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Immunomodulation produced by a green propolis extract on humoral and cellular responses of mice immunized with SuHV-1

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Abstract

Despite recent technological advances in vaccine production, most the vaccines depend on the association with adjuvant substances. This work evaluated the adjuvant capacity of an ethanol extract of green propolis associated to inactivated Suid herpesvirus type 1 (SuHV-1) vaccine preparations. Mice inoculated with SuHV-1 vaccine plus aluminum hydroxide and 5 mg/dose of propolis extract presented higher levels of antibodies when compared to animals that received the same vaccine without propolis. The use of SuHV-1 vaccine with propolis extract alone did not induce significant levels of antibodies, however it was able to increase the cellular immune response, evidenced by the increase in the expression of mRNA to IFN-γ. Besides, propolis increased the percentage of protected animals against challenge with a lethal dose of SuHV-1. The effect of green propolis extract on the humoral and cellular immune responses may be exploited for the development of effective vaccines.

Keywords: Green propolis; Adjuvant; Vaccines

1. Introduction

Vaccination has been a common practice for preventing or minimizing symptoms of diseases caused by infectious or parasitic agents in humans or animals. Traditionally, vaccines have been developed from live attenuated or inactivated pathogens [1], however synthetic peptides and recombinant proteins constitute the basis of new generation vaccines [2,3]. Despite representing an important technologic advance, the success of many of these subunit vaccines depends on their association with potent adjuvants, aiming at increasing their immunogenicity. In order to have an effective protection against the pathogen, it is necessary an efficient activation of specific effectors of the immune system, such as antibodies, cytotoxic or auxiliary T cells (Th1/Th2) [4]. Adjuvant substances when associated to an antigen modify or increase the potency of the humoral and/or cellular immune responses against that antigen [5,3]. Besides, they can be used to prolong the immune response or to promote mucosal immunity [4]. Even though a large number of adjuvants of several origins has been evaluated, most commercial vaccines continues to rely on the used of aluminum salts. This way, the development of new vaccines will be highly benefited if new substances capable of promoting and directing to an appropriate immune response were identified [4].

Propolis is a natural resinous substance, harvested by honeybees from different parts of plants such as shoots, buds and resinous exudates [6,7]. Chemically complex, propolis is composed by more than 300 different substances [7,8].

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The combination of these substances, probably resulting in a synergic effect, is essential for its biological activity [9]. Its constitutive characteristics, however, can vary according to the bee species, period of the year in which it is collected and, especially botanic origin [10]. Green propolis, characteristic from Brazil, is produced from a plant commonly known as "Alecrim do Campo" (*Baccharis dracunculifolia*). This species is not adapted to the natural conditions of other countries [11], which confers to the green propolis chemical and biological characteristics different from the European propolis, produced predominantly from the exudates of buds of aspen (*Populus* sp.) [8].

Used by bees against microorganisms like bacteria, virus and fungi [7,8], propolis has several other bioactive properties such as anti-inflammatory [12,13], antioxidant [14], antiparasitary [15] and anticarcinogenic action [16]. Although its immunostimulator and immunomodulator activities have been demonstrated [17–19], the mechanism of action remains unknown.

Suid Herpesvirus type 1 (SuHV-1), from *Herpesvirida*e family, Alphaherpesvirinae subfamily, is the causing agent of Aujeszky disease, an infecto-contagious disease responsible for high economical losses in the swine production due to high mortality and reproductive disorder [20]. The pathogenicity of this virus in mice allows its experimental use in tests to certify the efficiency of the vaccines [21].

The aim of this work was to evaluate the adjuvant properties of an ethanolic extract of green propolis, when added to inactivated SuHV-1 vaccine, used to immunize mice. Parameters of the humoral (neutralizing antibodies titer) and cellular immunity (levels of IFN-γ mRNA), as well as the protection (experimental challenge) afforded by vaccine preparations were determined.

2. Material and methods

2.1. Ethanolic extract of green propolis

Green propolis was obtained from Nectar Farmacêutica Ltda. (Brazil) and stored at −20 °C. The ethanolic extract was prepared as previoulsy described [13]. Briefly, the propolis was ground and macerated with an extract solution containing absolute ethanol, with 10 min daily agitation, for 10 days. Then, the solvent was evaporated and the resulting dried matter was dissolved in phosphate buffer solution (pH 6.2), in a final concentration of 40 mg/ml.

