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Anti-feedant activity of *Pachygone laurifolia* (DC.) L.Lian & Wei Wang bark extracts in tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) is mediated through biochemical responses and pathological damage

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Abstract

Spodoptera litura is a severe polyphagous insect pest, causing extensive damage to agricultural crops all over the world. The present study was undertaken to investigate the response of *S. litura* to polarity gradient fractions of *Pachygone laurifolia* bark extract. The fraction IV and V of *P. laurifolia* bark possess a significant anti-feedant activity and growth inhibitory effect on *S. litura*. The active fractions inhibited carboxylesterase activity, whereas no significant difference in total protein content was observed. Alteration in glutathione S- transferase activity and inhibition of acetylcholinesterase activity were noted on exposure of fraction V. The histopathological studies of the midgut region of the exposed larvae exhibited structural loss, damage in peritrophic membrane and longitudinal muscles, disintegration of goblet cells, oedema and lysis of the epithelial cells compared to control even at minimum concentration of exposure. Gas chromatographic – mass spectrometric analysis was carried out to ascertain the active constituents with the anti-insect properties. Overall, our findings revealed that fractions of *P. laurifolia* has significant anti-feedant activity mediated through biochemical mechanisms involving detoxification, oxidative stress and pathological damage. This plant extract has the potential to be developed as biopesticide formulation.

Keywords: insecticidal activity, biopesticides, detoxification enzymes, histopathology, *Cocculus laurifolius*

1 Introduction

The enhancement of agricultural product quality through the reduction of insect damage is one of the most important challenges in agriculture sector. *Spodoptera litura* is one of the most damaging polyphagous insects in the world (Chunxian *et al*., 2004), commonly known as the tobacco cutworm, and belongs to the order Lepidoptera and family Noctuidae. Organophosphates, organochlorines, carbamates and pyrethroids are among the synthetic insecticides being used to protect crops against damage caused by *S. litura* because they are very effective, quick-acting and simple to use (Saleem *et al*., 2016, Ruttanaphan *et al*., 2018). However, the pesticides leaves its residues which causes adverse effects both to environment and human health (Aktar *et al*., 2009). Due to the negative impacts of the use of chemical insecticides, the world is now searching for biodegradable, ecologically safer chemicals and developing new integrated pest management (IPM) techniques.

Since ancient times, plants are being explored for medicines (Bellakhdar, 1997) and use of bio-insecticides are also in place for thousands of years (Farzaei *et al*., 2013). Secondary metabolites such as terpenoids, alkaloids, flavonoids, phenolics play a major role in plant - insect interactions and are mainly responsible for plant resistance to insects (Isman, 2002). They are known to function as effective botanical pesticides that are completely biodegradable and safe for the environment and other non-target organisms (Koul *et al*., 2008).

Plants develop the ability to produce a variety of secondary metabolites in response to various environmental factors,

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stress conditions, insect attacks or even a long-term association with insects (Pan *et al*., 2016). Secondary metabolites may not directly be involved in the normal physiological functions or growth of the plant, but play a pivotal role in insect defence by killing, repelling, inhibiting food intake, growth inhibition, growth regulation, reduced fecundity and sterility (Schmutterer, 2002, Koul & Wahab, 2004, Niroumand *et al*., 2016). Alkaloids, flavonoids, phenolics, terpenoids and other secondary metabolites found in plants are responsible for bioactivities (Isman, 2002). These secondary metabolites also have a significant impact on insect host plant selection (Wang & Qin, 2007). During co-evolution, plants evolved a chemical defence system of secondary metabolites to impede insect attack and insects evolved detoxifying enzymes to overcome this plant chemical defence system (Peng *et al*., 2010).

Some of the plant extracts are found to be toxic to many agricultural insect species such as lepidopteran larvae, aphids, thrips, and many other cosmopolitan pests (Schmutterer, 2002, Koul & Wahab, 2004). Though many studies proved the use of plant-based extracts or compounds as insecticides, the mechanism of action of many of these were not examined in detail. There might be number of biochemical reactions like altering activities in digestive enzymes, anti-oxidants and inhibitions of detoxifying enzymes which could play key roles in the insecticidal activities of the above extracts. The entry of a xenobiotic molecule to an organism generates oxidative stress leading to an imbalance in the generation and removal of reactive oxygen species leading to the alteration in detoxification mechanism in the organism ultimately killing or deterring the insects (Rahman & Adcock, 2006). The toxicity of plant chemicals includes interruption of the insect gut digestive enzymes (α-amylases, proteases) (Duffey & Stout, 1996) and inhibition of detoxifying enzymes (Smirle *et al*., 1996). Crude plant extracts and their bioactive molecules are reported to be toxic to *S. litura* larvae (Nobsathian *et al*., 2019, Tharamak *et al*., 2020).

