JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

# The effect of propolis on CCL5 and IFN-$\gamma$ expression by peripheral blood mononuclear cells from leishmaniasis patients

Marla Karine Amarante[a], Maria Angélica Ehara Watanabe[a], Ivete Conchon-Costa[a], Leandra Lopes Fiori[a], Julie Massayo Maeda Oda[a], Michelle Cristiane Búfalo[b] and José Maurício Sforcin[b]

[a]Department of Pathological Sciences, UEL, Londrina, PR and [b]Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, SP, Brazil

**Keywords**
CCL5; IFN-$\gamma$; *Leishmania*; propolis

**Correspondence**
José M. Sforcin, Department of Microbiology and Immunology, Biosciences Institute, UNESP, 18618-970, Botucatu, SP, Brazil.
E-mail: sforcin@ibb.unesp.br

Received May 26, 2011
Accepted September 22, 2011

doi: 10.1111/j.2042-7158.2011.01385.x

## Abstract

**Objectives** Mucocutaneous leishmaniasis is associated with a strong Th1 immune response to *Leishmania*, which modulates chemokines and their receptors expression, affecting their migratory capacity. There are no antileishmanial vaccines available and chemotherapy still relies on the potentially toxic pentavalent antimonials. Propolis is a bee product with immunomodulatory and antiparasite activities, and researchers have been attracted to its potential for the development of new drugs. This work investigated the effects of propolis on CCL5 and IFN-$\gamma$ expression by peripheral blood mononuclear cells (PBMC) in order to evaluate a possible immunomodulatory action of propolis in patients with leishmaniasis compared to healthy control subjects.

**Methods** PBMC were incubated in the absence or presence of propolis and the evaluation of a possible cytotoxicity of propolis was carried out using MTT assay. The expression level of CCL5 and IFN-$\gamma$ was determined by real-time PCR.

**Key findings** Our data indicated that propolis modulates the immune response of leishmaniasis patients *in vitro*, affecting CCL5 and IFN-$\gamma$ expression by PBMC.

**Conclusions** Data suggested that propolis drives an anti-inflammatory response depending on concentration. Although propolis is a potential source of new and selective drugs for the treatment of leishmaniasis, its usefulness in the therapeutics should be further investigated.

## Introduction

Leishmaniasis is a significant cause of morbidity and mortality in several countries and is still an increasing health problem, affecting people in 88 countries with about 350 million individuals living in endemic areas. Approximately 2 million new cases are reported every year.[1,2]

American cutaneous leishmaniasis (ACL), conventionally known as localized cutaneous leishmaniasis (LCL) and/or mucocutaneous leishmaniasis (MCL), is a widespread disease in several countries of Latin America, caused by different species of the genus *Leishmania* and leading to a large spectrum of clinical forms of disease.[3,4] *Leishmania braziliensis* is regarded as the most important parasite associated with localized cutaneous leishmaniasis in the Americas[5] and as the most frequent and widely distributed *Leishmania* parasite in Brazil.[6]

MCL is associated with a strong Th1 immune response to *Leishmania* antigen, although the exact pathogenic mechanism of MCL has not yet been elucidated. It has been proposed that *Leishmania major* modulates chemokines and their receptors expression by dendritic cells, affecting their migratory capacity.[7] Thus, chemokines have been thought to be good candidates for therapeutic strategies.[8]

CCL5, also known as RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted), plays a primary role in the inflammatory/immune response due to its ability to attract and activate T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils to sites of inflammation and infection. Although this cytokine was initially considered a T cell-specific protein, it may also be produced by platelets, macrophages, eosinophils and

fibroblasts, as well as by endothelial, epithelial and endometrial cells.[9]

CCR5 ligands as CCL5/RANTES are involved in the development of Th1 cells. IFN-$\gamma$ is the prototypical Th1 helper type 1 (Th1) cytokine, inducing cell-mediated immunity by promoting Th1 over Th2 differentiation of T cells, inducing IgG class-switching to cytophilic isotypes, and activating phagocytes.[10] It is produced predominantly by NK and NKT cells, $\gamma\delta$ T and $\alpha\beta$ T cells, and by cells of the myeloid lineage.[11,12] Its production is largely dependent on IL-12 and IL-18 production by activated antigen-presenting cells,[13,14] in addition to signals directly activating lymphocytes themselves. High levels of pro-inflammatory cytokines such as IFN-$\gamma$ may impair wound healing in leishmaniasis.[15]

