Biological Forum – An International Journal **15(3): 798-804(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Larvicidal Effect of *Asparagus racemosus* and Induced Biochemical Changes in *Rhynchophorus ferrugineus* (Olivier)

***Chandana J.S.*[1] *and Ajitha V.S.*[2*]**
[1]*Research Scholar, Department of Zoology,*
University College University of Kerala, Thiruvananthapuram (Kerala), India.
[2]*Assistant Professor, Department of Zoology,*
University College University of Kerala, Thiruvananthapuram (Kerala), India.

(Corresponding author: Ajitha.V.S.)*
(Received: 23 January 2023; Revised: 17 February 2023; Accepted: 22 February 2023; Published: 15 March 2023)
(Published by Research Trend)

ABSTRACT: The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier), is a serious insect pest which attacks coconut trees. Control of RPW is difficult due to the concealed nature of the life cycle of the pest. The present study investigates the insecticidal potential of of *Asparagus racemosus* extracts against this serious pest of the coconut palm. The ethanolic extract of *Asparagus* was found to be most effective in causing a high rate of mortality in fourth-instar larvae. The LD_{50} value of plant extract was 1041.08. After treating with sublethal concentration of the extract, the larvae showed significant alterations in enzyme assays and nucleic acid level. The results of this study obviously indicate the presence of phytochemicals having insecticidal potential in this plant extract. Qualitative analysis of plant extract revealed presence of several secondary metabolites. Botanical pesticides can be an effective alternative to chemical pesticides to reduce environmental as well as health hazards.

Keywords: *Asparagus racemosus*, *Rhynchophorous ferrugineus*, LD $_{50}$, Enzyme assay, Botanical pesticide.

INTRODUCTION

Plant resources are useful alternatives to synthetic chemical pesticides in the control of insect pests. They constitute a rich resource of bioactive compounds. The exploitation of plant derived substances for the management of insect pests has achieved greater significance in recent years. Currently worldwide demand for plant-derived substances are increasing due to their ecofriendly nature. Globally greater awareness has been created on them as they have less non-target effects and possess more insect control properties through multifarious effects on insect body.

The Red Palm Weevil is one of the most destructive pest of coconut causing severe socioeconomic problems in our state. Conventionally, control of this pest is mostly based on the use of chemical insecticides. Environmental and non-target effects argue for development of more environment friendly and sustainable management practices. Improvement in understanding of plant allelochemicals offers new prospect in developing comparatively safe crop protection strategies (Regnault-Roger *et al*., 2005).

Most of the botanical pesticides employ a chemical mode of action in the biological control of pests. The mode of action of a botanical pesticide is a critical component in the commercial success of the product. It determines efficacy of pest control, efficiency of use, consistency of response, host target and non-target susceptibility. Typically, the most prominent mode of action of a biopesticide is biochemical means by disrupting biochemical, genetic or structural functions in the targeted pest (Hubbard *et al.*, 2014).

Insect haemolymph is a pathophysiological reflector of the whole body and hemolymph enzyme assays are important in diagnosing the structural and functional status of organisms exposed to toxicants (Adhikari *et al*., 2004). Several enzymes of hemolymph have been considered as relevant stress indicators, important among them are Acetylcholine esterase, AST and ALT. There are reports that the changing biochemical activities of insect cells during development may be caused by the differential activation or suppression of different sets of genes, which in turn is reflected in nucleic acid content (Kroeger, 1960). The present study focuses on the larvicidal potential of a well-known medicinal plant *Asparagus racemosus* on the fourth instar larvae of major coconut pest *Rhynchophorus ferrugineus* and the significant biochemical changes induced by the plant extracts.

MATERIALS AND METHODS

A. Insect culture

Adult *Rhynchophorus ferrugineus* (Olivier), coming under insect Order Coleoptera, Family Curculionidae, were collected from locally affected coconut palms. The collected insects were paired and kept in small plastic containers for mating. Sugarcane pieces were supplemented as diet. After the eggs were hatched, the newly emerged larvae were transferred into fresh plastic bottles containing coconut husk. At the early stage of larval development only one larva was kept inside each bottle to avoid cannibalism. Actively feeding 4th instar larvae were selected for the experiment.

