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Quantitative and qualitative analysis of protein in haemolymph of sixth instar larvae of *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae) treated with leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata*

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Abstract

Orthaga exvinacea is an agriculturally important major pest of mango crop. In caterpillar stage the yield of crop is reduced as they defoliate leaves. Eradication of this pest by using synthetic insecticide is creating serious health hazards to non-target organisms and severe environmental pollution. In such a scenario, use of Botanical insecticides are effective, ecologically acceptable and safe instead of uncontrolled used of insecticide. As a result, the purpose of this study was to see how methanolic leaf extracts of Clerodendrum infortunatum and Chromolaena odorata affected protein content and protein profile in haemolymph of the sixth instar larval stage of Orthaga exvinacea under laboratory circumstances. Sixth instar larvae of the pest were fed with the 1-5 different concentrations of both leaf extracts treated mango leaves. The larvae were sacrificed after 48 hours to collect haemolymph and bioassay was carried out. The quantitative study of protein showed that there was some considerable decrease in the amount of protein with the increase in concentration of botanicals. In addition to this, electrophoretic analysis of haemolymph protein showed some alterations in protein profile of botanical treated samples compared to control. Both appearances of new protein bands and disappearances of some protein bands were noticed in protein profile of treated larvae with increase in botanical concentrations. The study discovered that C. odorata had a higher efficacy than C. infortunatum in altering the physiology of larvae, and that both botanicals have the potential to be utilized as new natural Biopesticides.

Keywords: *Orthaga exvinacea*, Haemolymph, Protein, *Clerodendrum infortunatum*, *Chromolaena odorata*.

Introduction

Mango, 'The National Fruit of India' is the most popular and nutritionally rich fruit grown in the tropics. Due to its delicious taste and nutritional richness mango is treated as the 'King of fruits'. Mango is the leading fruit crop of India and India is ranked first among mango-producing countries, accounting for over half of global production. So, the cultivation of mango has played an inevitable role in Indian agro-economy. This crop is facing enormous losses due to ravages of wide range of pests and among them, 492 species of insects were reported as causing serious threat to the crop[1]. Among these, the leaf webber *Orthaga exvinacea* has gained a serious pest status due to their severe infestation causing tremendous losses of crop yield.

The mango leaf-webber, *Orthaga exvinacea:* The mango leaf-webber, *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae) has been identified as a serious mango pest in Kerala[2] and heavy infestation by this pest adversely affects the flowering as well as the growth of new flush[3]. The heavily infested trees result incomplete failure of flowering and severe infestation present a burnt look [4]. The caterpillars cause defoliation and reduction in crop yield. The caterpillars web the leaves and terminal shoots into clusters and feed within. A webbed cluster of leaves may harbour several caterpillars and their severe attack give burnt appearance to leaves.

Life Cycle: Life cycle of *O. exvinacea* consists of egg, larval stage of 6 instars, a prepupal stage which is inactive, pupal stage and adult stage. The entire life cycle of this species may take 45 to 52 days. Female moths lay between 60 to 400 eggs on mango leaves, which are ordinarily smaller than 0.5 mm and not sticky.

The eggs hatch in 2 to 4 days, the larval stage which includes 6 instars, last for about 27 days, and the prepupal stage which is usually inactive and starts constructing cocoon, last for 3 days. The pupal stage lasts for about 12 days. Adult male has 5-day life span and female has 6-day life span.

Pest status and management: *O. exvinacea* infestations have been found in India's several agro-climatic zones, particularly in Uttaranchal, Uttar Pradesh, Andhra Pradesh, and Kerala[5].

It was once considered a minor pest of mango, but in recent years, it has been reported as a serious pest of mango due to its acute infestation and the extent of destruction it has caused. The active season is reported from June to February, the activeness gets decreased in summer season.

Synthetic pesticides are, in general, the quickest way to manage insect pests. But the continuous use of synthetic organic insecticides in crop pest control programs around the world has reported and resulted in disturbance of the environment, pest resurgence, pest resistance to insecticides, lethal effects on non-target organisms and environmental pollution. In spite of the effective attribute of synthetic pesticides, repeated usage has its challenges such as development of pesticide resistant pests[6].

