ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Pentacyclic Triterpenoids and a Linear Alkane from the Milky Mangrove Tree (*Excoecaria agallocha* L.) are toxic to the larva of *Helicoverpa armigera* Hubner. (Lepidoptera: Noctuidae)

Satyan Ramachandran Santhanam[*2], Malarvannan Subramanian[1], Meseret C. Egigu[2] and Ajay Parida[1]

1 Tissue Culture & Bioprospecting Laboratory, M. S. Swaminathan Research Foundation, 3rd Cross Street, Taramani, Chennai - 600 113. India.

2 Department of Biology, College of Natural & Computational Sciences (CNCS), Haramaya University, P O Box: 138, Dire Dawa, Ethiopia (East Africa).

Manuscript Info

Manuscript History:

Received: 12 April 2014
Final Accepted: 22 May 2014
Published Online: June 2014

Key words:
E. agallocha, H. armigera, n-hentriacontane, taraxerone, taraxerol and midgut.

Corresponding Author

Satyan Ramachandran Santhanam

Abstract

Throughout the world, as a part of the Integrated Pest Management (IPM), botanicals are evaluated for its efficacy against the polyphagous Pink Bollworm *Helicoverpa armigera* Hubner. In our present study, hexane extract of the dry leaves of the milky mangrove tree *Excoecaria agallocha* L. exhibited dose-dependent growth inhibition of late 2nd & early 3rd instars larvae of *H. armigera* under in vitro conditions. 0.1% of crude hexane extract of *E. agallocha* leaf caused 50% mortality of early 3rd instars larvae of *H. armigera* (LC_{50}-0.0001g/kg body weight), compared to synthetic pesticide Ekalux® (90%) & the solvent control (12%). 2% extract dosage reduced the healthy adult moth emergence to 2% compared to Neem® control (14%). Out of the 7 column fractions (SiO_2 60-120 mesh, Eluent: Petroleum Ether: Ethyl Acetate) tested, fractions 1, 4 & 5 suggested growth inhibition (75, 90 & 94%). The bioactive fractions were characterized using FT-IR, NMR, Mass Spectrum & X-ray Crystallography as known linear alkane n-Hentriacontane (fraction-1), pentacyclic triterpenoids Taraxerone (fraction-4) & Taraxerol (fraction-5). Purified n-hentriacontane, exhibited 80% mortality with a reduced larval weight (280 mg) compared to crystalline taraxerone (75%, 151.7 mg), while taraxerol exhibited 80% larval mortality (Mean larvae weight: 64 mg), compared to the controls (416.5 mg on average). Transverse Sections (T.S.) of the midgut of *H. armigera* larvae treated with the bioactive fractions suggested drastic shrinkage & lysis of the gut wall, leakage of exocrine secretions & the micronuclei. Further studies would suggest the possibility of formulating a potential phytochemical pesticide from *E. agallocha* against lepidopteron pests.

INTRODUCTION

The American bollworm, *Helicoverpa armigera* (Hubner.) (Lepidoptera: Noctuidae) is a polyphagous pest that is widely distributed in Europe, Africa, Asia and South-Pacific regions. Larvae of *H. armigera* are voracious foliar feeders as early instars and later shift to the developing seeds, fruits, or bolls, leading to drastic reduction in yield in Pigeon Pea (Reed and Pawar, 1982). It has a wide host range of 360 plant species including crop plants such as cotton, maize, sorghum, sunflower, tomato, okra and legumes (Singh and Singh, 1996). In a wider global scenario,

its major outbreaks were on cotton in Virgin islands, America (Wilson, 1923) which busted into prominence as a major threat to cotton in South America, particularly Peru (Wille, 1940; Hambleton, 1994), Chick pea in Uganda (Ogengalatigo et al., 1994), sorghum and tobacco in Queensland (Jallow and Zalucki, 1996), grapevine in Hungary (Voros, 1996), maize in Argentina (Hamity et al., 1998), apples and tobacco in China (Li et al., 1998; Yi, 1998), beans in Malawi (Ross, 1998) and lettuce in Japan (Kashiyama et al., 1999). In India, heavy loss was observed during 1983-84 in Punjab, 1987- 88 in Andhra Pradesh and 1989- 90 in Tamil Nadu and Karnataka (Fitt, 1989; Guo, 1997). Annual yield loss attributed to this pest in India is around one million Indian Rupees (INR). Chemical control of *H. armigera* has become less feasible due to the development of pesticide resistance in insects and inherent possible environmental hazards when used at high doses (Armes et al., 1996).

An array of systemic, contact and broad-spectrum synthetic pesticides and other formulations were used to control *H. armigera*. Chloropyriphos, phosalone and malathion effectively reduced the pest incidence (Biradar et al., 2001). Among the group of carbamate and pyrethroid insecticides tested against 3rd instars larva of *H. armigera* in cotton under in vitro conditions, LORSBAN® 40 EC showed maximum efficacy of 100% mortality followed by LARVIN® 80 DF (72%) and LANNATE® 40 SP (52%) (Zahid and Hamed, 2003). Indoxacarb, an artificially synthesized pyrazoline from DUPONT®, under the trade names STEWARD® and AVAUNT® has been having a real impact on the lives of farmers (Lahm, 2004).

Despite such a strict regimen *H. armigera* has developed several fold resistance to several insecticides in almost all the countries. These include endosulphan, thiodicarb and methomyl (Gunning et al., 1994) in Australia; cyhalothrin, cypermethrin, methomyl, monocrotophos and phoxin (Rui et al., 1999) in China; cyfluthrin, alpha-cypermethrin, zeta-cypermethrin, lambda-cyhalothrin and bifenthrin (Ahmad, et al., 1997a); monocrotophos, ethion, chloropyrifos and profenphos (Ahmad, et al., 1997b) in Pakistan; and fenvalerate in Thailand (Burikam, et al., 1998). In India, *H. armigera* has shown resistance to cypermethrin (Kranthi and Kherde, 1998), Endosulphan, monocrotophos, phosalone, fenvalerate and deltamethrin (Manikandan, 1998). The unscrupulous use of synthetic chemical pesticides has created an alarming condition that includes the genetic resistance of pests, toxic residual poisoning in food produce, expensive application costs and handling hazards etc. In the context of increasing environmental awareness and due to the residual poisoning of the synthetic pesticides, there is a need for alternative pest control strategies that are eco-friendly, viable and biologically safe.