B. Preparation of Plant extract

Fresh aerial parts of the medicinal plant, *Asparagus racemosus* (order Asparagales, Family Lilliaceae) were collected from local areas of Thiruvananthapuram. The plant material was washed and shade dried for two weeks. The dried material was ground to a fine powder and subjected to soxhlet extraction (8-10 hours) using different solvents such as acetone, ethanol and water (Harborne, 1998). The crude extract was evaporated to a viscous, dark green residue.

C. Larvicidal effect testing

The larvicidal effect of the plant extracts was studied using uniform-sized 4th instar larvae of *Rhynchophorus ferrugineus*. The larvae were kept inside glass bottles with sugar-cane pieces. The larvae were exposed to different concentrations of the plant extracts (1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm, 5000 ppm, 6000 ppm, 7000 ppm, 8000 ppm) by soaking the sugarcane pieces in each concentration of the plant extract for 6 hours and then dried in room temperature (Salama and Abdel-Razek 2009). Carefully observed and the percentage mortality was recorded. The experiment was done in six replicates and an untreated control was maintained for each experiment.

D. LD_{50} calculation

The LD_{50} for each extract was determined by using Proboit analysis, using the software SPSS 20 for windows (Sokal and Rohlf 1973)

E. Acetyl choline esterase assay

Haemolymph samples were collected from both treated and control larvae. Enzyme assay buffer (45µL), choline oxidase enzyme mix (2µL), and AChE Probe (2µL) were taken in a test tube and a haemolymph(10µL) samples were added to it. Blank was also set. Samples were incubated for 20-30 minutes at 37°C and absorbance was read at 570nm (Ellman *et al*., 1961)

The AChE activity (mU/mL) was calculated based on the equation

AChE Activity (mU/mL) = B/(T × V) × D

Where B – Choline amount from the standard curve

T – reaction time

V – sample volume

D – Sample dilution factor

F. Aspartate Aminotransferase AST(IFCC Method, kinetic)

The activity of AST was calculated using the SGOT Erba kit. 100µL of the sample was mixed well with 1mL of working reagent and aspirated. The samples were incubated for 3 minutes. Absorbance was measured at 340nm. The initial absorbance was taken and the change in absorbance per minute was calculated. The AST activity was measured using the following equation

Activity of AST = Absorbance /minute × 1768

G. Alanine Amino Transferase ALT (IFCC Method, kinetic)

The activity of ALT was calculated using SGPT, Erba Kit. 100µL of the sample was mixed well with 1mL of working reagent and aspirated and incubated for 3 minutes. Absorbance was measured at 340nm. The initial absorbance was taken and the change in absorbance per minute was calculated. The ALT activity was measured using the following equation.

The activity of ALT = Absorbance /minute × 1768

H. Estimation of nucleic acid (Schneider 1946)

Estimation of DNA. DNA from the hemolymph sample is initially depurinated quantitatively followed by the dehydration of sugar to - hydroxylevulinylaldehyde. This aldehyde condenses in an acidic medium with diphenylamine to produce a deep blue color condensation product with an absorption maximum at 600nm.100µL of sample was taken and made up to 3mL with H_2O. In addition to that a tube containing 3mL H_2O was set as blank. 6mL of diphenylamine reagent was added to each tube, and after mixing, the tubes were heated in a boiling water bath for 10 minutes. The tubes were cooled and read the absorbance of the blue solution at 600nm against the blank. The concentration of DNA was calculated using a standard calibration curve.

Estimation of RNA. The RNA estimation was done by the Orcinol method. The method depends on the conversion of the pentose sugar, Ribose in the presence of hot acid to furfural, which then reacts to yield a green color. 100µL of the sample was taken and made up to 3mL with distilled water. In addition to that a tube containing 3mL of water was set as blank. 6 mL of orcinol reagent was added to each tube 0.4mL of 6.0% alcoholic orcinol was added to each tube. The tubes were shaken to mix the contents and then heated in a boiling water bath for 20 minutes. The tubes were cooled, and read the absorbance at 660nm against blank. The concentration of RNA was calculated using the standard graph.

