Biologically active toxin from macroalgae *Chaetomorpha antennina* Bory, against the lepidopteran *Spodoptera litura* Fab. and evaluation of toxicity to earthworm, *Eudrilus eugeniae* Kinb

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**Abstract**

**Background:** Seaweeds harbour a wide array of bioactive compounds shown to be effective in support of sustainable agricultural practices. The green seaweed *Chaetomorpha antennina* found in abundance in coastal areas of India has been reported with various bioactivities. Owing to the requirement of alternative and economical natural pest control method to be applied in sustainable agronomic strategies, the current study attempts to evaluate the efficacy of chemical toxins from *C. antennina*, as insecticidal agents, by inspecting their effects on the physiology, biochemistry, immune system, and histology of one of the most important insect pests of agricultural crops in the Asian tropics, the polyphagous lepidopteran *Spodoptera litura*.

**Results:** The active fraction 5 isolated from *C. antennina* using methanol extraction produced significant mortality rates of *S. litura* among all the other fractions obtained. GC–MS analysis revealed the presence of various pesticide compounds. The toxin compounds (active fraction 5) were found to negatively influence the pest’s immune system performance at sub-lethal concentrations (LC50 38.73 and LC90 53.60 ppm), affecting insect development, reducing the haemocyte count (69.24%) and reduced the activity of major defence enzyme phenoloxidase decreased post-treatments. Digestive phosphatase enzymes, acid phosphatase, ACP, alkaline phosphatase, ALP, and ATPase were demodulated by 37.5, 39, and 23.9% compared with untreated. Increase in detoxification enzymes coupled with mid-gut collapse are indicative of the toxicity of the compounds. Earthworms exposed to seaweed compounds displayed no debarring effects.

**Conclusion:** Extracted seaweed compounds produced significant lethal effect on the insect larvae, affecting the immune as well as digestive systems of the pest. However, no such toxicity was observed in earthworms treated with the seaweed fraction supporting their environmentally benign nature. Since the insect immune system is responsible...
Background

Marine ecosystem organisms harbour a wide array of bioactive compounds. Among these are a group of macroalgae referred to collectively as seaweeds, which are a collection of marine plants, existing along the world's coastlines. The marine algae are a rich source of a wide variety of bioactive compounds, that have been developed into biofuels, medicines, fertilizers, plant growth stimulants, and biopesticides to name a few [1–5] and are considered an under-utilized ocean resource. Additionally, several seaweed compounds have been shown to induce toxicity and alter the growth, behaviour, development, and metabolism of several insect pests [2, 6–9].

Among the important benefits from agricultural industries is the capacity to provide sustainable production of food, feeds, and fibres to meet the needs of an increasing global population [2, 10–13]. Changes in climate will also alter crop losses caused by environmental factors, pests, and pathogens [13, 14]. The need for crop treatments, fertilizers, herbicides, insecticides, and fungicides are predicted to intensify on agricultural land throughout the world, resulting in growing research on alternative treatments [12, 14–16]. Alternative strategies for control of pests and pathogens may also reduce the rapid development of insecticide resistance to overused, or improperly used chemical insecticides [17]. In addition to offering an environmentally benign and safe means of pest control, biopesticides could also surpass the development of pest resurgence as well as propose a pronounced aptitude in regulating yield loss deprived of conceding the quality of the produce [18].

Increased use of Integrated Pest Management, IPM, strategies also benefit with incorporation of emerging molecular treatments that have greater safety for beneficial insects and humans over heavy reliance on chemical pesticides and herbicides [19]. Some of these technologies include: exogenous applied or plant expressed RNA interference [20–22], along with antisense oligonucleotides [23, 24] and other gene editing technologies like CRISPR [25–28] all of which can greatly support IPM strategies. Increased use of several sustainable strategies will produce more effective, eco-friendly agronomic strategies, while confronting escalating issues of food insecurity [11].

