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Effect of botanical extracts on the biological activity of granulosis virus against *Pieris brassicae*

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Abstract A granulosis virus strain infecting *Pieris brassicae* (PbGV) was isolated from the dry temperate region of northwestern Himalayas as a potential microbial agent for its management. The effect of different botanicals (having insecticidal action against *P. brassicae*) on the bioefficacy of PbGV was evaluated under laboratory conditions using leaf disc bioassays on cabbage for improving the insecticidal performance of the PbGV. The synergistic action of different botanical extracts was evident in terms of reduction in LC_{50} values against different botanical extracts. Among different extracts, petroleum-ether extract of neem seed kernel (NSK) when combined with PbGV resulted in maximum reduction of LC_{50} value (4.39×10^2 occlusion bodies [OBs] ml^{-1}) followed by methanolic extract (7.38×10^2 OBs ml^{-1}) and aqueous extract (9.36×10^3 OBs ml^{-1}) as compared with PbGV alone (1.85×10^4 OBs ml^{-1}) for 2nd instar larvae of the test insect. These trends were found analogous in cases of 3rd and 4th instars of *P. brassicae* with different solvent extracts of NSK. The other botanicals evaluated, viz., *Eupatorium* and *Artemesia,* also resulted in reduction of LC_{50} values for 2nd, 3rd and 4th instars as compared with PbGV alone when different extracts were combined with virus for bioassays. The studies suggest that the PbGV in combination with botanical pesticides could be more useful as a bio-pesticide against cabbage butterfly (*P. brassicae*) in IPM programs.

Keywords *Artemesia* · Bioassay · Botanicals · Cabbage butterfly · *Eupatorium* · Granulovirus · Neem · PbGV

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Introduction

Cabbage butterfly, *Pieris brassicae* (Linn.) (Lepidoptera: Pieridae), is a serious pest of cabbage, cauliflowers and many crucifers found along temperate, tropical and subtropical regions of the world (Feltwell 1978). The pest is widely distributed along the Himalayan region including Pakistan, Nepal and throughout the plains except in the southern states of India (Chuang-Lang 1976; Lal and Ram 2004; Nair 1975; Vevai 1973; Younas et al. 2004). It passes the winter in the plains and migrates to hilly regions during summer in India (Gupta 1984). It breeds on the rapeseed mustard in the month of September and remains active until April. It causes extensive damage at all the growing stages of cole crops such as the seedling, vegetative and flowering stages (Ali and Rizvi 2007; Sachan and Gangwar 1980; Younas et al. 2004). The young caterpillars feed gregariously on leaves, defoliating the plants (Ali and

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Rizvi 2007; Younas et al. 2004), making insecticidal application mandatory for the cultivation of cole crops.

During the past six decades the efforts to manage the insect pests were dominated by chemical preparations. This has led to environmental pollution, destruction of the natural enemy complex and development of resistance against toxicants in over 500 species of insects and mites (Thomas 1999). Protective measures using different chemicals result in undesirable side effects to human health, as the cole crops are used as fresh vegetables in human diet (Matter et al. 2002). Thus, there is a need to look for safer eco-friendly alternatives in view of the pressing need to protect the environment and natural enemies.

The successful development and utilization of several microbial pesticides for the management of pests have made them promising for this use. Baculoviruses have shown a great potential against Lepidopteran pests because of their virulence and aggressiveness (Murphy et al. 1995). Entomopathogenic viruses possess distinct advantages over other microbial bioagents because of their horizontal and vertical transmission from one generation to the next host generation. They cause deformities, thereby making them very effective at all stages of the host, even at sublethal doses of the pathogen (Burden et al. 2002). The safety of such insect pathogens to vertebrates—non-target fauna, their host-specificity and high pathogenicity, has in fact made them more attractive as bioinsecticides (Entwistle and Evans, 1985).