I. Statistical analysis

In order to determine the significance of individual differences between the control and treatment groups, one-way analysis of variance (ANOVA) and Duncan's multiple range test were performed using GraphPad Prism version 5 software. In Duncan's test, P=0.05 was considered significant (Manimaran *et al*., 2022).

J. Qualitative phytochemical analyses (Harborne, 1998)

For qualitative analysis the plant extract was dissolved in distilled water (100mg/ml) by keeping at 60°C in a water bath for 2 hours. The samples were subjected to various qualitative analyses to identify the phytochemical present in it (Harborne, 1998).

RESULTS AND DISCUSSIONS

Ethanol, aqueous, and acetone extracts of *A. racemosus* were taken and screened for their insecticidal potential against RPW. The Larvicidal activity of these plant extracts was studied in detail. Mortality percentage, LD_{50} value with each plant extract against RPW larvae were elucidated in detail. After screening plant extract *Asparagus racemosus* for larvicidal effect on *Rhynchophorus ferrugineus*, the most effective one was found to be *Asparagus racemosus* extract in ethanol with LD_{50} value.

As the dose level rises, so does the risk of death. The effect of *Asparagus racemosus* ethanol extract is discovered to be larvicidal, resulting in substantial mortality and unusual anomalies in the developmental phases of the fourth instar stage (Table 1). After screening the plant extract in different solvents the insects effective one well found *Asparagus racemosus* extract in ethanol with LD ^{50} value1041.08 (Table 2). Various plant extracts have larvicidal activity against *S. litura* and inhibited the moulting process at the level of new cuticle synthesis, resulting in mortality at exuviations. The effect of plant extracts on the body of the insect varies depending on the amount of consumed food material added to the plant extract. Disruption in development and growth results in increased mortality due to toxic action and death during moulting (Lall *et al*., 2013). The next larval instars is being suppress. Similar findings in Ellman were revealed that plants' secondary metabolites may contribute individually or collectively to generate larvicidal, pupicidal adult emergence inhibition, and other bioactivities against insects (Ellman *et al.,* 1961).

Mohammed *et al*. (2000) reported that the larval stages are more susceptible to the toxicity of plant extracts compared to pupae and adult stages. In this study fourth instar larvae of RPW were selected for screening purposes. When the fourth instar larvae were treated with ethanol leaf extracts of *Asparags racemosus*, mortality was increased in a dose-dependent manner in *Rhynchophorus ferrugineus*. Similar observations were reported by Mona (2020) on the insecticidal activity of cardamom *Elettaria cardamomum* and clove *Eugenia caryophyllus* plant extracts on RPW. It was reported that a large number of terpenes, including eugenol present in plant extracts are well known for their insecticidal potentials against a large number of insects (Huang *et al*., 2004; Waliwitiya *et al.,* 2005).

Two natural insecticides rotenone and limonine were already reported with anti-feedant and growth inhibition in *Rhynchophorus ferrugineus* larvae and adults (Mohammed *et al*., 2003). They also observed that the adult stages were more tolerant than the larvae. Increasing concentrations of rotenone and limonene reduced food consumption and resulted in larval and adult mortality in RPW. A similar result was obtained in the study of Saxena and Khan (1986) on electronically recorded disturbances in the feeding behavior of *Nephotettix virescens* (Homoptera: Cicadellidae) on Neem Oil-treated rice plants. Phloem feeding by the insect on plants sprayed with neem oil at different concentrations was significantly reduced compared with control insects. Neem materials do not kill the pest, but incapacitate or neutralize it via varied cumulative behavioral, physiological and cytological effects ranging from repellency to feeding deterrence, growth disruption, sterilizing effects, mating disruption, oviposition inhibition, reduced hatchability of eggs, etc. At times elucidation of the precise mode of action is difficult because the synergistic effects of neem compounds present in the leaves, bark, seed, oil, cake, or extracts. Similar studies were observed when the essential oil of *B. officinalis* (L.) showed good larvicidal potential after 48hrs of exposure period against *Anopheles gambiae* (Kweka *et al.,* 2012). Essential oil of *L.nobilis* showed strong fumigant toxicity against *Trogoderma granarium* larvae (Tayoub *et al*., 2012).

