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Botanicals against Nuclear Polyhedrosis Virus infecting three breeds of mulberry silkworm, *Bombyx mori* L.

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ABSTRACT

Mulberry silkworm, *Bombyx mori* L, most valued for silk and other medicinal products is prone to various biotic stresses among which the disease caused by Nuclear Polyhedrosis Virus (BmNPV) is most serious as it occurs throughout the year causing losses to the tune of 30-40 per cent. Studies conducted on eco friendly management of the disease using botanicals revealed that 800 ppm hexane leaf extract of *Psoralea corylifolia, Plectranthus amboinicus* and the standard, gentamycin (50 ppm) were found to be effective against BmNPV infecting three different silkworm breeds, *viz.*, cross breed, PM X CSR 2 (28.50 %, 24.00 %, 23.50 %), bivoltine hybrid, CSR 2 x CSR 4 (36.0 %, 33.50 %, 30.50 %) and double hybrid (CSR 6 x CSR 26) x (CSR 2 x CSR 27) (31.50 %, 29.00 %, 26.50 %) recording lowest larval mortality. Treated control (BmNPV@10^7 POBs/ml) recorded the highest mortality of 60.00 %, 75.50 % and 68.50 % respectively against cross breed, bivoltine hybrid and double hybrid. Besides the disease reduction, administration of botanicals also enhanced the economic parameters, *viz.*, larval weight, cocoon weight, shell weight and shell ratio. Isolation of active principles from hexane extract of *P. corylifoli*a using Thin Layer Chromatography studies (TLC) resulted in seven partially purified fractions. From the seven partially purified fractions, two fractions possessing antiviral properties were further purified using High Performance Liquid Chromatography (HPLC). Characterization of these purified fractions using Liquid Chromatography Mass Spectroscopy (LCMS) revealed the presence of two flavanoid compounds, *viz.*, Bakuchicin and Bavacoumestan.

Key words: *Bombyx mori,* Nuclear Polyhedrosis Virus, Botanicals, *Psoralea corylifolia, Plectranthus amboinicus,* silkworm breeds, mortality, economic parameters, flavanoid compounds.

INTRODUCTION

Mulberry silkworm, *Bombyx mori* L. is prone to viral, bacterial, fungal and protozoan infections due to its domestication for about 5000 years. The loss due to various diseases is 30 – 40 per cent. The viral disease, caused by nuclear polyhedrosis virus is most serious as it occurs throughout the year. More than 15 per cent crop loss is reported. Several prophylactic and curative measures are aimed for managing the disease. Though chemicals play an important role, taking into account the ecofriendly nature and cost effectiveness, plant products are given importance, nowadays. Hence, identification of suitable botanicals for disease management will be of great use to sericulture industry for realizing higher yield of cocoons.

MATERIALS AND METHODS

Laboratory experiments were conducted to study the efficacy of botanicals against Nuclear Polyhedrosis Virus (BmNPV) against three silkworm breeds, *viz.*, PM x CSR 2, CSR 2 x CSR 4 and (CSR 6 x CSR 26) x (CSR 2 x CSR 27) with five treatments replicated four times with 50 larvae per replication. TLC, HPLC and LCMS studies were conducted to isolate the antiviral principles from *P. corylifolia.* The polyhedral occlusion bodies were extracted from the infected larvae and purified by gradient centrifugation method (Sugumari *et al.* 1990). The number of polyhedra in the suspension was counted using a haemocytometer in a phase contrast microscope.

Fresh plant material was thoroughly washed with running tap water followed by rinsing twice with distilled water. The plant material was then dried in shade and powdered. The powdered leaf sample was weighed and extracted with hexane in a soxhlet apparatus for six hours (Khatune, 2000). Solvent free filtrate was collected on a rotary evaporator at 30°C (Jacobson *et al.*, 1975). The residue was weighed and dissolved in equal volume of acetone (W/V) to get a working stock solution. From the working standard, required concentration of 800 ppm was prepared with water and used for laboratory assays.

Fresh mulberry leaves were dipped in viral suspension of 10^7 POBs/ml and shade dried. The worms after second

moult were fed with mulberry leaves treated with virus @ 10^7 POBs/ml. The treated leaves were provided during the first feed on first day and thereafter the larvae were fed with normal leaves. On the next day, the leaves administered with different treatments were fed to worms. Fresh leaves were dipped in required concentration of extracts and shade dried before feeding it to silkworms. Treatments were administered twice, once on the second day of third instar and the other on the first day of fourth instar. The worms fed with BmNPV alone served as treated control. Untreated control was also maintained. Observations were made on larval mortality, larval weight, cocoon weight and shell weight and shell ratio.

