Parasitol Res (2016) 115:1557–1566
DOI 10.1007/s00436-015-4890-4

ORIGINAL PAPER

Propolis reduces *Leishmania amazonensis*-induced inflammation in the liver of BALB/c mice

Suelen S. da Silva[1] · Sandra S. Mizokami[1] · Jacqueline R. Fanti[1] · Milena M. Miranda[1] · Natalia Y. Kawakami[1] · Fernanda Humel Teixeira[2] · Eduardo J. A. Araújo[2] · Carolina Panis[3] · Maria A. E. Watanabe[1] · José M. Sforcin[4] · Wander R. Pavanelli[1] · Waldiceu A. Verri Jr[1] · Ionice Felipe[1] · Ivete Conchon-Costa[1]

Received: 13 June 2015 / Accepted: 11 December 2015 / Published online: 28 December 2015

Abstract Experimental models of mouse paw infection with *L. amazonensis* show an induction of a strong inflammatory response in the skin, and parasitic migration may occur to secondary organs with consequent tissue injury. There are few studies focusing on the resolution of damage in secondary organs caused by *Leishmania* species-related cutaneous leishmaniasis. We investigated the propolis treatment effect on liver inflammation induced by *Leishmania amazonensis* infection in the mouse paw. BALB/c mice were infected in the hind paw with *L. amazonensis* (10^7) promastigote forms. After 15 days, animals were treated daily with propolis (5 mg/kg), Glucantime (10 mg/kg), or with propolis plus Glucantime combined. After 60 days, mice were euthanized and livers were collected for inflammatory process analysis. Liver microscopic analysis showed that propolis reduced the inflammatory process compared to untreated infected control. There was a decrease of liver myeloperoxidase and N-acetyl-β-glucosaminidase activity levels, collagen fiber deposition, pro-inflammatory cytokine production, and plasma aspartate transaminase and alanine transaminase levels. Furthermore, propolis treatment enhanced anti-inflammatory cytokine levels and reversed hepatosplenomegaly. Our data demonstrated that daily low doses of Brazilian propolis reduced the secondary chronic inflammatory process in the liver caused by *L. amazonensis* subcutaneous infection in a susceptible mice strain.

Keywords *Leishmania amazonensis* · Propolis · Liver · Glucantime · Inflammation

✉ Suelen S. da Silva
suelenbiomedica@gmail.com

[1] Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina 86057-970, Paraná, Brazil

[2] Departamento de Histologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina 86057-970, Paraná, Brazil

[3] Laboratório de Mediadores Inflamatórios, Universidade do Oeste do Paraná, UNIOESTE, Francisco Beltrão 85605-010, Paraná, Brazil

[4] Departamento de Microbiologia e Imunologia, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Botucatu 18618-970, São Paulo, Brazil

Introduction

Protozoa of the genus *Leishmania* are the causative agent of leishmaniasis, a neglected disease with high morbidity, mortality, and therapeutic failure, constituting a public health problem, and can cause skin lesions or visceral involvement (Grevelink and Lerner 1996; Desjeux 2004). American cutaneous leishmaniasis is characterized by ulcerative skin lesions, localized or mucosal, and disseminated lesions (nonulcerated nodules) (Reithinger et al. 2007).

Leishmania amazonensis is one of the main etiologic agents responsible for cutaneous leishmaniasis in Brazil. This parasite may cause the localized or diffuse clinical forms of the disease, depending on the host immune response and parasitic virulence (Barral et al. 1991; Jones et al. 2000; Ji et al. 2003).

The classic lesions in cutaneous leishmaniasis are most often ulcerated lesions in the skin, with a granular base and raised borders (Bittencourt and Barral 1991). However, in experimental infections with *L. braziliensis*, *L. tropica*, *L. mexicana*, *L. major*, and *L. amazonensis*, cases of migration to secondary organs differing from the site of infection have been described. These species were initially described as parasites with a unique tropism for skin and mucosa (Walton et al.

1977; Barral et al. 1986; Magill et al. 1993; Mohareb et al. 1996; Abreu-Silva et al. 2004; Wilson et al. 2005; Soliman 2006; Ribeiro-Romão et al. 2014). In addition, *L. amazonensis* has been reported as an etiologic agent of human visceral leishmaniasis (Roberts et al. 1989; Barral et al. 1991).

Visceralization due to hematogenous dissemination via phagocytic cells such as monocytes has resulted to histological damage to lymph nodes, liver, spleen, and bone marrow (Duarte and Corbett 1987). Splenomegaly is usually observed, with many macrophages parasitized by amastigotes. Liver disorders result from hypertrophy and hyperplasia of Kupffer cells, as well as intracellular fibrosis, and complications of fulminant hepatitis (Engwerda and Kaye 2000; Baranwal et al. 2007).

The host immune response is essential for disease control and elimination of the parasite, but an uncontrolled inflammatory response is a common mechanism involved in the majority of cases of clinical visceral leishmaniasis, resulting to secondary tissue damage with granulomatous changes and fibrosis (Gutierrez et al. 1984; Grevelink and Lerner 1996; Leite and Croft 1996; Nylén and Gautam 2010; Gupta et al. 2013).

Propolis has been widely used in popular medical practice and has shown promising results in a range of experimental models, including activity against some trypanosomatids of medical importance. For instance, propolis kills promastigote and amastigote forms of varied *Leishmania* species (Machado et al. 2007; Ayres et al. 2007; Duran et al. 2008; Pontin et al. 2008; Ozbilge et al. 2010; Ayres et al. 2011; da Silva et al. 2013).

In addition, several studies have shown that propolis has anti-inflammatory properties and accelerates tissue regeneration as well as exhibits an antimicrobial action and shortens healing time (Barbosa et al. 2009; Khorasgani et al. 2010; Ikeda et al. 2011; Olczyk et al. 2013a, b).

Studies have also demonstrated that the ethanolic extract of propolis has anti-inflammatory properties in both chronic and acute inflammation and exerts protective effects against hepatotoxicity (Seo et al. 2003; Batista et al. 2012). The molecular mechanisms involved in the immunomodulatory and anti-inflammatory activities of this natural compound include the capacity of inhibiting T cell activation by affecting mainly IL-2, NF-κB, MAP, STAT 3, and IL-6 (Okamoto et al. 2012; Búfalo et al. 2013). Other studies have also reported a decrease in myeloperoxidase (MPO) and NADPH-oxidase activities (Frenkel et al. 1993; Volpert and Elstner 1996) and ornithine decarboxylase, tyrosine protein kinase, and hyaluronidase activities (Miyataka et al. 1997).

Considering the antimicrobial and anti-inflammatory activity of propolis, the aim of the present study was to evaluate the effect of low dose propolis on liver inflammation in an experimental model of subcutaneous infection in a susceptible murine strain with *L. amazonensis*.