The treatment of leishmaniasis is a serious problem since there are no antileishmanial vaccines available and chemotherapy still relies on the potentially toxic pentavalent antimonials. However, these drugs are not orally active, requiring long-term parenteral administration, which causes serious side effects.[16] The emergence of drug-resistant parasites presents an additional and major problem.[17]

Natural products have displayed a major role in drug discovery because of antimicrobial resistance and some limitations of new drugs. Propolis, a honeybee product with plenty of biological properties such as immunomodulatory, antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal and antiparasite activities, among others,[18,19] has been considered a promising antimicrobial agent due to its potential for the development of new drugs.[20] Propolis antimicrobial activity has been well documented against different bacteria,[21] yeasts,[22] viruses[23,24] and parasites.[25] *In vitro*, propolis may act directly on microorganisms, and *in vivo* it may stimulate the immune system, activating the mechanisms involved in the microorganism killing. The knowledge of propolis' mechanisms of action on the immune system *in vitro* and *in vivo* has advanced in recent years.[26]

Since MCL is associated with a Th1 immune response, the aim of this study was to investigate the effects of propolis on CCL5 and IFN-$\gamma$ expression by peripheral blood mononuclear cells (PBMC) in order to evaluate a possible immunomodulatory action of propolis in patients with leishmaniasis compared to healthy control subjects.

## Subjects and Methods

### Patients and blood samples

Peripheral blood was collected from normal healthy donors and from leishmaniasis patients with clinical and laboratory diagnosis for *Leishmania braziliensis* (Montenegro skin test and indirect immunofluorescence assay) and with negative serology for HIV, HBV and HCV. All donors and patients ($n = 5$) were admitted to the Clinical Hospital of the State University of Londrina, Brazil. A term of free consent was signed by all donors and researchers prior to blood collection. This work was approved by the Human Ethics Committee of the State University of Londrina.

### Cell culture

Human PBMC obtained from the heparinized blood of donors were separated on Ficoll-Hypaque (Sigma) and maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen-Life Technologies) and penicillin-streptomycin (100 U/ml, Sigma). PBMC ($1 \times 10^6$ cells/ml) were incubated in the absence or presence of propolis at 5, 10, 25, 50 and 100 μg/ml[24] for 24 h at 37°C and 5% $CO_2$.

### Propolis extract

Propolis, collected in the Beekeeping Section, UNESP, was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, making up the volume to 100 ml with 70% ethanol) in the absence of bright light at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight was calculated (120 mg/ml).[26] Propolis chemical composition was investigated using thin-layer chromatography (TLC), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-MS) analysis.

### Quantitative determination of lactic dehydrogenase activity

The Dimension® (DADE Behring, Newark, USA) clinical chemistry system was used for lactic dehydrogenase (LDH) activity determination. The LDH method is a modification of the enzymatic lactate to pyruvate procedure.[27] A reference interval at 37°C was 100–190 U/l.

### Cytotoxicity assay

The evaluation of the possible cytotoxicity of propolis was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MTT-based assay kit, Sigma Chemical Co., St Louis, MO) according to the manufacturer's instructions. PBMC were grown in 24-well microplates (Nunc A/S, Roskilde) at 37°C and 5% $CO_2$. After stabilization, the medium was replaced by fresh medium containing different concentrations of propolis (5–100 μg/ml) and incubated for 24 h at 37°C. The 50% cytotoxic concentration (CC50) was calculated as the concentration of propolis able to reduce the optical density of MTT by 50% in relation to the control, by regression analysis.

### RNA isolation and reverse transcriptase reaction

Total cellular RNA was extracted from three healthy donors and two leishmaniasis patients with TRIzol LS reagent

(Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified total RNA was measured and assessed for purity by determining absorbance at 260 and 280 nm and then was stored at −20°C until testing. Reverse transcriptase reaction was performed using 500 ng of RNA, cloned moloney murine leukemia virus reverse transcriptase (M-MLV RT, 20 U; Invitrogen™) and recombinant ribonuclease inhibitor (RNaseOUT™, 4 U; Invitrogen™) under the following conditions: 2.5 μM oligo dT, 50 mM Tris HCl pH 8.3, 75 mM KCl, 1.5 mM $MgCl_2$ and 1.25 mM dNTP, at 42°C for 60 min in a Hybaid PCR Sprint Thermal Cycler (Biosystems, Guelph, Ontario, Canada).

