Impact of phytoecdysone fractions of the ferns *Cyclosorous interruptus, Christella dentata* and *Nephrolepis cordifolia* on the biology of *Spodoptera litura* (Fab.)

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ABSTRACT

The preliminary phytochemical analysis of ferns *viz*., *Cyclosorus interruptus* (Willd.) H.Itô (Theylepteridaceae), *Christella dentata* (Willd.) (Forssk.) Brownsey et Jermy (Theylepteridaceae), and *Nephrolepis cordifolia* (L.) Presl revealed the presence of the secondary metabolites like alkaloids, steroids, tannins, flavonoids, terpenoids, cardiac glycosides, phenolic compounds and terpenoids in crude extract. The characteristic colour change and the UV- Vis spectrum obtained for the silver nanoparticles (AgNPs) formulation of the experimental ferns confirmed the synthesis of nanoparticles (433.50nm, 450.00nm and 444.00 nm for *C. interruptus, C. dentata* and *N. cordifolia*, respectively). The pesticidal property of the crude extracts and ferns-AgNP (Fe-AgNP) were evaluated in the laboratory on *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) third instar larvae. The crude extract and Fe-AgNP formulations exerted their influence on the developmental period, pupation rate, pupal weight and adult emergence which were reduced significantly. They also caused larval, pupal and adult deformities that confirm the insecticidal activity of the plant.

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INTRODUCTION

Man competes with a number of insects for food and he strives to protect his cultivated crops and commodities. 15% of the annual crop yield is lost due to insect herbivory (David, 1992, Swaminathan, 1998, Carolyn Mitchell *et al.,* 2016). The larval forms of lepidopterans are the most dangerous pests of agriculture (Swaminathan, 1998). As the agrochemicals used in crop protection pollute the environment and cause hazards to non-target organisms including man, there is a search for eco-friendly pesticides as an alternative to the chemical pesticides (Muraleedharan and Sheeladevi, 1992 and Ravi and Verma, 2002). Many plants are being screened for potential compounds with pesticidal and antimicrobial activity.

The common cutworm, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) is an economically important pest of cultivable crops in southeast Asia, India, China, and Japan (Wheeler and Isman, 2001; Xu *et al.,* 2015). *Spodoptera litura* is a polyphagous and cosmopolitan pest of numerous types of agriculturally important plants, feeding on approximately 150 plant species from 40 families (Zhang *et al.,* 2012). Although insecticides remain the most widely used approach for controlling this pest, synthetic pesticides pollute the environment, affect non-target insects, and accumulate in the food chain. The extensive use of chemical pesticides has contributed to the development of resistance in a number of insect species, including *S. litura* (Chandrayudu *et al.,* 2015; Saleem *et al.,* 2016). Although several new alternatives have been suggested, botanicals are the best option for this pest management.

The toxic action of many secondary metabolites against insects are well known and used for the development such pesticides. These include mimics of important developmental hormones and chemicals of communicative significance. Among the pteridophytes, *Cheilanthes tenuifolia* (Faux *et*

al., 1970), *Polypodium vulgare, Pteridium aquilinum, Seratula tinctora* (Chandrakala *et al*., 1998) and *Cheilanthes farinosa* (Rajkumar *et al*., 2000) *Christella parasitica, Pteridium aquilinum* and *Hemionitis aurifolia* (Selvaraj, 2002) were reported to contain phytoecdysones.

Previously, impacts of ferns against various insect pests were studied (Muraleedharan, 1988, Rajkumar *et al*., 2002, Selvaraj, 2002, Selvaraj and Sahayaraj**,** 2005, Sahayaraj *et al*., 2003, 2007, Sahayaraj and Selvaraj 2013) and suggested to utilize for pest management. Hence this study was undertaken 1) to screen the phytochemical constituents of *C. dentata, C. interruptus* and *N. cordifolia*, and 2) synthesise and formulate a silver nano-liquid and to assay their insecticidal, biological traits and morphogenetic properties against the caterpillars of *S. litura.*

MATERIALS AND METHODS

Collection of ferns

The ferns *C. interruptus, C. dentata* and *N. cordifolia* were chosen for the present study. *Christella dentata* and *C. interruptus* were collected from a pond at Kandervillagam, Colachel (8.178620° N and 77.256096° N), Kanyakumari District of Tamil Nadu state. *Nephrolepis cordifolia* was collected from Athur, Thiruvattar (8.3348° N, 77.2664° E), Kanyakumari District (Plate 1).