Materials and methods

L. amazonensis

L. amazonensis (MHOM/BR/1989/166MJO) was obtained from homogenate of popliteal lymph nodes of infected BALB/c mice. The promastigote forms were cultured in 199 medium (Invitrogen-GIBCO) and supplemented with 10 % fetal bovine serum (Invitrogen-GIBCO), 1 M Hepes, 0.1 % human urine, 0.1 % L-glutamine, 1 % penicillin/streptomycin solution (Invitrogen-GIBCO), and 10 % sodium bicarbonate. Cultures were incubated at 25 °C in 25-cm^2 flasks. Promastigote forms, in stationary the growth phase (5 culture days), were used for experimental infection of the animals.

Propolis extract

The propolis sample was collected in the Beekeeping Section of Lageado Farm, UNESP, Botucatu Campus, Brazil, from honeybee (*Apis mellifera L.*) colonies. The method of extraction as well as the chemical composition has already been documented in previous studies, where propolis was analyzed by gas chromatography (GC), gas chromatography–mass spectrometry (GC-MS), and thin layer chromatography (TLC) (Sforcin 2007). The final concentration of the ethanol solvent in the experiments did not exceed 0.1 %. It is noteworthy that we used the same batch of propolis extract in all experiments to avoid differences in the active products and solvents.

Animals and experimental infection

Male BALB/c mice (20–25 g), 4–6 weeks old, were obtained from the Fundação Osvaldo Cruz, FIOCRUZ, Curitiba, Brazil. Mice were kept under pathogen-free conditions and used according to protocols approved by the Ethics Committee of the Universidade Estadual de Londrina (protocol No. 09/11).

Mice were divided into five groups with eight animals each. The groups were (1) control group (without infection and without treatment); (2) infected control group (with infection and without treatment); (3) propolis group (with infection and treatment with propolis); (4) Glucantime group (received infection and treatment with Glucantime); (5) propolis + Glucantime group (received infection and treatment with propolis and Glucantime combined).

Mice were infected subcutaneously in the right hind paw with *L. amazonensis* (MHO/BR/1989/166MJO) promastigote forms (10^7/20 μL). Daily treatment with propolis (5 mg/kg, orally [p.o.]) or Glucantime® (10 mg/kg, intraperitoneally [i.p.]), or propolis (5 mg/kg, orally [p.o.]) plus Glucantime (10 mg/kg, intraperitoneally [i.p.]) combined started 15 days after infection. After 60 days of treatment, mice were

euthanized, and blood was collected for biochemical tests and liver for histological and immunological analysis.

AST and ALT levels

The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as markers of hepatocellular damage and determined in blood plasma by a colorimetric assay using a diagnostic kit from Labtest Diagnóstica™ (Lagoa Santa, MG, Brazil) (Hohmann et al. 2013).

Liver histological analysis

The liver of each animal was removed, perfused with saline, and sectioned into standardized fragments. One of the fragments was fixed in Bouin's solution for 48 h. Subsequently, the tissue was subjected to routine histological processing to obtain 4-μm sections, which were stained with hematoxylin-eosin and examined by light microscopy (Olympus, Miami, FL, USA). The analysis was performed according to quality and quantitative parameters of inflammation.

Histological variation was classified according to the level of lesions found by the following criteria: no histological alteration (−); isolated inflammatory foci, presence of up to 1 intralobular granuloma, and up to 5 Kupffer cells per microscopic field (+); isolated or coalescent area of histological changes including inflammation, 2–4 intralobular granulomas, and 5–10 Kupffer cells per field (++); disseminated histological changes including inflammation, over 4 intralobular granulomas, and 10 Kupffer cells per field (+++).

Hepatic fibrosis analysis

Collagen quantification was determined in Sirius red-stained liver sections under polarized light using a photomicroscope (CARL ZEISS Axio imager A1) with a camera (HBO 100) coupled to a computer using AxioVision software, at a final magnification of 200×. Eight images of four sections from each mouse were considered for the study and analyzed by Image Pro Plus (version 4.5). The results were expressed as the mean of area with presence of total collagen and percentage of area with type I and III collagen.

Myeloperoxidase activity and N-acetylglucosaminidase activity

Neutrophil migration to the liver was evaluated by the MPO kinetic-colorimetric assay. N-acetylglucosaminidase (NAG) assay was used for evaluating the infiltration of macrophages in the liver. Samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5 % HTAB and were homogenized using a Polytron® (PT3100).

After the homogenates were centrifuged (16,100 × *g*, 2 min, 4 °C), the resulting supernatant was assayed spectrophotometrically for MPO or NAG activity at 450 nm (Spectra max) with three readings within 1 min. MPO activity of the samples was compared with a standard curve of neutrophils, and MPO activity results were presented as the number of neutrophils × 10^4/mg of tissue. NAG activity in the samples was compared with a standard curve of macrophages, and NAG activity results were presented as the number of macrophages × 10^3/mg of tissue) (Hohmann et al. 2013).

Cytokine measurement

Cytokines present in liver fragments were analyzed by enzyme-linked immunosorbent assay (ELISA). Samples were homogenized in 500 μL of buffer containing protease inhibitors (1 mM Phenylmethanesulfonyl fluoride, Sigma Aldrich), and IFN-γ, TNF-α, IL-17, IL-6, IL-12, IL-10, TGF-β, and IL-13 levels were determined by ELISA, according to the manufacturer's instructions (eBiosciences®, USA). Plates were read at 450 nm, using an ELISA plate reader (Thermo Plate—TP-Reader). Results were expressed as pg cytokine/mg tissue.

Statistic analysis

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Significant differences between treatments were determined by ANOVA, followed by Tukey's test for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

Results

Effect of propolis extract on blood plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and weight of liver and spleen

Blood samples were collected after 60 days of treatment for the assessment of AST/ALT levels. The infection with *L. amazonensis* induced a significant increase in AST (Fig. 1a) and ALT (Fig. 1b) levels. These enzyme levels were reduced by propolis or by its combination with Glucantime by 85 and 87 %, respectively.

Liver and spleen weights increased after 75 days of infection (Fig. 2a, b). Data showed that the liver and spleen of animals treated with propolis alone or combined with Glucantime did not differ when compared with the uninfected group (Fig. 2a, b). However, the weight of the liver and spleen of animals treated with propolis or propolis and Glucantime combined decreased when compared to infected control (Fig. 2a, b).