### Molecular analysis of β-actin, CCL5 and IFN-γmRNA

PCR for β-actin cDNA was determined as previously described.[28] Briefly, cDNA synthesis was carried out as previously described, and PCR conditions were 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 5°C for 30 s, 72°C for 1 min and finally 72°C for 10 min in a MG96–Biocycler (imported by Biosystems, Curitiba, PR, Brazil).

Real-time PCR using SYBR green fluorescence was performed with 80 ng of cDNA in a total volume of 20 μl. Quantitative real-time PCR reaction was carried out using Platinum®SYBR Green qPCR SuperMix UDG (Invitrogen ™) and 0.25 nM of each sense and antisense primers (described below). The PCR reaction was performed for 40 cycles as follows: 95°C for 30 s, 54°C for 30 s and 72°C for 30 s in a Chromo4™ Real Time PCR Detection (Bio-Rad, Hercules, USA). The quantitative real-time PCR conditions are found in Table 1.

In quantitative RT-PCR analysis, the expression level of CCL5 and IFN-γ mRNA was calculated as previously described,[29] and Ct values for the target gene were the mean fold change + SEM for three independent determinations corrected by the human 18S rRNA small ribosomal subunit (18S) Ct values from control samples, considering efficiency values.

### Statistical analysis

SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA) was used, and a $P$ value < 0.05 was considered statistically significant. The Kruskal–Wallis test and correlation analysis by two-tailed Spearman's rank correlation were used for mRNA expression and for LDH assays.

## Results

### Propolis chemical composition

Our propolis sample was analysed by GC, GC-MS and TLC, identifying phenolic compounds (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, and essential oils, among others.

The main constituents of our propolis sample were isolated and identified to be flavonoids (kaempferid, 5,6,7-trihydroxy-3,4′-dimethoxyflavone, aromadendrine-4′-methyl ether), a prenylated p-coumaric acid and two benzopyranes (*E*- and *Z*-2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes), essential oils (spathulenol (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones), aromatic acids (dihydrocinnamic acid, p-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-p-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzopyran), and di- and triterpenes.[18]

### Propolis affects cell viability in a dose-dependent way

PBMC from healthy blood donors were incubated in the presence of propolis (5–100 μg/ml) and propolis cytotoxicity was analysed by quantification of LDH activity (U/l). No differences were seen in LDH activity between control (28.0 ± 1.41) and propolis (5 μg/ml: 31.50 ± 6.36; 10 μg/ml: 32.0 ± 2.82; 25 μg/ml: 99.0 ± 104.65). Cell toxicity was seen after PBMC incubation with propolis (50 and 100 μg/ml: 278.0 ± 7.07 and 258.0 ± 7.07, respectively) ($P < 0.05$; Figure 1). Thus, only 5, 10 and 25 μg/ml of propolis were used in the quantitative real-time PCR for CCL5 and IFN-γ.

The CC50 of propolis for PBMC was determined by MTT assay to be 78 μg/ml. No effect on cell viability was detected for propolis concentrations up to 50 μg/ml (Figure 2).

### Viability and integrity of the RNA samples and cDNA quality

RNA was extracted from the cultures in the presence or absence of propolis for 24 h at 37°C and 5% $CO_2$. The

**Table 1** Quantitative real-time PCR conditions

| Gene | GenBank acession number | Primer | Sequence (annealing T: 54°C) |
|---|---|---|---|
| CCL5 mRNA | NM_002985 | *Foward* | 5 × CCTACCCCACCCGCTCCT 3× |
| | | *Reverse* | 5 × TTGATGTACTCCCGAACCCA 3× |
| IFNγmRNA | NM_000619 | *Foward* | 5′ AAT TGT CTC CTT TTA CTT CA 3′ |
| | | *Reverse* | 5′ GTC ATC TCG TTT CTT TTT GT 3′ |
| 18S mRNA | NR_003286 | *Foward* | 5′ GTA ACC CGT TGA ACC CCA TT 3′ |
| | | *Reverse* | 5′ CCA TCC AAT CGG TAG TAG CG 3′ |