The insecticidal activity of *Asparags racemosus* on other insects was also reported. Govindarajan and Sivakumar (2014) reported the ovicidal, larvicidal and adulticidal activities of methanol extract of *Asparagus racemosus* against *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi*. Among the extracts tested, the highest adulticidal activity was observed in methanol extract against *Anopheles stephensi* followed by *Aedes aegypti* and *Culex quinquefasciatus*. This work also depicts the insecticidal potential of the extracts of *Asparagus racemosus. Asparagus racemosus* (Asparagaceae) is an important medicinal plant in tropical and subtropical India. Its medicinal usage has been reported in the Indian and British Pharmacopoeias and in traditional systems of medicine such as Ayurveda, Unani and Siddha. *Asparagus racemosus* has been reported to use as antioxidant, immune stimulant, anti-dyspepsic and anti-tussive effects. It is also useful in treatment of epilepsy, kidney disorders, chronic fevers, excessive heat, stomach ulcers and liver cancer, increases milk secretion in nursing mothers and regulates sexual behaviors (Mishra and Verma 2017). These reports clearly demonstrated the fact that the phytochemicals present in the plant *Asparagus racemosus us* are nontoxic and safe to humans and have many medicinal properties also. So we can assume that the plant is a potential source of human-safe botanical pesticides but this necessitates further characterization studies.

The biochemical analyses conducted after treatment with sub-lethal concentration *Asparagus racemosus* extract revealed significant changes in the concentration of major enzyme assays such as SGOT and SGPT when compared with that of control. Significant increase in the amount of acetylcholine esterase activity was observed in plant extract-treated larvae as they develop a strong resistant stress. Sublethal quantities of any stress agent will be stimulatory to the organism by providing it increased sensitivity to respond the changes in the environment (Table 3). Acetylcholine esterase activity was also found to decrease after plant extract treatment in *Rhynchophorus ferrugineus* larvae. This may be due to inhibition of Acetylcholine esterase, thus preventing the breakdown of acetylcholine. Accumulation of acetylcholine can cause “jam” in the nervous system. Majority of chemical pesticides especially organophosphates act in this way. Acetylcholine esterase (AChE) is a key enzyme involved in the neurotransmission of many organisms including insects. Researchers substantiated that Acetyl choline esterase is a potential target for many chemical as well as botanical pesticides. In this study also it is found that the treated larvae showed a significant reduction in the Acetyl choline esterase enzyme compared to that of control. Similar alteration of the enzyme was reported in the cockroach, *Periplaneta Americana* L. (Shafeek *et al.,* 2004) and the snail,

Limnaea acuminate treated with neem oil (Singh *et al.*, 1996). Further, Rattan (2010) reviewed the mechanism of action of essential oils on the body of insects and documented several physiological disruptions, such as inhibition of AChE. The insect repellant activity of many botanical pesticides including essential oil is attributed to its AChE inhibitory activity (Houghton and Howes 2006). The inhibition of AChE leads to interruption in normal neurotransmission and causes paralysis and death of the insect pest (Hollingworth *et al*., 1984). From this study it is clear that ethanol extract of *Asparagus racemosus* is as efficient as other known botanical pesticides like essential oils in inhibiting AChE and causing significant insect mortality.

In this study the activities of two enzymes AST (Aspartate amino transferase) ALT (Alanine amino transferase) involved in transamination process of amino acid metabolism were treated. AST catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and an important enzyme in amino acid metabolism. The action of AST was found to be decreased in treated larvae than in control ones. ALT catalyzes the two parts of the alanine cycle which catalyzes the transfer of amino group from L-glutamate. The action of ALT was found to be decreased in treated larvae than in control. The concentration of both AST and ALT in treated larvae found to be decreased in this study (Table 3). Insect herbivores can boost their detoxifying activities against a specific plant poison/toxin after ingesting the same components for a long or short period of time. Esterase, oxidase, alanine aminotransferase, and aspartate aminotransferase all play a role in phase II of enzyme detoxification, according to research.