Among the phytochemicals, neem seed extract have been widely used for effective control of *H. armigera* (Neoliya et al., 2005; Sahayaraj and Amalraj, 2006). Foliar and twig extracts of three species of the genus Aglaia showed less feeding and growth impairment in *H. armigera* (Koul et al., 1997; Satasook et al., 1994). Ethyl acetate extract of *Delphinium cultorum* (Miles et al., 2000), *Artemisia annua* and *Ageratum conyzoides* displayed antifeedant activity, larval mortality and failure of normal adult emergence in *H. armigera* (Padmaja and Rao, 2000). Among several leaf extracts i.e., *Achyranthes aspera, Acorus calamus, Chrysanthemum cinerariefolium, Derris elliptica, D. alba, Annona squamosa* tested, 60% antifeedant activity was observed in *A. squamosa* (Singh et al., 2001).

Among mangroves, plants of Excoecaria genus (Family: Euphorbiaceae) comprise nearly 40 species that are distributed throughout tropical Asia, Africa and northwest Australia (Wiriyachitra et al., 1985). Chemical examination of the air-dried stem wood yielded a novel piperidine alkaloid (Prakash et al., 1983). A novel phorbol ester was isolated as the anti-HIV principle of the leaves and stems (Erickson et al., 1995). Excoecarins M and N were purified and confirmed by X-ray crystallography (Konishi et al., 2000). Chemical examination of the hexane extract of the roots of *E. agallocha* resulted in the identification of eleven diterpenoids of which five (Agallochins A-E) were novel (Anjaneyulu and Rao, 2000). Two novel ent-isopimarane diterpenoids (Agallochaols A and B) were characterized from the dried stems and leaves (Wang and Guo, 2004). Nevertheless, none of the reports discussed the insecticidal property of these di- and triterpenoids. Thus, the present study is focused on the extraction & characterization of bioactive terpenoids from the dried leaves of *E. agallocha* that are inhibitory to the larvae of *H. armigera.*

1. Materials and Methods

2.1 Collection and maintenance of *H. armigera*

Third and Fourth instars larvae of *H. armigera* were collected in plastic vials from Lady's finger (*Abelmoschus esculentus* L.) fields in Kancheepuram district (Tamil Nadu, India). They were brought to the laboratory within 2 hrs

and reared in semi-synthetic diet, as described by Shorey and Hale (1965) for 3 generations. The semi-synthetic diet consisted of Chick pea (dried powder)- 100 g, brewer's yeast- 30 g, methyl-4-hydroxy benzoate- 2 g, ascorbic acid- 3.2 g, sorbic acid- 1 g, 40% formaldehyde- 1 ml, ABDEC Vitamin solution- 10 ml, Streptomycin Sulphate- 0.2 g, Agar- 12.8 g & distilled water- 750 ml. 100 g of chick pea powder and 12.8 g of agar were boiled in separate vessels, with 360 ml of distilled water. The chick pea paste was blended thoroughly with 30 g of yeast and 2 g of methyl-4-hydroxy benzoate. The boiled agar was added to this mixture and blended for 2- 3 minutes. Finally, the remaining ingredients were added, blended well and poured into small plastic vials (4.5 cm diameter) with screw caps. The cap was bored with a needle for air circulation. Approximately, 10 ml of the diet was added to plastic vials & cooled for 30 min. The larvae (1 larva/ vial) were reared under captivity (22±2°C, 70-75% Relative Humidity). Fresh diet was replaced intermittently to maintain a regular supply & minimize microbial infection.

After pupation, the pupa were cleaned in running water and gently rolled over cotton. They were air dried and placed in petridishes of 10 cm diameter that was kept inside the adult emergence cage. The emergence of moths was recorded daily. The newly emerged adults were paired adopting a sex ratio of 1:1 and released for mating and oviposition. The oviposition cage consists of a small mud pot (12 x 17 cm diameter) covered with sterile wet muslin cloth. A cotton swab dipped in 10% sugar solution was kept in a small petridish (3 cm diameter) over a glass vial that served as adult feed. Fresh lady's finger (Variety: *Arka anamika*) fruits were placed inside the pot to facilitate oviposition. After releasing the adults the mud pot was covered with the black muslin and was tightened by a rubber band. The cage was kept in a dark place to enhance proper mating. Adult food was changed daily and the whole set up was maintained at 22±2 °C and 70-75% relative humidity respectively.

The female adults laid fertile eggs on the muslin cloth and okra from 2^{nd} to 13^{th} day after mating. The egg cloths were collected daily and fresh ones were laid for subsequent egg collection. The collected egg cloths were preserved inside a plastic box (12x9cm) and checked daily for hatching. The newly hatched larvae were transferred with a camel hairbrush to semi-synthetic diet in plastic boxes (9 cm diameter) and reared until the 2^{nd} instar stage. To prevent cannibalism, they were transferred into vials (1 larva/ vial) and reared until pupation. The pupae were washed in a sieve, dried in tissue paper and kept inside the adult emergence cage. Deformed and dead moths were discarded. Healthy moths were utilized for mating to yield the next generation. The 3^{rd} generation larvae were utilized for the bioassays.

2.2 Plant material collection & Extraction

Fresh *E. agallocha* L. plant material (100kg) was collected from the Pichavaram mangroves Chidambaram district (Tamil Nadu, India). The plants were identified by Dr. P. Eganathan (Botanist, M. S. Swaminathan Research Foundation-MSSRF, Chennai, India). A voucher specimen of the whole plant (E.A/09/03) was deposited at the herbarium in (MSSRF). Shade dried leaves (Temperature: 41°C, Relative Humidity: 76%) of *E. agallocha* were pulverized in an electronic mixer. 10 kg of the dried powder was and distributed in 5L conical flasks and soaked in hexane (25 L each) at room temperature for 3 days. The process was repeated thrice to get the maximum extractable. The extract was condensed in a rotary evaporator (Model: Buchi® R-200) to a viscous paste (Yield: 120.03g/ 1000g powder) and refrigerated until use.

