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Full Length Research Paper

Virucidal activity of green propolis against *avipoxvirus* in chorioallantoic membrane of embryonated chicken eggs

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Recent pandemics caused by virus like *influenzavirus* (H1N1, H5N1) reaffirm the importance of studies aiming at obtaining new virucidal and/or antiviral substances, once its prolonged use can lead to resistance to the active principles. Green propolis, which has several scientifically proven bioactive properties, was evaluated in this study as an ethanol extract regarding its virucidal capacity against *avipoxvirus* (APV) inoculated in chorioallantoic membrane of chicken embryos (CAM). Eggs inoculated with virus and 2400 µg/dose of propolis, previously incubated for four hours, presented reduction in the pox lesions number ($p<0.05$) in relation to the positive control, besides reduction in the number of intracytoplasmatic inclusion bodies and in the vacuolar degeneration score of epithelial cells from mesoderm CAM. After eight hours of incubation with the virus, the same concentration of propolis completely inactivated APV ($p<0.0001$) and in concentrations ten times lower (240 µg/dose) significantly reduced the pox lesion numbers and the histopathology findings ($p<0.05$). This product from bees presented virucidal activity depending on the dose and the incubation time with the virus before the inoculation. Although further research is needed, the activity of green propolis against APV can represent a new approach to virucidal or antiviral drugs development.

Key words: Green propolis, virucidal activity, *avipoxvirus,* chorioallantoic membrane.

INTRODUCTION

Vaccination remarkable success, reducing the incidence or even allowing the eradication of several diseases, represents one of the greatest achievements of modern human and veterinarian medicine (Amanna and Slifka, 2009). However, an efficient vaccine may not be available for several months after the outbreak of a pandemic, highlighting the importance of using virucidal or antiviral drugs as tools for prevention and treatment of this kind of infection (Arino et al., 2009).

The use of virucidal or antiviral drugs are not completely innocuous (van Boven et al., 2008) and, in large scale, has the potential of inducing new resistant viral strains to appear (McCaw et al., 2008; Handel et al., 2009), reducing the efficacy of this medicines and jeopardizing their use for epidemic or pandemic control. According to Mahy and Kangro (1996), virucidal agents inactivate virus due to their physicochemical characteristics. These agents are usually more effective in viruses that are outside of their host cells.

During the last years a reduced number of virucidal drugs has been approved, despite the significant effort

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that has been put into the development of efficient therapies against infections caused by viruses (Graci and Cameron, 2008). In addition, some viruses like RNAs have extremely high mutation levels, contributing even more to a fast appearance of resistant viral strains, highlighting the need for new research searching for alternative substances that can destroy these microorganisms. Therefore, natural and phytotherapic compounds have raised the interest in searching for new pharmaceuticals. Propolis, a resinous substance produced by honey bees from exudates collected from different parts of plants (Fischer and Vidor, 2008), presents a several biological activities (Fischer et al., 2007a, b; Vatansever et al., 2010; Gregoris and Stevanato, 2010), even though many of its action mechanisms are unknown. Propolis pharmacological activity against several viral infections has been evaluated in studies with influenza virus (Serkedjieva et al., 1992), HIV (Ito et al., 2001), adenovirus (Amoros et al., 1992), and herpes simplex viruses (Schnitzler et al., 2010; Nolkemper et al., 2010). The wide spectrum of propolis biological activities together with the need for new virucidal substances, renew the interest for this bee product regarding its antimicrobial potential (Nolkemper et al., 2010).

Poxviruses, from *Poxviridae* family, are double stranded DNA viruses which infect humans as well as animals. *Avipoxvirus* (APV), when inoculated in the chorioallantoic membrane (CAM) of embryonated chicken eggs, causes a whitish color lesion called pox lesion, which is essentially an area of inflammatory response from the invasion of the virus in the membrane epithelial cells (Mahy and Kangro, 1996). Therefore, CAM of chicken embryos is an excellent material to study the development of a large number of different viruses like APV, what makes it a reliable and useful biological model as well as accepted by the antivivisectionists (Nóbrega et al., 2008). This study aims at evaluating *in vitro* virucidal activity of green propolis ethanol extract against APV, after incubation for different periods and inoculated in the CAM of chicken embryonated eggs.