Separation of the active principles from botanicals

Thin layer chromatographic studies were carried out to separate the nature of active principles from the plant extracts (Sadasivam and Manickam, 2005). The solvent dichloromethane was used to separate the compounds. The TLC plate was dried and observed under bright light for the presence of phenols, flavanoids and alkaloids. All the spots were marked and Rf (Relative front) values were calculated using the formula.

$$R_f \text{value} = \frac{\text{Distance moved by the solute from the orgin}}{\text{Distance moved by the solvent from the orgin}}$$

Compounds in TLC purified retention factors were separated using Shimadzu LC 8A RP –HPLC with C18 column. Two pumps, pump A consisting of 80 per cent methanol and pump B consisting of 20 per cent water were used to run the TLC purified compounds. The LCMS of the HPLC purified sample was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer having a JASCO PU-980 HPLC pump connected to it.

RESULTS AND DISCUSSION

The 800 ppm hexane extract of *Psoralea corylifolia, Plectranthus amboinicus* and gentamycin @ 50 ppm (standard) were found to be effective against BmNPV infecting three different silkworm breeds, PM X CSR 2 (28.50 %, 24.00 %, 23.50 %), CSR 2 x CSR 4 (36.0 %, 33.50 %, 30.50 %) and (CSR 6 x CSR 26) x (CSR 2 x CSR 27) (31.50 %, 29.00 %, 26.50 %) recording lowest larval mortality compared to control (60.00 %, 75.50 % and 68.50 %) (Table 1). All the treatments recorded significantly higher larval weight, cocoon weight, shell weight and shell ratio compared to untreated control in all the silkworm breeds tested (Table 2).

The present result on the efficacy of botanicals against BmNPV is strengthened with the works of Padma and Manimegalai (2007) who reported that the aqueous extract of *P. amboinicus* and *P. corylifolia* were effective in suppressing grasserie with mortality of 24.00 and 25.33

Table 1. Effect of botanicals against BmNPV infecting silkworm breeds

Treatments	Silkworm breeds		
	PM x CSR2	CSR 2 x CSR 4	(CSR 6 x CSR 26) x CSR 2 x CSR 27)
Psoralea corylifolia	28.50^{b}	36.00^{c}	31.50^{c}
Plectranthus amboinicus	24.00^{b}	33.50^{c}	29.00^{bc}
Gentamycin	23.50^{ab}	30.50^{b}	26.50^{b}
Treated control	60.00^{c}	75.50^{d}	68.50^{d}
Untreated control	0.00^{a}	0.00^{a}	0.00^{a}
SEd	11.02	1.38	1.22
CD (0.05)	23.50	2.94	2.61

In a column, means followed by common small letter(s) are not significantly different at 5 % by LSD

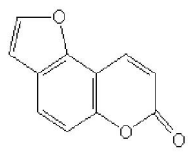
per cent in the cross breed, PM x CSR 2. The per cent mortality was found to be higher in bivoltine single hybrid than the bivoltine double hybrid. This may be due to the robust nature of double hybrid.

Separation of active principles from *P. corylifolia*

In *P. corylifolia*, five spots with Rf values of 0.98, 0.89, 0.84, 0.40 and 0.20 were obtained under visible light. Two spots with Rf values of 0.94 and 0.92 was visualized under UV light. Upon injection of 20 µl of the effective sample solution from TLC fraction of *P. corylifolia* into HPLC column, two peaks were obtained from each of the two effective fractions (Rf 4 and Rf 5). The HPL chromatogram of *P. corylifolia* yielded one major peak with a retention of 2.807 min and a few minor peaks.

A total of six different mass spectra were obtained. The compound present in the major peak was identified as Bakuchicin with a molecular weight of 186 Dalton. The $(M+H)^+$ ion was observed at m/z 187 as a base peak (100% intensity). A peak at m/z 171 is due to the loss of oxygen from (M+H) ion. Another peak at m/z 143 is due to the loss of CO_2 from (M+H) ion. Another compound was identified from the HPLC peak with a retention time of 13.029 as Bavacoumestan with a molecular weight of 352. The mass spectrum shows a peak at m/z 353 due to (M+H) ion.

