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The Antiviral Effect of the Propolis from Turkey on the Replication of Autographa californica nuclear polyhedrosis Virus (AcNPV) Developed in Spodoptera frugiperda Cell Culture

Spodoptera frugiperda Hücre Kültüründe Geliştirilen Autographa californica nükleer polihedrosis Virüsünün (AcNPV) Replikasyonu Üzerine Türkiye Propolisinin Antiviral Etkisi

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Abstract

In this study, the influence of propolis extracts gathered from Turkey on the replication of *Autographa californica nuclear polyhedrosis* virus (AcNPV) developed in *Spodoptera frugiperda* culture was researched through examining changes in cytopathic influences, hydroxyl radical mediated DNA damage and progeny virus concentration. By making use of propolis extract, we found inhibiting effect on cytopathic changes and DNA damage. It was found that propolis extracts on the cytopathic changes and hydroxyl radical-mediated DNA damage were similar to the control. The results showed that propolis has a protective effect on DNA exposed with H_2O_2. In terms of the production of occlusion bodies in Sf9 cells, no important difference was found between the average percentage of contaminated cells in contaminated control cells when the concentration of propolis is 3.125, and 1.5625 µg/mL (4.018 (Plaque forming units (PFU) pfu/mL and 5.145 pfu/mL respectively) ($p<0.05$) and no significant difference was found between the average number of occlusion bodies in the contaminated control cells and the contaminated cells in the concentration of propolis. Respectively 98.70% and 98.67% reduction were found in virus titter with propolis extracts when the concentration of propolis extracts 100, 50 µg/mL were included 1 h p.i. ($p<0.05$). However, different concentrations of propolis extract reduced the number of progeny viruses significantly.

Keywords: *Baculovirus*, Propolis, Antiviral activity, Virus replication

Özet

Bu çalışmada, *Spodoptera frugiperda* kültüründe geliştirilen *Autographa californica* nükleer polihedrozis virüsünün (AcNPV) replikasyonu üzerine Türkiye'den elde edilen propolis ekstraktlarının etkisi, sitopatik etkiler, hidroksil radikal aracılı DNA hasarı ve progeni virüs konsantrasyonundaki değişiklikler incelenerek araştırılmıştır. Propolis özü kullanarak sitopatik değişiklikler ve DNA hasarı üzerinde engelleyici etki bulduk. Propolis ekstraktlarının sitopatik değişiklikler ve hidroksil radikal aracılı DNA hasarı üzerinde kontrole benzer olduğu bulundu. Sf9 hücrelerinde emilim bölgesi üretimi açısından, propolis konsantrasyonu 3.125 ve 1.5625 µg/mL olduğunda kontamine kontrol hücrelerinde kontamine hücrelerin ortalama yüzdesi arasında önemli bir fark bulunmadı (sırasıyla 4.018 (plak oluşturan birim) (PFU) pfu/mL ve 5.145 pfu/mL) ($p<0.05$) ve kontamine kontrol hücrelerindeki ortalama emilim bölgesi sayısı ile propolis konsantrasyonundaki kontamine hücreler arasında anlamlı bir fark bulunmadı. 100, 50 µg/mL propolis ekstraktlarının konsantrasyonu 1 h pi dahil edildiğinde propolis ekstraktları ile virüs titresinde sırasıyla %98.70 ve %98.67 azalma bulundu. ($p<0.05$). Bununla birlikte, farklı propolis özütü konsantrasyonları, virüslerin sayısını önemli ölçüde azaltmıştır.

Anahtar Kelimeler: *Baculovirus*, Propolis, Antiviral aktivite, Virüs replikasyonu