A granulovirus strain, PbGV, isolated and characterized from Sangla Valley of Himachal Pradesh, India (situated at an altitude of 2590 m a.s.l., 31°25′ 56″ N latitude and 78°15′4″ E longitude), has been highly effective against *P. brassicae* (Sood 2004). Some local botanical extracts (collected from different regions of northwestern Himalayas) were also found effective in reducing the incidence of *P. brassicae* (Mehta et al. 2005). Management of the pest under field conditions, however, requires an integrated approach utilizing all the available options. However, there is a lack of information on the stressor or synergistic effects of botanicals on the virulence of granulosis virus (GV) against *P. brassicae,* which restricts their utilization. Hence, the present studies were undertaken to study the effects of different solvent extracts of three botanicals, namely, neem (*Azadirachta indica* A. Juss; family: Meliaceae), cypress weed (*Eupatorium adenophorum* Spreng; family: Asteraceae) and big sage brush (*Artemisia brevifolia* Wall; family: Asteraceae) on the bioefficacy of GV infecting *P. brassicae*.

Materials and methods

Rearing and selection of P. brassicae for bioassay The initial culture of *P. brassicae* was started from field-collected eggs. The eggs were kept in sterilized petri plates (7.5 cm diam) over a UV irradiated filter paper moistened with sterile distilled water (SDW) to prevent desiccation under laboratory conditions (temp. 25±2°C and 75–80% r.h.). Newly hatched larvae were transferred to fresh cabbage leaves surface-sterilized with aqueous solution of sodium hypochlorite (0.05%) followed by several washings with SDW. The cabbage leaves were kept in an ethanol-washed and UV-sterilized cage (15×15×15 cm^3). The first three larval instars were reared in the small cages (15×15×15 cm^3) while the later instars were reared in large cages (45×45×55 cm^3). Caterpillars in cages were provided with surface-sterilized fresh cabbage leaves daily. The fully grown caterpillars were transferred to the new cages for pupation. Two-day-old pupae were detached from the walls of the cage and kept separately with a batch of 20 pupae in the cage (60×60×70 cm^3) over a thick layer of UV-irradiated filter paper for adult emergence. The adults were held in cages (60×60×70 cm^3) provided with cotton swabs soaked in honey solution and SDW. Some flowering shoots of mustard were also provided as pollen source. Potted cabbage plants were kept in each cage for egg laying whenever needed.

Pre-starved (24 h) healthy larvae of 2nd, 3rd and 4th instars surface-sterilized with 70% ethanol were selected for the bioassay to facilitate feeding and reducing the chances of mortality due to other factors.

Preparation of viral OBs (occlusion bodies) and botanicals suspensions

PbGV OBs suspension The *Pieris brassicae* granulovirus (PbGV) used in the study was the local strain isolated and characterized from the dry temperate region of Himachal Pradesh by Sood (2004). The

PbGV was produced in the larvae of *P. brassicae* in the Postgraduate Laboratory of the Department of Entomology, CSK HP Agricultural University, Palampur, India (1200 m a.s.l.). The purified virus was used in the present studies, and the concentration of OBs in stock suspensions was determined by direct count using a Helber bacteria haemocytometer (0.02 mm depth). A stock solution of PbGV with a strength of 2×10^{12} OBs ml^{-1} was prepared for bioassay studies and stored in amber colored bottles at 4°C. Serial dilutions (10^3–10^9 OBs ml^{-1}) were prepared from the quantified virus stock solutions in sterile distilled water.

Aqueous, methanol and petroleum-ether extracts of botanicals Neem (*Azadirachta indica*) seed kernels, cypress weed (*Eupatorium adenophorum*) and big sage brush (*Artemisia brevifolia*) leaves were collected, dried in the shade, and ground into powder form. For aqueous extracts, 100 g of required material in powdered form of each plant was mixed in 1 *l* of distilled water to make 10% stock concentration (*w*/*v*), soaked for 48 h and filtered through four layers of muslin cloth to obtain the final working concentrations. The solvent (methanol and petroleum ether) extraction of plant material was done using a Soxhlet apparatus. The solvent was then evaporated under reduced pressure. Finally the amount of active material obtained was worked out by pre- and post-evaporation weighing of petri plates. The crude extracts obtained above were dissolved in acetone (10% *w*/*v*) to make stock solutions, which were then stored at 4°C. Further dilutions were prepared by addition of the appropriate amounts of SDW.

