



OPEN Enhanced polyphenolic production in tissue culture-derived *Opuntia ficus-indica* increases larvicidal activity against *Spodoptera exigua*: biochemical, histological and molecular docking evidence

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The accelerating resistance of *Spodoptera exigua* to synthetic insecticides demands the development of eco-friendly botanical alternatives. Plant tissue culture provides a controlled means to enhance the biosynthesis of defense metabolites. This study explored how in vitro micropropagation can enhance the polyphenolic profile and insecticidal efficacy of *Opuntia ficus-indica* cladode ethanolic extracts. These extracts, cultured on Murashige and Skoog medium without (Op1) or with plant growth regulators (Op2), were compared with greenhouse-grown (Op3) extracts for activity against second- and fourth-instar *S. exigua* larvae. Phytochemical analyses revealed differences among the extracts. The Op2 extract exhibited the highest total phenolic content (61.1 mg GAE g⁻¹) and flavonoid content (131.95 mg QE g⁻¹). HPLC profiling revealed that Op2 had higher levels of gallic acid (26.94 µg mg⁻¹) and kaempferol (17.26 µg mg⁻¹), whereas the greenhouse-grown extract was dominated by catechin and gallic acid. Bioassays showed that Op2 was the most toxic, with LC₅₀ values of 4.87 and 2.70 mg mL⁻¹ for the second and fourth instars, respectively. Biochemical assays revealed metabolic disruption, indicated by a significant elevation in lactate dehydrogenase (LDH) activity (up to 5.20-fold) and a suppression of glutathione peroxidase (GPx) activity (up to 82.18%). Histopathological examination confirmed severe damage to the integument and midgut tissues. Molecular docking analysis indicated that kaempferol exhibited the highest binding affinity for LDH and GPx (−6.32 and −6.72 kcal mol⁻¹, respectively), while ferulic acid showed strong binding (−6.12 and −6.15 kcal mol⁻¹, respectively). These findings identify tissue culture-enhanced *O. ficus-indica* as a promising biopesticide for pest management.

Keywords Cladode extracts, Tissue culture, Polyphenols, HPLC profiling, Molecular docking

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is among the most economically significant polyphagous agricultural pests worldwide. This species infests a broad range of crops, including potato, tomato, legumes, asparagus, tobacco, cotton, and cereals, causing substantial quantitative and qualitative yield losses¹. Currently, chemical insecticides are the primary management strategy against this pest^{2,3}. However, sustained and indiscriminate use of synthetic insecticides has led to escalating environmental contamination, harmful effects on non-target organisms, and potential public health concerns^{4,5}. Insecticide resistance in *S. exigua* populations is also rapidly developing across diverse agroecosystems⁶. This resistance is mainly associated

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with enhanced detoxification enzyme systems and increased oxidative metabolic activity, which reduces insecticide efficacy and shortens their operational lifespan^{7–10}.

These limitations have driven the search for environmentally sustainable, biologically derived alternatives for pest management¹¹. Botanical insecticides and biological insecticide formulations have emerged as promising candidates because of their ecological compatibility, reduced residual toxicity, and potential suitability for integrated pest management programs^{11–16}. Plant tissue culture (PTC) provides a controlled biotechnological platform for producing plant-derived bioactive metabolites under aseptic, reproducible conditions¹⁷. These methods enable large-scale micropropagation, genetic transformation, pathogen elimination, and long-term preservation of germplasm¹⁸, thereby contributing to agricultural sustainability and industrial applications.

The prickly pear cactus, *Opuntia ficus-indica* (L.) Mill. is a perennial succulent species distinguished by flattened photosynthetic cladodes, high drought tolerance, and remarkable adaptability to arid and semi-arid environments^{19,20}. While primarily valued as a food and forage crop, this species has recently attracted scientific interest for its rich phytochemical composition, including phenolic acids, flavonoids, betalains, vitamins, and other antioxidant constituents^{21–24}. These metabolites have been associated with a wide range of biological activities, including antioxidant, antimicrobial, anti-inflammatory, and insecticidal properties^{25–31}. The abundance of phenolic compounds in *O. ficus-indica*, together with its medicinal and agricultural value, makes it an attractive candidate for the development of environmentally friendly pest management products.

Plants utilize several distinct defense mechanisms against herbivorous insects. These defenses include mechanical barriers—such as thickened cuticles and structural reinforcements—and chemical deterrents mediated by secondary metabolites^{32,33}. Secondary metabolites, synthesized from primary metabolic intermediates, act as natural toxicants, repellents, or growth inhibitors against herbivores. Compared with conventional field-based extraction, which is often limited by seasonal variability, environmental fluctuations, and low metabolite yields³⁴, in vitro culture systems can provide more consistent and standardized production of bioactive compounds under controlled laboratory conditions^{35–37}.

Among plant secondary metabolites, polyphenols, terpenes, alkaloids, and steroids are major classes defined by their biosynthetic origin and chemical structure¹⁶. Polyphenols are broadly classified into phenolic acids and flavonoids, which differ in molecular complexity and structural characteristics^{38,39}. These compounds play crucial physiological roles, including pigmentation, lignification, and protection against abiotic and biotic stresses^{40,41}. Polyphenolic compounds can interfere with insect feeding behavior, growth, development, digestion, and oxidative balance, making them promising candidates for botanical insecticide development⁴². Accordingly, improving their biosynthesis through tissue culture represents a potentially efficient strategy for developing sustainable pest management agents, particularly when natural supplies are unstable or production costs are high^{43–47}.

Recent advances in plant tissue culture have demonstrated that controlled in vitro conditions can enhance secondary metabolite biosynthesis by manipulating nutrient composition and plant growth regulators. Cytokinins such as benzyladenine and kinetin have been reported to stimulate phenylpropanoid metabolism and increase the accumulation of phenolic acids and flavonoids in several medicinal and economically important plant species^{44,47}. Despite the recognized phytochemical richness of *O. ficus-indica*, the extent to which tissue culture-mediated metabolite enhancement can improve larvicidal efficacy against economically important pests such as *S. exigua* remains insufficiently investigated.

In insects, oxidative stress and energy metabolism represent two interconnected physiological systems that are highly sensitive to xenobiotic exposure. Disturbances in antioxidant enzymes may impair cellular redox homeostasis, whereas alterations in metabolic enzymes can reflect physiological adaptation to toxic stress. Therefore, evaluating both antioxidant and metabolic biomarkers provides valuable insight into the mechanisms underlying insecticidal activity and the physiological responses of exposed insects^{48–51}.

Although the phytochemical composition and medicinal properties of *O. ficus-indica* have been extensively studied, the interrelationships among tissue culture-induced phytochemical enrichment, larvicidal activity, biochemical disruption, histopathological damage, and potential molecular interactions remain unexplored. Therefore, a comprehensive evaluation combining phytochemical, biological, and computational approaches is necessary.

The present study was designed to test whether tissue culture-mediated enhancement of polyphenolic compounds in *O. ficus-indica* could improve its larvicidal activity against *S. exigua*. To address this hypothesis, the insecticidal efficacy of in vitro-derived cladode extracts produced under different culture conditions was compared with that of extracts from greenhouse-grown plants. In addition, phytochemical profiling, biochemical biomarkers, histopathological examination, and molecular docking analyses were integrated to investigate potential mechanisms underlying the observed biological activity.

Materials and methods

Insect culture

A laboratory colony of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) was obtained from the Vegetable Pests Department, Plant Protection Research Institute, Giza, Egypt. The colony was maintained for several generations under controlled laboratory conditions at 27 ± 2 °C, $65 \pm 5\%$ relative humidity (RH), and a 10 h light/14 h dark photoperiod. Larvae were reared on fresh, insecticide-free castor leaves (*Ricinus communis* L.), which were replaced daily^{52,53}. Castor leaves were selected because they were readily available, insecticide-free, and suitable for maintaining a stable laboratory colony under standardized conditions, thereby minimizing variability among larvae used in the bioassays⁵⁴. Only healthy, synchronized second- and fourth-instar larvae were used in bioassays and subsequent biochemical and histological analyses.

Plant material and extract preparation

Seed germination and micropropagation of *Opuntia ficus-indica*

Seeds of *O. ficus-indica* (L.) Mill. were obtained from the Agricultural Research Center in Giza, Egypt, which served as the botanical authentication for the source material. Seeds were thoroughly rinsed under running tap water and surface-sterilized in 20% (v/v) sodium hypochlorite with a few drops of Tween-20 for 5–10 min with gentle agitation. Subsequently, seeds were rinsed three times with sterile distilled water. To overcome seed dormancy, seeds were pretreated with hydrogen peroxide (H_2O_2) and gibberellic acid (GA_3) before culture. Seeds were inoculated onto half-strength Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar⁵⁵. The pH was adjusted to 5.8, and the culture was incubated at 25 ± 2 °C under a 16 h light/8 h dark photoperiod at a light intensity of $40\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Cultures were maintained in cylindrical glass jars (7–8 cm in diameter and 10–12 cm in height; approximately 300 mL volume). Micropropagation was conducted by culturing germinated shoots on MS medium either without plant growth regulators (designated in vitro Op1) or supplemented with 1 mg L^{-1} kinetin and 2 mg L^{-1} benzyladenine (designated as in vitro Op2) (Fig. S1). The experiment was conducted in ten replicates, with one explant per culture jar. The regeneration success rate was 100%.

Cladode extract preparation

To prepare the tested cladode extracts, *O. ficus-indica* plantlets were micropropagated through successive subculture cycles. Small in vitro-derived cladodes were excised from shoots and transferred to MS medium supplemented with 0.5 mg L^{-1} indole-3-butyric acid (IBA) to induce rooting^{56,57}. After four months of growth, cladodes from both in vitro-derived plantlets and greenhouse-grown plants were harvested, chopped into small pieces, shade-dried at room temperature (27 ± 2 °C) until constant weight, and ground into a fine powder.

Extraction was performed by macerating 10 g of dried cladode powder in 100 mL of 70% ethanol (1:10 w/v). The mixture was incubated in a water bath at 35 °C for 15 min, then agitated continuously at room temperature for 72 h. The extract was filtered through Whatman No. 1 filter paper and concentrated by allowing the solvent to evaporate at room temperature under a gentle airstream until a dried crude extract was obtained⁵⁸. The extraction yield was 18% (1.8 g dried extract from 10 g powder). The dried extract was stored at 4 °C for one week prior to use.

Phytochemical analyses of *Opuntia ficus-indica* extracts

Total phenolic and flavonoid contents and HPLC profiling were performed for each of the three cladode extracts: in vitro Op1, in vitro Op2, and greenhouse Op3.

Determination of total phenolic content (TPC)

The phenolic content was determined by the Folin–Ciocalteu colorimetric method⁵⁹. Briefly, 0.5 mL of the extract (dissolved in methanol) was mixed with 2.5 mL of tenfold-diluted Folin–Ciocalteu reagent and incubated at room temperature for 5 min. Subsequently, 2.5 mL of sodium carbonate solution (75 g L^{-1}) was added. The reaction mixture was vortexed for 10 s and incubated at 25 °C in the dark for 2 h. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer (Unicam UV 300).