Pachygone laurifolia, popularly known as laurel-leaved snail tree, a member of the Menispermaceae family is a tiny tree or shrub that grows to a height of 1 to 2 metres and has striate, glabrous branches and branchlets (Ajaib & Khan, 2012). The antibacterial and antioxidant potential of this plant has already been well studied against two gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*), two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), and two fungal strains (*Aspergillus niger* and *Fusarium solani*) (Ajaib *et al*., 2017). Our previous study had established the insecticidal properties of the whole extract of bark and leaves of this plant (Paul & Jayaraj, 2020). The study revealed the pesticidal properties of the crude extracts of *Pachygone laurifolia* and few other plant crude extracts against *Spodoptera litura*. So far, no study was carried out to understand the mode of action or biochemical events behind the pesticidal activity of this plant.

The present study reports the insecticidal activity of the purified fractions of *P. laurifolia* bark extract against the third instar larvae of *Spodoptera litura* along with the possible biochemical targets or events. The crude bark extracts were purified leading to isolation of active fractions. The insecticidal properties of these fractions along with their mode of action were studied through enzyme studies and histopathological analysis. The major phytochemical constituents responsible for the bioactivity was elucidated with Gas Chromatography – Mass Spectrometry analysis.

2 Materials and methods

2.1 Chemicals and reagents

All the chemicals were purchased from M/s. Merck Specialties Private Limited, Mumbai, India. Whatman No #3 paper was procured from M/s. GE Healthcare Life Sciences.

2.2 Plant materials, extraction and purification of fractions

The bark of *P. laurifolia* was collected from foothills of south western Ghats, Mattupetti, India (N 10°07'11.0" E 77°10'24.4") during the month of December 2019. Voucher specimens were deposited in Kerala Forest Research Institute Herbarium (KFRI), India with accession number 18026. The bark was thoroughly washed, shade dried and then powdered using a mixer grinder. Powdered samples (10 g) were hot extracted with 200 ml of methanol using a Soxhlet apparatus for 6–8 h (4–5 repeat refluxes). The extracts were concentrated using a rotary vacuum evaporator and stored at −20 °C until use.

The fractionation of the samples was carried out using a glass column packed with 100–200 mesh silica gel. The samples were eluted using different solvents as the mobile phase in the sequence hexane (H), chloroform (C), methanol (M) and water (W) with a flow rate of 1 mLmin-1. Six major fractions were collected. Fraction - I (H-100 %), Fraction - II (H 50 %: C 50 %), Fraction - III (C -100 %), Fraction - IV (C 50 %: M 50 %), Fraction -V (M-100 %) and Fraction - VI (W 100 %). The collected fractions were evaporated and the dry weights were recorded. These fractions were used for the bioactivity studies.

2.3 *Culture and maintenance of* Spodoptera litura

The larvae of *Spodoptera litura* (Lepidoptera) were collected from banana fields of Ernakulam (Kerala), India (N 9°58'35" E 76°26'20") and their subsequent generations were maintained at 25 °C ± 2 °C temperature and 60 ± 5 % relative humidity with a 14:10 h (L : D) photoperiod. During the pupation stage, it was shifted to jars containing moist, sterilised sand covered with filter paper. On emergence of adults, they were moved to oviposition jars and given honey solution with few drops of multivitamin. Neonates, upon hatching from eggs, were transferred to glass jars containing fresh, thoroughly washed with *Ricinus communis* (castor) leaves. This process was continued, and the insect culture was maintained throughout the study period.

2.4 *Pesticidal properties of fractions*

2.4.1 *Anti-feedant activity*

Anti-feedant activities of fractions of bark sample were studied using leaf disc no-choice bioassay method (Isman *et al*., 1990). Fresh castor leaf discs (4.5 cm diameter) were dipped with different concentrations (0.5, 1.0 and 2.5 %) of all the fractions tested against *S. litura* in individual boxes. Methanol in water was used as control. Wet cotton was placed in boxes to maintain the moisture. After 24 h, the consumption of leaves by the larvae were recorded using a graph paper. Three replications were maintained for each treatment. The percentage of anti-feedant activity in the no choice method was calculated based on following formula (Singh & Pant, 1980).

$$\text{antifeedant activity (\%)} = \frac{(\%\ \text{protection in treatment} - \%\ \text{protection in control})}{(100 - \%\ \text{protection in control})} \times 100$$

2.4.2 *Weight loss or growth inhibition*

Insect growth regulatory activity was evaluated by diet incorporation method. Different concentrations of active fractions were spread in castor leaves and provided to third instar larvae of *S. litura*. Larval weight reductions were calculated after 24 h of treatment (Arora *et al*., 2017).

$$\text{reduction in larval weight (\%)} = \frac{\text{weight gain in control} - \text{weight gain in treatment}}{\text{weight gain in control}} \times 100$$

2.5 *Evaluation of biochemical mechanisms*

Potential biomarker enzymes involved in the biochemical pathways of detoxification and oxidative stress were analysed to understand the mechanism of action of active fractions with insecticidal properties. Major enzymes such as glutathione-S-transferase, carboxylesterase, and acetylcholinesterase were analysed along with total protein levels.