2.3 Bioassay with the crude extract

0.1, 0.2, 0.5, 1 & 2% of crude hexane extract were separately emulsified in 250-300 mg of triton X-100 & solubilized in methanol. The extract was added to the semi-synthetic diet, distributed in plastic vials & cooled before the release of the larvae. Late 2^{nd} or early 3^{rd} instars of *H. armigera* were exposed to the treatments (5 replications of 20 larvae/ rep.). Larval mortality, pupation, adult moth emergence were recorded regularly. Standard synthetic insecticides EKALUX® (0.02%), natural insecticide VIJAY NEEM® (3%) were used as positive controls. Methanol (negative control) and diet (blank) were also parallel maintained. The experimental vials were kept in plastic tubs (1 tub/ treatment) and were arranged in wooden racks inside the laboratory (Temperature: 27°C, shaded condition) (Balaji et al., 2007)

2.4 Column fractionation & Bioassay

15 g of crude hexane extract was dissolved in 12.1 ml of hexane. 65 g of SiO_2 (60-120 mesh, 1: 4) was added to this solution to prepare the admixture. The admixture was dried in the air draft overnight for binding. SiO_2 (1.8 kg, 60-

120 mesh) was slurried with petroleum ether and packed for 12 hrs to form a column of fractionation (bed length- 50 cm, column diameter- 5.5 cm). Elution of mixtures was done using petroleum ether and ethyl acetate mixtures in 9.8:0.2, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7:3, 6:4 and 5:5 (v/v) ratios to yield 7 fractions (1:1.43 g, 2: 2.7 g, 3: 1.9 g, 4: 3.4 g, 5: 1.4 g, 6: 2.2 g, 7: 0.7 g) according to their thin-layer chromatographic (SiO_2 230-400 mesh with F_{254}, MERCK®) profiles. The fractions were transferred to pre-weighed glass vials, dried and yield details were recorded. TLC profile was standardized for the fractions using petroleum ether: ethyl acetate (7:3, v/v), recorded under UV (254 and 366 nm) and terpenoid-specific spray reagents i.e., 2% MeOH- H_2SO_4 (2 ml MeOH in 98 ml H_2SO_4) & anisaldehyde-H_2SO_4 (1 ml conc. H_2SO_4 in 50 ml acetic acid containing 0.5 ml anisaldehyde reagent) were used to detect the presence of respective compound classes. Each of the 7 fractions was dissolved in methanol to get 0.5% (w/v) solution. Thereafter, 100µl of each fraction was coated on the surface of the larval diet and dried. The diet was then offered to late 2nd or early 3rd instars of *H. armigera* (50 nos., 5 replications of 10 larvae/ rep.) in plastic vials. Observations were made as per the previous assay procedure (Section: 2.3)

2.5 Purification of bioactive fractions & bioassay with compounds

Fractions 1, 4 & 5 were found to be inhibitory to *H. armigera* larvae. Hence, they were separately subjected to purification.

Fraction 1: 1.43 g of the waxy fraction was dissolved in 1.15 ml of hexane. 6 g of SiO_2 (60-120 mesh, 1: 4) was added to this solution to prepare the admixture. The admixture was dried in the air draft overnight for binding. SiO_2 (170 g, 60-120 mesh) was slurried with petroleum ether and packed for 12 hrs to form a column of fractionation. Elution was done using petroleum ether & ethyl acetate (9.5:0.5 v/v) ratio. TLC was repeated & similar fractions were pooled & dried.

Fractions 4: 3.4 g of the amorphous fraction was subjected to column chromatography in SiO2 (60-120 mesh, 13.5 g) with mobile phase petroleum ether: ethyl acetate (8:2) to yield colorless crystals (slates) that were recrystallized by the procedure mentioned below.

Fraction 5: 1.4 g of the semi-crystalline fraction was purified in a preparatory plate (SiO2 230-400 mesh), using petroleum ether: ethyl acetate (8:2) as the mobile phase

Crystal growth of fractions 4 & 5

Crystals were separately washed for 5 times with methanol and the final crop was dried on a glass slide. 10 mg of each type of crystal was added to thin glass tubes (7 cm length, 4 mm diameter) and supersaturated with hexane. 2 drops of methanol is added to the sample and kept undisturbed at 20°C for 2 weeks. The resulting crop yielded pure single crystals that were analyzed by X-ray Diffraction Crystallography (XRD). Bioassay with compounds was performed as per Section 2.4. Periodic (day: 1, 3, 6, 9 & 12) changes in the length & weight of the existing larvae were measured & tabulated.

2.6 Histopathological studies of *H. armigera* larvae

Morbid late 2nd or early 3rd instar larvae treated with fractions 1-5 (10 larvae/ fraction treatment) of *H. armigera* were fixed in Bouin's fluid for 24 hr & washed in distilled water. Then, they were successively transferred to ethyl alcohol solutions of different strength (50, 60, 70, 80, 90 and 100%) for 4 hrs and were cleaned with xylol for 8 hrs. The larvae were then embedded in wax and sectioned (transverse) into 8-9 µm thickness. The larval specimens were mounted on microscopic slides, dewaxed in xylol for 5 min., hydrated in a series of concentration of ethyl alcohol & finally cleaned with distilled water. The sections were stained with Ehrlich's hematoxylin for 15 min followed by acid-alcohol (hydrochloric acid in 50% alcohol) destaining. Then the slides were washed & the tissues were dehydrated with alcohol. Finally, a few drops of dilute eosin were added to 100% alcohol for counter staining. The sections were again treated with xylol and mounted with Canada balsam for microscopic sectioning.

2.6 Analysis of compounds

Composition of purified compounds 1, 4 & 5 were determined using advanced analytical instruments at Sophisticated Analytical Instrumentation Facility (SAIF), Indian Institute Of Technology (IIT- Chennai, India). For

the amorphous bioactive compound-1 Infra Red (IR) spectra was recorded at 400-4000 cm^{-1} using KBr pellet and analyzed in FT-IR spectrophotometer (SHIMADZU®, Japan). ^{1}H and ^{13}C NMR spectra of the bioactive compounds were recorded at 400 and 100.4 MHz in $CDCl_3$ using FT-NMR spectrophotometer (JEOL®GSX 400 NB). A combined ^{1}H and ^{13}C probe is used for analysis. Ei-Mass Spectra for the bioactive compounds were recorded using JEOL®DX 303 Mass Spectrometer (FI). For crystalline compounds 3 & 4, intensity data for X-ray structure analysis were collected using ENRAF NONIUS® CAD4-MV31 and Bruker® Kappa APEX II Single Crystal Diffractometer.