1. INTRODUCTION

It is acknowledged that baculoviruses are a body of enveloped double-stranded DNA viruses affecting arthropods, especially insects belonging to the order Lepidoptera (Bilisard et al., 2000). The *Autographa californica* nuclear polyhedrosis virus (AcNPV) is an insect virus belonging to Baculoviridae family. In some Baculoviruses, virus enhancing factors (VEFs) in OBs improve infectivity (Bischoff & Slavicek, 1996; Wang et al.,1994). Following ODV envelope's direct blending to midgut columnar epithelial cells' plasma membrane (Granados and Lawler, 1981; Horton & Buand, 1993), the virions become uncoated and transferred to the nucleus and progeny nucleocapsids are formed in the nucleus. Later, progeny nucleocapsids move to the plasma membrane from the nucleus and grow into the hemocoel by means of basal sides of epithelial cells. In the hemocoel, before causing the death of the host, BVs first affect the tracheal epithelium and hemocytes and later affect the fat body, muscle, Malpighian tubules, and other tissues (Engehard et al., 1994). Due to its pharmaceutical characteristics, propolis (bee glue), a resinous beehive product, has been used since classical times (Matsuda, 1994). Bees use propolis to secure the walls and entrance of the hive and dead invaders. The best known and the most researched biological activities of propolis are antiviral, antifungal, anti-inflammatory, antibacterial, wound healing and antitumor activities (Marcucci, 1995). It is a natural remedy that has been in use for centuries (Castaldo & Capasso, 2002) and is widely applied in traditional medicine thanks to its pharmacological benefits of anticancer, antioxidant

(Kumazawa et al., 2004), antiviral, anti-inflammatory and antimicrobial properties (Banskota et al., 2002). Propolis is useful in foods. Propolis is as a natural preservative and a source of bioactive compounds for foods and drinks that help improve shelf-life and consumer health (Duman & Ozpo, 2015). The present study researched the influence of propolis extracts gathered from Turkey on the replication of *Autographa californica* nuclear polyhedrosis virus (AcNPV) in *Spodoptera frugiperda* cell culture through examining changes in cytopathic influences, hydroxyl radical mediated DNA damage and virus concentration (Bischoff & Slavicek, 1996; Wang et al.,1994).

2. MATERIALS and METHODS

2.1. Propolis Extract Preparation

Propolis extracts prepared such as represented by Boeru and Derevici (1978). Propolis sample was collected from colonies of honeybees located in the north-east Black Sea Region in Turkey. Hand gathered propolis was maintained in a dry place and reserved at -20°C until its used process. 200 grams of propolis were frozen to -20 °C, slaughter in small pieces, and ground in a frozen mortar then 20% ethanol extract propolis was prepared by supplementation 200 gram of propolis to 800 mL of 70% ethanol (1: 4 w/v) in a shaker at room temperature for 36 h. The ethanolic extract solution (EEP) was then filtered through 0.45µm membrane filter and then the solvent was evaporated. The crude extracts were stored at -20°C until used. (Ertürk et al., 2016).

2.2. DNA Cleavage Assay

The test was applied according to the method previously described by Asmafiliz et al. (2013) and Ayvaz et al. (2018). Plasmid DNA pUC18 was used to examine the useful effect of the propolis extracts on hydroxyl radical-mediated DNA damage (Asmafiliz et al., 2013; Ayvaz et al., 2018).

2.3. Cell Culture and Virus

For virus infection, *S. frugiperda* cell line (Sf900) was employed as the host cell. Cells were prepared in Gibco Sf-900 medium complemented with 5% fetal bovine serum (FBS), 100 µg/mL streptomycin in 25 cm^2 culture flasks at 28 °C (Petcharawan et al., 2012). Through inoculating in Sf9 cells and incubation at 28 °C, AcMNPV stock was prepared. A week post infection, the virus suspension was transported and stock virus titer was investigated as TCID50/mL (tissue culture infectious dose per mL) and converted to pfu/mL (plaque forming unit per mL) (Petcharawan et al., 2012; Reed & Muench, 1938).

2.4. Cytotoxicity Assay

By using Sf9 cell line by MTT assay, different Propolis extract concentrations were analysed in terms of in vitro cytotoxicity (Mosmann, 1983). Crude propolis extracts were first dissolved in 1 mL of dimethyl sulfoxide (DMSO) and in order to get a stock solution of 6 mg/mL concentration, 10 mL volume was attained with protected medium, later diluted to different concentrations (200, 100, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL) in DMSO. On a microtiter plate reader, absorbance of the solution in each well in the plates was estimated at 570 nm.