Preparation of working concentrations Five ml of aqueous, methanol and petroleum ether extracts of neem seed kernel, *E. adenophorum* and *A. brevifolia* leaves were mixed separately with 5 ml of different viral concentrations, *viz.,* 2×10^9, 2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4 and 2×10^3 OBs ml^{-1}, respectively, in order to obtain the final working concentration of 5% botanicals with 1×10^9, 1×10^8, 1×10^7, 1×10^6 and 1×10^5 OBs ml^{-1} for 4th instar; 1×10^8, 1×10^7, 1×10^6, 1×10^5 and 1×10^4 OBs ml^{-1} for 3rd instar; and 1×10^7, 1×10^6, 1×10^5, 1×10^4 and 1×10^3 OBs ml^{-1} viral concentrations for 2nd instar. The treatment combinations followed standard bioassay protocol.

Bioassay methodologies A leaf disc bioassay technique, as described by Ballard et al. (2000), was used to assess the effect of extracts of different botanicals on the bioefficacy of PbGV. Cabbage leaf discs (4.5 cm diam) were cut from 70% ethanol-washed fresh cabbage leaves collected from an insecticide-free plot. One hundred µl suspension of each treatment combination was used to treat both the surfaces of leaf discs, after which the discs were shade-dried. Treated discs were kept singly in the UV-irradiated petri dishes. Twenty-five pre-starved larvae each (24 h) of 2nd and 3rd instars were released in groups, and of 4th instars individually on treated cabbage leaf discs for each treatment separately. Each treatment was replicated thrice and an untreated control (DSW) was also kept. After 24 h of feeding, the larvae were transferred to 70% ethanol-washed untreated cabbage leaves. Mortality counts were recorded 48 h after release and thereafter. Observations on PbGV infection were recorded by identifying the peculiar viral symptoms (virosis). In case of uncertainty, larvae were prodded with a needle in order to provoke the characteristic disintegration of the larval tissue.

Statistical analysis The mortality data collected for different instars of *P. brassicae* with different treatments from the laboratory bioassay were subjected to probit analysis for calculation of LC_{50} (Finney 1971). Corrected mortalities were calculated using Abbott's formula (Abbott 1925). The slope of the probit line, regression equation and fiducial limits were also determined. The chi-square test was applied to confirm the data fit to probit model.

Results

Virulence of PbGV in laboratory bioassays Second instars of *P. brassicae* were the most susceptible to PbGV of the instars used in the bioassay. The LC_{50} of PbGV against 2nd instar *P. brassicae* larvae was 1.85×10^4 OBs ml^{-1}, whereas against 3rd and 4th instars the values were 1.86×10^6 and 5.89×10^7 OBs ml^{-1}, respectively. However, the LC_{50} of PbGV against 2nd instar larvae in combination with aqueous extract of neem seed kernel, *E. adenophorum* and *A. brevifolia* was 1.32×10^3, 9.36×10^3 and 1.38×10^4 OBs ml^{-1}, respectively (Table 1). The data

Table 1 Relative toxicity of PbGV (*Pieris brassicae* granulovirus) alone and in combination with different extracts of botanicals against second, third and fourth instar *Pieris brassicae* larvae