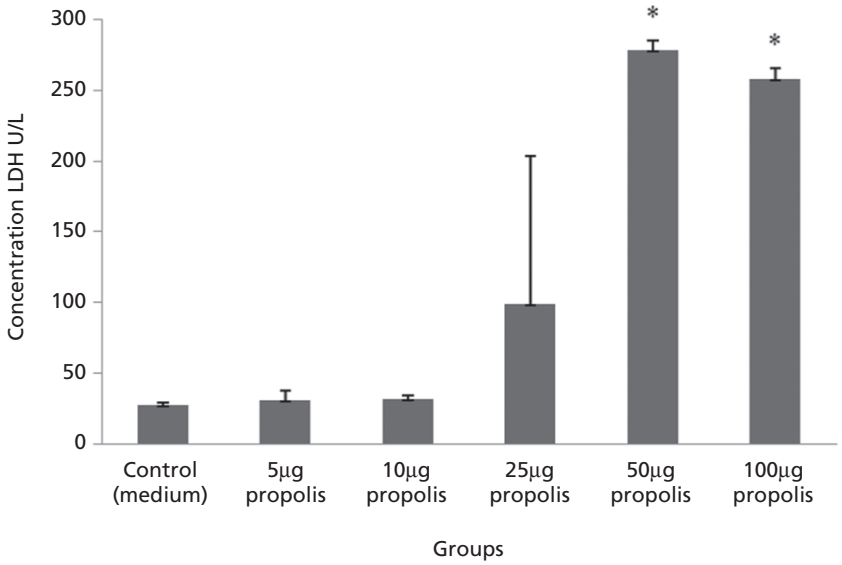


**Figure 1** Quantitative determination of lactic dehydrogenase activity (U/l) of healthy blood donors ($n = 5$). *Statistically different from control ($P < 0.05$).

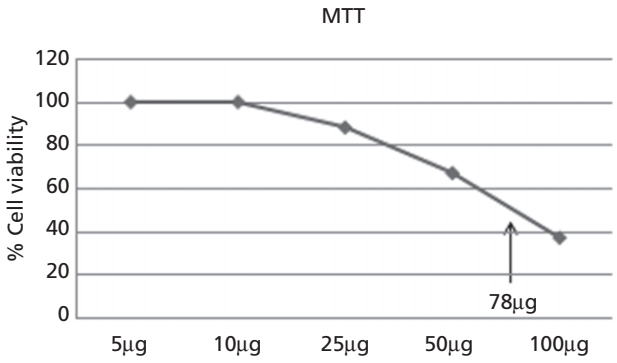


**Figure 2** The CC50 of propolis for PBMC determined by MTT assay.

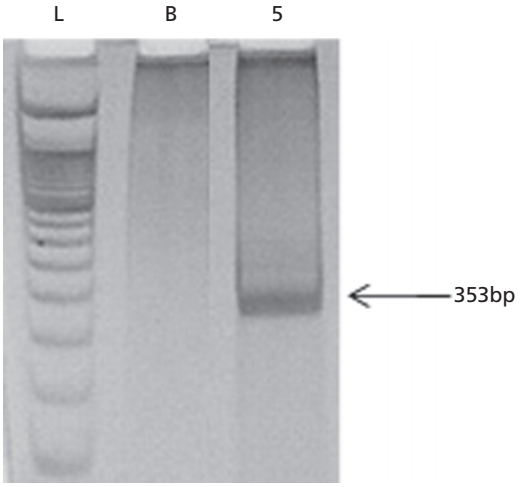


**Figure 3** Integrity of RNA samples and 18S expression. RT-PCR of $\beta$-actin mRNA of PBMC. L, ladder; B, blank; S, samples incubated with propolis.

viability and integrity of the RNA samples and cDNA quality were analysed by conventional PCR for $\beta$-actin, using specific primers. All the RNA samples presented detectable quantities of $\beta$-actin mRNA and acceptable integrity during amplification. No contamination with genomic DNA was verified since all the amplified products presented a fragment corresponding to 353 bp (Figure 3).

## Propolis modulates the expression of CCL5 and IFN-$\gamma$ mRNA by PBMC

Quantitative PCR was used to investigate the expression of IFN-$\gamma$ and CCL5 mRNA by human blood cells incubated with propolis.