Toxic compounds of synthetic pesticides have been reason to chronic human health disorders either due to consumption or exposure[7,8]. The toxic and non-biodegradable constituents of synthetic pesticides accumulate in the environment and cause serious pollution to soil and ground water in addition to depletion of the ozone layer[9,10]. This situation demands some alternate measures to overcome this problem.

Botanical insecticides have been identified as effective alternatives to synthetic insecticides for pest management. Plants are rich sources of natural substances that can be utilized in the development of environmentally safe methods for insect control[11,12]. Botanical pesticides are plant derivatives that act as repellent or growth inhibitors and kill pests[13].

The biological nature of botanicals makes their rapid degradation and therefore accumulation does not occur in the environment and thereby eliminating chances of pollution[14]. Most botanical pesticides are used to manage the insect pest and many research studies have focused majorly on insect pest management[15-20]. In the light of a number of similar research works, it was widely accepted that the botanicals used pest management system is environmentally acceptable.

Clerodendrum infortunatum and *Chromolaena odorata* are locally accessible weeds and many researchers proved its insecticidal property. Pesticidal effect of *C. infortunatum* on the fat body of *Oryctes rhinoceros* causes severe changes like depletion in the lobes of fat body, disintegration of cell membrane, nucleus that is shrunken and scattered[21]. In *Oryctes rhinoceros*, methanolic extracts of *E. odoratum* leaves triggered vitellogenesis and oocyte development disruption[22]. These results demonstrate the bioefficacy of both botanicals as natural biopesticides against the pest *O. exvinacea.*

The present investigation was an attempt to study the toxic effect of *Clerodendrum infortunatum* and *Chromolaena odorata,* through the quantitative and qualitative analysis of protein in haemolymph of sixth instar larvae of *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae) treated with botanicals.

Materials and Methods

Collection and culturing of *Orthaga exvinacea:* The pupae and larvae of *O. exvinacea* were collected from the infected mango field and maintained in laboratory conditions. The larvae were reared in plastic troughs covered with muslin clothes and kept inside rearing cages. The culture of *O. exvinacea* was maintained in laboratory under optimum conditions of temperature at 28± 3ºC and the relative humidity was about 70-85%. Fresh mango leaves were fed until the pupation of the larvae.

After pupation of larvae, adult moths that emerged were sorted out for their sexes and kept in plastic jars in the ratio of 1:1 and fed with 50% honey. Within 2-4 days, creamy white eggs were laid and the colour changed to brown and later black which shows its readiness for hatching. Young larvae were given fresh delicate mango leaves after the eggs hatched. In initial stage, the larvae were kept in 1500 ml of capacity containers (18×12cm) and later they were transferred to plastic troughs (40×13cm) covered with muslin cloths.

Preparation of leaf extracts and stock solution for biochemical studies: *Clerodendrum infortunatum* and *Chromolaena odorata* fresh leaves were collected from the field, washed, and shade dried at room temperature and grounded into fine powder with an electric mixer grinder and sieved through a muslin cloth. 50gm of leaf powder was extracted using 500ml methanol in Soxhlet apparatus at 70-80ºC temperature.

The extract was transferred to a pre-weighed Petri dish and it was allowed to evaporate in a microwave oven at 40-50ºC. After the solvent completely evaporated the weight of dried extract was taken and it was dissolved in appropriate volume of methanol to prepare 10% stock solution and stored in air-tight glass containers in a refrigerator. From this stock, different desirable concentrations of botanicals were prepared by diluting with methanol.

Mode of application- Food treatment method: The application of botanicals on the larvae was carried by food treatment method. For this fresh mango leaves were uniformly smeared with equal volume (1.0 ml) of different concentrations of extract and kept open at room temperature for the complete evaporation of the solvent methanol. The sixth in star larvae were provided with these treated leaves for 48 hrs and control larvae were provided with mango leaves treated with same volume of methanol alone.