A standard calibration curve was prepared using gallic acid solutions at concentrations of 15.62, 31.25, 62.5, 125, 250 and 500 mg L^{-1} in methanol: water (50:50, v/v) (Fig. 1a). The regression equation derived from the calibration curve was used to calculate total phenolic content, expressed as mg of gallic acid equivalents (GAE) per gram of dry extract, using the following equation:

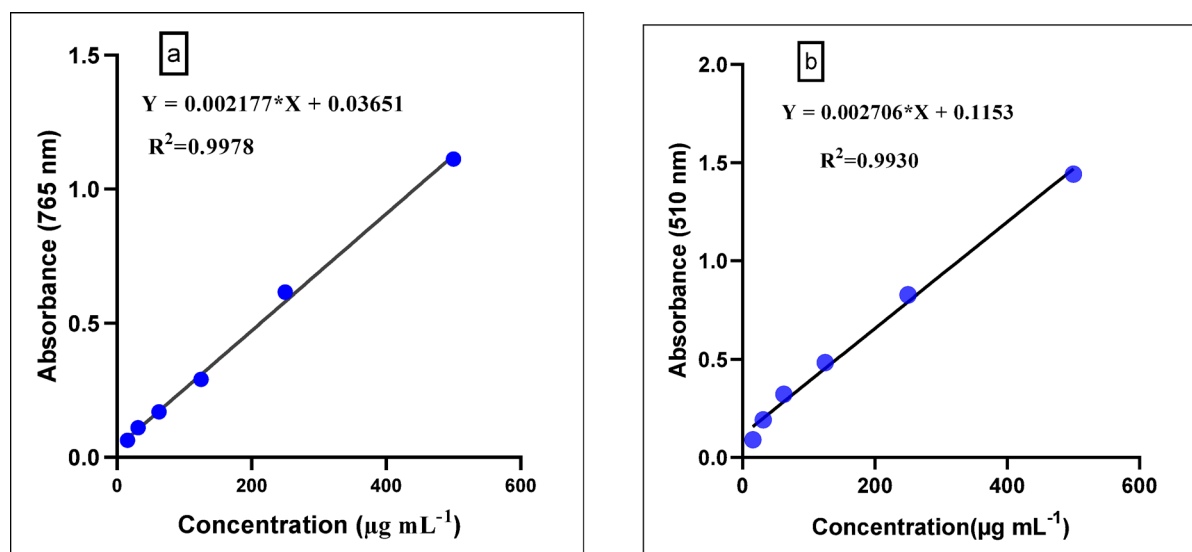


Fig. 1. Calibration curves for gallic acid (a) and quercetin (b).

$$PC = \frac{GAE \times V \times D}{W}$$

where GAE is the gallic acid equivalent (mg mL⁻¹), V is the total volume of the extract (mL), D is the dilution factor, and W is the sample weight (g). All measurements were performed in triplicate.

Determination of total flavonoid content (TFC)

The flavonoid content (FC) was determined using a modified aluminum chloride (AlCl₃) colorimetric method⁶⁰. Briefly, 0.5 mL of each extract (dissolved in methanol) was mixed with 75 µL of 5% (w/v) sodium nitrite (NaNO₂) and incubated at room temperature for 5 min. Subsequently, 1.25 mL of 10% (w/v) AlCl₃ was added, and the mixture was stirred for 5 min. Then, 0.5 mL of 1 M sodium hydroxide (NaOH) was added, and the final volume was adjusted to 5 mL with distilled water. The mixture was vortexed and incubated at room temperature for 5 min. Absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Unicam UV 300).

A standard calibration curve was constructed using quercetin solutions at concentrations of 15.62, 31.25, 62.5, 125, 250 and 500 µg mL⁻¹ prepared in methanol (Fig. 1b). The regression equation obtained from the calibration curve was used to calculate total flavonoid content, which was expressed as mg quercetin equivalents (QE) per gram of dry extract according to the following equation:

$$FC = \frac{QE \times V \times D}{W}$$

where QE denotes the quercetin-equivalent concentration (mg mL⁻¹), V is the total extract volume (mL), D is the dilution factor, and W is the sample weight (g). All measurements were performed in triplicate.

High-performance liquid chromatography (HPLC) analysis of phenolic compounds

Each crude cladode extract, namely in vitro Op1, in vitro Op2, and greenhouse Op3, was analyzed separately for phenolic profiling by HPLC on an Agilent 1260 Infinity system (Agilent Technologies, USA), following the procedures described by^{61,62}. The analysis was performed at the HPLC Unit, Central Laboratory, National Research Center (NRC), Dokki, Giza, Egypt, using a validated HPLC method routinely applied for phytochemical analysis. Samples were dissolved in methanol and filtered through a 0.45 µm membrane filter prior to injection. Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm). The mobile phase comprised two solvents: water with 0.05% (v/v) trifluoroacetic acid (TFA) (Solvent A) and acetonitrile with 0.05% TFA (Solvent B), delivered at a flow rate of 0.9 mL min⁻¹. Gradient elution was performed as follows: 82% Solvent A (18% Solvent B) from 0 to 1 min; linearly decreasing to 75% Solvent A (25% Solvent B) from 1 to 11 min; further decreasing to 60% Solvent A (40% Solvent B) from 11 to 18 min; and returning to the initial conditions of 82% Solvent A (18% Solvent B) from 18 to 24 min to re-equilibrate the column. The column temperature was set at 40 °C. The injection volume was 5 µL per sample. Detection was performed using a diode-array detector (DAD) set to 280 nm. Phenolic compounds were identified by comparing their retention times and UV spectra at 280 nm with those of authentic standards analyzed under identical chromatographic conditions. The standards included gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringic acid, rutin, ellagic acid, p-coumaric acid, vanillin, ferulic acid, naringenin, rosmarinic acid, daidzein, quercetin, cinnamic acid, and kaempferol. Calibration curves were constructed for the target analytes using analytical-grade reference standards across appropriate concentration ranges and were used to quantitatively determine them in the plant extracts.

Larvicidal bioassay

Stock solutions of in vitro Op1, in vitro Op2, and greenhouse Op3 extracts were prepared by dissolving each crude extract in distilled water containing 0.1% Tween-80 as an emulsifier. Working concentrations were prepared by serial dilution of the stock solution to 2.5, 5, 7.5 and 10 mg mL⁻¹ for each extract type. Fresh castor leaf discs were cut into uniform sections, immersed in the respective treatment solutions for 20 s, and then dried at room temperature. Ten larvae were placed on each treated leaf disc, with five replicates per concentration (n = 50 larvae per treatment). Mortality data were corrected using Abbott's formula when control mortality was observed. The tested concentrations were selected based on preliminary range-finding assays and were intended to span a dose range that produced 0–100% mortality. Larval mortality was recorded at 24, 72, 120 and 168 h post-treatment. Larvae were considered dead if they showed no movement upon gentle probing. Azadirachtin (Neemix® 4.5 EC) served as the positive control, while distilled water with 0.1% Tween-80 served as the negative control.

Biochemical assays on *Spodoptera exigua*

Second- and fourth-instar *S. exigua* larvae were exposed to the previously determined LC₅₀ concentrations of in vitro Op1, in vitro Op2, and greenhouse Op3 cladode extracts. Seventy-two hours after treatment, surviving larvae were selected for hemolymph collection. Hemolymph was obtained by excising the forelegs of live larvae and immediately transferred into Eppendorf tubes lightly coated with crystalline phenylthiourea to prevent melanization⁶³. For each treatment and larval instar, hemolymph from 20 larvae was pooled, and three independent replicates were prepared. Samples were homogenized in chilled distilled water at a concentration of 50 mg mL⁻¹ and centrifuged at 7000 rpm for 15 min to obtain the supernatant for biochemical analyses¹⁴. Absorbance was measured using a UV-visible spectrophotometer (BioSystem 310, Biosystem, Spain).

Each enzymatic assay was performed in three technical replicates using commercial kits according to the manufacturers' instructions. LDH activity was determined using a Lactate dehydrogenase Liquizyme kit

(Spectrum Diagnostics, Egyptian Company for Biotechnology, Egypt; Cat. No. 283001), while GPx activity was measured using a Glutathione Peroxidase UV method kit (Biodiagnostic, Egypt; Cat. No. GP 2524).

Determination of total protein content

Total protein content in the hemolymph of *S. exigua* larvae was determined using the Bradford method⁶⁴. The Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 mL of 95% ethanol, then adding 100 mL of 85% (w/v) phosphoric acid. The final volume was adjusted to 1 L with distilled water, and the solution was filtered before use. A 10 μ L aliquot of hemolymph was mixed with 200 μ L of Bradford reagent, and absorbance was measured at 595 nm after 2 min of incubation at room temperature and within 1 h of the reaction. A reagent blank containing phosphate buffer and Bradford reagent was used for background correction. Protein concentrations were calculated using a calibration curve constructed with bovine serum albumin as the standard and were expressed as mg mL⁻¹. For enzyme normalization, the activity value obtained for each sample was divided by its corresponding total protein concentration. Therefore, LDH and GPx activities were expressed as U mg⁻¹ protein.

Lactate dehydrogenase (LDH) activity

LDH activity was determined by monitoring the oxidation of NADH at 340 nm according to the manufacturer's protocol. The assay was based on the conversion of pyruvate and NADH to L-lactate and NAD⁺. The decrease in absorbance at 340 nm is directly proportional to LDH activity.

Briefly, 1.0 mL of working reagent was mixed with 20 μ L of enzyme sample in a cuvette at 37 °C. After mixing, the initial absorbance was read after 30 s, and the decrease in absorbance was then recorded at 1-min intervals for 3 min against a reagent blank. LDH activity was calculated according to the manufacturer's equation:

$$\text{LDH activity U L}^{-1} = 8095 \times \Delta A_{340}/\text{min}$$

where $\Delta A_{340}/\text{min}$ represents the mean decrease in absorbance per minute at 340 nm. The obtained LDH activity was then divided by the total protein concentration of the corresponding sample and expressed as U mg⁻¹ protein.

Glutathione peroxidase (GPx) activity

GPx activity was measured by monitoring NADPH oxidation at 340 nm, following the manufacturer's instructions. The assay was based on the oxidation of NADPH to NADP⁺ as oxidized glutathione was reduced by glutathione reductase. The decrease in absorbance at 340 nm is directly proportional to GPx activity. The reaction mixture contained 1.0 mL of assay buffer, 0.1 mL of NADPH reagent, 0.01 mL of enzyme sample, and 0.1 mL of diluted hydrogen peroxide substrate. After mixing, the decrease in absorbance at 340 nm was recorded over 3 min, with deionized water as the blank. GPx activity was calculated using the following equation:

$$\text{GPx activity} = (\Delta A_{340}/\text{min}/0.00622) \times 121$$

where 0.00622 is derived from the NADPH extinction coefficient at 340 nm, and 121 is the assay dilution factor calculated from the total reaction volume to sample volume ratio of 1.21/0.01. The calculated GPx activity was then normalized to the total protein concentration and expressed as U mg⁻¹ protein.

Histological analysis

Tissue fixation and processing

Histological examination of the integument and midgut was performed on second- and fourth-instar *S. exigua* larvae that survived exposure to the previously determined LC₅₀ concentrations of in vitro-derived Op1 and Op2, as well as greenhouse Op3 -derived cladode extracts. Control larvae of the same instars remained untreated. Larvae were dissected in Ringer's solution, and tissues were carefully excised and immediately fixed in aqueous Bouin's solution. After fixation, tissues were rinsed in 0.9% NaCl to remove excess picric acid and then placed in 10% neutral buffered formalin (NBF) for further preservation. The tissues were prepared for paraffin sectioning. Then, tissues were placed in cassettes and processed through a series of steps, including refixation in 10% NBF for 3 h, followed by dehydration in an ascending ethanol series (70%, 90%, and 95%) for 1 h each. Further dehydration was achieved in absolute ethanol for 3 h, followed by clearing in two changes of xylene for a total of 3 h. Finally, the tissues were infiltrated with molten paraffin wax for 2 h and embedded in blocks using a Paraffin Embedding Center (EC-350).

Staining of histological sections

Paraffin-embedded tissues were sectioned at 3–6 μ m with a semi-automatic microtome and mounted on clean glass slides. Staining was performed as described in⁶⁵. Sections were deparaffinized with two changes of xylene (3 min each) and rehydrated through a descending ethanol series (100, 90, 80 and 60%; 15 dips each), followed by a 2-min rinse in distilled water. Nuclei were stained with hematoxylin for 1–2 min, then washed under running tap water for 5 min to allow proper blueing. Cytoplasmic structures were counterstained with phloxine/eosin for 1 min. After staining, sections were dehydrated through an ascending ethanol series (2 min each), cleared in two changes of xylene (2 min each), and mounted in DPX medium. Ten larvae per treatment were randomly selected for histological evaluation. For each larva, five non-consecutive sections were analyzed to ensure the consistency of the observed pathological alterations. Sections were examined and photographed at 10 $\times$ magnification using a LEICA DM 750 light microscope equipped with LAS X software version 3.0, and the most representative images were selected for presentation.

