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Antiviral Activity of Some Plant Extracts on the Replication of *Autographa californica* Nuclear Polyhedrosis Virus

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Abstract: The effect of crude extracts, obtained from *Aconitum nasutum, Daphne glomerata, Hypericum androsaemum, Laurus nobilis, Nerium oleander, Olea europaea, Prunus laurocerasus, Punica granatum, Rhododendron caucasicum,* and *Urtica dioica* on the replication of *Autographa californica* nuclear polyhedrosis virus (AcNPV) grown in *Spodoptera frugiperda* cell culture were investigated by observing changes in cytophatic effects, progeny virus concentration and viral protein (polyhedrin) synthesis.

We determined an inhibitory effect on cytophatic changes, virus concentration and synthesis of polyhedrin by utilizing the extract of A conitum nasutum, *Hypericum androsaemum, Laurus nobilis, Rhododendron caucasicum*, and *Urtica dioica* plants. On the other hand, the effects of *Daphne glomerata, Nerium oleander, Olea europaea, Prunus laurocerasus* and *Punica granatum,* extracts on the cytophatic changes and synthesis of polyhedrin were determined to be similar to the control. However, they enhance the virus concentration significantly.

Key Words: Baculovirus, plant extract, antiviral, virus replication.

Bazı Bitki Özütlerinin *Autographa californica* Nüklear Polihedrozis Virus Replikasyonuna Etkileri

Özet: Bu çalışmada, *Aconitum nasutum*, *Daphne glomerata*, *Hypericum androsaemum*, *Laurus nobilis*, *Nerium oleander, Olea europaea, Prunus laurocerasus, Punica granatum, Rhododendron caucasicum*, ve *Urtica dioica* bitkilerinden elde edilen özütlerin *Autographa californica* nüklear polihedrozis virüs (AcNPV)'ün *Spodoptera frugiperda* hücre kültürlürindeki replikasyonlarına etkileri: sitopatik oluşumlar, üretilen virüs konsantrasyonu ve virüse ait polihedrin protein sentezi yönünden incelendi.

Sonuç olarak, *Aconitum nasutum*, *Hypericum androsaemum, Laurus nobilis, Rhododendron caucasicum*, ve *Urtica dioica* bitki özütlerinin, sitopatik etkiler, üretilen virüs konsantrasyonu ve polihedrin proteinin sentezi üzerinde kontrole oranla engelleyici bir etki gösterdiği gözlenirken, buna karşın, *Daphne glomerata, Nerium oleander, Olea europaea, Prunus laurocerasus* ve *Punica granatum*, bitki özütlerinin sitopatik etkiler ve polihedrin proteinin sentezi bakımından kontrole benzer etki gösterdiği, fakat virüs konsantrasyonunu arttırdıkları tespit edildi.

Anahtar Sözcükler: Baculovirüs, Bitki özütü, Antiviral, Virüs replikasyonu.

Introduction

The *Autographa californica nuclear* polyhedrosis virus (AcNPV) is an insect virus which belongs to baculoviridae. The size of AcNPV is 25x250 nm, and contains approximately 128

kbp double-stranded DNA (1). The replication of AcNPV occurs in the nuclei of infected cells and takes place in two phases. In the first phase, nuclecapsids are formed in the nucleus. These nucleocapsids reach the cytoplasm by passing through nuclei pores. The nucleocapsids gain envelope during the budding through the plasma membrane, and particles released from the cell (2). In the second phase, after nucleocapsids acquire envelope (*apparently* de novo) within the nucleus, viral occlusion bodies form and gain envelope and nucleocapsids are embedded in a crystalline protein matrix (3). The occlusion bodies of nuclear polyhedrosis virus (NPV) are know as polyhedral inclusion bodies (PIBs), and they are infective particles among insects in nature (4).