MATERIALS AND METHODS

The experiments were carried out in the Virology and Immunology Laboratory of Veterinary Faculty of Federal University of Pelotas (UFPel – Brazil) in collaboration with the Regional Laboratory of Diagnosis of the Veterinary Faculty of UFPel and were approved by the Ethical Committee in Animal Experimentation from UFPel (process 7962).

Green propolis ethanol extract

The ethanolic extract utilized was identical to that previously described (Fischer et al., 2007a). After evaporation of the solvent, the resulting dried matter was dissolved in phosphate solution (pH 6.2), in a final concentration of 240 mg/ml. During the experiment the solution was kept under refrigeration at 4°C. The chemical composition of the green propolis extract was determined by high performance liquid chromatography (HPLC), according to Fischer et al. (2007b).

Virus

A vaccine sample of APV (strong strain, pigeon virus), produced in embryonated chicken eggs, was kindly supplied by Laboratório Bio-Vet S/A (Vargem Grande Paulista, SP – Brasil). The lyophilized virus was reconstituted in 1 ml of diluent according to the supplier instructions, at the moment of its use. The initial titre was 1×10^5 pfu/egg ml.

Embryonated eggs

To determine the cytotoxic effect of the ethanol extract of green propolis, as well as the definition of the APV dilution to be used in the experiments, it was used has one hundred embryonated eggs incubated for nine, from heavy 62 week-old hens, not vaccinated for APV. The embryonated eggs supplied by Conjunto Agrotécnico Visconde da Graça (CAVG – UFPel - Brazil), were kept in an incubator at 37°C, with humidity controlled at 60%. The experiments were approved by the Ethics Committee for Animal Experimentation (CEEA/UFPel).

Propolis cytotoxicity tests and determination of APV infecting dose

Tests were performed with the aim of evaluate the green propolis ethanolic extract toxicity to the embryo and to the CAM of the embryonated chicken eggs. In order to do that, different propolis concentrations were tested: 2400, 240 and 24 µg/dose. These doses were based on studies about antiviral activity previously performed by our group (G. Fischer, Federal University of Pelotas, Brazil, personal communication). CAM inoculation was carried out as previously described (Lierz et al., 2007). After opening the egg shell and displacement of the membrane, the propolis test concentrations were inoculated in triplicate in a volume of 100 µl/egg. After incubation for 5 days at 37°C, the eggs were opened for evaluation of possible lesions to the CAM and to the chicken embryo.

In order to determine the APV concentration to be used in the evaluation of the propolis virucidal activity, the vaccine sample was reconstituted according to the manufacturer's recommendations and tested in the dilutions 1:100 (1×10^3 pfu/egg ml), 1:1000 (1×10^2 pfu/egg ml) and 1:2000 (0.3×10^2 pfu/egg ml), in triplicate. Thus, it was determined a viral concentration in which the embryo did not suffer hemorrhage or death, but which allowed the observation of the pox lesions in the CAM. APV dilutions were inoculated in embryonated eggs following the method previously described (Lierz et al., 2007).

In vitro virucidal activity of the green propolis ethanol extract

APV sample in the concentration 1×10^2 pfu/egg ml was incubated with propolis extract at the following concentrations: T1 = 2 400 µg/dose, T2 = 240 µg/dose, T3 = 24 µg/dose and T4 = 0 µg/dose (negative control), for zero, four and eight hours, at 22°C. Then, each treatment was inoculated on the CAM of six eggs, in a final volume of 200 µl (100 µl of APV + 100 µl of propolis in the treatments 1, 2 and 3 or 100 µl of APV + 100 µl PBS in treatment 4). The eggs were incubated at 37°C for five days and then opened for CAM evaluation. The virucidal activity was determined macroscopically through the observation of pox lesions on the

