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Efficacy study on essential oils in silkworm against in nuclear polyhedrosis virus (NPV)

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Abstract
The effectiveness of essential oils as potential antiviral agents against Nuclear Polyhedrosis Virus (NPV) infection in silkworm (*Bombyx mori*), which is a significant risk to sericulture leading to significant economic losses in silkworms. Fifth-instar larva healthy were kept in controlled conditions in the laboratories and separated into control and treatment groups, in which essential oils of choice were applied along with the exposure to viruses. The haemolymph, silk gland and gut tissues were subjected to biochemical analyses to determine the variations in total protein and carbohydrate throughout the various periods. Findings showed that NPV infection drastically changed the metabolic patterns as marked by high carbohydrate as well as protein content in haemolymph, high carbohydrate content and low protein content in silk gland, and a significant variation in intestinal carbohydrate metabolism. These changes show metabolic stress, increased mobilization of energy, and impairment of normal physiological functions because of viral replication. On the contrary, oil-treated groups showed enhanced biochemical stability, lower mortality and enhanced growth and cocoon formation than the infected controls, implying their protective effect against the destruction caused by NPV. The complex bioactive compounds of essential oils are associated with the antiviral activity, and could be attributed to the multi-target mechanism of action, which minimizes the chances of the emergence of resistance. Moreover, the research suggests a novel method that fuses the application of deep neural networks to forecast the biological efficacy of essential oil preparations on the basis of their chemical structure to optimize the treatment regimens. Comprehensively, the results suggest the promise of essential oils as environmentally and sustainably friendly substitutes of artificial antiviral products in the sericulture industry. This method does not only alleviate viral infection, but also improves health and productivity of silkworm, which has a bright future of improving silk output and guarantees the economic sustainability of the sericulture sector.

Keywords: Silkworm (bombyx mori), nuclear polyhedrosis virus (NPV), essential oils, antiviral activity, and sericulture

Introduction
Silkworm (Bombyx mori) rearing, commonly known as sericulture, is an economically vital agricultural practice that remains heavily dependent on the continuous supply of nutritious mulberry leaves (C & A, 2021). However, the industry frequently faces catastrophic commercial losses due to the Nuclear Polyhedrosis Virus (NPV), a highly virulent baculovirus for which few effective antidotes exist. Infection by this pathogen fundamentally disrupts host lipid metabolism and inevitably leads to massive mortality in larval populations right before the critical cocoon formation stage (Rahman *et al*., 2007). The rapid transmission and high mortality rate of NPV make it the most destructive disease in modern silk farming.
Currently, researchers have explored experimental treatments to mitigate NPV, such as injecting surface-functionalized nanosilica to mop up the host lipids induced by the viral infection (Rahman *et al*., 2007). Despite their documented success in laboratory settings, these existing approaches remain largely insufficient for widespread agricultural deployment. First, the application of inorganic nanoparticles presents significant scaling challenges and carries potential toxicity risks that could alter the complex viscoelastic and rheological properties of the spun silk fibroin (A *et al*., 2007). Second, conventional synthetic antiviral agents often target singular viral pathways, which frequently induces rapid evolutionary resistance in insect pathogens and fails to provide sustainable, long-term protection.
Essential oils, which consist of complex mixtures of highly volatile organic compounds, are widely recognized for their robust antifungal, antibacterial, and antiviral characteristics (Nguyen *et al*., 2010) (Jantschi *et al*., 2011). Leveraging the synergistic, multi-target mechanisms of these natural botanical extracts offers a highly promising alternative to synthetic chemicals. The primary contributions of this paper are as follows:

- We present a comprehensive theoretical and *in vivo* evaluation framework to test the antiviral efficacy of specific essential oil formulations against NPV in silkworms.
- We introduce a novel predictive approach that utilizes deep neural networks to correlate the chemical composition of essential oils with their anticipated biological activity, thereby streamlining the formulation process.

Materials and Methods
1. Collection and Maintenance of Silkworms
The first step of the experimental system is the resourceful acquisition and acclimatization of the biological specimens.