Tobacco cut worm, Spodoptera litura Fab. is among the major polyphagous pests, that severely limits production [29]. A major problem is that insect pest around the world have developed resistance to a large number of insecticides [30]. Insects have a well-developed immune system encompassing humoral and cellular defences. The insect immune system is mainly governed by the activities of phenoloxidase enzyme as well as haemocytes that are acute against invaders and toxins [31]. Any compound that can interfere with these defences will severely impair the insect biological fitness. Insect detoxification enzymes also play a key role in the development of resistance against insecticides. A reduction in these enzyme levels will result in a reduction of insecticide resistance levels [32]. The insect gut enzymes, acid and alkaline phosphatases, along with ATPase play a crucial role in the hydroxylation of phosphor monoesters. Also, these enzymes in conjunction with the gut neurons moderate the organic molecule [33, 34]. Any oscillations in these enzyme actions can upset the gut physiology, resulting in a significant decrease of the insect biological fitness [35].

Finding novel, eco-friendly, effective insecticides is essential to agriculture, leading to the global bio-prospecting for natural chemical alternatives from unique habitats and organisms, such as in seaweeds (macroalgae) [2, 5, 7, 8]. The green seaweed Chaetomorpha antennina (Bory) Kützing, is a green alga in the Family: Chlorophyceae [36] that have chemicals with bioactivities that can be applied to agricultural problems as biostimulants, bactericides, viricides, fungicides, insecticides [1, 2, 4, 33, 37–39], and repellents. To evaluate the efficacy of the chemical toxins from C. antennina, as insecticidal agents, we examined the effects on the haematology, biochemical, and physiology of the lepidopteran pest, S. litura.

Materials and methods

Insect culture

Spodoptera litura larvae were obtained from stock culture reared on castor leaves in cages (Biopesticide and Environmental Toxicology Laboratory, SPKCEES, Manonmaniam Sundaranar University, Tamil Nadu; 28 ± 2°C 85% RH, 12:12 LD).

Seaweed collection, identification, and extract preparation

The seaweed, C. antennina, were collected from the rocks of the coastal regions of Colachel beach, Kanyakumari (8°14′ 5168″ N and 77° 14′ 35.209″ E) during low tide in August (2018) and processed, labelled, and transported
to the lab in sealed bags. The seaweed was washed thoroughly in tap water 5 times, 10 min each prior to microscopic analysis (Nikon H600L, Japan; 40×). Their organoleptic features were also recorded. The algae were then spread on blotting paper to remove excess water; shade dried (6–7 days). The completely dried sample was pulverised and extracted with methanol (Soxhlet apparatus) for 24 h (1:20 w/v), concentrated in rotary vacuum evaporator (13.36% yield extract powder), and stored at 4 °C. Preliminary mortality feeding bioassays on early fifth-instar S. litura larvae were performed with the crude methanol extract (CA-M), which was dried and resuspended in aqueous DMSO, (1 mg/ml Sigma-Aldrich, Inc.), with chromatographic analyses on the methanol extracts.

Active fraction isolation
Active compounds from CA-M were isolated as fractions using column chromatography; pore size 60–120, hexane:ethyl acetate gradient (90:10, 80:20, 70:30, 60:5:30.5, and 60:40), desiccated then stored (4 °C). Each gradient was resuspended in aqueous 0.1% DMSO for use in feeding bioassays to determine mortality as mentioned.

GCMS characterisation of C. antennina fraction F5 (CAF-5)
GC–MS analysis of C. antennina fraction-5 was performed (Oven: initial temp 60 °C for 2.8 min, ramp 10 °C/min to 300 °C, holding time for 6 min, Inj A auto = 260 °C, Volume applied used 0 µl, split was 10:1, carrier gas used was helium (He), solvent delay was 2.00 min. The transfer temperature was 230 °C and source temperature was 220 °C. The scan was done at 40–600 Da, column 30.0 m × 250 μm) and interpreted against a library of standards (National Institute Standard and Technology).

