CELL BIOCHEMISTRY & FUNCTION

CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2012; **30**: 1–10.
Published online 24 October 2011 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/cbf.1810

Protective effect of brown Brazilian propolis against acute vaginal lesions caused by *herpes simplex* virus type 2 in mice: involvement of antioxidant and anti-inflammatory mechanisms

Gláubia Sartori[1], Ana Paula Pesarico[1,2], Simone Pinton[1], Fernando Dobrachinski[1], Silvane Souza Roman[1], Fernanda Pauletto[2], Luiz Carlos Rodrigues Junior[2] and Marina Prigol[1]*

[1]*Laboratório de Síntese, Reatividade e Avaliação Farmacológica e Toxicológica de Organocalcogênios, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS Brazil*
[2]*Laboratório de Biologia Molecular e Cultivo de Células, Centro Universitário Franciscano, Santa Maria, RS, Brazil*

Propolis has been highlighted for its antioxidant, anti-inflammatory and antiviral properties. The purpose of this study was to investigate if brown Brazilian hydroalcoholic propolis extract (HPE) protects against vaginal lesions caused by herpes simplex virus type 2 (HSV-2) in female BALB/c mice. The treatment was divided in 5 days of pre-treatment with HPE [50 mg·kg^{-1}, once a day, intragastric (i.g.)], HSV-2 infection [10 µl of a solution 1×10^2 plaque-forming unit (PFU·ml^{-1} HSV-2), intravaginal inoculation at day 6] and post-treatment with HPE (50 mg·kg^{-1}) for 5 days more. At day 11, the animals were killed, and the *in vivo* analysis (score of lesions) and *ex vivo* analysis [haematological and histological evaluation; superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO) activities; reactive species (RS), tyrosine nitration levels, non-protein thiols (NPSH) and ascorbic acid (AA) levels] were carried out. HPE treatment reduced extravaginal lesions and the histological damage caused by HSV-2 infection in vaginal tissues of animals. HPE was able to decrease RS, tyrosine nitration, AA levels and MPO activity. Also, it protected against the inhibition of CAT activity in vaginal tissues of mice. HPE promoted protective effect on HSV-2 infected animals by acting on inflammatory and oxidative processes, and this effect probably is caused by its antioxidant and anti-inflammatory properties. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—HSV-2; herpes; propolis; antioxidant; anti-inflammatory

INTRODUCTION

Propolis is a natural bee product originated from various plant sources. It has been used in folk medicine for a long time.[1] The raw propolis mainly consists of resins (40–55%), bee waxes and fatty acids (20–35%), essential oils (about 10%), pollen (about 5%), minerals, vitamins and some other components such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids. The chemical components of propolis are quantitatively and qualitatively variable, depending on the geographical region, vegetation, collection time and type of bees.[2,3] The color is dependent on its origin and may vary from dark brown to red brown, passing through a green color, and odor could vary from one sample to another; others may not present it.[3]

It is well known that propolis has a large spectrum of biological properties such as antioxidant,[4,5] antibacterial,[6] antiinflammatory,[7] neuroprotective,[8] anticancer [9] and antiviral.[10] The antiviral activity is especially against influenza,[11] human immunodeficiency virus,[12] adenovirus,[13] respiratory tract infections,[14] herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2).[15]

Herpes simplex virus type 2 is a sexually transmitted pathogen infecting human genital tract mucosa and is the most common cause of genital ulcer disease in humans. HSV-2 infects the genital epithelium and can, following vaginal replication, be transmitted to the nervous system via uptake and retrograde transport in sensory neurons. The virus may establish latency in lumbosacral nerves and can therefore give rise to lifelong infection.[16] The symptoms of initial genital HSV-2 infection are poorly characterized; patients usually present signals several days after the infection. Herpetic lesions are characterized by erythematous papules, ulcers typically superficial with an erythematous outline and a greyish base, which are intensely painful, mainly during primary infection.[17]

Some studies have demonstrated that both herpes virus (HSV-1 and HSV-2) induce production of proinflammatory mediators, recruitment and activation of phagocytic macrophages and neutrophils, and induction of rapid and robust production of reactive oxygen species (ROS), which

*Correspondence to: Marina Prigol, Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, 97105–900, Santa Maria, RS, Brazil. E-mail: marinaprigol@mail.ufsm.br

Received 18 May 2011
Revised 25 July 2011
Accepted 24 August 2011

facilitate damage to the invading pathogens. These factors also can cause irreparable harm through bystander damage to crucial host cells.[18] Indeed, studies have reported an increase in inflammatory mediators and ROS formation during HSV-1 brain infection and their association with tissue damage and neurotoxicity associated with herpes encephalitis.[19]

Based on the considerations mentioned, the aim of the present study was to investigate the antioxidant and anti-inflammatory effects of brown Brazilian hydroalcoholic propolis extract (HPE) against acute vaginal lesions caused by HSV-2 in female mice.

MATERIALS AND METHODS

Chemicals

The reagents thiobarbituric acid (TBA), dichlorofloureseine diacetate (DCFH-DA), N,N,N',N'-tetramethylbenzidine, hexadecyltrimethylammonium bromide, *p*-dimethylaminobenzaldehyde, epinephrine, dinitrophenyl hydrazine, Ellman's reagent (DTNB), 3-nitrotyrosine (3-NT) and tyrosine were purchased from Sigma (St. Louis, MO). All other chemicals were obtained of analytical grade or from standard commercial suppliers.HPE was prepared in our laboratory according to the methodology below described.

HPE preparation

The propolis produced by *Apis mellifera* L. bees was collected in mid-January to February at Santa Flora City (RS-Brazil), and then, 300 g were subjected to hot extraction by Soxhlet apparatus using about 1000 ml of 70% ethanol. Afterward, it was concentrated in rotary evaporator at low pressure, obtaining the dry extract. HPE was diluted in a hydroalcoholic solution (95:5) to be given to the animals (50 $mg \cdot kg^{-1}$). The dose of 50 $mg \cdot kg^{-1}$ was chosen based on previous study [20] and on a pilot study carried out in our laboratory.

Determination of total phenolic and flavonoid contents

The determination of phenolic compounds was based on the Folin–Ciocateau using gallic acid as standard.[21] HPE sample (200 µl, diluted 1:10) was added in test tubes containing 1000 µl of folin and 800 µl of 7·5% calcium carbonate; the same procedure was performed for the standard. Readings were taken after 2 h in the dark on a spectrophotometer UV/VIS using a wavelength of 765 nm. Total phenolic content was expressed as mg of gallic acid per g of HPE.