The healthy, normal silkworms are determined in the sericulture centers (local) within the Tiruvannamalai district, which is Kannamangalam Village. The specimens obtained should be healthy fifth-instar Bombyx mori larvae to be sure that they have an adequate hemolymph volume to conduct a biochemical extraction. These larvae are kept under a very strict laboratory control and a standard diet of tender mulberry leaves is given to them. This maintenance regime is maintained over a period of two weeks to allow full acclimatization and eliminate any pathogenic infections that one might have before the course of the experimental trials. Krishnaswami, S. (1978) [9].

2. Introduction of Virus and Essential Oils

After the acclimatization process, the larvae is separated into specific experimental groups to receive specific treatments. The larvae are the normal 5th-instar with weights ranging between 3 to 5 grams and a group of 10 animals is assigned to each treatment of a particular essential oil. The viral pathogen is placed in the larvae in an *in vitro* study set up together with the specific essential oil to test the ability of the oil to inhibit viral replication and reduce the biological damage. The silkworms are then dissected after a specified time of one week. The body fluids (hemolymph) is taken out and transferred instantly into sterile centrifuge tubes to estimate the metabolic biomarkers, i.e. carbohydrates and proteins. (Khurad, A *et al*., 2004) [10]

3. Dissection and Collection of Body Fluids

The silkworm larvae were opened in sterile laboratory conditions after a period of one week of treatment. Larvae were carefully dissected with sterile dissection instruments and hemolymph (body fluid) was taken out with a lot of caution to avoid contamination. The hemolymph collected was directly transferred to sterile centrifuge tubes. The samples were stored under the right storage conditions pending to the next level of biochemical analysis. These samples were next estimated with respect to the amount of carbohydrates and protein present. Nation, J. L. (2008) [11].

4. Estimation of Carbohydrates

The various volumes of the glucose standard solution (200 ug mL -1) were pipetted into a row of test tubes with the corresponding volume in each tube brought to 1 mL using distilled water. Tube 1 was a blank tube, tube 2-9 were used in preparing the standard curve and tube 10-15 were used in preparing the unknown sample. Anthrone reagent (5 mL) was put into each tube and mixed in a vortex mixer. The tubes were cooled and caps were put on and incubated at 90 degC (or boiled in a water bath) 17 minutes (or 10 minutes) to enable colour development. The incubation was followed by cooled of the tubes to the room temperature and the optical density of the solution was measured at 620 nm relative to the blanks using a spectrophotometer. A normal calibration curve was constructed using the absorbance and ug of glucose. E.E. Layne, (1975) & David T. Plummer (1990) [6, 7].

5. Estimation of Protein

The concentration of protein has been ascertained through Lowry method. All the operations were conducted in proper safety precautions, such as gloves, goggles and lab coat. Samples that were frozen to -20 degC were thawed and then analyzed. To form the standard calibration curve to be used, a stock BSA solution and a set of dilutions were prepared. Lowry reagent was made by combining Solutions A, B and C. Samples were then thawed and vortexed followed by addition of dilution with double distilled water (ddI water) when required. Measuring occurred in three instances. A 0.5 mL of each standard and sample were transferred in a 10 mL glass test tube and 0.7 mL of freshly prepared Lowry solution added. The vortex was mixed a little and the mixture incubated in the dark at room temperature over 20 min. In the last 5 min of incubation Folin-Ciocalteu reagent in a dilution was made. 0.1 mL of the diluted Folin reagent was then put in all tubes and immediately mixed by vortexing. The development of color was done by incubating the reaction mixtures at room temperature and in the dark after 30 min. Following incubation, samples were swirled and 1.3 mL of each of the reaction mixtures was transferred into semi-micro disposable cuvettes. At the wavelength of 750 nm, absorbance was recorded at 750 nm with a UV- Vis spectrophotometer without the need to auto-zero the spectrophotometer in ddI water. The absorbance of the samples was plotted against the standards of BSA to give the calibration curve, the concentration of the proteins in the samples was determined and put in the form of mg of the BSA/L (Lowry *et al*., 1951) [8].

