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Phytochemical screening and pesticidal efficacy of phytoecdysteroid fraction of a fern *Pityrogramma calomelanos* (L.) Link against *Spodoptera litura* (Fabricius.)

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ABSTRACT

Lepidopteran insect pests voraciously feed the vegetable crops and causes yield loss every year. Chemical insecticides widely used to control the destructive pest incidence which heavily affects the beneficial and non-target organisms and in the mean time, pests developed resistance. Biopesticides are being developed to minimize these ill effects. Plants synthesize secondary metabolites to prevent from biotic and abiotic stresses. More than 27 families of pteridophyta (ferns) are reported with phytoecdysteroids (PEs). PEs are analogues of insect moulting hormone and acts as insect growth regulators (IGRs). The present study aimed to isolate the phytoecdysteroid fraction from the fern, *Pityrogramma calomelanos*, preliminary screening of phytochemicals using standard protocols including UV-Visible spectrophotometric analysis and survival, growth and developmental periods of *Spodoptera litura* treated with phytoecdysteroid fraction. Steroids and phenolic compounds were present in the fraction and six prominent peaks were recorded between the wavelength of 200-800nm. *Spodoptera litura* larvae treated with phytoecdysteroid fraction showed maximum larval mortality (68%) in 2000ppm (LC_{50} = 1473ppm; F = 48.65; p = 0.049). Pupation, pupal weight and adult emergence were decreased in higher concentrations. Pupal mortality was relatively high in treated group than control (2.04%). Larvae treated with minimal concentrations exhibited developmental deformities includes larval - pupal, pupal - adult intermediates, deformed pupae, ecdysial failure, adultoids, early/late developmental periods and it may due to the interaction of phytoecdysteroid fraction to the insect endocrine system. Therefore, phytoecdysteroid mediated biopesticide formulation could be the better alternative to commercial chemical insecticides under IPM programme.

Key words: *Pityrogramma calomelanos*, Phytochemical analysis, UV-Visible Spectrophotometer, Bioassay, Developmental period and Larval mortality

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INTRODUCTION

Insect pests cause great harm to the agricultural crops and reduce their productivity. Tobacco armyworm, *Spodoptera litura* (Fab.) a serious polyphagous lepidopteran insect pest which infests about 115 plant species across the globe and out of that 60 species are known from India (Gandhi *et al*., 2016). Application of chemical insecticides is the most relied strategy to prevent yield loss. Though the chemical pesticides enhanced economic potential in terms of productivity, indiscriminate and rampant use of chemical pesticides under inappropriate condition causes potential risk to humans and other non-targeted organisms. Due time, insect pests developed resistance towards different synthetic pesticides (Senthil-Nathan, 2013). To curb the consequences, biopesticides with

certain standards become the key to solve pest problems and promote sustainable production. Such biopesticides are gaining widespread acceptance due to their cost effectiveness, reduced toxicity, target specificity, biodegradability and environmental safety (Souto *et al*., 2021). Secondary metabolites of plants are toxic to insect pests and acts as toxicants, repellents, anti-feedents, insect growth regulators (IGRs) and oviposition deterrents (Upadhyay *et al*., 2006). Phytoecdysteroids (PEs) are a class of chemicals synthesized by plants for defense against phytophagous insects and causes severe adverse effects including weight loss, moulting disruption and mortality. Approximately 6% of all plant species synthesis phytoecdysteroids and it have been detected in 27 families of pteridophyta (Arif *et al*., 2022). Phytoecdysteroids found in more than 50% of fern families such as Pteridaceae, Polypodiaceae and Blechneaceae (Savchenko *et al*., 2022).

Pityrogramma calomelanos (L.) Link (Pteridaceae) known as silver fern, shows several medicinal properties such as astringent, analgesic, anti-hemorrhagic, anti-hypertensive, anti-pyretic, anti-tussive and used to treat urinary problems (Lans, 2006). Ferns are promising candidates as a source of green insecticides and several species produce steroids (Xavier *et al*., 2016) which have insecticidal potential including *Dicksonia sellowiana* and *Nephrolepis cordifolia*. The ferns, *Christella parasitica, Pteridium aquilinum* and *Hemionitis arifolia*, efficiently controlled the incidence of insect pests in Groundnut field (Sahayaraj and Selvaraj, 2013). However, studies on biological activity of ferns remain scarce. Therefore, this study was designed to examine the bioactive phytochemicals present in the phytoecdysteroid fraction of fern, *P. calomelanos* and its pesticidal potential over the phytophagous pest, *Spodoptera litura*.