One may verify in Figure 4 that the level of IFN-$\gamma$ mRNA expression in the presence of propolis (5, 10 and 25 μg/ml) was downregulated by 4.21; 1.97 and 5.79 ($P < 0.01$) fold respectively, in PBMC from normal healthy donors compared to PBMC in the absence of propolis.

The levels of IFN-$\gamma$ mRNA expression by PBMC from leishmaniasis patients in the presence of propolis (5 μg/ml) was increased 1.74 fold, whereas using 10 μg/ml the levels of IFN-$\gamma$ expression was downregulated 1.65 fold ($P < 0.01$). The expression for 25 μg/ml of propolis was not affected (Figure 4).

There was no difference on CCL5 expression by PBMC from healthy donors in the presence of propolis (5, 10 and

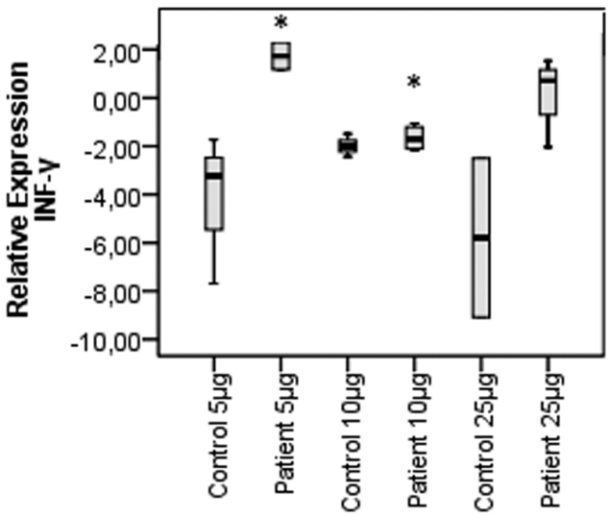


**Figure 4** INF-$\gamma$ expression by PBMC incubated with propolis. PfafII values from control and patient group were compared by Spearman's statistic test using 5, 10 and 25 µg/ml of propolis. *Statistically different from control in the same concentration ($P < 0.001$).

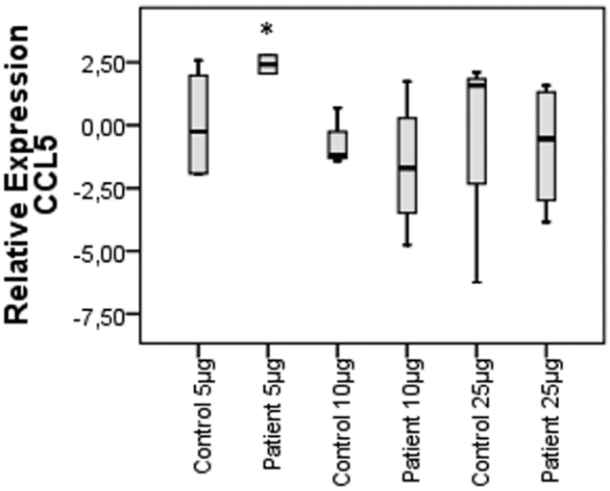


**Figure 5** CCL5 expression by PBMC incubated with propolis. PfafII values from control and patient group were compared by Spearman's statistic test using 5, 10 and 25 µg/ml of propolis. *Statistically different from control in the same concentration ($P < 0.001$).

25 µg/ml) compared to control ($P > 0.05$). However, the level of CCL5 expression by PBMC in the presence of propolis (5 µg/ml) was upregulated 2.42-fold in the patient group ($P < 0.001$) (Figure 5). No alteration was seen on CCL5 expression using 10 and 25 µg/ml of propolis.

## Discussion

The spectrum of clinical and immunopathological manifestations of ACL has been the subject of several investigations in an attempt to fully understand the host immune mechanisms that play a crucial role in the pathogenesis of this disease.[4]

Natural products have been the major sources for drug discovery and the development of novel antileishmanial agents. The efficacy of propolis samples has been demonstrated, especially Bursa propolis against *L. infantum* and *L. tropica in vitro*, showing a promising activity.[30] Herein, propolis presented toxicity and lytic activity for PBMC using 50 and 100 µg/ml, as assessed by LDH assay. MTT assay was also performed to determine the CC50 of propolis for PBMC, and the concentrations of 50 and 100 µg/ml were not used in the PCR due to their cytotoxic action.

