countries which are inclined to a demographic spurt in the near future.[1]The COVID-19 pandemic caused a large-scale global impact this year especially in Indian major crops.[2,3]India is an agrarian economy where agriculture and its allied activities act as a main source of livelihood for more than 80 per cent of the rural population. The contribution of the agriculture sector in the economy has significantly decreased from 51% in 1951 to 19% in 2011, and further to 14.8% in 2019-20.[4]Okra or Bhendi (*Abelmoschus esculentus* (L.) Moench), commonly known as lady's finger, belongs to the family Malvaceae. It is one of the important summer vegetables grown widely in the sub-tropical regions of the world for its tender pods. Okra is one of the most important vegetable crops grown extensively throughout the country during the summer and rainy seasons due to its high adaptability over a wide range of environmental conditions. It is one of the economically important vegetable crops grown in India. As a vegetable in the tender stage, okra is nutritious and it finds an important place in the Indian diet.[5]The widespread use of chemical fertilizers has major side effects, including pollution, the development of pest resistance, and a decrease in food safety. Because of the need for sustainable agriculture development, more research is being done on using plant-beneficial microbes to partially replace chemical fertilizer use.[6] Organic fertilizers are naturally available mineral sources that contain a moderate amount of plant-essential nutrients. They are capable of mitigating problems associated with synthetic fertilizers. They reduce the necessity of repeated application of synthetic fertilizers to maintain soil fertility. They gradually release nutrients into the soil solution and maintain nutrient balance for healthy growth of crop plants.[7]Applying organic matter to the soil would help to improve soil quality and sustain crop production. In addition, the small molecular organic matters could be active in influencing soil nutrient cycling and crop development.[8]*Manihot esculenta* Syn. *M. utilissima*; (Eng-Tapioca; Verna-Sakarkanda) - A small shrub; native of Brazil now grown in Kerala, Chennai and Mysore. Cassava *(Manihot esculenta*), also called yuca or manioc, is a woody shrub of the Euphorbiaceae (spurge family) native to South America Olsen *et al.*[9]*Manihot* leaves (and seeds) are used in folk medicine to alleviate fever, headache, rheumatism, and hemorrhoids. In Nigeria, they are also utilized in the treatment of ringworms, tumor, conjunctivitis, sores and abscesses.[10]*Bacillus thuringiensis* (*Bt*) is a gram-positive, spore-forming bacteria known for its ability to produce crystal proteins (Cry). Cry protein is believed to be toxic to many insects and that is why *Bt* is used as a microbial insecticide for improved resistance in plants by genetic modification.[11]Azadirachtin is neem based insecticide derived from extracts of *Azadirachta indica* (Meliaceae). This product has played a vital role in crop protection in the last decade. This insecticide is a very complex terpenoid, and has been successfully used not only for the management of pest, but against more than 400 species of insects. Azadirachtin has proved to be one of the most promising plant products for integrated pest management.[12-16]On the basis of the above scientific facts, an attempt has been made to evaluate the impact of methanolic seed extract of *Manihot esculenta*, Azadirachtin (AZA) and *Bacillus thuringiensis* var.*kurstaki* (*Btk*) on the fruit and shoot borer of bhendi, *Earias vittella* and their gut.

MATERIALS AND METHODS

Collection of Insect and Site of Investigation

The test insect, *Earias vittella*, was collected from the Bhendi Garden in and around Namakkal District, Tamilnadu, India. The insects were used for further evaluation at the laboratory.

Preparation of Extracts

M. esculenta

The fresh seeds of *M. esculenta* were collected from the Kolli Hills, Namakkal District. The collected seeds were identified by Dr. Raju, Taxonomist, Associate Professor and Head, PG and Research Department of Botany, Kandaswami Kandar's College, Namakkal-638 182, Tamil Nadu, India and the voucher of the specimen was stored in the herbarium of PG and Research Department of Botany, Kandaswami Kandar's College, Namakkal-638 182, Tamil Nadu, India. The seeds were shadow dried for a week. The dried seeds were grounded in a mixer grinder and stored in an airtight container. About 501.506g of the powdered material was subjected to soxhlation and exhaustively extracted with 90% Methanol for 48 hrs. The extracted materials were concentrated in a rotary evaporator under reduced pressure. Finally, a dark brown coloured material was obtained. It was stored in the refrigerator.

Azadirachtin

5.265 mg of Azadirachtin (95% purity) was dissolved in 50 ml of distilled water to obtain a stock solution of 100% concentration. Desired concentrations ranging from 0.01 to 10% were prepared from the stock solutions using distilled water as solvent.

Bacillus thuringiensisVar. kurstaki

B. thuringiensis was a Delfin WG product which contained *Bt kurstaki* serotype 3a, 3b, 85% and dispersing agents at 15%, potency: min 53,000 SU/mg (Sandoz (India) Limited). The required concentrations ranging from 0.1 to 10.0mg/ml were prepared by diluting with distilled water.

GC-MS Analysis of Methanolic Seed Extract of M. esculenta

Preparation of extract

Seeds of *M. esculenta* were shade dried. 20g of the powdered seeds were soaked in 95% methanol for 12h. The extracts were then filtered through Whatman filter paper No.41 along with 2gm sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulfate was wetted with 95% methanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phyto components of the plant material and 3µl of these solutions was employed for GC/MS analysis.[17]

GC-MS Analysis

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column(30 x 0.25mm ID x 1EM df, composed of 100% Dimethylpolysiloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1m1/min and an injection volume of 0.5 El was employed (split ratio of 10:1) injector temperature 250°C ion – source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5s and fragments from 40 to 550 Da. Total GC running time was 36 min. The spectrums of the components were compared with the database of spectrum of known components stored in the GCMS National Institute of Standards and Technology (2008) library[18].

Mass Rearing of Test Insect

The stock culture of *E. vittella* was maintained under laboratory conditions. For this purpose, egg clusters were collected from the Bhendi fruit in the field and kept in petri plates over the filter papers. Newly hatched larvae were transferred to Bhendi plants which had their stems dipped in water filtered glass bottles and were placed inside the wooden rearing cages (36 cm x 34 cm x 26 cm with glass panes on three sides and the top and wire mesh on the front door). Fresh Bhendi were provided daily to the larvae till pupation. One-day-old pupae were collected from the walls of the rearing cages and were sexed as suggested by Gupta[19]). The pupae of both the sexes were kept separately in glass jars over a piece of filter paper. In each jar, a resting place was provided for the newly hatched adults for normal expansion of their wings. The adults were provided with sugar solution soaked in cotton swabs and some shoots of Bhendi.

Biological Assays

Mortality Bioassay

The sliced bhendi pods were treated with different concentrations of methanolic seed extract of *M. esculenta ranging from* 1% to 4%, 0.10% to 0.50% of AZA and *Btk*, ranging from 0.5µg/ml to 3.0 µg/ml. Control leaves were treated with distilled water alone. The leaves were allowed to dry at room temperature for 10min and then placed on a wet filter paper disc in 10cm diameter plastic petri dishes. The experiments were carried out with newly moulted, 3 hr starved I, II, III, IV, and V instar larvae using one larvae per dish in five replicates (30 larvae/concentration). After 4 days, the larvae were transferred to fresh untreated sliced bhendi pods and maintained until they moulted into adults or died. The total number of normal adults that survived was noted. The larvae were observed for mortality and morphological changes associated with growth disrupting effects. After corrections, the percent mortality data were subjected to probit analysis to calculate the mean lethal concentration (LC_{50}).