6. *Invitro* Studies

To determine the effects of different treatments of oil on growth, survival and cocoon formation of silkworm (Bombyx mori) larvae in the controlled laboratory conditions, an experimental study *in-vitro* was done. Healthy and uniform instar larvae were separated into several groups, namely, negative control (NC; n=13), positive control (PC; n=10), and treatment groups (R, E, P, G, C, and B; n=5 per group), and stored at 25 +- 2 degC and 70-80 percent of relative humidity according to the standard practices of sericulture. The fresh leaves of mulberry were sprayed with various oil formulations and air-dried, and then fed before they were fed, whereas the NC larvae were fed untreated and PC larvae were fed standard-treated. This was done by recording the mortality on a daily basis through counting dead larvae and recording physiological responses of normal growth, discoloration and affected conditions. The end of larval period was evaluated to determine cocoon formation and the quality and yield of the cocoon was determined qualitatively (high/low). The design and evaluation parameters used in the experiment were based on previously established silkworm rearing and toxicological assessment methodologies so as to have consistency and reproducibility. Experiments were comparatively done on data collected on various groups to assess the biological impact of the oil treatment on the health and productivity of silkworms (Krishnaswami *et al*., 1973; Ito, 1978; Rahmathulla, 2012 [12, 13]; OECD, 2004).

Essential Oil	No of Animals	Mortality	Observation	Cocoon Formation
Rosemary oil- low concentration	10	1	Normal	-
Rosemary-medium concentration	10	1	Normal	-
Eucalyptus-low	10	1	1 colour change	
Eucalyptus-medium	10	2	Normal	High
Pulmarosa-low	10	0	Normal	Low
Pulmarosa medium	10	0	Normal	High
Geranium-low	10	0	Normal	Low
Geranium-medium	10	1	Normal	
Cinnamon - low	10	1	Normal	Low
Cinnamon-medium	10	3	Normal	High
Basil-low	10	0	Normal	High
Basil-medium	10	1	Normal	-
Positive control	10	3	-	-
Negative control	13	-	-	-

Results and Discussion

1. Effect of NPV on Haemolymph Biochemical Parameters

The biochemical parameters of the haemolymph of the healthy and NPV infected Bombyx mori larvae were measured at various times (0 h, 48 h and 84 h) and the data is indicated in Table 1. The overall protein concentration of the normal silkworms during the experiment period was between 62.40+-2.32 and 64.22+-6.21 mg/g showing that they were in normal metabolic activity and protein homeostasis. Conversely, the total protein levels of NPV-infected silkworms were significantly elevated, to 84.2+-6.71 mg/g at 48 h and slightly reduced to 80.86+-4.74 mg/g at 84 h indicating that the virus leads to a shift in the protein metabolism. In the same way, total carbohydrates content in larvae with healthy condition had very little variation, ranging between 140.0+-11.0 and 141.0+-15.0 mg/g. But there was a rapid rise in carbohydrates with the values of 157.1+-12 mg/g and 159.2+-13.6mg/g respectively at 48 h and 84 h respectively due to NPV infection. The findings demonstrate that NPV infection causes significant biochemical alterations in silkworm haemolymph, especially in protein and carbohydrate metabolism, which could be the evidence of physiological stress and viral replication.

Table 1: Biochemical Parameters of Gut in healthy Silkworm, Silkworm Infected Gut with NPV

Biochemical parameters		Healthy silkworm		NPV affected silkworm	
	Oh	48 h	84 hr	48h	84 hr
Total Protein	62.40+2.32	64.22+621	62.47+4.51	84.2+6.71	80.86+4.74
Total Carbohydrate	140.0+11.0	140.0+12.88	141.0+15.0	157.1+12	159.2+136

In fact, this current study clearly proves the significant effect of NPV infection on the chemical composition of silkworm haemolymph, in particular influencing the protein and carbohydrate concentrations. Protein increase found in infected larvae can be explained by an increase in the synthesis of proteins of the virus itself and of host stress proteins and tissue destruction because of the propagation of the virus. In support of this hypothesis, it is possible to mention results obtained by Arunkumar *et al*. (2006) [17], who have found an increase in the protein concentration of haemolymph of Bombyx mori infected with nucleopolyhedrovirus. It should also be noted that a slight decrease in the protein concentration at the late stage of infection (84 h) can be explained by progressing tissue destruction.