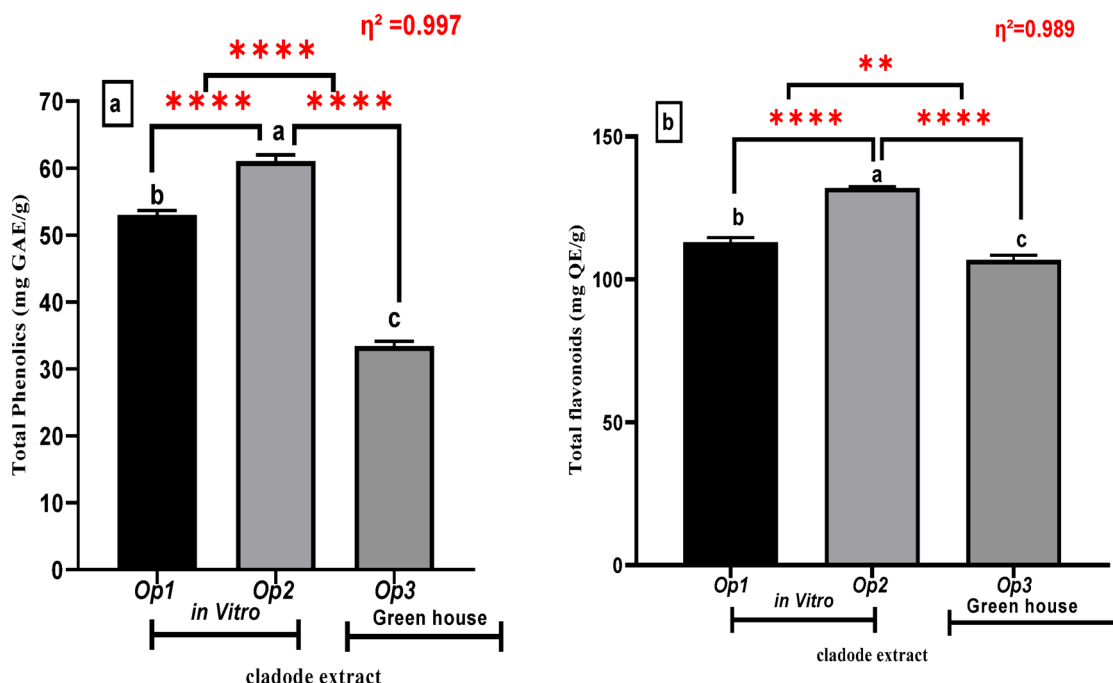


Fig. 2. Total phenolic (a) and flavonoid (b) contents in the tested cladode extracts. Different letters above the bars indicate significant differences according to Tukey's HSD test. (**) and (****) indicate significance levels at $P \leq 0.01$ and $P \leq 0.0001$, respectively. F value = 1004.49 df = (2,6) $P < 0.0001$ for a & F value = 261.37 df = (2,6) $P < 0.0001$ for b η^2 = eta squared (effect size; values ≥ 0.14 indicate a large effect).

Molecular docking analysis

Molecular docking analysis was performed to evaluate the potential binding affinity and interaction patterns of selected compounds with *S. exigua* LDH and GPx target proteins. The docked compounds were selected from the HPLC-identified phenolic and flavonoid constituents, with an emphasis on compounds abundant in the Op2 extract that were previously reported to possess insecticidal or enzyme-modulating activity. The amino acid sequences of the target proteins were retrieved from the UniProt database using the accession numbers A0A922SB74 for LDH and A0A835G3S5 for GPx. Since experimentally resolved three-dimensional structures were not available for these *S. exigua* proteins, AlphaFold-predicted models were used for docking analysis. The reliability of the predicted protein structures was evaluated using AlphaFold confidence metrics, including per-residue predicted Local Distance Difference Test (pLDDT) scores and Predicted Aligned Error (PAE) maps. Structurally reliable regions, particularly those with pLDDT scores ≥ 70 , were considered suitable for docking analysis.

The protein structures were prepared by removing water molecules and other non-essential components, followed by protonation, correction of incomplete valence states, energy minimization, and saving in PDBQT format⁶⁶. Similarly, the tested compounds were prepared by protonation and energy minimization, and the resulting structures were saved as PDBQT files⁶⁷. Docking simulations were performed using AutoDock Vina version 1.5.7⁶⁸. A blind docking approach was applied, and the best-scoring ligand conformations were selected for further analysis. Because no experimentally confirmed active sites were available for the selected *S. exigua* LDH and GPx targets, no separate active-site prediction tool was used. Instead, the docking grid was defined to cover the target protein structure, allowing the docking algorithm to explore potential ligand-binding regions without prior bias toward a specific binding pocket.

The grid box parameters used for docking were as follows: for *S. exigua* LDH, the grid center was set at $x = -0.479$, $y = -2.959$, and $z = -7.781$, with box dimensions of $30 \times 30 \times 30$ Å and spacing of 0.381 Å. For *S. exigua* GPx, the grid center was set at $x = 0.589$, $y = -13.263$, and $z = -9.532$, with box dimensions of $30 \times 30 \times 30$ Å and spacing of 0.375 Å. The docking scores of the most favorable ligand poses were recorded, and the corresponding three-dimensional (3D) and two-dimensional (2D) interaction visualizations were generated using Discovery Studio 2024⁶⁹.

Standard RMSD validation through re-docking of a co-crystallized ligand could not be performed because no experimentally determined protein–ligand complexes or native ligands are currently available for the selected *S. exigua* LDH and GPx targets. Therefore, the docking results were interpreted comparatively using binding-affinity rankings and the structural plausibility of ligand–protein interactions, including hydrogen bonding, hydrophobic contacts, and electrostatic interactions.

Statistical analysis

Lethal concentration values, including LC_{50} , LC_{90} , and their 95% lower and upper confidence limits (LCL–UCL), were estimated using LDP-Line Probit Analysis according to Finney⁷⁰. The probit analysis was used to

Peak	RT*	Polyphenolic Comp	In vitro				Greenhouse		Molecular formula	Insecticidal activity
			Op1		Op2		Op3			
			Area %	Conc. µg mg ⁻¹	Area %	Conc. µg mg ⁻¹	Area %	Conc. µg mg ⁻¹		
1	3.56	Gallic acid	51.5	20.17	32.25	26.94	57.1	107.1	C ₇ H ₆ O ₅	71
2	4.24	Chlorogenic acid	5.74	4.35	2.32	3.74	0.83	2.99	C ₁₆ H ₁₈ O ₉	72
3	4.54	Catechin	2.63	3.30	3.28	8.77	24.96	149.9	C ₁₅ H ₁₄ O ₆	73
4	5.63	Methyl gallate	6.10	1.87	10.23	6.69	2.50	3.67	C ₈ H ₈ O ₅	74
5	6.04	Caffeic acid	3.38	1.09	5.65	3.90	1.53	2.37	C ₉ H ₈ O ₄	75
6	6.45	Syringic acid	7.33	2.54	1.80	1.33	4.52	7.48	C ₉ H ₁₀ O ₅	76
7	6.86	Rutin	3.65	2.87	1.72	2.89	0.21	0.80	C ₂₇ H ₃₀ O ₁₆	77
8	7.36	Ellagic acid	3.97	2.44	2.32	3.04	0.06	0.16	C ₁₄ H ₆ O ₈	78
9	8.39	Coumaric acid	2.86	0.57	6.12	2.58	0.21	0.20	C ₉ H ₈ O ₃	79
10	9.39	Vanillin	1.38	0.25	0.70	0.27	4.21	3.69	C ₈ H ₈ O ₃	80,81
11	9.69	Ferulic acid	3.72	1.16	3.65	2.43	ND	ND	C ₁₀ H ₁₀ O ₄	82
12	10.52	Naringenin	1.26	0.66	1.91	2.13	0.93	2.34	C ₁₅ H ₁₂ O ₅	83
13	12.28	Rosmarinic acid	1.88	0.90	3.41	3.49	1.02	2.35	C ₁₈ H ₁₆ O ₈	84
14	16.83	Daidzein	0.82	0.25	5.52	3.64	0.19	0.27	C ₁₅ H ₁₀ O ₄	85
15	17.41	Quercetin	2.80	1.96	3.10	4.63	1.22	4.10	C ₁₅ H ₁₀ O ₇	86,87
16	19.73	Cinnamic acid	ND**	ND	1.40	0.35	0.16	0.09	C ₉ H ₈ O ₂	88
17	20.65	Kaempferol	1.01	0.56	14.61	17.26	0.29	0.77	C ₁₅ H ₁₀ O ₆	89

Table 1. Compounds identified in in vitro-derived (Op1 and Op2) and greenhouse Op3 cladode extracts. *RT, Retention time; and **ND, not detected.

calculate the slope $\pm$ SE, chi-square (χ^2) goodness-of-fit values, significance levels, regression coefficients (R^2), and regression equations. Normality of continuous data was assessed using the Kolmogorov–Smirnov test, while homogeneity of variance was evaluated using Levene's test before performing ANOVA. Calibration curves for gallic acid and quercetin were generated using linear regression analysis. Total phenolic and flavonoid contents were analyzed using one-way analysis of variance (ANOVA) with SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Larval mortality percentages were arcsine square-root transformed before ANOVA to satisfy parametric assumptions; however, untransformed mortality means were presented in the figures and supplementary tables for biological clarity. Mortality data were analyzed separately for each larval instar using a three-way factorial ANOVA to evaluate the main effects of extract type, concentration, and exposure time, as well as their two- and three-way interactions. LDH and GPx enzyme activities were analyzed using one-way ANOVA across the eight treatment–instar groups, including the second- and fourth-instar controls and larvae treated with the LC₅₀ concentrations of the tested cladode extracts. When significant differences were detected, means were separated using Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. Highly significant differences were indicated at $P \leq 0.01$ or $P \leq 0.0001$, where applicable. For enzyme assays, percentage change was calculated as: [(treated value – control value) / control value] $\times$ 100. The enzyme activity ratio was calculated as the treated mean / the control mean. Effect size was expressed as eta squared (η^2), with values ≥ 0.14 considered indicative of a large effect. Results are presented as mean $\pm$ standard error (SE).

Results

Assessment of phenolic and flavonoid contents

The phenolic and flavonoid contents of the cladode extract from in vitro-derived (Op1, Op2) and greenhouse Op3 derived plants are presented in Fig. 2. Phenolic content was determined using the regression equation derived from the standard curve ($Y = 0.002177X + 0.03651$), which showed a high regression coefficient ($R^2 = 0.9978$) (Fig. 1a). ANOVA indicated highly significant differences among the tested cladode extracts in total phenolic levels [$F(2, 6) = 1004.49$, $P < 0.0001$, $\eta^2 = 0.997$] (Fig. 2a). The in vitro Op2 extract had the highest total phenolic compound concentration (61.1 mg g⁻¹), followed by Op1 (53.0 mg g⁻¹), while the greenhouse Op3 cladode extract had the lowest value (33.4 mg g⁻¹).

Total flavonoid levels in the extracts were quantified using the regression equation derived from the calibration curve ($Y = 0.002706X + 0.1153$; $R^2 = 0.9930$) (Fig. 1b). As shown in Fig. 2b, there were highly significant differences in flavonoid content among the tested cladode extracts [$F(2, 6) = 261.37$, $P < 0.0001$, $\eta^2 = 0.989$]. The in vitro Op2 plants exhibited the highest flavonoid content (131.95 mg g⁻¹), followed by in vitro Op1 (112.98 mg g⁻¹) and the greenhouse Op3 extracts (106.8 mg g⁻¹).