Results were corrected for control mortality by using Abbott's[20] Formula.

$$P_t = (P_o - P_c / 100 - P_c) \times 100$$

Where,

P_t = Corrected percent mortality
P_o = Observed mortality
P_c = Observed mortality in control

From the mean lethal concentration (LC_{50}) the physiological doses were selected for biological, nutritional and biochemical studies.

Quantitative Food Utilization Efficiency Measures

The newly moulted I, II, III, IV, and V instar larvae were starved for 3 hours. After measuring the initial weight of the larvae they were individually introduced into separate containers. The larvae (10 larvae/concentration, five replicates) were allowed to feed on weighed quantities of *M. esculenta*, AZA, and *Btk* treated sliced bhendi pods for a period of 24 hrs. The difference in weight of the larvae gives the fresh weight gained during the period of study. Sample caterpillars were weighed, oven dried, and reweighed to establish a percentage dry weight of the experimental caterpillars. The sliced bhendi pods remaining at the end of each day were oven dried and re-weighed to establish a percentage dry weight conversion value to allow estimation of the dry weight of the diet given to the larvae. The quantity of food ingested was estimated by subtracting the diet remaining at the end of each experiment from the total dry weight of diet provided. Faeces were collected daily and weighted, then dried and and reweighed to estimate the dry weight of excreta. The experiment was continued for four days and observations were recorded every 24 hours. Consumption, growth rates and post ingestive food utilization efficiencies (all based on dry weight) were calculated in the traditional manner.[21-24]

Consumption Index (CI)	=	E / TA
Relative Growth Rate (RGR)	=	P / TA
Approximate Digestibility (AD)	=	100 (E-F)/E
Efficiency of Conversion of Ingested Food (ECI)	=	100 P/E
Efficiency of Conversion of Digested Food (ECD)	=	100 P/(E-F)

Where,

A = mean dry weight of anima1 during T
E = dry weight of food eaten
F = dry weight of faeces produced
P = dry weight gain of insect
T = duration of experimental period.

Faecal Pellet Egestion Efficiency Measures

Groups of fifty larvae in ten separate containers were fed with *M. esculenta*, AZA, and *Btk*-treated sliced bhendi pods. The time required for egestion of the first faecal pellet was noted. The total number of faecal pellets egested by each group of larvae was noted. The experiment was carried out every 6 hours.

Histological Studies

The gut region of the IV instar E. vittella larvae was studied histologically. A slight modification of the Martoja and Martoja25 Method, the histological techniques aim at obtaining thin and coloured sections of biological material, observable under an optical microscope. Control and treated larvae, placed in distilled water, were sampled at the same time intervals and were also fixed. Before initiating the histological technique steps, the guts were cut and separated with a scalpel blade. The parts were then placed in small boxes and subjected to a series of ethanol baths ranging from 70 to 100°C to dehydrate them, or remove intra- and extracellular water. They were then immersed in three xylene containers in order to remove traces of ethanol and lighten the parts. Once dehydrated, the parts were enclosed in paraffin to achieve permeation into the studied tissue as complete as possible. The impregnation of paraffin takes place in an oven at a temperature of 58 to 60°C. The blocks were then prepared and cut at a thickness of 5 with a microtome. The obtained sections were stained, fixed between slide and cover slip and observed under an optical microscope to determine the various anomalies that may have appeared in the gut of the treated E. vittella larvae.

STATISTICAL ANALYSIS

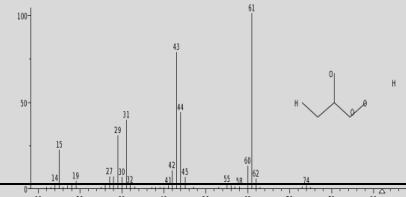
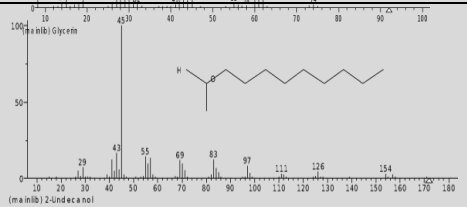
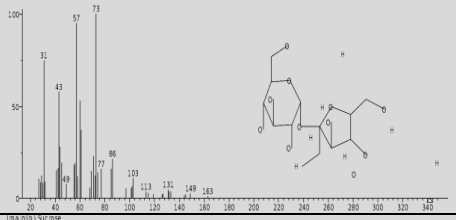
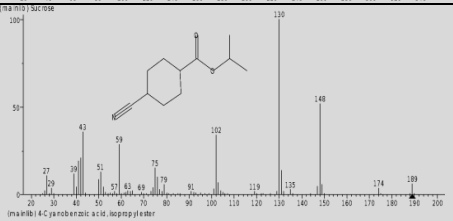
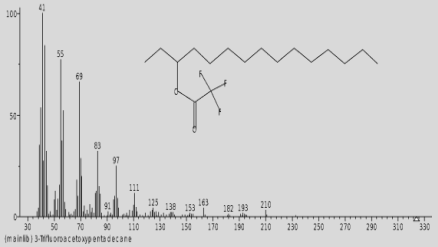
The SPSS software package, version 16.0 was used for all analyses. Data from larvicidal and pupicidal experiments were analyzed by probit analysis, calculating LC50 and LC90.26 Statistical analysis has been carried out by one way in which ANOVA used the SPSS Software Programme. The different treatment means were compared by Duncan's Multiple Range Test. The means, standard errors and other parameters were estimated as described by Snedecor and Cochran.27

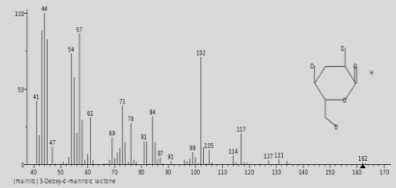
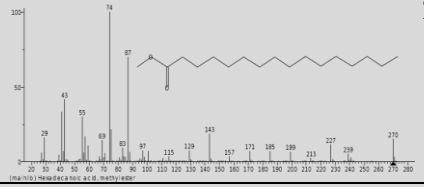
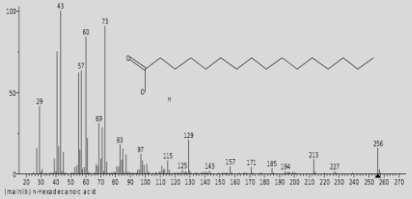
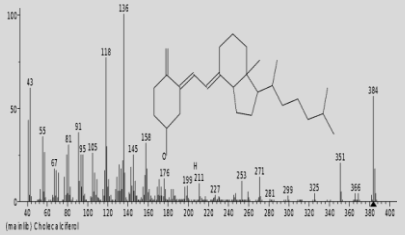
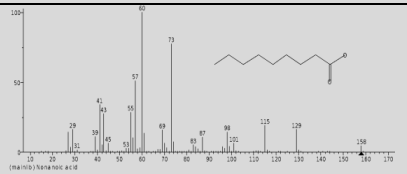
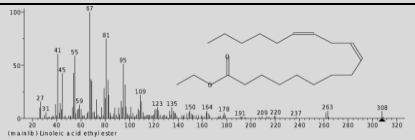
RESULTS