2.7 Statistical Analysis

The results were statistically analyzed using AGRES package version 4. Critical Difference (CD) with single analysis of variance was performed. In addition, the similarity was checked using Least Standard Deviation (LSD)

3. Results

3.1 Toxic effect of hexane extract & fractionates on *H. armigera* pupation and moth emergence

Crude hexane extract of *E. agallocha* leaf exhibited a dose-dependent growth inhibition of H. armigera larvae. 0.1% dosage arrested 50% of the larval population leading to 18% adult emergence (Table 1.). Deformed moths exhibited thorax shrinkage, limb and wing deformities. Of the 20% of the pupae that survived the 2% extract treatment, only 2% emerged into adult moths. Affected pupae were black in color with crushed appearance that led to the leakage of hemolymph. Larvae reared in hexane (control) showed 88% pupation proceeding to 78% successful moth emergence, while EKALUX® control showed 0%. Diet control showed maximum emergence of healthy adults (86%).

LC_{50} dose: The LC_{50} value of crude hexane extract was 0.1%. Hence, the value based on 1 kg (1000 mg) body weight of *H. armigera* larvae was fixed as 0.0001g/ kg body weight i.e., (0.1/1000 = 0.0001 g). Visual observations done during the first day of the bioassay revealed the avoidance of the feed fortified with 0.5, 1 and 2% of hexane extract.

Out of the 7 fractions assayed, fractions 1, 4 & 5 suggested drastic reduction in the growth of *H. armigera* (Table 2.). Only 5% of the adult moths emerged healthy after treating them with fraction-1.Whereas, fractions 4 & 5 reduced the moth emergence to 2%. Fractions 6 & 7 did not influence the growth of *H. armigera* larvae. Larvae reared in the diet control were healthy leading to 85% moth emergence. Ekalux® proved to be the best among the treatments curbing 97% of the larval population.

3.2 Characterization of bioactive compounds

n-Hentriacontane (Fraction-1)

Pet. ether: EtOAc (9.8:0.2) (Fig. 1) was a white semi-solid compound with a melting point at 67-68ºC. This semisolid wax was characterized by ^{1}H NMR, ^{13}C NMR, IR and FAB Mass spectrometry techniques as an acyclic hydrocarbon with molecular formula $C_{31}H_{64}$. FAB mass spectrum showed a peak at m/z 436 [M^+] (6) corresponds to the molecular formula $C_{31}H_{64}$. Other peaks were observed at m/z 407 (M^+-C_2H_5) and 393, 379, 365, 351, 337, 323, 309, 295, 281, 267, 253, 239, 225, 211, 197, 183, 169, 155, 141, 127, 113, 99, 85, 71 are due to the consecutive loss of CH_2. Base peak was observed at m/z 57 (100) is due to $C_4H_9^+$ and also m/z 43 (65) for $C_3H_7^+$. Absence of characteristic absorption band in IR spectrum indicated that no functional group was present in the hydrocarbon system. The ^{1}HNMR ($CDCl_3$, 400 MHz) showed three proton triplets at δ 0.88 (J = 7.2 Hz) and 0.85 (J = 7.1 Hz) ppm assigned to C-1 and C-31 primary methyl protons. A broad signal at δ 1.25 ppm associated with the rest of the methylene protons. The signal at δ 14.1 ppm in ^{13}C-NMR ($CDCl_3$, 100 MHz) assigned for methyl carbons (C-1 and C-31) and signals that are in the region δ 19.7 - 37.1 ppm corresponded to the rest of methylene carbons. Based on these spectral evidences the isolated fraction was characterized as n-Hentriacontane, a linear alkane found in plant waxes.

Taraxerone & Taraxerol (Fractions 4 & 5)

Both the crystals (colorless slates & needles) were analyzed by X-ray crystallography. Comparison of the unit cell parameters and the ORTEP image with the Cambridge Crystallographic Database Center (CCDC) suggested that the compounds were pentacyclic triterpenoids Taraxerone (colorless slates) & Taraxerol (colorless needles). IUPAC nomenclatures for the identified compounds are mentioned (Fig. 2 & 3)

3.3 Effect of n-Hentriacontane, Taraxerone & Taraxerol on *H. armigera* larvae

Triterpenoid taraxerone caused 50% mortality on 14th day of treatment while Taraxerol showed gradual mortality of the larval population to 78% at the end of 15th day. Hexane solvent control exhibited 5% mortality on the 6th day. From the 7th day onwards mortality was not observed among the larval population. 95% of the larvae reared in the diet control were healthy up till the 15th day of experiment. EKALUX® was the most effective among all the treatments, causing 100% mortality on the 12th day of the bioassay. Apart from mortality, significant observations were seen in the weight of the live larvae on 3rd, 6th, 9th and 12th day. On the 3rd day of the assay, triterpenoid Taraxerone effectively reduced the weight of the larvae (8.3 mg) when compared with other fractions. Whereas on the 6th day, the increase in weight of the larvae in n-Hentriacontane treatment came down to 24.3 mg. Taraxerone exerted similar activity on the larvae. Diet and hexane control augmented the larval weight to 56.1 mg and 52.3 mg. Hexane and diet control showed more than 3-fold (75 mg to 241.5 mg on average) increase that was comparable to the normal diet control. Larvae treated with EKALUX® exhibited similar weight gain nevertheless with high mortality. On the 9th day, existing larvae bred on hexane and diet controls crossed the average weight of 350 mg (Table 3.). The larval length in all the treatments did not exhibit drastic reduction but had differences in accordance to its weight (Table 4.)

3.4 Histopathology

Morbid larvae from the assay with column fractions of *E. agallocha* treatments were studied for the pathogenesis. Transverse sections (T. S) of the whole 3rd instars larvae of *H. armigera* treated with *E. agallocha* fractions were compared with the larvae reared on the plain diet and hexane control. Midgut of the larvae treated with the fractions exhibited conspicuous shrinkage when compared with that of the controls. Shrinkage of the gut was generally observed during starvation of insect larvae. But in the present study, larvae fed with the treated diet did not exhibit prolonged starvation due to captivity. The diameter of the gut was drastically reduced to one-sixth of the normal larvae. The contour of the gut wall was irregular compared with the clear circular contour observed in the controls. Lysis of the gut wall was observed leading to release of the secretions and the micronuclei. The exocrine secretions from the gut epithelium were visible in the normal (healthy larva) midgut sections but were damaged in the treated samples (Fig: 4 a & b).