MATERIALS AND METHODS

Collection and extraction of *P. calomelanos*

Pityrogramma calomelanos (L.) Link collected from Parassala, (8.3394^{o} N, 77.1517^{o} E) Trivandrum, Kerala, India and identified by Dr. V. Irudayaraj (Rtd), Department of Botany, St. Xavier's College (Autonomous), Palayamkottai. Voucher specimen (SXC/CPRC/FN/32) was prepared and deposited in Crop Protection and Research Centre (CPRC), St. Xavier's College, Palayamkottai. The fern was washed thrice in tap water to remove dust and debris and were shade dried for two weeks. The dried materials were partially ground in a domestic grinder and stored in refrigerator for further use. From the powder, 50g of each was taken separately for the Soxhlet extraction (45±5^{o}C) using ethanol solvent for about 8 hrs. The extract was concentrated and stored in refrigerator for further use.

Partial purification of phytoecdysteroid fraction

A common method for the isolation of phytoecdysteroid fraction from the dried plant materials (Rajkumar *et al.,* 2000) is used. In brief, extracts were dried in vacuum and residue partitioned between hexane and 75% ethanol (50mL of each phase). Hexane fraction (HF) was discarded. Ethanol phase was concentrated in vacuum and partitioned between chloroform: ethanol: water (10mL of each phase). Aqueous fraction (AF) was discarded. The chloroform phase (CP) was concentrated to dryness and dissolved in ethyl acetate: ethanol (2:1) as a 5% solution and filtered through neutral alumina (10%, 2g) and eluted with further solvent (25mL). The total elute was evaporated to dryness and considered as crude phytoecdysteroid fraction (PE).

Preliminary screening of phytochemicals

Phytoecdysteroid fraction of *P. calomelanos* was used for the phytochemical analysis (Brinda *et al*., 1981). The fraction was concentrated under room temperature and stored at 4°C for further use and then redissolved in distilled water to get the solution of 1mg/1mL which was subjected to phytochemical analysis (Dhivya and Kalaichelvi, 2017).

UV-Visible Spectrophotometry analysis

The phytoecdysteroid fraction of fern was examined under UV-Visible spectrophotometer for proximate analysis. The sample was centrifuged at 3000rpm for 10min and filtered

through Whatmann No.1 filter paper then the sample is diluted to 1:10 with the same solvent. The fraction was scanned at the wavelength ranging from 200-800nm using Shimadzu UV – 1601 spectrophotometer and the characteristic peaks were detected (Dhivya and Kalaichelvi, 2017).

Insect rearing

A single egg mass with 250-300 eggs of *S. litura* were collected from Bhendi field at Veeravanallur, Tirunelveli, Tamil Nadu. Eggs were hatched after 3days of incubation in room temperature (27±2ºC) with 14:10 hrs (Photophase:Scotophase) exposure and 75% relative humidity. Freshly hatched neonate larvae were fed with tender castor leaves (*Ricinus communis*) up to pre-pupal stage. Utmost care was taken to avoid overcrowding and strict sanitation was maintained to prevent any infection. After pupation, the pupae were collected and placed inside the oviposition chamber for adult emergence and oviposition. Cotton soaked with 10% (w/v) sugar solution fortified with multivitamins was provided as feed for the emerging adult. Castor leaves were kept inside the chamber for laying eggs. Adult female laid creamy white eggs covered with scales under the leaves. Eggs were carefully taken out using brush with soft bristles and kept in petridish with tender castor leaves and cotton soaked with water. Eggs were hatched into neonates and the laboratory reared larvae were used for the study.

Preparation of phytoecdysteroid and bioassay

Stock solution of 5000ppm (0.5%) of phytoecdysteroid fraction (PE) of *P. calomelanos* was prepared in ethanol, from which different concentrations (250, 500, 1000, 1500 and 2000ppm) were prepared to evaluate the effect of phytoecdysteroid fraction on the survival, growth and developmental parameters of *S. litura* by using leaf dip bioassay method (Kaur *et al*., 2019).