Chemokines display an important role in the acute inflammatory response,[31–33] inducing cell migration and activation by binding to specific G-protein-coupled cell-surface receptors on target cells. CCR5 ligands as CCL5/RANTES are involved in the development of Th1 cells.[34] In our work, a reduction of IFN-$\gamma$ mRNA expression by PBMC incubated with propolis (5, 10 and 25 µg/ml) in the control group as well of CCL5 expression (10 µg/ml) was seen, suggesting that these concentrations of propolis drive an anti-inflammatory response. This fact is very important since MCL is a disease associated with a strong Th1 immune response to *Leishmania* antigen.

The main constituents of our propolis sample were aromatic acids (dihydrocinnamic acid, p-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-p-coumaric acid, 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-1-benzo-pyran), di- and triterpenes, a prenylated p-coumaric acid and two benzopyranes (*E*- and *Z*-2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes), essential oils (spathulenol (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones), and flavonoids (kaempferid, 5,6,7-trihydroxy-3,4′-dimethoxyflavone, aromadendrine-4′-methyl ether), among others.[18] Nevertheless, it is not possible to predict which constituents of propolis are responsible for its effects, and further research should evaluate isolated compounds in order to investigate their involvement in propolis action.

In ACL caused by *L. braziliensis*, a mix of cytokine profiles may be found in the lesions since this parasite induces an inflammatory response mediated by Th1 cytokines to control the infection, whereas TGF-$\beta$ and IL-10 have been correlated with persistent infection and chronic lesions. Although IFN-$\gamma$ is crucial for leishmaniasis clinical resolution, it has been reported that IFN-$\gamma$ can have a 'pro-proteolytic' impact. The first months are the most important in establishing an effective immune response that may result in the success or failure of wound healing.[35]

Although propolis seems to exert potential antileishmanial activity, only a few studies have been carried out in this research field.[36–39] Brazilian green propolis possesses an antileishmanial activity against *L. braziliensis in vitro* and *in vivo*, suggesting that it is a promising source of natural compounds

for the development of new chemotherapeutic agents to leishmaniasis treatment.[39] Our preliminary data suggest that propolis exhibits a modulatory action in the immune response of leishmaniasis patients *in vitro*, affecting CCL5 and IFN-$\gamma$ expression by PBMC. This work was an attempt to understand propolis effects *in vitro* in patients with leishmaniasis compared to healthy control subjects. In-vitro approaches reveal an aspect of the scientific investigation, and PBMC interaction with chemokine *in vivo* would complement the in-vitro results, opening perspectives for new investigation in this research field. Although propolis is a potential source of new and selective drugs for the treatment of leishmaniasis,[20] its usefulness in therapeutics should be further evaluated.

## Conclusion

CCR5 ligands as CCL5/RANTES are involved in the development of Th1 cells, which produce pro-inflammatory cytokines such as IFN-$\gamma$ that may impair wound healing in leishmaniasis. Our findings point to an anti-inflammatory action of propolis since PBMC incubation with propolis was followed by a reduced CCL5 and IFN-$\gamma$ mRNA expression in the control group. These data are relevant and bring new insights because MCL is a disease associated with a strong Th1 immune response to *Leishmania* antigen.

## Declarations

### Conflicts of interest

The authors report no conflicts of interest related to this study.

### Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Londrina State University Coordination for Postgraduation (PROPPG-UEL). Marla Karine Amarante was a fellow of CAPES. The authors wish to express their gratitude to GENOPAR for supplying laboratory equipment and Finep for supplying Chromo4™ Real Time PCR Detection. The entire article was revised by a British-born scientific text editor.