2.2. Vaccine preparations and inoculations

Two experiments were carried out: the first one aimed at evaluating the adjuvant capacity of the ethanolic extract of green propolis through neutralizing antibody titration and determination of 50% protective dose of experimental vaccine preparations. The second experiment was carried out to determine the levels of IFN-γ mRNA expression by reverse transcription-PCR (RT-PCR). All experimental vaccine preparations were produced from a SuHV-1 isolate obtained from an outbreak of Aujeszky disease and maintained in the Virology and Immunology Laboratory, UFPel (Pelotas – Brazil). After growing the virus in Rabbit Kidney cell line - RK_{13} (ATCC), viral suspensions with titer of $10^{5.75}$ $CCID_{50}$/25 μl (cell culture infections dose 50%/25 μl), were inactivated with bromoethylamine – BEI ($C_2H_7Br_2N$ - Merck), 20 mM, pH 7.5.

In the first experiment, 120 female BALB/c mice, 6–8 week-old, allocated into three groups, were inoculated at day 0 and 14, subcutaneously (SC) with 0.5 ml of the inactivated vaccine preparations. Group 1 received vaccine with aluminum hydroxide ($Al(OH)_3$), group 2 received vaccine with $Al(OH)_3$ and propolis extract (5 mg/dose) and group 3 received vaccine with propolis (5 mg/dose). In the fourth group (negative control), 10 animals were kept without vaccination. In order to estimate protection afforded by each treatment, using the statistic method of Reed and Muench [22], each vaccine preparation was diluted 2, 4, 8 and 16 times and inoculated in 10 individually identified mice. In all cases the initial titer of the virus suspension was the same.

In the second experiment, 25 mice distributed into five groups were subcutaneously inoculated with 0.5 ml at day 0 and 14. Group one received 5 mg/dose of propolis extract (positive control); group two was inoculated with saline solution (PBS, pH 7.2 – negative control); group three received vaccine with $Al(OH)_3$; group four received vaccine with propolis extract (5 mg/dose), and group five received vaccine with $Al(OH)_3$ and propolis extract (5 mg/dose). All animals, supplied by Biotério Central of UFPel (Pelotas – Brazil), remained isolated, in controlled environment with temperature between 22-24 °C, receiving feeding and water *ad libitum*. The experiment was approved by the UFPel Committee of Ethics in Animal Experimentation.

2.3. Humoral immunity and protection

For titering neutralizing antibodies against SuHV-1, blood samples were collected from four animals from each experimental group, 21 days after the second inoculation. After total bleeding, the blood was processed and the serum was stored at −20 °C. Antibodies were titered by the serum neutralization method [21]. Briefly, each serum was serially diluted from 1:2 to 1:256, and distributed (25 μl) in quadruplicate in polystyrene plate (TPP). A volume of 25 μl of SuHV-1 virus suspension containing 100 CCID50% was then added. After incubation for 1 h at 37 °C in an environment with 5% CO_2, approximately 30,000 RK_{13} cells were added per well. The microplates were then returned to the incubator until being read in an inverted microscope when the 100 CCID50% was observed in the control cells. The absence of cytopathic effect was indication of viral neutralization, while its presence resulted from the absence of neutralizing antibodies. Antibody titers were calculated by the Behrends & Kärber method [22].

In order to evaluate protection afforded by the experimental vaccine preparations, 21 days after the second vaccination the remaining animals were inoculated subcutaneously with 0.1 ml containing 31.6 lethal doses (LD) of SuHV-1 (strain used in vaccine preparation) [21]. Daily, until the tenth day after the challenge, the number of dead animals in each experimental group was recorded for analysis by the statistical method of Reed and Muench [22]. For the LD calculation, starting from a viral suspension with $10^{5.75}$ $CCDI_{50}$/25 μl, the same statistical methodology was used.