HPLC profiling of polyphenolic compounds in the tested cladode extracts

HPLC analysis showed clear variation in the phenolic and flavonoid profiles of the three *O. ficus-indica* cladode extracts. The identified compounds included gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringic acid, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, rosmarinic acid, daidzein, quercetin, cinnamic acid, and kaempferol. The retention data, peak areas, and concentrations are presented

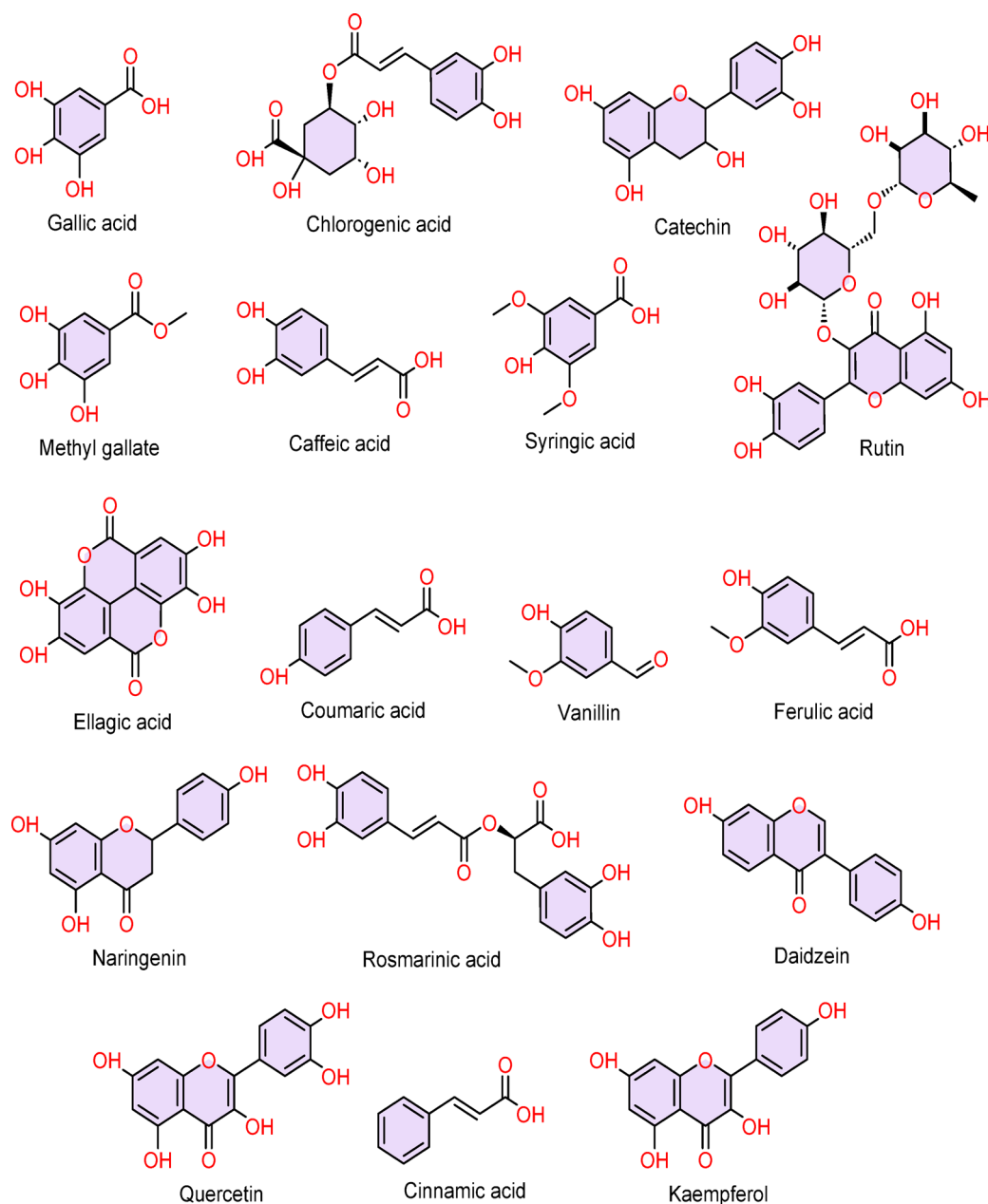


Fig. 3. Chemical structures of the polyphenols identified in the tested cladode extracts.

in Table 1, while the representative chromatograms are shown in Fig. S2a–c. The chemical structures of the identified compounds are illustrated in Fig. 3.

The *in vitro* Op1 extract showed a high gallic acid level, reaching $20.17 \mu\text{g ml}^{-1}$, with a peak area of 51.5%. However, the other active compounds were detected at lower levels, including catechin $3.30 \mu\text{g ml}^{-1}$, quercetin $1.96 \mu\text{g ml}^{-1}$, ferulic acid $1.16 \mu\text{g ml}^{-1}$, and kaempferol $0.56 \mu\text{g ml}^{-1}$. Cinnamic acid was not detected in this extract, as shown in Fig. S2a and Table 1. The *in vitro* Op2 extract contained a more balanced profile of the major active compounds. Gallic acid was detected at $26.94 \mu\text{g ml}^{-1}$ with a peak area of 32.3%, followed by kaempferol at $17.26 \mu\text{g ml}^{-1}$, catechin at $8.77 \mu\text{g ml}^{-1}$, quercetin at $4.63 \mu\text{g ml}^{-1}$, and ferulic acid at $2.43 \mu\text{g ml}^{-1}$. This extract also showed a markedly higher kaempferol content than the other two extracts, as shown in Fig. S2b and Table 1. The greenhouse Op3 extract was mainly dominated by gallic acid and catechin. Gallic acid reached $107.08 \mu\text{g ml}^{-1}$ with a peak area of 57.1%, while catechin showed the highest concentration among the detected compounds at $149.91 \mu\text{g ml}^{-1}$ with a peak area of 24.9%. In contrast, kaempferol was present only at a very low level ($0.77 \mu\text{g ml}^{-1}$), and ferulic acid was not detected, as shown in Fig. S2c and Table 1.

Toxicity bioassay

The insecticidal bioassay of *in vitro*-derived Op1 and Op2 extracts and the greenhouse-derived Op3 cladode extract against the second- and fourth-instar larvae of *S. exigua* is presented in Fig. 4a and b, with results

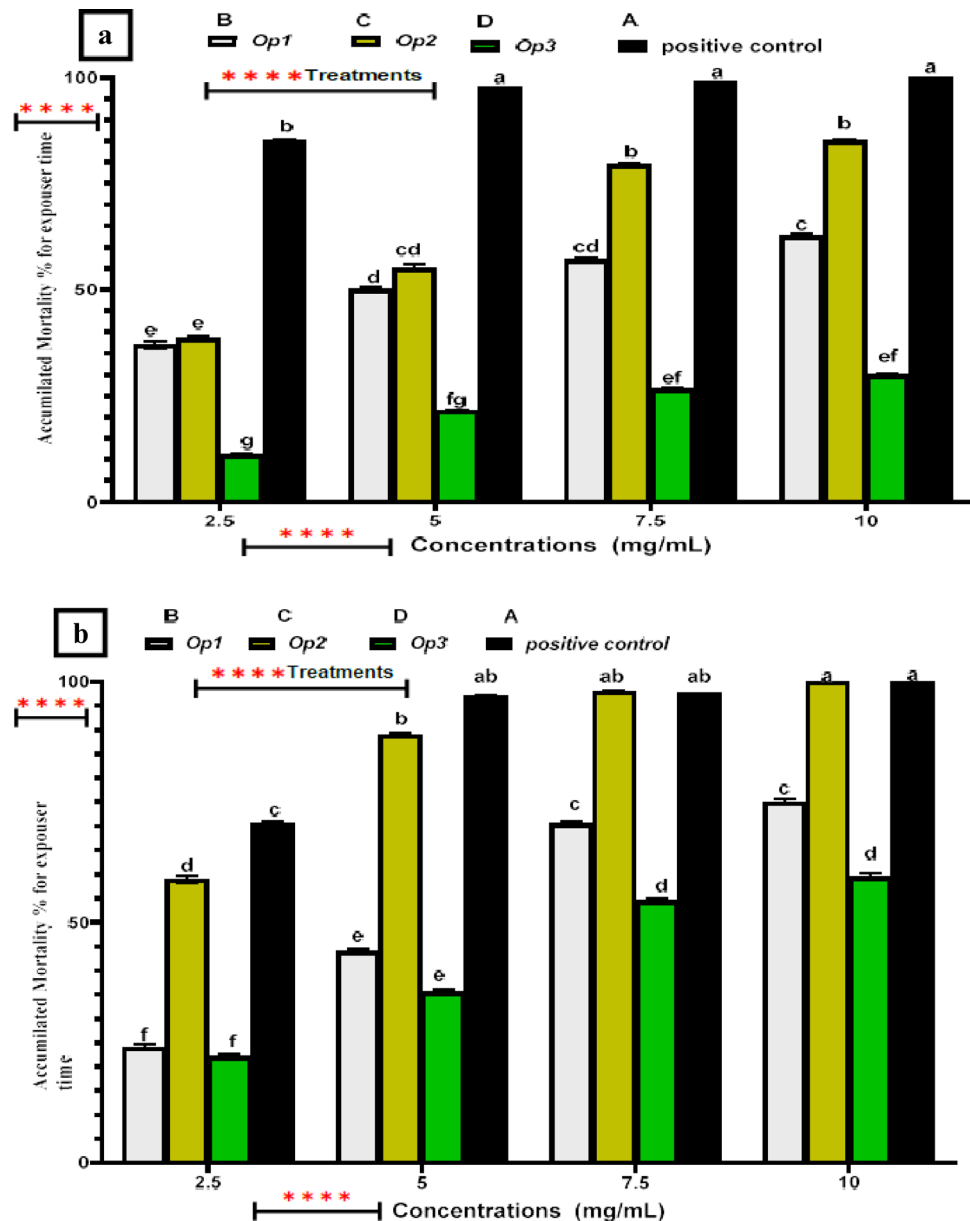


Fig. 4. Mortality (%) of *Spodoptera exigua* (a) second- and (b) fourth-instar larvae. Different letters above the bars indicate significant differences according to Tukey's HSD test at $P \leq 0.05$. **** indicates a highly significant difference ($P \leq 0.0001$).

compared to the positive control Neemix 4.5 EC. All tested extracts exhibited larvicidal activity, though with varying efficacy.

For the second larval instar, three-way factorial ANOVA revealed highly significant effects of plant extract type [$F(3, 256) = 649.56, P < 0.0001, \eta^2 = 0.884$], concentration [$F(3, 256) = 99.37, P < 0.0001, \eta^2 = 0.538$], and exposure time [$F(3, 256) = 241.97, P < 0.0001, \eta^2 = 0.739$].

Significant interaction effects were also observed for plant extract $\times$ concentration [$F(9, 256) = 9.176, P < 0.0001, \eta^2 = 0.244$], plant extract $\times$ treatment time [$F(9, 256) = 28.511, P < 0.0001, \eta^2 = 0.501$], concentrations $\times$ treatment times [$F(9, 256) = 5.854, P < 0.0001, \eta^2 = 0.171$], and the three-way interaction among plant extract, concentration, and exposure time [$F(27, 256) = 2.524, P < 0.0001, \eta^2 = 0.210$]. At 10 mg mL^{-1} , Op2 exhibited the strongest larvicidal activity against the 2nd instar, causing $85.00 \pm 0.39\%$ mortality, followed by Op1 ($62.50 \pm 0.69\%$), whereas the greenhouse Op3 extract produced the lowest mortality ($30.00 \pm 0.23\%$) (Fig. 4a, Table S1).