2.5.1 *Enzyme preparation*

The surviving larvae after the treatment were used for enzyme preparation to determine the enzyme activities. After the treatment regime, the third instar larvae of *S. litura* was homogenised in buffer (Potassium phosphate buffer, 100 mM, pH 7.2) containing ethylene diamine tetra acetic acid (EDTA, 1 mM). The homogenate of larvae was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was used as enzyme source for the assays (Feyereisen, 2005).

2.5.2 *Estimation of total protein content*

Protein estimation of the samples was carried out with modified Lowry's method (Lowry *et al*., 1951) using bovine serum albumin (BSA, 1 mg ml^{-1}) as standard.

2.5.3 *Glutathione-S-Transferase*

Modified method of Oppenoorth *et al*. (1979) was used to estimate the glutathione-S-transferase (GST, EC 2.5.1.18) activity of the larvae. The test solution was prepared from sample supernatant (30 μL), potassium phosphate buffer (pH 7.2, 50 mM, 370 μL), glutathione (10 mM, 20 μL) and 1-chloro-2, 4'-dinitrobezene (CDNB, 10 mM, 200 μL). The GST activity was measured spectrophotometrically (Lambda 650, M/s. Perkin Elmer) at 340 nm. The extinction coefficient of CDNB as 9.6 $mM^{-1}cm^{-1}$ was used for calculating GST activity.

2.5.4 *Carboxylesterase*

Bullangpoti *et al*. (2012) method with some modifications was used to examine the carboxylesterase (CE, EC 3.1.1.1) activity. The assay mixture was prepared by mixing the sample supernatant (20 μL), potassium phosphate buffer (pH 7.4, 50 mM, 580 μL) and p-nitrophenyl acetate (pNPA) in dimethyl sulfoxide (10 mM, 120 μL). The carboxylesterase activity was measured spectrophotometrically using a kinetic mode at 410 nm for 60 sec at 37 °C. The extinction coefficient of pNPA as 176.4705 was used for calculating the CE activity.

2.5.5 *Acetylcholinesterase*

The activity of acetylcholinesterase (AChE, EC.3.1.1.7) was determined following Scaps *et al*., 1997. Assays were performed in a reaction mixture containing; 0.5 ml 50 mM potassium phosphate buffer (pH - 8), 40 μl 0.01 M Ellman's Reagent (5, 5'-dithio-bis-[2-nitrobenzoic acid]) in phosphate

buffer (50 mM pH - 7), 0.075 M acetylthiocholine iodide in 50 mM phosphate buffer (pH 7.4) and 20 μl sample supernatant. Spectrophotometric measurements were carried out at 410 nm, for 5 min at 25 °C. The activity of AChE was expressed in mM acetylthiocholine iodide/min/mg protein, using the coefficient of extinction 1.36×10^4 $M^{\check{}1}$ $cm^{\check{}1}$ (Dauberschmidt *et al*., 1997).

2.6 *Histopathological analysis in the midgut of* Spodoptera litura

Histopathological analysis was carried out in the midgut region of *S. litura* fed with *P. laurifolia* active fractions by leaf disc no choice method (Isman *et al*., 1990). The treated larvae were kept for 24 hrs and after the incubation time, the larvae were fixed using Bouin's fluid. The midgut sections of the tissue were prepared by microtome with a size of 4 microns (Thermo scientific, USA) with Patho cutter-HP-R, Erma microtome blades (KAI, Japan) and histopathology was done following standard methods (Mohan, 2007). The midgut histology was observed through Leica DM 1000 LED microscope (Germany) and images were captured. The changes in the midgut due to the effect of treatments were compared with control organisms.

2.7 *Phytochemical profiling of active fractions using gas chromatography mass spectrometry analysis*

The chemical composition of the partially purified fractions of *P. laurifolia* having anti-feedant activity were analysed using GC–MS (QP-2010-Shimadzu) equipped with Rxi-5Sil column. The active fractions were filtered through 0.22 μm syringe filter before analysis. Helium was used as carrier gas at a constant flow of 1 mL min^{-1}. Oven temperature was programmed (80 °C for 4 min, increased to 280 °C at 5 °C/min ramp rate). The injection port was 260 °C and 1 μL sample (splitless) used for the analysis. EI mode was at 70 eV, while mass spectra were recorded in the 50–500 amu range and ion source temperature was maintained at 200 °C. The peaks of components in the gas chromatography were subjected to mass-spectral analysis. The active components are elucidated by comparing the retention times of chromatographic peaks and interpretation of mass spectrum (quadrapole detector) using database of National Institute of Standard and Technology (NIST) and Wiley library.

2.8 *Statistical analysis*

The results were expressed as mean ± SE of three replicates per each treatment. Data were analysed by one-way analysis of variance followed by Dunnet's test for comparison between respective control and treatment groups. The level of significance was set at $p \leq 0.05$. Data of all the results in this study were obtained from at least three independent experiments with similar pattern.