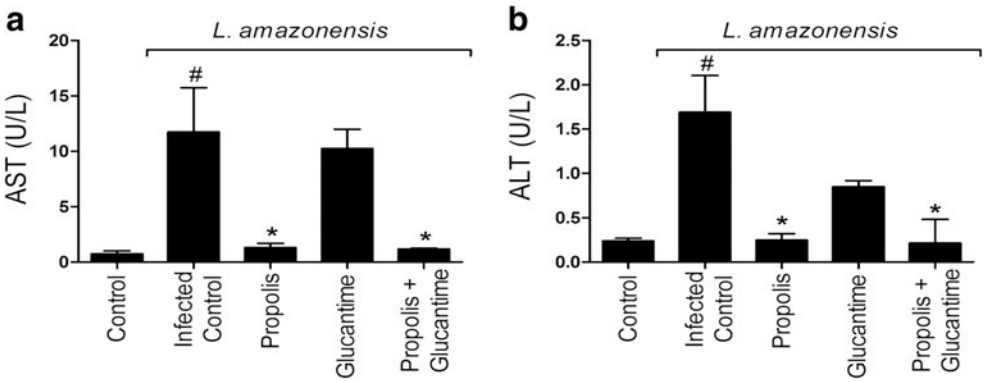

Fig. 1 Effect of propolis extract on blood plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Blood samples were collected for the determination of AST (**a**) and ALT (**b**) levels. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

The liver and spleen weights of animals treated with Glucantime alone were significantly increased compared to infected control animals (Fig. 2a, b).

Propolis promoted an anti-inflammatory effect in liver reducing granuloma formation and deposition of type I and III collagen fibers

The liver sample collected for histological analysis showed that *L. amazonensis* infection (10^7/20 μL/paw, s.c injection) induced histological changes. These alterations included increased infiltration of Kupffer cells, intralobular granuloma formation, and inflammation in the portal tracts (Fig. 3a–c). These changes were classified as disseminated histological changes (+++), which were reduced after treatment with 5 mg/kg propolis, classified as isolated inflammatory foci (+). Glucantime also reduced the changes in liver tissue to a level classified as isolated or coalescent area of histological changes (++). Glucantime in combination with propolis resulted in isolated inflammatory foci (+) (Table 1).

Infection induced the deposition of collagen fibers type I and III (Fig. 4a). Treatment with propolis, Glucantime, and the two combined reduced the deposition of total collagen fibers when compared with the infected control group (Fig. 4a).

The prevalence of type III collagen fibers was seen in all groups (Fig. 4b). Type I collagen fibers appeared less than 15.53 % in the control group without infection and in groups treated with propolis and propolis combined with Glucantime (Fig. 4b). The percentage of this type collagen fiber in infected control and Glucantime-treated groups was 35.38 and 28.18 %, respectively (Fig. 4b).

Propolis reduced *L. amazonensis*-induced myeloperoxidase and n-acetylglucosaminidase activities in liver tissue

After 60 days of treatment, the liver samples were collected for evaluation of MPO and NAG activities. *L. amazonensis* infection induced a significant increase in MPO (Fig. 5a) and NAG (Fig. 5b) compared to control. Treatment with propolis decreased MPO activity by 27 % and NAG activity by 31 % compared with infected control. Combined treatment also produced similar changes in enzyme activity.

Propolis reduced *L. amazonensis*-induced IFN-γ, TNF-α, IL-17, and IL-6 production and increased production of anti-inflammatory cytokines in the liver

After 60 days of treatment, liver samples were collected for the assessment of cytokine production. *L. amazonensis* infection increased the production of IFN-γ, TNF-α, IL-17, and IL-6 in liver (Fig. 6a–d). However, these levels were reduced by treatment with 5 mg/kg propolis (Fig. 6a–d). On the other hand, propolis increased IL-10, TGF-β, and IL-13 production

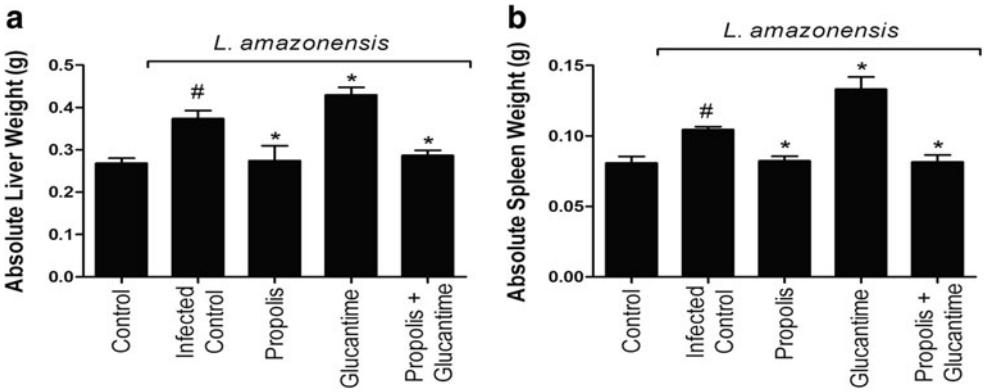

Fig. 2 Effect of propolis extract on weight of liver and spleen. Absolute weight of liver (**a**) and spleen (**b**) was evaluated as markers of hepatosplenomegaly. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

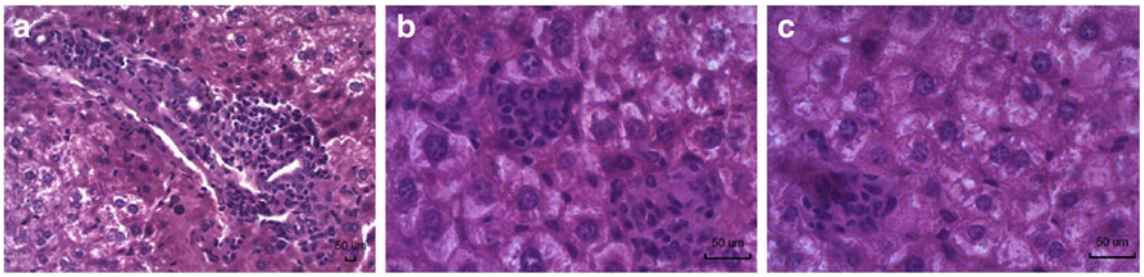

Fig. 3 Photomicrograph showing the main histological changes found in the liver of animals infected with *L. amazonensis*. Liver fragment samples were collected for analysis of the inflammatory process. Portal tract inflammation (**a**). Intralobular granuloma (**b**). Hyperplasia and hypertrophy of Kupffer cells (**c**). Histological sections (4 μm) were analyzed by optical microscope (magnification of ×100 for panel **a** and ×400 for panels **b** and **c**) (Olympus, Miami, FL, USA) after hematoxylin and eosin staining

(Fig. 7a–c) and did not affect IL-12 production (Fig. 6e). Furthermore, propolis combined with Glucantime also enhanced the production of IL-10 and IL-13 (Fig. 7a, c) and reduced TNF-α, IL-17, and IL-6 production when compared to infected control (Fig. 6b–d).