Bakuchicin



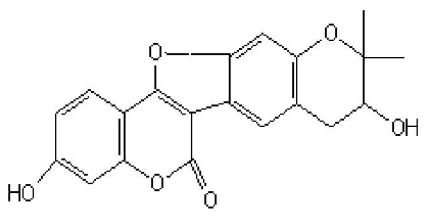
Molecular formula : $C_{11}H_6O_3$
Molecular weight :186, $(M+H)^+$: 187
$(M+H-O)^+$: 171
$(M+H-CO_{2)}$:143

Table 2. Effect of botanicals on larval and cocoon parameters of silkworm breeds

Treatments	Economic parameters											
	Larval Weight (g)			Cocoon Weight (g)			Shell Weight (g)			Shell Ratio (%)		
	PM x CSR2	CSR 2 x CSR 4	(DH 1)	PM x CSR2	CSR 2 x CSR 4	(DH 1)	PM x CSR2	CSR 2 x CSR 4	(DH 1)	PM x CSR2	CSR 2 x CSR 4	(DH 1)
Psoralea corylifolia	3.47[a]	2.93[a]	4.78[a]	1.72[a]	1.74[a]	1.92[a]	0.32[a]	0.43[a]	0.47[a]	18.60[ab]	24.71[a]	24.48[a]
Plectranthus amboinicus	3.50[a]	3.30[a]	4.85[a]	1.72[a]	1.76[a]	1.96[a]	0.33[a]	0.43[a]	0.47[a]	19.19[a]	24.43[a]	23.98[a]
Gentamycin	3.49[a]	3.15[a]	4.83[a]	1.74[a]	1.82[a]	1.94[a]	0.32[a]	0.41[a]	0.47[a]	18.39[ab]	25.50[a]	24.23[a]
Treated control	2.49[c]	2.40[b]	2.82[b]	1.43[b]	1.51[b]	1.63[b]	0.26[b]	0.32[b]	0.33[b]	18.18[b]	21.20[b]	20.25[b]
Untreated control	3.30[b]	3.20[a]	4.80[a]	1.70[a]	1.72[a]	1.95[a]	0.29[a]	0.40[a]	0.46[a]	17.06[b]	23.20[a]	23.59[a]
SEd	0.06	0.81	0.06	0.06	0.58	0.06	0.06	0.63	0.06	0.06	7.90	0.58
CD (0.05)	0.12	1.73	0.12	0.12	1.25	0.12	0.12	1.35	0.12	1.23	1.43	1.23

DH 1 - (CSR 6 x CSR 26) x (CSR2 x CSR27) In a column, means followed by common small letter(s) are not significantly different at 5 % by LSD.

Bavacoumestan



Molecular formula : $C_{20}H_{16}O_6$
Molecular weight : 352
$(M+H)^+$: 353

In the present study, the plant products were found to be effective against BmNPV in all the silkworm breeds tested. The antiviral effect of these compounds may be due to their ability to form complexes with viral DNA, altering the pH of the gut, binding with proteinaceous virions or blocking the pores in the peritropic membrane of the gut. The affinity of psoralen compounds for nucleic acid has been demonstrated by Scott *et al.* (1976); Song and Tapley (1979). Mason and Wasserman (1987) attributed that, the mechanism behind phenolic toxicity to microorganisms is due to enzyme inhibition by oxidized compounds possibly through reaction with sulf-hydryl group or through more non specific interaction with proteins. The disruption of microbial membranes by the flavanoids was reported by Tsuchiya *et al.* (1996).

According to Ya *et al.* (1988) the mode of action of phenol are related to the ability of plant compounds to inactivate microbial adhesion, enzymes, cell envelope, transport protein and forming complexes with polysaccharides. Keating *et al.* (1989) reported that phenols may bind directly the proteinaceous virions and subsequently interfere with host cell interactions. Felton and Duffey (1990) found that chlorogenoquinone, a powerful oxidizing agent covalently binded to the occlusion bodies of NPV and significantly reduced the solubility ultimately impairing the release of infective virions in the midgut.

Samuel Manohar Raj (1994) reported that the leaf extracts of *P. corylifolia* possessed some phenolics, which may have acted as viral inhibitors in avoiding infection by BmNPV. Similarly, Manoharan (1996) reported that leaves of *A. suma, C. coriaria* and *T. tomentosa* had higher amount of tannins and phenols and the polyhedra exposed to these extracts showed higher rate of aggregations with lesser mortality due to BmNPV.

Administration of botanicals improved the economic parameters *viz.*, larval weight, cocoon weight, shell weight and shell ratio in the treatments compared to treated control in the present study. The improvement of economic parameters due to administration of botanicals were documented by several authors (Rajasekargouda, 1991; Murugan *et al.*, 1998; Manimegalai and Chandramohan, 2005).

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