Bioassay: Amputated thoracic legs gave 0.2 ml of haemolymph, which was placed into pre-chilled Eppendorf tubes containing Sodium tungstate solution. Five replicates of samples were collected for each concentration and the total protein in samples were estimated by the method of Lowry *et al.*[23].

Protein profile: The protein profiling of haemolymph was performed by Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS – PAGE) according to Laemmle[24].

Results and Discussion

The results obtained show that the treatment of *C. infortunatum* and *C. odorata* produced significant changes in both qualitative and quantitative studies in the haemolymph of *O. exvinacea* sixth instar larvae.

The treatment caused a serious decline in protein content of haemolymph of larvae. The protein concentration in the haemolymph of untreated larvae was 23.84mg/ml, and it gradually dropped from 21.88mg/ml to 11.80mg/ml as the botanical content of *C. infortunatum* increased (Table-1). At 5% treatment, a 50.5 percent drop in protein content was observed. With a rise in botanical concentration, protein content dropped significantly from 19.68 mg/ml to 3.74mg/ml in the case of *C. odorata* treatment (Table-1). The treatment caused 84.31% of reduction in protein content at 5% of treatment. The common tendency noticed in both botanical treatments, was a gradual decline in protein concentration in relation to increased botanical concentrations and *C. odoarata* had the maximum efficacy in decreasing the protein content in all tissues than that of *C. infortunatum* (Figure-1).

Effect of botanicals on the protein profile of haemolymph: The treatment of *C. infortunatum* made some changes in the protein profile of haemolymph of larvae. The control larvae exhibited 7 protein bands with molecular weights ranging from 30 kDa to 100 kDa (Figure-2). Commonly 4 protein bands were noticed at 1%, 2% and 4% treatment ranging from 30 kDa to 60 kDa. The maximum appearance of protein bands has been noticed at 5% of treatment (Figure-2).

In the case of *C. odorata* treatment, 7 protein bands with molecular weights ranging from 20 kDa to 70 kDa were observed in control larvae (Figure-3). The maximum number of protein expressions were noticed in both 1% and 5% of treatment. The appearance and disappearance of protein bands were noticed in differently treated profiles (Figure-3).

The results of present investigation unfolded the strength of both leaf extracts to reduce the total protein and to alter the protein profile in haemolymph. These results emphasized the toxicity of phytochemicals present in both botanicals which interfered the physiology of larvae. Many parallel research works were published with alike outcomes and those findings can be correlated with this study.

In insects, proteins are very essential for the proper development and growth including much vital physiological processes like cell division, metabolism, transport of ions across membranes, cuticle melanisation and sclerotization. The studies related to both quantitative and qualitative changes of protein can reveal the condition of the physiological state of an insect. Proteins are important biological molecules that play an inevitable role in insect growth, development and various other physiological processes[25]. The protein content in an insect is dependent upon its synthesis, breakdown, water movement between tissues and haemolymph, so the reduction of protein content in larvae might be due to the reduction in synthesis of protein or increase in breakdown to detoxify the active principles present in the plant extracts[26]. Similar studies were carried out by Kiran Morya *et al.* and observed that the leaf extracts of *Lantana camara* reduced the protein content in *Corcyra cephalonica*[27]. Under stress condition an insect requires higher metabolic energy and this energy demand may have led to the protein catabolism to detoxify the toxic principles present in the *C. infortunatum* and *C. odorata*, but the lesser consumption of food for synthesis of protein might also be the reason for the reduction of protein.

