

In Vitro ACTIVITY OF PROPOLIS ON DOMESTIC ANIMAL VIRUSES: A REVIEW

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SUMMARY

Propolis is a natural resinous mixture produced by bees, based on substances collected from plants and its exudates. The ancient Greeks, Romans and Egyptians were aware of the healing properties of propolis and granted it extensive use in medicine. Propolis varies in components and proportion of active substances depending on the flora of each region where it is produced. Important biological properties of propolis as a bactericide, anti-

ti-parasitic, fungicide, immunomodulatory, antioxidant and antiviral agent have been demonstrated. The objective of this work was to explore the uses of propolis in studies of animal viral diseases through a thorough search of information in the main available databases. The paper reviews the main reported in vitro effects of propolis on viruses that cause domestic animal diseases, showing the prospects in this emerging field of study.

 Propolis is a resinous material produced by bees (*Apis mellifera*) using wax and plant exudates. The word propolis is believed to have been given by Aristotle to indicate that propolis is used to protect and defend the hive (Alves *et al.*, 2013). Propolis means 'before the city' or 'defender of the city' (Greek *pro*: in defense, and *polis*: city). In the beginnings of Greek civilization, Aristotle observed that propolis had the ability to defend a city with thousands of residents, the hive.

Propolis was useful for both structural repair and for maintenance of the species through the preparation of aseptic places for the deposit of eggs of the queen bee (Kuropatnicki *et al.*, 2013). Propolis is important for the bees, since it is an adhesive material used to seal openings and cracks in the hive (Bankova *et al.*, 2012), and is also used to smooth internal walls (Burdock, 1998) and to protect the colony from diseases and cover the corpses of intruders that have died in the hive, preventing their decomposition (Bankova

et al., 2000). It has been observed that bees collect the protective resin from flowers, leaves and buds with their jaws and then take them to the hive on its hind legs, its color varying from green to reddish brown depending on its botanical source (Kuropatnicki *et al.*, 2013).

Propolis has been widely used since antiquity. The Egyptians took advantage from the anti-putrefaction properties of propolis to embalm their dead and the Greek and Roman physicians used it as an antiseptic and healing

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medicine (Sforcin and Bankova, 2011). In the last decades numerous articles have been published describing different aspects of the biological properties of propolis (Rocha *et al.* 2013. However, in the majority of them, the information is limited (Sforcin and Bankova, 2011). Currently, propolis is used as a folk remedy and is available in different pharmaceutical forms (capsules, gel, powder, mouthwash, cream and powder) and combinations thereof (Castaldo and Capasso, 2002). Propolis has become popular constituent that complements health care products, as a natural ingredient in the pharmaceutical industry and in food preparation. Antimicrobial, antioxidant, antiviral, antifungal, anti-inflammatory, immunomodulatory and anti-tumoral activities are among the therapeutic properties that have been studied (Monzote *et al.*, 2012; Chan, 2013).

The most common methods for propolis extraction uses ethanol as solvent in different mixtures of water and ethanol (Rocha *et al.*, 2013). The precise composition of propolis can be determined through chemical analysis by HPLC (Castro, 2001). As with honey, propolis composition varies with a variety of factors, such as the source of the exudates, the climate and environmental conditions (Chen and Wong, 1996). The presence of at least 300 compounds has been identified, out of which approximately 50% correspond to resins, 30% to wax, 10% to essential oils, 5% to pollen and 5% to other organic compounds (Gómez *et al.*, 2006). Among these organic compounds, it is possible to find phenolic compounds, esters and flavonoids (flavonols, flavones, flavonones, dihydroflavonols and chalcones), terpenes, steroids, aromatic beta-aldehydes, alcohols, sesquiterpenes and stilbene (Aga *et al.*, 1994; Russo *et al.*, 2002).

Propolis has been used in veterinary medicine in different fields and pharmaceutical forms and in different animal species, among which have been reported: solutions for the prevention and control of foot diseases in sheep (Bogdanov, 2012), mammary infusion for treating mastitis, antidiarrheal powders, boluses and injectable solutions in genitourinary diseases such as endometritis (Bogdanov, 2012; Martos *et al.*, 2008), eyedrops and ointments for keratitis and keratoconjunctivitis infectious; tinctures and pomades for wounds, disinfectant solutions and repellents as therapy in calf omphalitis (Burdock, 1998), and it has also been used against rabbit infections by *Pasteurella multocida* (Gutiérrez, 2011).