To determine flavonoid content, quercetin was used as standard. HPE samples were diluted 1:10 in water. An aliquot of the sample (0·5 ml) was transferred to a test tube and was added 4·3 ml of 80% ethanol, 0·1 ml of aluminum nitrate at 10% and 0·1 ml of potassium acetate 1 $mol \cdot l^{-1}$. After 40 min of rest under the light, readings were made on a spectrophotometer UV/VIS using a wavelength of 415 nm. Blank tubes without the addition of aluminum nitrate were used in the same conditions. Total flavonoids content was expressed as mg of quercetin per g of HPE.[22]

Animals

Female adult BALB/c mice (20–25 g) from our own breeding colony were used. The animals were kept on a separate animal room, on a 12-h light/dark cycle, at a room temperature of $22 \pm 2\,^{\circ}C$, with free access to food (Guabi, RS, Brazil) and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil. All efforts were made to minimize animal suffering, to reduce the number of animals used.

Virus production and titration

The HSV-2 strain 333 was produced in African green monkey kidney (Vero) host cells. The cells were grown in 1X Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum in incubator with 5% CO_2 and temperature of 37 °C. The cells at the stage of monolayer were infected with HSV-2 (0.01 PFU per 100 cells) for a period of 3 h in DMEM without serum, after the medium was replaced by DMEM 1X with 10% fetal calf serum and culture incubated at sterilizer. After 72 h, the virus was extracted by freezing and thawing immediately. The viral extract was aliquoted and stored in a freezer at −80 °C. For titration, duplicate 200-µl aliquots of dilutions of each sample were plated on Vero cells grown to confluence in 24-well plates at 37 °C in 5% CO_2 for 1 h 30 min. Medium was aspirated, and 50 µl of 2x DMEM plus 1% low-melting point agarose was added to each well. Titers were calculated as $\log_{10}$ $PFU \cdot ml^{-1}$.[23]

In vivo experiments

Virus infection. Mice were anaesthetized with ketamine (150 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$) injected intraperitoneally (i.p.) and inoculated intravaginally by scratching small areas of the skin with the needle of a syringe containing 1×10^2 $PFU \cdot ml^{-1}$ of HSV-2 in a 10-µl volume. The dose between 2×10^2 and 3×10^2 $PFU \cdot ml^{-1}$ is normally used to induce lesions with HSV-2 333.[24] However, a standardization of the dose was necessary because we needed that the infected mice were alive, even with lesions, for longs periods to conduct the analyses. We tested 1×10^2, 2×10^2, 3×10^2 and 1×10^3, and the lowest dose produced better results for our mouse model.

Experimental protocol. The animals were divided into four groups: group I, control ($n = 4$); group II, HSV-2-infected mice ($n = 8$); group III, HPE (50 $mg \cdot kg^{-1}$)-treated mice ($n = 5$) and group IV, HPE (50 $mg \cdot kg^{-1}$)-treated and HSV-2-infected mice ($n = 5$). Five days prior to infection with HSV-2, all groups were pre-treated per oral route by gavage once a day. Groups I and II received hydroalcoholic solution (10 $ml \cdot kg^{-1}$), groups III and IV received HPE (50 $mg \cdot kg^{-1}$). At day 5, mice of groups II and IV were submitted to HSV-2 infection according to the above-mentioned item. Groups I and III were submitted to the same procedure but were not infected with HSV-2. All groups were submitted to vehicle or HPE (50 $mg \cdot kg^{-1}$) treatment for more than 5 days after HSV-2 infection.

At day 11, extravaginal lesions were recorded for each animal and scored according to a six-point scale as follows: 0—no sign of infection, 1—slight genital erythema and edema, 2—moderate genital inflammation, 3—severe exudative genital lesions, 4—hind limb paralysis and 5—death.[25] Three mice of HSV-2 group died between the fourth and fifth day after HSV-2 infection and have not been included in *ex vivo* experiments. Afterwards, all mice were anesthetized for blood collection by heart puncture for haematological analysis. Then, they were killed by cervical dislocation, and the vaginal tissues were removed for *ex vivo* experiments (histopathology and biochemical analysis).

Ex vivo experiments

Tissue preparation (S1). Vaginal tissue was immediately homogenized in cold 50 mmol·l^{-1} of Tris–HCl, pH 7·4 (1/10, w/v). The low-speed supernatant (S1) was used to determine reactive species (RS), 3-nitrotyrosine (3-NT), non-protein thiols (NPSH) and ascorbic acid (AA) levels; superoxide dismutase (SOD) and catalase (CAT) activities.

RS levels. To estimate the level of tissue homogenate RS production, an aliquot of S1 (10 µl) was incubated with 10 µl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA;1 mmol·l^{-1}). The RS levels were determined by a spectrofluorimetric method. The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) is measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480-nm excitation) 30 min after the addition of DCHF-DA to the medium. RS levels were expressed as units of fluorescence (UF).[26]

Tyrosine nitration levels. Determination of 3-nitrotyrosine (3-NT) and tyrosine was performed by High-performance liquid chromatography coupled to ultraviolet (HPLC-UV) detection method.[27] Briefly, S1 aliquots of each sample were hydrolysed in HCl (12 N; 1:1 v/v) at 60 °C for 24 h. Digested samples were filtered through a membrane (0.45-µm pore size) Millipore® before injection on to the HPLC instrument. Samples were analysed on a Shimadzu® HPLC apparatus. The analytical column was a 5-µm-pore size Spherisorb ODS-2 C_{18} reverse-phase column (4.6 × 250 mm). The mobile phase was 50 mmol·l^{-1} of sodium acetate, 50 mmol·l^{-1} of sodium citrate and 8% (v/v) methanol, pH 3·1 (corrected with HCl 12 N). HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml·min^{-1} and UV detector set at 274 nm. Tyrosine nitration levels were expressed as 3-NT (µmol·l^{-1})/tyrosine (µmol·l^{-1}).

NPSH levels. To determine NPSH, S1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded, and free SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 mol·l^{-1} of potassium phosphate buffer, pH 7·4, and 10 mmol·l^{-1} of DTNB.[28] The color reaction was measured at 412 nm. NPSH levels were expressed as mmol g^{-1} tissue.

AA levels. S1 was precipitated in 10 volumes of a cold 4% trichloroacetic acid solution.[29] An aliquot of its supernatant at a final volume of 1 ml of the solution was incubated for 3 h at 38 °C, then 1 ml of H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg·ml^{-1} of dinitrophenyl hydrazine and $CuSO_4$ (0·075 mg·ml^{-1}) at 520 nm. The content of AA is related to tissue amount (µmol AA/g wet tissue).