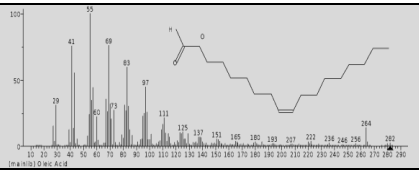
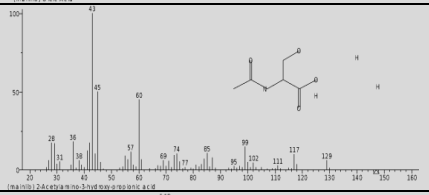
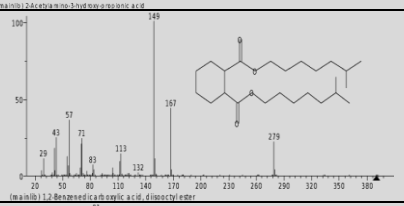
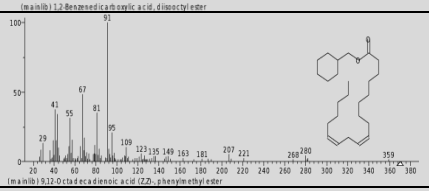
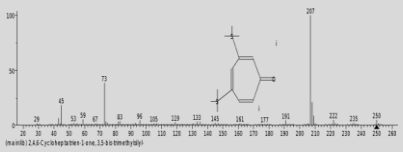
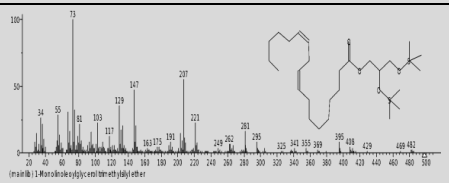
Phytochemical Analysis of M. Esculenta by GC-MS Method

During the investigations seventeen different phytoactive compounds were identified from our study material seed extract of M. esculenta by GC-MS analysis. All the compounds name, its retention time (RT), molecular formula, molecular weight and its peak area % where given in the table 1 and GC-MS Chromatogram of seed extract of M. esculenta is given in the figure 1.Their identification and characterization were based on their elution order in a GC-MS column. The elution time, molecular formula and the amount of these bioactive compounds were also presented. Based on abundance, there were eighteen major compounds present in the methanolic seed extract of M. esculenta were Glycerin (2.26%), 2-Undecanol (6.47%), Sucrose (7.87%), 3-Trifluoroacetoxypentadecane (8.92%), 4-Cyanobenzoic acid, isopropyl ester (9.14%), 3-Deoxy-d-mannoic lactone (10.01%), Hexadecanoic acid, methyl ester (12.73%), n-Hexadecanoic acid (13.26%), Vitamin d3 (13.57%), Nonanoic acid (13.92%), Linoleic acid ethyl ester (14.92%), Oleic Acid(15.01%), 2-Acetylamino-3-hydroxy-propionic acid (15.39%), 1,2-Benzenedicarboxylic acid, diisooctyl ester (21.64%), 9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester (24.29%), 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl (26.39%), 1-Monolinoleoylglycerol trimethylsilyl ether (34.80%), respectively (Structures of the identified compounds are given in Table 1).

Table1. Phyto-Components identified in the Methanolic Seed Extract of *M. esculenta* GC-MS study

No.	RT	Name of the compound	Molecular	Structure and MF	MW	Peak Area %	Compound Nature	** Activity
1.	2.26	Glycerin	C_3H8O_3		92	5.37	Alcoholic compound	Antimicrobial Preservative
2.	6.47	2-Undecanol	$C_{11}H_{24}O$		172	0.62	Alcoholic compound	Antimicrobial Preservative
3.	7.87	Sucrose	$C_{12}H_{22}O_{11}$		342	41.64	Sugar moiety	Preservative
4.	8.92	3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$		324	0.56	Fluro compound	Antimicrobial
5.	9.14	4-Cyanobenzoic acid, isopropyl ester	$C_{11}H_{11}NO_2$		189	11.40	Cyano compound	Insecticide

6.	10.01	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$		162	17.48	Lactone compound	No activity reported
7.	12.73	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$		270	0.62	Palmitic acid methyl ester	Insecticide Pesticide
8.	13.26	n-Hexadecanoic acid	$C_{16}H_{32}O_2$		256	1.54	Palmitic acid	Insecticide Pesticide
9.	13.57	Cholecalciferol-D3	$C_{27}H_{44}O$		384	0.27	Vitamin compound	Nutrient
10.	13.92	Nonanoic acid	$C_9H_{18}O_2$		158	1.60	Fatty acid	Herbicide
11.	14.92	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$		308	3.48	Fatty acid ester	Nematicide, Insectifuge

12.	15.01	Oleic Acid	$C_{18}H_{34}O_2$		282	0.81	Fatty acid	Insecticide
13.	15.39	2-Acetylamino-3-hydroxy-propionic acid	$C_5H_9NO_4$		147	0.19	Amino compound	Antimicrobial
14.	21.64	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$		390	6.62	Plasticizer compound	Antimicrobial Anti fouling
15.	24.29	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester	$C_{25}H_{38}O_2$		370	1.62	Linoleic acid ester	Nematicide, Insectifuge
16.	26.39	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	$C_{13}H_{22}OSi_2$		250	1.23	Ketone compound	No activity reported
17.	34.80	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$		498	3.20	Steroid compound	Antimicrobial

***Activity source: Dr. Duke's Phytochemical and Ethnobotanical Databases*

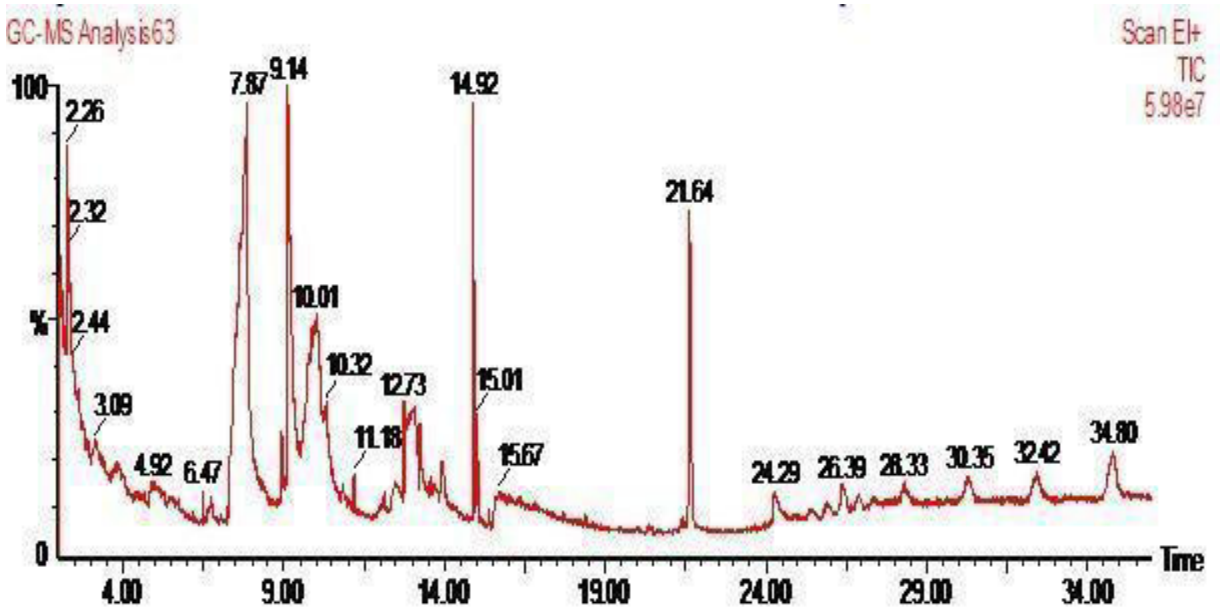


Fig 1. GC-MS Chromatogram of methanolic seed extract of *M. esculenta*

This study was undertaken to determine the effects of methanolic seed extract of *M. esculenta,* Azadirachtin (AZA), *Bacillus thuringiensis* var. *kurstaki* (*Btk*) and a synergistic combination (*M. esculenta* + AZA + *Btk*) at different concentrations on the fruit and shoot borer of Bhendi, *E. vittella* (Fabricius). The results obtained are presented below:

Larvicidal Activity

The percentage of mortality showed a decreasing trend from early to later instars. As a result, mortality was dose-dependent with age. **Table 2** provides the values of LC_{50}, (LC_{90}) and Chi-square values of the botanicals and bio-pesticides for the larval form of *E. vittella*. The LC_{50} (LC_{90}) values of methanolic seed extract of *M. esculenta* for I, II, III, IV and V instars larvae of *E. vittella* were 1.294% (5.268%), 1.398% (5.539%), 1.546% (5.827%), 1.719% (6.164%) and 1.948% (6.548%). **Table 3** shows that the LC_{50} (LC_{90}) values of AZA for I, II, III, IV and V instars larvae of *E. vittella* were 0.122% (0.586%), 0.131 (0.629%), 0.150 (0.685%), 0.157% (0.746%) and 0.168% (0.832%), respectively. All the LC_{50} (LC_{90}) and Chi-square values are significant at the $P< 0.05$ level for all larval instar.

Table 2: Effect of methanolic seed extract of *M. esculenta* on Larval, pupal and adult mortality of *Earias vittella*

Life stages of *E. vittella*	Larval, pupal and adult mortality (%)			LC_{50} (LC_{90})	95% Confidence Limit		(χ^2)
	Concentration (%)				LC_{50} (LCL- UCL)	LC_{90} (LCL- UCL)	
	I	2	4				
I instar	45.31	60.37	80.44	1.294 (5.268)	(0.496- 1.781)	(4.326- 7.279)	0.122*
II instar	44.04	58.97	78.48	1.398 (5.539)	(0.603- 1.892)	(4.512- 7.800)	0.162*
III instar	42.29	57.25	76.30	1.546 (5.827)	(0.780- 2.042)	(4.712- 8.344)	0.216*
IV instar	40.27	55.52	73.74	1.719 (6.164)	(0.989- 2.225)	(4.941- 9.010)	0.333*
V instar	37.80	53.27	70.76	1.948 (6.548)	(1.273- 2.476)	(5.202- 9.774)	0.460*

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, pupae and adult, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, pupae and adult, χ^2-Chi square value, Significant at $P<0.05$ level. Within a column means followed by the same letter(s) are not significantly different at the 5% level by DMRT.

Table 3: Effect of AZA on Larval, pupal and adult mortality of *Earias vittella*

Life stages of *E. vittella*	Larval, Pupal and adult mortality (%)			LC_{50} (LC_{90})	95% Confidence Limit		(χ^2)
	Concentration (%)				LC_{50}	LC_{90}	
	0.10	0.25	0.50		(LCL- UCL)	(LCL- UCL)	
I instar	46.60	66.10	85.20	0.122 (0.586)	(0.029- 0.179)	(0.483- 0.792)	0.130*
II instar	45.50	64.48	82.95	0.131 (0.629)	(0.035- 0.191)	(0.514- 0.868)	0.193*
III instar	44.00	61.50	80.26	0.150 (0.685)	(0.053- 0.212)	(0.554- 0.968)	0.130*
IV instar	43.50	60.58	77.51	0.157 (0.746)	(0.049- 0.223)	(0.593- 1.100)	0.253*
V instar	43.35	58.48	74.36	0.168 (0.832)	(0.045- 0.240)	(0.645- 1.313)	0.190*

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, pupae and adult, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, pupae and adult, χ^2-Chi square value, Significant at $P<0.05$ level. Within a column means followed by the same letter(s) are not significantly different at the 5% level by DMRT.

Table 4 provides the LC_{50} (LC_{90}) values of *Btk* for I, II, III, IV and V instars larvae of *E. vittella* were 0.606mg/ml (3.287mg/ml), 0.664mg/ml (3.495mg/ml), 0.740mg/ml (3.777mg/ml), 0.829mg/ml (4.121mg/ml) and 0.933 mg/ml (4.549mg/ml), respectively. For I, II, III, IV, and V instars larvae of *E. vittella*, the LC_{50} (LC_{90}) values of the synergistic combination of (*M. esculenta* + AZA + *Btk*) were 1.434ppm (6.414ppm), 1.469ppm (6.888ppm), 1.531ppm (7.460ppm), 1.701ppm (8.206ppm), and 1.908ppm (9.211ppm), respectively. All the LC_{50} (LC_{90}) and Chi-square values are significant at $P< 0.05$ level for all larval instars **given in table5**.

Table 4: Effect of *Btk* on Larval, pupal and adult mortality of *Earias vittella*

Life stages of *E. vittella*	Larval, pupal and adult mortality (%)			LC_{50} (LC_{90})	95% Confidence Limit		(χ^2)
	Concentration mg/ml				LC_{50}	LC_{90}	
	0.5	1.5	3.0		(LCL- UCL)	(LCL- UCL)	
I instar	47.80	67.60	87.65	0.606 (3.287)	(0.061- 0.950)	(2.719- 4.381)	0.000*
II instar	46.40	66.40	85.69	0.664 (3.495)	(0.106- 1.016)	(2.875- 4.713)	0.037*
III instar	44.90	64.68	83.18	0.740 (3.777)	(0.164- 1.105)	(3.082- 5.190)	0.112*
IV instar	43.60	62.58	80.36	0.829 (4.121)	(0.228- 1.210)	(3.325- 5.817)	0.164*
V instar	42.45	60.21	77.16	0.933 (4.549)	(0.299- 1.337)	(3.613- 6.676)	0.184*

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, pupae and adult, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, pupae and adult, χ^2-Chi square value, Significant at $P<0.05$ level. Within a column means followed by the same letter(s) are not significantly different at the 5% level by DMRT.

Table 5: Synergistic effect of seed extract of *M. esculenta,* AZA and *Btk* on Larval, pupal and adult mortality of *Earias vittella*

Life stages of *E. vittella*	Larval, pupal and adult mortality (%) Concentration [(ppm / (%)]			LC_{50} (LC_{90})	95% Confidence Limit		(χ^2)
	1% + 0.10% + 0.50% µg/ml	2% + 0.25% + 1.50% µg/ml	4% + 0.50% + 3.00% µg/ml		LC_{50} (LCL- UCL)	LC_{90} (LCL- UCL)	
I instar	52.0	72.0	94.00	1.434 (6.414)	(0.283- 2.176)	(5.522- 7.907)	0.016*
II instar	51.38	71.53	92.05	1.469 (6.888)	(0.260- 2.220)	(5.801- 8.907)	0.000*
III instar	49.88	70.66	89.70	1.531 (7.460)	(0.228- 2.331)	(6.240- 9.793)	0.111*
IV instar	47.91	68.59	86.93	1.701 (8.206)	(0.331- 2.540)	(9.793- 6.809)	0.278*
V instar	46.11	65.99	83.42	1.908 (9.211)	(0.430- 2.803)	(7.541- 12.696)	0.426*

LC_{50} – Lethal concentration that kills 50% of the exposed larvae, pupae and adult, LC_{90} – Lethal concentration that kills 90% of the exposed larvae, pupae and adult, χ^2-Chi square value, Significant at $P<0.05$ level. Within a column means followed by the same letter(s) are not significantly different at the 5% level by DMRT.