2.4. Cellular immunity

The IFN-γ mRNA level in splenocytes from mice inoculated in the second experiment was used as a parameter for cellular immunity evaluation. The splenocytes were processed according to Bastos et al. [23]. Briefly, a splenocyte suspension was obtained from a pool of spleens of mice from each treatment, 21 days after the second inoculation. After lysing the red blood cells with NH_4Cl, the spleen cells were counted and 10^7cells/ml were then suspended in Minimum Essential Medium – MEM (Gibco-BRL), supplemented with 10% of bovine fetal serum (Gibco-BRL) and plated in a 96 well plate. After 24 h of incubation at 37 °C in an environment with 5% CO_2, the supernatant was removed and the cells were stimulated in triplicate, with MEM (negative control), 0.1 multiplicity of infection (MOI) of SuHV-1 or 5 μg/ml of Concanavalin A (Sigma). Twenty-four or 48 h after stimulation, total RNA was extracted with Trizol (Invitrogen), according to the manufacturer's protocol. The cDNA synthesis was performed from 5 μg of total RNA, in a 25 μl reaction containing 0.5 μl (150 ng) of random primers (Invitrogen), 1 μl of desoxynucleoside triphosphates (dNTP - 10 mM), 1 × First Strand buffer (New England Biolabs), 0.1 M DDT, 40 U of RNaseOUT (Invitrogen) and 50 U of M-MuLV Reverse Transcriptase (New England Biolabs), following previously described methodology [24]. After incubating for 10 min at 25 °C, the samples were incubated at 42 °C for 50 min, followed by 70 °C for 15 min, in an thermocycler (Eppendorf Mastercycler Gradient). The resulting cDNA was stored at −20 °C. PCR reactions were carried out in triplicate with 2 μl of cDNA, 200 μM of dNTPs, 1 × reaction buffer, 1.5 U of Taq DNA polymerase (Invitrogen), 1 μM of each primer, 3 mM $MgCl_2$ for IFN-γ or 1.5 mM for β-actin, and RNase free water (Gibco-BRL) in a final volume of 25 μl. The thermocycler parameters were as follows: 95 °C for 2 min, followed by 30 cycles of 94 °C for 50 sec, 60 °C for 50 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min. The primers used in this experiment, described in the literature [24] and synthesized by MWG-Biotech Inc. (USA), were: IFN-γ forward 5′-AGCGGCTGACTGAACTCAGATTGTAG; IFN-γ reverse 5′-GTCACAGTTTTCAGCTGTATAGGG; β-actin forward 5′-TGGAATCCTGTGGCATCCATGAAAC; β-actin reverse 5′-TAAAACGCAGCTCAGTAACAGTCCG. PCR reactions using primers for β-actin and PCR reaction without cDNA was carried out as a controls. PCR products were visualized under UV light after electrophoresis in 2% agarose gel containing ethidium bromide. Analysis of the data was done using Scientific Imaging System software (Kodak).

2.5. Statistical analysis

Antibody titers, expressed in $\log_2$, were compared using variance analysis (ANOVA). The LSD test was used to determine significant differences ($p < 0.05$) among the mean of each treatment using the SAS program. The protection afforded by the experimental vaccine preparations was evaluated through the statistical method of Reed and Muench [22].

3. Results

3.1. Humoral immunity

The mean ± S.E.M. of the antibody titers of each experimental group are presented in Fig. 1. As it can be observed, the association of 5 mg/dose of ethanolic extract of green propolis to an inactivated vaccine against SuHV-1 containing aluminum hydroxide showed significant adjuvant effect. In all the dilutions of this vaccine preparation there was an increment in the antibody titers when compared to the vaccine with aluminum hydroxide alone. This effect was more evident in the intermediate dilutions ($p < 0.05$) where the titer went from 3 to 4.49 when the vaccine was diluted four times and doubled, going from 2.18 to 4.48 in the 1:8 dilution. In the 1:16 dilution, probably due to the low quantity of antigen present in each dose, antibody titers were similar in all treatments.

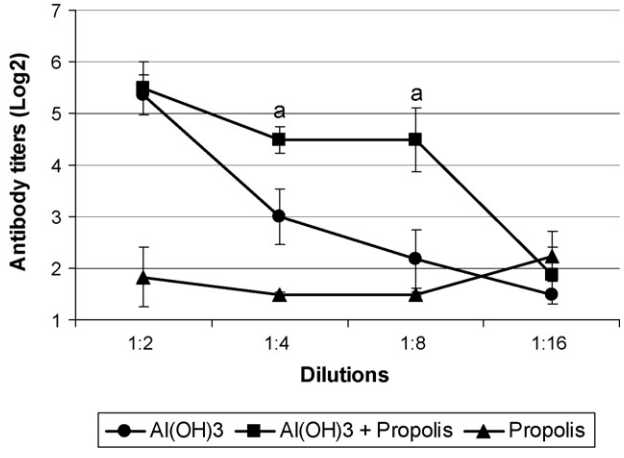

Fig. 1. Antibody titers ($\log_2$) of mice immunized with SuHV-1 associated to $Al(OH)_3$, $Al(OH)_3$ + 5 mg/dose of ethanolic extract of green propolis or 5 mg/dose of ethanolic extract of green propolis. The titer was determined by the serum neutralization test, 21 days after the second inoculation. All the vaccines were diluted 2, 4, 8 or 16 times. The data represents the mean ± S.E.M. Different letters indicate significant statistical difference ($p < 0.05$).