Third instar larvae (8-day old) were taken for the study. Ten grams of castor leaves each were soaked in five different concentrations (wt/v) separately *viz*., 250, 500, 1000, 1500 and 2000ppm of phytoecdysteroid fraction of *P. calomelanos*. The treated and control category leaves were air dried for 5minutes. The healthy, pre-starved newly moulted *S. litura* larvae (ten each) were taken in plastic containers of 500mL capacity and were provided with respective concentrations soaked castor leaves and the containers were covered with muslin cloth. Five replicates (n=50) were maintained for each concentrations and control respectively. Containers were cleaned on daily basis to avoid any infection. The left-over leaves and excreta were collected for further studies and replaced with fresh leaves. After the stipulated period of the experimental exposure (4days) the animals were fed with normal diet (untreated castor leaves). Larval mortality and developmental deformities were observed. The percentage mortality was calculated using Abbott's formula (Abbott, 1925);

Mortality (%) = No. of dead individuals / No. of treated individuals × 100

Survival and development of *S. litura*

The pupated insects were collected, cleaned and placed on moist cotton swabs in petridishes and kept in the adult emergence cages for further observation until adult emergence. The pupae were examined daily to note mortality or developmental abnormalities if any. This was continued up to the adult emergence or death of all the experimental animals. The emerged adult moths were collected and released into the oviposition chamber. Pupation, pupal mortality, adult emergence (%), pupal weight (mg), larval, pupal period and adult longevity (days) were observed and recorded (Pandey *et al*., 2020).

Statistical analysis

Data were subjected to one way - analysis of variance (ANOVA) and means were separated by Tukey's HSD (honestly significant difference) at $p \leq 0.05$. Values were represented as mean ± SE. Lethal concentrations (LC_{30}, LC_{50} and LC_{90}) were calculated using probit analysis and statistical analysis of experimental data was carried out using SPSS software for Windows version 16.0 (Kaur *et al*., 2019).

RESULT

Phytochemical analysis

Phytochemicals are naturally occurring, physiologically active chemical substances found in plants, which protect plants from pests,

diseases and favours the plant's colour, aroma and flavour. In this study, phytoecdysteroid fraction of *P. calomelanos* was analysed qualitatively for the secondary metabolites and tested positive only for steroids and phenolic compounds.

UV- Visible Spectrophotometry analysis

UV-Vis spectrum profile of phytoecdysteroid fraction of *P. calomelanos* showed the peaks with proper baseline at the wavelength of 326.5nm, 353nm, 364nm, 505.5nm, 539nm, 606nm and 666nm along with the absorbance of 3.92, 4, 4, 0.73, 0.57, 0.45 and 1.76 respectively (Fig. 1).

Wavelength (nm)	Absorbance
666	1.756
606	0.447
539	0.571
505.5	0.729
364	4
353	4
326.5	3.923

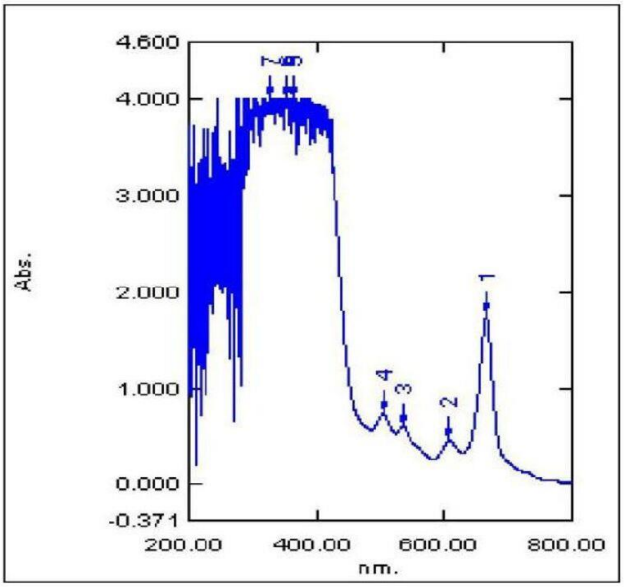


Fig. 1. UV-Visible spectrophotometric analysis of phytoecdysteroid fraction of *P. calomelanos*.

The precise range and relative absorbance of the peak maxima provide key information about the nature of phytochemicals in the phytoecdysteroid fraction.