3 Results

Through the present study, the crude extracts of the *P. laurifolia* bark extract were fractionated and the fractions with significant anti-feedant activity were taken up to understand the biochemical and pathological events during the process.

3.1 *Fractionation of active ingredients and anti-feedant activity of fractions*

Column chromatography was performed to isolate active compounds from methanol extract of *P. laurifolia* (bark). Six fractions were collected for the bioactivity studies (Table 1). The fractions isolated from *P. laurifolia* bark were tested for feeding deterrence activity. Among the fractions tested, fraction IV and V of bark extract showed feeding deterrence. The maximum anti-feedant activity was showed by fraction IV (chloroform : methanol – 50:50) of *P. laurifolia* (bark) with feeding deterrence of 60.01 ± 7.33 %, 75.03 ± 2.05 % and 89.09 ± 0.488 % at exposure of 0.5 %, 1.0 % and 2.5 % extracts respectively. Along with this, fraction V (methanol - 100 %) also showed maximum feeding deterrence of 90.94 ± 4.09 % at its 2.5 % extract exposure (Fig. 1). Though the fractions VI and III showed feeding deterrence less than 50 %, but exhibit significant anti-feedant activity when compared to control. All other fractions (F-I and II) of *P. laurifolia* bark extracts doesn't exhibit any feeding deterrence effect when compared to the control. The active fractions IV and V were taken up for further studies.

Table 1: *Percentage yield of fractions of* Pachygone laurifolia *bark extract in column chromatography.*

Fraction no.	*Solvents*	*Ratio (V/V)*	*% W/W**
I	Hexane	100	0.373
II	Hexane : Chloroform	50:50	0.457
III	Chloroform	100	6.503
IV	Chloroform : Methanol	50:50	64.21
V	Methanol	100	16.48
VI	Water	100	9.4571

*Percentage yield of active fractions

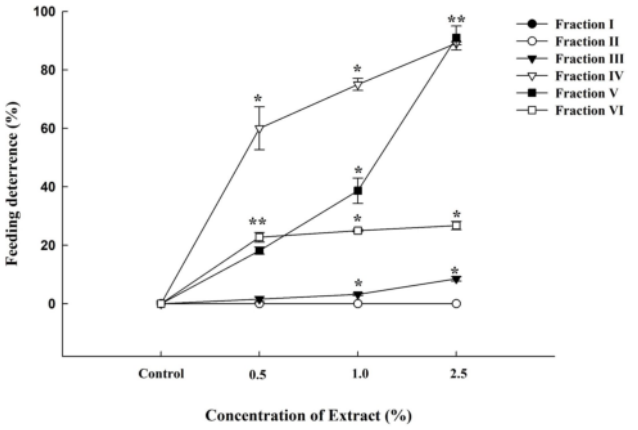

Fig. 1: *Anti-feedant activity of fractions of* Pachygone laurifolia *bark extract on third instar larvae of* Spodoptera litura*. The values are expressed as mean ± SE of three replicates per experiment. *Significantly different from respective controls at* $p \leq 0.05$ *by Dunnet's test.*

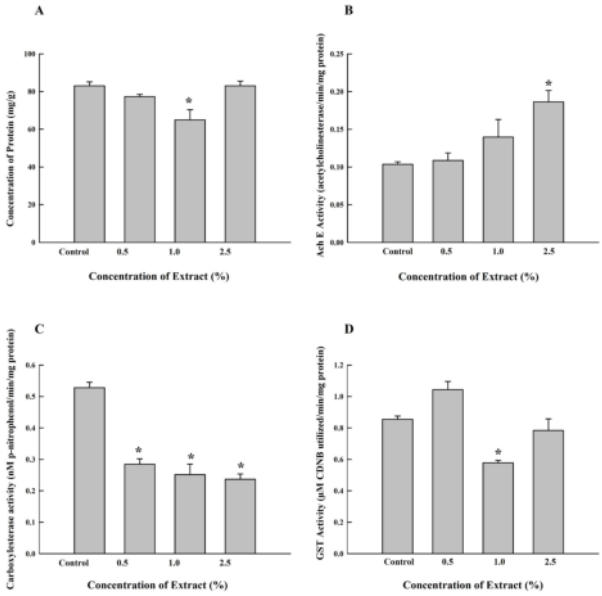

Fig. 2: *Effect of Fraction-IV of* Pachygone laurifolia *bark extract treatment on total protein (A), ACh E (B), CE (C), and GST (D) activities in live third instar* Spodoptera litura *larvae after 24 h treatment. The values are expressed as mean ± SE of three replicates per experiment. *Significantly different from respective controls at* $p \leq 0.05$ *by Dunnet's test.*

3.2 Effect of active fractions on body weight of exposed organisms

The present study has evaluated the growth inhibitory effects of different methanolic extract fractions of *P. laurifolia* in *S. litura*. The active fractions – fraction-IV and fraction-V - showed strong growth inhibition in terms of decreased body weight against *S. litura* within 24 hrs of exposure. The exposure of high concentrations of (1.0 and 2.5 %) fraction-IV and V of *P. laurifolia* has caused significant weight loss in exposed organisms compared to control (Table 2).