Discussion

In this study, *L. amazonensis* infection in the paw spreads to other tissues such as the liver. There are histological changes in the liver including inflammation of the portal tract, hyperplasia, and hypertrophy of Kupffer cells and fibrosis. These histological changes were corroborated by the increase in MPO and NAG activities as well as by increased levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, and IL-6 in the liver. Moreover, liver injury was associated with the classic increase in AST and ALT levels in the blood and hepatosplenomegaly. Importantly, treatment with propolis in a therapeutic protocol reduced all liver inflammatory responses induced by *L. amazonensis* paw infection.

Similar to the present murine model, there are morphological changes in the liver during human visceral leishmaniasis, characterized mainly by hypertrophy and hyperplasia of Kupffer cells with granuloma formation and intralobular portal and diffuse intralobular fibrosis (Murray 2001).

It is known that wound healing response accounts for liver fibrosis in varied acute and chronic conditions. Liver fibrosis may occur due to increased synthesis and deposition of collagen (Friedman 2008).

Few studies have demonstrated the importance of the liver fibrosis process in murine models of experimental infection with *Leishmania*. However, it is known that infection with *L. donovani* in BALB/c causes an increase in the deposition of collagen, particularly type III, in granuloma formation regions (Leite and Croft 1996).

Liver fibrosis involves qualitative and quantitative changes in the composition of the extracellular matrix in the portal and sinusoidal space and is characterized by substantial deposition of fibrillar type I and III collagen, proteoglycans, fibronectin, and hyaluronic acid in the scar regions (George and Chandrakasan 1996; George et al. 2004; Zeisberg et al. 2006).

Table 1 Liver histological analysis (hematoxylin-eosin staining) of control mice and *L. amazonensis* experimental infected mice

Liver histologic analysis	Experimental groups				
	Control	Infected control	Propolis	Glucantime	Propolis + Glucantime
Portal tract inflammation	0	3	3	0	0
Intralobular granuloma (1)	0	0	4	1	1
Intralobular granuloma (2–4)	0	4	0	1	0
Intralobular granuloma (>4)	0	1	0	0	0
Kupffer cells (4)	0	1	4	3	3
Kupffer cells (5–10)	0	1	0	0	0
Kupffer cells (>10)	0	3	0	0	0
Total score	−	+++	+	++	+

Results are the number of animals in each group with lesions. No histological alteration (−); isolated inflammatory foci, presence of up to 1 intralobular granuloma, and up to 5 Kupffer cells per microscopic field (+); isolated or coalescent area of histological changes including inflammation, 2–4 intralobular granulomas, and 5–10 Kupffer cells per field (++); disseminated histological changes including inflammation, over 4 intralobular granulomas, and 10 Kupffer cells per field (+++). The number of animals in each group that presented histological change was also taken into consideration for the classification score (400× magnification)

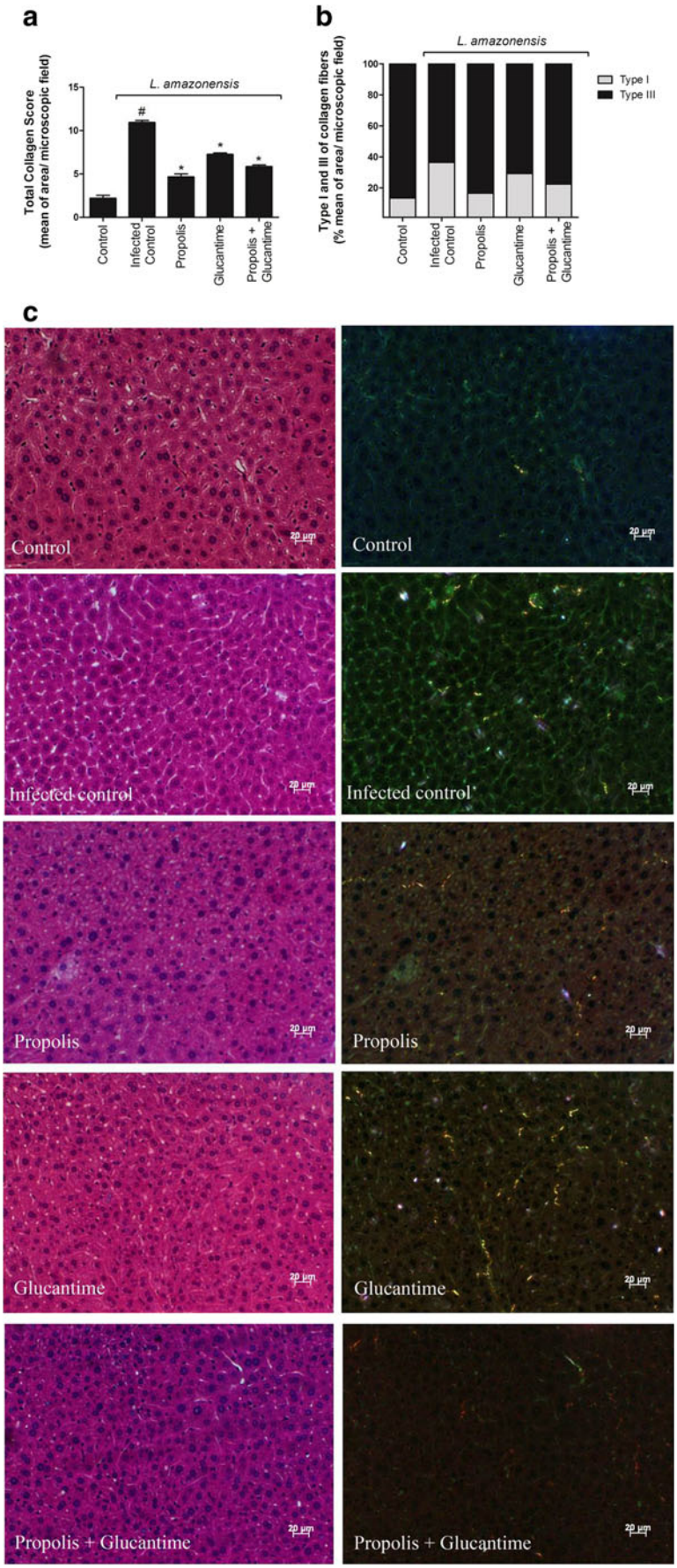

Fig. 4 Propolis reduced the deposition of type I and III collagen fibers. Liver fragment samples were collected for analysis of total collagen score (**a**) and percentage of Type I and III fiber collagen (**b**). Photomicrograph of hepatic histological sections with Sirius red staining in light and polarized microscopy (**c**). (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