Association of the propolis extract on its own with inactivated SuHV-1, however, did not induce a significant production of antibodies (Fig. 1). The lowest titers in this experiment were obtained from mice inoculated with this vaccine preparation.

3.2. Protection afforded by the vaccine preparations

The percentage of animals protected after inoculation with the experimental vaccine preparations in the different dilutions, and challenged with 31.6 DL of SuHV-1, can be observed in Fig. 2. Only the vaccine preparation with aluminum hydroxide, in the 1:2 dilution, protected 100% of the animals. However the association of green propolis extract to this vaccine preparation increased the percentage of animals protected in the higher dilutions (1:4, 1:8 and 1:16). In this case, the higher the dilution of the vaccine, the bigger the percent difference of animals protected, suggesting immunostimulant action of the propolis extract. These data is compatible with the antibody titers of the same animals. Surprisingly, the vaccine preparation in which propolis was associated on its own with the antigen allowed good levels of protection in all the dilutions evaluated (1:2–92%; 1:4–85%; 1:8–80% and 1:16–68%), contrary to the serologic results. By the 10th day after challenge, all animals from the control group were dead, suggesting that the protection in the other groups occurred due to the immunizations.

3.3. Cellular immunity

In order to evaluate the effect of the ethanolic extract of green propolis on the cellular immune response [25], a semi-quantitative method of measurement (RT-PCR) of the IFN-γ mRNA expression was used. As it can be observed in Fig. 3A, 24 h after splenocyte stimulation with 0.1 MOI of SuHV-1, there was a significant increase in the IFN-γ mRNA expression in the animals inoculated with the vaccine preparation containing aluminum hydroxide and propolis (sample 3), in relation to the vaccine preparation without propolis (sample 2). In addition, when propolis alone was associated with the antigen, expression of IFN-γ mRNA was even higher, superior then the other treatments (sample 4).

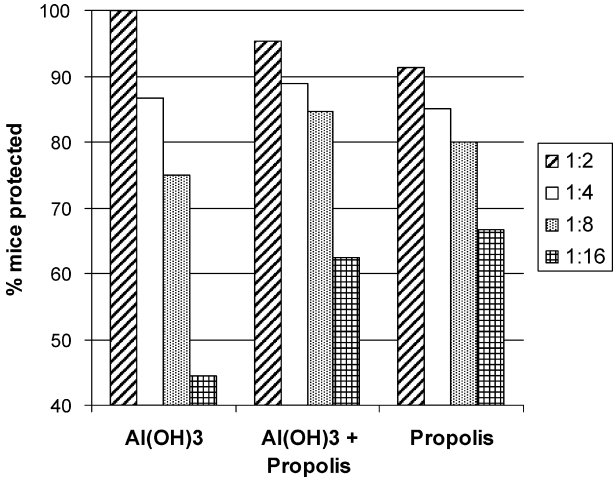

Fig. 2. Percentage of mice protected in the test of protection capacity afforded by the experimental vaccines. Each animal was immunized with two doses of a vaccine containing SuHV-1 associated to $Al(OH)_3$, $Al(OH)_3$ + 5 mg/dose of ethanolic extract of green propolis or 5 mg/dose of ethanolic extract of green propolis, diluted from 1:2 to 1:16. Twenty-one days after the second inoculation the animals were challenged with 31.6 LD of SuHV-1. All animals from the negative control (non-vaccinated) died (result not shown). The number of dead and alive in each experimental group was analyzed by the Reed and Muench [22] statistical method.