Plate 1. Experimental ferns *C. interruptus, C. dentata* and *N. cordifolia*.

The ferns were washed thoroughly thrice with tap water to remove the dust and debris and were shade dried in room temperature for two weeks. The dried ferns were partially ground and it was stored for further use. From this 100 gms of powder were used for extraction using Soxhlet apparatus with methanol as solvent between 40-60^{O}C. The extracts were concentrated and stored for further use.

Phytochemical analysis

Preliminary phytochemical analysis of methanol extract and fractions obtained during the process of phytoecdysone isolation were used for preliminary phytochemical analysis (Brindha *et al.,* 1981).

Isolation of phytoecdysteroids

A common method for the isolation of ecdysteroids from the dried plant materials (Rajkumar *et al.,* 2000) was used. In brief dried and milled sample (10 g) was extracted with 96% ethanol (750 ml). Extract dried in vacuum and residue partitioned between hexane and 75% ethanol (50 ml of each phase). Hexane extract was discarded. The ethanol phase was concentrated in vacuum and partitioned between chloroform: ethanol: water (10 ml of each phase). Aqueous phase was discarded. The chloroform phase 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 (25 ml). The total eluate was evaporated to dryness and considered as crude ecdysteroids (CESs).

Preparation of and characterization of silver nano formulations

17 mg of silver nitrate (10^{-3} M $AgNO_3$) was taken and dissolved in 100 mL of distilled water. To induce the synthesis of silver nano particles 5 ml of silver nitrate solution was mixed with the plant extract in different concentrations (25-150µl at pH 7). The appearance of brown colour was considered as an indication of the synthesis of silver based nano particles. The best concentration in which the brown colour was observed was chosen for further studies. The silver nano particles formed at the concentration of 150µl of crude extract of *C. interruptus, C. dentata* and *N. cordifolia* were used for the UV- Vis Spectroscopic analysis between 200–450 nm.

Pest collection and rearing

A laboratory stock culture of *S. litura* was established at Crop Protection and Research Center, St. Xavier's College, Palayamkottai using adult males and females collected from groundnut fields around Tirunelveli. The adults were introduced into the ovipositor chamber for egg laying. The eggs laid were carefully taken in a petridish and are maintained in a laboratory condition of temperature 28 ± 2° C, RH 85% and Light period 14 L:10 D. The adult female laid creamy white eggs that hatched after three days of incubation. The young larvae were fed with fresh tender castor (*Ricinus communis*) leaves and were reared under laboratory conditions.

Bioassay

Five grams of Castor leaves were soaked in four different concentrations (wt/v) (*viz*., 0.5%, 1.0%, 1.5%, 2.0%) of crude extract phytoecdysone fractions and silver nano formulations of *C. interruptus, C. dentata* and *N. cordifolia* separately for five minutes. The crude extracts were prepared by dissolving the required amount of dry paste of phytoecdysone in 10 ml distilled water to get the desired percentage of extract. For the control, leaves were soaked in methanol solvent and after five minutes, the leaves were air dried for another 10 min and was used for the bioassay. The experimental *S. litura* larvae (five each) were taken in plastic containers of 500ml capacity and the containers were covered with muslin cloth. Three replicates each were maintained for each concentration and control respectively. The larvae were allowed to feed on the treated leaves for a period of 4-days and the mortality was recorded at regular intervals of 24 hrs up to 96 hrs. After 96 hrs the animals were fed with normal diet (untreated castor leaves). The pupated insects were placed on moist cotton swaps in petridishes and kept inside wire cages for further observation until adult emergence. The pupae were checked daily to note their mortality. This was continued up to death of all the experimental animals. Observations such as mortality, developmental deformities (larval pupal intermediates and abnormal adults) and adult emergence were observed and recorded. The adult moths which emerged were collected and released into the oviposition wire mesh cage for oviposition.

Statistical analysis

Statistical analysis and graphical representation of the experimental data was

carried out statistically using Microsoft Excel and SPSS 16.6.

RESULTS AND DISCUSSIONS

The present study was designed to identify secondary metabolites especially the active ingredient phytoecdysone and to evaluate the insecticidal property of the ferns *viz*., *Cyclosorous interruptus, Christella dentata* and *Nephrolepis cordifolia.*

Phytochemical analysis

The results of the preliminary phytochemical analysis revealed the presence of alkaloids, steroids, tannins, flavonoids, terpenoids, cardiac glycosides, phenolic compounds and terpenoids in ethanolic extracts. The test for steroids gave positive result in the hexane fractions. Alkaloids, flavonoids, cardiac glycosides, compounds and carbohydrates were present in aqueous fractions. The tests for steroids, tannins terpenoids and phenolic compounds reported positive results in phytoecdysone fractions (Table 1).