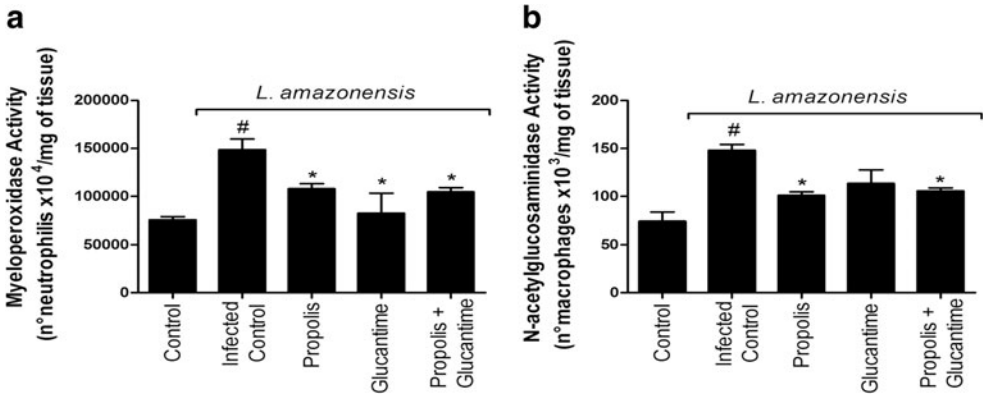

Fig. 5 Propolis reduced *L. amazonensis*-induced myeloperoxidase activity (MPO) and n-acetylglucosaminidase (NAG) on liver tissue. MPO (**a**) and NAG (**b**) were evaluated as markers of the inflammatory infiltrate on liver. BALB/c mice were infected with *L. amazonensis* (10^7) promastigote forms by subcutaneous (s.c.) injection in the hind paw. After 15 days, animals were treated daily with propolis (5 mg/kg, p.o) or Glucantime (10 mg/kg, i.p.) or combination of propolis plus Glucantime for 60 days. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

Interleukin-13 is associated with inducing fibrosis in chronic infectious and autoimmune diseases like schistosomiasis and chronic asthma. However, the pathogenesis of fibrous tissue involves IL-13 associated with a cascade of events that involve an increase of TGF-β and TNF-α, regulating collagen synthesis and collagen catabolism (Wynn 2004; Fichtner-Feigl et al. 2006).

In our study, the Sirius red staining method with polarized light microscopy was used, allowing the characterization of type I and III of collagen in tissues. Three colors can be distinguished: green, characteristic of thin collagen fibers and reticular type III, and the yellow to red spectrum, indicating dense type I fibers (Montes and Junqueira 1991). Thus, it was possible to evaluate the total collagen

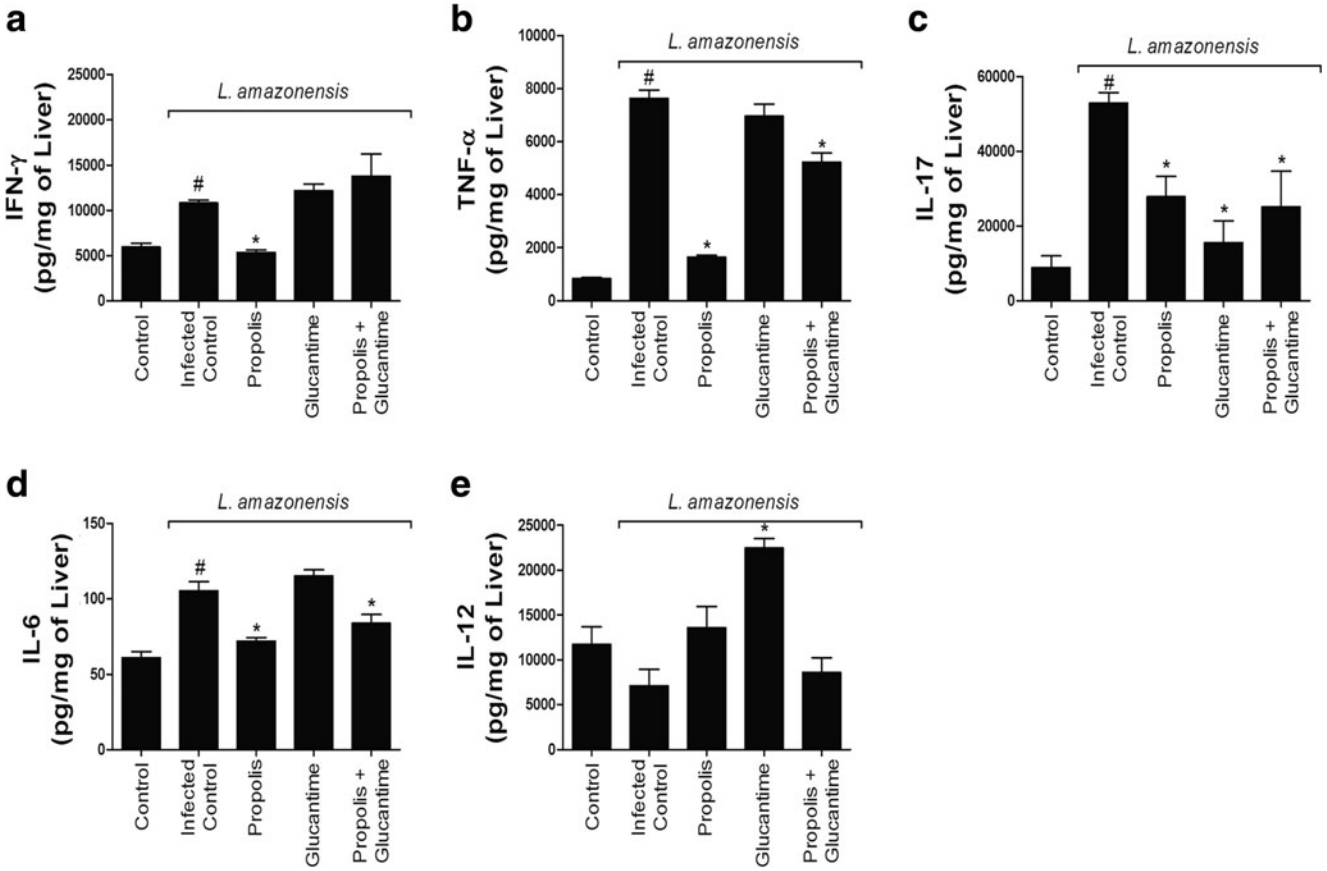

Fig. 6 Propolis reduced *L. amazonensis*-induced pro-inflammatory cytokine production in liver. IFN-γ (**a**), TNF-α (**b**), IL-17 (**c**), IL-6 (**d**), and IL-12 (**e**) were evaluated in homogenate liver. (*Number sign*) Significantly different from control. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

