Green propolis phenolic compounds act as vaccine adjuvants, improving humoral and cellular responses in mice inoculated with inactivated vaccines

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Adjuvants play an important role in vaccine formulations by increasing their immunogenicity. In this study, the phenolic compound-rich J fraction (JFR) of a Brazilian green propolis methanolic extract stimulated cellular and humoral immune responses when co-administered with an inactivated vaccine against swine herpesvirus type 1 (SuHV-1). When compared to control vaccines that used aluminium hydroxide as an adjuvant, the use of 10 mg/dose of JFR significantly increased ($p < 0.05$) neutralizing antibody titres against SuHV-1, as well as the percentage of protected animals following SuHV-1 challenge ($p < 0.01$). Furthermore, addition of phenolic compounds potentiated the performance of the control vaccine, leading to increased cellular and humoral immune responses and enhanced protection of animals after SuHV-1 challenge ($p < 0.05$). Prenylated compounds such as Artepillin C that are found in large quantities in JFR are likely to be the substances that are responsible for the adjuvant activity.

Key words: propolis - phenolic compounds - vaccines - humoral immunity - cellular immunity

Vaccination is a practice that aims to stimulate strong and lasting immune responses in the defence against infectious and parasitic agents (Aguilar & Rodríguez 2007). Unlike live attenuated vaccines, inactivated or highly purified vaccines, such as subunit vaccines, generally require the addition of an adjuvant to be effective (Petrovsky & Aguilar 2004).

Several compounds have been investigated for their adjuvant activity, including microbial products, mineral salts, emulsions, liposomes and immunostimulating complexes, as well as natural substances such as plant extracts (Cleff et al. 2008, Mosca et al. 2008). Propolis has been suggested to be a promising adjuvant substance in duck inactivated vaccines (Cai et al. 2001). Our group has recently focused on the adjuvant properties of green propolis (Fischer et al. 2007a, b). Total ethanolic extracts of this particular propolis have been shown to improve humoral and cellular immune responses in mice inoculated with an inactivated vaccine against bovine herpesvirus type 5. However, this resinous substance, which is collected from plant exudates by *Apis mellifera* bees and to which salivary enzymes are added (Cai et al. 2001), may contain over 200 different compounds (Shimazawa et al. 2005) that can both stimulate and suppress the immune system (Sforcin 2007, Fischer & Vidor 2008). Phenolic compounds such as flavonoids, aromatic acids and diterpenes are the main components that account for the numerous biological activities of propolis (Galal et al. 2008), which include antioxidant, antifungal, antiviral, antimutagenic and immunomodulatory activities (Ansorge et al. 2003, Jasprica et al. 2007, Paulino et al. 2008). In fact, the total amount of phenols is a parameter used in quality control for propolis (Gardana et al. 2007). Moreover, phenolic compounds have been identified as major substances in several Brazilian propolis samples, especially green propolis (Barros et al. 2008, Fischer & Vidor 2008, Paulino et al. 2008). Thus, the aim of this study was to evaluate the effect of green propolis phenolic compounds on the humoral and cellular immune response of mice immunized with an inactivated vaccine against swine herpesvirus type 1 (SuHV-1).

MATERIALS AND METHODS

Separation of green propolis fractions - Green propolis (30 g) was frozen, macerated and stored in a paper filter cartridge for Soxhlet extraction with 250 mL methanol. Extraction was carried out at 50ºC for 8 h and the wax was separated by cold precipitation. The supernatant was evaporated in a rotoevaporator at 40ºC to obtain the green propolis alcohol-free methanolic extract (PME) which was dissolved in a minimum amount of ethanol and fractionated by gel filtration chromatography on an LH-20 Sephadex column (Amersham Pharmacia, G&E, USA) into six parts designated as O, Q, U, J, A and I. After concentrating the fractions, the six collected fractions plus the original sample were injected into a high performance liquid chromatography apparatus (L-7100, Merck-Hitachi, Darmstadt, Germany)

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with a photodiode network and an automatic injector. The chromatographic conditions used were Lichrochat 100 RP-18 reverse phase column (12.5 x 0.4 cm, particle diameter 5 µm) (Merck, Darmstadt, Germany) and formic water-acid (95:5, solvent A) and methanol (solvent B) for the mobile phase. Elution was carried out at 1 mL/min flow through a linear gradient and the injected volume amounted to 20 µL. The maximum analysis time was 50 min and detection was performed at 280 and 340 nm wavelengths. The software used for data analysis was the Model D-7100 Merck-Hitachi Chromatography Data Station-DAD Manager (Darmstadt, Germany). In the present study, only the phenolic compound-rich J fraction (JFR) extracted from green propolis was used.