2.5. Antiviral Assay of Propolis Extract at Different Concentration

Into each well of 24-well culture plates, $2.5x10^4$ cells in 5 % fetal bovine serum (FBS) added 0.5 mL Gibco Sf-900 medium were seeded and cultured for 4 h at 28 °C. To each well of 24-well plates, 6 mg/mL dissolved crude extract (diluted in unsupplemented Gibco Sf-900 medium, at concentrations of 200, 100, 25, 12.5, 6.25) was added and incubated for 1.5 h at room temperature for adsorption. Later, adsorption, the virus, and extract mixture were removed, phosphate buffer saline (PBS) was used to wash the cells and complete TNMFH medium containing 5 % FBS and 100 units/mL gentamicin, 100 µg/mL streptomycin medium solution was added to 24-well plates. 24 well culture plates were incubated at 28 °C for 4 days. When the period of incubation ended, the cells were first scraped and counted, and later in order to separate the the supernatant and the pellet, they were centrifuged at 4000 rpm for 20 min (Sökmen, 2001).

2.6. Statistical Analysis

For the estimation of cytotoxicity curves and CC50, GraphPad Prism 5.0 program was used. SPSS statistics 17.0 software was used to evaluate the data (Motulsky, 2007).

3. RESULTS and DISCUSSION

3.1. DNA Cleavage Assay

In this study, the DNA protection activity of propolis sample was investigated by using DNA interaction assay. Gel electrophoresis can be used to analyze DNA damage resulting from conformational change when H_2O_2 interact with pUC18 plasmid DNA. When the, the due to the can be investigated via agarose gel. Propolis extracts were dissolved in THF and pUC18 plasmid DNA was treated with different concentrations extracts, respectively. According to analysed gel electrophoresis results; Lane 1 and lane 2 were managed with untreated pUC18 plasmid DNA as a control, while lanes 3-6 pointed out plasmid DNA contacted with increasing

concentrations of the extracts in H_2O_2 condition (Figure 1). The results showed that propolis has a protective effect on DNA exposed with H_2O_2.

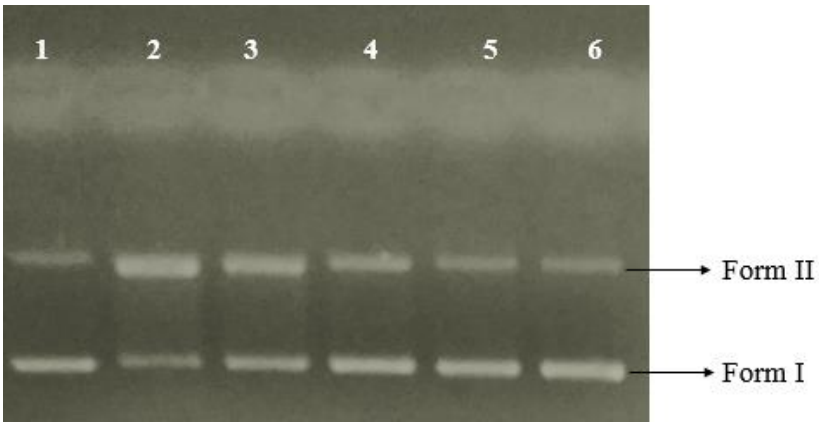

Figure 1. Gel electrophoretograms, Lane 1: control DNA; Lane 2: H_2O_2 and plasmid DNA; Lane 3-6: H_2O_2 + plasmid DNA and 12.5, 25, 50, 100 mg/mL extract

Plasmid DNA has three different forms as a supercoiled DNA form I, open circular form II and linear form III. These forms can be shown as the different lines on gel electrophoresis. Among these forms, the supercoiled form has faster mobility than other forms. In case of damage to one strand (nicking), as a result of relaxing to the open circular, the supercoil moves slower. In case of damage to both strands, linear form III appears between form I and form II (Akbaş et al., 2013; Asmafiliz et al., 2015).

3.2. Cytotoxicity Assay

Table 1 shows cytotoxicity assessment results of this concentration of propolis extracts against the Sf9 cell line. Based on the 60-hour exposure results with the prepared concentrations of propolis, at the concentrations tested (1.56 g / mL, 3.12 µg / mL, 6.25 µg / mL, 12.50 µg / mL, 25 µg / mL, 50 µg / mL, 100 µg / mL and 200 µg / mL), the decreases in cell viability were statistically significant compared to control ($p < 0.05$) (Table 1). It was also seen that the applied concentrations decreased cell viability depending on the dose (Figure 2). The concentrations of propolis have been shown to increase cytotoxicity due to dose increase (Figure 3) (Custódio, 2011; Gad 1999). Increases in cytotoxicity of all concentrations tested were statistically significant compared to control ($p<0.05$) (Table 1).