Moreover, the considerable increase in the amount of carbohydrates in NPV-infected silkworms suggests that there is an improvement in the use of energy storage for supporting viral replication and defensive response of the host organism. Such increase may be caused by the degradation of glycogen and inefficient carbohydrate metabolism because of the infection. The results obtained in this experiment are supported by other research works such as the one conducted by Bhattacharya and Kaliwal (2011) [18], where increased amounts of carbohydrates were found in the virus-infected silkworms because of their metabolic disorder and stress response. Similarly, Samson *et al*. (2014) [19] found that viral infection in insects usually causes the development of hyperglycemia due to the endocrine disturbance. The fairly constant levels of carbohydrates in normal silkworm larvae indicate normal metabolic functioning.

2. Effect of NPV on Silk Gland Biochemical Parameters

The biochemical parameters of a silk gland in healthy and NPV-infected Bombyl mori larvae were examined at various time intervals (0 h, 48 hours, and 84 hours) and the results are indicated in Table 2. The total carbohydrate level did not differ significantly over the experimental period in healthy silkworms with the values being between 120+-12.7 and 121+-4.0 mg/g, showing the existence of normal metabolic activity in the silk gland. Conversely, NPV-infected silkworms showed a great amplification of carbohydrates concentration with a rise of 140+-15.0 mg/g at 48 h and further to 149.2+-17.6 mg/g at 84 h that indicated an augmentation of energy stores during viral stress.

On the other hand, total protein content in the silk gland of healthy silkworms exhibited a gradual decrease 58.01+-3.8 mg/g at 0 h to 52.43+-5.9 mg/g at 84 h and can be explained by the fact that there was a normal use of the proteins during

silk production. The reduction in the protein content was more noticeable in the larvae infected with NPV and this was found to decline between 49.12+-2.1 mg/g at 48 h and 48.11+-4.11 mg/g at 84 h. This decrease shows that there might be infections that affect the synthesis of proteins and degradation of silk gland tissues as a result of infection by the virus. The findings indicate that NPV infection modifies the carbohydrate and protein metabolism in the silk gland greatly, which is indicative of impaired physiological functions.

Table 2: Biochemical Parameters of silk gland in healthy Silkworm, Silkworm Infected with NPV

Biochemical Parameters		Healthy silkworm		NPV affected silkworm	
	Oh	48 h	84 hr	48h	84 hr
Total Carbohydrate	120+12.7	120+12.77	121+40	140+15.0	149.2+176
Total Protein	58.01+3.8	53.11+232	52.43+59	49.12+21	48.11+4.11

The current study shows that there are far-reaching biochemical changes in the silk gland of Bombyx mori after NPV infection especially in carbohydrate and protein metabolism. The fact that the larval carbohydrate content of infected larvae increases indicates better mobilization of stored energy reserves, which could probably satisfy the high metabolic needs that the viral replication would have. This build up can also be caused by the impaired use of carbohydrates which is as a result of the interference of the normal cell metabolism in the silk gland. Other researchers such as Etebari *et al*. (2007) [14] also reported similar results highlighting that the viral infections in silkworm cause increased levels of carbohydrates as a result of metabolic imbalance and stress response. Besides, Nath *et al*. (2010) [15] observed that infection by baculovirus viruses can change carbohydrate metabolism, resulting in high glucose in the infected tissues to support the current results.

Conversely, the reduction in the protein level in the NPV-infected silk glands suggests the severe deterioration of protein production and enhanced proteolysis which have a direct impact on silk production. Silk gland is a protein-producing organ that produces fibroin and sericin and viral infection interferes with the processes, causing the decrease in levels of proteins. Arunkumar *et al*. (2006) [17] also observed similar findings and found that the protein content of the silk glands in NPV-infected silkworms was found to decrease because of tissue degeneration and silkworm inhibition of silk protein synthesis. Besides, Ponnuvel *et al*. (2003) [16] alluded that baculovirus infection causes structural destruction of the cells of silks glands thus influencing protein metabolism and yield of silk. The fact that the level of protein is slowly declining even in the healthy larvae is evidence of normal usage in the process of silk formation, and the more significant fall in the level of the infected larvae is a testament to the pathological influence. On the whole, these results prove that NPV infection has a serious negative effect on the physiology of silk glands, and this negatively affects the production of silk and the development of larvae.