Replication of the virus can be determined by observing cytophatic changes, virus concentration and various groups of macromolecules which belong to the virus (5). The cytophatic effect that occurs in the AcNP-infected Sf cells are as follows: granulation and membrane shrinking observed in the infected cells, the nuclei also enlarge and are flat and ellipsoid. Many cells lose cytophatic extension and include a swollen nuclei. Also the most characteristic cytophatic effect of baculovirus infection that can be observed with an inverted light microscope is the presence of crystalline like polyhedral inclusion bodies (PIBs) in the nuclei of infected cells. Although many AcNPV specific proteins were determined (6) polyhedrin is the most abundant (90% of total viral protein) protein of AcNPV. This polypeptide can easily be used as a sign for virus replication. Virus replication can also be determined by assaying the concentration of the progeny (7).

In this study, the effect of the crude extracts obtained from *Aconitum nasutum* Fisch. *(Ranunculaceae)*, *Daphne glomerata* Lam. *(Thymelaeaceae)*, *Hypericum androsaemum* L. *(Guttiferae) Laurus nobilis* L. *(Lauraceae)*, *Nerium oleander* L. *(Oleaceae)*, *Olea europaea* L. *(Oleaceae)*, *Purunus laurocerasus* L. *(Rosaceae)*, *Punica granatum* L. *(Punicaceae) Rhododendron caucasicum* L. *(Ericaceae)*, and *Urtica dioica* L. *(Urticaceae)* on the replication of *Autographa californica* nuclear polyhedrosis virus (AcNPV) in *Spodoptera frugiperda* cell culture was investigated by observing changes in the cytophatic effects, the virus concentration and the viral synthesis of polyhedrin.

Plants used in this study are also used in medicine (8). However, this is the first study regarding the antiviral effect of these plants. In this paper, we report the result of studies on the anti-AcNPV activity of the ethanol-soluble extract.

Materials and Methods

Preparation of the plant extracts:

Fresh leaves and twigs of *Aconitum nasutum* Fisch. *(Ranunculaceae)*, *Daphne glomerata* Lam. *(Thymelaeaceae)*, *Hypericum androsaemum* L. *(Guttiferae) Laurus nobilis* L. *(Lauraceae)*, *Nerium oleander* L. *(Oleaceae)*, *Olea europaea* L. *(Oleaceae)*, *Prunus laurocerasus* L. (*Rosaceae)*, *Punica granatum* L. *(Punicaceae) Rhododendron caucasicum* L. *(Ericaceae)*, and *Urtica dioica* L. *(Urticaceae)* were collected between March and January (1995) in the Black Sea region (Turkey). The identification of these specimens was carried out using Flora of Turkey (9-18).

The specimens are kept in the Herbarium, Department Biology of Karadeniz Technical University, (KTUB). Fresh leaves and twigs of the plants were dried at 45°C for 5-6 hours. The extract of the plants were prepared according to the methods described by Alkkofahi et al. (19) and Holopainen et al. (20), with slight modification. Dried leaves and twigs of the plants were extracted with 95% ethanol (50 g 1/5 ethanol) at room temperature. The extracts were kept at 4°C for a day, and they were filtered through 45 µm membrane filter, and then the solution was dried with an evaporator (BIBBY RE 100 B). The crude extracts were stored at -20°C until used.

Cell and viruses:

Vaughn's Sf-IPLB-21 (*Spodoptera frugiperda*, Sf) cell culture was used as the host cell (21). These cells were grown in TNMFH medium (22) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) in 25 cm^2 culture flasks at 28°C.

Acal 5 strain of *Autographa californica* nuclear polyhedrosis virus (AcNPV, baculoviridae) was used.

Cytotoxicity assay:

In order to test the effects of the crude extracts on Sf cells, 2.5x104 cells (in 0.5 ml TNMFH medium, supplemented with 10% FBS) were seeded into each well of 24-well plates, cultured for 6 h at 28°C, and cells were allowed to grow for additional 48 h in the presence of increasing amounts of extract (24, 74, 500 and 666 µg/ml). The cytotoxicity of extracts were determined by a conventional haemocytometer using the trypan blue-exclusion method (23). The highest noncytocidal (on Sf cells) concentration of plant extract was determined to be 74 µg/ml. Therefore, 74 mg/ml concentration of extracts was used for the determination of antiviral activities.