Table 1. Effect of crude extracts of *E. agallocha* on *H. armigera*.

Treatments*	Conc. (%)	Pupation (%)	Adult (Moth) Emergence (%)	
			Healthy	Deformed
***E. agallocha* Crude hexane leaf extract**	0.1	50 ± 1.9^{fgh}	18 ± 2.20^{ef}	10 ± 0.02^{cdef}
	0.2	40 ± 1.2^{hi}	16 ± 1.25^{ef}	10 ± 0.90^{cdef}
	0.5	42 ± 0.45^{ghi}	12 ± 1.45^{de}	10 ± 0.45^{cdef}
	1	30 ± 1.92^{ij}	6 ± 1.47^{ab}	4 ± 0.99^{efg}
	2	20 ± 1.38^{jk}	2 ± 0.45^{ab}	2 ± 0.95^{fg}
	C	88 ± 1.85^{a}	78 ± 1.27^{n}	0 ± 0.00^{g}
Diet Control	-	94 ± 0.23^{ij}	86 ± 0.57^{n}	4 ± 0.36^{efg}
VIJAY NEEM®	3%	30 ± 1.26^{kl}	14 ± 0.35^{de}	8 ± 1.64^{defg}
EKALUX®	0.02%	10 ± 0.46^{i}	-	-
CD (P=0.05)		19.924	11.3525	8.5004

*The average of 5 replications (20 larva/ rep.) is mentioned. Values within a column followed by the same letter do not differ significantly (P > 0.05), based on 5% LSD. CD- Critical Difference. C- solvent control

Table 2. Effect of fractions 1-7 of hexane extract on *H. armigera*

Treatments*	Pupation (%)	Adult (Moth) Emergence (%)	
		Healthy	Deformed
Fraction-1	25±1.9^{c}	5±2.1^{cd}	6±0.2^{bc}
Fraction-2	60±1.2^{b}	40±1.45^{b}	10±0.5^{b}
Fraction-3	20±1.38^{cd}	42±0.42^{c}	2±0.95^{c}
Fraction-4	10±0.25^{cde}	2±1.3^{d}	10±0.35^{b}
Fraction-5	6±1.92^{de}	2±1.72^{d}	6±0.25^{bc}
Fraction-6	80±1.85^{a}	78±1.45^{a}	20±0.52^{a}
Fraction-7	85±1.85^{a}	75±1.57^{a}	22±0.55^{a}
Diet Control	90±0.23^{a}	85±0.55^{a}	4±0.36^{bc}
3% VIJAY NEEM®	20±1.22^{cd}	8±0.35^{cd}	10±1.16^{b}
0.02% EKALUX®	3±0.33^{e}	-	-
CD (P=0.05)	**16.07**	**17.24**	**6.18**

*The average of 5 replications (10 larva/ rep.) is mentioned. Values within a column followed by the same letter do not differ significantly (P > 0.05), based on 5% LSD. CD- Critical Difference.

Table 3. Differences in the average larval weight between treatments with compounds

Treatments*	Day-1	Day-3	Day-6	Day-9	Day-12
n-Hentriacontane	25.5±0.61	71.2±0.04^{g}	95.6±0.05^{c}	217.7±0.03^{f}	279.9±0.15^{f}
Taraxerone	23.7±0.53	61.9±0.23^{e}	166.0±0.22^{f}	178.2±0.19^{e}	156.4±0.07^{e}
Taraxerol	24.6±0.11	47.2±0.80^{b}	78.0±0.12^{b}	100.2±0.02^{b}	64.0±0.06^{a}
Hexane Control	27.9±0.23	71.3±0.13^{g}	236.7±0.17^{g}	356.0±0.02^{i}	462.0±0.09^{h}
Diet Control	22.8±0.07	78.8±0.17^{h}	255.1±0.90^{h}	424.7±0.08^{j}	471.7±0.12^{i}
EKALUX® (0.03%)	23.5±0.12	58.1±0.01^{d}	146.7±0.12^{d}	305.5±0.19^{h}	321.3±0.11^{g}
CD (P=0.05)	N. S	1.3233	3.3439	3.0192	0.8723

* Fractions were tested at the conc. of 0.5% (w/v). The average of 5 replications (10 larva/ rep) is mentioned. The numbers indicate the average weight of the live and dead larva in milligrams (mg) Values within a column followed by the same letter do not differ significantly (P > 0.05), based on 5% LSD. CD- Critical Difference.

Table 4. Differences in the average larval length between treatments with pure compounds

Treatments*	Day-1	Day-3	Day-6	Day-9	Day-12
n-Hentriacontane	1.0±0.01	1.43±0.03	1.80±0.01	2.23±0.07	2.35±0.04
Taraxerone	1.2±0.09	1.32±0.03	1.50±0.02	2.45±0.01	2.45±0.01
Taraxerol	1.0±0.11	1.80±0.04	2.00±0.01	2.10±0.01	2.10±0.02
Hexane Control	1.2±0.02	1.90±0.08	2.25±0.04	2.63±0.05	2.72±0.03
Diet Control	1.3±0.05	2.0±0.02	2.23±0.05	2.63±0.06	3.15±0.05
EKALUX® (0.03%)	1.2±0.01	1.40±0.05	1.80±0.05	2.10±0.04	2.20±0.01
CD (P=0.05)		5.670	N. S	3.258	2.237

* Fractions were tested at the conc. of 0.5% (w/v). The average of 5 replications (10 larva/ rep) is mentioned. The numbers indicate the average length of the live and dead larva in centimeters. CD- Critical Difference.