Similarly, for the fourth larval instar, ANOVA results revealed highly significant effects of plant extracts [$F(3, 256) = 523.510, P < 0.0001, \eta^2 = 0.860$], tested concentrations [$F(3, 256) = 295.503, P < 0.0001, \eta^2 = 0.776$], and exposure times [$F(3, 256) = 307.429, P < 0.0001, \eta^2 = 0.783$]. Significant interaction terms were also detected for plant extracts $\times$ concentrations [$F(9, 256) = 9.356, P < 0.0001, \eta^2 = 0.248$], plant extracts $\times$ exposure times [$F(9, 256) = 22.091, P < 0.0001, \eta^2 = 0.437$], concentrations $\times$ exposure times [$F(9, 256) = 14.415, P < 0.0001, \eta^2 = 0.336$],

Treatment	Instar	LC ₅₀ (mg mL ⁻¹) (LCL–UCL) ^b	Slope ± SE	χ ² (Sign.) ^c	R ^{2d}	RE	TI ₅₀ ^e	RP _{50f}
In vitro	Op1	2nd	7.494 (7.494–8.882)	2.28 ± 0.32	1.12(0.57)	0.97	– 1.31 + 1.71x	13.48
		4th	6.346 (5.796–6.966)	3.50 ± 0.40	3.81(0.14)	0.97	– 2.83 + 3.43x	37.96
	Op2	2nd	4.873 (4.416–5.329)	3.75 ± 0.34	1.18(0.55)	0.96	– 2.07 + 3.57x	20.72
		4th	2.702 (2.423–2.952)	5.34 ± 0.58	1.83(0.40)	1.00	– 2 + 5x	89.16
Greenhouse	2nd	15.965 (12.159–27.683)	2.19 ± 0.41	3.07(0.22)	0.93	– 3.36 + 2.86x	6.33	0.06
	4th	10.446 (9.105–12.863)	2.96 ± 0.42	3.80(0.15)	0.97	– 3.57 + 3.57x	23.02	0.23
PC ^a	2nd	1.010 (0.326–1.542)	2.87 ± 0.70	2.20(0.33)	1.00	0.575 + 1.75x	100.0	1.00
	4th	2.409 (2.127–2.641)	5.74 ± 0.77	0.35(0.84)	1.00	– 1.5 + 5x	100.0	1.00

Table 2. Probit regression analysis of the tested cladode extracts against the second and fourth instar larvae of *Spodoptera exigua*. ^aPC: positive control (Neemix[®] 4.5 EC). ^bLCL: lower 95% confidence limit; UCL: upper 95% confidence limit. ^cχ²: chi-square value (significance level). ^dR²: regression coefficient; R.E.: regression equation. ^eToxicity index (TI) for LC₅₀ = (LC₅₀ of the most toxic insecticide / LC₅₀ of the tested insecticide) × 100, following ⁹⁰. ^fRelative potency (R.P.) = LC₅₀ of the standard / LC₅₀ of the tested plant extract following ⁹¹.

			Lactate dehydrogenase (LDH)			F value (<i>P</i>)	Glutathione peroxidase (GP _x)			F value (<i>P</i>)
			Mean	Change%	Activity ratio		Mean	Change%	Activity ratio	
In vitro	Op1	2nd	34.64 ± 0.69 ^c	+ 130.42	2.30	2336.8**** (<i>P</i> = 0.00) <i>df</i> (7,16)	17.06 ± 0.35 ^d	– 50.56	0.49	570.5**** (<i>P</i> = 0.00) <i>df</i> (7,16)
		4th	71.27 ± 0.38 ^b	+ 277.12	3.77		9.38 ± 0.82 ^c	– 74.64	0.25	
	Op2	2nd	42.73 ± 0.58 ^d	+ 184.21	2.84		9.54 ± 0.34 ^c	– 72.37	0.28	
		4th	98.30 ± 0.46 ^a	+ 420.11	5.20		6.59 ± 0.42 ^f	– 82.18	0.18	
Greenhouse	2nd	33.28 ± 0.99 ^e	+ 121.35	2.21	21.71 ± 0.25 ^c		– 37.10	0.63		
	4th	65.67 ± 0.67 ^c	+ 247.48	3.47	19.31 ± 0.61 ^d		– 47.79	0.52		
Cont	2nd	15.03 ± 0.09 ^g	–		34.50 ± 0.40 ^b		–			
	4th	18.9 ± 0.45 ^f	–		36.97 ± 0.38 ^a		–			

Table 3. Percentage change and activity ratio of selected enzymes in second- and fourth-instar *Spodoptera exigua* larvae treated with the LC₅₀ concentrations of the tested cladode extracts after 72 h. Different letters in the same column indicate significant differences according to Tukey's HSD test at $P \leq 0.05$; **** indicates highly significant at $P \leq 0.0001$. Activity ratio of the enzyme = treated mean / control mean following ⁹². Change percentage of enzyme activity = (treated value – control value / control value) × 100; (+) indicates an increase and (–) indicates a decrease relative to the corresponding control.

and the three-way interaction [F (27, 256) = 4.621, $P < 0.0001$, $\eta^2 = 0.328$]. Additionally, Op2 achieved complete mortality ($100 \pm 0.00\%$), outperforming Op1 ($75.00 \pm 0.55\%$), while the greenhouse-grown extract produced a moderate effect, inducing $59.50 \pm 0.63\%$ mortality (Fig. 4b, Table S1). Regression parameters, toxicity indices, and relative potency values are presented in Table 2. Probit analysis confirmed insecticidal activity of all tested extracts against both larval instars, though toxicities varied markedly. For the second instar, Op2 was the most potent extract (LC₅₀ = 4.873 mg mL⁻¹; LC₉₀ = 11.136 mg mL⁻¹), followed by Op1 (LC₅₀ = 7.494 mg mL⁻¹; LC₉₀ = 27.339 mg mL⁻¹) and the greenhouse Op3 extract (LC₅₀ = 15.965 mg mL⁻¹; LC₉₀ = 61.534 mg mL⁻¹). A similar trend was observed for the fourth-instar, with Op2 showing the highest toxicity (LC₅₀ = 2.702 mg mL⁻¹; LC₉₀ = 4.698 mg mL⁻¹), followed by Op1 (LC₅₀ = 6.346 mg mL⁻¹; LC₉₀ = 14.752 mg mL⁻¹) and the greenhouse OP3 extract (LC₅₀ = 10.446 mg mL⁻¹; LC₉₀ = 28.276 mg mL⁻¹).

Effect of cladode extracts on *S. exigua* enzymatic activity

Glycolytic enzyme lactate dehydrogenase (LDH)

As shown in Table 3 and Fig. 5a, treatment with LC₅₀ concentrations of the tested cladode extracts significantly altered LDH activity in both second- and fourth-instar *S. exigua* larvae [F(7, 16) = 2336.76, $P < 0.0001$, $\eta^2 = 0.999$]. In second-instar larvae, LDH activity increased markedly with all extracts. The in vitro Op2 extract produced the strongest stimulation, increasing enzyme activity by 184.21% relative to the control (2.84-fold). This was followed by in vitro Op1 and the greenhouse-grown extract, which induced increases of 130.42% (2.30-fold) and 121.35% (2.21-fold), respectively. A similar trend was observed in fourth-instar larvae, with all treatments significantly increasing LDH activity. The in vitro Op2 extract again showed the highest induction, with a

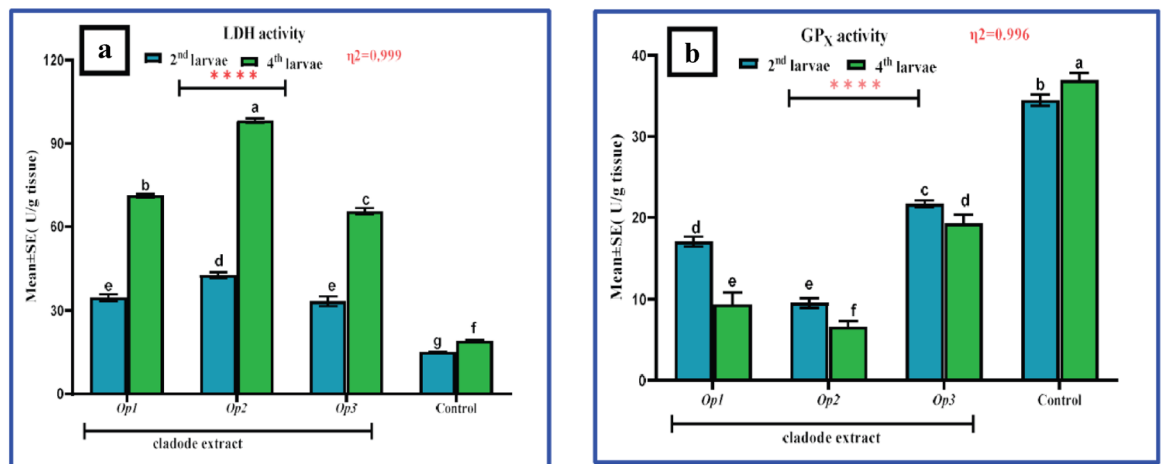


Fig. 5. Changes in (a) lactate dehydrogenase (LDH) and (b) glutathione peroxidase (GPx) activities in second- and fourth-instar *Spodoptera exigua* larvae 72 h post-treatment with the LC₅₀ concentrations of the tested cladode extracts. Bars represent mean ± SE. Different letters above bars indicate significant differences among treatment–instar groups according to Tukey’s HSD test at $P \leq 0.05$. **** indicates highly significant differences at $P \leq 0.0001$. η^2 = eta squared effect size; values ≥ 0.14 indicate a large effect.

420.11% increase (5.20-fold). In comparison, in vitro Op1 and the greenhouse Op3 extract caused moderate but significant increases of 277.12% (3.77-fold) and 247.48% (3.47-fold), respectively, relative to the control.

Antioxidant enzyme glutathione peroxidase (GPx)

Changes in GPx activity are presented in Table 3 and Fig. 5b. In contrast to LDH, GPx activity was significantly reduced in both larval instars after treatment with the tested extracts [$F(7, 16) = 570.46$, $P < 0.0001$, $\eta^2 = 0.996$]. In second-instar larvae, the greatest suppression was observed with the in vitro Op2 extract, which decreased GPx activity by 72.37% (activity ratio = 0.28). The in vitro Op1 extract caused a 50.56% reduction (activity ratio = 0.49), while the greenhouse Op3 extract produced a moderate decrease of 37.10% (activity ratio = 0.63) relative to the control. Similarly, in fourth-instar larvae, GPx activity was significantly inhibited by all treatments. The strongest reduction was again induced by in vitro Op2 (82.18%), followed by the in vitro Op1 (74.64%) and the greenhouse Op3 extract (47.79%), with corresponding activity ratios of 0.18, 0.25, and 0.52, respectively.

Histological analysis

Histological analysis of the integument and midgut of *S. exigua* second- and fourth-instar larvae treated with cladode extracts of *O. ficus-indica* (in vitro Op1, Op2, and greenhouse Op3) was conducted at the LC₅₀ concentration (Figs. 6, 7, 8 and 9).

Integument histology

Histological characteristics of untreated larvae showed normal integument architecture, with well-organized cuticular layers, including distinct epicuticle and endocuticle, an intact hypodermis, clearly defined muscle fibers, a compact fat body, and uniformly distributed cytoplasmic granules, indicating normal structural integrity of the integument. In contrast, larvae treated with the tested *O. ficus-indica* extracts showed marked histopathological alterations, including disruption and separation of cuticular layers, degeneration of the hypodermis, and degradation of the fat body. In addition, treatment with in vitro Op2 extract exhibited the highest toxic potency, resulting in fragmentation of the endocuticle, folding and circular protrusions in the epicuticle, dispersion of cytoplasmic granules, and deformation of muscle tissues in both second- and fourth-instars, while in vitro Op1 and greenhouse Op3 extracts induced moderate structural disorganization, as shown in Figs. 6 and 7.