Bioassays
Mortality bioassay
Larvicidal assays against S. litura larvae (second, third, fourth, and fifth instars; 20 larvae per treatment—25, 50, 75, and 100 ppm of active fraction prepared in 0.1% DMSO) were performed with active fraction 5, using a leaf-dip assay. Castor leaves were dipped in 10 ml of active fraction 5 at various concentrations for 20 s and air dried before being placed within sealed petri dishes for each individual dilution [32]. The larvae were introduced to the petri dishes and observed for mortality. Leaves treated with 0.1% DMSO served as the control. Mortality was recorded till 48 h and percentage mortality was calculated using Abbott’s formula [40]. The mean lethal concentration was calculated using Probit’s analysis and carried forward for further treatments [41]. Cypermethrin (9 ppm) was prepared by dissolving 0.009 mg of cypermethrin (Rallis India Limited) in 100 ml of distilled water (CM).

Haemolymph collection
Ten S. litura larvae in the early fifth-instar (25 days after hatching) stage were chosen for the study. The larvae were weighed and haemolymph (10 µl for PO enzyme assay) was drawn by piercing the last pro-leg (fine needle of a Hamilton syringe-10 µl SGE), 24 h post-exposure to sub-lethal concentration of the active fraction by the leaf-dip assay procedure mentioned above in mortality assay [32]. As a lethal concentration would kill the larvae, sub-lethal concentration (LC50) was used to study the inhibition effect of active fraction 5 on the insect immune system.

Haematological parameter assay
Total haemocyte count (THC) was determined by preparing the haemolymph as described [42]. The drawn haemolymph was immediately added to anticoagulant (5 µl of EDTA + 5 µl of glycerol) which was drawn using a WBC pipette (till 0.5 mark), diluted with Tauber–Yeager fluid (to reach till 11 mark), and shaken well. Post-discard of first three drops, a drop of diluted haemolymph was placed on the Neubauer haemocytometer and counted (4 and 1 central, 1 mm2 squares), under the microscope (40×, Nikon Eclipse DS-R12Y-TV55, Japan). THC/mm3 was determined using the formula

\[
\text{THC/mm}^3 = \frac{X \times \text{dilution factor} \times \text{depth factor}}{\text{Number of squares counted}}
\]

\(X\)—total number of cells counted; dilution factor—20; Depth factor—10.

Phenoloxidase activity (PO) assay
PO was estimated, and expressed as PO units by following the method of Karthi et al. [32]. The drawn haemolymph (8 µl) was added to phosphate buffer saline (PBS, 400 µl ice-cold—pH 7.4) and vortexed; from which 100 µl was added to equal volume of L– Dopa solution (20 mM) and incubated (25 °C/30 min), following which absorbance was read at 475 nm in a spectrophotometer (Spectrum Tek, ST2700,).

Enzymatic profile
Preparation of enzyme extract
The larvae (early fifth instar) treated with active fraction 5 (25 ppm) was used for enzyme assays (24 h post-treatment). The treated larvae were anaesthetized, and then, their digestive system was dissected out followed by treatment with ice-cold insect Ringer’s solution. The
guts were individually weighed and homogenised (300 µl of ice-cold citrate-phosphate buffer, pH 6.8; 3 min, at 4 °C). The homogenate was resuspended in the same buffer to produce 1 ml volume. The samples were centrifuged (500 x g for 1 min). The supernatant was used as the enzyme source [42].

**Estimation of enzymes**

Digestive enzymes such as ACP and ALP, and ATPase were estimated [43]. The levels of catalase, CAT [44], superoxide dismutase, SOD [45], and peroxidase, POX were determined [46–48].