Table 1. Cell viability and cytotoxicity values calculated from data obtained as a result of 60-hour exposure of propolis concentrations

Dose	**%Cell viability**	**Cytotoxicity**
Control	100	0.00
1.56 µg/mL	0.14	99.86
3.12 µg/mL	-15.21	115.21
6.25 µg/mL	-22.72	122.72
12.5 µg/mL	-34.92	134.92
25 µg/mL	-43.81	143.81
50 µg/mL	-48.11	148.11
100 µg/mL	-48.31	148.31
200 µg/mL	-47.17	147.17

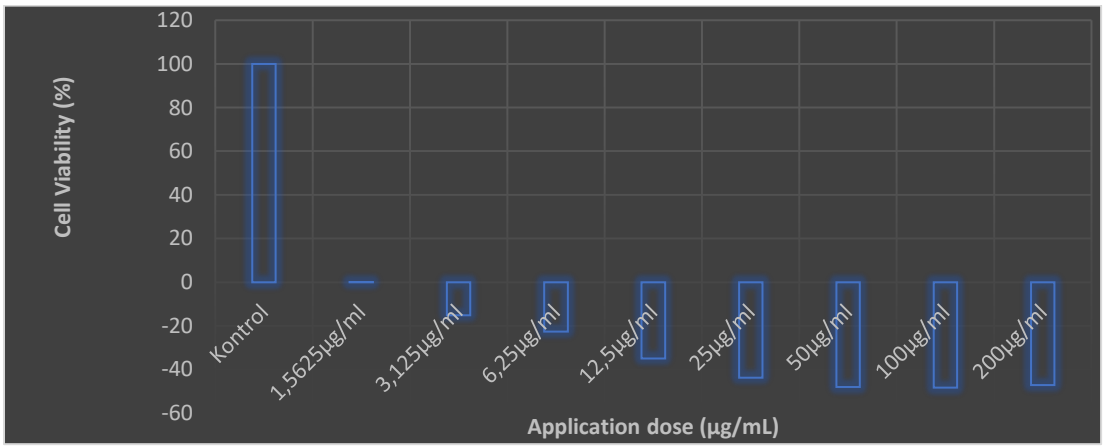

Figure 2. The effect of propolis on cell viability of cells after 60 hours of exposure Sf Cell (*: p<0.05)

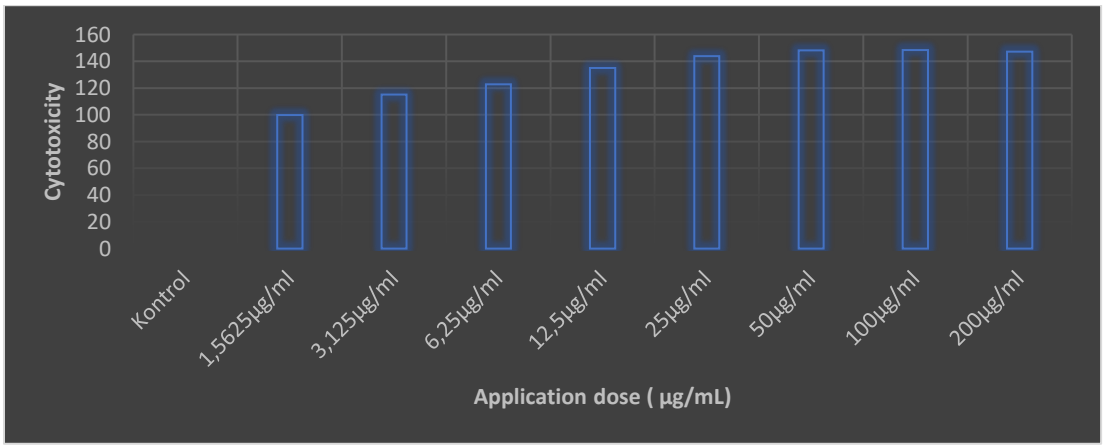

Figure 3. Effect of propolis on cytotoxicity of cells after 60 hours of exposure Sf cells (*: p<0.05)

3.3. Antiviral Activity

In the present study, according to antiviral activity result of ethanol extracts concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL against AcMNPV were shown in Table 2. In order to find out the antiviral activity of propolis extract on the replication of AcMNPV, all propolis extract concentrations were used. 1 h after contamination with AcMNPV, extracts were included at a multiplicity of infection of 1 and incubated at 28 °C for 60-72 h. Viruses were counted, especially when (OBs) were seen in the control group (Figure 4).