Midgut histology

Healthy larvae exhibited typical midgut architecture comprising a monolayer of columnar epithelial cells resting on a well-defined basement membrane and surrounded by circular and longitudinal muscle layers. The epithelium consisted of columnar cells, goblet cells, and regenerative cells. The apical surface was densely packed with microvilli, and the gut lumen was enclosed by an intact peritrophic membrane, indicating normal digestive structure and epithelial renewal. Treated larvae showed pronounced histopathological alterations, including epithelial disintegration, damage to the peritrophic membrane, and loss of cellular integrity. These effects were more severe after treatment with the in vitro Op2 extract across both larval instars, causing complete fusion of midgut epithelial cells and epithelial layers and resulting in the loss of normal cellular boundaries (Figs. 8 and 9). In comparison, the in vitro Op1 and greenhouse Op3 extracts induced moderate structural disorganization.

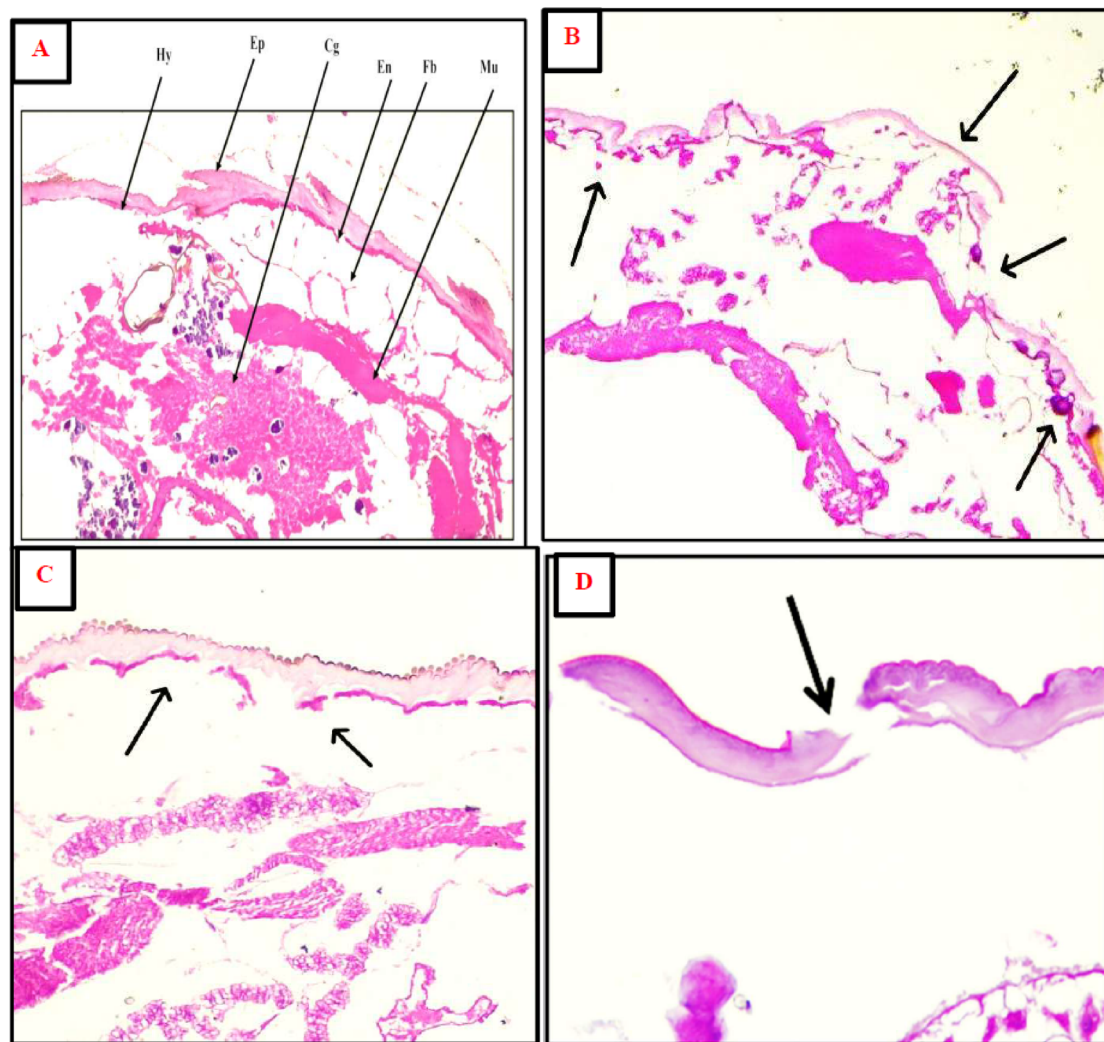


Fig. 6. Histological sections of the integument of second-instar *Spodoptera exigua* larvae showing the effects of the tested cladode extracts at LC_{50} concentrations. **(A)** Control larvae showing normal cuticular architecture with intact epicuticle and endocuticle layers (**Ep**: epicuticle, **En**: endocuticle, **Hy**: hypodermis, **Mu**: muscle fibers, **Fb**: fat body, **Cg**: cytoplasmic granules). **(B)** Larvae treated with in vitro Op1 extract showed rupture and separation of the endocuticle from the hypodermis, irregular epidermal thickness, and disintegration of the fat body. **(C)** Larvae treated with in vitro Op2 extract show severe histopathological damage, including fragmentation of the endocuticle, folding of the epicuticle, dispersion of cytoplasmic granules, and deformation of muscle tissues. **(D)** Larvae treated with the greenhouse Op3 extract show wide folds in the epicuticle, partial separation of cuticular layers, and generalized structural disorganization. Arrows indicate histopathological alterations. Magnification: 10×10 .

Molecular docking analysis

The constituents identified in the tested cladode extracts were docked against *S. exigua* LDH and GPx to evaluate their potential binding affinities and interaction patterns (Table 4). Among the evaluated compounds, ferulic acid, kaempferol, rutin, and quercetin exhibited the most favorable docking scores against the target proteins.

Ferulic acid showed strong binding toward *S. exigua* LDH with a binding affinity of $-6.12 \text{ kcal mol}^{-1}$. The interaction involved two hydrogen bonds with Arg160 (2.93 Å) and Ser153 (2.28 Å), along with five hydrophobic π -alkyl interactions with Ala242, Ala157, Ala243 and Arg246 (Fig. 10A). Similarly, kaempferol displayed a promising binding mode with lactate dehydrogenase, with an affinity score of $-6.32 \text{ kcal mol}^{-1}$. This interaction was stabilized by four hydrophobic π -alkyl interactions with Ala157, Ala243 and Ala242, in addition to five hydrogen bonds formed with Arg246, Val258, Arg160 and Ser153 at distances of 2.46, 2.49, 2.21, 2.86 and 3.04 Å, respectively (Fig. 10B).

Regarding GPx, ferulic acid demonstrated a binding affinity of $-6.15 \text{ kcal mol}^{-1}$, forming six hydrophobic π -alkyl interactions with Ile5, Cys117, Val21 and Ile109, together with three hydrogen bonds involving Ala116 (2.64 Å) and Asn6 (2.86 and 2.32 Å) (Fig. 10C). Kaempferol exhibited the highest affinity toward glutathione peroxidase ($-6.72 \text{ kcal mol}^{-1}$), stabilized by four π -alkyl interactions with Cys117, Ile5, Ile109 and Ile4, as well as two hydrogen bonds with Thr115 and Asp118 at distances of 2.81 and 2.24 Å, respectively (Fig. 10D).

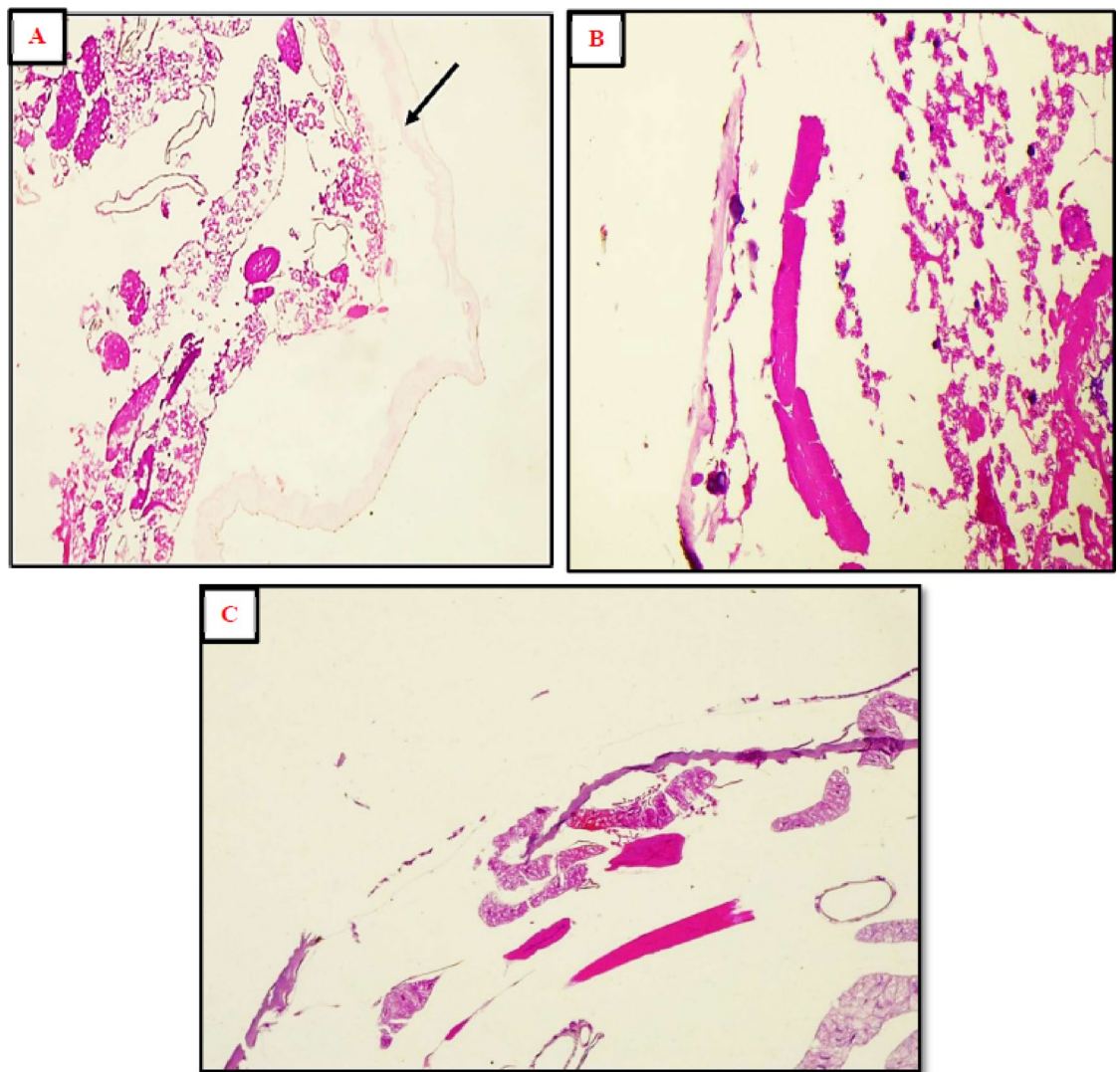


Fig. 7. Histological sections of the integument of fourth-instar *Spodoptera exigua* larvae treated with the LC₅₀ concentrations of the tested cladode extracts. (A) Larvae treated with in vitro-derived Op1 extract show weakening and increased transparency of the integument layers, disintegration of the fat body, and destruction of cytoplasmic granules. (B) Larvae treated with in vitro Op2 extract exhibit severe destruction and fragmentation of both epicuticle and endocuticle layers, along with dispersion and separation of cytoplasmic granules. (C) Larvae treated with the greenhouse Op3 extract show partial detachment of the epicuticle, dissolution of the hypodermis, semi-partial decomposition of integument layers, and marked muscle deformation. Arrows indicate histopathological alterations. Magnification: 10 × 10.