Vaccine preparation and immunization protocol - Experimental vaccines were produced from a SuHV-1 sample isolated from an Aujeszky's disease outbreak provided by the Virology and Immunology Laboratory, Federal University of Pelotas (UFPel), Rio Grande do Sul, Brazil, following standard methodology (Fischer et al. 2007a). After virus cultivation in RK13 rabbit renal cells, viral suspensions at a cell culture infectious dose ($CCID_{50}$) of $10^{5.50}$ per 25 µL were inactivated by 20 mM bromoethylamine BEI ($C_2H_7BR_2N$; Merck, Brazil), pH 7.5 (Bahnemann 1975).

Evaluation of JFR adjuvant activity was performed using six groups of 10 BALB/c 6-8-week-old mice as follows: group 1, 1 mg JFR; group 2, 10 mg JFR; group 3, aluminum hydroxide [$Al(OH)_3$] (positive control); group 4, $Al(OH)_3$ + 1 mg JFR; group 5, $Al(OH)_3$ + 10 mg JFR; group 6, phosphate buffered saline (PBS) (negative control). Animals were inoculated intramuscularly at days zero and 21 with 200 µL of the experimental vaccines (130 µL of inactivated SuHV-1 antigen plus adjuvant combinations described above at 30% of antigen volume). The negative control group (group 6) was inoculated with the antigen (130 µL) plus PBS (70 µL). All vaccines were prepared from suspensions with the same initial infectious titre.

Humoral immunity and protection - For measurements of neutralizing antibody titres against SuHV-1, blood samples were collected by retro-ocular venous plexus puncture at days 0, 21 and 42. The serum was processed and stored at -20ºC until use. Animals obtained from the UFPel animal house remained isolated in an environment with a temperature between 22-24ºC and they received food and water *ad libitum*. The experiment was approved by the Ethical Committee in Animal Experimentation from UFPel (process 2253/2008-91).

Antibodies were titrated by the serum-neutralization technique (Fischer et al. 2007a). Each serum sample was serially diluted from 1:2 down to 1:256 and tested in quadruplicate. Antibody titres were calculated by the Behrends and Kärber method (Mayr et al. 1982).

To evaluate the protection afforded by experimental vaccines, six animals from each experimental group were challenged subcutaneously with 0.1 mL of SuHV-1 containing lethal doses (LD) 50% of SuHV-1 21 days after the second vaccination. The number of dead animals was recorded daily until the 10th day after the challenge.

Cellular immunity - IFN-γ mRNA levels in splenocytes were measured by reverse transcription-polymerase chain reaction (RT-PCR) (Bastos et al. 2002) and used as a measure of the cellular immune response. Briefly, a splenocyte suspension was obtained from the spleens of four mice per treatment. Following erythrocyte lysis with ammonium chloride (NH_4Cl; Casquímica, Brazil), the cells were cultured at a concentration of 10^7 cell/mL in tissue culture plates 96-wells (TPP-Switzerland). After a 24-h incubation period at 37ºC in a 5% CO_2 environment, the supernatant was removed, the cells were stimulated in triplicate with minimum essential medium (negative control; Gibco, Invitrogen, USA) and SuHV-1 at a multiplicity of infection of 0.1 or 5 µg/mL Concanavalin A (positive control; Sigma-Aldrich Inc, USA). After 24 h or 48 h of stimulation, total RNA was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNA synthesis was performed with 5 µL of total RNA in a 25-µL reaction containing 0.5 µL (150 ng) random primers (Invitrogen, USA), 1 µL deoxynucleotide triphosphates (dNTP, 10 mM), 1 × First Strand buffer (New England Biolabs, Ipswich, MA, USA), 0.1 M DDT, 40 U RNaseOUT (Invitrogen, USA) and 50 U of M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) following standard methodology (Ulett et al. 2000). After incubation for 10 min at 25ºC, the samples were incubated at 42ºC for 50 min, followed by 70ºC for 15 min in a thermocycler (Eppendorf Mastercycler gradient). The resulting cDNA was stored at -20ºC. PCR reactions were performed in triplicate with 2 µL of cDNA, 200 µM dNTPs, 1 × reaction buffer, 1.5 U Taq DNA polymerase (Invitrogen, USA), 1 µM of each primer, 3 mM $MgCl_2$ for IFN-γ or 1.5 mM $MgCl_2$ for ß-actin and RNase free water (Gibco, Invitrogen, USA) in a final volume of 25 µL. The thermocycler settings were as follows: 95ºC for 2 min, followed by 30 cycles at 94ºC for 50 s, 60ºC for 50 s and 72ºC for 1 min and then a final extension at 72ºC for 7 min. The primers used in this experiment were described by Ulett et al. (2000) and synthesized by MWG-Biotech, Inc, USA: IFN-γ forward 5'-AGCGGCTGACTGAACTCAGATTGTAG; IFN-γ reverse 5'-GTCACAGTTTTCAGCTGTATAGGG; ß-actin forward 5'-TGGAATCCTGTGGCATCCATGAAAC; ß-actin reverse 5'-TAAAACGCAGCTCAGTAACAGTCCG. Control PCR reactions were performed using primers for ß-actin or without cDNA. PCR products were visualized under ultraviolet light after electrophoresis in 2% agarose gels containing ethidium bromide.