3.3 Biochemical changes induced by fractions IV and V in exposed organisms

The total protein content in an organism is directly linked with its metabolic activities. The exposure of 1.0 % extract of fraction-IV had shown a significant decrease in total protein compared to control, however all other exposures did not show any significant difference (Fig. 2A). The exposure of fraction-V does not show any significant effect on the total protein concentration of the organisms (Fig. 3A). Over all, there is no significant alterations in the total protein were observed in *S. litura* on exposure to active fractions of *P. laurifolia* extract compared to control. The inhibitory impact of *P. laurifolia* extract fraction-IV and V on acetylcholine esterase (AChE) was examined in order to assess their role in synaptic transmission. During the exposure of fraction-IV, no inhibition of AChE was noted rather an increasing trend was seen and a significant increase was observed in

Table 2: *Growth inhibitory activity of* Pachygone laurifolia *active fractions against* Spodoptera litura*.*

		Weight of the larvae (g)			
Fractions	*Treatment*	*initial*	*final*	*Weight gain/loss (g)*	*Weight loss (%)*
P. laurifolia Fraction IV	Control	0.469 ± 0.004	0.729 ± 0.0097	0.2603 ± 0.0087	0
	0.5 %	0.472 ± 0.008	0.590 ± 0.006	0.1185 ± 0.0028	54.47
	1.0 %	0.465 ± 0.010	0.566 ± 0.009	0.1014 ± 0.0011	61.04
	2.5 %	0.4415 ± 0.007	0.437 ± 0.008	−0.0046 ± 0.0005	101.76
P. laurifolia Fraction V	Control	0.4287 ± 0.006	0.8017 ± 0.004	0.3753 ± 0.0052	0
	0.5 %	0.4305 ± 0.006	0.445 ± 0.006	0.0146 ± 0.0005	96.11
	1.0 %	0.4321 ± 0.0048	0.2507 ± 0.0007	−0.1814 ± 0.0055	148.33
	2.5 %	0.4397 ± 0.0062	0.3786 ± 0.004	−0.062 ± 0.0048	116.52

The values are expressed as mean ± SE. -ve values indicate reduction in body weight when compared to control

the highest concentration of 2.5 % (0.186 ± 0.015 mM acetylthiocholine iodide/min/mg protein) (Fig. 2B). In case of fraction-V, though there was a decreasing trend in AChE activity with increase in concentration of fractions, significant inhibition was noted only in the highest concentration, i.e., 2.5 % and was 0.070 ± 0.011 mM acetylthiocholine iodide/min/mg protein (Fig. 3B).

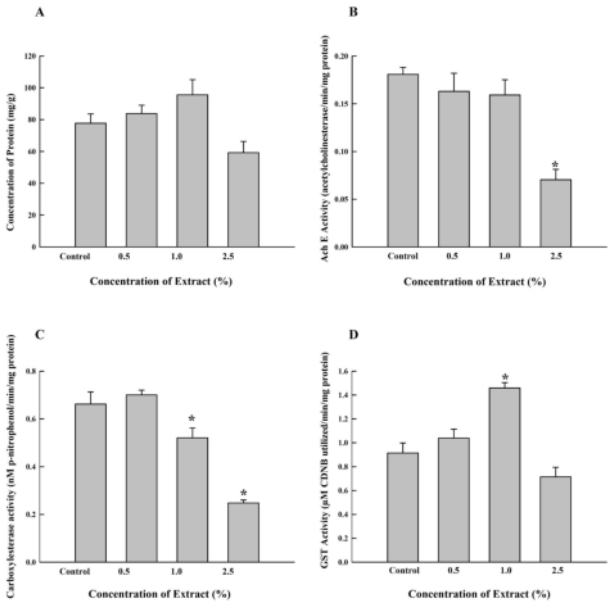

Fig. 3: *Effect of Fraction–V of* Pachygone laurifolia *bark extract treatment on Total protein (A), ACh E (B), CE (C), and GST (D) activities in live third instar* Spodoptera litura *larvae after 24 h treatment. The values are expressed as mean ± SE of three replicates per experiment. *Significantly different from respective controls at $p \leq 0.05$ by Dunnet's test.*

The compounds present in two active fractions had the potential to inhibit carboxyl esterase (CE) activity (Fig. 2C and 3C). A significant reduction of CE activity was noted in insects treated with fraction IV (0.28 ± 0.017, 0.25 ± 0.03 and 0.24 ± 0.017 nM p-nitrophenol/min/mg protein) at its concentration of 0.5, 1.0 and 2.5 % respectively (Fig. 2C). Similar trend was observed in fraction V with significant inhibition noted in 1.0 and 2.5 % concentrations with activity of 0.52 ± 0.04 and 0.25 ± 0.01 nM p-nitrophenol/min/mg protein respectively.