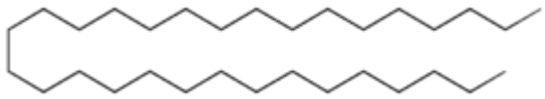

Fig 1. n-Hentriacontane

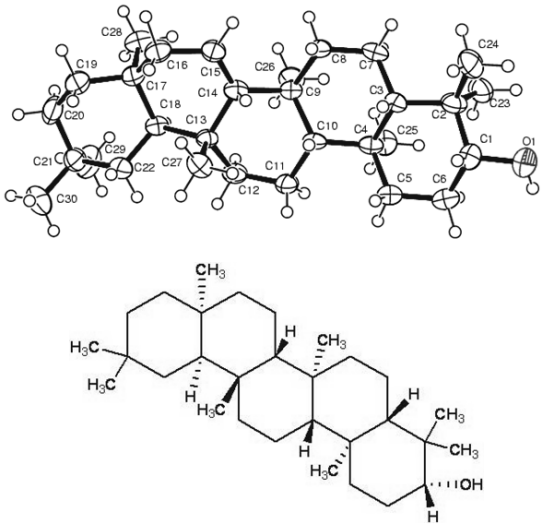

Fig 2. Taraxerol

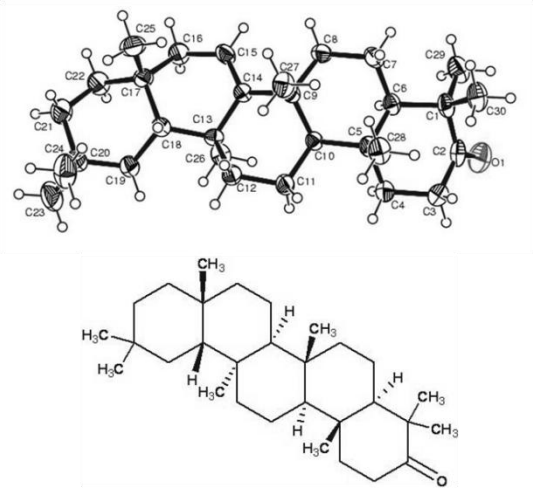

Fig 3. Taraxerone

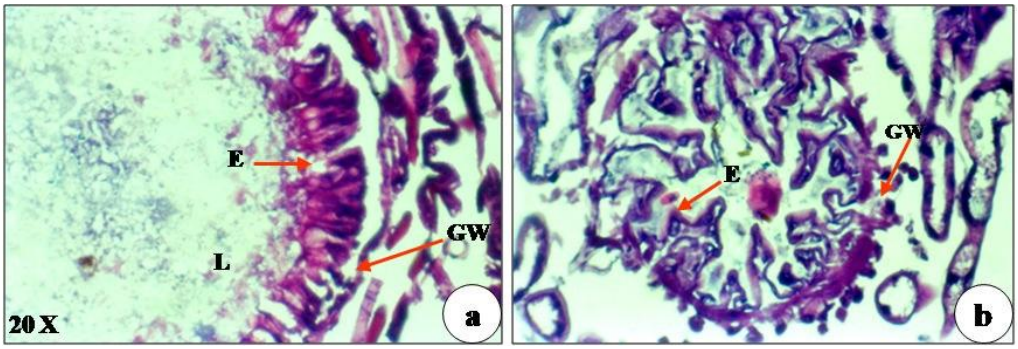

Fig 4. Effect of bioactive fractions on the midgut of 3rd instars larva of *H. armigera*

a. Healthy midgut of *H. armigera* (Control larva) depicting intact gutwall (GW); b. *H. armigera* larva treated with bioactive compounds depicting shrunken midgut and lumen (L) with damaged gutwall and the release of exocrine secretions (E) and micronuclei

Discussion

Novel & known di- and triterpenoids are continuously evaluated against *H. armigera*. Three novel norleucosceptroids A-C (1-3), isolated from the leaves & flowers of *Leucosceptrum canum* (Labiatae) has exhibited moderate antifeedant activity against *H. armigera* (Luo et al., 2012). In another study, Cantharidin, a terpenoid compound of insect origin was proved to be highly toxic to *H. armigera* larvae. Real-time qPCR showed the downregulation of *H. armigera* glutathione S-transferase gene (HaGST) at the mRNA transcript, ranging from 2.5-12.5 folds, while biochemical assays showed in vivo inhibition of GST's in the midgut & *in vitro* inhibition of rHaGST (Khan et al., 2013).

In our present study, triterpenoids taraxerone & taraxerol from *E. agallocha* exhibited prominent growth inhibition of H. armigera. These triterpenoids have also been proved to be bioactive against several insect & parasite systems. Hernández-Chávez et al. (2012) have proved the anti-giardial activity of taraxerone (IC_{50}: 11.33µg) and taraxerol (IC_{50}: 16.11µg) crystallized from *Cupania dentata* DC. (Sapindaceae) against the trophozoites of *Giardia lamblia* IMSS:0696:1. Taraxerone isolated from the stem and root bark of *Ficus tsiangii* Merr. Ex Corner. exhibited the highest mortality rate of 83.33% (LC_{50} value of 89.82 µg/g sugar) after 48 hrs of treatment in *Musca domestica vicina* adults. The toxicity of taraxerone was comparable to that of the control rotenone (LC_{50} value of 67.58 µg/g sugar) (Wang et al., 2010). Molluscicidal activity of taraxerol with the acetone extract of *Euphorbia tirucalli* L. against the harmful snail *Lymnaea acuminata* suggested the inhibitory activity of the molecule both singly and as a binary (1:1) combination (Chauhan & Singh, 2011). Among mangroves, the insecticidal activity of taraxerol and other triterpenes from *Rhizophora mangle* L. (Family: Rhizophoraceae) has been documented (Williams, 1999). Hentriacontane, the long-chain, linear alkane hydrocarbon is also one of the important epicuticular waxes present in Eucalyptus globulus Labill. that is resistant to *Mnesampela privata* Guénée (Autumn gum moth) defoliation (Jones et al., 2002).

Similar to our pathological observations in *H. armigera*, the insecticidal activity of protein toxins had been reported on *H. armigera*, with the damage being observed only in the midgut & fat bodies (Brown et al., 2004). 3d-Cry toxins from *B. thuringiensis* have similar effect on Lepidopterans. It involves sequential interactions with an array of insect midgut proteins that facilitate the formation of an oligomeric structure. These hydrolyzed toxins subsequently bind to the insect midgut cells at high affinity, where they interfere with potassium-ion dependent active amino acid symport mechanism. This disruption caused the formation of large pores, increasing the water permeability of the cell membrane leading to disintegration of the midgut lining and cell lysis (Pardo-Lopez et al., 2012; Kumar and Venkateswerlu, 1998). According to our observation, the mode of action of *E. agallocha* compounds was similar, causing lysis of midgut cells of *H. armigera* larvae. Nevertheless, further studies are to be conducted to thoroughly understand the mode of action.