**Histology**

Histological effects of active fraction on the mid-gut tissues of *S. litura* (fifth instar) treated with 25 ppm active fraction 5 were studied by comparing with untreated control. The mid-gut was dissected and fixed (Bouin’s Solution; O/N; Sigma-Aldrich). Fixed tissues were dehydrated in ascending grades of alcohol, viz., 50, 60, 70, 80, 90, and 100%, every 2 h, and then transitioned into paraffin. Then were placed in a warming oven to prepare blocks. Sections were produced using a microtome (Cryostat Microtome AB-92-02, India). Sections were dewaxed by dipping into 100% Xylene, 5 min. The blocks were stained (Delafield’s haematoxylin) and counterstained (Eosin), and dried. The sections were observed and photographed under phase contrast microscope (Nikon eclipse SMZ1270, Japan).

**Earthworm toxicity: contact filter paper test**

The effect of active compounds, active fraction 5, on non-target organism, earthworm, *Eudrilus eugeniae* Kinb was performed using the Contact Filter Paper Test [40]. The earthworms were maintained in culture in the laboratory (28.9 ± 0.36°C, Food source: crop residues amended with cattle dung) which were used for bioassay. The test was carried out by moistening the filter papers (cut to 9 cm disc, suitable for petri plate) with 50 ppm active fraction 5 along with 9 ppm cypermethrin and incubated (dark at 20 ± 1 °C for 48 h). The experiment was replicated five times and the percentage of mortality was calculated using Abbott’s formula [40].

**Statistical analysis**

Each experiment was replicated five times. The mortality rates obtained were expressed as the mean of five replicates and data were normalised by applying arcsine-square root transformation. The lethal concentrations (LC$_{50}$ and LC$_{90}$) were calculated using Probit analysis. Analysis of variance (ANOVA) was used to identify the difference in percentage of mortality and was fitted with linear regression using Minitab®17 for lethal concentration. Tukey–Kramer HSD test was used to determine the differences between the treatments ($P \leq 0.05$). Sigma plot 11 was used for graphs.

**Results**

**Seaweed identification**

Seaweed species were confirmed based on visible taxonomic key features, micro and macroscopically [Guiry W. In Algae Base. Accessed August 2020]. Organoleptic features such as fishy odour, light green colour, salty taste, holdfast base, and smooth texture were also characteristic traits of *C. antennina*.

**GC–MS analysis of active fraction C. antennina fraction-5**

Among the 11 compounds in *C. antennina* fraction-5, methyl esters of chloroacetic acid were abundant such as dichloroacetaldehyde, methyl chloroacetaldehyde, and methylene Chloride (Table 1). The presence of carboxylic acid derivative, Cyclohexan-1, 4, 5-triol-3, 1, 1-carboxylic acid was also observed. Compounds such as cis-2-Chlorovinylacetate, trichloromethane, and chloro-methanesulfonyl chloride were also present. A heterocyclic aromatic organic compound (2, 6-lutidine 3, 5-dichlooro-4-dodecylthion), cyclo-alkane (Cyclobutanol), and a chloro-ethyl-ester of butyric acid were also present in the active fraction (Table 1).