Antiviral activity

Propolis is capable to inhibit viral propagation. Most of the studies published describe activities against viruses that affect humans. *In vitro* studies demonstrate the effects of propolis over both DNA and RNA viruses. The observed effects include reduction in viral multiplication and even a virucidal action (Amoros *et al.*, 1992), but the mechanism of the antiviral action of propolis is still unclear. The first hints about the mechanism of antiviral action of propolis was given by Selway (1986) by mentioning that the flavonoids compounds can be related to the inhibition of viral polymerase and the binding of viral nucleic acid or viral capsid proteins (Selway, 1986). The mechanism of action could suggest a receptor cell membrane blockage by the propolis, or propolis interactions that induce internal changes in the host cells, which in turn affects the virus replication cycle (Amoros *et al.*, 1992). Other authors state that the effect is due to the flavonoids and other phenolic compounds that are present in propolis and interact with viral proteins, forming complexes unstable compounds and therefore altering the stages of adsorption and penetration (Selway, 1986; Amoros *et al.*, 1992).

Different components of propolis appear to act synergistically, which would explain the fact that honey and propolis possess a greater antiviral activity than their individual components (Kujumgiev *et al.*, 1999). The antiviral activity of propolis has been tested, with promising results, against some pathogenic viruses to humans (Gutiérrez, 2011) and animals (Amoros *et al.*, 1992), such as the herpes simplex virus type 1 (HSV-1; Hegazi *et al.*, 2001) and type 2 (HSV-2), human immunodeficiency virus (HIV) (Gekker *et al.*, 2005) and avian influenza (Bogdanov, 2012). However, few studies have been published regarding the use of propolis in viral diseases of veterinary interest. This review describes the biological properties of propolis antiviral mentioned specifically in veterinary medicine widening the prospect of use.

Uses and Perspectives of Propolis in Animal Viral Diseases

Poultry

Hegazi *et al.* (2001) used propolis of four different origins (Egypt, Austria, France and Germany) to prove the antiviral activity of propolis on Infectious Bursal Disease Virus (IBDV)

and Avian Reovirus (ARV). The active components of propolis were identified by mass spectrometry. To assess the infectivity of the virus and the antiviral effect of propolis, primary cultures of chicken embryo fibroblasts (CEF) were used. All the propolis varieties used caused a reduction in the titer of IBDV and ARV, as determined by its TCID₅₀. Further, when the cytopathic effect (CPE) was evaluated, they found that the degree of reduction was different in each of the samples, but all of them reduced viral infectivity at different degrees. In this study the Egyptian propolis showed the greatest activity against ARV and IBDV. Authors suggest that propolis show qualitative similarities between them and the quantitative differences obtained could be due to the participation of different popular species, therefore the reduction of infectivity is completely dependent on the chemical composition of each propolis (Hegazi *et al.*, 2001; Bankova *et al.*, 2002; Sforcin and Bnkova, 2011).

The components of three propolis originating from different regions of Egypt (Dakalia, Ismailia and Sharkia) were determined by mass spectrometry and gas chromatography. The antiviral activity against IBDV and ARV was also evaluated by determining the viral titer (TCID₅₀) in cultures of chicken fibroblasts. The sample from Dakalia showed the presence of aliphatic acids, aromatic acids, esters of aromatic acids, flavonoids and some triterpenoids; a total of 65 compounds were identified. The composition of propolis from Sharkia does not vary significantly from the Dakalia propolis, while propolis from Ismailia does not contain aromatic acids, aromatic esters (except phthalate esters) and flavonoids (except hexamethoxyflavone). The propolis that produced the largest reduction in viral titer were Dakahlia against IBDV and Ismailia versus ARV. On the other hand, propolis from Sharkia showed moderate activity against both viruses. Reduced infectivity depends on the chemical composition presented by the propolis samples from the three provinces. A reduction in cytopathic effect was observed, but this changed with the chemical composition of each of the samples (Abd El Hadya and Hegazi, 2001).

The activity of propolis has also been tested in conjunction with other components, as is the case of the studies carried out using the Newcastle disease virus (NDV) and IBDV (Kong *et al.*, 2006). They analyzed eight extracts of propolis from Egypt in which 42 polyphenolic components were determined by HPLC. Thirteen aromatic acids were found, as well as esters and alcohols, and

29 flavonoids, 6 of which had not been previously reported. Viral titration was performed in cultures of fibroblasts. The determination of antiviral activity was made by mixing an equal volume of serial dilutions of each virus with stock solutions of propolis, and the antiviral activity of the samples was determined by the decrease in the TCID₅₀ in chicken embryo fibroblasts. All propolis samples led to a reduction in TCID₅₀ of IBDV and NDV, and the reduction varied according to the origin of propolis. Similar results were reported with herpes simplex virus (Abd El Hadya *et al.*, 2007), avian influenza virus (Amoros *et al.*, 1992) and infectious bursal disease virus and reovirus (Abd El Hadya *et al.*, 2007).