Activity of Nutritional indices

The nutritional indices, consumption index (CI) and relative growth rate (RGR), Approximate Digestibility (AD), Efficiency of Conversion Ingested Food (ECI) and Efficiency of Conversion of Digested Food (ECD) against III instar larvae, IV instar, and V instars of *E. vittella*, were provided in Tables 4, 5, and 6 and figure 1-6, respectively. The control treatment of CI, RGR, AD, ECI, and ECD against III instar larvae were 0.348 mg/day, 0.076 mg/day, 40.90%, 22.39%, and 54.76%, respectively. After the treatment of methanolic seed extract of *M. esculenta* (4% highest concentration), the rate of CI and RGR was significantly reduced to 0.216 mg/day and 0.030 mg/day, and the percentage of AD, ECI, and ECD were 44.00%, 15.07%, and 34.27% respectively. Similarly, after AZA (0.50%) treatment, they observed CI=0.196 mg/day, RGR= 0.021 mg/day, AD= 44.07%, ECI=12.06%, and ECD= 29.04%.the *Btk* (3 g/ml) treatment, CI=0.170 mg/day, RGR=0.023 mg/day, AD=44.54%, ECI=11.35%, and ECD=26.32% were discovered. Significant reductions were observed after the synergistic treatment (*M. esculenta* + AZA + *Btk*) explored CI and RGR reduced CI by 0.123 mg/day and 0.010 mg/day, respectively, and the percentage of AD, ECI, and ECD were calculated at 45.61, 9.46, and 20.75%, respectively **(given in table 6)**.

Table 6: Effect of methanolic seed extract of *M. esculenta,* AZA and *Btk* on the nutritional indices of III instars larvae of *E. vittella*

Treatment	CI (mg/ mg/day)	RGR (mg/ mg/day)	AD (%)	ECI (%)	ECD (%)
Control	0.348± 0.030^{a}	0.076± 0.007^{a}	40.90± 3.95^{b}	22.39± 1.96^{a}	54.76± 4.82^{a}
***M. esculenta* (%)** 1.0	0.308± 0.028^{a}	0.056± 0.005^{b}	41.70± 4.04^{b}	18.85± 1.77^{b}	45.22± 4.67^{b}
2.0	0.273± 0.020^{b}	0.045± 0.004^{c}	42.62± 4.05^{b}	17.32± 1.64^{c}	40.67± 3.98^{c}
4.0	0.216± 0.018^{c}	0.030± 0.003^{c}	44.00± 4.15^{b}	15.07± 1.45^{c}	34.27± 3.75^{d}
AZA (%) 0.10	0.294± 0.027^{a}	0.053± 0.007^{b}	41.82± 4.06^{b}	18.81± 1.78^{b}	45.00± 4.60^{b}
0.25	0.219± 0.015^{b}	0.034± 0.003^{c}	42.89± 4.05^{b}	16.39± 1.65^{c}	38.19± 3.80^{d}
0.50	0.196± 0.014^{c}	0.021± 0.002^{d}	44.07± 4.15^{b}	12.06± 1.36^{d}	29.04± 2.86^{e}
***Btk* (µg/ml)** 0.5	0.259± 0.019^{b}	0.053± 0.005^{b}	41.94± 4.08^{b}	18.82± 1.80^{b}	44.89± 4.65^{bc}
1.5	0.202± 0.017^{bc}	0.034± 0.003^{c}	43.05± 4.02^{b}	15.27± 1.48^{c}	35.46± 3.85^{d}
3.0	0.170± 0.013^{c}	0.023± 0.002^{d}	44.54± 4.04^{b}	11.35± 1.30^{d}	26.32± 2.65^{c}
***M. esculenta* + AZA + *Btk*(2% + 0.25% + 1.50 µg/ml)**	0.123± 0.012^{d}	0.010± 0.001^{e}	45.61± 4.04^{a}	9.46± 0.98^{e}	20.75± 2.08^{c}

Within a column means followed by the same letter(s) are not significantly different at 1% level by DMRT.

(Table 7) shows the methanolic seed extract of *M. esculenta,* AZA, *Btk* and the synergistic effect of the nutritional indices of CI, RGR, AD, ECI, ECD against IV instar larvae of *E. vittella*. The highest concentration of *M. esculenta* (4%) exhibited CI=0.276 mg/day and RGR=0.064 mg/day, and AD, ECI, and ECD were calculated 57.71%, 23.76%, and 41.19%,

respectively. AZA (0.50%) investigated 0.241 and 0.052 mg/day, and AD=58.69%, 22.39%, and 38.12%, respectively. After the treatment of *Btk*(3 g/ml),CI=0.218 mg/day, RGR=0.044 mg/day, AD=59.28%, ECI=21.11% and ECD=35.62% and synergistic effects exhibited CI=0.095 mg/day, RGR=0.011 mg/day, AD=59.00%, 13.17%, and 22.34%, respectively.

Table 7: Effect of methanolic seed extract of *M. esculenta*, AZA and *Btk* on the nutritional indices of IV instars larvae of *E. vittella*

Treatment	CI (mg/ day)	RGR (mg/ day)	AD (%)	ECI (%)	ECD (%)
Control	0.490± 0.045^{a}	0.157± 0.012^{a}	54.19± 4.35^{c}	32.38± 2.98^{a}	59.76± 5.20^{a}
***M. esculenta* (%)** 1.0	0.359± 0.038^{a}	0.096± 0.009^{b}	55.07± 4.16^{c}	27.39± 2.05^{b}	49.74± 4.63^{b}
2.0	0.322± 0.033^{b}	0.080± 0.008^{b}	55.96± 4.52^{c}	25.29± 1.95^{b}	45.21± 4.51^{b}
4.0	0.276± 0.025^{b}	0.064± 0.007^{bc}	57.71± 5.02^{bc}	23.76± 1.68^{c}	41.19± 3.93^{c}
AZA (%) 0.10	0.319± 0.026^{b}	0.084± 0.009^{b}	55.98± 4.61^{c}	27.07± 2.00^{b}	48.34± 4.92^{b}
0.25	0.292± 0.032^{b}	0.071± 0.006^{b}	56.41± 4.83^{b}	24.92± 1.93^{bc}	44.19± 4.28^{bc}
0.50	0.241± 0.025^{bc}	0.052± 0.004^{c}	58.69± 5.28^{b}	22.39± 1.20^{c}	38.12± 3.96^{cd}
***Btk* (µg/ml)** 0.5	0.302± 0.022^{b}	0.080± 0.007^{b}	56.25± 4.62^{b}	26.37± 1.98^{b}	46.91± 4.69^{b}
1.5	0.261± 0.027^{b}	0.060± 0.003^{c}	57.07± 4.98^{c}	22.97± 1.30^{c}	40.25± 3.03^{c}
3.0	0.218± 0.016^{c}	0.044± 0.002^{c}	59.28±5.62^{b}	21.11± 1.02^{c}	35.62± 2.82^{d}
***M. esculenta* + AZA + *Btk*(2% + 0.25% + 1.50 µg/ml)**	0.095± 0.008^{d}	0.011± 0.001^{d}	59.00± 5.08^{b}	13.17± 0.92^{d}	22.34± 1.96^{e}

Within a column means followed by the same letter(s) are not significantly different at 1% level by DMRT.