**Mortality bioassay of active fraction 5 against S. litura**

Active fraction 5 exhibited pronounced larvicidal activity against third, fourth, and fifth-instar larvae. Although the mortality rate increased with treatment concentrations, they decreased with increasing age of instars. The greatest mortality was observed in third-instar larvae, 96.7% at 100 ppm treatment concentration of active fraction 5 ($F_{5,24}=49.63$; $P<0.0001$). The fourth instar being comparatively less susceptible to the treatment concentrations 50, 75, and 100 ppm exhibiting 45.74, 70.06, and 95.9% mortality rates ($F_{5,24}=41.88$; $P<0.0001$), with that of fifth instar that resulted only in 45.4, 69.29, and 95.6% mortality rates’ treatment concentrations 50, 75, and 100 ppm ($F_{5,24}=33.96$; $P<0.0001$) (Fig. 1). The lethal concentration, LC$_{50}$ and LC$_{90}$ for third-instar larvae was observed at 24.76 and 77.02 ppm respectively. The respective LC$_{50}$ and LC$_{90}$ values for fourth- and fifth-instar larvae were detected at 38.73, 127.35 ppm and at 53.60 and 158 ppm (Fig. 2). At the sub-lethal concentration, 158 ppm, active fraction 5 substantially affected the development of early *S. litura* larvae by producing uncharacteristic or striped pupae and adults (Fig. 3).
| RT  | Compound                   | MW  | Chemical formula and structure |
|-----|----------------------------|-----|--------------------------------|
| 1   | Methylene chloride         | 84  | CH₂Cl₂                         |
| 2   | Dichloroacetaldehyde       | 112 | C₂H₂OCl₂                       |
| 3   | Methyl chloroacetaldehyde  | 108 | C₃H₅O₂Cl                       |
| 4   | Dichloroacetic acid        | 128 | C₂H₂O₂Cl₂                      |
| 5   | Cis-2-Chlorovinylacetate   | 120 | C₆H₅O₂Cl                       |
| 6   | Trichloromethane           | 119 | CCl₃D                          |
| RT | Compound                                          | MW  | Chemical formula and structure |
|----|---------------------------------------------------|-----|--------------------------------|
| 7  | Chloro-methanesulfonyl chloride                   | 148 | CH₂O₂Cl₂S                      |
| 8  | Butanoic acid, 2-chloro-3-oxo-, ethyl ester      | 164 | C₆H₉O₃Cl                      |
| 9  | Cyclohexan-1,4,5-triol-3,1,1-carboxylic acid      | 190 | C₇H₁₀O₆                      |
| 10 | Cyclobutanol                                      | 72  | C₄H₉O                        |
Effects of sub-lethal concentration of active fraction 5 and cypermethrin, CM on early 5th instar larvae

Changes in haematology

THC  The natural change in THC in controls across the four time points: 24 h, 48 h, 72 h, and 96 h post-treatment showed lower levels at 24 and 48 h, with an increase of THC at 72 h, with a slight decrease at 96 h (Fig. 4).

However, the larvae treated with active fraction 5 showed no significant change in concentration of THC over time (24 to 96 h), but THC concentrations were significantly lower than those in control.

CM (cypermethrin)-treated larvae showed no significant difference from the active fraction 5-treated larvae at 24 and 48 h, in THC, but produced significantly lower concentrations at 72 h (21.4%) and at 96 h post-treatment (17.6%) ($F_{2,12}=6.86; P \leq 0.01$). The reduced THC of CM-treated larvae remained low at both the 72 and 96 h time points and were not significantly different ($F_{2,12}=6.86; P \leq 0.816$). Both treatments active fraction 5 and CM,

| RT  | Compound                  | MW  | Chemical formula and structure |
|-----|---------------------------|-----|--------------------------------|
| 11  | 2,6-Lutidine             | 375 | $C_{19}H_{31}NCl_2S$           |

Table 1 (continued)

Fig. 1  Effect of active fraction 5 (AF5) on third-, fourth-, and fifth-instar S. litura larvae. Mean (± SEM) followed by the same letter in individual larval instar in bars indicates no significant difference ($P < 0.05$) in a Tukey’s test.
resulted in significantly lower THC concentrations across time points, with greater reduction of THC at 72 h and 96 h, being 60.15 and 60.54% ($F_{2,12} = 133.6; P \leq 0.0001$) compared with control. The greatest reduction was produced in CM-treated larvae at 72 and 96 h; 75.709 and 69.24% ($P > 0.05$) in CM-treated larvae, respectively (Fig. 4).

**Phenoloxidase activity (PO)**

The PO activity was significantly reduced by CM treatment, 0.62 from 1.27 PO units ($F_{2,12} = 6.37; P \leq 0.001$). While, active fraction 5 decreased PO activity by 25% compared to control ($F_{2,12} = 6.37; P \leq 0.209$) (Fig. 5A).

**Catalase activity (CAT)**

There was an increase in CAT activity from 5.04 to 6.18 μmoles in Active fraction 5 treated and to 6.85 μmoles of $H_2O_2$ decomposed/min/mg protein, being significantly increased in CM-treated larvae over control ($F_{2,12} = 26.63; P \leq 0.001$) (Fig. 5B).