Table 1. Phytochemical analysis on different fractions of the experimental ferns, *C. dentate, C. interruptus* and *N. cordifolia* extractions: E ethanol, H- Hexane, A- Aqueous, P – phytoecdysone; +Indicates present and – indicates absent

Secondary metabolites	*C. dentate*				*C. interruptus*				*N. cordifolia*			
	E	**H**	**A**	**P**	**E**	**H**	**A**	**P**	**E**	**H**	**A**	**P**
Alkaloids	+	-	-	-	+	-	-	-	+	-	-	-
Steroids	+	-	-	+	+	+	-	+	+	+	-	+
Tannins (a)	+	-	-	-	+	-	+	+	+	-	+	+
Tannins (b)	+	+	-	+	+	-	+	-	+	+	-	-
Saponoids	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	+	-	+	-	+	+	+	-	+	+	-	-
Terpenoids	+	-	+	+	+	-	+	+	+	-	+	+
Cardiacglycosides	+	-	+	-	+	-	+	-	-	-	-	-
Phenolic compounds	+	+	-	+	+	+	-	+	+	+	-	+
Aromatic acids	-	-	-	-	-	-	_	-	-	-	-	-
Xanthoproteins	-	-	-	-	-	-	-	-	-	-	-	-

Similar studies have also been carried out by various authors. Britto and Manickam (1992) and Jesudass (1997) studied the phytochemical constituents of ferns and they reported the presence of tannins, phenolics, flavonoids, terpenoids, and saponins. Ferns are reported to contain secondary metabolites such as steroids, tannins, lignins, flavonoids and phenolics compounds (Murakami, 1989; Tanaka *et al.*, 1981; Veit *et al.*, 1995; Kamaya *et al.*, 1996; Jesudas, 1997). Among these secondary metabolites tannins and steroids are well known for their insecticidal properties (Suganthy, 2000; Sundararajan and Kumuthakalavalli, 2000; Ananthakrishnan, 2002; Selvaraj, 2002).

Isolation of phytoecdysone and UV-Vis spectrophotometric analysis

The crude phytoecdysone fractions isolated were dried and were used for further studies. The spectra (UV-vis. Spectrophotometer-Shimadzhu 1800) obtained for the crude phytoecdysone fractions revealed the presence of phytoecdysones. Distinct peaks of absorption were obtained at 280nm, 283nm and 286nm recorded in *C. dentata*, *C. interruptus* and *N. cordifolia*, respectively (Fig. 1).

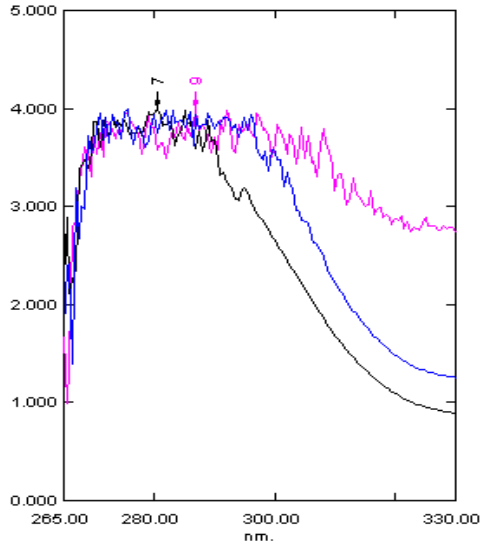

Fig. 1. UV -Vis spectrum of phytoecdysone fractions of *Christella dentata* (a)(286.50nm), *Cyclosorous interruptus* (b) (283.50 nm) and *Nephrolepis cordifolia* (c) (280.50nm).