Assay of antiviral activities:

$2.5x10^4$ cells in 0.5 ml TNMFH medium supplemented with 10% FBS were seeded into each well of 24-well plates and cultured for 3 h at 28°C. After the removal of medium, the cells were washed with phosphate-buffered saline (NaCl 0.8 g., KCl 0.2 g., Na_2HPO_4 1.15 g., KH_2PO_4 0.2 g. in 1 litre H_2O pH 7.2). One milliliter dissolved crude extract (in unsupplemented TNMFH medium, at a concentration of 74 mg/ml) was added to each well of 24-well plates, cultured for 1h at 28°C, infected with AcNPV at an m.o.i of 20 (the number of infectious virus particles for each cell) and incubated for 1.5 h at room temperature for adsorption. After adsorption, the virus and extract mixture were removed, the cells were washed with phosphate buffer saline (PBS), and complete TNMFH medium containing 10% FBS and 100 µg/ml gentamicin solution was added to 24-well plates. At this point, the time was recorded as "0" h post infection. Each panel included toxicity control (cells plus extract), and uninfected cell control (cells only, Control 1) and infected cell control (virus plus cells, Control 2).

Inverted light microscopy: After "0" h.p.i. of Sf cells infected with AcNPV and mock infected Sf cells were prepared as mentioned above for each plant extract, incubated at 28°C. At 0, 6, 12, 24, 36, 48, h.p.i., each sample was observed under a Prior-England inverted microscope

for cytophatic effects. The pictures of the cytophatic effects were taken with a Centon camera attached to inverted microscope.

Assay for virus titer: At 72 h.p.i. medium from all samples was removed as mentioned above and tested for determination of the change in the titer of extracellular virus. The titer of virus samples was determined by the Reed and Muench dilution method (8) using six wells of a 60-cell micro plate (Nunclon) per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses per milliliter ($TCID_{50}$). All of the samples were assayed. The total virus concentration of these cultures (treated with plants extracts) was compared with the control (Sf cells which had been infected with AcNPV).

Analysis of proteins: Sf cells, with or without pretreatment of the plant extract for 1 or 2 h were mock infected or infected with AcNPV at an m.o.i. of 20. Cells were washed with PBS, 5 ml of complete TNMFH medium was added to a T-25 cm^2 flask, and culture incubated at 28°C for 60 h. The protein samples were prepared according to the procedure described by King and Possee (24). The samples were harvested with a lysing buffer (containing 0.062 M. Tris-HCl pH 7, 2% SDS, 10% gliserin, 5% 2-mercaptoethanol, 1 nM PMSF, 2 µg/ml Leupeptin, 1 mg/ml Pepsitain, the final volume adjusted with dd H_2O). The virus and cell lysates were analyzed by 12% SDS-polyacrylamide gel electrophoresis (25). Gels were stained with silver nitrate according to the method described by Hames et al. (26).

Results

In order to determine the effects of plant extracts on the replication of AcNPV, uninfected Sf cells (Fig. 1A= Control 1) and Sf cells infected with AcNPV (Fig. 1B= Control 2) were observed as controls.

Since the best chemotherapeutic index was determined to be 74 mg/ml, we used this concentration in all of the experiments to test the effects of plant extract on virus replication. No deterioration observed in morphological state of uninfected cells indicates that the physical and chemical environments in which the cell growth occurred were appropriate for the proliferation of Sf cells (Control 1). Infected cells showed the crystal-like polyhedral inclusion bodies (PIBs, one of the most definitive cytophatic effect) and other cytopathic effects (the enlargement of cells, becoming ellipsoid and flattened, granulation, membrane shrinking, loss of cell extensions) resulting from the infection of Sf cells with AcNPV (Control 2).