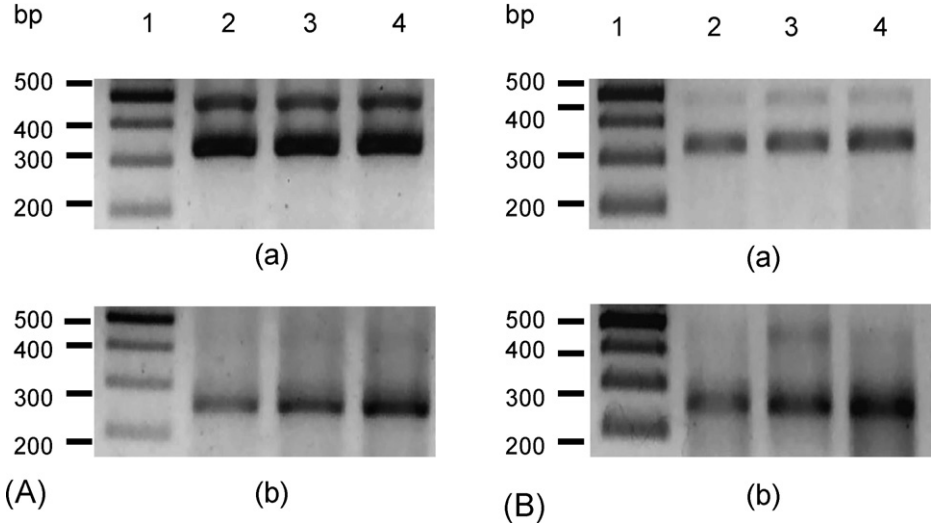

Fig. 3. Agarose gel electrophoresis of RT-PCR products resulting from the amplification of IFN-γ mRNA from mice splenocytes collected 21 days after the second inoculation with the different experimental vaccine preparations. (A) Twenty-four hours after stimulating the splenocytes with 0.1 MOI of SuHV-1: (a) β-actin used as internal control; (b) IFN-γ – 1, GeneRuler 100 pb DNA Ladder Plus (Invitrogen); 2, $Al(OH)_3$; 3, $Al(OH)_3$ + propolis (5 mg/dose); 4, propolis (5 mg/dose). (B) Forty-eight hours after stimulating the splenocytes; (a) β-actin used as internal control; (b) IFN-γ – 1, GeneRuler 100 pb DNA Ladder Plus (Invitrogen); 2, $Al(OH)_3$; 3, $Al(OH)_3$ + propolis (5 mg/dose); 4, propolis (5 mg/dose).

The expression of β-actin mRNA did not vary in the different treatments, indicating a specific stimulus in the IFN-γ expression. Forty-eight hours after splenocyte stimulation, the results remained the same (Fig. 3B). The highest IFN-γ mRNA expression occurred when propolis alone was inoculated with the antigen, followed by the association of propolis and aluminum hydroxide. The lowest expression was when aluminum hydroxide alone was used with the antigen, suggesting an immunostimulant effect of propolis on the cellular immune response to SuHV-1.

4. Discussion

Several strategies have been pursued aiming at inhibiting the growth and dissemination of pathogenic microorganisms due to economical losses caused in different productive systems. Priority has been given to the development of vaccines which generate an appropriate immune response [26]. Despite technological advances in molecular biology and in genetic engineering, allowing identification of antigens with immunogenic potential [3], the majority of vaccines requires association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [5,3].

In this study, the adjuvant capacity of an ethanolic extract of Brazilian green propolis was evaluated when associated to inactivated SuHV-1 with or without aluminum hydroxide, in BALB/c mice. The addition of 5 mg/dose of this extract [26,27] to the vaccine with aluminum hydroxide increased the potency of the humoral immune response when compared to the vaccine without propolis, determined by neutralizing antibody titers, 21 days after the second dose. This adjuvant effect was more evident when the vaccine was diluted (1:4 and 1:8 – $p < 0.05$), suggesting that the lowest the antigenic mass in the vaccine or less immunogenic the antigen, more pronounced is the propolis effect. According to Sforcin et al. [28], the ability of modulating the synthesis of antibodies is part of the propolis adjuvant activity. Despite the fact that the precise mechanism of action of propolis on the immune system cells remains unknown [12], stimulation of macrophages to produce cytokines such as IFN-γ, with further general amplification of the immune response [16,29], can be one of its main adjuvant mechanisms.