Similar results reported in the use of insecticides caused multiple sublethal effects on the enzyme activities in insect pests (Sabri *et al*., 2017). As reported by Yousef *et al.* (2013) biological and biochemical studies on the effect of some botanical extracts on cotton leaf worm, *Spodoptera littoralis* the activities of the tissue-specific enzymes have been used to diagnose the damage of specific tissues and organs resulting from a chemical toxicity. Upadhyay (2013) also noted that acid and alkaline phosphatase have been examined as enzymes important in detoxification for the first time. Amino transferases, according to Zibaee *et al.* (2008) are crucial components of amino acid catabolism, as they are engaged in transferring an amino group from one amino acid to a keto acid. Furthermore, according to Etebari *et al*. (2005) both the AST and the ALT act as a key link between carbohydrate and protein metabolism and are known to be altered under a variety of physiological and pathological circumstances. Sahayaraj and Antony (2006) found the reverse tendency in *S. litura*, indicating that the insect is vulnerable to the plant extracts they investigated. According to another study, *Tephrosia purpurea* seed extract stimulated the formation of AST and ALT in the fat body and intestine of *Dysdercus cingulatus* (Upadhyay, 2013). In the present study, the treatment with *Asparagus* extract was found to be inhibitory on the enzyme activities in the fourth instar larvae of *Rhynchophorus ferrugineus*, depending on the concentration level.

The nucleic acid content of the treated larvae (both DNA and RNA) is reduced significantly which may be due to inhibition of nucleic acid synthesis at the cellular level and catabolism get increased which results in low availability of nucleic acids (Table 4). Similar observations were reported by Qari *et al*. (2017) on the DNA damaging effect of essential oils of *Citrus aurantium*, *Eruca sativa*, *Z. officinale* and *Origanum majorana* against *R. dominica*. In *Bombyx* during embryogenesis, both RNA and DNA increase several-fold during the early phases of growth and cell multiplication (Chino, 1956). Both these compounds are reduced in relation to body weight during larval life because most of the food is directed into nutrient reserves for metamorphosis (Niemierko *et al.*, 1959.) From the results it appears that the amount of DNA and RNA is decreased after treating plant extract. Protein synthesis is inextricably linked to RNA production, and that RNA plays a direct role in protein synthesis. Changes in RNA metabolism are closer to the location of ecdysone action than changes in protein synthesis. The cytological observations of Wigglesworth (1972) who revealed that after injecting ecdysone into *Rhodnius* larvae, the nucleoli in the epidermal cells began to grow and the amount of RNA increased within a few hours. These observations, together with current biochemical parameters related to nucleic acid that is heavily oriented towards most of the metabolism related to protein synthesis, have prompted a greater focus on nucleic acid metabolism during metamorphosis.

Ethanol extract of *Asparagus racemosus*, which showed potential insecticidal activity against RPW larvae were qualitatively analysed for the presence of various phytochemicals. The result indicates that the extract is rich in various secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, phenols, saponins and quinons (Table 5). Terpenoids, coumarins and phenol present in the methanol extracts of *Gliricidium sepium* exhibited significant antifeedant activity (Jose and Sujatha 2017). Reports are available on the larvicidal activity of many secondary metabolites, such as saponins, Phenolics, isoflavonoids, essential oil, alkaloids and tannin compounds (David *et al.*, 2000; Khanna and Kannabiran 2007; Pelah *et al*., 2021).

Botanical pesticides (essential oils, flavonoids, alkaloids, glycosides, esters and fatty acids) have various chemical properties and modes of action against insects. In this study it is proved that the plant extract is a potential source of botanical insecticides against RPW. The insecticidal property of the plant extract can be attributed to its various phytochemicals. The observations and results of this study substantiate the potential of the ethanol extract of *Asparagus racemosus*as a potential insecticide against the red palm weevil, *Rhynchophorus ferrugineus*.