Statistical analysis - Antibody titres, expressed in log_2, were compared by means of variance analysis. The least square difference test and Statistix software were used to determine significant differences ($p < 0.05$) among the averages in each treatment. Protection provided by experimental vaccines was evaluated by the Reed and Muench statistical method (Mayr et al. 1982).

RESULTS

JFR characterization - Six green propolis fractions were extracted by gel filtration chromatography on a Sephadex column. Only the JFR was rich in phenolic

compounds and was therefore used in this study. This fraction represented 14.74% of the green PME (Fig. 1). Artepillin C and its derivatives (Fig. 1, peaks 2, 3, 5, 7-11) were the most abundant substances (5.61%), followed by 3-prenyl-4-hydroxycinnamic acid (4.975%) and p-coumaric acid (3.097%).

Phenolic compounds increased antibody titres and the percentage of protected animals after SuHV-1 challenge - The mean ± standard error of the means titre obtained for each experimental group is shown in Fig. 2. SuHV-1 plus PBS (negative control) did not induce significant antibody titres. The combination of SuHV-1 plus $Al(OH)_3$, which was used as the control adjuvant, induced the second lowest antibody titre against SuHV-1 21 days after the second inoculation. Although not statistically significant, inclusion of green propolis JFR (at 1 mg and 10 mg/dose) in the SuHV-1 and aluminium hydroxide vaccine resulted in an increase in antibody titre from 1.93 $\log_2$ to 2.49 and 2.68 $\log_2$, respectively. The adjuvant activity of JFR was most evident when JFR was used as the only adjuvant. Inclusion of JFR at 1 mg/dose resulted in an increase of about 1 $\log_2$ in the anti-SuHV-1 antibody titre (2.88 $\log_2$) when compared to the $Al(OH)_3$-containing vaccine. JFR used at 10 mg/dose resulted in an even higher humoral response (4.4 $\log_2$), which was twice that elicited by the $Al(OH)_3$-containing vaccine ($p < 0.05$).

The percentage of protected animals after challenge with 50 SuHV-1 LD (Vidor et al. 1991) correlated with antibody titres against SuHV-1, as shown in Fig. 3. The use of 1 mg/dose JFR, alone or at 10 mg/dose with $Al(OH)_3$, resulted in 32% protection ($p < 0.05$). The use of 10 mg/dose JFR resulted in 66% protection ($p < 0.01$). On the 10th day after challenge, all six mice from the negative control group that had been inoculated with vaccine plus PBS died, suggesting that the protection observed was due to immunization.

Increase in IFN-γ mRNA expression - IFN-γ mRNA levels were measured by RT-PCR and used as a measure of the cellular immune response induced by JFR. As shown in Fig. 4B, IFN-γ mRNA expression was higher in splenocytes from mice immunized with vaccine plus phenolic compounds (samples 2 and 3) compared to splenocytes from mice in which $Al(OH)_3$ was used as the only adjuvant (sample 4). This increase in IFN-γ mRNA levels was also evident in splenocytes from mice immunized with phenolic compounds plus $Al(OH)_3$ (samples 5 and 6)