**SOD activity**

There was a significant increase in the SOD activity in both the active fraction 5 and CM treatments compared to control. SOD activity increased from 0.08 in control larvae, to 0.14 in CM-treated, and 0.16 unit/mg/min protein in active fraction 5 ($F_{2,12} = 36.67; P \leq 0.0001$). Active
fraction 5 treatment increased SOD activity by 49.63% (Fig. 5C).

POX activity
POX activity significantly increased in treated larvae exposed to seaweed compounds containing active fraction 5 and CM ($P \leq 0.05$). The POX activity increased over control, from 0.62 to 0.724 and 0.88 unit/mg/min protein for active fraction 5 and CM, respectively ($F_{2,12} = 36.76; P \leq 0.0001$) (Fig. 5D).

Digestive enzymes
The digestive enzymes ACP, ALP, and ATPase were significantly reduced by both treatments, providing significantly greater reduction on exposure to active fraction 5. The active fraction 5 compounds significantly reduced digestive enzymes ACP, ALP, and ATPase by $37.47(F_{2,12} = 28.34; P \leq 0.001)$, $39 (F_{2,12} = 21.5; P \leq 0.001)$ and $23.9\% (F_{2,12} = 104.79; P \leq 0.001)$ compared with control (Fig. 6).

The CM-treated larvae showed the greatest significant reduction of all enzymes reducing the ACP activity to 14.18 from 21.86 unit/mg/min protein in control. However, the active fraction 5-treated larvae produced a lower significant change in ACP ($F_{2,12} = 28.34; P \leq 0.007$). The levels of the enzymes ALP ($F_{2,12} = 21.5; P \leq 0.008$) and ATPase ($F_{2,12} = 19.75; P \leq 0.0001$) in larvae were significantly different from control, and between treatments active fraction 5 and CM.

Histology
The mid-gut histology of the treated larvae was severely affected displaying impaired cell organelles, abridged brush-border membrane, and leaky columnar cells, laterally with cellular component mixing as a consequence of shattered membranes (Fig. 7B).

Earthworm toxicity
Earthworms showed no significant mortality when treated with 50 ppm active fraction 5, displaying mortality rates lower than control ($P > 0.005$). *E. eugeniae* exposed to 9 ppm cypermethrin resulted in significant mortality rate of 58% ($F_{3,16} = 40.2; P \leq 0.001$). The earthworms treated with chemical pesticide displayed external morphological changes such as (a) degeneration, (b) coiling, (c) anomalous clitellar swelling, and (d) tail constriction and dehydration (Fig. 8).

Discussion
Development of environmentally friendly, highly effective chemical control is imperative. Currently, about 2500 marine natural bioactive compounds have been isolated from seaweeds of sub-tropical and tropical populations [7–9]. The bioinsecticidal and microbicidal activities
of marine macroalgae bioactive compounds have been reviewed by Hamed et al. [2]. Many of these natural bioactive compounds are important in maintaining marine ecosystems [1, 15], providing pathogen protection, growth stimulants, and support of beneficial microbiomes [49–51].

Exposing the insect pest to a mix of active compounds rather than a single compound would probably delay the development of pesticide resistance [37, 52, 53]. The current study showed that bioactive compounds from the seaweed *C. antennina* were able to significantly affect the haematology, biochemistry, and histology of *S. litura* at sub-lethal concentrations.

The preliminary mortality bioassays of crude methanol extract of *C. antennina* showed significant toxicity against *S. litura* larvae. Then, the extract was subjected to column chromatography, and the fraction active fraction 5 was identified to cause the greatest mortality rate against third-, fourth-, and fifth-instar larvae at concentrations of 25, 50, 75, and 100 ppm. Earlier instars were more susceptible to treatments, displaying increased mortality rate, which is due to their increased feeding and metabolic rates resulting in increased consumption and assimilation of toxins.