The IC_{50} value determined by logarithmic regression after 60 hours of propolis exposure was determined as 1.26 µg / mL (Table 2).

Table 2. The IC_{50} value obtained by treatment of cells with propolis.

Bee propolis	**Concentration** **60 h**
IC_{50}	1.26 µg/mL

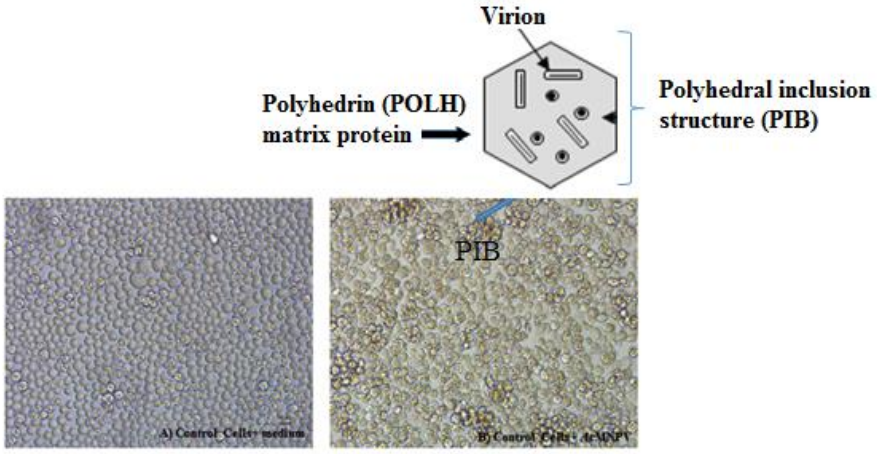

Figure 4. Phase-contrast micrographs of Sf9 cells (A), Sf9 cells after 60 h exposure in Gibco Sf-900 medium (B) Sf9 cells after 60 h post infection with AcMNPV Note, blue arrow shows polyhedral inclusion structure (PIB)

This process takes about 60 hours. Total number of occlusion bodies (OBs)/mL, the virus titter and the corrected percent reduction of virus titter were calculated. The results showed extremely important variation in the average virus titter between contaminated control cells (12.481) and contaminated cells in propolis extract of different concentration solutions (Figure 5).