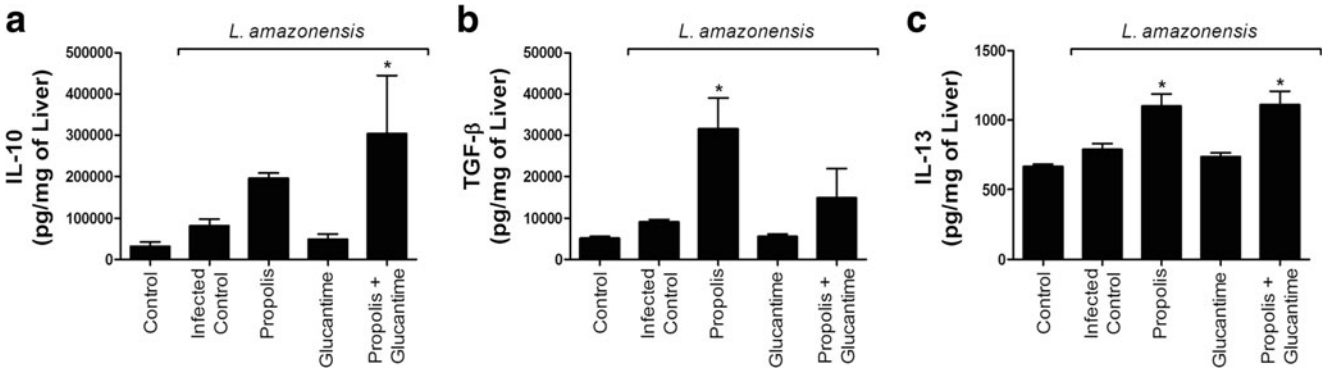

Fig. 7 Propolis increased anti-inflammatory cytokine production in liver. IL-10 (**a**), TGF-β (**b**), and IL-13 (**c**) were evaluated in homogenate liver. (*Asterisk*) Significantly different from infected control ($P < 0.05$)

score as well as the percentage of deposition of fibrillar type I and III collagen.

Some authors suggest that oxidative stress also participates in the process of fibrosis, which can progress to necrosis or apoptosis of hepatocytes. The increase in pro-fibrogenic response with consequent increased expression and deposition of type I collagen fibers may be evidenced by reduction of antioxidant defenses such as glutathione (GSH), catalase, or superoxide dismutase (SOD), along with an increase in lipid peroxidation (George 2003; Bataller and Brenner 2005; Nieto 2006).

Propolis-treated groups showed reduced chronic inflammation, with a decrease in the deposition of type I and III collagen; lower levels of the pro-inflammatory cytokines TNF-α, IL-17, and IL-6; decreased AST and ALT levels; and reduction in liver and spleen weights.

Hepatosplenomegaly is another important factor observed as a clinical sign in visceral leishmaniasis. Thus, to evaluate possible changes resulting from infection or treatment, the liver and spleen weights of the animals were evaluated. *L. amazonensis* infection increased liver and spleen weights. However, the treatment with propolis with or without Glucantime reversed this process.

Previous studies with different propolis extracts have shown antiprotozoal activity, as well as immunomodulatory and anti-inflammatory effects (Dimov et al. 1992; Ramos and Miranda 2007; Ayres et al. 2007; Sforcin 2007; da Silva et al. 2013). In addition, the wound healing activity of propolis has attracted attention (Olczyk et al. 2013a, b).

The sample of Brazilian propolis used in our experiments has exhibited leishmanicidal, fungicidal, antimicrobial, and immunomodulatory effects in different experimental models (Orsi et al. 2000, 2012; Murad et al. 2002; Sforcin 2007; Missima and Sforcin 2008; da Silva et al. 2013; Miranda et al. 2015). Some studies have suggested that the immunomodulatory action of propolis may occur through the inhibition of T cell activation by inhibiting mainly IL-2, NF-κB, MAPK, STAT 3, and IL-6. In addition, other studies have shown a decrease in MPO and NADPH-oxidase activities (Frenkel et al. 1993; Volpert and Elstner 1996; Okamoto et al. 2012; Búfalo et al. 2013).

The available drug in Brazil for the treatment of leishmaniasis is an antimonial, a complex of $Sb^{(V)}$ with N-methyl-D-glucamine (meglumine antimoniate or Glucantime®). However, this drug has serious side effects and limitations in its use and shows therapeutic failures (Sundar and Chakravarty 2013).

Here, we found that animals treated with Glucantime did not show an improvement of inflammatory responses induced by infection with *L. amazonensis*. However, when Glucantime was combined with propolis, the anti-inflammatory effects became more evident. Furthermore, studies have suggested the use of propolis combined with Glucantime to decrease the side effects of Glucantime in the host (Ayres et al. 2011; Ferreira et al. 2014).

Considering the antioxidant and anti-inflammatory role that propolis has shown in several models, our data indicated that the daily treatment in mice susceptible to infection with *L. amazonensis* is able to prevent the progression of lesions in the liver. This effect may be due to immunomodulatory effects with consequent reduction of inflammatory infiltrate, granuloma formation, and fibrosis in liver tissue.

Conclusion

Our study demonstrated the anti-inflammatory effect of propolis in the liver, when given at low daily doses. Propolis promoted immunomodulation with efficacy by reducing cellular recruitment, which prevented inflammatory processes in the liver due to infection with *L. amazonensis*. Furthermore, these data encourage further studies to determine the value of combining this apitherapeutic agent with Glucantime to increase the treatment efficacy in leishmaniasis and reduce the side effects of Glucantime.

Acknowledgments Dr. A. Leyva helped with English editing of the manuscript.

This work was supported by Brazilian grants from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Secretaria da Ciência, Tecnologia e Ensino Superior (SETI)/Fundação Araucária, and Governo do Estado do Paraná.

