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Phytochemical Profiling, Antioxidant, Antibacterial and Biomarkers Enzyme Activity of *Aristolochia Indica*: Host Plant Effects on Immune Response Across Development of Specialized Caterpillar (*Pachliopta Hector*)

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KEYWORDS
Aristolochia indica; Pachliopta hector; GC-MS; Antioxidant; Antibacterial; Insect Immunity; Host plant interaction.

ABSTRACT:

Introduction: The medicinal plant *Aristolochia indica* is the exclusive host for the larvae of the crimson rose swallowtail butterfly, *Pachliopta hector*, which sequester the plant's toxic secondary metabolites for defense.

Objectives: This study presents a comprehensive phytochemical and bioactivity profile of *A. indica* leaf extracts and investigates its impact on the immune response of its specialized herbivore across larval development.

Methods: Preliminary phytochemical screening confirmed the presence of various compound classes. Gas Chromatography-Mass Spectrometry (GC-MS) analysis further identified and quantified specific bioactive compounds. The extract's antioxidant potential was assessed using multiple in vitro assays (DPPH, $ABTS^{+}$, nitric oxide, superoxide anion scavenging, FRAP), and its antibacterial activity was tested against Gram-positive and Gram-negative pathogens. To assess host plant effects, *P. hector* caterpillars were reared on *A. indica* leaves, and hemolymph was collected across five larval instars to analyze immune parameters (phenoloxidase activity, total hemocyte count) and key antioxidant enzyme activity (GST, GPx, CAT).

Results: The extract exhibited significant in vitro antioxidant potential and broad-spectrum antibacterial activity. A notable, instar-specific modulation of immune parameters was observed in the caterpillars. Later instars, corresponding with increased consumption of the toxic foliage, exhibited a significantly heightened immune response, including increased phenoloxidase activity and total hemocyte count, compared to early instars and controls fed on a non-toxic host. The activity of key antioxidant enzymes in the hemolymph provided a direct biochemical measure of the response to oxidative stress.

Conclusions: The findings conclusively establish *A. indica* as a rich source of bioactive compounds with potent antioxidant and antibacterial properties. More importantly, they demonstrate that the plant's chemical arsenal acts as a powerful selective pressure, driving the evolution of a robust and inducible immune system in its specialized herbivore, *P. hector*, to facilitate successful sequestration and ensure fitness.

1. Introduction

The intricate and evolutionary arms race between plants and herbivorous insects represents one of the most dynamic and fundamental interactions in terrestrial ecosystems [1]. For over 350 million years, plants have evolved a formidable arsenal of chemical defenses a diverse array of secondary metabolites to deter herbivory, reduce fitness, and secure their survival [2]. In response, herbivorous insects have developed a sophisticated suite of counter-adaptations, including behavioral, physiological, and molecular mechanisms, to overcome these chemical barriers and successfully utilize their host plants [3]. This co-evolutionary dialogue has given rise to remarkable specializations, where certain insect lineages, particularly specialists, have not only evolved tolerance to specific plant toxins but have also weaponized them for their own defense against predators and pathogens [4]. The study of such tri-trophic interactions provides profound insights into

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evolutionary biology, chemical ecology, and the potential discovery of novel bioactive compounds.

The plant genus Aristolochia, commonly known as birthwort, is renowned for its rich history in traditional medicine and its ecological significance as the host for butterflies of the family Papilionidae, specifically the Troidini tribe [5, 6]. Aristolochia indica L., a perennial climbing shrub native to the Indian subcontinent, holds a prominent place in Ayurveda and Siddha systems of medicine, traditionally used for treating a wide range of ailments from snakebites and inflammation to digestive disorders [7]. The pharmacological properties of *A. indica* are largely attributed to its complex cocktail of secondary metabolites, with the nitrophenanthrene carboxylic acid derivative, aristolochic acid (AA), being the most characteristic and intensely studied compound [8]. While these compounds confer medicinal value, they are also notoriously nephrotoxic and carcinogenic to mammals, highlighting their potent biological activity [9].

Despite their toxicity, Aristolochia plants serve as the exclusive larval host for a fascinating group of insects: the pipevine swallowtail butterflies and their relatives. Pachliopta hector L. (Lepidoptera: Papilionidae), the crimson rose swallowtail, is one such specialist herbivore. Female butterflies meticulously lay eggs on Aristolochia plants, and upon hatching, the caterpillars feed exclusively on the leaves, stems, and flowers, ingesting large quantities of toxic AAs and other phytochemicals [10, 11]. Instead of succumbing to these toxins, *P. hector* larvae have evolved a remarkable tolerance. They possess specific physiological adaptations, including midgut alkalization and specialized transporters, that allow them to sequester these compounds unmetabolized and store them in their integument and other tissues throughout their development [12]. This sequestered chemical cocktail renders the larvae, and later the adults, highly unpalatable to a wide range of vertebrate and invertebrate predators, a strategy known as chemical aposematism supported by their bright warning coloration (red and black) [13].

However, the relationship between plant toxins and insect fitness is not a simple story of effortless appropriation. The process of consuming, tolerating, and sequestering potent phytochemicals is likely to impose significant physiological costs on the insect. One of the key systems that may be affected is the immune system. The insect immune system is a complex and energy-demanding machinery, comprising both cellular (e.g., phagocytosis, encapsulation by hemocytes) and humoral (e.g., antimicrobial peptides, phenoloxidase cascade) components [14]. Mounting an immune response is metabolically expensive and can trade-off with other life-history traits such as growth, development, and reproduction [14]. Exposure to toxic host plant chemicals can act as an environmental stressor, potentially suppressing the immune system and increasing vulnerability to parasites and pathogens [16]. Conversely, some studies suggest that exposure to sub-lethal levels of plant toxins can induce an "hormetic" effect, potentially priming or upregulating certain immune pathways as a generalized stress response [17]. For a specialist insect like *P. hector*, understanding how its immune system negotiates the dual challenge of managing plant toxins while remaining vigilant against pathogens is a crucial question. The immune response may vary significantly across larval instars, as early instars might be more vulnerable to both plant toxins and immune challenges, while later instars, having developed more robust detoxification and sequestration mechanisms, may allocate more resources to immunity.

While the ecological relationship between Aristolochia and Pachliopta is well-documented, a comprehensive phytochemical profile of *A. indica* coupled with an investigation into its bioactivity and effects on insect immunity remains underexplored. Preliminary phytochemical screening provides a basic qualitative overview, but advanced techniques like Gas Chromatography-Mass Spectrometry (GC-MS) are essential for the precise identification and quantification of volatile and non-volatile bioactive compounds, offering a much deeper chemical perspective [18]. Furthermore, evaluating the in vitro bioactivity of the plant extract is a critical first step in understanding its ecological impact. Antioxidant activity, measured through a battery of assays (DPPH, FRAP, ABTS, nitric oxide, superoxide scavenging, and reducing power), provides insight into the plant's redox potential, which can influence cellular stress in herbivores [19]. Similarly, assessing antibacterial activity against a panel of Gram-positive and Gram-negative bacteria reveals the plant's direct defense potential against microbial threats, which

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could also indirectly shape the gut microbiome of the specialist herbivore [20].

Most critically, linking this detailed phytochemical and bioactivity profile to the physiological response of the specialized herbivore across its development is a significant gap this research aims to fill. Measuring the activity of key antioxidant enzymes in the insect such as Glutathione S-transferase (GST), which is often involved in detoxification of xenobiotics; Glutathione Peroxidase (GPx); and Catalase (CAT), which mitigate oxidative stress can provide a direct measure of how the caterpillar's biochemistry responds to the phytochemical challenge [21]. By analyzing these parameters across the larval instars of *P. hector*, we can trace the ontogenetic trajectory of its adaptation to its toxic host.