The activity of taraxerone and taraxerol against *H. armigera* was not worked upon by other research groups. In the present study, as the late 2nd instars larvae of *H. armigera* showed drastic weight loss & mortality to both these

phytochemicals, it is understood that the secondary alcohol in Taraxerol and the ketone group in Taraxerone are the main functional groups that interact with the gut proteases to bring about the mortality. Further, docking studies would give an in-depth view about the molecular targets and a possibility of formulating them as an effective botanical pesticide against *H. armigera*. Even though *E. agallocha* is considered as a threatened mangrove associate, it is abundantly seen in the mangrove basin, throughout the world. Moreover, the micropropagation of *E. agallocha* has been successfully accomplished (Rao et al., 1998). This technique would enable mass propagation of the species for bulk production of the formulation for large-scale field application.

ACKNOWLEDGMENTS

We thank the Department of Biotechnology (DBT), Government of India, for their support. We express our sincere thanks to Dr. Babu Varghese (IIT- Madras, India) for the XRD analysis & interpretation and Mr. Shankar S. (SASTRA University, Tanjore, India) for critically reviewing the manuscript.

REFERENCES

1. Anjaneyulu, A.S.R. & Rao, V.L. (2000): Five diterpenoids (agallochins A-E) from the mangrove plant Excoecaria agallocha Linn. Phytochem., 55: 891-901.
2. Armes, N.J., Jadhav, D.R., DeSouza, K.R. (1996): A survey of insecticide resistance in Helicoverpa armigera in the Indian subcontinent. Bull. of Entomol. Res., 86: 499-514.
3. Wilson, C.E. (1923): Insect pests of cotton in St. Croix and means of combating them. Virgin Isles Agri. Exp. Stat. Bull. 3, Virgin Isles.
4. Wille, J.E. (1940): Observations sobre Heliothis virescens F. como plaga del algodonero en el Peru (lepidoptera). Rev. de Entomol., 11: 584-588
5. Ogengalatigo, M.W., Obuo, J.E. and Orotin, P. (1994): Infestation and Pod damage by Helicoverpa armigera (Hubner) on Chickpea (Cicer arietinum L.) in Uganda. International J. of Pest Management, 40: 245-248.
6. Hambleton, E.J. (1994): Heliothis virescens as a pest of cotton, with notes on host plant in Peru. J. of Eco. Entomol., 37: 660-666.
7. Hamity, M.G.A., Guerra, V.Q., Domenech, P. and Montero, E. (1998): Susceptibility of six collections of maize to attack from Helicoverpa zea and Euxesta cluta in Jujuy, Argentina. Manejo-Integrado-de-Plagas, 50: 73-77.
8. Jallow, M.F.A. and Zalucki, M.P. (1996): Within- and between population variation in host plant preferences and specificity in Australian Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). Aus. J. of Zool., 44: 503-519.
9. Li, Y.E., Zhu, Z., Chen, Z.X., Wu, X., Wang, W. and Li, S.J. (1998): Obtaining transgenic cotton plant with cowpea trypsin inhibitor. Acta Gossypii Sinica, 10: 237-243.
10. Voros, G. (1996): Damage by cotton bollworm (Helicoverpa armigera Hubner) in grapevine. Novenyvedelem, 32: 229-234.
11. Yi, B. (1998): Investigation and control of the tobacco pests in the middle-western region of Jilin province. J. of Jilin Agri. Univ., 20: 19-25.
12. Ross, S. (1998): Farmer's perception of bean pest problems in Malawi. Network on Bean research in Africa, CIAT, kampala, Uganda. Occasional Publication Series, pp. 31.
13. Kashiyama, T., Fujinaga, M. and Arai, Y. (1999): Seasonal prevalence and occurrence of cotton bollworm, Helicoverpa armigera and its control in lettuce producing areas in Nagano prefecture. Ann.Rep. of Kanto-Tosan Plant Protect. Soc., 46: 105-107.
14. Guo, Y.Y. (1997): Progress in the researches on migration regularity of cotton bollworm and relationship between the pest and its host plants. Acta Entomol. Sin., 40: 1-6.
15. Fitt, G.P. (1989): The ecology of Heliothis species in relation to agro eco-systems. Annual Rev. of Entomol., 34: 17-52.
16. Biradar, A.P., Balikai, R.A. and Teggelli, R.G. (2001): Chemical control of pod borer, Helicoverpa armigera (Hübner) on pigeonpea. Karnataka J. of Agri. Sci., 14: 500-502.
17. Lahm, G. (2004): Killing the very hungry caterpillar. Chem. World, 1(4): 35-39.
18. Zahid, M. and Hamed, M. (2003): Comparative efficacy of insecticides against the American bollworm, Heliothis armigera "Hüb" of cotton. Songklanakarin. J. of Sci. and Tech., 25(2): 169-173.