The nutritional indices of V instars of *E. vittella* larvae after the treatment of *M. esculenta* + AZA + *Btk* are provided in **table 8**. The CI, RGR, AD, ECI and ECD of *M. esculenta* were 0.504 mg/day, 0.210 mg/day, 71.61%, 41.81% and 58.37%, respectively. Similarly, after treatment, AZA revealed that CI, RGR, AD, ECI and ECD were 0.378 mg/day, 0.141 mg/day, 72.00%, 37.39% and 51.92%, respectively. *Btk* was followed by 0.357 mg/day, 0.102 mg/day, 73.67%, 28.42%, and 38.56%, respectively, and synergistic effect showed that CI, RGR, AD, ECI, and ECD were 0.193 mg/day, 0.038 mg/day, 74.50%, 19.57%, and 26.27%, respectively.

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Table 8: Effect of methanolic seed extract of *M. esculenta*, AZA and *Btk* on the nutritional indices of V instars larvae of *E. vittella*

Treatment	CI (mg /day)	RGR (mg/day)	AD (%)	ECI (%)	ECD (%)
Control	0.623± 0.065^{a}	0.312± 0.029^{a}	66.41± 5.21^{b}	50.28± 4.03^{a}	75.72± 6.29^{a}
***M. esculenta* (%)** 1.0	0.601± 0.060^{a}	0.273± 0.026^{ab}	67.45± 5.87^{c}	45.75± 3.90^{ab}	67.84± 5.40^{b}
2.0	0.569± 0.057^{a}	0.237± 0.022^{b}	67.96± 5.95^{c}	41.61± 3.08^{b}	61.22± 5.06^{bc}
4.0	0.504± 0.049^{a}	0.210± 0.019^{c}	71.61± 6.58^{b}	41.81± 3.09^{b}	58.37± 4.42^{c}
AZA (%) 0.10	0.590± 0.059^{a}	0.247± 0.024^{b}	67.91± 5.94^{c}	43.34± 3.16^{b}	65.31± 5.27^{b}
0.25	0.412± 0.039^{b}	0.162± 0.015^{c}	68.07± 6.10^{bc}	38.95± 2.45^{c}	57.24± 4.36^{c}
0.50	0.378± 0.036^{b}	0.141± 0.013^{c}	72.00± 6.65^{b}	37.39± 2.36^{c}	51.92± 4.10^{c}
***Btk* (µg/ml)** 0.5	0.561± 0.056^{a}	0.222± 0.021^{b}	68.29± 6.21^{bc}	37.71± 2.38^{c}	55.20± 4.26^{c}
1.5	0.410± 0.039^{b}	0.119± 0.012^{cd}	69.52± 6.58^{b}	29.15± 1.46^{c}	41.94± 3.10^{d}
3.0	0.357± 0.034^{b}	0.102± 0.011^{d}	73.67± 6.88^{a}	28.42± 1.42^{c}	38.56± 2.43^{d}
***M. esculenta* + AZA + *Btk*(2% + 0.25% + 1.50 µg/ml)**	0.193± 0.018^{c}	0.038± 0.009^{e}	74.50± 7.01^{a}	19.57± 0.95^{d}	26.27± 1.31^{e}

Within a column means followed by the same letter(s) are not significantly different at 1% level by DMRT.

The efficiency of faecal pellet egestion of IV instar larvae of *E. vittella* fed along with methanolic seed extract of *M. esculenta*, AZA, and *Btk* is provided in **table 9**. The control larvae egested out the first fecal pellet at 16 minutes. It ejected 16 fecal pellets in total over the course of about 149 minutes. A delayed first faecal pellet egestion of approximately 25 minutes was observed in larvae fed *M. esculenta* dosage. It has caused a highly significant reduction in the total number of faecal pellets ejected to about 10 numbers. To defecate its 10th pellet took longer time duration of about 240 minutes when compared to control. The AZA dosage evoked a significant 50% reduction in the total faecal pellets when

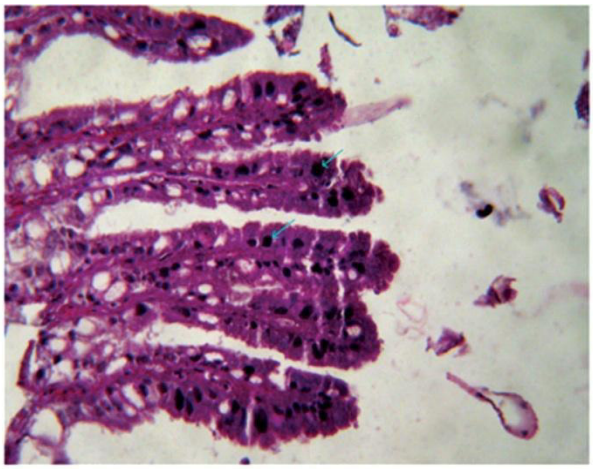
compared to control. Furthermore, it took more time for it to egest out its 8th pellet, about 268 minutes. The treatment with *Btk* resulted in a significant reduction in the total number of faecal pellets to about 8, as well as an increase in time duration to about 228 minutes. The synergistic combination evoked a highly significant reduction in the total number of faecal pellets, 4 numbers, with an increase in defecation time duration of about 160 minutes.

Table 9: Efficiency of faecal pellet egestion of IV instars larvae of *E. vittella* feed along with methanolic seed extract of *M. esculenta,* AZA and *Btk*

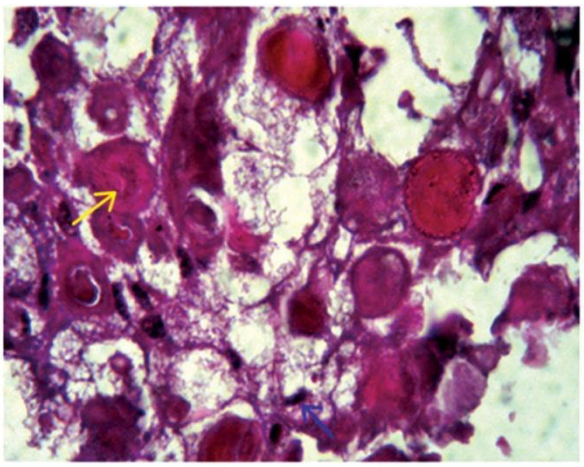
No. of faecal pellets	Time (Minutes)								
	Control	*M. esculenta*		AZA		Btk		Synergistic Combination	
		2%	4%	0.25%	0.50%	1.5 µg/ml	3.0 µg/ml	2% + 0.25% + 1.50 µg/ml	4% + 0.50% + 3.00 µg/ml
1	16	22	25	27	32	29	35	32	40
2	23	32	36	40	49	44	54	52	65
3	32	45	49	58	62	62	68	78	100
4	41	61	66	81	91	86	98	119	160
5	49	78	88	107	124	113	132	160	-
6	58	100	113	142	167	150	177	212	-
7	68	126	143	183	216	193	228	-	-
8	75	154	172	232	268	244	-	-	-
9	81	185	203	250	-	-	-	-	-
10	89	220	240	-	-	-	-	-	-
11	97	261	-	-	-	-	-	-	-
12	107	307	-	-	-	-	-	-	-
13	115	-	-	-	-	-	-	-	-
14	125	-	-	-	-	-	-	-	-
15	136	-	-	-	-	-	-	-	-
16	149	-	-	-	-	-	-	-	-