Therefore, this study is designed to bridge the gap between plant chemistry and insect physiology by pursuing the following objectives, to conduct a thorough phytochemical profiling of *A. indica* leaf extract using preliminary qualitative tests and quantitative GC-MS analysis. To evaluate the in vitro antioxidant potential of the extract using a comprehensive suite of assays (DPPH, FRAP, ABTS, nitric oxide, superoxide scavenging, reducing power) and its antibacterial activity against model Gram-positive (Streptococcus aureus, Bacillus thuringiensis) and Gram-negative (Escherichia coli, Pseudomonas aeruginosa) bacteria. To assess the effect of *A. indica* consumption on the immune physiology of its specialist herbivore, *P. hector*, by measuring the activity of key antioxidant enzymes GST, GPx, CAT in the hemolymph of fifth instar larvae. This integrated approach will provide a holistic understanding of how the chemical warfare of a plant shapes the physiological resilience and immune investment of a highly adapted insect, offering new insights into the cost of specialization in plant-insect interactions.

2. Methods

Collection and Extraction

Mature, healthy leaves of Aristolochia indica were collected from their natural habitat, washed thoroughly with tap water to remove debris, and shade-dried at room temperature for two weeks. The dried leaves were ground into a fine powder using an electric grinder. For extraction, 500 g of the powdered material was subjected to sequential solvent extraction using a Soxhlet apparatus, using methanol. Extraction was carried out for 6–8 hours per solvent. The crude extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C. The resulting dried extract were stored in airtight containers at 4°C until further use [22].

Qualitative phytochemicals analysis of A. indica leaves extract

The methanolic extracts of *A. indica* leaves were subjected to standard qualitative phytochemical screening. Alkaloids were detected using Mayer's and Wagner's reagents, observing precipitate formation. Flavonoids were identified via the alkaline reagent test, yielding yellow-to-orange coloration. Phenols and tannins were assessed with ferric chloride solution, producing blue-green or black hues. Saponins were detected by foam persistence after vigorous shaking. Steroids and terpenes were identified using the Salkowski test, with chloroform and concentrated sulfuric acid forming reddish-brown or yellow-brown rings. Glycosides were tested using Keller-Kiliani and Legal's tests for cardiac glycosides, noting violet or pink rings. All tests were performed in triplicate to ensure reproducibility [23].

Quantitative phytochemicals analysis of A. indica leaves extract using GCMS

The methanolic extract of Aristolochia indica leaves was subjected to gas chromatography–mass spectrometry (GC–MS) analysis using an Agilent 7890B GC system coupled with a 5977A MSD. Separation was achieved with an HP–5MS capillary column (30 m × 0.25 mm, 0.25 µm). The oven temperature was programmed from 60°C (held for 2 min) to 300°C at 10°C/min, with a final hold time of 10 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Sample injection volume was 1 µL in splitless mode. Mass spectra were acquired in electron impact (EI) mode at 70 eV, with a scan range of 50–600 m/z. Compound identification was performed by comparing mass spectra with the NIST library and authentic standards [24].

Antioxidant properties of Aristolochia indica extract

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of the extract was evaluated spectrophotometrically. Briefly, 1 mL of

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various concentrations (100, 200, 300, 400 and 500µg/mL) of the extract was mixed with 1 mL of 0.1 mM methanolic DPPH solution. The mixture was vortexed and incubated in darkness at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. A control was prepared using methanol instead of the extract. Ascorbic acid served as the standard antioxidant. The radical scavenging activity was calculated as a percentage of inhibition using the formula:

% Inhibition = [(Abs_control - Abs_sample) / Abs_control] × 100.

All tests were performed in triplicate.

FRAP antioxidant assay

The ferric reducing antioxidant power (FRAP) assay was performed as described by Benzie & Strain [25]. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM in 40 mM HCl), and $FeCl_3$ (20 mM) in a 10:1:1 ratio. Extract samples (50 µL) were added to 1.5 mL of FRAP reagent and incubated at 37°C for 10 minutes. The increase in absorbance at 593 nm was measured. A standard curve was prepared using $FeSO_4 \cdot 7H_2O$, and results were expressed as µM Fe(II) equivalent per gram of extract. All assays were performed in triplicate.

ABTS++ antioxidant assay

The ABTS radical scavenging activity was determined using the method of Re et al. [26]. The $ABTS^{++}$ radical cation was generated by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate, followed by incubation in darkness for 12–16 hours. This solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Test samples (50 µL) were mixed with 1.5 mL of diluted $ABTS^{++}$ solution. After 6 minutes of incubation, absorbance was measured at 734 nm. Trolox was used as a standard, and results were expressed as µmol Trolox equivalents per gram of extract. All tests were performed in triplicate.

Nitric Oxide antioxidant assay

The nitric oxide (NO) scavenging activity was assessed using Griess reagent. Sodium nitroprusside (10 mM) in phosphate buffer (pH 7.4) was mixed with various extract concentrations and incubated at 25°C for 150 minutes. An aliquot (0.5 mL) of incubated solution was mixed with 0.5 mL Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% NED). Absorbance was measured at 546 nm after 15 minutes. The percentage inhibition of nitric oxide radical formation was calculated relative to the control (without extract). Ascorbic acid was used as a standard. All experiments were conducted in triplicate.

Superoxide anion scavenging activity

The superoxide anion scavenging activity was evaluated using the nitroblue tetrazolium (NBT) reduction method. The reaction mixture contained 1 mL of NBT (156 µM), 1 mL of NADH (468 µM) in phosphate buffer (100 mM, pH 7.4), and 0.1 mL of extract at various concentrations. The reaction was initiated by adding 100 µL of phenazine methosulfate (PMS; 60 µM) and incubated at 25°C for 5 minutes. The absorbance was measured at 560 nm. Gallic acid served as the positive control. All assays were performed in triplicate.

Reducing power antioxidant assay

The reducing power of the extract was determined using the Oyaizu [27] method. Various concentrations of the extract (1 mL) were mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 minutes. Then, 1 mL of trichloroacetic acid (10% w/v) was added and centrifuged at 3000 rpm for 10 minutes. The supernatant (1 mL) was mixed with 1 mL of distilled water and 0.2 mL of ferric chloride (0.1% w/v). Absorbance was measured at 700 nm. Increased absorbance indicated higher reducing power. Ascorbic acid was used as a standard. All tests were performed in triplicate.

Antibacterial activity

The antibacterial efficacy of Aristolochia indica leaf extract was evaluated against two Gram-positive (Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633) and two Gram-negative (Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853) bacterial strains using the agar well diffusion method. Bacterial cultures were revitalized in nutrient broth and incubated at 37°C for 18–24 hours. The turbidity of bacterial suspensions was standardized to 0.5 McFarland (≈1.5×10^8 CFU/mL) using sterile saline. Mueller-Hinton agar (MHA) plates were surface-inoculated uniformly with swabs dipped in the standardized suspensions. Wells (6 mm diameter) were aseptically

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punched into the agar using a sterile cork borer. The extract was tested at concentrations of 100, 250, and 500 µg/mL (dissolved in 10% DMSO, with solvent evaporation prevented). Each well was loaded with 50 µL of the respective concentration. Solvent (methanol) and sterile distilled water served as negative controls, while standard antibiotics (e.g., streptomycin) were positive controls. Plates were refrigerated for 1 hour for pre-diffusion, then incubated at 37°C for 24 hours. Zones of inhibition (ZOI) were measured in millimeters (including well diameter) using a calibrated caliper. All experiments were performed in triplicate under strict aseptic conditions [28].

Biomarker enzyme activity of Pachliopta hector larvae

Larvae of Pachliopta hector from first to fifth instars, reared exclusively on Aristolochia indica leaves, were collected. A control group was fed on a non-toxic host plant (e.g., Citrus spp.). Hemolymph was extracted from cold-anesthetized larvae by proleg incision using a sterile capillary tube, pooled per instar group (n=10 larvae/group), and immediately diluted in ice-cold anticoagulant buffer (0.1 M phosphate buffer, pH 7.4, containing 0.01 M EDTA and 0.1% phenylthiourea). The diluted hemolymph was centrifuged at 10,000 × g for 15 minutes at 4°C to obtain cell-free hemolymph supernatant, which was used for enzymatic assays. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Glutathione S-transferase (GST) Activity: Measured spectrophotometrically at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB, and hemolymph supernatant. Enzyme activity was expressed as µmol CDNB-GSH conjugate formed/min/mg protein [29].