Cytopathic Effects:

The most definitive cytophatic effect of AcNPV (production of PIB) was a marker point to test the effect of plant extract on virus replication. We also observed other cytophatic changes during virus replication in the presence of plant extracts.

PIBs were not observed in the nuclei of infected cells after the application of *A. nasutum, H. androsaemum, L. nobilis, R. caucasicum, U. dioica,* and extracts at 74 µg/ml concentration (Fig. 2 A-E). Since other concentrations either showed a toxic effect on cells or were not effective on virus replication, the related data are not shown. After the application of all the extracts,

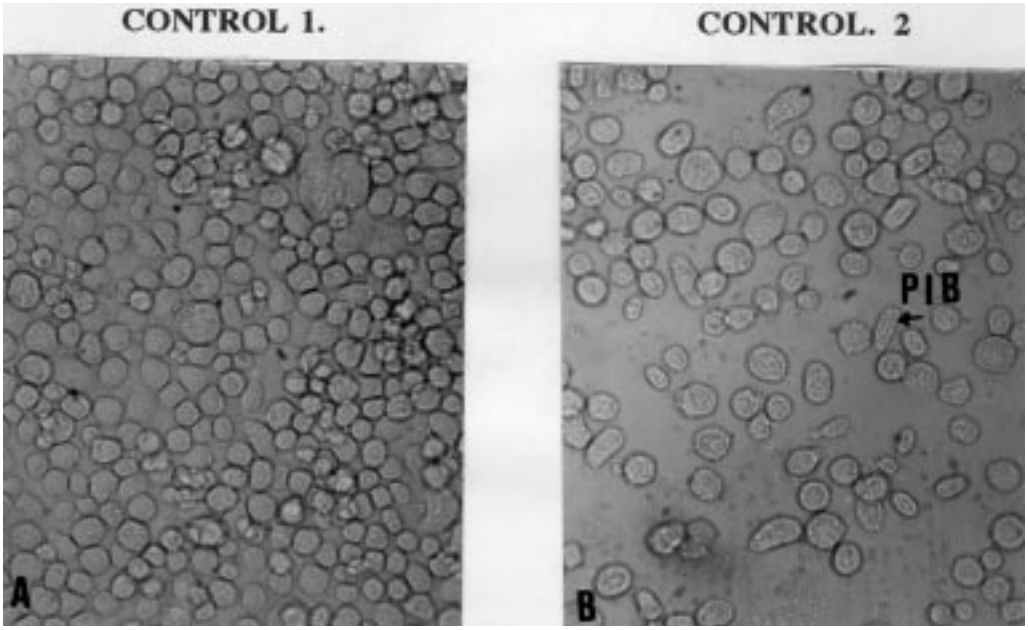

Figure 1. A) Control 1: Mock infected *Spodoptera* frugiperda (Sf) Cells B) Control 2: Sf cells infected with *Autographa californica* nuclear polyhedrosis virus (AcNPV), arrow shows polyhedral inclusion bodies (PIB).

cell density and cell morphology were quite similar to uninfected cells (Control 1). However, after the application of *U. dioica* extract, some of the cells showed membrane shrinkage, most of the cells covered the bottom of the culture plate and did not undergo any significant morphological or volume change (Fig. 2 B). Samples to which *R. caucasicum* was applied were also observed, and in some of these cells cytoplasmic extensions were determined (Fig. 2 E). Furthermore, the figures related to the cytopathic effects of *D. glomerata, N. oleander, O. europaea P. laurocerasus* and *P. granatum* applied samples are not given because they were similar to those of AcNPV-infected Sf cells (Control 2).