Larval mortality of *S. litura*

The mortality rate of *S. litura* treated with phytoecdysteroid fraction of *P. calomelanos* exhibited good larvicidal activity (Table. 1; Fig. 2). The significant highest mortality was observed at the concentration of 2000ppm against *S. litura* larvae as compared to control category (2%). The obtained LC_{50} value was 1473ppm (LC_{30} = 821.35ppm; LC_{90} = 6144ppm; F = 48.65; p = 0.049) and the mortality rate (%) was directly proportional to the concentrations. In later instars, larval mortality took place due to failure of moulting.

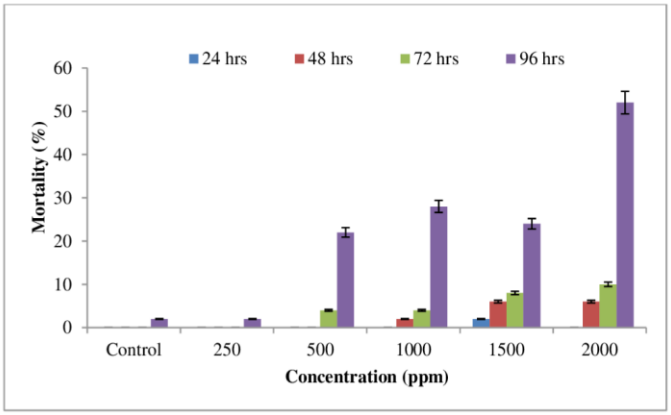
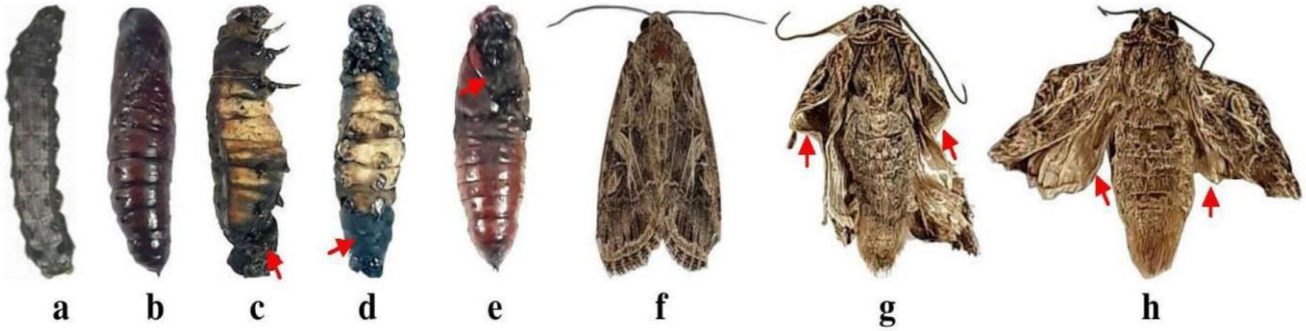


Fig. 2. Larval mortality (%) of *S. litura* larvae treated with phytoecdysteroid fraction of *P. calomelanos.*

Survival and development of *S. litura*

Percentage of pupation and adult emergence was decreased significantly at higher concentration (2000ppm). Under the influence of phytoecdysteroid fraction of *P. calomelanos*, maximum pupal mortality (PM) was observed in 1500 and 2000ppm (Table 1). Compared to control, reduced larval period (LP) and adult longevity (AL) were observed such as 15days (2000ppm) and 3.6days (1500 and 2000ppm) respectively (Table 2). Extended pupal period (PP) was observed in 2000ppm (7.8days) as against to control (7days). Phytoecdysteroid fraction of *P. calomelanos* heavily influenced the pupal weight (PW) and resulted in decreased pupal weight of 280.60mg (2000ppm) and followed by 286.80mg (1500ppm) compared to control (306.60mg). Consumption of phytoecdysteroid fraction of *P. calomelanos* amended diet interrupts regular life cycle of *S.*

Fig. 3. Developmental deformities of *S. litura* treated with phytoecdysteroid fraction of *P. calomelanos*



a) Normal 3rd instar larva, **b)** Normal pupa, **c, d)** Ecdysial failure and Larval - pupal intermediates, **e)** Deformed pupa with reduced thoracic region, **f)** Normal adult moth, **g, h)** Dorsal view of adultoids with twisted and crumbled wings. ↗ red arrows shows abnormalities