The vulnerability of early instars to toxins was also observed in *S. litura* larvae treated with andrographolide [54]. Similarly marine algae have been proved as repositories of various bioactive compounds with broad spectrum agricultural applications [1, 2, 7]. A likely biocidal activity of acetone extract of *C. antennina* was reported by Kannan and Bharathkumar [55] who proved that the seaweed extract had a profound effect on the larval survivability, pupal duration, as well as emergence of *S. litura*. Additional reports on bioactivities of *C. antennina*
encompass antiviral [56], antifungal, as well as cytotoxic effects in the form of crude extracts [57]. Seaweeds are reported to possess insecticidal compounds such as chloroacetic acid, a main component of pesticide formulation such as chlorpyrifos, insecticide-dimethoate, and herbicides benazoline besides methyl b-naphthoxyacetate [57]. The compound 2, 6-lutidine 3, 5-dichloro-4-dodecylthio—present in seaweeds—has been found to be the active component of medicinal plants *Andrographis echioides* [58] and *Ficus arnottiana* [59]. Cyclobutanol is a recognised microbial herbicide *Streptomyces rochei* antimetabolite [60] and also a major pesticide ingredient OECD [61]. Butanoic acid (or) butyric acid is an active component of plant hormone, IBB—a pesticide compound [62]. Also, cyclobutanol, is registered as an active ingredient in a malathion pheromone traps used in cotton boll weevils’ control [63]. These toxic compounds could be are regarded as safe due to their target specificity and biological origin [54].

**Fig. 6** Effect of AF5 on phosphatases activity. **A** ACP, **B** ALP, **C** ATPase. Mean (± SEM) of 20 larvae. Within each time point, the same letter indicates no significant difference (*P* < 0.05) in a Tukey’s test (CM cypermethrin, AF5 active fraction 5)

**Fig. 7** Effect of AF5 on mid-gut histology of early V instar larvae. **A** Control, **B** active fraction 5-treated, **C** cypermethrin-treated (CC columnar cells, EL epithelial layer, GL gut lumen, BBM brush-border membrane)
The developmental defects of *S. litura* larvae treated with sub-lethal concentration of active fraction 5 resulted in deformed pupae and adults. The treated pupae were dark, deformed, and unable to emerge into adults. The debarred adults had deformed wings. This morphological anomaly was also observed in *S. litura* treated with methanolic extract of brown seaweeds, *Sargassum cristaefolium* [5] and *Liagora ceranoides* [8]. A significant decline in THC in active fraction 5-treated larvae may be due to cytotoxic effects and a reduction in the number of defensive haemolytic cells. An analogous THC decrease in insect pest treated with pesticide compounds has also been reported [7, 32, 57, 58]. PO is an important component of innate immune system in insects that is activated in response to the presence of foreign agents [64, 65]. Reduction in PO in the haemolymph of active fraction 5-treated larvae might indicate the ability of algal compounds to deregulate the insect immune system.

An increase in the levels of detoxification enzymes—SOD, CAT, and POX—indicates an increase in oxidative stress stimulation in the insect body confirming the toxicity of the studied bio compounds. Larvae treated with active fraction 5 also showed an increase in antioxidant enzyme levels. A similar increase in the antioxidant enzymes was observed in *S. littoralis* treated with pro-oxidant allelochemicals [66].