Raimana *et al.* (2008) reported a typical ecdysteroid spectrum with a maximum at 242 nm. That they also reported two unusual compounds with peaks at 294 and 317 nm in addition to the major absorbance at 240 nm, which are indicative of the presence of an aromatic conjugating moiety. Li *et al.* (2007) developed a high-performance liquid chromatographic method for the detection of phytoecdysone and the detection was carried out at 242 nm. They also determined the identities by comparing the retention time and UV spectrum with those of reference compounds. However other studies reported hundreds of phytoecdysones and its analogues. Among the pteridophytes *Cheilanthes tenufolia* (Faux *et al.*, 1970), *Polypodium vulgare*, *Pteridium aquilinum*, *Seratula tinctora* (Chandrakala *et al.*, 1998) and *Cheilanthes farinosa* (Rajkumar *et al.*, 2000). *Christella parasitica*, *Pteridium aquilinum*, and *Hemionitis aurifolia* (Selvaraj, 2002) were reported to contain phytoecdysones.

Characterization of fern nano formulations

Among the experimental concentrations 150µl concentrations of phytoecdysone fractions of the experimental ferns showed the characteristic colour change (formation of brown colour). The above samples were used for further studies. The UV-Vis spectrophotometric studies showed a characteristic peak at around 430-450nm. Among the three ferns *C. dentata* recored an absorbance of 2.087 at 450nm followed by *C. interruptus* recorded a peak of 1.109 at 433nm with an absorbance, whereas, *N. cordifolia* recorded the peak at 444 nm with absorption 0.296 (Fig. 2).

Mortality of *S. litura* larva

Irrespective of the experimental ferns, the phytoecdysone fractions recorded a concentration dependent mortality during the experimental period (Table 2) as observed by Sahayaraj and Mary Jeeva (2012); Ventrella *et al.* (2016). Among the experimental concentrations of phytoecdysone fractions, the highest mortality was observed in the highest concentration of *C. dentata* at 96-hrs (LC_{50}=0.765%), followed by *C. interruptus* (LC_{50}= 0.890%) and *N. cordifolia* (LC_{50}= 0.9177%). However, the Fe-AgNPs formulations recorded very low level of toxicity (20% mortality) against the *S. litura* larvae. The comparisons between 72 and 96 hours data was significant for *C. dentate* (F=41.415, p=0.008) and *N. cordifolia* (F=72.079, p=0.0003). *Cyclosorous interruptus* between 24 and 96 hours data was weekly significant (F=24.276; p=0.01), but 72 and 96 strongly (F=10.500, p=0.05).

Biological traits of *S. litura* larva

Phytoecdysone fraction treated categories recorded the lowest percentage of adult emergence. No adults emerged from the 1.5% and 2% concentrations followed by 6.67% in 0.5% and 1% of *C. dentata*. Among the experimental categories the highest adult emergence was observed in *N. cordifolia* in 1% *C. interruptus* against the maximum of 46.67% adult emergence recorded in the control (Table 3).

The larval stages showed characteristics changes in the experimental larvae. The dead larvae showed shrunken body retention of moult skin (Plate 2a, b); translucent body, leakage of body fluid (Plate 2c, d). Larval pupal intermediate with improperly hardened pupal case retained larval appendages (Plate 2e-g). The adults which emerged out from the treated categories of the experiment also showed improper metamorphosis. The adults that emerged from the phytoecdysone treated categories had crumbled and deformed wings.

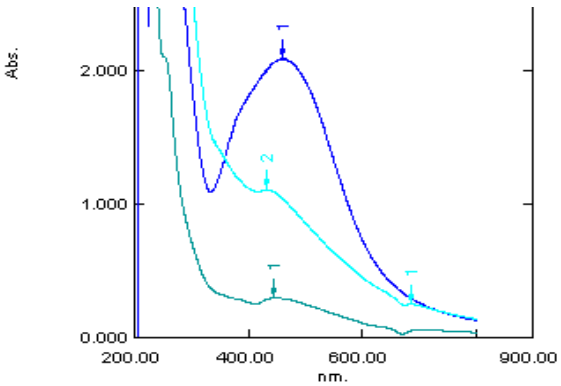

Fig. 2. UV-spectrum of AgNPs of *Christella dentata* (a)(450.00nm), *Cyclosorous interruptus* (b) (433.50nm) and *Nephrolepis cordifolia* (c) (444.00 nm).

Table 2. Impact of phytoecdysone fractions and their Nano formulations on the larval mortality (%) of *Spodoptera litura* larvae exposed after 24, 48, 72 and 76 hours. Profit analyses was carried out for 96 hours data.