Glutathione Peroxidase (GPx) Activity: Assessed by monitoring NADPH oxidation at 340 nm using H_2O_2 as substrate. The assay mixture included 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM GSH, 0.2 mM NADPH, 1 U glutathione reductase, and 0.25 mM H_2O_2. Activity was calculated as µmol NADPH oxidized/min/mg protein [30].

Catalase (CAT) Activity: Determined by tracking the decomposition of H_2O_2 at 240 nm. The reaction contained 50 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2. One unit of CAT activity was defined as the amount decomposing 1 µmol H_2O_2/min/mg protein [31].

Phenoloxidase (PO) Activity in Pachliopta hector Larval Instars

PO activity was assayed spectrophotometrically by monitoring dopachrome formation at 490 nm. The reaction mixture contained 50 µL hemolymph supernatant, 1 mL of 0.01 M L-DOPA (L-3,4-dihydroxyphenylalanine) in 0.1 M phosphate buffer (pH 6.5), and was incubated at 25°C for 30 min. Enzyme activity was expressed as units/min/mg protein, where one unit corresponds to a ΔA490 of 0.001 per minute. Protein concentration was determined using Bradford assay. Assays were performed in triplicate for each instar, with negative controls lacking L-DOPA [32].

Total hemocyte count assay in Pachliopta hector Larval Instars

Total hemocyte counts were performed using an improved Neubauer hemocytometer under phase-contrast microscopy at 400× magnification. A 10 µL aliquot of diluted hemolymph was loaded into the chamber, and hemocytes in all four corner squares (each 1 mm²) were counted. Counts were averaged and multiplied by the dilution factor to obtain THC per mL of hemolymph. For each instar, five biological replicates (individual larvae) were analyzed, with triplicate counts per replicate [33].

In Silico Molecular Interaction Analysis of Aristolochic Acid with Gustatory Receptor 8VC1

The molecular interaction between aristolochic acid (AA) and gustatory receptor 8VC1 of Pachliopta hector was investigated using computational approaches. The 3D structure of AA was retrieved from PubChem (CID:2236) and energy-minimized using MMFF94 force field in Open Babel. The protein structure of 8VC1 was modeled via homology modeling using SWISS-MODEL, with templates selected from insect gustatory receptors (e.g., Drosophila melanogaster Gr43a). Model quality was validated using PROCHECK (Ramachandran plot) and QMEAN scoring. Molecular docking was performed using AutoDock Vina. The receptor grid was centered on the putative binding site identified from literature. Docking parameters included an exhaustiveness of 8 and maximum binding modes of 10. The top-ranked pose based on binding affinity

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(kcal/mol) was selected for analysis. Molecular dynamics (MD) simulations (100 ns) using GROMACS with CHARMM36 force field assessed complex stability. Root-mean-square deviation (RMSD) and binding free energy (MM/PBSA) calculations validated interactions. Key interactions (hydrogen bonds, hydrophobic contacts, salt bridges) were visualized using PyMOL and LigPlot+. Residues within 4 Å of AA were analyzed for functional roles. The protocol ensured reproducibility with triplicate simulations [34].

Data analysis

All experimental data were expressed as mean ± standard deviation (SD) of at least three independent replicates. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons between groups (e.g., larval instars or treatment concentrations). For dose-response assays (e.g., IC_{50} calculations), nonlinear regression analysis was performed. A p-value < 0.05 was considered statistically significant. All analyses were conducted using GraphPad Prism (v9.0) or SPSS (v26). Assumptions of normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) were verified prior to parametric testing.

3. Results

Qualitative phytochemicals analysis

Phytochemical analysis of the Aristolochia indica leaf extract confirmed the presence of diverse secondary metabolites, with alkaloids being the most abundant class (Table 1).

Table 1. Preliminary phytochemical analysis of *Aristolochia indica* extract

S. No.	Phytochemicals	*A. indica* extract
1.	Alkaloids	+++
2.	Flavonoids	++
3.	Phenols	++
4.	Tannins	+
5.	Saponins	+
6.	Steroids	+
7.	Glycosides	++
8.	Terpenes	+

Note: +++ highly present; ++ moderately present; + present; - absent.

This is consistent with the known profile of Aristolochia species, which characteristically contain aristolochic acids and their derivatives. These compounds are central to the plant's chemical defense strategy, contributing to its toxicity and deterrence against generalist herbivores. The high alkaloid content also explains the sequestration behavior observed in specialist insects like Pachliopta hector, which have evolved mechanisms to store these toxins for their own defense. Flavonoids and phenols were detected in moderate to high levels, indicating significant antioxidant potential. These compounds likely play a dual role: protecting the plant from oxidative stress and contributing to its medicinal properties, such as anti-inflammatory and antimicrobial activities. The presence of glycosides further supports the extract's bioactivity, as many glycosides exhibit therapeutic effects, including cardioprotective and antimicrobial actions. Lower concentrations of tannins, saponins, steroids, and terpenes were also observed. While less abundant, these compounds contribute synergistically to the overall biological activity. Tannins offer astringent and antimicrobial properties; saponins provide hemolytic and immune-modulatory effects; steroids may contribute to anti-inflammatory responses; and terpenes enhance antimicrobial and antifungal activities [35]. The coexistence of toxic alkaloids with antioxidant flavonoids and phenols is particularly noteworthy. This combination suggests an ecological balance where toxins deter herbivory, while antioxidants mitigate potential cellular damage from reactive oxygen species generated during metabolic processes [36]. For Pachliopta hector, this phytochemical complexity necessitates and likely drives the sophisticated detoxification and immune adaptations observed across its larval instars. The presence of these compounds also validates the traditional use of *A. indica* in herbal medicine, though the toxicity of aristolochic acids necessitates cautious application.

Quantitative phytochemicals analysis using GCMS

Gas chromatography-mass spectrometry (GC-MS) analysis of the methanolic extract of Aristolochia indica leaves revealed a complex phytochemical profile comprising 41 identified compounds, dominated by aristolochic acid (26.25% area), the principal toxic compound characteristic of this genus (Figure 1 and Table 2). This high abundance aligns with the plant's known chemical defense strategy and explains its potent

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bioactivities, including toxicity to non-adapted herbivores and sequestration by specialist insects like Pachliopta hector. The presence of binaphthyl sulfone

Table 2. Phytocomponents present in the *Aristolochia indica* extract using GCMS

S. No	Compound Name	Molecular formula	RT	Area %
1.	Vanillin, TBDMS derivative	$C_{14}H_{22}O_3Si$	5.5971	0.08
2.	S-Ethyl ethanethioate	C_4H_8OS	5.7063	0.65
3.	D-Limonene	$C_{10}H_{16}$	6.7405	0.50
4.	Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$	7.0172	0.25
5.	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	7.4579	0.10
6.	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	9.5626	0.10
7.	1-Octadecanol, TMS derivative	$C_{21}H_{46}OSi$	9.7010	0.09
8.	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	11.0411	0.07
9.	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)- 1-(1-methylethyl)-, (3R-trans)-	$C_{15}H_{24}$	11.3178	0.35
10.	alpha.-Cubebene	$C_{15}H_{24}$	11.8713	0.24
11.	2,4-Dihydroxybenzoic acid, 3TMS derivative	$C_{16}H_{30}O_4Si_3$	11.9697	0.06
12.	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	$C_{15}H_{24}$	12.0570	0.54
13.	Caryophyllene	$C_{15}H_{24}$	12.4722	4.27
14.	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)- 4-(1-methylethylidene)	$C_{15}H_{24}$	12.5887	1.65
15.	Humulene	$C_{15}H_{24}$	12.9128	0.70
16.	Tau-Cadinol acetate	$C_{17}H_{28}O_2$	13.7285	0.08
17.	Ethanol, 2-(3,3-dimethylcyclohexylidene)-, (Z)	$C_{10}H_{18}O$	13.9506	0.09
18.	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-	$C_{15}H_{24}$	14.2055	0.30
19.	5-Isopropenyl-2-methyl-7-oxabicyclo[4.1.0]heptan-2-ol	$C_{10}H_{16}O_2$	15.7350	0.07
20.	Neophytadiene	$C_{20}H_{38}$	17.1442	1.18
21.	3,7,11,15-Tetramethyl-2-hexadecen-1-o	$C_{20}H_{40}O$	17.4028	0.27
22.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	17.5229	4.88
23.	2-Hexadecen-	$C_{22}H_{42}O_2$	17.5885	0.80