Discussion

The present study integrates phytochemical characterization, biological assays, enzymatic analyses, histopathology, and molecular docking to elucidate the insecticidal activity of *O. ficus-indica* cladode extracts against *S. exigua*. Overall, variations in phytochemical composition among the tested extracts were closely associated with differences in biological efficacy and physiological responses observed in treated larvae. This integrated approach provides evidence that tissue culture conditions can influence the accumulation of bioactive metabolites and, consequently, the biological performance of the resulting extracts.

Assessment of phenolic and flavonoid contents showed that the in vitro-derived extracts (Op1 and Op2) had higher total phenolic levels than the greenhouse-grown extract. Similarly, HPLC profiling revealed qualitative differences among extracts, with the in vitro Op2 extract showing the most balanced and concentrated mixture of bioactive polyphenolic compounds, particularly a markedly higher kaempferol content. These differences in phytochemical composition appear central to determining bioactivity, as polyphenols are recognized as key contributors to plant defense mechanisms^{93–95}. However, the present findings should be interpreted at the extract level because the individual purified compounds were not tested separately. Therefore, the enhanced activity of Op2 is most appropriately attributed to its enriched and diversified polyphenolic profile rather than to a single compound alone.

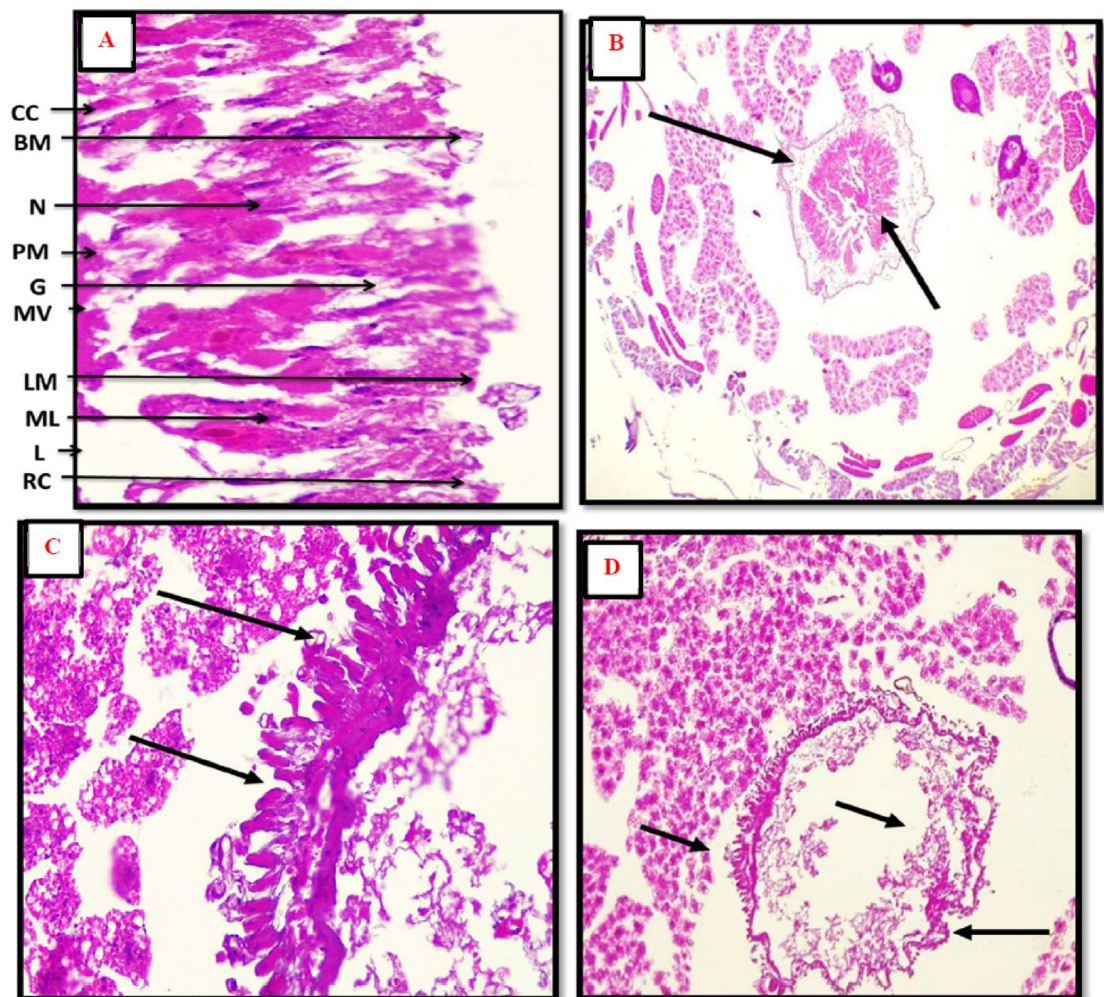


Fig. 8. Histological sections of the midgut of second-instar *Spodoptera exigua* larvae showing normal architecture and alterations following treatment with the LC_{50} concentrations of the tested cladode extracts. (A) Control larvae stained with hematoxylin–eosin (H&E) showing normal midgut organization (CC: columnar cells, BM: basement membrane, N: nuclei, PM: peritrophic membrane, G: goblet cells, MV: microvilli, ML: muscular layer, LM: longitudinal muscle, L: lumen, RC: regenerative cells). (B) Larvae treated with in vitro Op1 extract show disintegration of the lumen, segmental separation of the peritrophic membrane, and partial detachment of epithelial layers. (C) Larvae treated with in vitro-derived Op2 extract exhibit increased spacing between the peritrophic membrane and regenerative cells, resulting in epithelial disruption and the dispersion of cellular components into the lumen. (D) Larvae treated with the greenhouse Op3 extract showed a partial loss of columnar, goblet, and regenerative cells in some regions. Arrows indicate histopathological alterations. Magnification: 10×10 .

All tested extracts exhibited significant insecticidal activity against *S. exigua*, with efficacy varying by extract type, concentration, larval stage, and exposure time. The observed insecticidal activity followed the order in vitro Op2 > in vitro Op1 > greenhouse Op3 extract, underscoring the clear influence of culture conditions on bioefficacy. The enhanced toxicity of the in vitro Op2 extract was closely associated with elevated levels of phenolic acids and flavonoids, particularly kaempferol, quercetin, gallic acid, catechin, and ferulic acid, which are widely recognized for their roles in plant defense against insect herbivores^{93–96}. This increased accumulation of polyphenols in the in vitro Op2 treatment may be mechanistically linked to cytokinin supplementation in the culture medium, as cytokinins such as benzyladenine and kinetin are known to stimulate phenylpropanoid metabolism and enhance flavonoid and phenolic acid biosynthesis^{97,98}.

In contrast, the moderate toxicity of in vitro Op1 might correspond to the lower concentrations of these compounds, whereas the weaker activity of the greenhouse-grown extract may be explained by the absence of ferulic acid and by a markedly lower kaempferol content. These findings suggest that insecticidal activity depends not only on total phenolic concentration but also on the qualitative composition of bioactive compounds⁹⁹. Accordingly, differences in insecticidal efficacy among treatments can be attributed to both quantitative and qualitative differences in phenolic and flavonoid profiles^{96,99}. Although the observed trends suggest an association between phytochemical enrichment and larvicidal activity, no formal correlation analysis was performed;

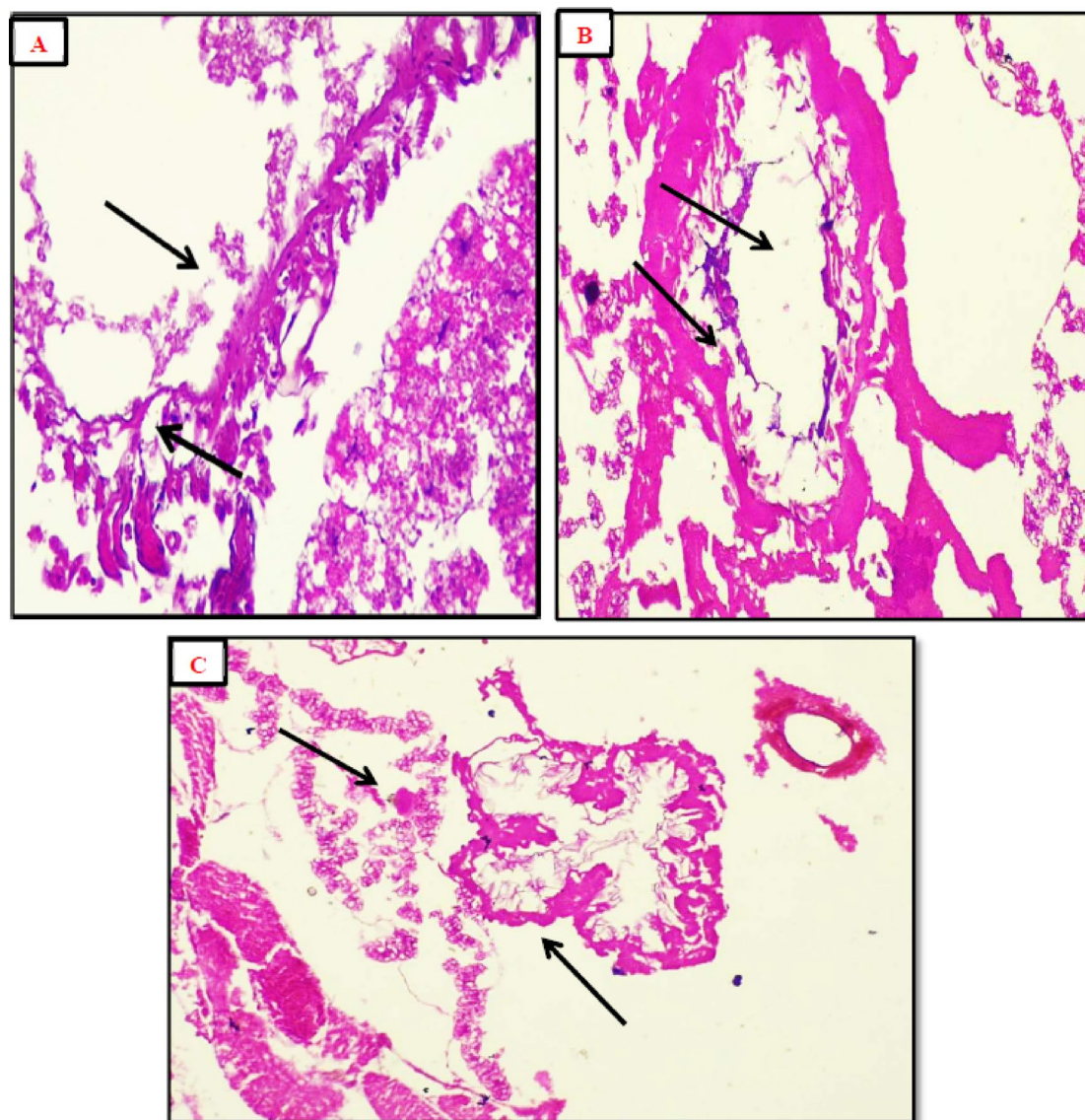


Fig. 9. Histological sections of the midgut of fourth-instar *Spodoptera exigua* larvae treated with the LC₅₀ concentrations of the tested cladode extracts. (A) Larvae treated with in vitro Op1 extract showed goblet cell hyperplasia, lysis of the muscular and longitudinal muscle layers, and disintegration of regenerative cells within the lumen. (B) Larvae treated with in vitro Op2 extract show severe histopathological damage characterized by complete fusion of midgut cells and loss of normal layer organization. (C) Larvae treated with the greenhouse Op3 extract showed narrowing of the gut lumen, distortion and fusion of epithelial layers and cells, disruption of tissue architecture, and leakage of luminal contents. Arrows indicate histopathological alterations. Magnification: 10 × 10.

therefore, the relationship should be interpreted cautiously and warrants further statistical evaluation in future studies. Nevertheless, further bioassays with purified compounds and defined compound mixtures would be required to confirm the relative contributions of each metabolite and any potential synergistic effects.