On the other hand, (Kong *et al.*, 2006), determined the effectiveness of four components of a prepared Chinese herbal product (CHI) in chickens, both *in vitro* and *in vivo*. The product used included astragalus polysaccharide (ASP), isatis root polysaccharide (IRP), propolis polysaccharide (PP) and epimedium flavone (EF). Animals distributed in 10 groups were used, with a total of 200 animals. Chickens were inoculated with the IV strain of NDV by intranasal and intraocular administration. Other groups were administered for 3 consecutive days with 0.5ml of CHI subcutaneously, corresponding to different doses/kg body weight of APS and IRP. Control groups (CHI-free control and a vaccination control and CHI-free) of chickens were injected with physiological saline. The results indicate that most of the CHI used in appropriate concentrations were effective. The administration of CHI to the vaccinated chickens increased the concentration of antibodies anti-Newcastle in serum, as determined by hemagglutination inhibition, compared to single administration of Newcastle. The effect on the production of antibodies was observed 21 days after the start of vaccinations.

Bovines

There are studies performed on cattle about the antiviral activity of propolis experimentally used to counteract the causal agents of diarrhea syndromes, as are the presence of enterotoxigenic bacteria *Escherichia coli*, *Cryptosporidium* sp. and rotavirus in groups of calves. Two schemes of treatment were applied: group A was treated orally with a propolis solution, while the group B was subjected to standard therapy with oximicine. The results revealed that animals treated with propolis showed a better recovery at 24, 48, and 72h. When comparing the percentage of reco-

vered animals with those obtained in group B it was determined that these differences were statistically significant (Bernal, 1991).

Porcines

An *in vitro* evaluation was carried out of the antiviral effect of a Mexican propolis on pseudorabies virus (PRV) by infecting cultures of MDBK cells (González, 2015). In order to infect these cultures with the Shope strain of PRV, an infective dose was determined and, subsequently, an ethanol extract of propolis (EEP) was allowed to interact with the virus (2h before, during and 2h after infection). Also, in order to determine the effects of EEP on virus, samples from cell cultures subjected to the different previously mentioned treatments were processed for transmission electron microscopy (TEM). It was observed that administration of EEP two hours before infection resulted in a reduction in the number of plaque forming units compared to the other treatments or with the infected culture without treatment. The difference found was statistically significant. Under TEM, viral particles with altered structure were observed, suggesting the occurrence of damage to proteins of the viral envelope, causing structural destabilization, and an electron-dense layer was also observed around the membrane of cells in which viral particles were found, so that propolis seems to affect both penetration and the viral replication cycle. In another work, the extract aqueous of Israel propolis was tested on cultured Vero cells infected with Herpes Simplex Virus type 1 (HSV-1), treating the cultures with propolis at various concentrations for 2h before and 2h after completion of the infection. The cultures were maintained for 10 days and resulted in a lower CPE with the more concentrated extract of propolis, showing that the treatment applied prior to infection resulted in less penetration of the virus into the host cell. In this same study, the effect of the virus was evaluated when applied after the treatment with propolis, without changes in the cytopathic effect. On the other hand, when virus and propolis were applied simultaneously to the culture, similar results were obtained as in the pre-treatment, in which a lower cytopathic effect was shown to take place (Mahmoud y Isanu, 2002).

Similar results were obtained by Schnitzler *et al.* (2010), who studied the antiviral effect in cell culture of aqueous and ethanolic extracts of propolis, as well as that of caffeic, p-coumaric and benzoic acids, galangin, pino-

cembrin and chrysin, against Herpes Simplex Virus type 1 (HSV-1). The two extracts of propolis showed high levels of antiviral activity against HSV-1, as plaque formation was significantly reduced when applied before viral infection. The compounds galangin and chrysin showed significant antiviral activity when compared to the rest. The study shows that extracts differ quantitatively and qualitatively, indicating that the ethanolic extract has more flavonoids and therefore its greater antiviral capacity. The activity of each propolis is reflected in the decrease in the cytopathic effect in relation to the exposure time and the concentration at which they are used in the tests (Schnitzler *et al.* 2010).

Dog and cat

The antiviral activity of two ethanol extracts of propolis (EP1 and EP2) from two different sources against feline calicivirus (FCV), canine adenovirus type 2 (CAV-2) and Bovine Viral Diarrhea Virus (BVDV) was determined by Cueto *et al.* (2011). The choice of these viruses is justified because the CAV-2 and FCV are etiologic agents of respiratory diseases in the respective species, while BVDV has been widely used as a model to study the antiviral activity against hepatitis C. In the analysis of propolis extract by high pressure liquid chromatography, the presence of flavonoids such as rutin, quercetin and gallic acid was detected. In the chromatogram, it is possible to visualize the presence of different peaks between samples of the commercial propolis (EP2) and the propolis extract obtained in the laboratory (EP1). There were quantitative differences in the chromatographic peaks, especially in the rutin fraction. For evaluation of antiviral activity of EP1 and EP2 diverse cells (MDBK, MDCK and CRFK) were infected with the viruses (25 µl/well of viral dilution), incubated for 2h and the inoculum was removed. A negative control (cells and medium) and a positive control (viruses, cells and medium) were included. The extracts were added: I) before viral inoculation (interaction of cells-extract for 1 h); II) after the viral inoculation (the extracts were added immediately after inoculation) and III) before and after viral inoculation. After three days of incubation, the culture medium was removed and cell viability was quantified by determining the degree of metabolization of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan. Under these conditions the median effective concentration (EC₅₀), which is the concentration of propolis extract that can