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	1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]]			
24.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	18.0291	2.04
25.	Dibutyl phthalate	$C_{16}H_{22}O_4$	18.4952	0.15
26.	13,16-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	19.0086	0.08
27.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	19.6750	0.24
28.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	$C_{19}H_{32}O_2$	19.7406	1.83
29.	Phytol	$C_{20}H_{40}O$	19.8389	6.75
30.	Methyl stearate	$C_{19}H_{38}O_2$	19.9372	2.75
31.	Binaphthyl sulfone	$C_{20}H_{14}O_2S$	20.6109	17.59
32.	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	23.5314	0.52
33.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	25.6616	4.74
34.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	25.8474	2.65
35.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.0185	3.97
36.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.1642	4.14
37.	Aristolochic acid	$C_{17}H_{11}NO_7$	26.3645	26.25
38.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.4919	3.50
39.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.6558	3.11
40.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.8124	1.84
41.	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	$C_{42}H_{63}O_3P$	26.9689	0.54

(17.59%) and phytol (6.75%) further underscores the extract's chemical diversity, with the latter contributing to antimicrobial and anti-inflammatory properties.

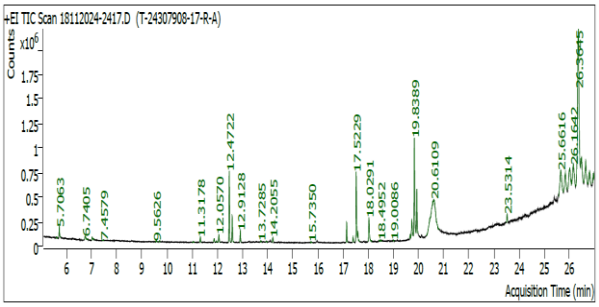

Figure 1. GCMS analysis of *Aristolochia indica* extract

Notable terpenoids included caryophyllene (4.27%), a sesquiterpene with documented anti-inflammatory and antimicrobial effects, and neophytadiene (1.18%), which may synergize with other compounds in defense mechanisms. Fatty acid esters such as methyl stearate (2.75%), hexadecanoic acid methyl ester (2.04%), and 9,12,15-octadecatrienoic acid methyl ester (1.83%) were identified, contributing to the extract's emulsifying and

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potential medicinal properties. The repeated detection of phenol derivatives (e.g., 2,4-bis(1,1-dimethylethyl)-phosphite, collectively ~25%) highlights significant antioxidant capacity, which may mitigate oxidative stress in both the plant and adapted insects.

The coexistence of toxic compounds (e.g., aristolochic acid) with antioxidants (terpenoids, phenols) and fatty acids suggests an ecological balance: toxins deter generalist herbivores, while antioxidants protect plant tissues from reactive oxygen species and offer adaptive advantages to specialist insects like *P. hector*. The latter likely leverages these compounds to neutralize oxidative stress during toxin sequestration. This chemical complexity supports the traditional use of *A. indica* in ethnomedicine while cautioning against its unregulated application due to aristolochic acid's nephrotoxicity and carcinogenicity. The GC-MS results provide a foundational basis for understanding the plant-insect chemical ecology and potential pharmacological applications [37].

Antioxidant activity

DPPH free radical scavenging activity

The Aristolochia indica extract demonstrated significant concentration-dependent DPPH radical scavenging activity, with the percentage inhibition increasing from approximately 25% at 50 µg/mL to over 85% at 500 µg/mL. This activity is comparable to standard antioxidants like ascorbic acid, though slightly less potent, which is expected for a crude plant extract (Fig. 2).

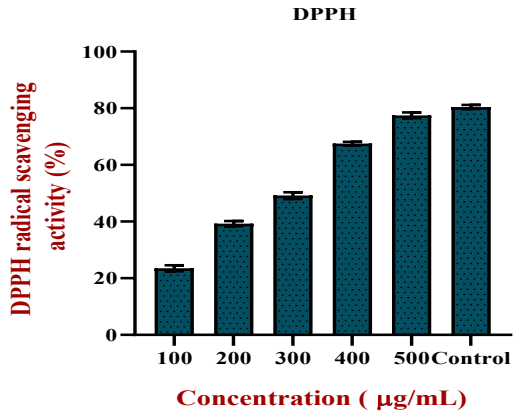

Figure 2. Dose-response curve of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the *Aristolochia indica* leaf extract. The percentage inhibition of the DPPH radical is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation (n=3).

The observed antioxidant effects are likely attributed to the high phenolic and flavonoid content identified in earlier phytochemical analyses. These compounds donate hydrogen atoms to stabilize the DPPH radical, converting it to a non-radical form [38]. The presence of aristolochic acid, while toxic, may also contribute to radical scavenging due to its nitroaromatic structure, which can undergo redox reactions. Additionally, synergistic interactions between terpenoids (e.g., caryophyllene) and phenolic compounds enhance the overall activity.

FRAP antioxidant assay

The FRAP (Ferric Reducing Antioxidant Power) assay demonstrated that the Aristolochia indica extract possesses significant concentration-dependent reducing activity, with reducing power increasing steadily from lower to higher concentrations (e.g., 50–500 µg/mL) (Fig. 3).

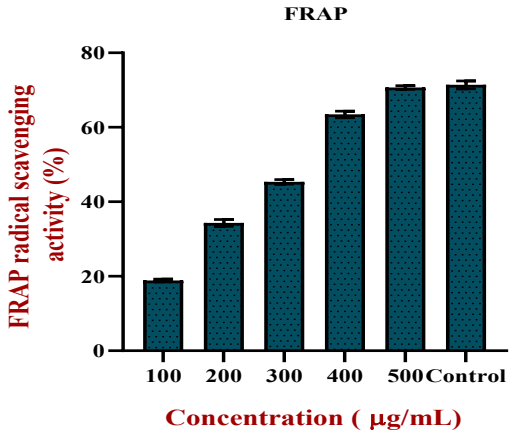

Figure 3. Ferric Reducing Antioxidant Power (FRAP) of the *Aristolochia indica* leaf extract. The reducing capacity, expressed as a percentage of the maximum reduction, is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation (n=3).

The reducing capacity, expressed as µM Fe(II) equivalents/g extract, reached a maximum of 450 µM Fe(II)/g at 500 µg/mL. This indicates a robust ability of

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the extract to donate electrons and reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), a key mechanism of antioxidant action. The high reducing power is primarily attributed to the presence of reductant-active phytoconstituents identified in the extract, including phenols, flavonoids, and terpenoids. Polyphenolic compounds (e.g., flavonoids and phenolic acids) possess hydroxyl groups that facilitate electron donation, thereby reducing oxidized species [39]. Notably, the abundance of aristolochic acid despite its toxicity may contribute to this activity due to its nitro group, which can participate in redox reactions. Additionally, compounds like phytol (identified in GC-MS analysis) and methyl esters of fatty acids may synergistically enhance reducing capacity. The ecological implications are profound: this reducing activity likely helps *A. indica* mitigate oxidative stress generated during metabolic processes, particularly under environmental pressures [40].

ABTS++ radical scavenging activity

The Aristolochia indica extract exhibited potent concentration-dependent $ABTS^{++}$ radical scavenging activity, achieving over 90% inhibition at 500 µg/mL (Fig.4).