SOD activity

Superoxide dismutase activity was assayed spectrophotometrically and based on the capacity of SOD to inhibit autoxidation of epinephrine to adrenochrome. Enzymatic reaction was initiated by adding an S1 aliquot (150 µl) of the homogenized tissue and the substrate (epinephrine) to a concentration of 4 mmol·l^{-1} in a medium containing 50 mmol·l^{-1} of bicarbonate buffer, pH 10·3. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26 °C.[30]

CAT activity. Catalase activity was assayed spectrophotometrically, which involves monitoring the disappearance of H_2O_2 in the homogenate at 240 nm. Enzymatic reaction was initiated by adding an aliquot of 20 µl of S1 and the substrate (H_2O_2) to a concentration of 0.3 mmol·l^{-1} in a medium containing 50 mmol·l^{-1} of phosphate buffer, pH 7·0. The enzymatic activity was expressed in Units (one Unit decomposes 1 µmol of H_2O_2 per min at pH 7 at 25 °C)/mg protein.[31]

Myeloperoxidase activity

The vaginal tissues were homogenized in potassium phosphate buffer (20 mmol·l^{-1}, pH 7·4) containing ethylenediaminetetraacetic acid (0.1 mmol·l^{-1}). After the homogenization, the samples were centrifuged at 2000 *g* at 4 °C for 10 min to yield a low-speed supernatant fraction (S2). Then, the S2 fraction was centrifuged again at 20 000 *g* at 4 °C for 15 min to yield a final pellet that was resuspended in potassium phosphate buffer (50 mmol·l^{-1}, pH 6·0) containing hexadecyl trimethyl ammonium bromide (0.5%). The samples were finally frozen and thawed three times for the posterior enzymatic myeloperoxidase (MPO) assay. Besides, aliquots of vaginal tissue preparations were frozen (−20 °C) for 1-week posterior analysis.[32]

For the MPO activity measurement, an aliquot of S2 (20 µl) was added to a medium containing potassium phosphate buffer (50 mmol·l^{-1}; pH 6·0), hexadecyl trimethyl ammonium bromide (0·5%) and N,N,N',N'-tetramethylbenzidine (1·5 mmol·l^{-1}). The kinetic analysis of MPO was started after H_2O_2 (0·01%) addition, and the color reaction was measured at 655 nm at 37 °C. Results are expressed as mmol MPO/mg of protein.

Haematological parameters. Haematological parameters (total leukocytes, neutrophils and lymphocytes) were determined in MICROS 60 (HORIBA ABX Diagnostics) equipment.

Histopathology. Three mice per group were subjected to a detailed necropsy evaluation. Small pieces of vaginal tissue from individual mice were fixed in 10% buffered formalin solution for 24 h after they were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Serial sections of 4 μm in thickness were cut and stained with haematoxylin and eosin, and evaluated under light microscopy. The sections were analysed by a histologist who was not aware of sample assignment to experimental groups. Only qualitative histology was performed. The morphological changes in the layers of the epidermis and the loose and dense connective tissue in the dermis were observed. The histological features in injury of skin were as follows: granulation tissue, vascular congestion, haemorrhage and neutrophil leukocyte infiltration. Decidual morphology was assessed using light microscopy.

Protein quantification. Protein concentration was measured using bovine serum albumin as the standard.[33]

Statistical analysis

Statistical analysis of data was performed using a two-way analysis of variance (ANOVA), followed by *post hoc* comparisons using the Newman–Keuls multiple range test when appropriate. Main effects are presented only when the second-order interaction was not significant. Experiments were expressed as means ± standard error of the mean. Differences between groups were considered statistically significant when $P < 0{\cdot}05$.

RESULTS

Determination of total phenols and flavonoids contents

The total phenolic and flavonoid contents presented in the HPE samples used in our study were 130 mg of gallic acid equivalent per g of HPE and 12·4 mg of quercetin equivalent per g of HPE, respectively.

Extravaginal lesions

Two-way ANOVA showed a significant main effect of HSV-2 infection ($F_{(1,\ 18)} = 22{\cdot}12$; $P < 0{\cdot}0002$) in vaginal tissues of mice. *Post hoc* comparisons showed that HSV-2 significantly increased the lesions of score means. Treatment with HPE protected against the increase in the lesion score means induced by HSV-2 infection. Animals of control and HPE groups did not present infection. Three mice of HSV-2 group died between the fourth and fifth day after HSV-2 infection and have not been included in *ex vivo* experiments (Table 1).

RS levels

Two-way ANOVA of RS levels revealed a significant HSV-2 × HSV-2 + HPE interaction ($F_{(1,\ 13)} = 5{\cdot}07$; $P < 0{\cdot}0422$). *Post hoc* comparisons showed that HSV-2 significantly increased RS levels in vaginal tissues of mice when compared with the control group. Treatment with HPE protected against the increase of RS levels in vaginal tissues caused by HSV-2 in mice. RS levels were not altered in uninfected mice treated with HPE (Figure 1).

Table 1. Effect of treatment with hydroalcoholic propolis extract on extravaginal lesions caused by herpes simplex virus type 2 in female mice

Groups	Score means*
Control	NS
HPE	NS
HSV-2	3·25 ± 0·559*
HSV-2 + HPE	1·4 ± 0·509

Data are reported as mean ± standard error of the mean (n = 4–8) and were analysed using two-way analysis of variance, followed by Newman–Keuls test when appropriated. *Denotes $P < 0{\cdot}05$ as compared with the control group. 'NS', animals which did not present infection.
HPE, hydroalcoholic propolis extract; HSV-2, herpes simplex virus type 2.
*Score means of extravaginal lesions observed at day 11 of experiment.

Tyrosine nitration levels

Two-way ANOVA of tyrosine nitration levels showed a significant HSV-2 × HSV-2 + HPE interaction ($F_{(1,\ 13)} = 20{\cdot}80$; $P < 0{\cdot}0070$). *Post hoc* comparisons showed that HSV-2 significantly increased tyrosine nitration levels in vaginal tissues of mice when compared with the control group. Treatment with HPE significantly protected against the increase of tyrosine nitration levels in vaginal tissues of mice caused by HSV-2 infection. HPE administration in uninfected mice caused a decrease of tyrosine nitration levels *per se* (Figure 2).

NPSH levels

Non-protein thiol levels were not altered in vaginal tissues of mice from all groups tested (data not shown).

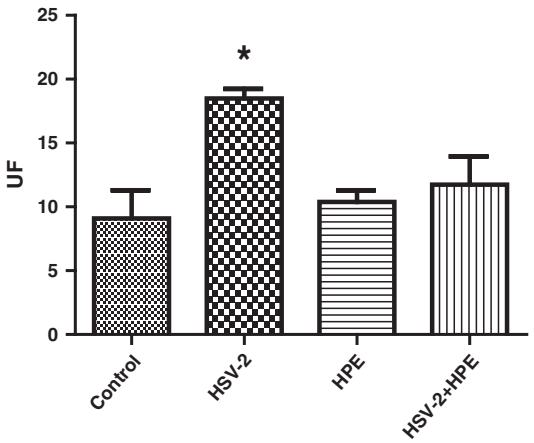

Figure 1. Effect of treatment with hydroalcoholic propolis extract (HPE) at the dose of 50 mg·kg^{-1} on reactive species (RS) levels in vaginal tissues of female mice infected with herpes simplex virus type 2 (HSV-2). Data are reported as mean ± standard error of the mean (SEM) ($n = 4$–5) and were analysed using two-way analysis of variance (ANOVA) followed by Newman–Keuls test when appropriate. Results are expressed as UF. *Denotes $P < 0{\cdot}05$ as compared with all other groups. *Abbreviations*: **Control**, control group; **HSV-2**, HSV-2-infected group; **HPE**, HPE-treated group; **HSV-2 + HPE**, HSV-2-infected and HPE-treated group