inhibit the cytopathic effect of the virus in the 50% of the cultures, was determined. The results (Cueto *et al.*, 2011) showed that when the extracts are added before infection an increased antiviral effect is observed. The activity of the extract obtained in the laboratory (EP1) was superior to the commercial one (EP2). Both propolis extracts showed higher activity against BVDV than to the other two viruses, although the differences between BVDV and CAV-2 were small. BVDV has a lipid envelope surrounding the nucleocapsid and this could constitute a structural difference with the other two viruses and could explain the greater sensitivity of this virus to the antiviral action of the extracts.

Conclusions

Propolis appears to be a promising alternative for the prevention and possible treatment of some viral diseases in several animal species. It is noted that all the explored antiviral activities of propolis have been performed *in vitro* (Table I). It is important to translate this knowledge into clinical practice and explore their properties and their mechanism of action. On the other hand, the adjuvant properties of propolis have been explored *in vivo* with promising results, but it is essential to conduct further studies in order to know which components are responsible for the different biological activities.

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TABLE 1
EFFECTS OF PROPOLIS ON ANIMAL VIRUSES

Species	Viruses	Evidence	References
Poultry	Avian Reovirus	<i>In vitro</i>	Hegazi, 2001.
	Infectious bursal disease virus	<i>In vitro</i>	Abd El Hadya, 2001; Hegazi, 2001; Abd El Hadya <i>et al.</i> , 2007.
	Newcastle disease virus	<i>In vitro</i>	Abd El Hadya <i>et al.</i> , 2007.
Bovines	Rotavirus	<i>In vivo</i>	Bernal, 1991.
Porcines	Swine herpesvirus 1	<i>In vitro</i>	Fischer <i>et al.</i> , 2001; Schnitzler <i>et al.</i> , 2010. González, 2015.
Dogs	Canine adenovirus type 2	<i>In vitro</i>	Cueto <i>et al.</i> , 2011.
Cats	Feline calicivirus	<i>In vitro</i>	Cueto <i>et al.</i> , 2011.

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ACTIVIDAD *In Vitro* DEL PROPÓLEO SOBRE VIRUS DE ANIMALES DOMÉSTICOS: UNA REVISIÓN

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RESUMEN

El propóleo es una mezcla resinosa natural producida por las abejas a base de sustancias recogidas de las plantas and sus exudados. Los antiguos griegos, romanos and egipcios eran conscientes de las propiedades curativas del propóleo and se le otorgó un amplio uso en la medicina. El propóleo varia en componentes and proporción de sustancias activas en función de la flora de cada región donde es producido. Se ha demostrado que el propóleo posee importantes propiedades biológicas

como bactericida, anti-parasitario, fungicida, inmunomodulador, antioxidante and antiviral. El objetivo de este trabajo fué explorar el uso del propóleo en estudios con virus de origen animal mediante una minuciosa búsqueda de información en las principales bases de datos disponibles. Se reportan los principales efectos encontrados con el uso del propóleo in vitro sobre virus de animales domésticos and se muestran las perspectivas en este campo de estudio emergente.

ATIVIDADE *In Vitro* DO PRÓPOLIS SOBRE VÍRUS DE ANIMAIS DOMÉSTICOS: UMA REVISÃO

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RESUMO

O própolis é uma mistura resinosa natural produzida pelas abelhas a base de substâncias recolhidas das plantas e seus exudados. Os antigos gregos, romanos e egípcios eram conscientes das propriedades curativas do própolis ao qual lhe foi outorgado um amplo uso na medicina. O própolis varia em componentes e proporção de substâncias ativas em função da flora de cada região onde é produzido. Tem sido demonstrado que o própolis possui importantes propriedades biológicas

como bactericida, anti-parasitário, fungicida, imunomodulador, antioxidante e antiviral. O objetivo deste trabalho foi explorar o uso do própolis em estudos com vírus de origem animal mediante uma minuciosa busca de informação nas principais bases de dados disponíveis. Relatam-se os principais efeitos encontrados com o uso do própolis in vitro sobre vírus de animais domésticos e se mostram as perspectivas neste campo de estudo emergente.