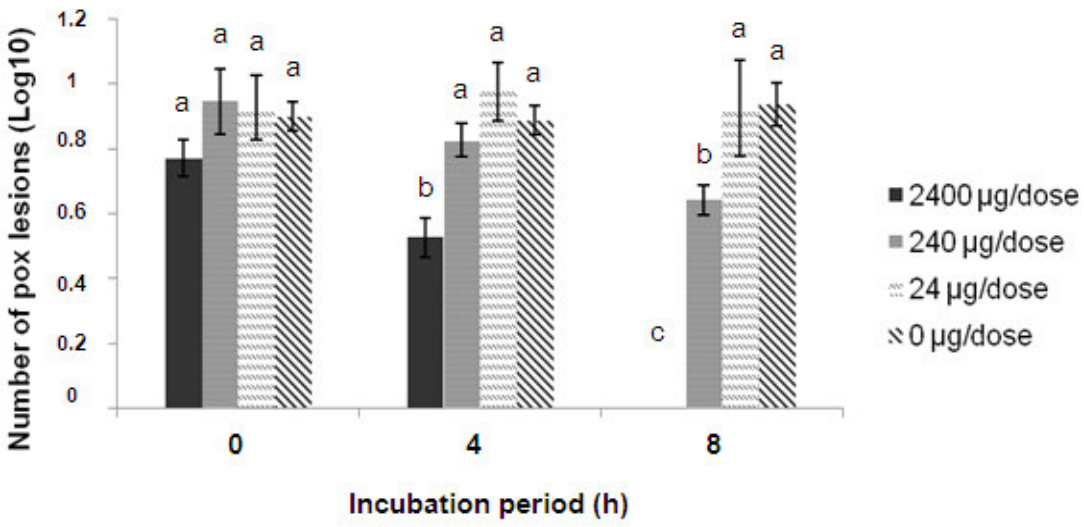

Figure 1. Mean ± standard deviation of pox lesions in chorioallantoic membrane (CAM) of embryonated chicken eggs (Log_{10}) after inoculation with *avipoxvirus* (APV) associated to 2400 µg/dose, 240 µg/dose, 24 µg/dose or 0 µg/dose of Green propolis ethanolic extract, after zero, four or eight hours of incubation of the virus and propolis at 22°C. Different letters in the same period of incubation represent statistical difference ($p<0.05$) by Tukey test.

membrane and by the histopathology analysis of the lesions.

Histopathology

A CAM of each treatment and in different periods of incubation, randomly chosen, was fixed in 10% formol for further inclusion in paraffin wax. Using a microtome, 5 µm thin cuts were obtained, which were placed on glass slides and stained by hematoxylin and eosin (Allen, 1994). Under optical microscope visualization, epithelial hyperplasia, vacuolar degeneration, inclusion corpuscles, inflammation and congestion/edema were analyzed and classified by lesion scores as: severe lesions (+++), moderate lesions (++), mild lesions (+) and absence of lesions (-).

Statistical analysis

The number of pox lesions was converted into Log_{10}. Variance analysis was performed by General Linear Models procedure using the statistics package SAS 8.0 (SAS, 2001). Variables that presented statistical differences to F test were submitted to Tukey´s test ($p<0.05$), in order to identify differences among the means of the treatments.

RESULTS

Propolis cytotoxicity and determination of the APV infecting dose

Macroscopic alterations in the CAM or damage to the embryos were not observed in any propolis concentration tested. Therefore, it was decided to use the tested concentrations to evaluate the virucidal capacity of the green propolis ethanolic extract.

The 1 x 10^2 pfu/egg ml APV concentration was the one that allowed better visualization of the pox lesions on the CAM, therefore it was selected for the subsequent evaluations. The eggs inoculated with 1 x 10^3 pfu/egg ml of the virus presented a much higher number of pox lesions, making impossible their counting and correct characterization. In the 0.3 x 10^2 pfu/egg ml concentration, the number of pox lesions was low or nonexistent, making its use to evaluate the virucidal capacity of green propolis ethanol extract impossible.

Virucidal activity of green propolis ethanol extract

No statistical difference was observed among the treatments ($p>0.05$) when APV and green propolis ethanol extract were associated and immediately inoculated on CAM (no incubation), as it can be observed in Figure 1. However, a reduction in the number of pox lesions on CAMs inoculated with the virus associated to 2400 µg/dose of propolis extract (Figure 1), was observed.