Table 1. Effect of phytoecdysteroid fraction of *P. calomelanos* on the survival of *S. litura*

Concentrations (ppm)	LM	P	PM	AE
Control	2.00 ± 0.00^{cdef}	98.00 ± 0.00^{cdef}	2.04 ± 0.38^{cdef}	97.96 ± 0.72^{cdef}
250	2.00 ± 0.42^{cdef}	98.00 ± 2.00^{cdef}	11.11 ± 0.00^{cdef}	88.89 ± 0.00^{cdef}
500	26.00 ± 0.87^{abf}	74.00 ± 4.00^{abf}	23.33 ± 0.68^{abdef}	76.67 ± 0.39^{abdef}
1000	34.00 ± 0.59^{abf}	66.00 ± 3.09^{abf}	36.90 ± 1.76^{abcef}	63.10 ± 1.86^{abcef}
1500	40.00 ± 0.00^{abf}	60.00 ± 0.00^{abf}	55.08 ± 3.75^{abcdf}	44.92 ± 1.38^{abcdf}
2000	68.00 ± 0.53^{abcde}	32.00 ± 0.32^{abcde}	67.86 ± 2.19^{abcde}	32.14 ± 1.73^{abcde}

Within each column, mean ± SE followed by the same letters show significant differences (ANOVA, Tukey's HSD test, $p \leq 0.05$); **LM**-Larval mortality, **P**-Pupation, **PM**-Pupal mortality, **AE**-Adult emergence

Table 2. Effect of phytoecdysteroid fraction of *P. calomelanos* on the developmental stages of *S. litura*

Concentrations (ppm)	LP	PP	AL	PW
Control	15.20 ± 0.74	7.00 ± 0.00^{bcde}	4.80 ± 0.24^{cdef}	306.60 ± 1.86^{bcdef}
250	15.00 ± 0.00	7.00 ± 0.00^{af}	4.60 ± 0.45^{cdef}	304.80 ± 3.17^{aef}
500	15.00 ± 0.00	7.00 ± 0.00^{af}	4.40 ± 0.00^{abdef}	297.20 ± 2.21^{af}
1000	15.00 ± 0.00	7.00 ± 0.00^{af}	4.20 ± 0.00^{abcef}	289.74 ± 1.20^{a}
1500	15.00 ± 0.00	7.40 ± 0.24^{a}	3.60 ± 0.40^{abcdf}	286.80 ± 0.87^{ab}
2000	15.00 ± 0.00	7.80 ± 0.20^{bcd}	3.60 ± 0.55^{abcde}	280.60 ± 0.93^{abc}

Within each column, mean ± SE followed by the same letters show significant differences (ANOVA, Tukey's HSD test, $p \leq 0.05$); **LP**-Larval period, **PP**-Pupal period, **AL**-Adult longevity, **PW**-Pupal weight

litura (Fig.3 a, b, f) and resulted in larval - pupal intermediates (Fig. 3c, d), deformities in pupae (Fig. 3e) and adults (Fig.3g, h).

DISCUSSION

Qualitative screening of phytochemicals in the phytoecdysteroid fraction of *P. calomelanos* showed the presence of steroids and phenolic compounds. Plant steroids are well known for their insecticidal and anti-microbial properties. Plant phenolic compounds possess anti-microbial, anti-inflammatory, anti-feedent, anti-viral, anti-cancer, vasodilatory actions and insecticidal activities (Mandal *et al*., 2005). Similarly, Xavier *et al*. (2016) reported the phytoecdysteroid fraction of *C. dentata* and *N. cordifolia* with the occurrence of tannins, steroids, phenolic compounds and terpenoids. Among the pteridophytes, *Cheilanthes tenufolia, Polypodium vulgare* (Lafont *et al*., 2021), *Pteridium aquilinum, Seratula tinctora, Cheilanthes farinosa* (Rajkumar *et al*., 2000), *Christella parasitica, Pteridium aquilinum* and *Hemionitis arifolia* (Sahayaraj and Selvaraj, 2013) were reported to contain phytoecdysones.