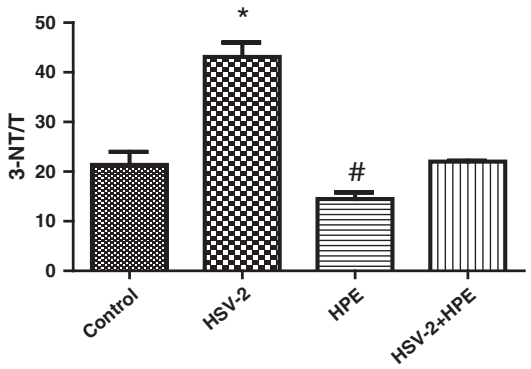

Figure 2. Effect of treatment with HPE at the dose of 50 mg·kg^{-1} on tyrosine nitration levels in vaginal tissues of female mice infected with HSV-2. Data are reported as mean ± SEM (n = 4–5) and were analysed using two-way ANOVA, followed by Newman–Keuls test when appropriate. Results are expressed as 3-nitrotyrosine (μmol·l^{-1})/tyrosine (μmol·l^{-1}) (3-NT/T). *Denotes $P < 0.05$ as compared with all other groups. #Denotes $P < 0.05$ as compared with the control group

AA levels

Two-way ANOVA of AA levels revealed a significant HSV-2 × HSV-2 + HPE interaction ($F_{(1, 15)} = 5.49$; $P < 0.0333$). *Post hoc* comparisons showed that HSV-2 significantly increased AA levels in vaginal tissues of mice when compared with the control group. Treatment with HPE protected against the increase of AA levels in vaginal tissues caused by HSV-2 in mice (Figure 3).

SOD activity

Superoxide dismutase activity was not altered in vaginal tissues of mice from all groups tested (data not shown).

CAT activity

Two-way ANOVA showed a significant main effect of HSV-2 infection ($F_{(1, 15)} = 16.70$; $P < 0.001$) and HPE treatment ($F_{(1, 15)} = 5.51$; $P < 0.0330$) on CAT activity. *Post hoc* comparisons revealed that the HSV-2 infected group had an inhibition of CAT activity. HPE treatment was effective in protecting against the inhibition of CAT activity induced by HSV-2 infection to control levels (Figure 4).

MPO activity

Two-way ANOVA of MPO activity yielded a significant HSV-2 × HSV-2 + HPE interaction ($F_{(1, 15)} = 18.21$; $P < 0.0007$). *Post hoc* comparisons showed that HSV-2 significantly increased MPO activity in vaginal tissues of mice when compared with the control group. Treatment with HPE protected against the increase of MPO activity in vaginal tissues caused by HSV-2 in mice. HPE administration in uninfected mice did not show effect *per se* (Figure 5).

Haematological parameters

Two-way ANOVA of total leukocytes [white blood cell (WBC)] showed a significant main effect of HSV-2 infection ($F_{(1, 13)} = 9.32$; $P < 0.0092$) and HPE treatment ($F_{(1, 13)} = 7.11$; $P < 0.0193$). *Post hoc* comparisons showed that HSV-2 significantly increased WBC levels in blood samples of mice when compared with the control group. Treatment with HPE protected against the increase of WBC in blood samples caused by HSV-2 infection in mice (Figure 6A). HPE treatment in uninfected mice did not demonstrate effect *per se*.

Two-way ANOVA of neutrophils yielded a significant HSV-2 × HSV-2 + HPE interaction ($F_{(1, 13)} = 6.19$; $P < 0.0272$). *Post hoc* comparisons showed that HSV-2 significantly increased neutrophils in blood samples of mice when compared with the control group. Treatment with HPE protected

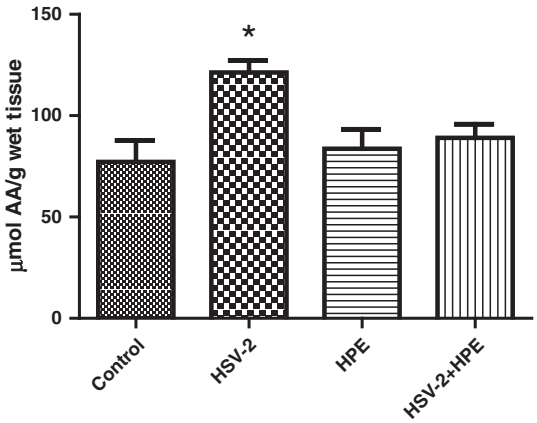

Figure 3. Effect of treatment with HPE at the dose of 50 mg·kg^{-1} on ascorbic acid (AA) levels in vaginal tissues of female mice infected with HSV-2. Data are reported as mean ± SEM (n = 4–5) and were analysed using two-way ANOVA, followed by Newman–Keuls test when appropriate. Results are expressed as μmol of AA per g of wet tissue. *Denotes $P < 0.05$ as compared with all other groups

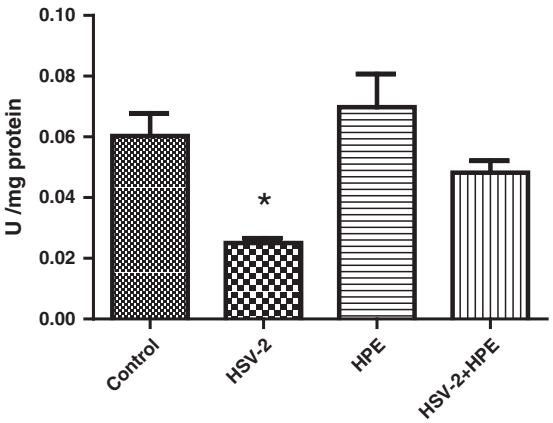

Figure 4. Effect of treatment with HPE at the dose of 50 mg·kg^{-1} on catalase activity in vaginal tissues of female mice infected with HSV-2. Data are reported as mean ± SEM (n = 4–5) and were analysed using two-way ANOVA, followed by Newman–Keuls test when appropriate. Results are expressed as U·mg^{-1} of protein. *Denotes $P < 0.05$ as compared with all other groups