The increase in the potency of the humoral immune response, however, was not detected when propolis alone was associated to inactivated SuHV-1. The neutralizing antibody titers, in this case, were the lowest among all experimental groups, compared only to the highest dilutions (1:16) of the other treatments. This fact can be understood if the propolis is classified as a nonparticulate adjuvant, as suggested by Cox and Coulter [30]. According to these authors, nonparticulate adjuvants acting as immunomodulators are those which the activity does not depend on any particle, and benefit from the association with a particulate adjuvant. These substances, as they do not attach to the antigen, are rapidly processed by the immune system cells (inducing a weak response) and do not impair the loss of their conformational integrity [30]. On the other hand, when the antigen is absorbed in a particulate adjuvant, such as aluminum hydroxide, the particle formed facilitates its direction to antigen present cells. Besides, this association allows a slow liberation of the antigen, increasing the exposure time to the immune system, extending the response [30,31]. This fact might explain the increase in the neutralizing antibody titers to SuHV-1 of the vaccine preparation in which propolis plus aluminum hydroxide were associated to the antigen.

The evaluation of humoral and/or cellular responses induced by a vaccine or even an adjuvant substance should not be the only parameter analyzed for its validation [32]. It is necessary an effective protection of the immunized animal in the event of exposition to the pathogen. In this sense, six animals from each experimental group were challenged with 31.6 LD of SuHV-1 [21], 21 days after the last vaccination. The results obtained highlight the adjuvant capacity of the Brazilian green propolis extract used. Comparing the vaccines with aluminum hydroxide, the addition of the extract resulted in an increase percentage of protected animals, especially in the higher dilutions. The high percentage of animals which survived the challenge in the immunized group with the antigen and the propolis extract was also surprising. The lack of correlation with the results obtained in the serological tests suggest the role of cellular immunity in the protection against SuHV-1. It is likely that activation of macrophages was one of the main adjuvant mechanisms in this case.

Activation of T lymphocytes causes a series of ordered interactions and events, including activation of transmembrane signals and expression of cytokine genes. Attributes such as stability and rate of synthesis of mRNA and proteins are altered. The result of this process is the proliferation and differentiation of T cells and production of cytokines [33]. An important functional attribute of the immune system cells is the capacity of synthesizing and secreting cytokines, which bind to specific receptors in the surface of a target cell. After binding, the cytokines act regulating the growth and/or the differentiation of these cells, optimizing the immune response [12]. The development of a cellular or humoral immune response depends on a wide range of cytokines produced by several cells, including CD4+ (Th1 and Th2) and CD8+ T cells. The IFN-γ produced by Th1 cells is an essential cytokine in the cellular immune response, classically described as a defense mechanism against viral infections [34].

Besides increasing the potency of the humoral immune response, the use of green propolis extract also allowed an increase in the cellular response, increasing the synthesis of mRNA of IFN-γ. The IFN-γ expression was higher in splenocytes of mice immunized with SuHV-1 plus propolis, explaining the higher percentage of animals protected in this group after challenge. These results are similar to the ones obtained by Blonska et al. [35] working with a European propolis sample, for whom the ethanolic extract acts regulating gene expression at transcriptional level. Other authors,

using either propolis of Brazilian or European origin, also reported increase in cytokine secretion in animals receiving propolis [29,35,36]. Ansorge et al. [12], using a Polish sample, reported that propolis has a regulatory effect directly on the basic functional properties of the immune system, which can be mediated by the ErK2 MAP-kinase signal involved in mechanisms that promote cellular growth.

In a previous study, multivariate analysis associating ethanol extracts of different samples with the levels of bioactive compounds determined by high performance liquid chromatography (HPLC) allowed the typing of Brazilian propolis [37]. Green propolis showed high levels of phenolic compounds such as artepillin C, in addition to cinnamic acid and flavonoids such as pinobanksin and kaempferol. The precise mechanism of action of propolis remains unknown [12], however, these compounds may have stimulated the immune system cells to produce cytokines like IL-1, IL-6, IL-8, IL-12 and IFN-γ [26,29,36], promoting an increase in the humoral as well as the cellular immune response.

In our study, an ethanolic extract of green propolis acted as a modulator of the immune system. In mice immunized with SuHV-1, the association of propolis and aluminum hydroxide increased the potency of the humoral and cellular responses. In addition, when used on its own with SuHV-1, green propolis extract promoted an increment of the cellular immune response which resulted in increased protection. The use of green propolis extract as an adjuvant might contribute for the efficacy of vaccines, especially those which depend on the cellular immune response for protective response. It can also increase the potency of vaccines when associated to particulate adjuvants such as aluminum hydroxide.

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