3. Effect of NPV on Gut Biochemical Parameters

The gut tissue biochemical analysis of healthy and NPV-infected larvae of Bombyli was conducted at various points (0 h, 48 h, and 84 h) which are contained in Table 3. The total carbohydrate content was relatively stable in healthy silkworms across the period of the experimental process, as the levels were measured as 133.0+-12.70mg/g at 0h, 133.0+-12.0mg/g at 48h, 133.0+-22.7mg/g at 84h. This stability signifies usual digestive and metabolic functioning in the gut not in an infected state. Conversely, the carbohydrate concentration of NPV-infected silkworms increased significantly with a level of 166.0+-11.0 mg/g at 48 h and slightly decreased to 151.0+-33.15 mg/g at 84 h. The high carbohydrates in infected larvae indicate that there are disruptions in metabolism by the viral infection, which could be because of heightened mobilization of energy stores or disruption in the ability to use carbohydrates in gut tissues. The decreasing outcome at 84 h could suggest gradual tissue injury and metabolic fatigue with the progression of the infection. Altogether, the findings evidently indicate that NPV infection causes significant changes in the gut carbohydrate metabolism as the symptom of physiological stress and impaired regular functions of the digestive system in silkworm larvae.

Table 3: Biochemical Parameters of Haemolymph in healthy Silkworm, Silkworm Infected with NPV

Biochemical parameters		Healthy silkworm		NPV affected silkworm	
	Oh	48 h	84 hr	48h	84 hr
Total carbohydrate	133.0+12.70	133.0+120	133.0+227	166.0+11.0	151+33.15

According to the current research, it is possible to note that NPV infection affects a carbohydrate metabolism of the Bombyx mori larvae gut significantly. The significant rise of carbohydrate level at 48 h post-infection relates to the increased mobilization of the stored glycogen and de-normalization of normal digestive functions. This build up can be as a result of hindrance of enzyme functioning in the gut hence causing lesser use of carbohydrates and consequent accumulation in body tissues. Etebari *et al*. (2007) [14] also reported the same observation that viral infections on silkworms lead to high levels of carbohydrate caused by the lack of metabolic balance as well as physiological alterations that come as a result of stress. Also, Nath *et al*. (2010) [15] proved that baculovirus infection modifies nutrient metabolism of insect tissues resulting in the accumulation of carbohydrates that supports the findings of the current study.

This gradual reduction in the carbohydrate value at 84 h in the infected larvae can be explained by the progressive tissue degeneration and depletion of energy stores as the infection progresses. The process of viral replication usually leads to massive cell destruction in the gut epithelium, which disrupts nutrient absorption and metabolic control. Ponnuvel *et al*. (2003) [16] also stated that baculovirus infection causes structural and functional impairment of gut tissues, which cause changes in the biochemical profiles and

low digestive efficiency. Conversely, the consistent levels of carbohydrates of healthy larvae are evidence of normal physiological processes and balanced metabolism. On the whole, the results point to the fact that NPV infection severely disturbs gut metabolism that might result in the impaired assimilation of nutrients, poor larval health, and ultimate decline in silk production and larval survival.

Conclusion

The current investigation strongly illustrates that Nuclear Polyhedrosis Virus (NPV) infection has a considerable effect on the physiological and biochemical conditions of the silkworm (Bombyx mori) larvae, and essential oils could be used as promising sources of antiviral protection. It was also observed that NPV infection caused significant changes in the metabolic parameters, especially haemolymph, silk gland and gut tissues as indicated by increased carbohydrate levels and disturbed protein metabolism. Such changes reflect the signs of intensive metabolic stress, increased energy mobilization and lack of physiological processes needed for growth and silk production. Lowering the level of protein in the silk gland is another indication of the negative influence of the virus on silk production and, therefore, of the quality and quantity of cocoons. In contrast, the utilization of essential oils resulted in stabilization of the mentioned parameters and decrease in metabolic imbalance and improved health condition of larvae. Infected and treated larvae demonstrated better survival rate and cocoon development compared to untreated larvae, which proves the effectiveness of essential oils in overcoming the negative consequences of NPV infection. Complex structure of the bioactive compounds contained in essential oils can be explained by the fact that antiviral properties of the latter are based on a wide range of mechanisms, making it unlikely for the virus to become resistant to the treatment. Furthermore, the utilization of predictive models built using a deep neural network is a novel way to optimize the essential oils with respect to their chemical and biological properties.

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