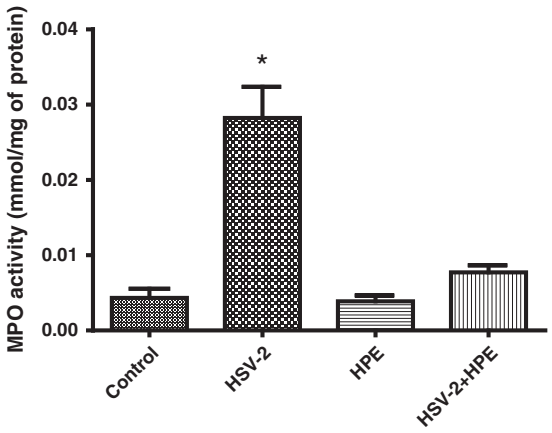

Figure 5. Effect of treatment with HPE at the dose of 50 mg·kg^{-1} on myeloperoxidase activity in vaginal tissues of female mice infected with HSV-2. Data are reported as mean ± SEM ($n = 4$–5) and were analysed using two-way ANOVA followed by Newman–Keuls test when appropriate. Results are expressed as mmol of MPO per mg of protein. *Denotes $P < 0.05$ as compared with all other groups

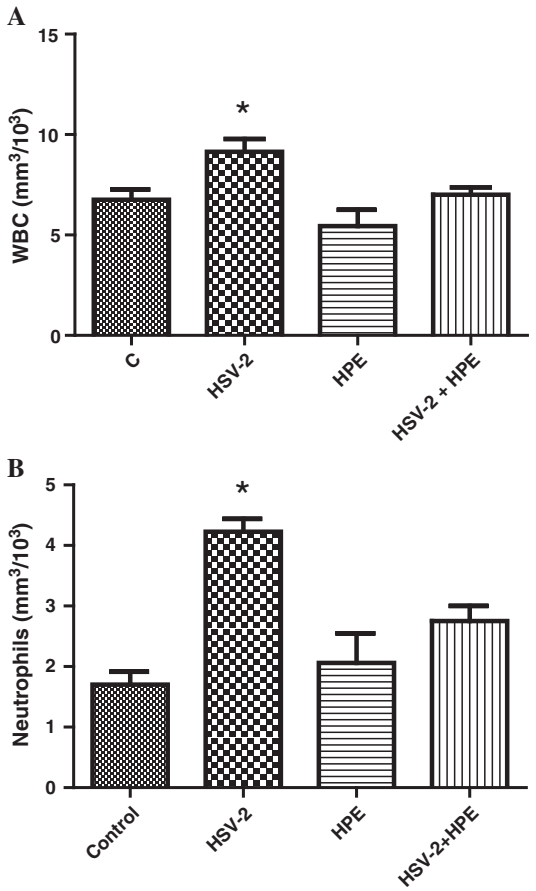

Figure 6. Effect of treatment with HPE at the dose of 50 mg·kg^{-1} on total leukocytes (white blood cell) (**6A**) and neutrophils (**6B**) in blood samples of female mice infected with HSV-2. Data are reported as mean ± SEM (n = 4–5) and were analysed using two-way ANOVA followed by Newman–Keuls test when appropriate. Results are expressed as mm^3 of WBC per 10^3 (6A) and mm^3 of neutrophils per 10^3 (6B). *Denotes $P < 0{\cdot}05$ as compared with all other groups

against the increase of neutrophils in blood samples caused by HSV-2 infection in mice (Figure 6B).

Lymphocytes were not altered in blood samples in all groups tested (data not shown).

Histopathology

In the control group, the histological evaluation showed a complete epithelialization in the epidermis. Note the loose and dense connective tissue of the dermis of normal aspect with the presence of cutaneous annexes as sebaceous glands (Figures 7A and 7B). The HSV-2-infected mice showed intense dense and loose connective tissue disorganization with marked leukocyte infiltration in the epidermis and dermis. The absence of all layers of the epidermis compared with the control was observed (Figures 7C and 7D). The treatment with HPE reduced inflammation in both epidermis and dermis of animals infected with HSV-2 (Figures 7E and 7F). The analysis demonstrated the presence of normal epithelialization without morphological alterations in the dermis. The histopathologic data of mice from HPE-treated group were similar to that of the control group (data not shown).

DISCUSSION

Propolis is an advantageous compound and its constituents, mainly phenols and flavonoids, have been responsible for several biological properties as anti-inflammatory and antioxidant.[3] In this study, we report, for the first time, the protective effect of HPE treatment against acute vaginal lesions induced by HSV-2 in female mice. It was observed that HPE treatment reduced extravaginal lesions and tissue damage of infected mice. We demonstrated that pro-inflammatory (total leukocytes, neutrophils and MPO activity) and oxidative stress (RS, tyrosine nitration, AA levels, MPO and CAT activity) markers are involved in HSV-2 tissue damage and that HPE protective effect is related to anti-inflammatory and antioxidant processes.

Animals infected with HSV-2 had an increase in the appearance of visual signals in vaginal area such as swelling, edema and inflammation. Additionally, 3 of 8 animals of HSV-2 group were found dead before the end of treatment. In fact, lesions in genital area are common in HSV-2 because the virus produces local replication infecting cell to cell. Primary infections by HSV are usually intense due to the lack of specific immunity. HSV obtains access to the nervous system and follows a retrograde movement along neuronal axons establishing latent infection in the lumbosacral sensory ganglia.[34] Therefore, may appear lesions, inflammatory ulcerations and death.[35] Our histological findings also are in accordance with the reports above because we observed intense dense and loose connective tissue disorganization with a marked leukocyte infiltration in the epidermis and dermis of HSV-2 infected mice.

The important point of this work is that HPE treatment was effective in attenuating the extravaginal lesions, preventing lesion progress and prolonging the life of the