Treatments	2nd instar LC_{50} (occlusion bodies per ml)	3rd instar	4th instar
PbGV alone	1.85×10^4 (1.62×10^3–2.10×10^5)[a]	1.86×10^6 (2.41×10^5–1.44×10^7)	5.89×10^7 (3.29×10^6–1.05×10^9)
PbGV + aqueous extract of neem seed kernel (NSK)	1.32×10^3 (0.53×10^2–3.28×10^4)	1.49×10^5 (1.63×10^4–1.37×10^6)	2.17×10^7 (1.24×10^6–3.78×10^8)
PbGV + aqueous extract of *Eupatorium adenophorum*	9.36×10^3 (9.72×10^2–9.02×10^4)	5.33×10^5 (7.89×10^4–3.59×10^6)	3.24×10^7 (2.86×10^6–3.66×10^8)
PbGV + aqueous extract of *Artemesia brevifolia*	1.38×10^4 (1.02×10^4–1.17×10^5)	6.44×10^5 (9.63×10^4–4.30×10^6)	3.90×10^7 (3.18×10^6–5.01×10^8)
PbGV + methanol extract of NSK	7.38×10^2 (0.18×10^2–2.95×10^4)	1.29×10^5 (1.02×10^4–1.63×10^6)	7.13×10^6 (5.26×10^5–9.64×10^7)
PbGV + methanol extract of *E. adenophorum*	7.21×10^3 (6.51×10^2–7.98×10^4)	2.57×10^5 (3.10×10^4–2.12×10^6)	1.23×10^7 (8.21×10^5–1.85×10^8)
PbGV + methanol extract of *A. brevifolia*	1.25×10^4 (1.83×10^3–2.30×10^7)	2.73×10^5 (3.36×10^4–2.22×10^6)	1.37×10^7 (8.82×10^5–2.12×10^8)
PbGV + petroleum ether extract of NSK	4.39×10^2 (0.30×10^2–6.91×10^4)	2.30×10^4 (1.56×10^3–3.45×10^5)	1.15×10^6 (3.76×10^4–3.52×10^7)
PbGV + petroleum ether extract of *E. adenophorum*	4.03×10^3 (5.03×10^2–3.23×10^4)	5.59×10^4 (3.56×10^3–8.75×10^5)	4.57×10^6 (2.60×10^5–8.06×10^7)
PbGV + petroleum ether extract of *A. brevifolia*	8.76×10^3 (1.02×10^3–6.81×10^4)	1.05×10^5 (8.32×10^3–1.33×10^6)	4.98×10^6 (4.21×10^5–5.89×10^7)

[a] Figures in parentheses are fiducial limits

revealed that botanicals increased the virulence of PbGV with a considerable reduction in the LC_{50} value. The order of efficacy of aqueous extract of botanicals combined with PbGV was neem seed kernel > *E. adenophorum* > *A. brevifolia*. A similar trend was observed for methanol ($7.38\times10^2>7.21\times10^3>1.25\times10^4$ OBs ml^{-1}) as well as for petroleum ether extracts ($4.39\times10^2>4.03\times10^3>8.76\times10^3$) of botanicals.

The order of efficacy of aqueous extracts of botanicals against 3rd instar larvae was neem seed kernel > *E. adenophorum* > *A. brevifolia* ($1.49\times10^5>5.33\times10^5>6.44\times10^5$ OBs ml^{-1}). Similarly, methanol ($1.29\times10^5>2.57\times10^5>2.73\times10^5$ OBs ml^{-1}) as well as petroleum ether extracts ($2.30\times10^4>5.59\times10^4>1.05\times10^5$ OBs ml^{-1}) of botanicals showed the same pattern of toxicity (Table 1).

Exposure of 4th instar *P. brassicae* larvae to PbGV revealed the LC_{50} value to be 5.89×10^7 OBs ml^{-1}, whereas PbGV combined with aqueous extract of neem seed kernel, *E. adenophorum* and *A. brevifolia* showed LC_{50} values of 2.17×10^7, 3.24×10^7 and 3.90×10^7 OBs ml^{-1}, respectively (Table 1). The comparative efficacy of aqueous extracts of botanicals was neem seed kernel > *E. adenophorum* > *A. brevifolia,* whereas a combination of methanol ($7.13\times10^6>1.23\times10^7>1.37\times10^7$ OBs ml^{-1}) as well as petroleum ether extracts of botanicals ($1.15\times10^6>4.57\times10^6>4.98\times10^6$ OBs ml^{-1}) showed the same pattern of efficacy against 4th instar *P. brassicae*. When comparing the efficacy of different extracts of botanicals in combination with PbGV against 2nd instar larvae of *P. brassicae*, petroleum ether extract of neem seed kernel was found to be the best (LC_{50} value 4.39×10^2 OBs ml^{-1}), followed by methanolic extract (LC_{50} value 7.38×10^2 OBs ml^{-1}) and aqueous extract (LC_{50} value 9.36×10^3 OBs ml^{-1}) of neem seed kernel. A similar trend was observed with 3rd and 4th instars of *P. brassicae*.