Table 1: Effect of *Asparagus racemosus* extracts on 4th instar larvae of *Rhynchophorus ferrugineus.*

Percentage mortality						
Concentration	**control**	**Aqueous Mean ± SE**	**Control2**	**Acetone Mean ± SE**	**Control3**	**Ethanol Mean ± SE**
1000ppm	0	29±0.01	0	24±0.120	0	36±0.03
2000ppm	0	33±0.00	0	27±0.041	0	39±0.040
3000ppm	0	41±0.07	0	31±0.001	0	43±0.021
4000ppm	0	44±0.05	0	35±0.032	0	47±0.000
5000ppm	0	47±0.02	0	39±0.021	0	51±0.007
6000ppm	0	51±0.05	0	41±0.132	0	56±0.0098
7000ppm	0	53±0.07	0	43±0.142	0	61±0.010
8000ppm	0	57±0.12	0	46±0.03	0	66±0.003

Mean in a column followed by the same letters are not significantly different ($p \leq 0.05$) each value represents the mean ± SE of six replicates of larvae dead at each concentration of the treated substance, controls mortality nil.

Table 2: LD_{50} of Selected Plant Extracts.

Plant	Extract	LD_{50} (ppm)
Asparagus recemosus	Ethanol	1041.08
	Aqueous	1110.727
	Acetone	1420.48

Table 3: Enzyme Assays (µG/ml mean ± SE).

t-test for Equality of Means				
Enzymes	**Experimental group**	**t value**	**Sig. (2-tailed**	**Mean ±SE**
AST	control	47.624	0.001***	63.94 ± 0.15
	Treated		0.001***	54.98 ± 0.11
ALT	control	56.697	0.001***	17.87 ± 0.01
	Treated		0.001***	4.70 ± 0.23
Acetylcholine esterase	Control	236.906	0.001***	206.53 ± 0.86
	treated		0.001***	101.19 ± 0.13

*** Highly significant t value ($P \leq 0.001$)

Since $p \leq 0.001$ is less than our chosen significance level $\alpha = 0.05$, we can reject the null hypothesis, and conclude that enzyme (AST, ALT & AchE) level of the control larvae and treated sample is significantly different

Table 4: Nucleic Acid Estimation (µG/ML MEAN ± SE).

t-test for Equality of Means				
Nucleic acid	**Experimental group**	**t value**	**Sig. (2-tailed)**	**Mean ±SE**
DNA	control	12.008	.000***	14.85 ± 0.24
	Treated		.001***	11.51 ± 0.14
RNA	Control	114.48	.001***	191.6 ± 40.23
	Treated		.001***	137.75 ± 0.41

*** Highly significant t value ($P \leq 0.001$)

Since $p \leq 0.001$ is less than our chosen significance level $\alpha = 0.05$, we can reject the null hypothesis, and conclude that Nucleic acid content of control larvae and treated sample is significantly different

Table 5: Qualitative phytochemical screening.

Sr. No.	Secondary metabolite	Test method	Result
1.	Alkaloids	Mayer's test	+
2.	Flavonoids	Ammonia test	+
3.	Tannins	$FeCl_2$ test	+
4.	Saponins	Froth test	+
5.	Terpenoids	Salkowski test	+
6.	Glycosides	Keller killani test	+
7.	Quinones	H_2SO_4 test	+
8.	Phenols	$FeCl_2$ test	+
+ indicates presence			

CONCLUSIONS

The results obtained in this study *Asparagus racemosus* extract-treated insects clearly indicate that the extract cause mortality in larvae by disrupting the normal metabolic pathway in the insect body. Enzyme assays conducted in both control and experimental larvae provide information regarding the stress induced by the plant extract, which eventually resulted in high larval mortality rate. Qualitative analysis of the ethanol extract of *Asparagus* revealed the presence of many phytochemicals which have known insecticidal properties.

FUTURE SCOPE

The practice of using botanical pesticides or plant extracts in agriculture can be a promising tool in Integrated Pest Management (IPM) in the coming years, so as to reduce the deleterious impact of chemical pesticides. The medicinal plant, *Asparagus racemosus* is proved to be a potent source of bioactive insecticidal compounds which is promising enough to get incorporated in IPM strategies.

Acknowledgement. We express our gratitude to the University of Kerala for providing Fellowship (JRF) to conduct the research work and the Principal and authorities of University College for providing the necessary facilities to carry out the work.
Conflict of Interest. None.

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How to cite this article: Chandana J.S. and Ajitha V.S. (2023). Larvicidal Effect of *Asparagus racemosus* and Induced Biochemical Changes in *Rhynchophorus ferrugineus* (Olivier). *Biological Forum – An International Journal, 15*(3): 798-804.