The glutathione–S-transferase (GST) activity was not much affected by treatment with any of the fractions, IV or V. In case of fraction-IV, a significant decrease in activity was noted at 1.0 % treatment (Fig. 2D). However, a contradictory result – significant increase in GST activity with 1.0 % treatment – was noted in the treatment with fraction-V (Fig. 3D). Overall, the results indicated that two active fractions (IV and V) possess anti-feedant activity and the exposure of these fractions caused significant alterations in the activities of acetylcholine esterase, carboxyl esterase and glutathione-S-transferase. This indicates the involvement of these enzyme systems in the detoxification mechanisms in *S. litura*.

3.4 Histological analysis

The study was carried out to understand and compare the effect of different concentrations of two active fractions of *P. laurifolia* on insect midgut.

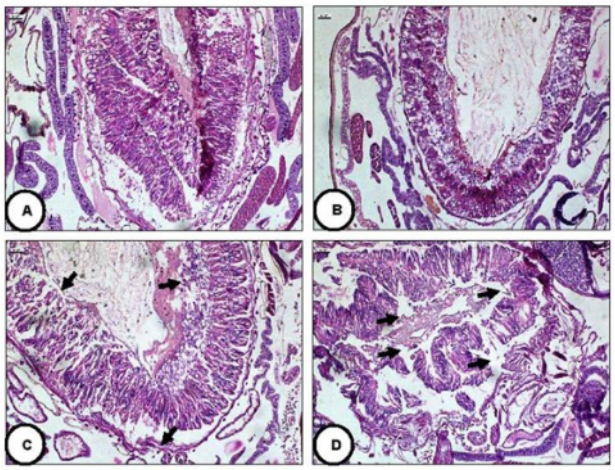

Fig. 4: *Midgut histological structure of* Spodoptera litura *third instar larva; Control (A), Concentration: 0.5 % (B), 1.0 % (C) and 2.5 % (D) of Fraction-IV of* Pachygone laurifolia *bark extract. Staining: Harris Hematoxylin-Eosin (HE).*

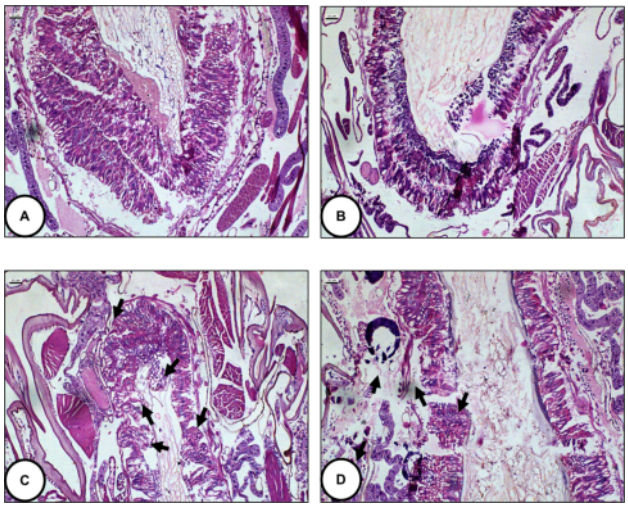

Fig. 5: *Midgut histological structure of* Spodoptera litura *third instar larva; Control (A), Concentration: 0.5 % (B), 1.0 % (C) and 2.5 % (D) of Fraction-V of* Pachygone laurifolia *bark extract. Staining: Harris Hematoxylin-Eosin (HE).*

Three concentrations of fractions – IV and V, 0.5 %, 1.0 % and 2.5 % - which were found to have anti-feedant activity,

were tested to evaluate the histological changes in the midgut region of third instar larvae of *S. litura*. In the untreated larvae, the midgut region showed an undamaged peritrophic membrane, well developed muscular layers and goblet cells indicating a healthy midgut region (Fig. 4A and 5A). Mild histological changes were noted in the midgut of *S. litura*, after the treatment with *P. laurifolia* fraction–IV at 0.5 % concentration, 24 hours after exposure (Fig. 4B). Damage to peritrophic membrane, irregular epithelial cells and gap formations between digestive and goblet cells were noted with increase in concentration (Fig. 4C). A complete disintegration of the midgut region with detachment of epithelial cells from basement membrane, collapse of lumen and damage of peritrophic membrane was noted in the highest dose of 2.5 % (Fig. 4D). The effect was observed to be dose dependent. Similarly on exposure to *P. laurifolia* fraction-V, the extent of damage was found to similar as that of the damage caused by the fraction-IV though with minor differences (Fig. 5).