Virus titer:

In order to test the effect of plant extracts on virus concentration, virus-suspension was collected from samples as explained above. The results are shown in Figure 3. Virus titer relative to that of control 2 (AcNPV-infected Sf cells) was as follows: 67.71% decrease for *L. nobilis*, 44.44% decrease for H. *androsaemum*, 25.15% decrease for *R. caucasicum*, 22.11% decrease for *U. dioica*, and 2.20% decrease for *A. nasutum*, 31.13% increase for *N. oleander*, 30.39% increase for *O. europaea*, 28.61% increase for *D. glomerata*, 8.28% increase for *P. laurocerasus* and 1.67% increase for *P. granatum*.

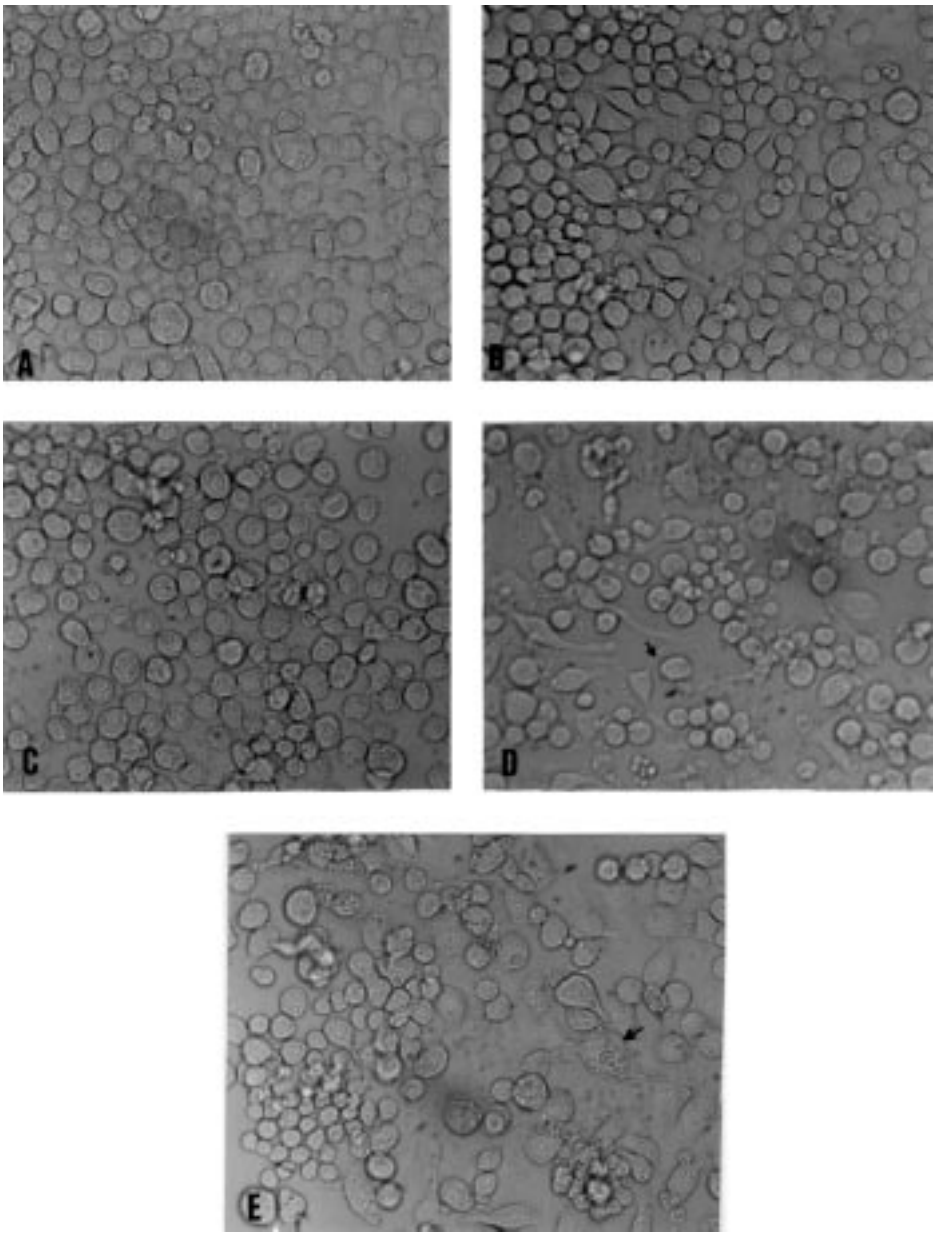

Figure 2. Effect of plant extracts on the cytophatology of *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus (AcNPV). A) *L. nobilis*, B) *H. androsaemum*, C) *U. dioica*, D) *A. nasutum* and E) *R. caucasicum*. Applied amount : 74 µg/ml. Arrow shows vacuoles.

Protein Synthesis:

Cellular and viral proteins prepared according to the method described by King and Possee (14) were separated using 12% SDS-PAGE (Fig. 4). Seven viral protein bands (56.1, 40.5, 31.8, 26.9, 26.8, 26.7 and 22.4 kD) were determined in control 2 (Sf cells infected with AcNPV) but not in control 1 (uninfected Sf cells). Among these proteins, polyhedrin (26.8 kD) was the most abundantly synthesized. A polyhedrin band was seen clearly in the case of application of *N. oleander* plant extract, and as very faint in the case of *P. granatum, P. laurocerasus* and *O. europaea* plant extract applications. A polyhedrin protein band was not observed in other lines in which the rest of the plant extracts were applied (Table 1).

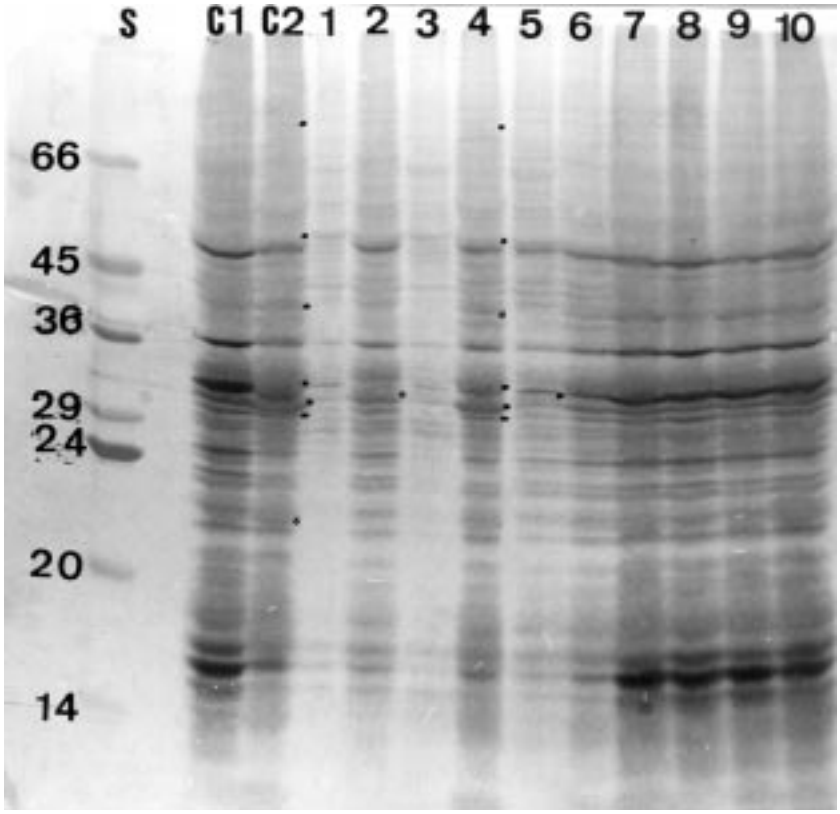

Figure 3. Changes in the number of progeny virus of AcNPV in *Spodoptera frugiperda* (Sf) cells in respect to applied cell extracts. (Sf+Ac) Control 2, Sf cells infected with AcNPV. Rest of the samples indicate Sf cells infected with AcNPV in the presence of plant extracts: 1) *L. nobilis*, 2) *U. dioica*, 3) *A. nasutum*, 4) *P. laurocerasus*, 5) *P. granatum*, 6) D. *glomerata*, 7) *N.oleander*, 8) *R. caucasicum*, 9) *H. androsaemum*, 10) *O. europaea*. Values are shown as anti-log.

