PROTECTIVE EFFECT OF A MEXICAN PROPOLIS ON MDBK CELLS EXPOSED TO AUJESZKY'S DISEASE VIRUS (PSEUDORABIES VIRUS)

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Abstract

Background: In this paper, the evaluation of the antiviral effect of a Mexican propolis on Pseudo Rabies Virus (PRV) was performed by infecting cell cultures of MDBK.

Materials and Methods: First, the level of cytotoxicity of the ethanol extract of propolis (EEP) was determined, subsequently, infective dose of PRV strain Shope was determined and finally, we performed the interaction of the virus with EEP (two hours before, during and two hours after infection) in MDBK cells. Also, in order to determine the possible effects of propolis on the virus, cell culture samples subjected to the different treatments mentioned above were processed for Transmission Electron Microscopy (TEM).

Also, cell culture samples with the same treatments were processed for Transmission Electron Microscopy (TEM) to observe possible alterations of propolis on the virus.

Results: It was determined that administration of 0.5 mg / ml of EEP two hours before infection caused a reduction in the number of plaque forming units compared to the rest of the treatments used or with the infected and untreated culture, it is important to note that the difference found was statistically significant ($p < 0.01$). By other hand, viral particles with altered morphology were observed at MET suggesting a possible damage to the viral envelope proteins. We also found virions in an electro-dense layer formed around the cell membrane. This seems to affect the penetration of the virus and its replication cycle.

Conclusion: This study demonstrated the antiviral activity of EEP used and its results open perspectives to explore the possibility of using propolis as a preventive method against pseudo rabies.

Key words: Mexican propolis, antiviral activity, Aujeszky's disease virus, Pseudo Rabies Virus (PRV).

Introduction

Propolis is a product that has been used by man since ancient times. It has remained popular through the years thanks to its medicinal properties and antifungal, antibacterial, antiviral, and anti-parasitic activities (Bogdanov, 2012; Sforcin, 2007). Therefore, it is used as a usual remedy in medicine and as a component of bio-cosmetics. Variations in its biological activity have been observed depending on their geographical origin (Cushnie et al., 2005; Londoño et al., 2010.). Although there is little information on the antiviral properties of propolis, its effect on avian influenza, herpes, HIV, Marek, among others is known. Particularly, only some few antiviral activity data of this compound against pseudo rabies disease have been reported (Huleiehl, 2012); hence assess the antiviral effect of propolis is important especially considering that viral diseases are completely different in their mechanism of action and, also, its effect at the cellular level (Bagla et al., 2012). Aujeszky's disease is also known as pseudo rabies disease (Pomeranz, 2005; Mettenleiter, 2009). It is caused by Herpes virus suis I. It is a contagious viral disease that affects mammals. Pseudo rabies is mainly observed in pigs. These animals are the natural hosts and reservoirs of the virus (Plonait and Blickhart, 2001), although a large number of species can be naturally or experimentally infected. The PRV is infectious for cattle, sheep, goats, dogs and cats (Mettenleiter et al., 2000). *In vitro*, pseudo rabies virus can be isolated via cultivation in susceptible cell monolayers (Fuchs et al, 2012), allowing to increase the knowledge about their biology. In cell cultures, replicative cycle is short (6-9 hours) (Echeverría, 1999) and causes massive destruction, producing a cytopathic effect (CPE) characterized by cell rounding (Vrublevskaya, 2002), syncytium formation and the presence of lytic plaques or "holes" in the cell monolayer. Moreover, it can also be seen as eosinophilic inclusion bodies depending on viral strain and infection status (Pejsak, 2006). Several cell lines have been used for the multiplication of PRV including PK-15 (porcine kidney epithelial cell line 15), RK-13 (Rabbit Kidney) and MDBK (Madin Darby Bovine Kidney) (Cedeño, 1998).

The objective of this study was to evaluate the antiviral action of ethanolic extract of propolis from the State of Mexico, Mexico, using monolayers of MDBK cells infected with PRV to perform *in vitro* the exposed virus-propolis, obtaining comparative number of plaque forming units (PFU) present in infected cultures and in cultures treated with propolis.

