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Antiviral Action of Certain Medicinal Plants against AmCPV and their Effect on Cellular and Biochemical Changes in Tasar Silkworm, *Antheraea mylitta* D.

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ABSTRACT

Among the diseases of tasar silkworm, virosis caused by Cytoplasmic Polyhedrosis Virus (CPV) is highly contagious and more prevalent. Thirteen antiviral plants were used to test their efficacy against *Antherae mylitta* cytoplasmic polyhedrosis virus (AmCPV) in tasar silkworm. The aqueous extracts of these plants in different concentrations were used for containment of virosis in virus-infected silkworm and mortality was recorded. The influence of best three phytoextracts which have shown good results in suppressing virosis were subjected for the study of cellular and biochemical changes. Two percent aqueous extracts of *Aloe barbedensis* (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) were found more effective in suppressing the virosis and reduced the mortality due to virus infection of 66.17, 64.47 and 57.19%, respectively. The total hemocyte count increased up to 6th day of post inoculation in phytoextract treated batches while in the inoculated control the increase was within 3 days indicating the positive hemocyte mediated response in silkworm treated with phytoextract. The hemolymph protein in *Aloe barbedensis* treated silkworm (35.27 mg mL^{-1}) was significantly higher than inoculated control (20.25 mg mL^{-1}). The gradual increase of total hemolymph proteins from 1st day (16.31 mg mL^{-1}) to 8th day (33.73 mg mL^{-1}) was observed in healthy control where as in inoculated control increasing trend was observed from day 1 (16.26 mg mL^{-1}) to day 3 (24.22 mg mL^{-1}) there after decreasing trend was observed and finally reached to 20.25 mg mL^{-1} (8th day). The plant extract of *Aloe barbedensis* (AKP 3) is more effective in suppressing virosis based on the results of mortality reduction against virosis cellular and biochemical changes.

Key words: *Antheraea mylitta*, cytoplasmic polyhedrosis virus, antiviral, phytoextracts, hemolymph

INTRODUCTION

Among the tasar silkworm pathogens, virus causes comparatively higher level of mortality in most of the tasarculture areas. Virosis accounts for 25-30% of the total crop loss from diseases (Singh *et al*., 2011) and thus pose a serious problem in tasar culture.

The control of infectious diseases is seriously threatened by the continuous increase in the number of microorganisms that are resistant to the chemical antimicrobial drugs (Nenaah and Ahmed, 2011). The chemical based disinfectants and drug formulations used for prevention/control

of this disease are not economic, eco-friendly and also have many limitations to be effective in open and outdoor rearing. Because of this reason disinfectants/drug formulations are ineffective to control this disease at field level. Chemical disinfectants and antibiotics have been used for managing the diseases in silkworm (Kagawa, 1980; Reddy *et al*., 1990). In view of high cost of chemicals and antibiotics and their hazardous consequences, plant extracts has been on the top priority for control of diseases (Jespers and de Waard, 1993; Kumar *et al*., 1999). Recently there has been a concerted effort to promote the use of botanicals as possible alternatives to treat infectious diseases (Mohsenzadeh, 2007; Jazani *et al*., 2009; Chanda *et al*., 2011). These natural products were found to possess promising antimicrobial activities when applied alone or in combination with conventional antimicrobial drugs (Jazani *et al*., 2007). Kumar *et al*. (1998) and Manimegalai *et al*. (2000) used plant products and succeeded to control grasserie disease (caused by nuclear polyhedrosis virus) in mulberry silkworm, *Bombyx mori*. Cellular responses are direct interactions between circulatory haemocytes and invading non self materials. Insects shows defense response through cellular and humoral components (Dunn, 1986; Gupta, 1986).

Effects of various pathogens have been reported in the insects (Horohov and Dunn, 1982; Lea, 1985) however, not much work has been published so far on pathological aspects of tasar silkworm (Reddy *et al*., 2010). Especially the influence of phytoextracts on haematological defense in the insects infected with CPV is not available. In the present study, the efficacy of the certain medicinal plants and the influence of top three phytoextracts (having antiviral properties) on the haemocyte mediated response was reported through study of total hemocyte count and estimation of total protein.

MATERIALS AND METHODS

The study was conducted during March, 2010 to March, 2011 in Central Tasar Research and Training Institute, Ranchi, India. Thirteen numbers of medicinal plants known for their antibiotic properties were selected and tested for their efficacy against virosis in tasar silkworm, *A. mylitta*. Medicinal plants available locally were collected for use in the experiment and coded as AKP1 to AKP 13. Aqueous extracts of plant/plant parts were prepared by grinding 50 g of clean and washed plant materials (leaf/bulb/rhizome) in a little water, filtered through muslin cloth, made the volume 100 mL and centrifuged at 3000 rpm for 15 min. Final volume was made to 100 mL with distilled water to bring the concentration of the stock to 50%. The stocks were further diluted to desired concentration with distilled water before use.