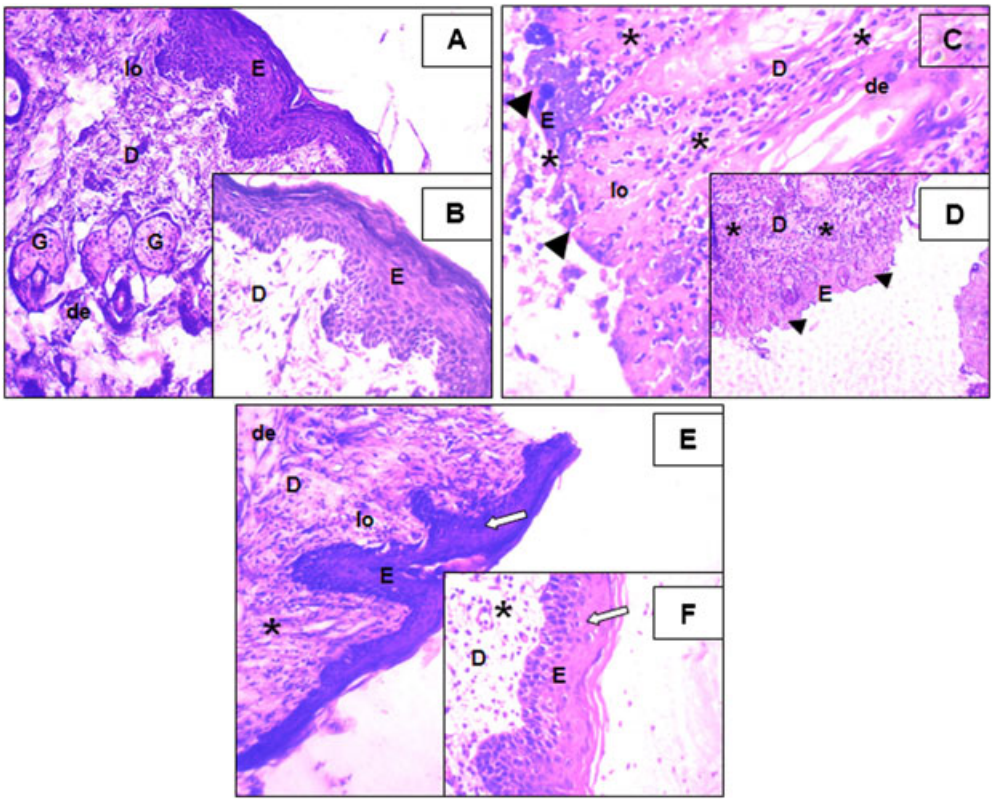

Figure 7. Photomicrography of the section of vaginal tissue with a detail on the right of (7A, 7B) an animal in the control group showed complete epithelialization in the epidermis. Note the loose (lo) and dense (de) connective tissue of the dermis of normal aspect. Note the presence of sebaceous glands (G). HSV-2-infected animal (7 C, 7D) showed an intense cellular infiltration (*) containing neutrophils and mononuclear cells in the epidermis and dermis. Observe the absence of all layers of the epidermis (arrowhead). HSV-2 + HPE group (7E, 7 F) showing intense reduction of inflammatory cells in the epidermis and dermis (*). Observe the presence of normal complete epithelialization (arrow) without morphological alterations in the dermis. Epidermis (E); Dermis (D). Haematoxylin and eosin; original magnification, $\times 100$ and $\times 400$, respectively

animals. Furthermore, it was able to reduce inflammation in both epidermis and dermis and to maintain a normal epithelialization without morphological alterations in the dermis, which is indicative of a decrease in viral infection. Concerning other biological properties of propolis, its extracts clearly have viricidal action. Amoros *et al.* (1992) investigated the *in vitro* effect of propolis on several DNA and RNA viruses including HSV 1 (an acyclovir resistant mutant), HSV 2, adenovirus type 2, vesicular stomatitis virus and poliovirus type 2.

The main bioactive compounds of propolis are phenolic compounds, especially flavonoids and phenolic acids.[36,37] For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases.[3] The chemical composition of HPE used in our study presented a great amount of total phenols ($130\,mg{\cdot}g^{-1}$) and flavonoid ($12{\cdot}4\,mg{\cdot}g^{-1}$) contents. These contents are similar to those observed by other authors in HPE samples derived from the southeastern region of Brazil and in methanolic extracts of propolis from Argentina.[38,39] These works also displayed the antioxidant and antibacterial activities of respective samples.

Our study demonstrated that HSV-2 infection increased total leukocytes and neutrophils in the bloodstream of mice. These, in turn, are related with inflammatory process in the vaginal tissue caused by herpetic lesions. It was well characterized by an increase in MPO activity and neutrophil infiltration in the vaginal tissue. The results showed that HPE treatment attenuated virus infection through reduction of total leukocytes and neutrophils in the bloodstream. However, lymphocytes were not altered. Our experimental model was an acute HVS-2 infection, and an increase of lymphocytes was not expected. During HSV-2 genital infection, the traffic of lymphocytes is mostly between vaginal epithelial cell and iliac lymph nodes, and they do not come back to the blood.[40] In the initial infection of genital mucosal surface, HSV-2 replicates primarily in the epithelium and in innate cells including dendritic cells and natural killer cells that become activated by viral structures.[41] Neutrophils are early and predominant innate immune cells that infiltrate the genital tract and contribute towards the resolution of HSV-2.[42,43] The migration of neutrophils is mediated by cytokines and chemokines (CXCL-1) released from epithelial cells during infection.[42] Previous studies have shown that oral treatment with propolis reduces the viral attachment to cell surface and, consequently, the infection.[20] Then, in our study, we showed that animals previously treated with HPE were more resistant to the development of HSV-2

lesions and probably did not produce enough mediators for neutrophil activation or migration as did untreated infected mice. In fact, neutrophils are one of the mainly participants of the immunity against HSV infection.[44]

Another important finding observed is that HPE treatment protected against the increase of MPO activity caused by HSV-2 infection, probably because of its constituents such as phenolic compounds quantified in our study. The decrease of MPO activity also may corroborate with the decrease on neutrophils, given that this pro-inflammatory enzyme can reflect the activation of neutrophils at the site of injury. In this context, we can suggest that MPO activity is a probable mechanism involved in the anti-inflammatory effect of HPE in this model.[45] Authors demonstrated that propolis treatment normalizes inflammatory infiltrates consistent with the ability to inhibit MPO activity in mice skin.[46] Moreover, other studies suggested that propolis has anti-inflammatory effects via inhibiting the release of prostaglandins, leukotrienes and histamine [47] or via inhibition of the release of arachidonic acid from cell membranes and suppression of COX-1 and COX-2 enzyme activities.[48,49]

The present results showed the involvement of oxidative stress in tissue injury induced by HSV-2 in female mice. It is characterized by an increase of RS, 3-NT and AA levels and by an inhibition in CAT activity. It is known that under inflammatory conditions, free radicals including ROS and reactive nitrogen species (RNS) are generated from inflammatory and epithelial cells.[50] Also, it is well documented that viral multiplication stimulates intracellular redox processes. Stimulation of the redox processes, in turn, promotes the production of various free radicals, which have a noxious influence on cell viability. Furthermore, we observed a notable increase of neutrophil infiltration into the vaginal mucosa of mice infected with HSV-2. The neutrophil cells also contribute to the production of ROS via activation of their nicotinamide adenine dinucleotide phosphate oxidase and secretion of myeloperoxidase into extracellular space.[51]

It was observed that HPE treatment was able to protect against tyrosine nitration, and we suppose that this protection is caused by its antioxidant properties. In this way, several authors demonstrate that some constituents present in propolis like flavonoids have important antioxidant function,[52] which may provide efficient peroxynitrite ($ONOO^-$) scavenger activity.[53,54] HSV-2 infection increased tyrosine nitration levels in vaginal tissues of female mice. This effect is probably caused by the inflammatory response associated with HSV-2 infection. Nitration of proteins represent key biologically relevant redox signaling and injury events. These processes involve the participation of nitric oxide-derived species (RNS) such as $ONOO^-$ and nitrogen dioxide ($\cdot NO_2$) generated during oxidative and nitrative stress. $ONOO^-$ is a highly reactive product of the reaction between superoxide anion ($O_2^{-\cdot}$) and nitric oxide ($NO\cdot$). Different cells, such as neutrophils and activated macrophages, produce significant quantities of $O_2^{-\cdot}$ and $NO\cdot$ during the inflammatory response, and these cells may be the major sources of peroxynitrite.