The aim of this study was to evaluate the antiviral activity of an ethanol extract of propolis (EEP) of the State of Mexico, Mexico. To do this, monolayers of MDBK cells were infected with PRV and treated with EEP, and we determined the number of units plaque forming (PFU) on infected cell cultures in contrast to cultures treated.

Materials and Methods
Ethanolic Extract of Propolis

The propolis produced by *Apis mellifera* bee was collected from the apiary of the Facultad de Estudios Superiores Cuautitlán, UNAM, located in the municipality of Cuautitlan Izcalli, State of Mexico. The collected material was cleaned from any physical contaminants such as chips, plastic, bee body parts, etc., and then split into smaller pieces. Subsequently, 70% ethanol was added to 150 g of propolis and was maintained 15 days in an amber bottle. During this period, it was daily subjected to slight agitation. After this time, it was filtered and a rotary evaporator solvent distillation was performed to get the ethanol extract of propolis (EEP).

Cell Culture and Virus

Monolayers of Madin Darby Bovine Kidney (MDBK) cells were used, which were grown within 100 mm polystyrene culture dishes using Dulbecco's Modified Eagle's Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin) and then they were maintained in an incubator at 37°C under a humidified atmosphere of 95% air and 5% CO_2. Infection assays were performed in 96-well plates. For this reason, the cells were counted and standardized in order to have the same number of cells in each of the wells (50×10^5 cells/well). The infection was performed using the Shope strain of pseudo rabies virus (PRV).

Cytotoxicity Assays

Cells were standardized and placed in 96-well plates and incubated for 24 hours at 37°C under a humidified atmosphere of 95% air and 5% CO_2. After, the culture medium was replaced by a fresh culture medium. On the other hand, EEP was 1:100 diluted with DMEM and dimethyl sulfoxide (DMSO). The cultures of MDBK cells were exposed to increasing doses (0.0625, 0.125, 0.25, 0.5, 1.0 and 4 mg/ml) of EEP. Wells that received only DMEM (200 µl) were used as a negative control and wells that received DMEM and DMSO (3 µl) were used as a positive control. After adding EEP, the plate was incubated for another 24 hours and then was observed under an inverted microscope for assessment. All assays were performed in triplicate (Nolkemper et al., 2010).

Viral Titer

The infective dose was determined by plaque assay. To do this, MDBK cell monolayers were used in polystyrene culture dishes 100 mm containing DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin). These cultures were incubated under the conditions already mentioned (Hierholzer and Killington, 1996). The viral stock used for infection assays had a title of 1×10^4 pfu/ml.

Propolis-Virus Interaction Assays

MDBK cell monolayers were used in 96-well plates. The medium was replaced at 24 hours of incubation. The assay was designed to perform the following assessments (Nolkemper et al., 2010): 1) treatment using EEP administered before viral infection, medium was removed from wells and then 50 µl of propolis solution were added at a concentration of 0.5 mg/ml, after two hours of incubation, the wells were inoculated with 50 µl of viral solution and supplemented with 100 µl of DMEM; 2) treatment with EEP administered simultaneously with the viral infection. Thereafter, 50 µl of the solution of propolis were added at a concentration of 0.5 mg/ml at the same time that the cell culture was infected with the viral dilution and 3) treatment with EEP administered after viral infection. First, cells were infected with 50 µl of the viral dilution, at the minimum infectious dose determined by a previous titration, it was allowed to incubate for 2 hours, after which 50 µl of EEP were added at a concentration of 0.5 mg/ml, the monolayers were supplemented with fresh DMEM. Cell cultures were observed at 24 hours post-infection in all treatments.

Quantification of Lytic Plaques

MDBK cell cultures in 100-mm Petri dishes were used. The cultures were treated with 0.5 mg/ml of propolis and infected with the determined viral dilution. After 24 hours of incubation, the cultures were fixed with 4% paraformaldehyde for 10 minutes and the cells were stained with crystal violet. Plaques formed in each of the treatments were observed and counted (before, during and after infection). Lytic plaques present were counted per field of 40x magnification - 10 fields were randomly counted and the average was obtained. This assay was performed in triplicate.