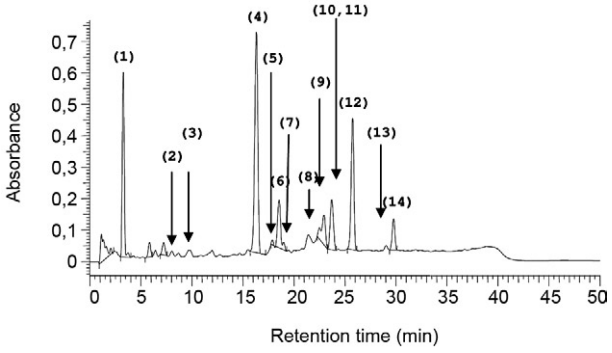

Fig. 1: identification and quantification of phenolic compounds in fraction J from green propolis methanolic extract by high performance liquid chromatography. 1: p-coumaric acid; 2: 3-[4-hydroxy-3-(oxobutenyl)-phenylacrylic] acid (propol); 3: dihydrokaempferide: 4: 3-prenyl-4-hydroxycinnamid acid; 5: 3-prenyl-4-(2-methylpropionyloxy)-cinnamic acid; 6: 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran; 7: 4-hydroxy-3(E)-(4-hydroxy-3-methyl-2-butenyl)-5-prenyl cinnamic acid; 8: 3,4-dihydroxy-5-prenylcinnamic acid; 9: 3-hydroxy-2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid; 10: betulethol; 11: (E)-3-{-4-hydroxy-3-[(E)-4-(2,3-dihydrocinamoyloxy)-3-methyl-2-butenyl]-5-prenyl-phenyl}-2-propenoic acid; 12: 3,5-diprenyl-4-hidroxycinnamic acid; 13: 3-prenyl-4-dihydrocinamoyloxy-cinnamic acid; 14: 6-propenoic-2,2-dimethyl-8-prenyl-2H-1-benzopiran acid. Peaks 2, 3, 5, 7-11: Artepillin C derivatives.

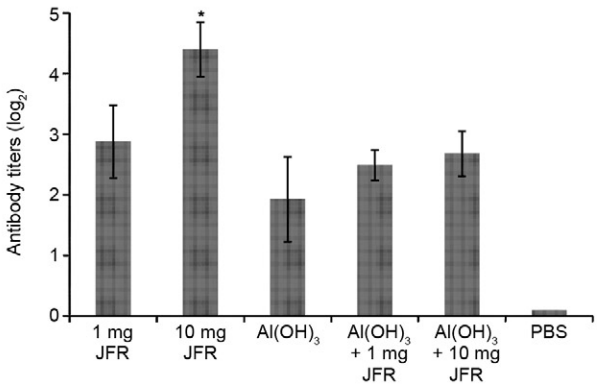

Fig. 2: antibody titers ($\log_2$) of mice inoculated with an inactivated vaccine against swine herpesvirus type 1 co-administered with 1 mg or 10 mg/dose of Brazilian green propolis phenolic compounds [J fraction (JFR)], aluminum hydroxide [$Al(OH)_3$], $Al(OH)_3$ + 1 mg/dose JFR or $Al(OH)_3$ + 10 mg/dose JFR. The titers were determined by serum neutralization 21 days after the last inoculation. The data represents the mean ± standard error of the means (n = 10). PBS: phosphate buffered saline. Asterisk means $p < 0.05$.

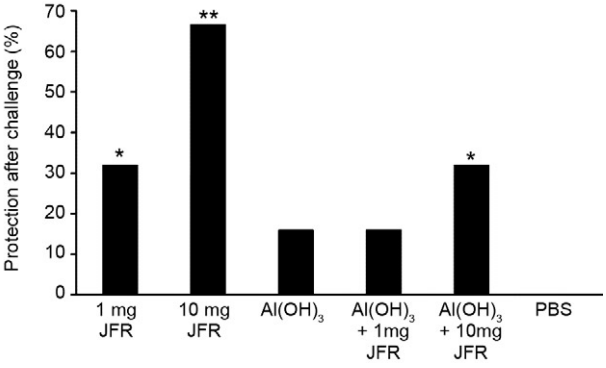

Fig. 3: percentage of protected animals after the challenge test. Twenty-one days after the last inoculation, six animals from each group were challenged with 50 lethal dose of swine herpesvirus type 1. The number of dead and alive animals in each experimental group was analyzed by the Reed and Muench statistical method. $Al(OH)_3$: aluminum hydroxide; JFR: J fraction; PBS: phosphate buffered saline; *: $p < 0.05$; **: $p < 0.01$.