UV-Visible spectrophotometry analysis of phytochemicals in the phytoecdysteroid fraction *P. calomelanos* showed six prominent peaks at the wavelength ranging between 326.5nm - 666nm. Various authors reported the absorption spectrum of different phytochemicals that include steroids (200-400nm) and polyphenols (240-650nm) (Zahari *et al*., 2016). UV absorbance of 20 - E (ecdysteroid analogue) recorded at 242nm and 310nm (Lafont *et al*., 2021). Raimana *et al*. (2008) reported a typical ecdysteroid spectrum with a maximum wavelength of 242nm and two unusual peaks at 294 and 317nm indicated the presence of an aromatic conjugating moiety.

Plants provide a rich source of secondary metabolites possessing attractive pesticidal properties and are mainly biodegradable and renewable. Pteridophytes are reported with high concentration of phytoecdysteroids which mimic like insect moulting hormone and play a role in disturbing the metamorphosis (Chaubey, 2018). In this experiment, phytoecdysteroid fraction of *P. calomelanos* treated 3rd instar *S. litura* larvae showed distinct larvicidal activity. At 96 hrs of treatment, maximum larval mortality (52%) was obtained in 2000ppm concentration. This could be due to the active compounds of secondary metabolites present in the phytoecdysteroid fraction of *P. calomelanos*. Rajkumar *et al*. (2000) concluded that active mixture of secondary metabolites along with phytoecdysteroids synergistically acts over the insect pests. Phytoecdysones of *Pteridium aquilinum* (bracken fern) plays a potent role against phytophagous insect attacks, feeding deterrents and disrupts the development and/or reproduction (Jones and Firn, 1978). Steroidal derivative of *Myrtillocactus geometrizans* (Bilberry cactus), showed insecticidal and insect growth regulatory activity against *S. frugiperda* (Cespedes *et al*., 2005). Jing *et al*. (2012) stated that plant sterols affect reproduction of *S. exigua*. Plant secondary metabolites (psoralen and 2-undecanone) responsible for the toxicity effect on *S. frugiperda* (Ayil-Gutiérrez *et al*., 2015). Phytoecdysteroids alone or in combination with other potent signaling molecules deters plant consumption (anti-feedents), disrupt invertebrate endocrine system, or cause death in phytophagous insects (Soriano *et al*., 2004).

Phytoecdysteroid fraction of *P. calomelanos* treated *S. litura* larvae showed several developmental abnormalities, shortened or delayed growth period. Higher larval and pupal mortality was observed in 2000ppm concentration as 68% and 67.86% respectively. The percentage of pupation and adult emergence was relatively high in control and 250ppm concentration. These results may due to the occurrence of IGRs (Insect Growth Regulators) and juvenile hormone analogues (JHAs) in the phytoecdysteroid fraction of ferns which inhibits the pupation and adult emergence (Ghoneim *et al*., 2007). *S. litura* larvae treated with minimal concentration of phytoecdysteroid fraction of *P. calomelanos* caused the deformities in pupae and were died with abnormal head capsule, shrunken body and larval - pupal intermediates with larval exuviae. 20-Hydroxyecdysone and its analogue ponasterone A inhibit ecdysis in pink bollworm, *Pectinophora gossypiella* (Jones and Firn, 1978). Aldhous (1992) found that imbalanced

moulting hormone titer attributed to larval - pupal, pupal - adult intermediates, deformed pupae and adults. During moulting, 20-hydroxy ecdysone circulates as free hormone in the haemolymph which emphasizes the morphological abnormalities in larvae and pupae. Phytoecdysteroids enhances abnormal moulting in several arthropods (Soriano *et al*., 2004). The larval period and adult longevity was decreased in 2000ppm (15days and 3.6days), when compared to the control (15.2days and 4.8days). Subsequently, delayed pupal period (7.8days) was observed in the higher concentration (2000ppm) than the control group (7days). Pupal weight was relatively high in untreated control (306.6mg). The phytoecdysteroid fraction treated group exhibits ecdysial failure, pupal malformation, shrunken pupa and adultoid with crumbled wings. Our findings heavily corroborated with the results of Freitas *et al*. (2014). Extracts of *Christella parasitica* caused aforementioned developmental abnormalities in lepidopteran pests such as *Achea janata*, *H. armigera* and *S. litura* (Sahayaraj and Selvaraj, 2013). Hence the phytoecdysteroid fraction of the experimental fern could be used as a reliable alternative biopesticide to control lepidopteran pests.

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