Histopathological changes in the control IV instar larvae are shown in **Figure 2**. In the control IV instar larvae, showing normal architecture of midgut epithelial cells (columnar cells), depicting normal cytoplasm and nucleus **(Figure 2a)**. *M. esculenta*(4%)treatment resulted in vacuolar changes in the cytoplasm and pyknotic changes in the nucleus **(Figure 2b)**.In the AZA treated IV instar larvae, midgut epithelial cells show necrotic changes in pyknosis and chromatolysis of the nucleus **(Figure 2c)**. The IV instar larvae treated with *Btk* (3.0g/ml) show sloughing of epithelial cells leaving the basement membrane.In the sloughed cells, there was severe necrosis and sloughing of epithelial cells, lysis of the plasma membrane, and breakdown of the nuclear membrane **(Figure 2d and 2e)**.In the synergistic combination (*M. esculenta*, AZA and *Btk* at 4% + 0.50% + 3.0 mg/ml), treated IV instar larvae showed severe necrosis and sloughing of midgut epithelial cells, and accumulation of sloughed cells in the lumen was noticed **(figure 2f)**.

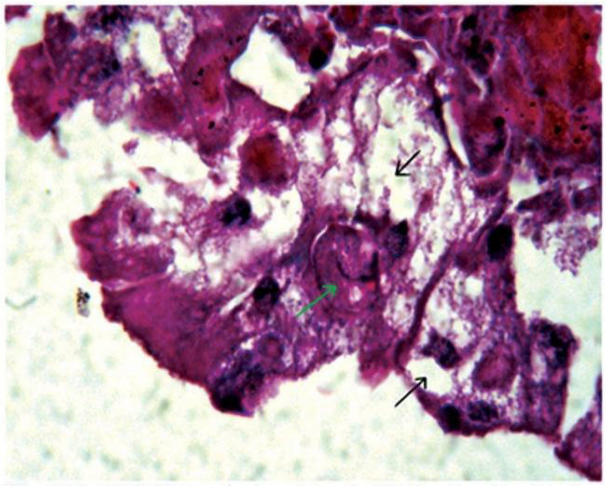
Figure 2.Histopathalogical changes in IV instar larvae of *E. vittella*



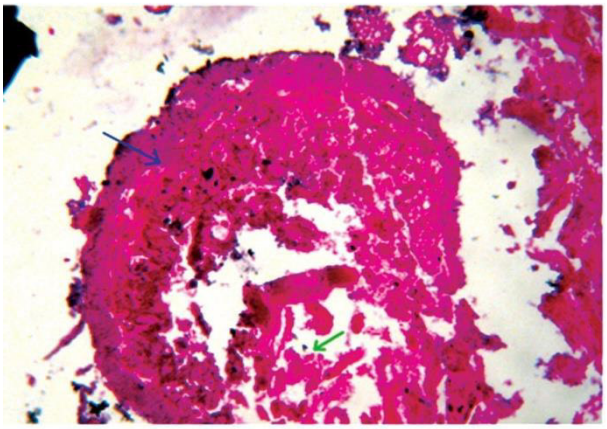
2a. Control - midgut of IV instar *E. vittella.* Normal architecture of midgut epithelial cells depicting normal cytoplasm and nucleus (Arrow) H & E 400 X



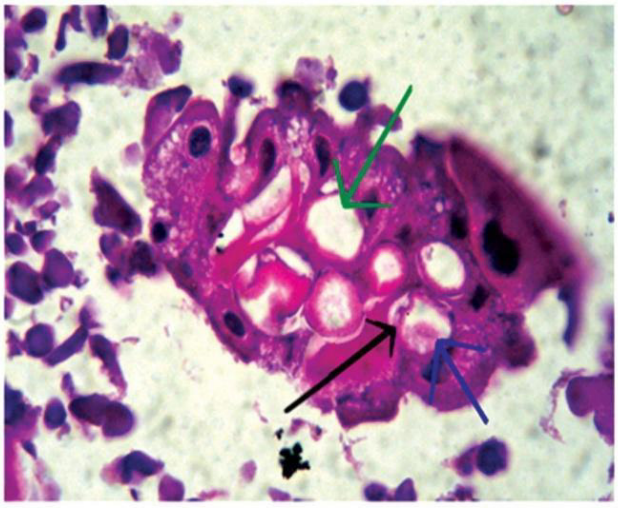
2b. *M. esculenta* treated midgut of IV instar *E. Vittella.* Midgut epithelial cells depicting necrotic changes of pyknosis (Blue Arrow) and chromatolysis of nucleus (Yellow Arrow) H & E 1000 X



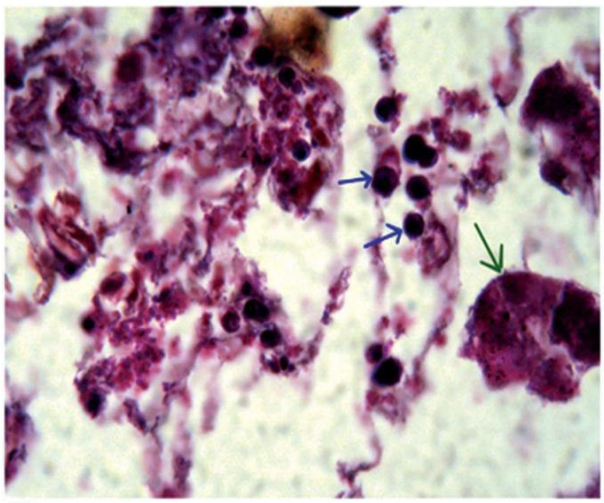
2c. AZA (0.50%) treated - midgut of IV instar *E. vittella.* Midgut epithelial cells showing vacuolar changes (Black Arrow) in the cytoplasm and chromatolysis of nucleus (Necrosis) (Green Arrow) H & E 1000 X



2d. *Btk* treated midgut of IV instar *E. vittella.* Complete necrosis of epithelial cells (Blue Arrow) and accumulation of cell debris in the lumen (Green Arrow) H & E 400 X



2e. *Btk* treated - midgut of IV instar *E. vittella.* Sloughed epithelial cells showing necrotic changes of chromatolysis (Blue Arrow), breakage of plasma membrane (Black Arrow) and megavaccuolation of cytoplasm (Green Arrow) H & E 1000X



2f. Synergistic combination treated-midgut of IV instar *E. Vittella.* Destruction of midgut epithelial cells, sloughing (Green Arrow) and infiltration by macrophages (Blue Arrow) H & E 1000 X