19. Rui, C.H., Meng, X.Q., Fan, X.L., Liang, G.M. and Li, Y.P. (1999): Resistance to insecticides in Helicoverpa armigera in Hebei, Henan, Shandong and Xinjiang. Acta Phytopar. Sin., 26: 260-264.
20. Manikandan, P. (1998): Comparative resistance of Heliothis armigera populations to some insecticides in different places of Tamil Nadu. Insec. Environ., 4: 9-10.
21. Kranthi, K.R. and Kherde, M. (1998): Status of insecticide resistance in cotton bollworms. Insec. Environ., 4(2): 35.
22. Gunning, R.V. and Easton, C.S. (1994): Endosulphan resistance in Helicoverpa armigera (Hübner.) (Lepidoptera: Noctuidae) in Australia. J. of Aus. Entomol. Soc., 31: 97-103.
23. Ahmad, M., Arif, M.I. and Ahmad, Z. (1997a): Patterns of resistance to organophosphorus insecticides in the field populations of Helicoverpa armigera in Pakistan. Pest. Sci., 55: 26-632.
24. Ahmad, M., Arif, M.I. and Attique, M.R. (1997b): Pyrethroid resistance of Helicoverpa armigera (Lepidoptera: Noctuidae) in Pakistan. Bull. of Entomol. Res., 87: 343-347.
25. Koul, O., Multani, J.S., Singh, G. and Wahab, S. (2002): Bioefficacy of Melia dubia (syn. M. azedarach) against gram pod-borer Helicoverpa armigera, Curr. Sci., 83(11): 1387-1391.
26. Lopenzolguin, J.F., Torredela, M.C., Vinuela, E. and Castanera, E. (1998): Activity of seed extracts of Trichilia havanensis Jacq. (Meliaceae) on the larvae of Helicoverpa armigera (Hübner) Boletin de Sanidad Vegetal Plagas, 24: 629-636.
27. Miles, J.E.C., Ramsewak, R.S. and Nair, M.G. (2000). Antifeedant and Mosquitocidal compounds from Delphinium cultorum cv. Magic fountain flowers. J. of Agri. and Food Chem., 48: 503-506.
28. Padmaja, P.G. and Rao, P.J. (2000): Efficacy of certain plant oils on the American Bollworm Helicoverpa armigera (Hübner.) Pest. Res., 12: 107-111.
29. Satasook, C., Isman, M.B., Ishibashi, F., Medbury, S., Wiriyachitra, P. and Towers, G.H.N. (1994): Insecticidal bioactivity of crude extracts of Aglaia species (Meliaceae). Biochem. and Sys. Ecol., 22: 121-127.
30. Singh, I.B., Singh, K. and Singh, H.N. (2001): Relative efficacy of certain plant extracts as antifeedants against gram podborer Heliothis (Helicoverpa) armigera (Hüb.). Bioved., 12: 41- 44.
31. Balaji P., Malarvannan S. and Hariharan G.N. (2007): Efficacy of Roccella montagnei extracts on Helicoverpa armigera (Lepidoptera:Noctuidae). J. of Entomol., 4: 248-252.
32. Brown, S.E., Cao, A.T., Hines, E.R., Akhurst, R.J., East, P.D. (2004): A novel secreted protein toxin from the insect pathogenic bacterium Xenorhabdus nematophilia. J. of Biol. Sci., 279(15): 14595-14601.
33. Erickson, K.L., Beutler, J.A., Cardellina II, J.H., McMahon, J.B. (1995): A novel Phorbol ester from Excoecaria agallocha. J. of Nat. Pro., 58(5): 769-772.
34. Hernández-Chávez, I., Torres-Tapia, L.W., Simá-Polanco, P., Cedillo-Rivera, R., Moo-Puc, P., Peraza-Sánchez, S.R. (2012): Antigiardial Activity of Cupania dentata Bark and its Constituents. J. Mex. Chem. Soc., 56(2): 105-108.
35. Jones, T.H., Potts, B.M., Vaillancourt, R.E., Davies, N.W. (2002): Genetic resistance of Eucalyptus globulus to autumn gum moth defoliation and the role of cuticular waxes. Can. J. of For. Res., 32(11): 1961-1969.
36. Konishi, T., Konishima, T., Maoka, T., Fujiwara, Y. (2000): Novel diterpenes, Excoecarins M and N from the resinous wood of Excoecaria agallocha. Tet. Lett., 41: 3419-3422.
37. Prakash, S., Khan, M.A., Khan, H., Zaman, A. (1983): A piperidine alkaloid from Excoecaria agallocha L. Phytochem., 22(8): 1836-1837.
38. Reed, W. and Pawar, C.S. (1982): Heliothis: a global problem. In: Proceedings of the International Workshop on Heliothis Management. Reed, W. and Kumble, V. (eds.) ICRISAT, Patancheru, India, pp. 9-14.
39. Shorey, H.H. and Hale, R.L., 1965. Mass rearing of the larvae of nine noctuid species on a simple artificial medium. J. of Eco. Entomol., 58, 522-524.
40. Singh, O.P. and Singh, K.J. (1996): Outbreak of gram pod borer Helicoverpa armigera on winter soybean and its effects on the grain yield in Madhya Pradesh. Ann. of Entomol., 14: 23-25.
41. Wang, J.D. and Guo, Y.W. (2004): Agallochaols A and B, two new Diterpenes from the Chinese mangrove Excoecaria agallocha L. Hel. Chimi. Acta, 87: 2829-2833.
42. Williams, L.A.D. (1999): Rhizophora mangle (Rhizophoraceae) triterpenoids with insecticidal activity. Naturwissenschaften, 86: 450-452.
43. Wiriyachitra, P., Hajiwangoh, H., Boonton, P., Adolf, W., Opferkuch, H. and Hecker, E. (1985): Investigations of medicinal plants of Euphorbiaceae and Thymelaeaceae occurring irritants of the diterpenes ester type from three Excoecaria species. Planta Med., 51: 368-371.
44. Zou, J.H., Dai, J., Chen, X. and Yuan, J.Q. (2006): Pentacyclic triterpenoids from the leaves of Excoecaria agallocha. Chem. and Pharm. Bull. 54(6): 920-921.

45. Zygadlo, J.A., Maestri, D.M., Grosso, N.R. (1994): Alkane distribution in epicuticular wax of some Solanaceae species. Biochem. Sys. and Ecol., 22: 203-209.
46. Pardo-Lopez, L., Soberon, M. and Bravo, A. (2012) Bacillus thuringiensis insecticidal three-domain cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol., Special Issue: Microbial toxins in the green world, 37(1): 3-22.
47. Kumar, N.S. and Venkateswerlu, G. (1998) Endogenous protease activated 66-kDa toxin from Bacillus thuringiensis subsp. kurstaki active against Spodoptera littoralis. FEMS Microbiol. Lett., 159(1): 113-120.
48. Luo S.H., Hua J., Li C.H., Jing S.X., Liu Y., Zhao X. and Li S.H. (2012) New antifeedant C20 terpenoids from Leucosceptrum canum. Org. Lett., 14(22): 5768-71.
49. Rao C.S., Eganathan P., Anand A., Balakrishna P. and Reddy T.P. (1998) Protocol for in vitro propagation of Excoecaria agallocha L., a medicinally important mangrove species. Plant Cell Rep., 17(11): 861-865.