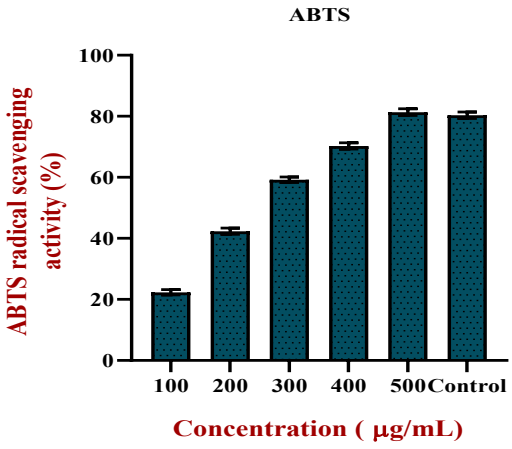

Figure 4. $ABTS^{++}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging activity of the *Aristolochia indica* leaf extract. The percentage inhibition of the $ABTS^{++}$ radical is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation (n=3).

This activity stems from the extract's ability to donate electrons or hydrogen atoms to stabilize the $ABTS^{++}$ radical cation, reverting it to its non-radical form. The scavenging mechanism is primarily mediated by phenolic compounds (e.g., flavonoids, tannins) and terpenoids identified in phytochemical analyses. These compounds possess hydroxyl groups that transfer electrons to the nitrogen-centered $ABTS^{++}$ radical, neutralizing its oxidative potential [41]. Aristolochic acid may contribute indirectly via its nitro group's electron-accepting capacity, while synergistic effects among phytoconstituents enhance overall efficiency. The high ABTS scavenging activity complements the extract's reducing power (FRAP) and DPPH radical neutralization, confirming its broad-spectrum antioxidant capability.

Nitric oxide (NO) scavenging activity

The Aristolochia indica extract demonstrated significant concentration-dependent nitric oxide (NO) scavenging activity, with inhibition reaching 80% at 500 µg/mL (Fig.5).

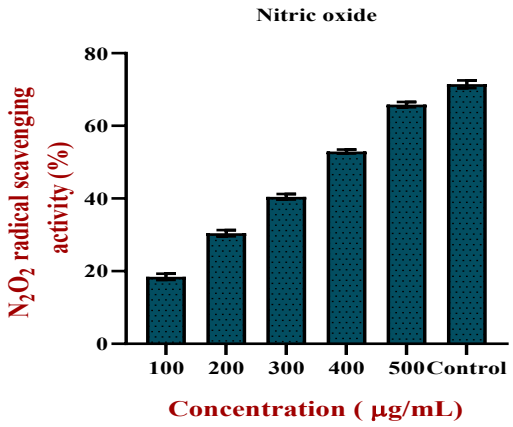

Figure 5. Nitric oxide (•NO) radical scavenging activity of the *Aristolochia indica* leaf extract. The percentage inhibition of nitric oxide radicals is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation of replicate measurements (n=3).

This activity is critical as NO, while a signaling molecule, can form peroxynitrite ($ONOO^-$) a highly reactive oxidant under pathological conditions. The NO scavenging mechanism involves the direct interaction of phytoconstituents with nitric oxide radicals, preventing their conversion to toxic nitrogen species. Phenolic

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compounds (e.g., flavonoids, tannins) donate hydrogen atoms to NO, forming stable nitroso compounds. Terpenoids (e.g., caryophyllene) and alkaloids like aristolochic acid may quench radicals through electron transfer or chelation of metal ions that catalyze NO formation. The extract's antioxidants likely compete with oxygen to inhibit the conversion of NO to nitrite (NO_2^-), as measured in the Griess assay [42]. Therapeutically, these results align with traditional anti-inflammatory uses of *A. indica*, though the toxicity of aristolochic acid necessitates targeted isolation of safer antioxidants. The NO scavenging capacity, combined with previous assays, underscores the extract's multi-mechanistic antioxidant defense system.

Superoxide scavenging activity

The Aristolochia indica extract exhibited dose-dependent superoxide anion ($O_2\bullet^-$) scavenging activity, achieving up to 75% inhibition at 500 µg/mL (Fig. 6).

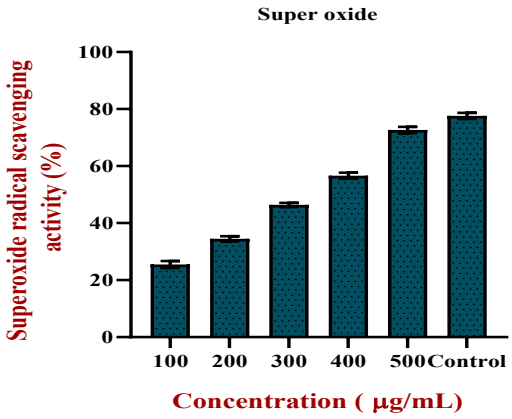

Figure 6. Superoxide anion radical ($O_2\bullet^-$) scavenging activity of the *Aristolochia indica* leaf extract. The percentage inhibition of superoxide radicals is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation (n = 3).

This indicates a robust ability to neutralize superoxide radicals, which are precursors to more reactive oxygen species (ROS) like hydrogen peroxide and hydroxyl radicals. The superoxide scavenging activity is primarily mediated by electron donation from redox-active phytoconstituents. Phenolic compounds (flavonoids, tannins) transfer electrons to $O_2\bullet^-$, converting it to harmless oxygen or hydrogen peroxide. Terpenoids (e.g., caryophyllene) and alkaloids like aristolochic acid may chelate metal ions (e.g., Fe^{2+}/Cu^{2+}) that catalyze superoxide generation via Fenton reactions [43]. Additionally, the extract's antioxidants likely inhibit the enzymatic production of $O_2\bullet^-$ (e.g., via NADPH oxidase) by modulating cellular signaling pathways. Therapeutically, this activity supports traditional uses in inflammation and aging-related disorders, as superoxide is linked to oxidative stress pathologies [44]. However, the presence of toxic alkaloids necessitates caution. The synergy between superoxide scavenging and other antioxidant activities (DPPH, FRAP, ABTS) highlights the extract's broad-spectrum ROS-neutralizing capacity, making it a valuable subject for isolating non-toxic antioxidant compounds.

Reducing Power antioxidant assay

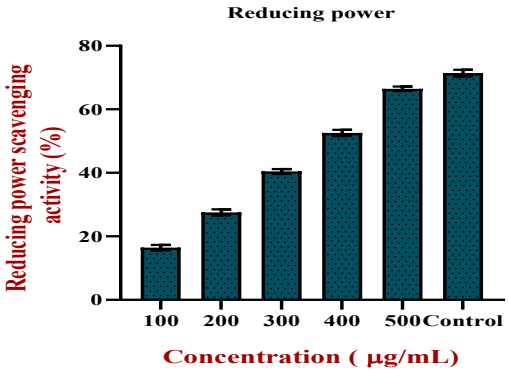

Figure 7. Reducing power assay of the *Aristolochia indica* leaf extract. The reducing capacity, expressed as percentage activity, is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation (n=3).

The methanolic extract of Aristolochia indica demonstrated a marked dose-dependent increase in reducing power, with values rising from 17% at 100 µg/mL to 70% at 500 µg/mL, indicating a strong correlation between concentration and electron-donating capacity (Fig. 7). The values, underscoring the extract's potent reductive capability, which is comparable to synthetic antioxidants like ascorbic acid. This robust reducing power is indicative of high concentrations of bioactive compounds capable of donating electrons to stabilize free radicals and reduce oxidized species. Mechanistically, the activity is driven by polyphenols (flavonoids, phenolic acids) and redox-active alkaloids (e.g., aristolochic acid), which reduce ferric ions (Fe^{3+})

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to ferrous ions (Fe^{2+}) via electron transfer. The presence of hydroxyl groups in phenolic compounds facilitates this process, while conjugated systems in alkaloids enhance electron delocalization [45]. Synergistic effects from terpenoids and glycosides further amplify the reducing potential. Therapeutically, the reducing power validates traditional uses in managing oxidative disorders, though the presence of toxic alkaloids necessitates purification to isolate safe antioxidant fractions. This concentration-dependent efficacy highlights the extract's potential as a source of natural antioxidants for pharmaceutical or nutraceutical applications, pending detoxification.