When APV and propolis were incubated for four hours at 22°C before being inoculated in embryonated eggs, the use of 2400 µg/dose of green propolis ethanolic extract presented significant reduction on the number of lesions ($p<0.05$), as shown in Figure 1. The number of lesions dropped from 0.888 (log_{10}) in the control treatment in which the virus was inoculated without propolis, to 0.527 (log_{10}). In addition, even though statistical difference was not observed, the use of 240 µg/dose of propolis allowed a reduction in the number of

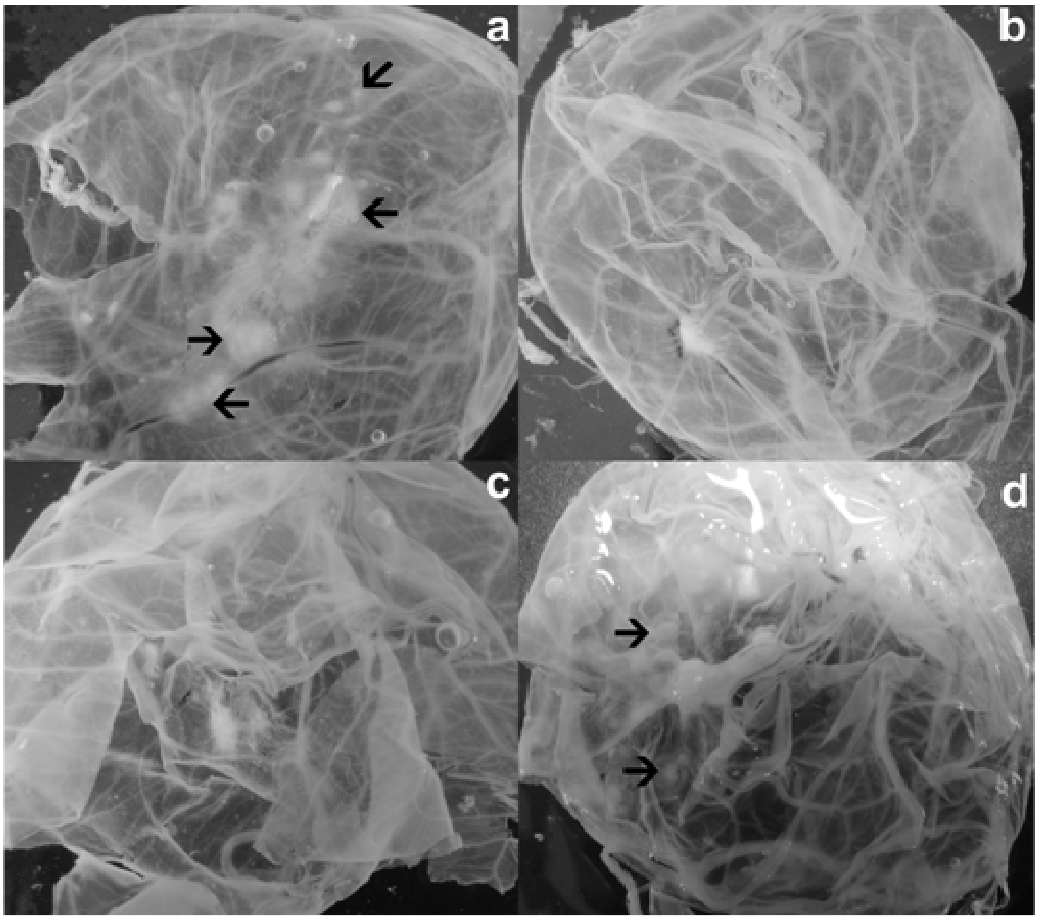

Figure 2. CAM of embryonated chicken eggs fixed in formaldehyde 10%, inoculated with APV and different concentrations of green propolis ethanolic extract, after eight hours of incubation: (a) 0 µg/dose of propolis; (b) 2400 µg/dose of propolis; (c) 240 µg/dose of propolis; (d) 24 µg/dose of propolis. ↑ = pox lesions on chorioallantoic membrane.

pox lesions to 0.827 (log_{10}). After eight hours of incubation of APV with propolis, its virucidal effect became more evident (Figures 1 and 2). While in the control treatment (Figure 2a) it was observed 0.938 (log_{10}) pox lesions, the use of higher concentration of propolis (2400 µg/dose) inactivated completely the virus (Figure 2b), once pox lesions on CAM of inoculated eggs were not observed ($p<0.0001$). Besides, there was a reduction ($p<0.05$) in the number of lesions between the control treatment and T2 (APV incubated with 240 µg/dose of propolis extract), which presented 0.640 (log_{10}) pox lesions.