Hydroalcoholic propolis extract showed a protective effect against oxidative stress because of decreased RS levels in vaginal tissues of HSV-2-infected mice. This effect also is attributed to flavonoids because scavengers of free radicals [55] could attenuate the oxidative stress, determined by oxygen free radicals, and consequently, promote an antiviral protection.[56,57] In line with the oxidative stress hypothesis, antioxidant molecules, such as CAT and AA, are involved in several infection processes.[58] CAT is a potent protective enzyme that metabolizes the excess of H_2O_2 producing $O_2 + H_2O$, decreasing the intracellular redox status. We found in this work a decrease of CAT activity in vaginal tissues of mice after HSV-2 infection, and propolis was able to protect this effect. Our results demonstrated an increase of AA, another antioxidant molecule, in HSV-2-infected group that probably occur by high levels of oxidative stress. HPE administration reduced the AA levels similar to the control group by the absence of the excessive oxidative stress. SOD activity and NPSH levels were not altered in our study; probably, they are not directly involved in HSV-2 infection.

The different properties from propolis are caused by a natural mixture of constituents like flavonoids, especially galangin and caffeic acid phenethyl ester (CAPE) because a single propolis constituent does not have an activity greater than that of the total extract.[6] The anti-inflammatory and antioxidant activities of propolis observed in our experiment probably can be caused by the presence of a mixture of constituents. Several investigations have pointed out that galangin (a type of flavonoid) has shown to inhibit cyclooxygenase (COX) and lipooxygenase activity. Furthermore, it has been reported that CAPE, another component of propolis, possesses anti-inflammatory activity by inhibiting the release of arachidonic acid from the cell membrane.[59]

Concerning our results on histological and biochemical data, the HSV-2 infection has a relationship with changes on inflammatory and oxidative markers, evidenced by an increase on neutrophil infiltration, MPO activity, RS, tyrosine nitration and AA levels and a decrease in CAT activity. Thus, it is possible that the effect of HPE is caused by its anti-inflammatory and antioxidant properties. However, we do not exclude the possibility of propolis to be protecting from HSV-2 infection by having antiviral properties. A recent study *in vitro* revealed that the addition of propolis extract to a cell culture 2 h before or at the time of viral infection completely blocked the development of the viral infection. The effect could be the result of blocking by propolis of the cell membrane receptors for HSV, where propolis interact with the cell membrane and could block the penetration of viral particles into the cells and/or could induce internal changes inside the host cells, which would in turn affect the virus replication cycle.[20]

Based on the above considerations, we conclude that the protective effect of HPE in this model is caused by a combination of effects. HPE revealed a reduction of the inflammatory damage and oxidative stress at the infected vaginal region, proving its anti-inflammatory and antioxidant actions in HSV-2 infected mice. These actions are probably caused by phenolic compounds, especially flavonoids

present in HPE. This indicates that propolis could be an important natural alternative therapy against infections caused by HSV-2.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

The financial support by FAPERGS, CAPES and CNPq is gratefully acknowledged.

M. Prigol is a recipient of PNPD/CAPES fellowship.

REFERENCES

1. Nolkemper S, Reichling J, Sensch KH, Schnitzler P. Mechanism of herpes simplex virus type 2 suppression by propolis extracts. *Phytomedicine* 2010; **17**: 132–138.
2. Banskota AH, Tezuka Y, Adnyana IK, *et al*. Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and China. *J Ethnopharmacol* 2000; **72**: 239–246.
3. Marcucci MC. Propolis - Chemical-Composition, Biological Properties and Therapeutic Activity. *Apidologie* 1995; **26**: 83–99.
4. Afaq F, Mukhtar H. Botanical antioxidants in the prevention of photocarcinogenesis and photoaging. *Exp Dermatol* 2006; **15**: 678–684.
5. Kumazawa S, Hamasaka T, Nakayama T. Antioxidant activity of propolis of various geographic origins. *Food Chemistry* 2004; **84**: 329–339.
6. Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J Ethnopharmacol* 1999; **64**: 235–240.
7. Wang L, Mineshita S, Ga I, Shigematsu T, Matsuno T. Antiinflammatory effect of propolis. *Jpn J Clin Pharmacol Therapeut* 1993; **24**: 223–224.
8. Nakajima Y, Shimazawa M, Mishima S, Hara H. Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. *Life Sci* 2007; **80**: 370–377.
9. Kimoto T, Aga M, Hino K, *et al*. Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. *Anticancer Res* 2001; **21**: 221–228.
10. Amoros M, Lurton E, Boustie J, Girre L, Sauvager F, Cormier M. Comparison of the anti-herpes simplex virus activities of propolis and 3-methyl-but-2-enyl caffeate. *J Nat Prod* 1994; **57**: 644–647.
11. Serkedjieva J, Manolova N, Bankova V. Antiinfluenza Virus Effect of Some Propolis Constituents and Their Analogs (Esters of Substituted Cinnamic-Acids). *J Nat Prod* 1992; **55**: 294–297.
12. Ito J, Chang FR, Wang HK, *et al*. Anti-AIDS agents. 48.(1) Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. *J Nat Prod* 2001; **64**: 1278–1281.
13. Amoros M, Simoes CM, Girre L, Sauvager F, Cormier M. Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. *J Nat Prod* 1992; **55**: 1732–1740.
14. Cohen HA, Varsano I, Kahan E, Sarrell EM, Uziel Y. Effectiveness of an herbal preparation containing echinacea, propolis, and vitamin C in preventing respiratory tract infections in children: a randomized, double-blind, placebo-controlled, multicenter study. *Arch Pediatr Adolesc Med* 2004; **158**: 217–221.
15. Debiaggi M, Tateo F, Pagani L, Luini M, Romero E. Effects of propolis flavonoids on virus infectivity and replication. *Microbiologica* 1990; **13**: 207–213.
16. Whitley RJ, Gnann JW. Viral encephalitis: familiar infections and emerging pathogens. *Lancet* 2002; **359**: 507–513.
17. Patel R. Genital Herpes. *Medicine* 2010; **38**: 276–280.
18. Armien AG, Hu S, Little MR, *et al*. Chronic cortical and subcortical pathology with associated neurological deficits ensuing experimental herpes encephalitis. *Brain Pathol* 2010; **20**: 738–750.
19. Schachtele SJ, Hu S, Little MR, Lokensgard JR. Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2. *J Neuroinflammation* 2010; **7**: 35.
20. Huleihel M, Isanu V. Anti-herpes simplex virus effect of an aqueous extract of propolis. *Isr Med Assoc J* 2002; **4**: 923–927.
21. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult* 1965; **16**: 144–158.
22. Park YK, Koo MH, Sato HH, Contado JL. Survey of some components of propolis which were collected by Apis mellifera in Brazil. *Arquivos de Biologia e Tecnologia* 1995; **38**: 1253–1259.
23. Spear PG, Roizman B. Proteins Specified by Herpes-Simplex Virus .5. Purification and Structural Proteins of Herpesvirion. *J Virol* 1972; **9**: 143-&.
24. Kwant-Mitchell A, Ashkar AA, Rosenthal KL. Mucosal Innate and Adaptive Immune Responses against Herpes Simplex Virus Type 2 in a Humanized Mouse Model. *J Virol* 2009; **83**: 10664–10676.
25. Hayashi K, Hayashi T, Miyazawa K, Tomoda A. Phenoxazine derivatives suppress the infections caused by herpes simplex virus type-1 and herpes simplex virus type-2 intravaginally inoculated into mice. *J Pharmacol Sci* 2010; **114**: 85–91.
26. Loetchutinat C, Kothan S, Dechsupa S, Meesungnoen J, Jay-Gerin J, Mankhetkorn S. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 20,70-dichlorofluorescein diacetate assay. *Radiat Phys Chem* 2005; **72**: 323–331.
27. Erdal N, Gurgul S, Tamer L, Ayaz L. Effects of long-term exposure of extremely low frequency magnetic field on oxidative/nitrosative stress in rat liver. *J Radiat Res (Tokyo)* 2008; **49**: 181–187.
28. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; **82**: 70–77.
29. Jacques-Silva MC, Nogueira CW, Broch LC, Flores EM, Rocha JB. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. *Pharmacol Toxicol* 2001; **88**: 119–125.
30. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; **247**: 3170–3175.
31. Aebi H. Catalase in vitro. *Methods Enzymol* 1984; **105**: 121–126.
32. Grisham MB, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. *Am J Physiol* 1986; **251**: G567–G574.
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
34. Szpara ML, Kobiler O, Enquist LW. A Common Neuronal Response to Alphaherpesvirus Infection. *J Neuroimmune Pharmacol* 2010; **5**: 418–427.
35. Stevens JG, Cook ML. Latent Herpes Simplex Virus in Spinal Ganglia of Mice. *Science* 1971; **173**: 843-&.
36. Bankova V. Chemical diversity of propolis and the problem of standardization. *J Ethnopharmacol* 2005; **100**: 114–117.
37. Sales A, Alvarez A, Rodriguez Areal M, *et al*. Content of flavonoids in the Argentinean propolis. *J Hazard Mater* 2006; **137**: 1352–1356.
38. Castro ML, Cury J, Rosalen PL. Própolis do Sudeste e Nordeste do Brasil: Influência da Sazonalidade na Atividade Antibacteriana e Composição Fenólica. *Quim. Nova* 2007; **30**: 1512–1516.
39. Lima B, Tapia A, Luna L, *et al*. Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the Province of San Juan (Argentina). *J Agric Food Chem* 2009; **57**: 2691–2698.
40. Nicolas E, Reichhart JM, Hoffmann JA, Lemaitre B. In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila. *J Biol Chem* 1998; **273**: 10463–10469.