Transmission Electron Microscopy

MDBK cell cultures were treated with 0.5 mg/ml of propolis in the different evaluations already mentioned. After 24 hours of incubation, were washed with PBS pH 7.2 and fixed in situ with 2.5% glutaraldehyde and 4% paraformaldehyde. The cells were separated from the dishes and washed with PBS. They were post-fixed with 1% osmium tetroxide and were again washed with PBS (Huang et al., 2011). The cell pellet was dehydrated through an ascending ethanol series (30%, 50%, 70%, 80%, 90% and absolute ethanol). Spurr´s resine was used the inclusion of the samples. For the polymerization of the resin, samples were incubated at 60°C for 36 hours, then were cut, stained and observed by electron microscopy. In parallel, a non-infected culture and another infected, but untreated with EEP were processed.

Statistical Analysis

The plaques produced by the PRV were observed and counted in each of the cases (infected culture, culture treated with propolis two hours before infection, during infection and two hours after infection). The data were processed using the Graph Pad Prism version 4 software, using ANOVA statistical test.

Results

Cytotoxicity Assay

In order to evaluate the possible toxic effects of EEP, MDBK cell monolayers were exposed to increasing doses of EEP. At the dose of 0.5 mg/ml, we did not observe any significant morphological alteration at the light microscope analysis, while at higher doses were observed

cells with cytoplasmic vesicles, loss of cell morphology and lysis. In this way, it was determined that the dose of 0.5 mg/ml is well tolerated by the cells so it was decided that it could be used in subsequent treatments.

Anti-viral activity of EEP

Antiviral activity of EEP was evaluated *in vitro* using the Shope strain of pseudo rabies virus through inhibition of number of PFU in infected culture (Fig. 1B), culture treated before infection (Fig. 1C), culture treated during the infection (Fig. 1D) and culture treated after the infection (Fig. 1E). Through interactions described, a difference was found between the negative control culture (Fig. 1) and PRV infected culture compared with cultures in which the EEP was applied at different times. The culture treated with propolis two hours before the viral infection showed a lower amount of PFU, with an average of 8.7 PFU. While cultures treated simultaneously to the infection showed a mean of 16.6 PFU and cultures treated two hours after the PRV infection had a mean of 15.4 PFU (Table 1).