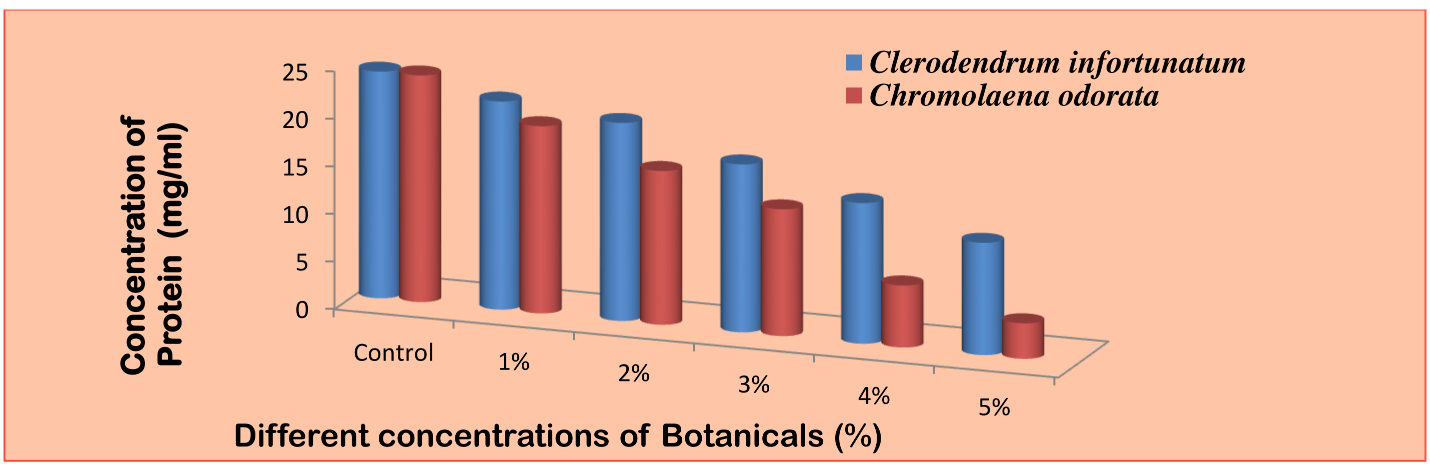
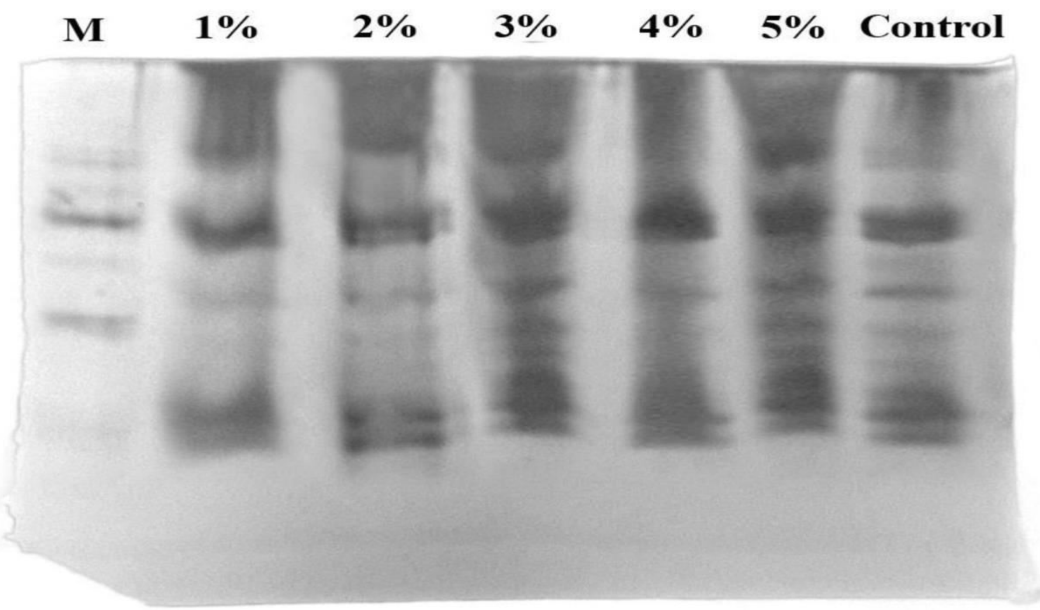
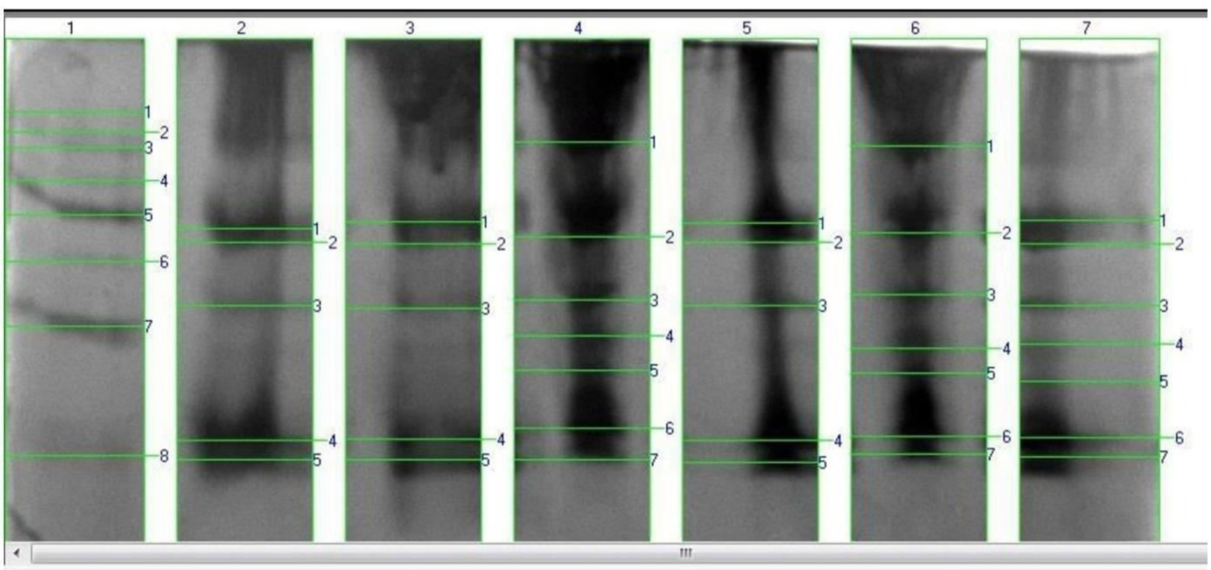