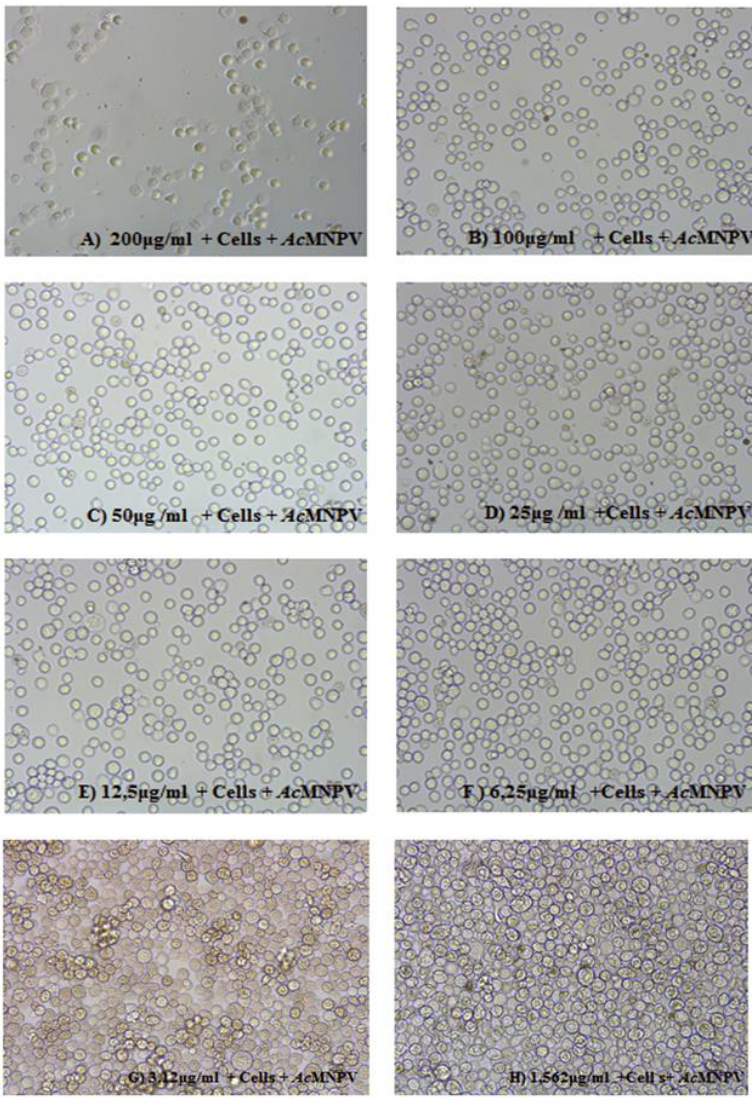

Figure 5. Phase-contrast micrographs of Sf9cells, Sf9 cells + various concentrations of propolis extract, A, B, C, D, E, F, H, G

When 1 h p.i was included in each concentration of propolis extract, virus concentration was found 1.586 pfu/mL for 100 µg/mL concentration of propolis extract and 1.601 pfu/mL 1.917 pfu/mL 2.570 pfu/mL 3.466 pfu/mL, for different concentration of propolis extract respectively. In terms of the production of occlusion bodies in Sf9 cells, no significant difference was found between the average percentage of contaminated cells in contaminated control cells when the concentration of propolis extract is 3.125, and 1.5625 µg/mL (4.018 pfu/mL and 5.145 pfu/mL respectively) ($p<0.05$) and no significant difference was found between the average number of occlusion bodies in the contaminated control cells and the contaminated cells in the concentration of propolis extract. Respectively 98.70% and 98.67% reduction were found in virus titter with propolis extracts when the concentration of propolis extracts 100, 50 µg/mL were included 1 h p.i. ($p<0.05$) (Table 3, Figure 6).

Table 3. Effects of propolis extract prepared in different concentrations on the offspring virus AcMNPV concentration

Cell + Different concentration of propolis extract Treatments	**Mean virus titer (PFU/mL)∗± S.D.**
Control Cells+ AcMNPV	$4.3x10^{9}$a ±5.42
200µg/mL propolis + Cells + AcMNPV	0
100µg/mL propolis + Cells + AcMNPV	$5.6x10^{7}$c±4.76
50µg /mL propolis + Cells + AcMNPV	$1.0x10^{8}$b±2.34
25µg /mL propolis +Cells + AcMNPV	$3.1x10^{8}$b±5.14
12.5µg/mL propolis + Cells + AcMNPV	$5.6x10^{8}$b±2.67
6.25µg/mL propolis +Cells + AcMNPV	$9.9x10^{8}$b±0.98
3.12µg/mL propolis + Cells + AcMNPV	$3.9x10^{9}$a±1.12
1.562µg/mL propolis+Cells+ AcMNPV	$3.9x10^{9}$a±4.42
Cell + Different concentration of propolis extract Treatments	**Mean virus titer (PFU/mL)∗± S.D.**
Control Cells+ AcMNPV	$4.3x10^{9}$a ±5.42
200µg/mL propolis + Cells + AcMNPV	0
100µg/mL propolis + Cells + AcMNPV	$5.6x10^{7}$c±4.76
50µg /mL propolis + Cells + AcMNPV	$1.0x10^{8}$b±2.34
25µg /mL propolis +Cells + AcMNPV	$3.1x10^{8}$b±5.14
12.5µg/mL propolis + Cells + AcMNPV	$5.6x10^{8}$b±2.67
6.25µg/mL propolis +Cells + AcMNPV	$9.9x10^{8}$b±0.98
3.12µg/mL propolis + Cells + AcMNPV	$3.9x10^{9}$a±1.12
1.562µg/mL propolis+Cells+ AcMNPV	$3.9x10^{9}$a±4.42