Samples		polyhedrin
Sf	(Control 1)	No
Sf + AcNPV	(Control 2)	Yes
Sf + AcNPV	+ L. nobilis	No
Sf + AcNPV	+ P. granatum	Yes
Sf + AcNPV	+ H. androsaemum	No
Sf + AcNPV	+ N. oleander	Yes
Sf + AcNPV	+ P. laurocerasus	Yes
Sf + AcNPV	+ O. europaea	Yes
Sf + AcNPV	+ D. glomerata	Yes
Sf + AcNPV	+ U. dioica	No
Sf + AcNPV	+ A. nasutum	No
Sf + AcNPV	+ R. caucasicum	No

Table 1. Presence of polyhedrin bands in the extracts of *Spodoptera frugiperda* (Sf) cell infected with *Autographa californica* nuclear polyhedrosis virus (AcNPV) in 12% SDS-polyacrylamid gel.

Discussion

This study indicates that some crude extracts prepared from various plants have significant inhibitory effects on the replication of AcNPV. These activities were demonstrated by inhibition of virus-induced cytopathogenicity, synthesis of infected cell protein and reduction on the virus titer.

Being intracellular parasites, viruses need living systems to proliferate. They replicate themselves by actively using the cellular systems of their host. This makes it difficult to fight with viruses. The antiviral agent to be used against viruses is desired to affect the virus but not to damage the host cell. There are several antiviral agents which disrupt the replication steps of viruses in infected cells, namely adhesion, endocytosis, decapsulation, nucleic acid synthesis, protein synthesis, fusion and leaving the cell. These antiviral agents are structurally purine or pyrimidine base analogous in general. They are known to inhibit adhesion of the virus on the cell surface, relieving from the protein coat, and some inhibit mRNA synthesis (27, 28). Among antiviral agents, amantadine inhibits adhesion of some viruses on the cell, idoxuridine inhibits replication of DNA viruses, and ribavirin most probably inhibits mRNA synthesis (27, 28). Hypericin and rose bengal substances are reported to inactivate replication of the Friend leukemia virus (FLV) (29). On the other hand, lamivudine, a nucleoside analog, significantly inhibits replication of HIV when used together with 3'-azido-3'-deoxythymidine (AZT) (30).

In the current study, when the information and figures in the results section were analyzed, differences among the cells of control 1 (Fig. 1), those of control 2 (Fig. 1) and of all others with which plant extracts utilized were observed. Although the same number of cells ($2.5x10^4$ cell/ml) were applied to each well, while the number of cells did not change in control 2, the number of cells increased in the cases where some plant extracts (Fig. 2 A-E) were applied. Similar results were also observed in the level of synthesis of polyhedrin (Fig. 4 and Table 1) and in the titer of the progeny virus (Fig. 3). Thus, when all the results are analyzed there are