Histopathology

The negative control CAM, without virus, did not present any histological alteration characteristic of APV proliferation (Figure 3a), whereas in the positive control it was observed proliferation of CAM epithelial cells with vacuolar degeneration and presence of viral eosinophilic inclusion bodies in the cytoplasm of epithelial cells (Figure 3b). As seen in Table 1, the lesions have higher scores when the virus and propolis were associated and immediately inoculated on CAM (without incubation). Regardless the propolis concentration used, histological lesions observed on CAM were similar. Using 2400 µg/dose of propolis extract, it was observed an accentuated proliferation of epithelial cells in the ectoderm and endoderm of the CAM, with vacuolar degeneration and viral eosinophylic inclusion bodies in the cytoplasm of epithelial cells (Figure 4a). In the mesoderm it was observed a diffused and pronounced inflammatory infiltrate, constituted of heterophyllous and some lymphocytes, besides edema, congestion and heterophyllous in the blood vessels. With 240 or 24 µg/dose of propolis extract, multifocal and moderate lesions were observed.

After four hours of incubation of APV with green propolis extract, the histological lesions observed were

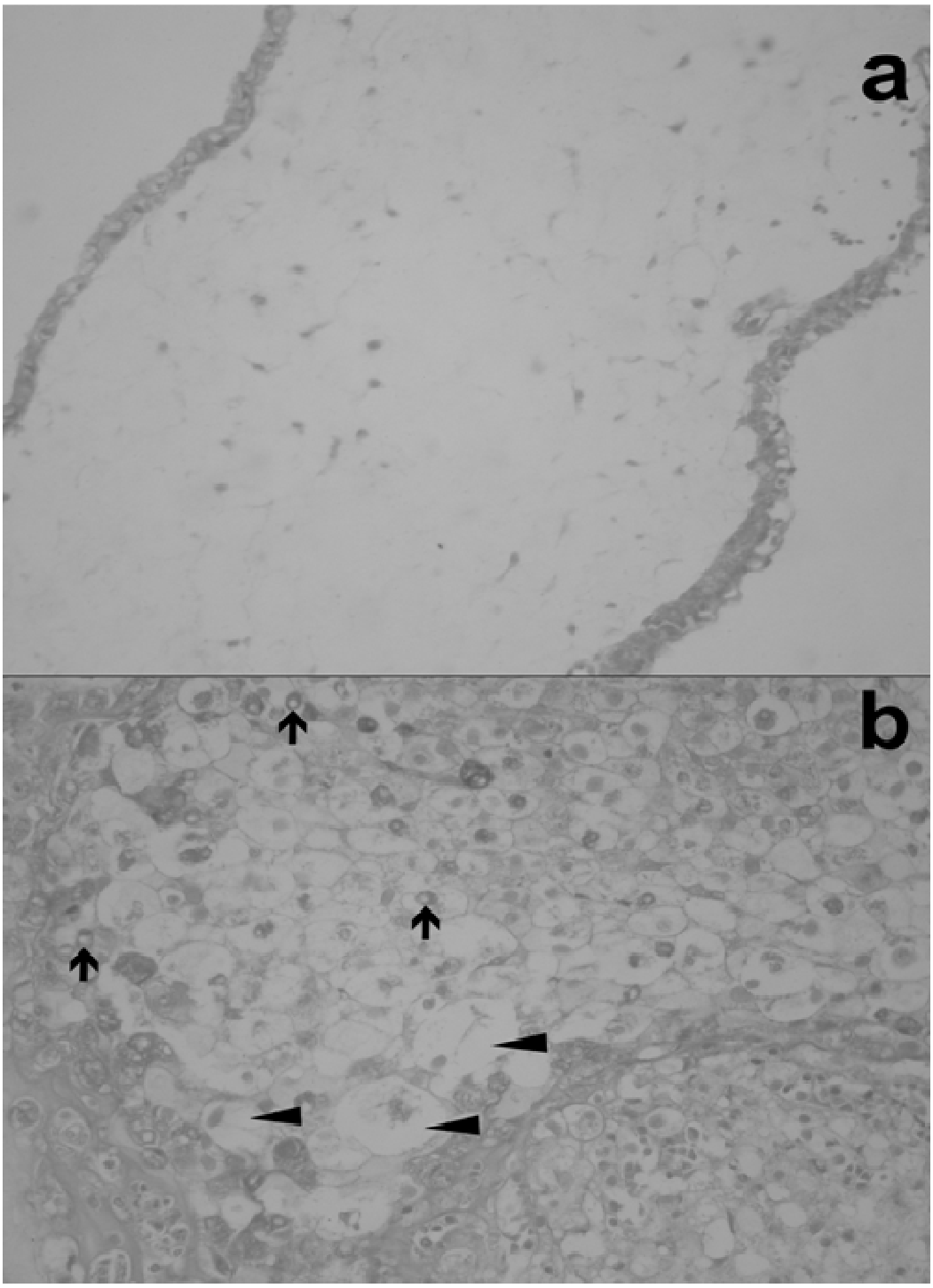

Figure 3. CAM of embryonated chicken eggs stained with hematoxylin eosin, observed under 40x optical microscope: (a) negative control; (b) positive control. ↑ = eosinophilic intracytoplasmic inclusion bodies of epithelial cells from mesoderm; ◄ = vacuolar degeneration of epithelial cells from mesoderm.

similar to those observed without incubation, but with lower scores (Table 1). The use of 2400 µg/dose of propolis allowed a reduction in score of the inflammatory process and the presence of eosinophylic inclusion bodies in the cytoplasm of epithelial cell, from severe to mild lesions (Figure 4b). In the 240 µg/dose there was a reduction in the score of congestion, edema and inflammation to mild lesion in comparison with the

Table 1. Characterization of histological lesions in the CAM of embryonated chicken eggs after inoculation of *avipoxvirus* associated to different concentrations of green propolis ethanol extract, in three periods of incubation.