Figure-1: Graphical representation of both botanical treatments showing the common tendency of decline in protein concentration in relation to increased botanical concentrations. Comparatively *C. odoarata* had the maximum efficacy in decreasing the protein content in haemolymph than that of *C. infortunatum*.



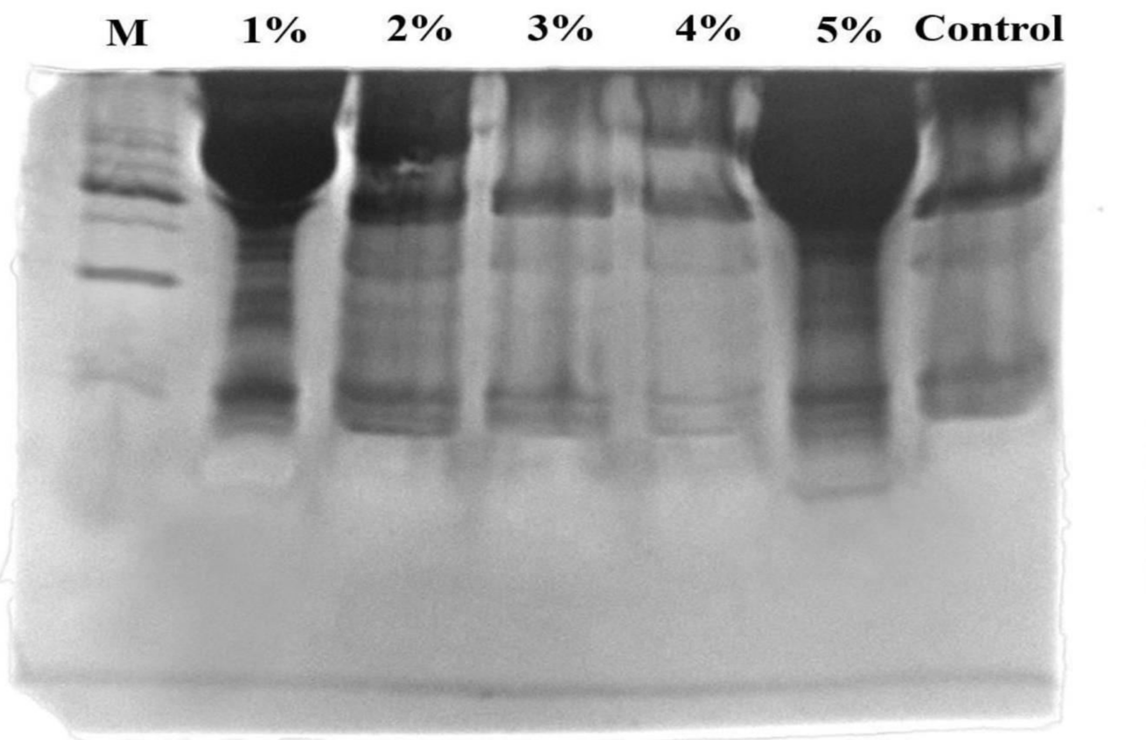
SDS- PAGE gel image showing protein bands of haemolymph treated with *Clerodendrum infortunatum*