DISCUSSION

Biopesticides may serve as suitable alternatives to chemical insecticides in the future as they are relatively safe, inexpensive and available everywhere in the world. This work demonstrates the potency *of M. esculenta*, AZA and *Btk* as an effective larvicide against *E. vittella* larvae. For instance, Shanmugapriyan and Kingsly[28] reported that neem oil at 1. 23% in III instar larvae of the gourd beetle *Epilachna vigintioctopunctata*. Murugesan[29] studied the effect of Neem oil on the *Semilooper* caterpillar, *Achaeaj anata*, infesting castor plants. A lot of evidence is available on the insect growth regulator activity of neem, where treatment with neem products caused physiological effects like reduction, delay, or absence of ecdysone and juvenile hormones during the last larval instars and nymphal periods.[30] *Azadirachtin* probably has more than one site of action, depending on the time and growth stage of test insects.[31] Several larvae displayed morphological deformities, bloated abdomens, and larva-pupa intermediates.[32] In the present study, during development, larvae also lost their body weight rapidly due to excessive defecation and feeding inhibition, and transformed into small, shriveled pupae. In many cases, larvae were unable to produce silken cocoons and transformed into naked pupae. Nearly 6 components namely (1) Glycerin, (2) 2-Undecanol, (3)3-Trifluoroacetyl Pentadecane, (4) 2-Acetylamino-3-hydroxy propionic acid, (5) 1, 2-Benzenedicarboxylic acid, diisooctyl ester (6) 1-Monalinaleoylglyceral trimethylsilyl ether possess Antimicrobial activities. Among the 18 components identified 2 components namely, (1) Linoleic acid ethyl ester, (2) 9, 12-Octadecadienoic acid, (Z, Z)-Phenylmethylester have nematicidal activities. Further we identified the presence of nonanoic acid, a potent herbicide in our study material. The activities of identified components were given in table-1. Similarly, Ruba *et al.*[33] reported that GCMS analysis of *Andrographis paniculata* 20 phytochemical compounds were identified especially, Benzene,1,2,3-trimethoxy-5-(2-propenyl)-, Cyclopenta[c] pyran-4-carboxylic acid, 7-methyl-, methyl ester and 9,12,15- Octadecatrienoic acid, methyl ester, (Z, Z, Z)- covered the high areas that might be responsible or can possess the antifungal activity against Tuber root rot causing fungal pathogens. Also Benoite *et al.*[34] reported that *Delonix regia* flower extract as a potential source of bioactive phytochemicals and can be used as a plant-based antioxidant, antidiabetic, anti-inflammatory and anticancer agent. The finding that the use of higher concentrations of *Btk* achieved 87.65% larval mortality after application shows that much higher concentrations of *Btk*. *Bacillus thuringiensis* (*Bt*) is a required in group of strains or isolates of naturally occurring soil bacteria which mainly exerts toxicity through the production of Crystal (Cry) toxins. Cry and Cyt toxins from Bt include pore formation in which Bt toxins induce cell death by forming ionic pores following insertion into the membrane, causing osmotic lysis of midgut epithelial cells in their target insects.[35-40] This may be the reason for the mortality seen in our investigation. the effect of Btk due to Cyt 1Aa is also reported to be toxic in coleoptera larvae *Chrysomela scripta*[41] and Cyt1C to the coleopteran pests *Leptinotarsa decemlineata*, *Tribolium castaneum*, and *Diabrotica* spp.[42] In the present study, the food utilization parameters of *E. vittella* were affected by the treatment of methanolic seed extract of *M. esculenta*, AZA and *Btk*, in a dose-dependent manner and that the efficiency measures were highly correlated with the gut enzymes activity of *E. vittella*. Instars fed with *M. esculenta*, AZA and *Btk* coated leaves pods consumed less food and were less efficient in converting the ingested and digested food into biomass compared to the controls. These results are similar to those reported for the larvae of the European corn borer, *Ostinia nublalis* and for the tobacco budworm, *Heliothis virescens*. Therefore, expenditure of a major fraction of the assimilated energy on metabolism for neutralization of the toxins has apparently lowered the conversion efficiency of the pests.[43,44] The reduced relative growth rate of the treated insects indicates that the *M. esculenta*, AZA, and *Btk* treatments have affected some physiological parameters of utilization. The CI and RGR decrease with increasing concentrations, as would be expected from the involvement of chemoreceptors.[45,46] The reduced ECI and ECD result from a reduction in the efficiency of converting food into growth, perhaps by a diversion of energy from the production of biomass into detoxification. The prolongation of food in the gut may increase exposure to digestive enzymes. Increases in Approximate Digestibility (AD) following treatment of larvae with AZA, as observed here, have been reported for the cabbage webworm *Crocidolomia binotalis* and *Heliothis armigera*.[47] It is reasonable to associate such increases in AD with an attempt by the treated insects to compensate for their reduced food consumption and utilization efficiencies to meet their meat body requirements and maintain growth and developmental processes.[48,49] In the present study, egestion of an increasing number of faecal pellets in control insects of *E. vittella* may be due to increased feeding and an increased rate of passage of food through the gut.[50] Hence, in this study, the slow rate of feeding and decreased rate of passage of food through the gut was due to the treatment, suggested that the higher level of accumulation of allelochemicals in the gut which might obstruct the digestive physiology was reflected in the reduced feeding activity and lower faecal pellet production on the *M. esculenta*, AZA and *Btk* treated slices of Bhendi fruits. In the present study, *M. esculenta*, AZA, and *Btk* evoked significant histopathological changes in the midgut of the IV instar form of *E. vittella*. After ingestion of *M. esculenta* AZA and *Btk*, the active toxin is known to bind to and destroy the midgut epithelium, resulting in rapid gut paralysis, which causes the larva to stop feeding within hours in the most sensitive species.[51] *Btk*-affected larvae die from starvation, which may take several days. Since *Btk* does not kill rapidly, users may incorrectly assume that it is ineffective if treatments are assessed a day or two after application. Similarly, Peric Mataruga *et al.*[52] reported that *R. pseudoacacia* leaves in gypsy moth caused vacuolization of cytoplasm, elongation of the columnar cells and partial loss of microvilli and also the nuclei were smaller than the control ones, which suggest the presence of degenerative processes in this cell group. Nasiruddin and Mordue[53] reported histological changes caused by AZA in the midgut of locusts. They revealed the necrosis of epithelial cells, enlargement of cytoplasmic inclusions, and small size of striated borders. Abo EL-Ghar *et al.*[54] provided histological effects of abamectin on the midgut of *Spodoptera littoralis,* resulting in shredding and erosion of the lining epithelium.

CONCLUSION

Overall, the study concluded that larvicidal, digestive indices and histopathology properties are achieved by our bio-pesticide methanolic seed extract of *M. esculenta.* Further study with the purified active principle from the methanolic seed extract of *M. esculenta* may shed light on its role as a biopesticide. When compared to synthetic pesticides, our experimental materials, *Manihot esculenta*, AZA, and *BTK,* are

very effective and eco-friendly. Botanical insecticides are harmless to mammals and other non-target animals.

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CONFLICT OF INTEREST

Conflict of interest declared none.

AUTHORS CONTRIBUTION STATEMENT

The corresponding author and third author of the research article contributed to experimental study, data acquisition, data analysis, statistical analysis, and manuscript preparation. The first author contributed to the concept and design of the manuscript. The second author contributed to the literature collection and the definition of intellectual content.

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