Hours	Treatment (µg/dose)	Epitelial hyperplasia	Vacuolar degeneration	Inclusion bodies	Inflamation	Congestion / edema
0	2400	+++[a]	+++	+++	+++	+++
	240	+++	++[b]	+++	+++	+++ Haemorrhage
	24	++	++	++	+++	+++
	0	+++	+++	+++	+++	+++
4	2400	++	++	+[c]	+	++
	240	++	++	++	+	+ Haemorrhage
	24	+	+	+	++	++
	0	+++	+++	+++	+++	+++
8	2400	+	+	-[d]	+	+
	240	+	+	+	+	+
	24	+	+	+	+	+
	0	+++	+++	+++	+++	+++

[a] severe lesions; [b] moderate lesions; [c] mild lesions; [d] absence of lesions.

treatment without propolis (T4), whereas when the 24 µg/dose was used, the lesions were classified as mild in the parameters epithelial hyperplasia, vacuolar degeneration and in number of intracytoplasmic inclusion bodies (Table 1). When the APV and the different concentrations of green propolis ethanol extract were incubated at 22ºC for eight hours before being inoculated on CAM of embryonated chicken eggs, there was a big reduction in the lesion scores. In all treatments it was observed focal and discrete epithelial hyperplasia, hydropic degeneration and discrete inflammatory reaction in the mesoderm. Rare inclusion bodies in the cytoplasm of epithelial cells were observed with the use of 240 and 24 µg/dose, while the use of 2400 µg/dose inhibited completely the formation of inclusion bodies characteristic of APV (Figure 4c).

DISCUSSION

The use of virucidalor antiviral drugs has been proposed as a strategy to avoid the risk of pandemia, like the recent one caused by the influenza A-H1N1 virus (Handel et al., 2009). However, the indiscriminate and extended use of these substances can cause toxicity problems and resistance to drugs, resulting reduction in its effectivity against the pathogen (Handel et al., 2009; van Rompay, 2010). Therefore, the success in the prevention and treatment of many diseases caused by viruses is intimately related to the development of new drugs, or even the improvement of the existing ones (Freestone, 1985). A very important source of new chemical compounds is the abundant number of molecules found in natural products, which have demonstrated virucidal properties.