Cytoplasmic polyhedrosis virus (AmCPV): Fresh cytoplasmic polyhedrosis virus inoculum was prepared from diseased silkworm. Completely whitened mid-gut obtained from cytoplasmic polyhedrosised silkworm at an advanced stage of infection was homogenized in sterile distilled water. The polyhedral suspension was filtered through a cheese-cloth and the filtrate was centrifuged at 3000 rpm for 15 min and the polyhedra were purified following Aizawa (1971) by repeated and differential centrifugation. The resultant pellet suspended in distilled water was examined by light microscope for purity. The polyhedral suspension in sterile distilled water was prepared to contain 1×10^5 PIB mL^{-1}.

Inoculation of PIBs and treatment of larvae with plants extracts: Two hundred microlitter suspensions containing 1×10^5 PIB mL^{-1}. was evenly smeared on to the Arjuna (*Terminalia arjuna*) leaves, air dried and fed to 2nd instar larvae of Daba eco-race 24 h after moult. After 6 h of virus

inoculation and once in 3rd, 4th and 5th instar larvae were allowed to feed on arjuna leaves treated with 1.0 and 2.0% plants extract. Three replications with 100 silkworm larvae each and fourth replication with 50 silkworm larvae (For cellular and biochemical estimation) were maintained separately for each treatment. Both treated and inoculated control batches were reared in two rearing seasons (July-August and September-October) in indoor. The observations were made on development of diseases symptoms and larval mortality. The dead larvae in different treatments during rearing were examined microscopically for presence of concerned pathogen.

Estimation of haemocytes count: Every day Total Hemocyte Counts (THC) estimation in the hemolymph of all treated and control batches was determined following the method described by Tauber and Yeager (1934 and 1935) using haemocytometer. The THC per mm^3 of haemolymph was estimated according to the formula suggested by Jones (1962). Every day from 1st to 8th day 6 larvae were collected from fourth replication of treated, inoculated and healthy control batches. Hemolymph from all the 6 larvae was collected in to two eppendorf tubes (3 larval hemolymph/tube) on ice and counted total hemocyte count; remaining hemolymph was utilized for estimation of total protein.

Estimation of total protein: The total protein content in hemolymph was estimated by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Efficacy of the medicinal plants: Results of larva mortality and percent of reduction in virosis in silkworm, *A. mylitta* in indoor rearing after inoculation with POBs of AmCPV and treatment with plant extracts are presented in Table 1. Two percent aqueous extract of AKP 3 with different concentrations was observed more effective where lowest larva mortality due to virosis was recorded 32.25 and 28.04% during 1st and 2nd crop rearing, respectively. Comparatively higher larva mortality (86.22 and 85.12%) was observed with the 1% treatment of AKP 4 followed with 2% treatment of AKP 4 (82.33 and 86.66%) and 1% treatment of AKP 7 (82.66 and 80.66%) during 1st and 2nd crops, respectively. In inoculated control (infected with AmCPV) larva mortality was 87.00 and 91.66 during 1st and 2nd crops, respectively.

Pooled analysis of data revealed maximum reduction in virosis 66.17 in the 2% treatments of AKP 3 followed by AKP 13 (64.47%), AKP 9 (57.19%) and AKP 1 (54.15%). Minimum reduction in virosis was with the 1% treatment of AKP 4 (4.02%).

The aqueous extract of plants AKP 3, AKP 13 and AKP 9 showed encouraging results in reduction of virus infection in silkworm larvae in indoor rearing. Cocoon parameters like cocoons harvested (51.00%), single cocoon weight (13.57%), single shell weight (1.540 g) and silk ratio percent (11.25%) were observed maximum in 2% aqueous extract AKP 3 followed by 1% extract of AKP 3, 2% extract of AKP 13, 1% extract AKP 13 and minimum values were observed in case of control batch (Table 2). Plant extract of all treated batches were on par with the control which indicated that these plant extracts have no adverse effect on the silkworm larvae.