3.5 Gas chromatography mass spectrometry analysis of Pachygone laurifolia *extract active fractions*

The compounds present in the fractions were identified using gas chromatography mass spectrometry analysis. Identification of phytochemicals was established on the basis of the molecular structure, molecular mass and calculated fragments. The correlative percentage of each component was calculated by comparing its average peak area in total area. The compounds were identified using NIST and Wiley library. The analysis of fraction -IV showed the presence of 13 compounds as listed in Annex 1 of the supplement. Four compounds were identified by the GC-MS analysis of methanol fraction (fraction V) of *P. laurifolia* bark exhibiting various phytochemical activities (Supplement). The chemical constituents with their retention time, area, area percentage, height, height percentage, mass to charge (m/z) ratio, chemical nature, identified properties and structure were shown in Annex 1 of the supplement. Among the identified compounds, hordenine is the major compound and has highest percent peak area (64.28 %).

4 Discussion

The plant extract and their derivatives play a significant role in pest management due to the presence of different class of chemical constituents such as alkaloids, phenolics, terpenoids, terpenes, tannins, flavonoids, steroids, coumarins and lignins (Chellappandian *et al*., 2018). Botanical anti-feedants are a group of compounds that inhibits the feeding of insects but do not kill them (Yasui *et al*., 1998). Different classes of secondary metabolites found in plants possess anti-feedant activity (Baskar *et al*., 2009). Both basic laboratory works as well as agricultural practices on anti-feedant behavior for insect control has been studied well and handful of plant based chemicals are currently in use. Botanical anti-feedants have little effect on the environment because they degrade quickly after use. The effects of plant derived compounds or their mixtures or essential oils on *S. litura* were studied in terms of anti-feedant activity by many researchers (Yooboon *et al*., 2019). The significant anti-feedant activity of secondary metabolites influences the insects' choice of plant host rather than killing the insects directly (Pan *et al*., 2016).

Various deformities in lepidopteran pests were caused by the toxic effects of plant-based pesticides (Baskar & Ignacimuthu, 2012). On certain pests, especially the tobacco cutworm, *S. litura*, the plant compounds exert growth-regulating effects (Attaullah *et al*., 2020, de Freitas *et al*., 2022). Our study also supports that chemical constituents present in plants play a major role in growth of target pest, *S. litura*. Various enzyme systems are used in the defence mechanisms that insects have developed to fend off xenobiotics especially pesticides and natural diseases (Yooboon *et al*., 2019). Naturally occurring compounds protect their host plants from insects also by disrupt the detoxification activity (Arash & Ali Reza, 2010). The fractions IV and V from the bark extracts of *P. laurifolia* showed significant feeding deterrence as showed in the results.

Acetylcholine esterase (AChE) is a key enzyme that involved in the transmission of nerve impulse conduction by quickly hydrolyzing the neurotransmitter acetylcholine in the synaptic cleft. Insecticides especially organophosphates and carbamates, work primarily by inhibiting acetylcholinesterase leading to disruption in the synaptic transmission (Ghosh *et al*., 2012). The hydrolysis of the neurotransmitter acetylcholine by acetylcholinesterase (AChE) leads to termination of nerve impulse transmission. AChE is a key enzyme in the insect nervous system in which the cholinergic system is essential. Fraction - V indicated the reduction of AChE activity up on increase in concentration of the active fraction of *P. laurifolia* extract, leading to feeding inhibition of *S. litura*. This result is in tune with previous studies on plant derived extracts or essential oils or pure compounds including triterpene glycosides against *S. litura* (Nobsathian *et al*., 2019); monoterpene linalool against *Aedes aegypti, Leptinotarsa decemlineata*, *S. litura* (Praveena & Sanjayan, 2011) and *S. exigua* (Rachokarn *et al*., 2008). However, a different trend was observed in fraction IV where in a significant increase in AChE activity was noted when compared with untreated group. Similar result was obtained in treat-

ment with thymyl cinnamate –treated *S. litura* larvae (Tharamak *et al*., 2020) and terpinen-4-ol and 1,8-cineole treated pests (Greenberg-Levy *et al*., 1993). This indirectly indicates the effect of *P. laurifolia* extracts in the nervous system of *S. litura*.

In the context of allelochemical metabolism and resistance, herbivorous insects always utilize enzymes, such as carboxylesterase (CE) and glutathione – S – transferase (GST), to metabolize potentially harmful secondary metabolites from plants. Carboxylesterases are responsible for the hydrolysis of carboxylesters into the corresponding alcohol and carboxylic acid (Namountougou *et al*., 2012). Esterase (EST) is a crucial enzyme for detoxification that breaks down the esteric link in synthetic toxins. In our study significant reduction in carboxylesterase activity were observed. Previous studies also demonstrated that many plant extracts or compounds may reduce the CE activity such as *Melia toosendan* Sieb. et Zucc. Pron. (Feng *et al*., 1995); *Melia asedarach* and *Amaranthus viridis* against *Spodoptera exigua* (Rachokarn *et al*., 2008); *Alpinia galanga* against *Bactrocera dorsalis* (Sukhirun *et al*., 2011); *Jatropha gossypifolia* against *S. frugiperda* (Bullangpoti *et al*., 2012), triterpene glycosides in *S. litura* (Nobsathian *et al*., 2019). The CE activity in *S. litura* was inhibited by active fractions of *P. laurifolia* extracts indicating its involvement in inhibition of detoxification mechanisms. According to several earlier studies, toxicity in insects may be linked to inhibition of detoxifying enzymes (Ishaaya *et al*., 1987).