In this study, the *in vitro* virucidal activity of green propolis was evaluated when an ethanolic extract was incubated with APV and then inoculated on CAM of chicken embryos. It was not observed virucidal activity when the different concentrations of propolis evaluated were added to the virus and immediately inoculated to CAM without incubation. According to Mahy and Kangro (1996) and Huleihel and Isanu (2002), virucidal agents inactivate viruses due to their physical and chemical characteristics and are usually more effective when the virus is out of its host cells. In this case, as the virus and propolis extracts were immediately inoculated after their association, there was not enough time for the propolis compounds to act on the virions, allowing infection of the CAM ectoderm epithelial cells and, consequently, the lesions observed in the histopathological analysis.

Virucidal activity of green propolis was clearly observed when APV and propolis were incubated for four hours at 22°C before inoculation on embryonated eggs. The effect was even more evident with the highest concentration propolis and the eight hour incubation period, when complete virus inactivation was observed, as shown in Figure 2b. Besides, there was a statistically significant reduction ($p<0.05$) in the number of lesions between positive control treatment and T2, in which APV was incubated with 240 µg/dose of propolis extract. These data corroborate with the ones obtained by Amoros et al. (1992), who detected virucidal activity of a French propolis sample against herpes simplex virus type 2 (HSV-2) and the vesicular stomatitis virus. According to these researchers, inactivation of viruses was dependent on the incubation time and concentration of the propolis