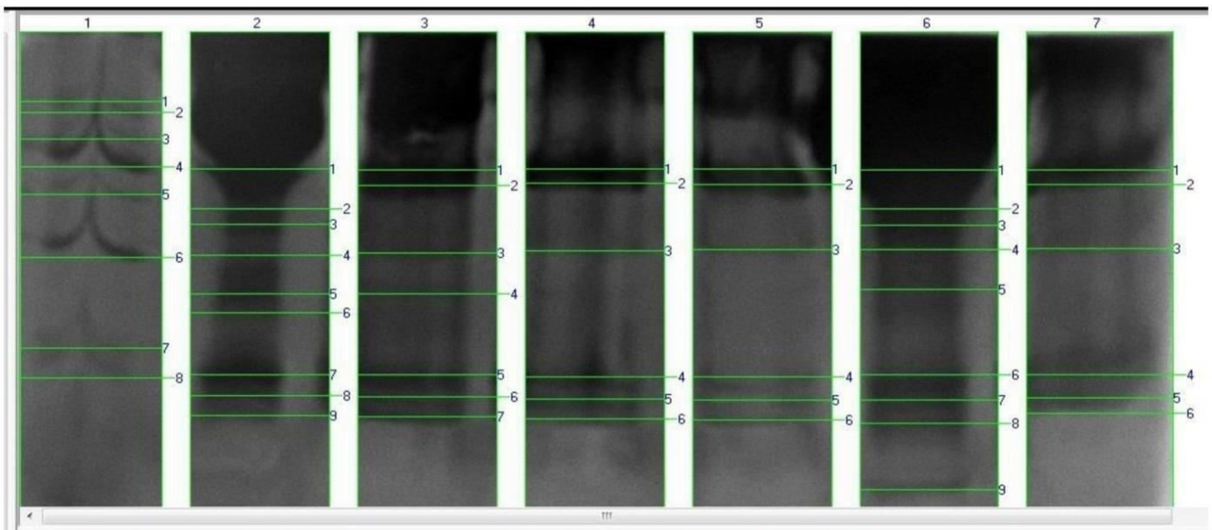
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	56.931	58.461	83.287	58.154	80.764	58.768
Band 2	90.000	53.934	53.641	55.119	53.934	56.021	53.641
Band 3	80.000	42.681	42.298	43.472	42.681	44.297	42.681
Band 4	70.000	30.856	30.934	38.894	30.856	37.593	38.012
Band 5	60.000	29.681	29.681	35.580	29.468	35.352	34.696
Band 6	50.000			31.574		31.092	31.013
Band 7	40.000			29.681		30.077	29.894
Band 8	30.000						

SDS- PAGE gel documentation showing molecular weights of different protein bands of haemolymph treated with *Clerodendrum infortunatum*

Figure-2: Showing both gel image and gel documentation of haemolymph treated with *Clerodendrum infortunatum*.