Biochemical analyses further supported these findings by revealing significant metabolic disruption in treated larvae. Lactate dehydrogenase, a key enzyme in glycolytic metabolism that maintains NADH/NAD⁺ balance and supports ATP production under stress, showed markedly elevated activity after treatment. In the present study, the pronounced elevation in LDH, particularly the 5.20-fold increase observed in *S. exigua* fourth-instar larvae treated with Op2, indicates a robust metabolic response to extract-induced stress. This increase likely reflects a compensatory shift in energy metabolism rather than direct enzymatic inhibition^{100–103}. The observed increase in LDH activity aligns with previous reports of similar responses in insects exposed to botanical or synthetic insecticides, including *Schistocerca gregaria*, *Chilo suppressalis*, *S. littoralis*, and *Pectinophora gossypiella*^{92,104,105}. These findings suggest that LDH induction serves as a common metabolic adaptation to the toxic stress and reflects the disruption of normal energy metabolism.

The tested compounds	LDH	GPx
	Affinity score (kcal mol ⁻¹)	Affinity score (kcal mol ⁻¹)
Gallic acid	− 4.65	− 4.55
Chlorogenic acid	− 6.10	− 5.70
Catechin	− 6.30	− 6.10
Methyl gallate	− 4.25	− 4.45
Caffeic acid	− 6.01	− 5.80
Syringic acid	− 5.17	− 5.37
Rutin	− 6.45	− 6.65
Ellagic acid	− 5.86	− 5.46
Coumaric acid	− 5.60	− 5.10
Vanillin	− 5.12	− 5.01
Ferulic acid	− 6.12	− 6.15
Naringenin	− 6.46	− 6.70
Rosmarinic acid	− 6.18	− 6.22
Daidzein	− 5.58	− 5.18
Quercetin	− 6.30	− 6.67
Cinnamic acid	− 5.14	− 5.25
Kaempferol	− 6.32	− 6.72

Table 4. Binding affinity scores of the major compounds identified in the tested cladode extracts docked against *Spodoptera exigua* lactate dehydrogenase (LDH) and glutathione peroxidase (GPx).

In contrast, GPx, a key antioxidant enzyme that detoxifies peroxides and maintains cellular redox homeostasis, was significantly suppressed in treated larvae, particularly after exposure to the in vitro Op2 extract. Glutathione peroxidase normally protects cells from oxidative damage by catalyzing the reduction of hydrogen peroxide and lipid peroxides^{106,107}. Its reduction may reflect impairment of antioxidant defense mechanisms and the development of oxidative imbalance in treated larvae, resulting in increased cellular susceptibility to oxidative damage^{108–112}. Although reactive oxygen species and lipid peroxidation markers were not directly measured in the present study, the marked reduction in GPx activity, particularly the 82.18% suppression induced by the in vitro Op2 extract, provides biochemical evidence of disrupted antioxidant capacity. Similar reductions in GPx activity have been reported in pesticide-exposed insects, underscoring the sensitivity of glutathione-dependent pathways to chemical stress^{113,114}. Together, the elevation of LDH and suppression of GPx indicate severe metabolic stress and oxidative imbalance as major mechanisms underlying extract-induced toxicity.

To contextualize these findings within the broader literature, several recent studies have demonstrated the potent insecticidal properties of flavonoid-rich plant extracts against various *Spodoptera* species. Recent comparative research highlights that plant-derived polyphenols and specific flavonoids—such as quercetin and rutin—induce oxidative stress, inhibit detoxification enzymes, and disrupt metabolic pathways in related pests, including *S. litura* and *S. littoralis*^{77,115,116}. The metabolic disruption and enzymatic imbalances observed in our study closely mirror these documented mechanisms of flavonoid-mediated toxicity. This alignment corroborates the hypothesis that the enhanced larvicidal efficacy of the in vitro Op2 extract is driven by its enriched polyphenolic profile, reinforcing its competitive potential alongside other established botanical insecticides.

At the tissue level, exposure to LC₅₀ concentrations caused extensive structural damage in both the integument and midgut, with the severity of the alterations varying by extract type and larval stage. Integumentary damage included disruption of cuticular layers, separation of the endocuticle and hypodermis, muscle deformation, fat body disintegration, and alterations in cytoplasmic granules, suggesting impairment of cuticle integrity, which is essential for protection and physiological regulation¹¹⁷. Such integumental disruption may compromise barrier function, increase vulnerability to dehydration and secondary stress, and interfere with normal movement and development. Similar integument damage has been reported in *S. litura* exposed to insecticidal compounds¹¹⁸.

The midgut, a primary target of insecticidal action, exhibited pronounced structural deterioration, including epithelial disorganization, disruption of microvilli, chromatin fragmentation, and altered cellular architecture, consistent with previous reports of insect growth regulator-induced damage¹¹⁹. Because the midgut relies on tightly regulated epithelial renewal for digestive efficiency, disruption of this balance inevitably leads to severe functional impairment¹²⁰. Damage to microvilli, which are critical for nutrient absorption¹²¹, has likely contributed to reduced feeding efficiency and larval mortality. In addition, disruption of the peritrophic membrane may increase epithelial exposure to toxic metabolites and digestive stress, thereby accelerating tissue degeneration. Comparable dose-dependent histopathological effects have been widely documented in lepidopteran larvae treated with plant-derived insecticides^{122–125}. It should be noted, however, that the histological assessment in the present study was qualitative; therefore, future morphometric analysis or lesion scoring would further strengthen the tissue-level interpretation.

Molecular docking simulations offered a potential mechanistic explanation for these physiological observations. The major polyphenolic compounds, particularly ferulic acid and kaempferol, exhibited strong binding affinities to the target enzymes. Ferulic acid bound strongly to LDH (− 6.12 kcal mol⁻¹) via hydrogen

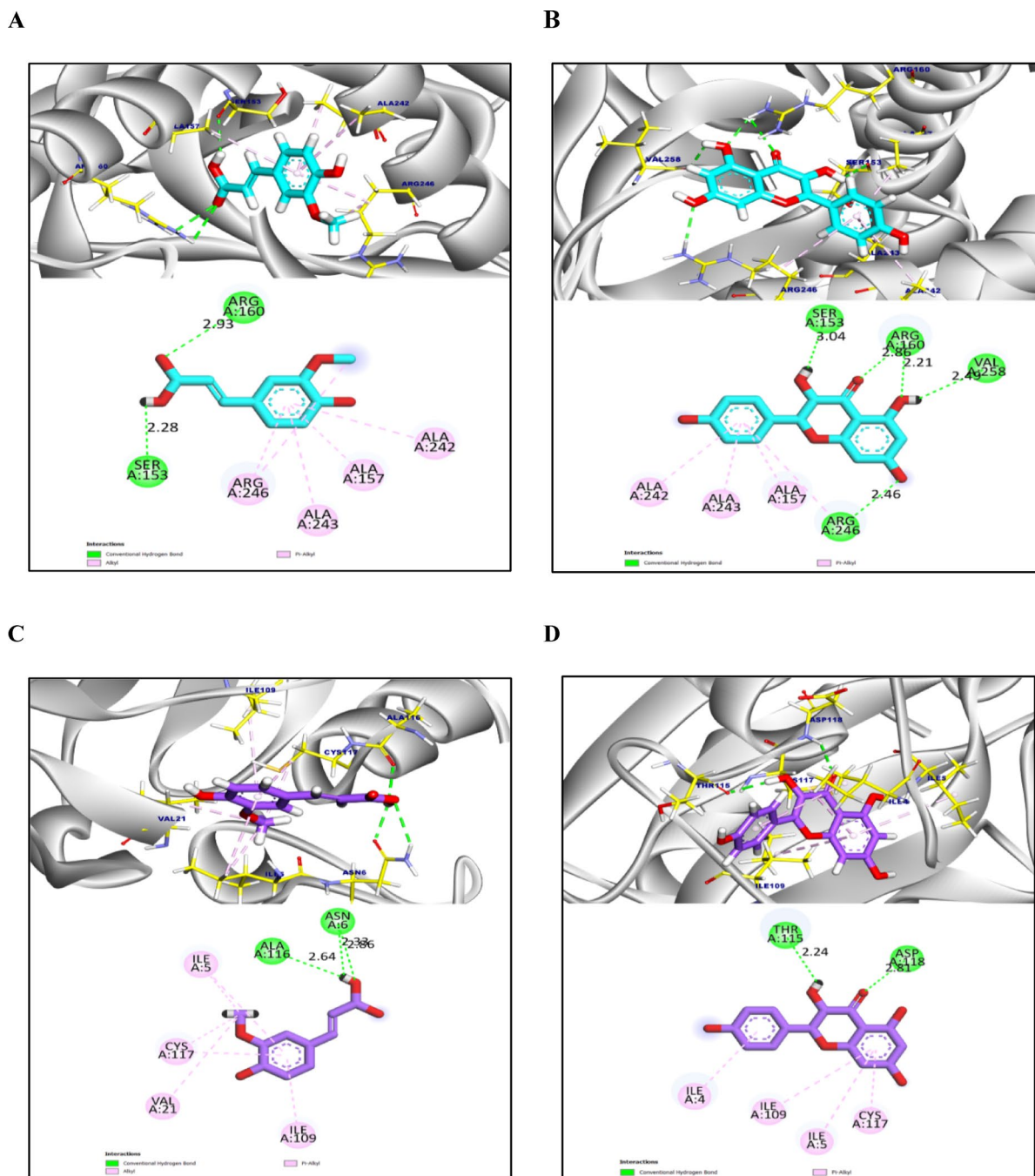


Fig. 10. Three-dimensional (3D) and two-dimensional (2D) representations of the proposed binding modes of selected compounds docked against *Spodoptera exigua* lactate dehydrogenase (LDH) and glutathione peroxidase (GPx). (A) Binding mode of ferulic acid with LDH. (B) Binding mode of kaempferol with LDH. (C) Binding mode of ferulic acid with GPx. (D) Binding mode of kaempferol with GPx. Interacting amino acid residues are shown in yellow, while ligands are depicted in turquoise for lactate dehydrogenase complexes and in purple for glutathione peroxidase complexes.

bonds and hydrophobic interactions, while kaempferol showed a slightly higher affinity ($-6.32 \text{ kcal mol}^{-1}$). Similarly, both compounds formed stable interactions with GPx, with kaempferol showing the strongest binding ($-6.72 \text{ kcal mol}^{-1}$). These docking results are consistent with the biochemical alterations observed experimentally; however, they should not be interpreted as direct evidence of enzyme inhibition. Molecular docking predicts possible ligand–protein interactions under computational conditions, whereas confirmation of enzyme inhibition requires targeted biochemical assays^{126,127}. The consistency between docking predictions and experimental enzymatic responses, therefore, suggests that these polyphenols may contribute to the physiological responses observed in treated larvae through potential interactions with metabolic and antioxidant enzymes.

Further validation using purified compounds, enzyme inhibition assays, and LC–MS/MS-based metabolite profiling is recommended to confirm the precise metabolites responsible for the observed toxicity and to clarify whether additive or synergistic interactions occur among the extract constituents.

Conclusion

In conclusion, tissue culture-mediated micropropagation of *Opuntia ficus-indica* significantly enhanced its polyphenolic profile and was associated with improved insecticidal efficacy against *S. exigua*. The in vitro Op2 extract, enriched through cytokinin supplementation, exhibited the highest larvicidal toxicity, primarily attributable to elevated concentrations of key bioactive metabolites, including kaempferol, gallic acid, quercetin, and ferulic acid. This enhanced phytochemical profile induced profound physiological disruption in the larvae, characterized by a 5.2-fold elevation in lactate dehydrogenase activity, an 82% suppression of glutathione peroxidase activity, and severe histopathological degradation of the midgut and integument. Finally, molecular docking suggested potential interactions between kaempferol or ferulic acid and *S. exigua* LDH and GPx. These findings underscore the in vitro Op2 extract as a highly potent, eco-friendly botanical insecticide, highlighting the value of plant tissue culture in sustainably optimizing secondary metabolite production for integrated pest management.

Data availability

Data will be made available on request.

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