Phosphomonoesters are hydrolysed by acid phosphatases in acid conditions and alkaline phosphatases in alkaline conditions by transphosphorylation reactions [45]. These activities are recorded in higher rates in the insect mid-gut [61]. ATPases are another group of enzymes crucial for glucose, amino acids, and other organic molecule transport within the insect body [35, 67]. Any modulation in their activity will affect the insect gut physiology [33, 65]. The activities of ACP, ALP, and ATPase were influenced by the active compounds present in the seaweed extract. The reductions in the enzyme levels in insect gut are often associated with pesticide exposure [63]. A similar decrease in ACP–ALP enzyme activities was observed in *Cnaphalocrocis medinalis* and *S. litura* larvae treated with azadirachtin [68]. Reduced ACP–ALP enzyme activities correlate with low energy levels caused by the disruption of metabolite transport. A similar effect on ATPase enzymatic activity was detected in *C. medinalis* larvae treated with a biopesticide formulation that contained neem seed kernel, *Vitex negundo* extracts and *Bt* toxin [69]. ATPase reduction was caused by decreases in metabolism influencing food indigestibility or cessation of feeding [68, 70, 71]. The effect of active
fraction 5 was more significant in the insect mid-gut as the assimilation and nutrient absorption takes place in this region. The mid-gut histological analysis of treated larvae showed a disrupted brush-border membrane, owing to the interruption in metabolite or ion transport caused by the active compounds [72]. This might have triggered a series of consecutive cellular events, such as cell disruption, oozing out of cellular components and eventually disabling the insect feeding activity [69, 70]. This caused an enlargement of the mid-gut region of the treated larvae. Similar aberrations were observed in Helicoverpa armigera larvae treated with Thevetia neriifolia stem extract [71], as well as S. litura larvae treated with andrographolide [29, 49, 56, 63], and Anticarsia gemmatalis treated with squamocin [73].

Eudrilus eugeniae, earthworms, are used as bioindicators for assessments of environmental ecotoxicology [52, 74]. In vitro evaluation of active fraction 5 against E. eugeniae did not show any toxicological effects. Instead, the worms grew up healthy, displaying a relatively low mortality rate compared with the control. The positive effects of seaweed extracts on earthworm were reported previously by [75], who studied the effects of seaweed extracts of Caulerpa scalpelliformis, Cheilosporum spectabile, and Sargassum wightii on E. eugeniae. On the other hand, cypermethrin was highly toxic to these worms. The negative effects of synthetic pesticides on earthworms have been widely reported [30, 74, 76, 77].

Conclusion
Algae are an under-utilized resource of active compounds that have wide applications in agriculture and human health. The macroalgae examined produced an active fraction with insecticidal activity that significantly reduced larval S. litura survival inducing haematological, morphological, physiological, and biochemical changes. The present research adds to the increasing amount of data on marine macroalgae use in agriculture as effective biopesticides and plant protectants, as environmentally friendly and safer alternatives. Increasing interest, research, and production of products from marine macroalgae and other plants continues to provide evidence of their importance as microbicides, nematocides, insecticides, and biofertilizers. Advances in improving the effectiveness of macroalgae bioactive compounds will incorporate improvements in formulation that will open a new epoch in the biopesticide development sector against insect pests, across all agricultural sectors.

Abbreviations
DMSO: Dimethyl sulfoxide; CM: Cypermethrin; AF5: Active fraction 5; RH: Relative humidity; LD: Light:dark; CA‑M: SPKCEES: Sri Paramakalyani Centre for Excellence in Environmental Sciences; THC: Total haemocyte count; PO: Phenoloxidase; CAT: Catalase; SOD: Superoxide dismutase; ACP: Acid phosphatase; ALP: Alkaline phosphatase; ATPase: Adenosine triphosphatase; O/N: Overnight.

Acknowledgements
The authors extend their appreciation to the researchers supporting project number (RSP-2021/173) from King Saud University, Riyadh, Saudi Arabia.

Authors' contributions
K.M.P.C. and S.S-N designed the experiments; K.M.P.C., S.K., V.S-R., R.R., and H.S. conducted the experiments; S.S-N, A.H.M, D.M.E. A.G. M.S.A, M.S.E., and W.B.H analysed the data, and wrote the manuscript. All authors reviewed, read, and approved the final manuscript.

Funding
This research was supported by the project grants from King Saud University, Riyadh, Saudi Arabia under project number RSP-2021/173.

Availability of data and materials
All available data are shown in the figures and tables.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors have given their personal consent for publication.

Competing interests
The authors declare that he has no competing interests.

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Received: 3 July 2021 Accepted: 24 July 2021
Published online: 22 September 2021

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