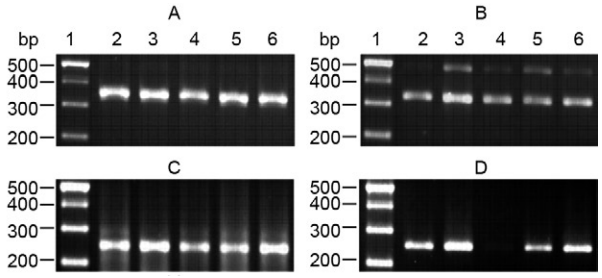

Fig. 4: agarose gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) products resulting from the amplification of IFN-γ mRNA from mice splenocytes, collected 21 days after the second inoculation with different experimental vaccines. A, B: twenty-four hours after splenocytes stimulation with 0.1 multiplicity of infection of swine herpesvirus type 1; A: ß-actin used as internal control; B1: Ladder 100 pb (Ludwig Biotec); 2: 1 mg/dose Brazilian green propolis phenolic compounds [J fraction (JFR)]; 3: 10 mg/dose JFR; 4: aluminum hydroxide [$Al(OH)_3$]; 5: $Al(OH)_3$ + 1 mg/dose JFR; 6: $Al(OH)_3$ + 10 mg/dose JFR; C, D: forty-eight hours after splenocytes stimulation; C: ß-actin used as internal control; D1: Ladder 100 pb (Ludwig Biotec); 2: 1 mg/dose JFR; 3: 10 mg/dose JFR; 4: $Al(OH)_3$; 5: $Al(OH)_3$ + 1 mg/dose JFR; 6: $Al(OH)_3$ + 10 mg/dose JFR.

compared to splenocytes from mice in which aluminium hydroxide was used as the only adjuvant (sample 4). The highest IFN-γ mRNA expression was observed when JFR was co-administered with the vaccine at 10 mg/dose (sample 3) and was consistent with serologic evaluations. mRNA expression of ß-actin (positive control) did not vary across the different treatments, indicating that changes in IFN-γ expression were not non-specific (Fig. 4A). Similar results were obtained after stimulation of splenocytes for 48 h (Fig. 4D), reinforcing the adjuvant activity of green propolis phenolic compounds. However, a significant reduction in IFN-γ mRNA expression was observed in splenocytes of animals that had been inoculated with vaccine plus $Al(OH)_3$ (sample 4). Again, mRNA expression of ß-actin (positive control) did not vary across different treatments (Fig. 4C).

DISCUSSION

The evaluation of compounds with adjuvant potential has been on going and aims to increase the list of substances that can be added to human or animal vaccines to increase their immunogenicity (Podda 2001). Studies have shown that the Brazilian green propolis total ethanolic extract has immunomodulatory activity (Fischer et al. 2007a, b). Some of the substances found in this kind of propolis are: phenolic compounds such as Artepillin C (3,5 diprenil-4-hydroxycinnamic acid), flavonoids and caffeic, ferulic, p-coumaric and cinnamic acids (de Funari et al. 2007, Barros et al. 2008, Medana et al. 2008). However, propolis has a complex composition and is composed of numerous immunostimulatory and immunosuppressive substances (Sforcin 2007, Fischer & Vidor 2008), thus highlighting the need to link individual components of propolis to specific biological activities (Bankova 2005). To this end, several studies have evaluated either propolis compounds or specific substance groups such as phenolic compounds (de Funari et al. 2007, Barros et al. 2008, Paulino et al. 2008).

In this study, Brazilian green propolis phenolic compounds were evaluated for their adjuvant activity when co-administered to an inactivated vaccine against SuHV-1 in the presence or absence of $Al(OH)_3$. Out of six fractions of the PME obtained by gel filtration chromatography, the JFR was selected for further study because it showed high levels of phenolic compounds (Fig. 1). The prenylated phenolic acid Artepillin C and its derivatives were the most abundant substances, followed by 3-prenil-4-hydroxycinnamic and p-couramic acids. The results were similar to those obtained by de Funari et al. (2007), which identified Artepillin C to be the most common substance in green propolis samples. In addition, chemical analyses from other studies support the abundance of phenolic compounds in green propolis (Barros et al. 2008, Paulino et al. 2008, Pontin et al. 2008, Viuda-Martos et al. 2008).