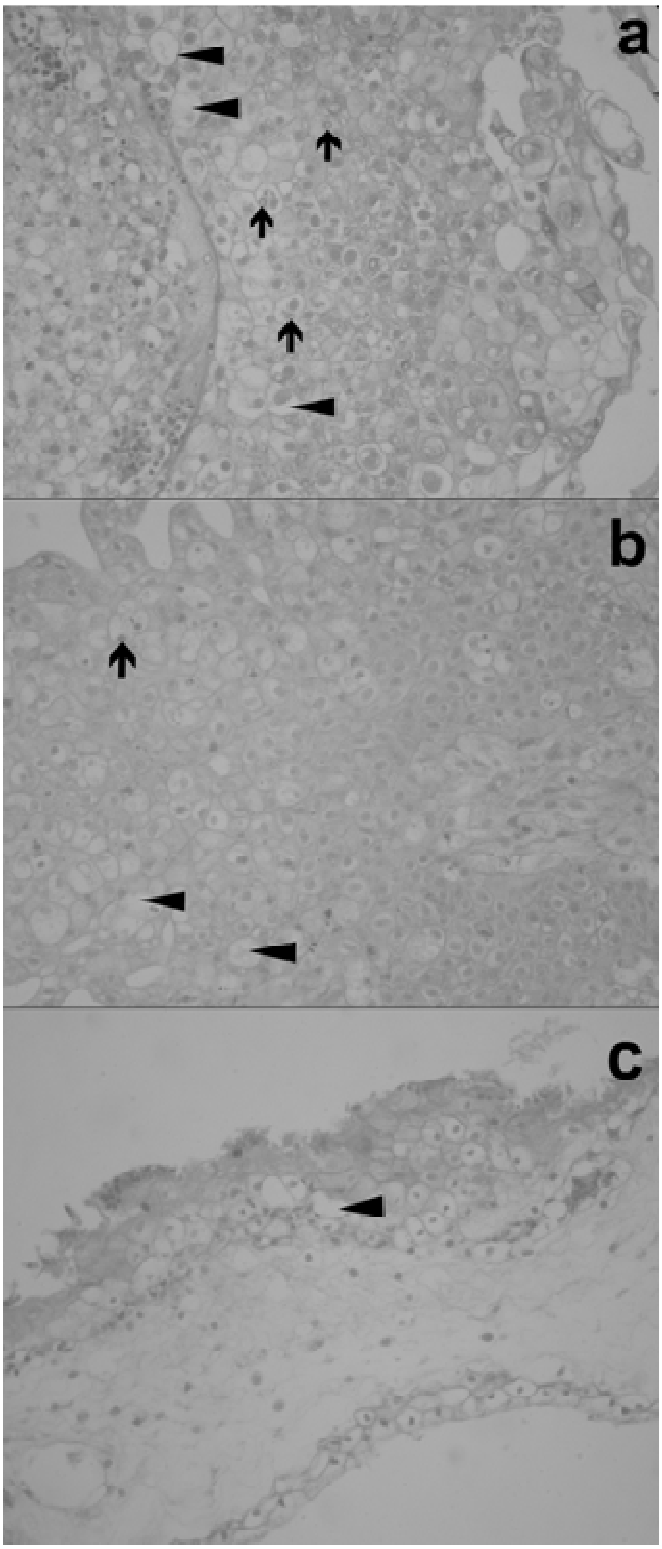

Figure 4. CAM of embryonated chicken eggs stained with hematoxylin eosin, observed under optical microscope: (a) APV + 2400 µg/dose, without incubation, 40x; (b) APV + 2400 µg/dose of propolis, 4 h incubation, 40x; (c) APV + 2400 µg/dose of propolis, 8 h incubatiion, 20x. ↑ = eosinophilic intracytoplasmic inclusion bodies of epithelial cells from mesoderm; ◄ = vacuolar degeneration of epithelial cells from mesoderm.

used. More recently, Schnitzler et al. (2010), using aqueous and ethanolic extracts of a Czech propolis sample detected virucidal activity against herpes simplex viruses. Both propolis extracts exhibited high anti-HSV-1 activity when the viruses were pretreated with these extracts prior to infection. In a study performed by our group (Fischer et al., 2007b), aiming at characterizing the immunomodulator effect of the same green propolis ethanol extract used in the present study, a chromatographic analysis high performance liquid chromatography (HPLC) showed high levels of phenolic compounds and cinnamic acid and its derivative. Flavonoids corresponded to 22.37% of the dry extract (Fischer et al., 2007b). It is likely that the virucidal activity of propolis are not caused only by a single chemical compound, but by a synergic action between the several constituents (Nolkemper et al., 2010). The virucidal activity observed in the present study is probably related to the high levels of phenolic compounds and flavonoids found in the green propolis extract used. Similar result was obtained by Nolkemper et al. (2010) who found strong virucidal activity against herpes simplex virus type 2 (HSV-2) using aqueous and ethanol extracts from a Czech propolis sample, rich in phenolic compounds and flavonoids.

The virucidal activity of green propolis ethanolic extract was also observed in the histopathological evaluation. Without a period of incubation of the APV and the extract before inoculation, there was no virucidal effect. However, after four hours of incubation, the virucidal effect became evident. The use of 2400 µg/dose of ethanol extract allowed a reduction in the scores of inflammation and inclusion bodies, presenting only mild lesions, while the control treatment had severe lesions for all the parameters analyzed. The use of lower propolis concentration (24 µg/dose) allowed a reduction in the scores of epithelial hyperplasia, vacuolar degeneration and inclusion corpuscle. The inflammatory process, the formation of intracytoplasmic eosinophilic inclusion bodies, as well as the proliferation of ectoderm and endoderm epithelial cells are histopathological lesions characteristic from APV. The reduction in the scores of these lesions demonstrates clearly the virucidal activity of the green propolis used.

The full virucidal action of the green propolis ethanol extract was observed after eight hours of incubation with APV. Besides avoiding the appearance of pox lesions characteristic of the virus, the use of 2400 µg/dose of propolis resulted in the absence of inclusion bodies (Figure 4c), pathognomonic of the virus. Moreover, the scores of all analyzed parameters (Table 1) were reduced.

In this study, a green propolis ethanol extract showed virucidal effect against APV when inoculated on CAM of chicken embryos. This effect was dependent on the concentration of propolis used, as well as the incubation time of the virus and the ethanol extract. The complete

inhibition of the virus was obtained with eight hours of APV incubation with 2400 µg/dose of propolis, expressed by the absence of pox lesions and intracytoplasmic inclusion bodies.

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