Statistical Analysis

The data were processed using the Grad Pad Prism version 4 software, using ANOVA statistical test. According to the results obtained by counting PFU, a significant difference ($p < 0.01$) was observed between the infected control culture and the culture treated with propolis two hours before the infection, which is a statistical evidence supporting that propolis has an antiviral effect on infected cultures with PRV (Figure 2).
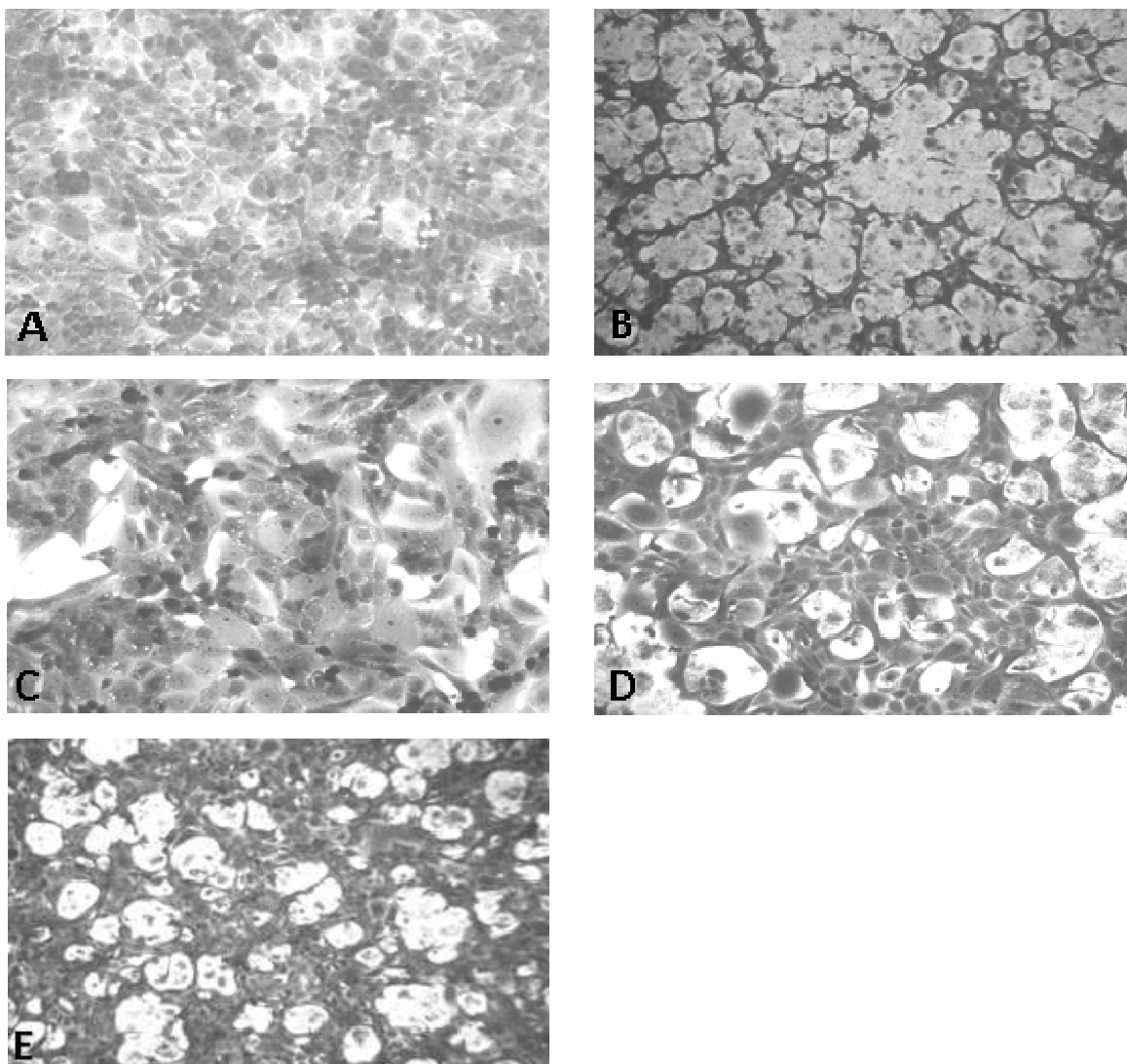
Figure 1: Cultures of MDBK cells stained with crystal violet. **A.** Not infected MDBK cell culture. **B.** Culture infected with PRV**. C.** Culture treated with propolis 0.5 mg/ml two hours before infection**. D.** Culture treated with 0.5 mg / ml of propolis and simultaneous infection. **E.** Culture treated with propolis two hours after of infection. 250×.

Table1: Mean of PFU in different treatments with propolis.