	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**	**Profit analyses**
			Control		
Concentrations (%)	-	-	6.67±1.15	6.67±1.15	
			C. dentata		
0.5	6.67±1.15	13.33±1.15	33.33±1.15	33.33±1.15	
1.0	13.33±1.15	26.67±0.00	26.67±1.15	40.00±0.00	LC_{50}=0.765%
1.5	13.33±1.15	20.00±0.00	53.33±1.15	66.67±1.15	χ^2 = 2.336
2.0	6.67±1.15	20.00±0.00	46.67±1.15	66.67±1.15	p = 0.05
			C. interruptus		
0.5	-	26.67±1.15	33.33±1.15	33.33±1.15	
1.0	-	13.33±1.15	26.67±1.15	46.67±1.15	LC_{50}= 0.890%
1.5	-	13.33±1.15	33.33±1.15	53.33±1.15	χ^2 = 3.469
2.0	33.33±1.15	40.00±20.00	53.33±1.15	60.00±20.00	p = 0.023
			N. cordifolia		
0.5	-	-	6.67±1.15	13.33±2.31	
1.0	6.67±1.15	20.00±2.00	20.00±2.00	26.67±1.15	LC_{50}= 0.9177%
1.5	-	20.00±2.00	40.00±17.32	46.67±3.05	χ^2 = 0.5480
2.0	-	-	33.33±1.15	46.67±1.15	p = 0.01
			Silver Nano formulations (150µl conc.)		
CD AgNPs	-	-	-	6.67±1.15	
CI AgNPs	-	-	-	20.00±2.00	
NCAgNPs	-	-	6.67±1.15	13.33±2.39	
Control ($AgNO_{3)}$	-	-	6.67±1.15	13.33±2.39	

- Indicates no mortality was observed

Table 3. Impact of phytoecdysone fractions and their nano formulations on biological traits- larval mortality, pupation, pupal mortality and adult emergence (%) of *Spodoptera litura*.

Concentration (%)	**Larval mortality**	**Pupation**	**Pupal mortality**	**Adult emergence**
Control	6.67	93.33	46.67	46.67
		C. dentata		
0.5	33.33	66.67	60.00	6.67
1.0	40.00	60.00	53.33	6.67
1.5	66.67	33.33	33.33	0.00
2.0	66.67	33.33	33.33	0.00
		C. interruptus		
0.5	33.33	66.67	46.67	20.00
1.0	46.67	53.33	40.00	13.33
1.5	53.33	46.67	46.67	0.00
2.0	60.00	40.00	40.00	0.00
		N. cordifolia		
0.5	13.33	86.67	60.00	26.67
1.0	26.67	73.33	66.67	6.67
1.5	46.67	53.33	46.67	0.00
2.0	46.67	53.33	46.67	0.00
		Silver Nano formulations (150µl conc.)		
CD Ag NP	6.67	93.33	53.33	40.00
CI Ag NP	20.00	80.00	60.00	20.00
NC Ag NP	13.33	86.67	6.67	0.00
Control ($Ag\ NO_{3)}$	13.33	86.67	33.33	53.33

Plate 2. Impact of chosen ferns *Christella dentate* (CD), *Cyclosorous interruptus* (CI) and *Nephrolepis cordifolia* (NC) at various concentrations (1, 1.5 and 2.0 %) on the life stages of *S. litura.*

PE CI 2%

PE NC 2%

PE CI 1%

PE CD 2%

PE NC 1%

PE CI 1.5%

PE NC 1.5%

They also retained the pupal case (Plate 2h-j). In the present study the phytoecdysone fractions and Fe-AgNPs resulted in moulting disruption, morphological anomalies and mortality in *S. litura* and it showed a dose-dependent response. All the fern extracts revealed a developmental disruption in which the insects died in pharate condition following initiation of apolysis, but before completion of ecdysis. The insect moulting cycle is visibly initiated when the cuticular epithelium separates from the overlying cuticle. Moreover, the newly moulted *S. litura* larvae died while shedding the old cuticle and head capsule (pharate condition) (Plate 2). The results of the present experiment were also in accordance with the results of Hori *et al.* (1984) and Koul *et al.* (2004) in *Bombyx mori* treated with azadirachtin. Extracts of *Christella parasitica* also caused similar effects on *Achaea janata* (Sahayaraj *et al.*, 2003). Muraleedharan (1988); Archana and Nath (1995); Rajkumar *et al.* (2000); Desai and Desai (2000); Sahayaraj and Paulraj (2000) and Sahayaraj *et al.* (2003) reported similar kind of developmental abnormalities in lepidopteran pests such as *Achea janata, H. armigera* and *S. litura.*

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