In the present study all the plant extracts tested were found effective in reducing the virosis. Plant extracts have also shown significant difference with each other in reduction of virosis in tasar silkworm. The treatments of aqueous extract of plant AKP 3 (2.00% conc.) was more effective and reduced the virus infection to the tune of 66.17% in indoor rearing. Similar studies were made by Manoharraj (1994) and Mallika (1997) where they observed 72.46 and 79.50% reduction in grasserie (caused by nuclear polyhedrosis virus) in mulberry silkworm, *Bombyx mori* by application

Table 1: Tasar silkworm mortality and reduction of virus infection after AmCPV inoculation and treatment with plant extracts (in indoor rearing)

		Mortality due to virusis			Reduction of virus infection from control (%)		
Plants	Concentration	1st crop	2nd crop	Pooled	1st crop	2nd crop	Pooled
AKP 1	1	35.67	53.63	44.65	59.00	41.49	50.25
	2	32.00	50.33	41.17	63.22	45.09	54.15
AKP 2	1	78.00	70.33	74.17	10.34	23.27	16.81
	2	74.33	72.66	73.50	14.56	20.73	17.65
AKP 3	1	40.66	47.00	43.83	53.26	48.72	50.99
	2	32.25	28.04	30.15	62.93	69.41	66.17
AKP 4	1	86.22	85.12	85.67	0.90	7.14	4.02
	2	82.33	86.66	84.50	5.37	5.45	5.41
AKP 5	1	42.33	55.00	48.67	51.34	40.00	45.67
	2	37.67	51.67	44.67	56.70	43.63	50.16
AKP 6	1	36.82	52.61	44.72	57.68	42.60	50.14
	2	33.04	50.12	41.58	62.02	45.32	53.67
AKP 7	1	82.66	80.66	81.66	4.99	12.00	8.49
	2	80.00	78.00	79.00	8.05	14.90	11.47
AKP 8	1	80.00	72.00	76.00	8.05	21.45	14.75
	2	77.66	74.33	76.00	10.74	18.91	14.82
AKP 9	1	63.33	56.66	60.00	27.21	38.18	32.70
	2	35.23	41.37	38.30	59.51	54.87	57.19
AKP 10	1	39.18	46.18	42.68	54.97	49.62	52.29
	2	41.67	53.33	47.50	52.10	41.82	46.96
AKP 11	1	42.33	55.33	48.83	51.34	39.64	45.49
	2	40.00	50.66	45.33	54.02	44.73	49.38
AKP 12	1	40.00	48.66	44.33	54.02	46.91	50.47
	2	39.33	46.00	42.67	54.79	49.81	52.30
AKP 13	1	40.00	46.33	43.17	54.02	49.45	51.74
	2	28.50	35.10	31.80	67.24	61.71	64.47
Inoculated control	87.00	91.66	89.33				

Table 2: Effect of plant extracts, Aloe barbedensis (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) on cocoon parameters in silkworm

Treatment	Conc. (%)	Cocoons harvested (%)	Cocoon wt. (g)	Shell wt. (g)	SR (%)
AKP 3	1	50.27	12.98	1.49	10.87
	2	51.00	13.57	1.54	11.25
AKP 13	1	44.84	12.30	1.37	10.33
	2	47.54	14.04	1.53	10.90
AKP 9	1	46.96	12.77	1.42	10.37
	2	44.03	13.58	1.32	10.49
Control		38.37	12.49	1.41	11.00

of leaf extract of *Psoralea coryleifolia*, respectively. Sivaprakasham (1994) reported that the aqueous leaf extract of *P. corylifolia* was better than gentamycin or calcium hydroxide in reducing the larval mortality due to grasserie. Manimegalai *et al.* (2000) observed 63% reduction in grasserie disease in *B. mori* with the application of turmeric and chalk powder. However, plant extracts tested in the present investigation have not been reported earlier against the virus disease in tasar silkworm.

Estimation of total hemocyte count: The data on the effect of aqueous extracts of *Aloe barbedensis* (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) on haemocyte count in CPV infected worms is presented in Table 3. In all the treatment sets and in the normal control the total haemocyte count increased significantly from 1st day to 6th days and decreased on 7th day. In AKP 3 batch the total haemocyte counts was 12650 mm^{-3} of haemolymph and increased to 15432 mm^{-3} by 6th day. On 7th day total haemocyte counts were 14903 mm^{-3}. On 8th day the counts was increased up to 15625 mm^{-3}. The trend was same in all the treatments and in normal control. While in inoculated control the counts were increased after inoculation with CPV up to 2nd day of infection. Then there was a decrease for a period ranging from 3-8 days. In normal control the total haemocyte counts was significantly low as compared to the treatment because the increase may represent the defense response of silkworm, *A. mylitta* against the invading pathogen.