Assay	Mean of pfu (±DS)
Culture uninfected	0
Culture infected and untreated	16.4 (± 11.8)
Propolis two hours before infection	8.7 (± 5.5)
Propolis and simultaneous infection	16.6 (± 10.9)
Propolis two hours after infection	15.4 (± 9.0)
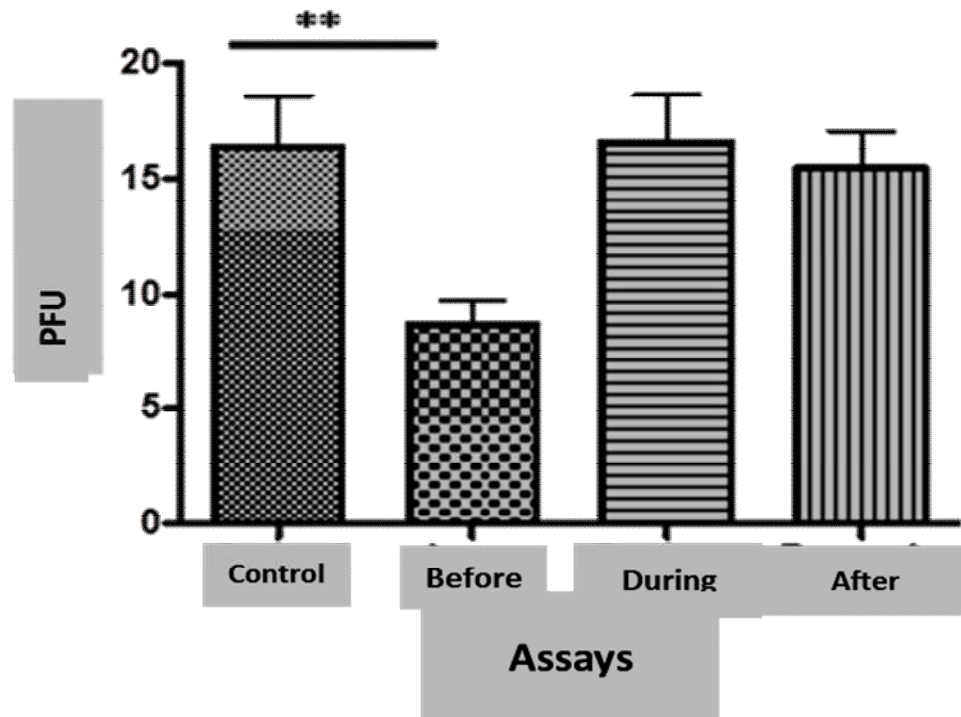
Figure 2: Average plaque forming units (PFU) in cell cultures. Control infected MDBK cell culture. **Before -** MDBK cell treated 0.5 mg / ml of propolis two hours before infection with PRV. **During**, MDBK cell culture treated with 0.5 mg / ml of propolis and simultaneously infected with PRV. **After -** MDBK cell culture treated with 0.5 mg / ml of propolis performed two hours after infection with PRV. The bars represent the mean + / - standard error (**p <0.01). ANOVA statistical test.

Transmission Electron Microscopy

In order to find if the EEP exerts an effect on the ultra-structural integrity of the virus, MDBK cell cultures subjected to different treatments previously mentioned were processed for transmission electron microscopy. In infected cells untreated with the EEP, the presence of virus was observed in the cytoplasm of cells (Fig. 3A). The images of the samples from cultures treated with EEP two hours before infection with PRV showed the formation of an electron dense coating on the cell membrane in which structural changes in the virus particles were observed. The presence of vesicles with lumpy appearance was also found in the cytoplasm of cells (Fig. 3B and 4).

Discussion and Conclusion

Several researchers have proposed the use of various cell lines in order to replicate the cytopathic effect of PRV. Additionally, they emphasize that the cytopathic effect is less noticeable in MDBK cells compared to other cell lines (Cedeño et al., 1998; Dezengrini, 2006). However, in this study, it was found that MDBK cells besides being a cell line easy to use and show a good growth rate to be infected with PRV showed an evident cytopathic effect at 24 hours post-infection. On the other hand, in the context of the assessment of the toxicity of propolis in the literature, it is mentioned that doses of 1, 10, 50 and 100 mg/ml do not cause changes or alterations in cell morphology when this test is performing this test with 1 hour of EEP adsorption (Nolkemper et al., 2010), while the concentration of 200 mg/ml can be toxic for the cell culture after 2 hour of EEP adsorption (Huleihel and Isanu, 2002). In this paper, the EEP was not removed until 24 hours after the cell incubation. Our results showed that the maximum concentration of EEP that does not cause morphological changes in the MDBK growing cells was 0.5 mg/ml while higher doses were toxic to the cells. This difference in dose, compared with those reported in the literature, is possibly due