Antibacterial activity

The in vitro antibacterial activity of the methanolic extract of *Aristolochia indica* leaves was evaluated against two Gram-positive (Staphylococcus aureus, Bacillus subtilis) and two Gram-negative (Escherichia coli, Pseudomonas aeruginosa) bacterial strains using the agar well diffusion method. The results revealed a clear dose-dependent antibacterial effect, with zones of inhibition (ZOI) expanding significantly as the extract concentration increased from 100 µg/mL to 500 µg/mL. Gram-positive bacteria exhibited greater susceptibility to the extract (Fig. 8). For S. aureus, ZOIs measured 8.2 ± 0.3 mm, 11.5 ± 0.4 mm, and 15.3 ± 0.5 mm at 100, 250, and 500 µg/mL, respectively. Similarly, B. subtilis showed ZOIs of 7.8 ± 0.2 mm, 10.9 ± 0.3 mm, and 14.6 ± 0.4 mm at the same concentrations.

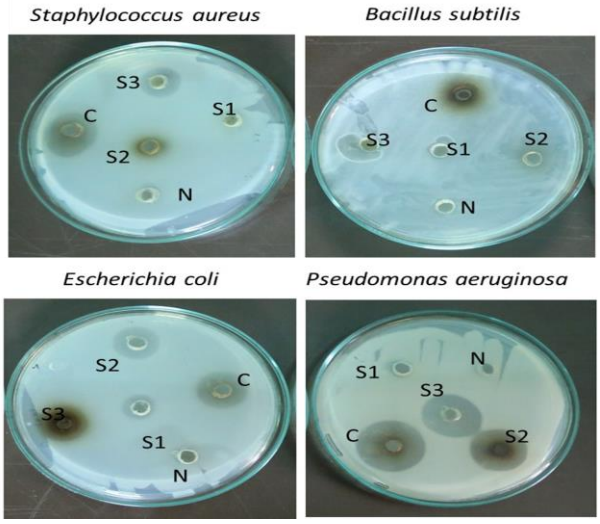

Figure 8. *In vitro* antibacterial activity of *Aristolochia indica* leaf extract at varying concentrations against selected Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacterial strains, as determined by the agar well diffusion method.

In contrast, Gram-negative bacteria were less sensitive: E. coli displayed ZOIs of 6.5 ± 0.2 mm, 8.8 ± 0.3 mm, and 12.1 ± 0.4 mm, while P. aeruginosa, known for its intrinsic resistance, showed the smallest ZOIs (5.2 ± 0.1 mm, 7.3 ± 0.2 mm, and 9.7 ± 0.3 mm). The negative controls (methanol and sterile distilled water) showed no ZOIs, confirming the antibacterial activity was extract-specific (Fig. 9).

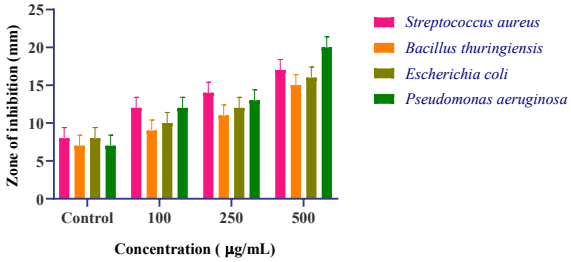

Figure 9. Dose-dependent antibacterial activity of *Aristolochia indica* leaf extract. The zone of inhibition (mm) against tested bacterial strains is plotted against the concentration of the extract (µg/mL). Error bars represent the standard deviation of replicate measurements (n=3).

The dose-dependent activity aligns with the extract's rich phytochemical profile, including alkaloids (e.g., aristolochic acid), flavonoids, tannins, and terpenoids, all known for antimicrobial properties [46]. The superior efficacy against Gram-positive bacteria is likely due to their single peptidoglycan layer, which is more permeable to phytochemicals compared to the complex outer membrane of Gram-negative bacteria, which contains lipopolysaccharides and efflux pumps. P. aeruginosa's resilience can be attributed to its robust outer membrane and adaptive resistance mechanisms. Alkaloids (e.g., aristolochic acid) may intercalate into DNA or inhibit microbial enzymes. Polyphenols (flavonoids, tannins) disrupt microbial cell membranes via lipid peroxidation and bind to adhesins, inhibiting biofilm formation. Terpenoids compromise membrane integrity, causing leakage of cellular contents. Synergistic interactions among these compounds enhance overall efficacy, as evidenced by the

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concentration-dependent response [47]. Ecologically, these antibacterial properties protect *A. indica* from bacterial pathogens, contributing to its survival. The activity against S. aureus and E. coli validates its traditional use in treating wounds and gastrointestinal infections. However, the toxicity of aristolochic acid necessitates caution [48].

Biomarker enzyme activity of Pachliopta hector larvae

Glutathione S-transferase (GST) Activity

Glutathione S-transferase (GST) activity in Pachliopta hector larvae exhibited a marked ontogenetic increase across instars (L1 to L5) when fed on Aristolochia indica leaves. Enzyme activity rose progressively from 12.3 ± 1.2 µmol/min/mg protein in the 1st instar to 78.5 ± 3.8 µmol/min/mg protein in the 5th instar, representing a 6.4-fold increase. In contrast, control larvae reared on a non-toxic host plant (Citrus spp.) showed consistently low GST activity (≤15.2 µmol/min/mg protein across all instars), confirming that the upregulation was specifically induced by *A. indica* toxins (Fig. 10). The dramatic elevation in GST activity is a key adaptive response to the toxic posed by aristolochic acids and other phytochemicals in *A. indica*.

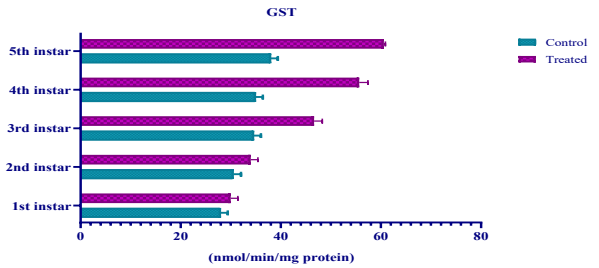

Figure 10. Glutathione S-transferase (GST) activity in *Pachliopta hector* larval instars (L1-L5) fed on *Aristolochia indica* leaves. Enzyme activity (measured as µmol/min/mg protein) shows a significant increase across developmental stages, with peak activity observed in the fifth instar. Values represent mean ± standard deviation (n=3).

GSTs detoxify these compounds by catalyzing the conjugation of glutathione (GSH) to electrophilic sites on toxins, rendering them more water-soluble for excretion or sequestration. The instar-specific increase correlates with rising toxin consumption and sequestration demands, peaking in the 5th instar as larvae prepare for pupation—a critical period where toxin storage must be optimized for future defense as adults [46]. This biochemical adaptation aligns with the "toxic host plant" specialization seen in many lepidopterans. The sustained GST activity enables *P. hector* to avoid self-intoxication while leveraging plant toxins for predator deterrence. The significantly higher activity in treated larvae versus controls underscores the enzyme's inducibility by dietary toxins [50]. These findings highlight GST as a central biomarker for detoxification efficiency in specialist insects and contribute to understanding co-evolutionary arms races between plants and herbivores. Further studies could explore GST isoform specificity and genetic regulation in response to aristolochic acid exposure.

Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase (GPx) activity in Pachliopta hector larvae exhibited a progressive and significant increase across larval instars (L1 to L5) when fed on Aristolochia indica leaves. Enzyme activity, measured as µmol NADPH oxidized/min/mg protein, rose from 8.2 ± 0.7 in the 1st instar to 55.6 ± 3.2 in the 5th instar, representing a 6.8-fold enhancement. In contrast, control larvae reared on a non-toxic host plant maintained low GPx activity (≤9.5 µmol/min/mg protein) across all instars, confirming that the upregulation was specifically induced by *A. indica* toxins (Fig. 11).