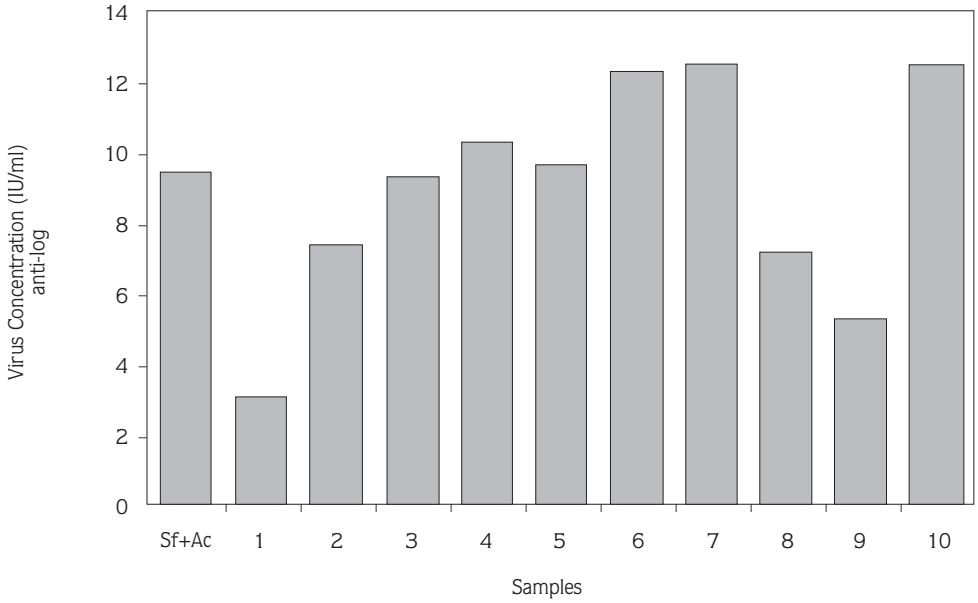

Figure 4. Protein synthesis of *Autographa californica* nuclear polyhedrosis virus (AcNPV) after application of plant extracts in 12 % SDS-PAGE. MW) molecular weight standards, C1) Sf cell, C2) Sf + AcNPV, 1) *L. nobilis*, 2) *P. granatum*, 3) *H. androsaemum*, 4) *N. oleander*, 5) *P. laurocerasus*, 6) *O. europaea*, 7) *D. glomerata*, 8) *U. dioica*, 9) *A. nasutum* and 10) *R. caucasicum*. 30 µg of protein samples were applied to each lane.

significant inhibition effects of extracts prepared from *L. nobilis, U. dioica, A. nasutum, R. caucasicum* and *H. androsaemum*. The results indicate that the replication steps of the virus were inhibited when plant extracts were applied. Therefore, the cells in these cases continued their normal growth and proliferation, but the replication of the virus was inhibited. This inhibition probably occurs at the level of DNA replication or transcription. Some studies have shown that synthesis of DNA, and RNA and virus-specific enzymes such as thymidine kinase, DNA polymerase, deoxyribonuclease, and ribonucleotide reductase are the targets for inhibitory actions (31-34) which is also supported by this study.

In the study conducted by Amoros et al. (35), saponin isolated from *Anagallis arvensis* plant was said to inhibit adhesion and penetration of the herpes virus to Vero cell, and it was suggested that this substance can be used for virus inactivation by competing with them to bind cell receptor proteins. In another study, conducted by Kyoko et al. (36) a subtance (Col 1-6) isolated from *Cordia salicifolia* was found to inhibit the adsorption and/or the penetration of HSV-1 to HeLa cells. The plant samples used in the current study affected virus replication because they probably contain saponin or other similar substances. However, at this level it is not clear what kind of subtances exactly affect virus replication in the studied plant extracts.

In conclusion, plant extracts from *U. dioica, H. androsaemum, L. nobilis, A. nasutum* and *R. caucasicum* showed a decreasing effect with respect to cytopathic effects, the concentration of virus produced and viral protein synthesis compared with control 2. On the other hand, the effects of *N. oleander, P. laurocerasus, P. granatum, O. europaea* and *D. glomerata* plant extracts with respect to cytopathic formations and viral protein synthesis were similar to those seen in control 2. They increased the concentration of progeny virus.

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 (37, 38). Since this is a screening study, it is better to utilize a nonpathogenic virus rather than pathogenic viruses of humans. Once any effective extract is determined, this substance can be purified and used on pathogenic DNA viruses. On the other hand, the plant extracts that showed an increasing effect on virus concentration can also be used when more virus is needed especially for biological control and in foreign gene expression (39) especially for baculovirus expression systems in order to produce more proteins.

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