to the fact that the chemical composition of propolis varies depending on its origin as it is known that the ratio of the components changes with this variable (Schnitzler et al., 2010).
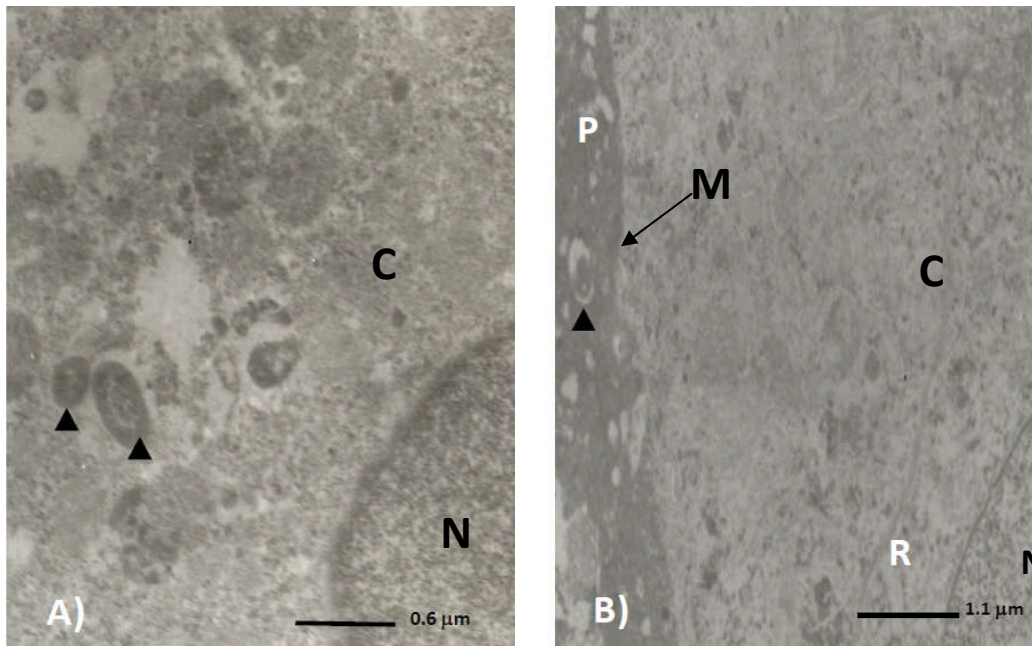
Figure 3: Ultra-structural analysis of infected MDBK cells. **A**. Infected cell with PRV and untreated Presence of viral particles (▲) in cytoplasm (C). **B**. Treated with EEP two hours before infection with PRV; viral particles (▲) are observed outside the cell membrane trapped in an electron-dense structure (P) that is coating to the cell. Nucleus (N) and (R) endoplasmic reticulum.
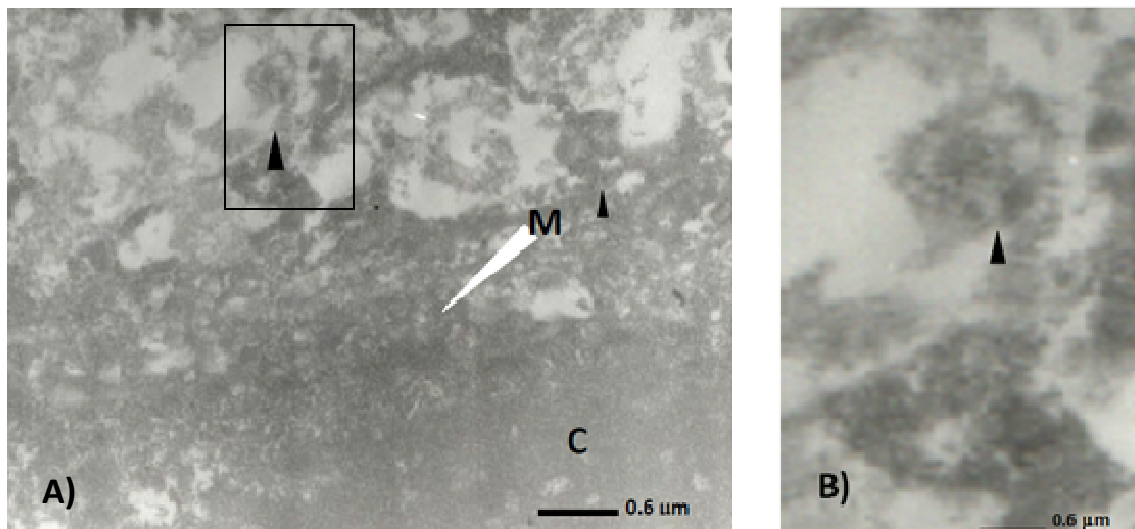
Figure 4: MDBK cell treated with propolis (0.5 mg/ml) two hours before infection with PRV. A. Part of plasma membrane is shown with presence of viral particles (▲) in cytoplasm (C), dense lumpy structure located on the cell membrane (M) was also observed in which viral particles are trapped. **B**. Amplified micrograph of the figure 4A in which deterioration is observed on the surface of a viral particle of PRV located outside the plasma membrane of the infected cell.