SDS PAGE gel image showing protein bands of haemolymph treated with *Chromolaena odorata*



M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	69.130	68.696	69.130	69.130	68.696	68.696
Band 2	90.000	57.302	62.880	63.663	63.269	57.302	63.269
Band 3	80.000	54.554	50.459	50.708	50.837	54.368	50.970
Band 4	70.000	50.222	47.531	30.407	30.407	50.837	31.220
Band 5	60.000	47.531	31.220	22.963	22.593	47.795	23.333
Band 6	50.000	46.091	23.704	16.296	15.926	31.220	18.148
Band 7	40.000	31.220	17.037			22.593	
Band 8	30.000	24.074				14.815	
Band 9		17.407					

SDS- PAGE gel documentation showing molecular weights of different protein bands of haemolymph treated with *Chromolaena odorata*

Figure-3: Showing both gel image and gel documentation of haemolymph treated with *Chromolaena odorata*.

Table-1: Table showing the bio-efficacy of both Clerodendrum infortunatum and Chromolaena odorata leaf extracts on protein content in haemolymph of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of protein in haemolymph		Percentage reduction of total protein concentration in Haemolymph (%)
		mg/ml	mg/larva	
Control		23.84±0.20	2.98±0.025	
C. infortunatum	1%	21.88±0.267	2.73±0.033	8.22
	2%	20.82±0. 124	2.59±0.016	12.67
	3%	17.65±0.332	2.20±0.041	25.96
	4%	14.78±0.159	1.85±0.020	38
	5%	11.80±0.549	1.47±0.068	50.5
	F value	1113.975	1111.724	----
C. odorata	1%	19.68±0.611	2.46±0.076	17.45
	2%	16.17±0.226	2.02±0.029	32.17
	3%	13.33±0.354	1.66±0.044	44.09
	4%	6.52±0.255	0.81±0.032	72.65
	5%	3.74±0.181	0.47±0.023	84.31
	F value	2575.919	2577.615	----

The changes in protein profile showed in all results indicated that the botanicals influenced the different protein expressions in haemolymph of *O. exvinacea*. Comparable results were reported by many researchers regarding the toxic effects of phytochemical on the protein profiling of different insect tissues. The active ingredients present in both leaf extracts might have induced protein profile and it was expressed either through the appearance of new polypeptides or through the disappearance of existing ones. The electrophoretic studies on the effect of azadirachtin on *S. litura* showed an altered protein profile of larvae with 10 protein bands that were affected[28]. The structural deformities and diminution of protein profile were reported in *Anopheles* larvae after the methanolic treatment of *A. indica*[29]. Huang *et. al.* investigated the bioactivities of azadirachtin on third instars of *Ostrinia furnacalis* and found that protein expression in pupae is correlated with azadirachtin treatment and affects insect growth[30]. Effects of azadirachtin on hemolymph protein expression in *O. furnacalis* showed the changes in two proteins in hemolymph lipid metabolism[31]. The possible reason for the appearance and disappearance of polypeptides in protein profile may be due to the alteration that occurred at molecular level of larvae. Al-Qahtani *et al.* reported that the treatment of some dried plants created transcriptional changes in *Oryzaphilus surinamensis* to develop resistance or detoxification mechanism[32]. Hence any foreign particle interacting with the cellular metabolism created stress in the cell. It may either up-regulate or down-regulate gene expression. The disappearance of protein bands at higher concentration might be due to the phytochemicals present in the botanicals which down- regulate some gene expressions. The appearance of many new small protein bands might be due to new peptides formed or it might be the peptides which are formed by the breakdown due to the action of protease.

Conclusion

The results of quantitative and qualitative analysis of protein content in haemolymph of larvae showed the maximum potency of *C. odorata* than that of *C. infortunatum.* According to the current study, the active components found in both botanicals may be the reason for lower protein concentration, as well as for the alterations in the protein profile of the haemolymph of larvae. Hence this study revealed the potency of *Clerodendrum infortunatum* and *Chromolaena odorata* to control the *Orthaga exvinacea* and thereby introduce these botanicals as new natural Biopesticides.

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