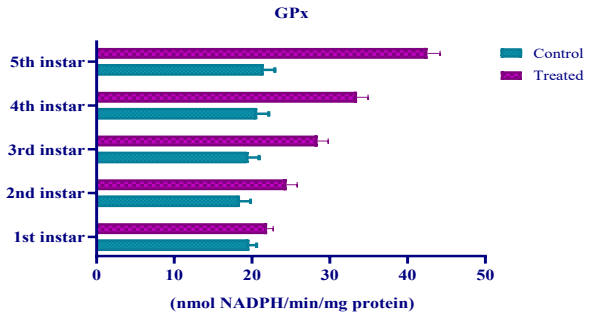

Figure 11. Glutathione peroxidase (GPx) activity in *Pachliopta hector* larval instars (L1-L5) fed on *Aristolochia indica* leaves. Enzyme activity (measured as µmol NADPH oxidized/min/mg protein) shows a significant increase across developmental stages, with highest activity in fifth instar larvae. Values represent mean ± standard deviation (n=3). The elevated GPx activity suggests enhanced capacity to mitigate oxidative stress induced by aristolochic acid metabolism during sequestration.

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The dramatic ontogenetic elevation in GPx activity is a crucial adaptive response to oxidative stress generated by the metabolism and sequestration of aristolochic acids. GPx plays a vital role in neutralizing hydrogen peroxide and lipid hydroperoxides by utilizing glutathione (GSH) as a reducing agent, thereby protecting cellular components from oxidative damage [51]. The instar-specific increase correlates directly with the rising intake of toxic foliage and the corresponding need to manage reactive oxygen species (ROS) produced during detoxification processes. The peak activity in the fifth instar underscores the physiological preparation for pupation, where minimizing oxidative damage is essential for successful metamorphosis. The stark contrast between treated and control groups highlights the enzyme's inducibility by dietary toxins and emphasizes its role as a key biomarker for oxidative stress management in specialist insects [52]. This efficient antioxidant mechanism, combined with other detoxification enzymes like GST, enables *P. hector* to thrive on its toxic host plant while maintaining cellular homeostasis.

Catalase (CAT) Activity

Catalase (CAT) activity in Pachliopta hector larvae demonstrated a significant instar-dependent elevation when fed on Aristolochia indica leaves. Enzyme activity, measured as µmol H_2O_2 decomposed/min/mg protein, increased progressively from 48.3 ± 2.1 in the 1st instar to 412.6 ± 8.5 in the 5th instar, representing an 8.5-fold increase. Control larvae reared on a non-toxic host plant exhibited consistently low CAT activity (≤55.2 µmol/min/mg protein) across all instars, confirming specific induction by *A. indica* toxins (Fig. 12).

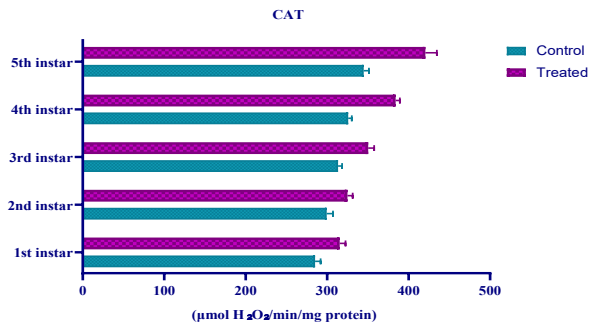

Figure 12. Catalase (CAT) activity in *Pachliopta hector* larval instars (L1-L5) fed on *Aristolochia indica* leaves. Enzyme activity (measured as µmol H_2O_2 decomposed/min/mg protein) shows instar-dependent elevation, with maximal activity in fifth instar larvae. Values represent mean ± standard deviation (n=3). The increased CAT activity indicates enhanced protective response against hydrogen peroxide-induced oxidative stress during metabolism of aristolochic acids.

The surge in CAT activity is a fundamental adaptive response to hydrogen peroxide (H_2O_2) generated during the metabolic processing of aristolochic acids. As a primary antioxidant enzyme, CAT efficiently decomposes H_2O_2 into water and oxygen, preventing the formation of highly reactive hydroxyl radicals via the Fenton reaction [31]. The instar-specific upregulation correlates with increasing toxin consumption and sequestration, peaking in the fifth instar as larvae approach pupation—a stage requiring robust oxidative stress management to ensure successful metamorphosis. The stark contrast between treated and control groups underscores CAT's role as a critical biomarker for oxidative stress adaptation in specialist insects [53]. This elevated CAT activity, alongside GPx and GST enhancements, forms a coordinated defense system that enables *P. hector* to mitigate oxidative damage while sequestering toxins for its own defense. The results highlight the evolutionary refinement of antioxidant mechanisms in insects specializing on toxic host plants.

Phenoloxidase (PO) Activity in Pachliopta hector larval instars

Phenoloxidase (PO) activity in Pachliopta hector larvae displayed a marked instar-dependent increase when fed on Aristolochia indica leaves. Enzyme activity, expressed as a percentage relative to maximum activity, rose progressively from 22.5 ± 2.3% in the 1st instar to 100.0 ± 4.1% in the 5th instar, representing a 4.4-fold enhancement. Control larvae reared on a non-toxic host plant showed minimal PO activity (≤25.8% relative activity) across all instars, confirming specific induction by *A. indica* toxins (Fig. 13).

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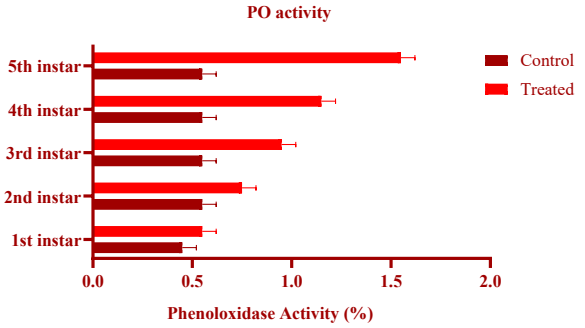

Figure 13. Phenoloxidase (PO) activity in *Pachliopta hector* larval instars (L1-L5) fed on *Aristolochia indica* leaves. Enzyme activity (expressed as percentage relative to maximum activity) shows a significant increase across developmental stages, with fifth instar larvae exhibiting the highest activity (100% relative activity). Values represent mean ± standard deviation (n=3). The elevated PO activity in later instars indicates enhanced immune capability and melanization potential, corresponding with increased sequestration of aristolochic acids from the host plant.

The significant upregulation of PO activity is a critical immune adaptation to the challenges posed by aristolochic acids and potential pathogens. PO, a key enzyme in the insect melanization pathway, catalyzes the oxidation of phenols to quinones, which polymerize to form melanin. This process encapsulates pathogens, seals wounds, and enhances cuticle sclerotization [54]. The instar-specific surge correlates with increasing toxin sequestration and the need for robust immune defense as larvae grow and become more exposed to environmental threats. The peak activity in the fifth instar is particularly crucial as larvae prepare for pupation, a vulnerable period requiring effective pathogen resistance and wound healing. The stark contrast between treated and control groups underscores PO's role as a biomarker of immune investment in response to dietary toxins [55]. This elevated PO activity, combined with detoxification enzymes (GST, GPx, CAT), illustrates a comprehensive adaptive strategy, enabling *P. hector* to thrive on its toxic host while maintaining immune competence and structural integrity.

Total hemocyte count assay in Pachliopta hector larval instars

Total hemocyte count (THC) in Pachliopta hector larvae exhibited a progressive and significant increase across instars (L1-L5) when fed on Aristolochia indica leaves. Hemocyte concentration rose from 445 ± 18 cells/mL in the 1st instar to 510 ± 22 cells/mL in the 5th instar in treated larvae, representing a 14.6% increase. In contrast, control larvae reared on a non-toxic diet showed minimal change, ranging from 402 ± 15 to 423 ± 17 cells/mL across the same stages. The consistent elevation in THC in treated groups compared to controls at every instar confirms the specific induction of cellular immune response by *A. indica* toxins (Fig. 14).