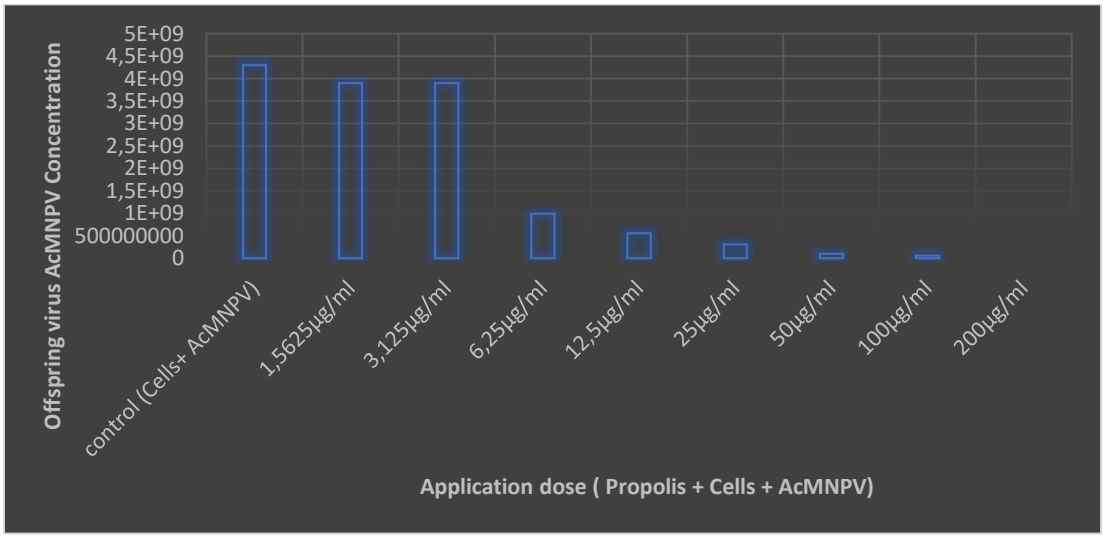

Figure 6. Effects of propolis extract prepared in different concentrations on the offspring virus AcMNPV concentration

The results showed that in Sf9 cell line, DNA replication of AcMNPV was restricted and antiviral influence was found in ethanol extract of propolis. Antiviral influence of extract from crude hexane extracts of *A. cathartica* plant was analyzed by Petcharawan et al. (2012). They also stated that higher antiviral effect was found from leaf extracts and virus concentration decreased by 66%. Humans are not infected by baculovirus; however, replication cycle of AcMNPV is similar to double-stranded DNA viruses causing infection in people. 100, 50, 25 µg/mL concentrations of propolis extracts were not found to influence the growth of Sf9 cells, just as 6.25, 3.125, and 1.5625 µg/mL concentrations. These concentrations were not found to have restricting effects on the replication of AcMNPV in Sf9 cell line, although extracts of the same concentrations were found to have a high significant restrictive effect on the replication of AcMNPV. Respectively 98.70 % and 98.67 % decrease were found in the virus concentration when 1 h p.i (post infection) was added in the stem and leaf extracts (Ertürk et al., 2000; Oktar, 2009). The results showed that when different concentrations of propolis extracts were applied, replication steps of the virus were restricted. As a result, while the replication of the virus was hindered, it was possible for the cells to continue their normal growth and proliferation (Vanhaelen & Vanhaelen-Fostre, 1979). It might be possible to explain the properties of propolis with high content of flavonoids. It was shown that quercetin and luteolin are virucidal. Viruses which were sensitive were only those that had membranes (Kaul et al., 1985). For this reason, flavonoids present in propolis might be the cause of extracellular inactivation of enveloped viruses. As shown by this study, some other studies have also shown that targets of inhibitory actions are synthesis of DNA, and RNA and virus-specific enzymes including thymidine kinase, DNA polymerase, deoxyribonuclease, and ribonucleotide reductase (Poncede et al., 1997).

4. CONCLUSION

The virus AcNPV used in the study is quite important because it is a good model for other pathogenic DNA viruses such as herpesvirus, adenovirus and vaccinia virus of humans. Since this is a screening study, it is better to utilize a nonpathogenic virus rather than pathogenic viruses of humans. Once any effective concentrations of propolis extract is determined, this substance can be purified and used on pathogenic DNA viruses. On the other hand, the concentrations of propolis extract, which has a reducing effect on virus replications, and the fact that it contains different compounds especially for the control of viral diseases, also provides the opportunity to use these compounds as raw materials in the pharmaceutical industry by purifying them.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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