41. MasCasullo V, Fam E, Keller MJ, Herold BC. Role of Mucosal Immunity in Preventing Genital Herpes Infection. *Viral Immunol* 2005; **18**: 595–606.
42. Milligan G. Neutrophils aid in protection of the vaginal mucosae of immune mice against challenge with herpes simplex virus type 2. *J Virol* 1999; **73**: 6380–6386.
43. Thapa M, Carr DJ. Chemokines and Chemokine Receptors Critical to Host Resistance following Genital Herpes Simplex Virus Type 2 (HSV-2) Infection. *Open Immunol J.* 2008; **1**: 33–41.
44. Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease - Herpetic stromal keratitis. *J Immunol* 1997; **158**: 1383–1391.
45. McLennan SV, Bonner J, Milne S, *et al*. The anti-inflammatory agent Propolis improves wound healing in a rodent model of experimental diabetes. *Wound Repair Regen* 2008; **16**: 706–713.
46. Beyer G, Melzig MF. Effects of propolis on hypoxanthine-xanthine oxidase-induced toxicity in cultivated human cells and on neutrophil elastase activity. *Biol Pharm Bull* 2005; **28**: 1183–1186.
47. Pascual C, Gonzalez R, Torricella RG. Scavenging action of propolis extract against oxygen radicals. *J Ethnopharmacol* 1994; **41**: 9–13.
48. Borrelli F, Maffia P, Pinto L, *et al*. Phytochemical compounds involved in the anti-inflammatory effect of propolis extract. *Fitoterapia* 2002; **73**(Suppl 1): S53–S63.
49. Rossi A, Longo R, Russo A, Borrelli F, Sautebin L. The role of the phenethyl ester of caffeic acid (CAPE) in the inhibition of rat lung cyclooxygenase activity by propolis. *Fitoterapia* 2002; **73**(Suppl 1): S30–S37.
50. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860–867.
51. Luk HH, Ko JK, Fung HS, Cho CH. Delineation of the protective action of zinc sulfate on ulcerative colitis in rats. *Eur J Pharmacol* 2002; **443**: 197–204.
52. Miguel MG, Nunes S, Dandlen SA, Cavaco AM, Antunes MD. Phenols and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve, South of Portugal. *Food Chem Toxicol* 2010; **48**: 3418–3423.
53. Fiala ES, Sodum RS, Bhattacharya M, Li H. (−)-Epigallocatechin gallate, a polyphenolic tea antioxidant, inhibits peroxynitrite-mediated formation of 8-oxodeoxyguanosine and 3-nitrotyrosine. *Experientia* 1996; **52**: 922–926.
54. Schroeder P, Zhang H, Klotz LO, Kalyanaraman B, Sies H. (−)-epicatechin inhibits nitration and dimerization of tyrosine in hydrophilic as well as hydrophobic environments. *Biochem Biophys Res Commun* 2001; **289**: 1334–1338.
55. Mathiesen L, Wang S, Halvorsen B, Malterud KE, Sund RB. Inhibition of lipid peroxidation in low-density lipoprotein by the flavonoid myrigalone B and ascorbic acid. *Biochem Pharmacol* 1996; **51**: 1719–1725.
56. Nakayama T, Yamada M, Osawa T, Kawakishi S. Suppression of active oxygen-induced cytotoxicity by flavonoids. *Biochem Pharmacol* 1993; **45**: 265–267.
57. Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* 1988; **37**: 837–841.
58. Lee YT, Don MJ, Liao CH, Chiou HW, Chen CF, Ho LK. Effects of phenolic acid esters and amides on stimulus-induced reactive oxygen species production in human neutrophils. *Clin Chim Acta* 2005; **352**: 135–141.
59. Jian-Hong C, Yu S, Mou-Tuan H, Chee-Kok C, Chi-Tang H. Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells. *Cancer Letter* 1996; **108**: 211–214.