References

Abreu-Silva AL, Calabrese KS, Cupolilo SMN, Cardoso FO, Souza CS, Gonçalves da Costa SC (2004) Histopathological studies of visceralized *Leishmania (Leishmania) amazonensis* in mice experimentally infected. Vet Parasitol 121:179–187. doi:10.1016/j.vetpar.2004.03.002

Ayres DC, Marcucci MC, Giorgio S (2007) Effects of Brazilian propolis on *Leishmania amazonensis*. Mem Inst Oswaldo Cruz 102:215–220. doi:10.1590/S0074-02762007005000020

Ayres DC, Fedele TA, Marcucci MC, Giorgio S (2011) Potential utility of hyperbaric oxygen therapy and propolis in enhancing the leishmanicidal activity of glucantime. Rev Inst Med Trop Sao Paulo 53:329–334. doi:10.1590/S0036-46652011000600006

Baranwal AK, Mandal RN, Singh R (2007) Fulminant hepatic failure complicating visceral leishmaniasis in an apparently immunocompetent child. Indian J Pediatr 74:489–491. doi:10.1007/s12098-007-0083-1

Barbosa MH, Zuffi FB, Maruxo HB, Jorge LLR (2009) Therapeutic properties of propolis for treatment of skin lesions. Acta Paul Enferm 22(3):318–322

Barral A, Badaró R, Barral-Netto M, Grimaldi G Jr, Momem H, Carvalho EM (1986) Isolation of *Leishmania mexicana amazonensis* from the bone marrow in a case of American visceral leishmaniasis. Am J Trop Med Hyg 35:732–734

Barral A, Pedral-Sampaio D, Grimaldi Júnior G, Momen H, McMahon-Pratt D, Ribeiro de Jesus A, Almeida R, Badaro R, Barral-Netto M, Carvalho EM, Warren Jr DJ (1991) Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. Am J Trop Med Hyg 44:536–546

Bataller R, Brenner DA (2005) Liver fibrosis. J Clin Invest 115:209–218. doi:10.1172/JCI24282

Batista LLV, Campesatto EA, de Assis MLB, Barbosa AP, Grillo LA, Dornelas CB (2012) Comparative study of topical green and red propolis in the repair of wounds induced in rats. Rev Col Bras Cir 39:515–520

Bittencourt AL, Barral A (1991) Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. Mem Inst Oswaldo Cruz 86:51–56

Búfalo MC, Ferreira I, Costa G, Francisco V, Liberal J, Cruz MT, Lopes MC, Batista MT, Sforcin JM (2013) Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages. J Ethnopharmacol 149:84–92. doi:10.1016/j.jep.2013.06.004

Da Silva SS, Thomé G d S, Cataneo AHD, Miranda MM, Felipe I, Andrade CG, Watanabe MA, Piana GM, Sforcin JM, Pavanelli WR, Conchon-Costa I (2013) Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med 2013:673058. doi:10.1155/2013/673058

Desjeux P (2004) Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 27:305–318. doi:10.1016/j.cimid.2004.03.004

Dimov V, Ivanovska N, Bankova V, Popov S (1992) Immunomodulatory action of propolis: IV. Prophylactic activity against gram-negative infections and adjuvant effect of the water-soluble derivative. Vaccine 10:817–823

Duarte MI, Corbett CE (1987) Histopathological patterns of the liver involvement in visceral leishmaniasis. Rev Inst Med Trop Sao Paulo 29:131–136

Duran G, Duran N, Culha G, Culha G, Ozcan B, Oztas H, Ozer B (2008) *In vitro* antileishmanial activity of Adana propolis samples on *Leishmania tropica*: a preliminary study. Parasitol Res 102:1217–1225. doi:10.1007/s00436-008-0896-5

Engwerda CR, Kaye PM (2000) Organ-specific immune responses associated with infectious disease. Immunol Today 21:73–78

Ferreira FM, Castro RAO, Batista MA, Rossi FMO, Silveira-Lemos D, Frézard F, Moura SAL, Rezende SA (2014) Association of water extract of green propolis and liposomal meglumine antimoniate in the treatment of experimental visceral leishmaniasis. Parasitol Res 113:533–543. doi:10.1007/s00436-013-3685-8

Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A (2006) IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med 12:99–106. doi:10.1038/nm1332

Frenkel K, Wei H, Bhimani R, Ye J, Zadunaisky JA, Huang MT, Ferraro T, Conney AH, Grunberger D (1993) Inhibition of tumor promoter-mediated processes in mouse skin and bovine lens by caffeic acid phenethyl ester. Cancer Res 53:1255–1261

Friedman SL (2008) Mechanisms of hepatic fibrogenesis. Gastroenterology 134:1655–1669. doi:10.1053/j.gastro.2008.03.003

George J (2003) Ascorbic acid concentrations in dimethylnitrosamine-induced hepatic fibrosis in rats. Clin Chim Acta 335:39–47

George J, Chandrakasan G (1996) Glycoprotein metabolism in dimethylnitrosamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol 28:353–361

George J, Tsutsumi M, Takase S (2004) Expression of hyaluronic acid in N-nitrosodimethylamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol 36:307–319

Grevelink SA, Lerner EA (1996) Leishmaniasis. J Am Acad Dermatol 34:257–272

Gupta G, Oghumu S, Satoskar AR (2013) Mechanisms of immune evasion in leishmaniasis. Adv Appl Microbiol 82:155–184. doi:10.1016/B978-0-12-407679-2.00005-3

Gutierrez Y, Maksem JA, Reiner NE (1984) Pathologic changes in murine leishmaniasis (*Leishmania donovani*) with special reference to the dynamics of granuloma formation in the liver. Am J Pathol 114:222–230

Hohmann MSN, Cardoso RDR, Pinho-Ribeiro FA, Crespigio J, Cunha TM, Alves-Filho JC, da Silva RV, Pinge-Filho P, Ferreira SH, Cunha FQ, Casagrande R, Verri Jr WA (2013) 5-lipoxygenase deficiency reduces acetaminophen-induced hepatotoxicity and lethality. Biomed Res Int 2013:627046. doi:10.1155/2013/627046

Ikeda R, Yanagisawa M, Takahashi N, Kawada T, Kumazawa S, Yamaotsu N, Nakagome I, Hirono S, Tsuda T (2011) Brazilian propolis-derived components inhibit TNF-α-mediated downregulation of adiponectin expression via different mechanisms in 3T3-L1 adipocytes. Biochim Biophys Acta 1810:695–703. doi:10.1016/j.bbagen.2011.04.007

Ji J, Sun J, Soong L (2003) Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. Infect Immun 71:4278–4288

Jones DE, Buxbaum LU, Scott P (2000) IL-4-independent inhibition of IL-12 responsiveness during *Leishmania amazonensis* infection. J Immunol 165:364–372

Khorasgani EM, Karimi AH, Nazem MR (2010) A comparison of healing effects of Propolis and silver sulfadiazine on full thickness skin wounds in rats. In: Pak Vet J 30(2).