The observed data agreed with the earlier workers as they investigated that once entomophagus fungi have penetrated in the host integument and gained access the nutrient-rich haemocoel, they are confronted with humoral and or cellular defenses. As humoral response, the phenoloxidase system will be activated to induce the phagocytic process and melanization which works as toxin to invading microorganism. The cellular responses to infection have been worked out in many insect by earlier workers (Horohov and Dunn, 1983). Similar type of result also obtained when the effect of systematic fungicide was studied on the total haemocyte count in *B. bassiana* infected silkworm, *Bombyx mori*. Haemocytes are extremely efficient in removing pathogens by accomplishing a series of reactions designated as phagocytosis, nodule formation or encapsulation. The observed data agreed well with the earlier investigation that their number may increase (Balavenkatasubbaiah *et al*., 2001; Al-Attar, 2010) and decrease to counter foreign body when infected. Recently stress has been induced to tasar silkworm to study its impact on Haemocytes count (Pandey *et al*., 2010). On the basis of the above findings of the earlier workers it evident that CPV induce the defense response through multiplication of haemocytes as is in dicated by the increase in total haemocyte counts of the haemolymph of the worms. When the treatment was given with aqueous solution of plants having antiviral activity is suppressing the multiplication of the pathogen and boosting the immunity level which may be the result of the high total hemocyte count than the healthy control.

Estimation of total protein content in hemolymph: The data on the effect of aqueous extracts of *Aloe barbedensis* (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) on protein content in CPV infected worms is presented in Table 4.

Table 3: Effect of aqueous extracts of Aloe barbedensis (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) on the total hemocyte count in virus infected tasar silkworm

	Days post inoculation							
Treatment	1	2	3	4	5	6	7	8
AKP 3	12650	14266	14810	15266	15407	15432	14903	15625
AKP 13	12750	14305	15000	15530	15833	15903	14856	15457
AKP 9	12500	14236	14607	15111	15527	15543	14230	15008
Inoculated control	12700	14480	14350	12671	9851	6203	5009	3812
Normal control	12333	12455	12782	13124	13690	13702	12480	13713
S.E.±	132.56	141.21	125.07	106.32	156.78	182.22	191.06	190.23
CD. at 5%	138.08	151.26	177.12	125.28	256.27	308.15	289.05	331.47

Table 4: Effect of aqueous extracts of *Aloe barbedensis* (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) on the total protein content in virus infected tasar silkworm

	Days post inoculation							
Treatment	1	2	3	4	5	6	7	8
AKP 3	16.17	20.26	23.75	28.55	31.95	36.75	36.03	35.27
AKP 13	16.28	20.21	23.61	27.05	29.56	34.27	34.14	34.05
AKP 9	16.33	20.05	23.58	26.88	29.12	34.69	34.19	33.96
Inoculated control	16.26	20.14	24.22	23.58	22.16	21.59	20.98	20.25
Healthy control	16.31	20.19	23.54	26.74	28.53	30.34	32.83	33.73

The hemolymph protein in aqueous extracts of *Aloe barbedensis* (AKP 3), *P. corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) silkworms increased gradually from day 1 to day 7 and decreased on day 8. In especially *Aloe barbedensis* (AKP 3) treatment the total protein increased from 16.17 to 36.03 mg mL^{-1} and reached to 35.27 mg mL^{-1} at the end of day 8. In inoculated control, the total hemolymph proteins have shown increasing trend from 1st (16.26 mg mL^{-1}) to 3rd day (24.22 mg mL^{-1}) and decreasing trend from 4th day onwards and reached 20.25 mg mL^{-1} by 8th day from the inoculation. In the healthy control study increase was observed from day 1 to day 8 and reached from 16.31 to 33.73 mg mL^{-1}.

Quantitative and qualitative changes in protein profile of various tissues of tropical tasar silkworm, *Antheraea mylitta* D. was recently studied (Kumar *et al*., 2011). The results indicated that changes occur in the hemolymph protein, during the course of AmCPV infection. The difference in total hemolymph protein in healthy silkworm, inoculated silkworm and antiviral plant solution treated becomes more pronounced as the diseases progresses. High weight of protein was observed in the case of *Aloe barbedensis* (AKP 3) at the end of 8th day from the AmCPV inoculation. This would probably indicate that suppression of pathogen and boosting of immunity, the synthesis of proteins were greatly increased.

CONCLUSION

Among the tested 13 antiviral plants, *Aloe barbedensis* (AKP 3) has given encouraging results in containment of AmCPV in tasar silkworm. The *Aloe barbedensis* plant product can be recommended to the farmers for the prevention of crop loss due to the AmCPV after conducting large scale field trials in the farmer's fields.

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