Discussion

The LC_{50} values increased with increasing host age from 2nd to 4th instar. This was due to amplified tolerance/resistance level in the host against pathogen with increased age, as neonate larvae are the most susceptible to the pathogen. These findings are in agreement with those of Matthews et al. (2002), who

reported that the LD_{50} values of tomato moth *Lacanobia oleracea* granulovirus were $10^{1.38}$ OBs in 1st instars and $10^{7.45}$ OBs in 5th instars, which increased with increasing host age in the 2nd, 3rd and 5th stadia. Grzywacz et al. (2002) had also observed that the LC_{50} value of *Plutella xylostella* granulovirus was 2.36×10^{6} OBs ml^{-1} for Nya-01 PlxyGV and 3.95×10^{7} OBs ml^{-1} for Nya-40 PlxyGV against 2nd instar larvae. The lower value of LC_{50} in the present studies in comparison with *P. xylostella* could be attributed to high susceptibility of the host to its GV and the pathogen virulence. The findings also get support from those of Sporleder et al. (2005), who reported that the LC_{50} value of *Phthorimaea operculella* granulovirus was 5×10^{6} granules ml^{-1} by the egg dip bioassay method; and of Forté et al. (1999), who observed that the LD_{50} value of granulovirus against 4th instars of *Choristoneura fumiferana* was 5.7×10^{5} granules $larva^{-1}$.

The present studies revealed that neem seed kernel extract enhanced the efficacy of PbGV, followed by *E. adenophorum* and *A. brevifolia,* against all the evaluated instars of *P. brassicae*. Enhancement of the virulence of a pathogen, i.e., PbGV, or disease occurrence in the host larvae suggests that latent infections or ingested viruses are sometimes activated by stress, both biotic and abiotic, resulting in the outbreak of the disease in the host insect populations (Aruga 1963). The role of stressors in activating latent viral infection has also been reported earlier. Stress as a factor in insect disease was reported by Steinhaus 50 years ago (1958). Muralibaskaran et al. (1999) observed that suspending nuclear polyhedrosis virus in different concentrations (0.10–1.00%) of neem oil and neem seed kernel extract reduced the LC_{50} value of *Spodoptera litura* nuclear polyhedrosis virus (NPV) by 1.05- to 1.43-fold and 1.03- to 1.33-fold, respectively. Reduction in the LC_{50} and LT_{50} of the HaNPV when combining neem products with different formulations of *Heliothis* nuclear polyhedrosis virus has been reported earlier (C. Muthiah, unpublished MSc thesis, 1988; R. J. Rabindra, T. Manoharan, & S. Jayaraj, unpublished paper, 1994; Kumar et al. 2008). Lingappa et al. (2000) also reported that Econeem (a neem-based formulation) in combination with HaNPV reduced damage by 70.36% to 71.99% and led to maximum cotton yield. Murugan et al. (1999), however, observed that combining neem products with *S. litura* NPV resulted in threefold enhancement of percent mortality even at reduced concentrations. In the present study with different botanicals, neem seed kernel extracts with different solvents were found to be superior to other botanicals, viz., *E. adenophorum* and *A. brevifolia*, because neem inflicts multiple stresses at the same time on the insect, signifying a greater reduction in LC_{50} values of PbGV. The results of the experiment suggest that the application of GV in combination with botanical insecticides will provide better management of the pest than GV alone. The findings may have implications in field usage of these baculoviruses as the botanical additives may result in better management of the pest even at lower virus inocula under field conditions countering the effects of UV irradiations.

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