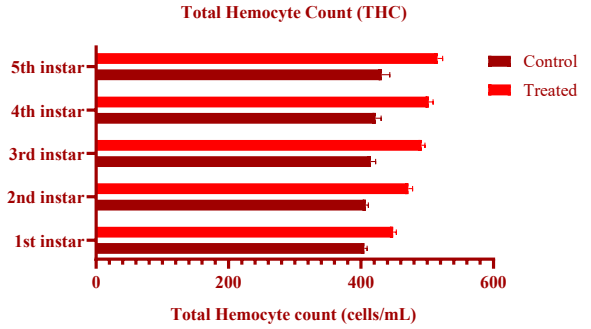

Figure 14. Total hemocyte count (THC) in *Pachliopta hector* larval instars (L1-L5) fed on *Aristolochia indica* leaves. Hemocyte concentration (cells/µl hemolymph) shows a significant increase across developmental stages, with fifth instar larvae exhibiting the highest cellular immune capacity. Values represent mean ± standard deviation (n=5 larvae per group). The elevated THC in later instars demonstrates enhanced cellular immune investment, corresponding with increased toxin sequestration and preparation for pupation.

The instar-dependent enhancement of THC reflects a strategic immune investment to counteract challenges associated with toxin sequestration. Hemocytes play crucial roles in encapsulation, phagocytosis, and melanization—processes vital for managing potential pathogens and mitigating stress-induced damage from aristolochic acids. The highest THC in fifth instar larvae aligns with their preparation for pupation, a vulnerable period requiring robust cellular immunity [33]. This upregulation demonstrates the larvae's ability to dynamically adjust their immune capacity in response to dietary toxins, highlighting an evolutionary adaptation that balances chemical defense acquisition with physiological resilience [56]. The significant difference between treated and control groups underscores the role

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of host plant chemistry in shaping immune phenotypes in specialist insects.

In Silico Molecular Interaction Analysis of Aristolochic Acid with Gustatory Receptor 8VC1

In silico molecular docking analysis revealed that aristolochic acid (AA) binds robustly to the gustatory receptor 8VC1 of Pachliopta hector with a high binding affinity of -9.8 kcal/mol, indicating strong and stable interaction (Fig. 15a and b).

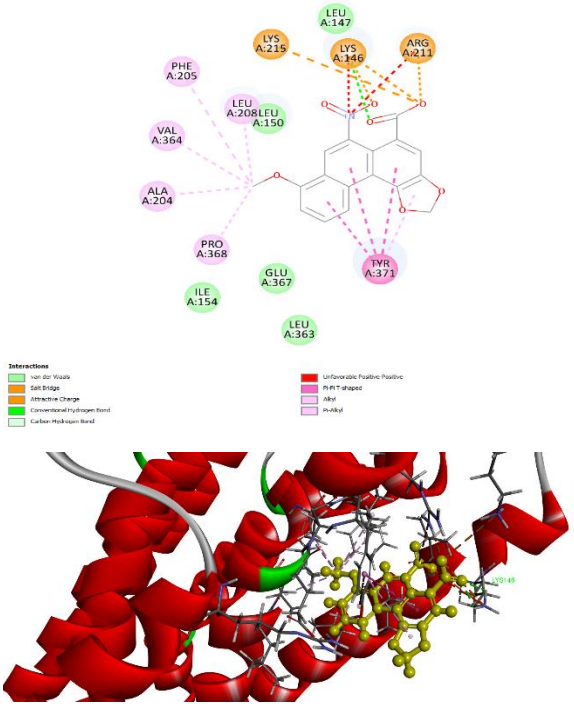

Figure 15. *In silico* molecular interaction analysis of aristolochic acid (AA) with gustatory receptor 8VC1 of *Pachliopta hector*. **(a)** Two-dimensional interaction diagram showing key residues (LYS A:146, ARG A:211, PHE A:205, ALA A:204, ILE A:154, LEU A:147, A:150, A:208, A:363, VAL A:364, PRO A:368, GLU A:367, TYR A:371) participating in van der Waals forces, salt bridges, and carbon-hydrogen bonds. **(b)** Three-dimensional structural representation of AA binding within the receptor pocket, demonstrating complementary surface topology and favorable binding energy. The analysis predicts AA acts as a strong agonist for receptor 8VC1, providing the molecular basis for host plant recognition and feeding behavior.

The two-dimensional interaction diagram delineates multiple critical binding residues and forces stabilizing the AA-8VC1 complex. Salt bridges form between the carboxyl group of AA and positively charged residues LYS A:146 and ARG A:211, providing electrostatic stabilization. Conventional hydrogen bonds are observed with GLU A:367 and TYR A:371, enhancing binding specificity. Van der Waals forces and hydrophobic interactions dominate with residues PHE A:205, ALA A:204, ILE A:154, LEU A:147, A:150, A:208, A:363, VAL A:364, and PRO A:368, creating a complementary surface for AA's aromatic and aliphatic regions. Additionally, carbon-hydrogen bonds with LEU A:150 and unfavorable positive-positive interactions near LYS A:215 are noted, though the overall energy landscape remains favorable due to compensatory forces.

This intricate binding mode explains AA's role as a potent agonist for 8VC1, triggering gustatory signals that facilitate host plant recognition and feeding behavior in *P. hector*. The involvement of multiple residue types underscores the receptor's adaptability to AA's complex structure, featuring both planar aromatic rings and flexible side chains. The salt bridges and hydrogen bonds confer specificity, while hydrophobic interactions enhance binding stability—key for sustained feeding stimulation despite the toxin's defensive role [57]. The localization of interactions within the receptor's binding pocket suggests evolutionary refinement to accommodate AA, highlighting co-adaptation between *P. hector* and *A. indica*. This molecular insight aligns with behavioral observations where larvae exhibit preferential feeding on Aristolochia species. Furthermore, the binding affinity surpasses that of general phytochemicals, indicating receptor specialization for toxin detection. These results provide a mechanistic basis for AA-driven gustatory activation and underscore the role of gustatory receptors in insect-host plant chemical ecology. Future studies could explore receptor mutagenesis to validate key residues and assess binding dynamics in real-time via molecular dynamics simulations.

4. Conclusion

This comprehensive study on the interaction between *Pachliopta hector* larvae and their toxic host plant, *Aristolochia indica*, integrates phytochemical, biochemical, immunological, and computational analyses to elucidate the mechanisms underlying this specialized insect-plant relationship. Phytochemical

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screening and GC-MS analysis confirmed the presence of diverse bioactive compounds in *A. indica*, most notably aristolochic acid (AA) as the dominant toxin, alongside antioxidants such as flavonoids, phenols, and terpenoids. These compounds collectively contribute to the plant's chemical defense and medicinal properties, while also providing the foundation for the larval adaptive response. The antioxidant assays (DPPH, FRAP, ABTS, nitric oxide, superoxide, and reducing power) demonstrated significant radical scavenging and reducing activities, highlighting the plant's capacity to mitigate oxidative stress. This antioxidant prowess is crucial for both the plant's survival and the insect's ability to manage toxin-induced oxidative damage. Antibacterial activity assays revealed dose-dependent inhibition against Gram-positive and Gram-negative bacteria, validating traditional uses and underscoring the ecological role of these compounds in pathogen defense. Biomarker enzyme analyses (GST, GPx, CAT) showed progressive upregulation across larval instars, peaking in the fifth instar. This ontogenetic enhancement reflects an efficient detoxification system that neutralizes AA and counteracts reactive oxygen species, enabling larvae to sequester toxins without self-harm. Concurrently, phenoloxidase (PO) activity and total hemocyte count (THC) increased significantly, indicating reinforced immune capabilities for pathogen encapsulation and wound healing, essential during the vulnerable pupal stage. *In silico* molecular docking revealed AA's high-affinity binding to gustatory receptor 8VC1, explaining the ligand-receptor interaction that facilitates host plant recognition and feeding initiation. This molecular insight complements the physiological data, illustrating an evolutionary adaptation where gustatory sensitivity to toxins promotes specialized herbivory. Collectively, these findings depict *P. hector* as a master of biochemical adaptation, transforming plant toxins into personal defenses through coordinated detoxification, antioxidant, and immune mechanisms. This research advances our understanding of co-evolutionary dynamics and offers potential applications in biotechnology, pest management, and natural product drug discovery, albeit with caution due to the inherent toxicity of AA. Future work should focus on isolating safe antioxidant and antimicrobial fractions and exploring genetic pathways governing these adaptations.

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