In the evaluation of new substances with adjuvant potential, aluminum compounds such as $Al(OH)_3$ have become a reference, as they are the only adjuvants regularly used in human vaccines (Gupta & Rost 2000). The use of 1 mg/dose of Brazilian green propolis JFR provided nearly a 1 $\log_2$ increase in neutralizing antibody titres compared to use of $Al(OH)_3$, whereas 10 mg/dose JFR doubled the humoral response when compared to the control adjuvant ($p < 0.05$). Moreover, these phenolic compounds induced an increase in antibody titres against SuHV-1 when co-administered with the vaccine and $Al(OH)_3$ and this effect was dose-dependent (Fig. 2), i.e., JFR potentiated the effects of the control vaccine and increased the humoral immune response. These results are different from those obtained when a total ethanol extract of green propolis was evaluated for its adjuvant activity (Fischer et al. 2007a). In that study, propolis increased humoral responses against SuHV-1 only when co-administered with a particulate adjuvant [$Al(OH)_3$]. The presence of both immunostimulatory and immunosuppressive substances (Sforcin 2007, Fischer & Vidor 2008) in total green propolis ethanolic extract might explain those results. Phenolic compounds are found at high levels in various propolis samples and have been shown to be the main substances in propolis bearing biological activity (de Funari et al. 2007, Galal et al. 2008). Their adjuvant and co-adjuvant actions have been attributed to flavonoids and cinnamic acid derivatives such as Artepillin C (Park et al. 1998, Tazawa et al. 1998).

The combined use of vaccines and immunostimulatory agents is a growing and innovative approach in adjuvant development (Gautam et al. 2008). In this study, co-administration of green propolis JFR to the inactivated vaccine against SuHV-1 was shown to be an effective strategy in inducing immunity against SuHV-1, as it increased the percentage of protected animals after challenge with 50 LD SuHV-1 21 days after the last vaccination (Fig. 3). Only 16% (1 animal) of the mice inoculated with the vaccine containing only $Al(OH)_3$ survived the challenge. This percentage doubled (32%, $p < 0.05$) and quadrupled (66%, $p < 0.01$) in the group of animals in-

oculated with vaccines containing 1 mg and 10 mg/dose green propolis JFR, respectively, which underscores the immunostimulatory capacity of JFR phenolic compounds. These data complement those obtained in the serological evaluations (Fig. 2), as the percentage of protected animals (Fig. 3) increased proportionally to the increase in antibody titre against SuHV-1.

In addition to stimulating the humoral immune response ($p < 0.05$), JFR phenolic compounds also increased cellular immune responses, as evidenced by the increase in IFN-γ mRNA expression (Fig. 4B, D). These results are consistent with the increase in percentage of protected animals after SuHV-1 challenge in treatments using JFR. These results are also consistent with the study by Wittmann (1982) that demonstrated that cellular immunity is responsible for protection against Aujeszky's disease, which is caused by SuHV-1 in pigs. Using a Polish sample, Ansorge et al. (2003) found that propolis has immunoregulatory effects that may be mediated by Erk2 MAP kinase (MAPK) signals that promote cellular growth.

Although the immunomodulatory effects of propolis are still unclear, these properties are attributed to the high concentrations of phenolic compounds. In a study performed by de Funari et al. (2007), the phenolic compounds of an ethanolic extract showed high absorption by the human skin as a result of their ability to form complexes with proteins via hydrogen bridges. The immunostimulatory effect of JFR on both humoral and cellular responses observed in this study can be linked to its effects on cells such as macrophages that induce production of cytokines such as IFN-γ (Ansorge et al. 2003). Furthermore, the increase in humoral and cellular immune responses induced by JFR is probably related to the mitogen activated kinase p38 (p38 MAPK) signalling pathway. Extracellular signals are transduced in immune cells by a series of protein kinases and are initiated by a group of Ras proteins with GTPase activity, which is activated by post-translational prenylation. Once these proteins are activated, several intracellular proteins, including p38 MAPK (Lee et al. 2003), are sequentially phosphorylated. In mammals, MAPK proteins regulate cell growth (Zhang et al. 1999, Mavropoulos et al. 2005). Our hypothesis is that the green propolis JFR, which is rich in prenylated compounds such as Artepillin C, acts by increasing Ras protein prenylation and activating the p38 MAPK pathway and activator factor 2 transcription factor, which ultimately increases cellular and humoral immune responses. Marcucci et al. (2001) found that many of the biological activities of Brazilian green propolis are associated with prenylated compounds such as Artepillin C.

The data presented in this paper clearly demonstrate the adjuvant activity of phenolic compounds obtained from Brazilian green propolis when co-administered in mice with an inactivated vaccine against SuHV-1. This adjuvant activity was evident in the increase in both cellular and humoral immune responses. Prenylated phenolic compounds such as Artepillin C that act at the cell signaling level are likely the main substances with adjuvant activity.

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