With respect to antiviral effect of the propolis, it was reported that administration of 50 mg / ml two hours before infection with herpes simplex virus type 1 confers 100% protection given the absence of cytopathic effect, it is also noted that the dose of 5 mg/ml gave 50% protection, while the dose of 100 mg / ml supplied two hours after infection provided 80% protection (Huleihel e Isanu, 2002). Another report indicates that concentrations of 0.0005 and 0.0004 mg / ml of propolis produced inhibition of the cytopathic effect of herpes simplex virus type 2 in cell culture (Nolkemper et al., 2010).

In this study, we found that the dose of 0.5 mg / ml of EEP supplied two hours before infection produced a significant reduction in the number of PFU (8.7). In contrast to the tests performed during and two hours post-infection where a significant decrease is not observed in the number of PFU compared to the infected and untreated culture (16.4 PFU). When comparing the doses determined in this work (toxic and antiviral effect) with those reported by other authors (Nolkemper et al., 2010; Huleieh, M. & Isanu, V., 2002) there is a big difference, which may be due to a variation in the chemical composition of propolis determined by their geographical origin as flora, each location is different (Londoño et al., 2010). Since we must consider that the EEP's reported come from different geographic regions as Czech Republic (Nolkemper et al., 2010) and Israel (Huleieh, M. & Isanu, V., 2002), whereas for our study, propolis was obtained from Cuautitlán Valley in the State of Mexico, Mexico. (Londoño et al, 2010).

To explain the mechanism of action of antiviral, effect of propolis is mentioned that flavonoids and other phenolic acids present in propolis interact with viral proteins forming complexes unstable therefore altering the stages of adsorption and penetration (Oliveira et al., 2013). Besides, inhibiting the viral polymerase therefore interfere with the synthesis of the viral genome and the production of complete virions (Carvalho et al., 2013; Schnitzler et al., 2010). In this work, it was observed by electron microscopy that the cells treated two hours before infection with PRV displayed an electron-dense layer on cell membrane, suggesting that the presence of this barrier prevents the penetration of the virus to host cell. Additionally, the ultra-structural changes observed in the viral particles also suggest a direct damage to the proteins of the virus envelope (Schnitzler et al., 2010; Huleihel and Isanu, 2002) which can lead to a destabilization of the entire viral structure and, therefore, affect its ability to penetrate the development of the viral cycle, as proposed by other researchers (Schnitzler et al., 2010; Huleihel and Isanu, 2002). In our findings, it was also observed in the cytoplasm that the presence of vesicles containing some lumpy aggregates, which suggests the formation of defective virions or virus destruction within the cell. The EEP used in this work contains flavonoids and other phenolic acids, so that they may be responsible for altering the glycoproteins of the virus or structural damage observed. Additionally, other components of the EEP used and which may also be acting on viral proteins are cinnamic acid and caffeic acid (Schnitzler, 2008).

Finally, it should be noted that there are no previous reports of studies about effect of EEP on the PRV and according to the results obtained; it can be considered that propolis has potential use in the prevention of this viral disease.

Acknowledgments

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