In insects, the defence against oxidative stress is provided by a network of protective enzymes including glutathione-S-transferase (GST), which operate to retain organisms in a state of dynamic equilibrium by keeping the level of reactive oxygen species (ROS) in check to prevent oxidative stress-related cellular damage (Felton & Summers, 1995). GST functions as a detoxifying enzyme that efficiently metabolizes external xenobiotic substances and is essential for both providing protection and preserving the body's regular physiological processes (Li *et al*., 2018). The present study revealed that the active fractions of *P. laurifolia* extracts did not have significant effect on the activity of GST. Previous studies have demonstrated that GST played a crucial role in defence mechanism of insects by inactivating exogenous and endogenous toxin molecules by enhancing the activity level (Bouayad *et al*., 2013, Pan *et al*., 2016). Similar result was found for *S. litura* on treatment with triterpene glycosides extracted from *Holothuria atra* (Nobsathian *et al*., 2019).

Histological data provides direct evidence for the damages caused by the xenobiotics to the internal organs in an organism and indicates the pathological levels during the toxic insult. In insects, one of the target areas is its midgut, because of the absence of chitin layer and it controls the insect physiology (Farder-Gomes *et al*., 2022). The toxic compounds cause damage to the larvae throughout its middle region of intestine (Suryani *et al*., 2020). A healthy lepidopteran larval midgut has four types of cells which are involved in the food digestion and nutrient absorption, namely columnar cells, goblet cells, endocrine cells and regenerative cells. These cells are involved in absorption of nutrients and secretion of enzymes, ion homeostasis, hormonal control and regeneration of new epithelial cells respectively (Franzetti *et al*., 2015). Once toxic compounds enter in to the midgut of *S. litura*, the peritrophic membrane is degraded within half an hour, after that epithelial cells will start swelling leading to hypertrophy. The goblet and columnar cells become elongated and the intercellular space widens. Finally causes destruction of midgut occurs with goblet and columnar cells becoming elongated, vacuolated and hypertrophied and nuclear material gets fully scattered (Pandey *et al*., 2009).

Once toxic compounds enter in to the midgut of *S. litura*, the peritrophic membrane is degraded within half an hour, after that epithelial cells will start swelling, hypertrophy, goblet and columnar cells become elongated and the intercellular space become widened and finally the almost destruction of midgut occurs with goblet and columnar cells becoming elongated, vacuolated, elongated and hypertrophied, and also the nuclear material gets fully scattered (Pandey *et al*., 2009).

The present study showed that the treatment of active fractions of the *P. laurifolia* caused significant effect on the midgut cells of third instar larvae of *S. litura* by the way of degrading the peritrophic membrane, collapse of lumen and affecting epithelial cells etc which in turn may affect the growth and development of the insect pest. Previous studies have shown that plant derived compounds resulted in midgut epithelial cell apoptosis of *S. litura* (Suryani *et al*., 2020, Muthu *et al*., 2023).

The results of GC-MS analysis are in-line with earlier findings of various methanolic crude extracts reported to have many biological effects including pesticidal properties. Among the identified compounds, 3,7,11,15- Tetramethyl-2-hexadecen-1-ol is a derivative of n- hexadecaanoic acid and it have anti-oxidant, hemolytic, anti-microbial, 5-alpha-reductase inhibitor, nematicidal, and pesticidal properties (Starlin *et al*., 2019). Olemide was another major compound of peak area 7.54 %, which induce sleep (Verdon *et al*., 2000). Hordenine is an alkaloid, with activities including, inhibition of pyruvate dehydrogenase kinase 3 (PKD 3), cytotoxic to lung cancer cells (Anwar *et al*., 2020), a norepinephrine and noradrenaline uptake inhibitor and an MAO-B inhibitor that raises norepinephrine content (Frank *et al*.,

1990), inhibit melanogenesis in human (Kim *et al*., 2013) and causes weight loss. The results of the current study showed that fractions of *P. laurifolia* bark extract have considerable anti-feedant, enzyme-inhibitory and toxic effects on important agricultural insect pest – *S. litura* - of ecological and economical importance. The naturally occurring bioactive compounds present in this plant are toxic, causing feeding deterrence effect or inhibiting larval growth. The compounds like alkaloids and terpenoids present in the plant may be responsible for the damaging effect on pest. Further purification, identification and comparison of the active ingredients causing larval mortality is essential to tap its commercial potential. Ideally, commercial botanical insecticides made from efficient active fractions or purified active ingredients can replace the current chemical pesticides, which are causing potential environmental damage and developing chemical resistance in a variety of insect pests.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplement

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