Leite VH, Croft SL (1996) Hepatic extracellular matrix in BALB/c mice infected with *Leishmania donovani*. Int J Exp Pathol 77:181–190

Machado GM de C, Leon LL, De Castro SL (2007) Activity of Brazilian and Bulgarian propolis against different species of *Leishmania*. Mem Inst Oswaldo Cruz 102:73–77

Magill AJ, Grögl M, Gasser RA, Sun W, Oster CN (1993) Visceral infection caused by *leishmania tropica* in veterans of operation desert storm. N Engl J Med 328:1383–1387. doi:10.1056/NEJM199305133281904

Miranda MM, Panis C, Cataneo AHD, da Silva SS, Kawakami NY, Lopes LGF, Morey AT, Yamauchi LM, Andrade CGTJ, Cecchini R, Silva JJN, Sforcin JM, Conchon-Costa I, Pavanelli WR (2015) Nitric oxide and Brazilian propolis combined accelerates tissue repair by modulating cell migration, cytokine production and collagen deposition in experimental leishmaniasis. PLoS ONE 10(5): e0125101. doi:10.1371/journal.pone.0125101

Missima F, Sforcin JM (2008) Green Brazilian propolis action on macrophages and lymphoid organs of chronically stressed mice. Evid Based Complement Alternat Med 5:71–75. doi:10.1093/ecam/nel112

Miyataka H, Nishiki M, Matsumoto H, Fujimoto T, Matsuka M, Satoh T (1997) Evaluation of propolis. I. Evaluation of Brazilian and Chinese propolis by enzymatic and physico-chemical methods. Biol Pharm Bull 20:496–501

Mohareb EW, Mikhail EM, Youssef FG (1996) *Leishmania tropica* in Egypt: an undesirable import. Trop Med Int Health 1:251–254

Montes GS, Junqueira LC (1991) The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Mem Inst Oswaldo Cruz 86(Suppl 3):1–11

Murad JM, Calvi SA, Soares AM, Bankova V, Sforcin JM (2002) Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. J Ethnopharmacol 79:331–334

Murray HW (2001) Tissue granuloma structure-function in experimental visceral leishmaniasis. Int J Exp Pathol 82:249–267

Nieto N (2006) Oxidative-stress and IL-6 mediate the fibrogenic effects of [corrected] Kupffer cells on stellate cells. Hepatology 44:1487–1501. doi:10.1002/hep.21427

Nylén S, Gautam S (2010) Immunological perspectives of leishmaniasis. J Glob Infect Dis 2:135–146. doi:10.4103/0974-777X.62876

Okamoto Y, Tanaka M, Fukui T, Masuzawa T (2012) Brazilian propolis inhibits the differentiation of Th17 cells by inhibition of interleukin-6-induced phosphorylation of signal transducer and activator of transcription 3. Immunopharmacol Immunotoxicol 34:803–809. doi:10.3109/08923973.2012.657304

Olczyk P, Ramos P, Komosinska-Vassev K, Stojko J, Pilawa B (2013a) Positive effect of propolis on free radicals in burn wounds. Evid Based Complement Alternat Med 2013:356737. doi:10.1155/2013/356737

Olczyk P, Wisowski G, Komosinska-Vassev K, Stojko J, Klimek K, Olczyk M, Kozma EM (2013b) Propolis modifies collagen types I and III accumulation in the matrix of burnt tissue. Evid Based Complement Alternat Med 2013:423809. doi:10.1155/2013/423809

Orsi RO, Funari SRC, Soares AMVC, Calvi SA, Oliveira SL, Sforcin JM, Bankova V (2000) Immunomodulatory action of propolis on macrophage activation. J Venom Anim Toxins 6:205–219. doi:10.1590/S0104-79302000000200006

Orsi RO, Fernandes A, Bankova V, Sforcin JM (2012) The effects of Brazilian and Bulgarian propolis *in vitro* against salmonella typhi and their synergism with antibiotics acting on the ribosome. Nat Prod Res 26:430–437. doi:10.1080/14786419.2010.498776

Ozbilge H, Kaya EG, Albayrak S, Silici S (2010) Anti-leishmanial activities of ethanolic extract of Kayseri propolis. In: African J Microbiol Res 4(7).

Pontin K, Da Silva Filho A a, Santos FF, Silva ML, Cunha WR, Nanayakkara NP, Bastos JK, de Albuquerque S (2008) *In vitro* and *in vivo* antileishmanial activities of a Brazilian green propolis extract. Parasitol Res 103:487–492. doi:10.1007/s00436-008-0970-z

Ramos AFN, Miranda JL (2007) Propolis: a review of its anti-inflammatory and healing actions. J Venom Anim Toxins incl Trop Dis 13:697–710

Reithinger R, Dujardin J-C, Louzir H, Pirmez C, Alexander B, Brooker S (2007) Cutaneous leishmaniasis. Lancet Infect Dis 7:581–596. doi:10.1016/S1473-3099(07)70209-8

Ribeiro-Romão RP, Moreira OC, Osorio EY, Cysne-Finkelstein L, Gomes-Silva A, Valverde JG, Pirmez C, Da-Cruz AM, Pinto EF (2014) Comparative evaluation of lesion development, tissue damage, and cytokine expression in golden hamsters (Mesocricetus auratus) infected by Inocula with different *Leishmania (Viannia) braziliensis* concentrations. Infect Immun 82:5203–5213. doi:10.1128/IAI.02083-14

Roberts M, Alexander J, Blackwell JM (1989) Influence of Lsh, H-2, and an H-11-linked gene on visceralization and metastasis associated with *Leishmania mexicana* infection in mice. Infect Immun 57:875–881

Seo KW, Park M, Song YJ, Kim SJ, Yoon KR (2003) The protective effects of Propolis on hepatic injury and its mechanism. Phytother Res 17:250–253. doi:10.1002/ptr.1120

Sforcin JM (2007) Propolis and the immune system: a review. J Ethnopharmacol 113:1–14. doi:10.1016/j.jep.2007.05.012

Soliman MFM (2006) The persistence, dissemination, and visceralization tendency of *Leishmania major* in Syrian hamsters. Acta Trop 97:146–150. doi:10.1016/j.actatropica.2005.09.007

Sundar S, Chakravarty J (2013) Leishmaniasis: an update of current pharmacotherapy. Expert Opin Pharmacother 14:53–63. doi:10.1517/14656566.2013.755515

Volpert R, Elstner EF (1996) Interactions of different extracts of propolis with leukocytes and leukocytic enzymes. Arzneimittelforschung 46:47–51

Walton BC, Intermill RW, Hajduk ME (1977) Differences in biological characteristics of three *Leishmania* isolates from patients with espundia. Am J Trop Med Hyg 26:850–855

Wilson ME, Jeronimo SMB, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microb Pathog 38:147–160. doi:10.1016/j.micpath.2004.11.002

Wynn TA (2004) Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol 4:583–594. doi:10.1038/nri1412

Zeisberg M, Kramer K, Sindhi N, Sarkar P, Upton M, Kalluri R (2006) De-differentiation of primary human hepatocytes depends on the composition of specialized liver basement membrane. Mol Cell Biochem 283:181–189. doi:10.1007/s11010-006-2677-8