## References

1. World Health Organization (WHO). 2011, http://www.who.int/leishmaniasis/burden/en/.
2. CDC. Centers of Diseases Control and Prevention. 2010, http://www.cdc.gov/parasites/leishmaniasis/.
3. Lainson R, Shaw JJ. New World Leishmaniasis – The neotropical *Leishmania* species. In: Cox FEG *et al.*, ed. *Topley & Wilson's Microbiology and Microbial Infections*. London: Arnold, 1998: 242–266.
4. Silveira FT *et al.* Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil – a review. *Mem Inst Oswaldo Cruz* 2004; 99: 239–251.
5. Lainson R, Shaw JJ. Evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Medicine*. 1: London: Academic Press, 1987: 1–120.
6. Coutinho SG *et al.* Immunologic patterns associated with cure in human American cutaneous leishmaniasis. *Braz J Med Biol Res* 1998; 31: 139–142.
7. Teixeira MJ *et al.* Distinct *Leishmania braziliensis* isolates induce different paces of chemokine expression patterns. *Infec Immun* 2005; 73: 1191–1195.
8. Pastore S *et al.* Chemokine networks in inflammatory skin diseases. *Eur J Dermatol* 2004; 14: 230–238.
9. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol* 2001; 22: 83–87.
10. Schroder K *et al.* Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; 75: 163–189.
11. Gessani S, Belardelli F. IFN-$\gamma$ expression in macrophages and its possible biological significance. *Cytokine Growth Factor Rev* 1998; 9: 117–123.
12. Frucht DM *et al.* IFN-$\gamma$ production by antigen-presenting cells: mechanisms emerge. *Trends Immunol* 2001; 22: 556–560.
13. Munder M *et al.* Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. *J Exp Med* 1998; 187: 2103–2108.
14. Otani T. Identification of IFN-$\gamma$-producing cells in IL-12/IL-18-treated mice. *Cell Immunol* 1999; 198: 111–119.
15. Maretti-Mira AC *et al.* Therapeutic failure in American cutaneous leishmaniasis is associated with gelatinase activity and cytokine expression. *Clin Exp Immunol* 2011; 163: 207–214.
16. Kayser O *et al.* Natural products as antiparasitic drugs. *Parasitol Res* 2003; 90: S55–S62.
17. Croft SL, Coombs GH. Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 2003; 19: 502–508.
18. Sforcin JM. Propolis and the immune system: a review. *J Ethnopharmacol* 2007; 113: 1–14.
19. Búfalo MC *et al.* In vitro cytotoxic activity of *Baccharis dracunculifolia* and propolis against HEp-2 cells. *Nat Prod Res* 2010; 24: 1710–1718.
20. Sforcin JM, Bankova V. Propolis: is there a potential for the development of new drugs? *J Ethnopharmacol* 2011; 133: 253–260.

 

21. Sforcin JM *et al*. Seasonal effect on Brazilian propolis antibacterial activity. *J Ethnopharmacol* 2000; 73: 243–249.
22. Sforcin JM *et al*. Seasonal effect of Brazilian propolis on *Candida albicans* and *Candida tropicalis*. *J Venom Anim Toxins* 2001; 7: 139–144.
23. Gekker G *et al*. Anti-HIV-1 activity of propolis in CD4(+) lymphocyte and microglial cell cultures. *J Ethnopharmacol* 2005; 102: 158–163.
24. Búfalo MC *et al*. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. *J Appl Microbiol* 2009; 107: 1669–1680.
25. Freitas SF *et al*. In vitro effects of propolis on *Giardia duodenalis* trophozoites. *Phytomedicine* 2006; 13: 170–175.
26. Sforcin JM *et al*. Effects of propolis, some isolated compounds and its source plant on antibody production. *J Ethnopharmacol* 2005; 98: 301–305.
27. Gay RJ *et al*. Optimum reaction conditions for human lactate dehydrogenase isoenzymes as they affect total lactate dehydrogenase activity. *Clin Chem* 1968; 14: 740–753.
28. Amarante MK *et al*. Expression of noncoding mRNA in human blood cells activated with synthetic peptide of HIV. *Blood Cells Mol* 2005; 35: 286–290.
29. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: 45–51.
30. Duran N *et al*. GC-MS analysis and antileishmanial activities of two Turkish propolis types. *Parasitol Res* 2011; 108: 95–105.
31. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* 1994; 12: 593–633.
32. Samson M *et al*. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* 1996; 35: 3362–3367.
33. Yang X *et al*. Analysis of the CC chemokine receptor 5 (CCR5) Delta32 polymorphism in Behçet's disease. *Eur J Immunogenet* 2004; 31: 11–14.
34. Berres ML *et al*. Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. *J Clin Invest* 2010; 120: 4129–4140.
35. Ji J *et al*. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. *Infect Immun* 2003; 71: 4278–4288.
36. Ayres DC *et al*. Effects of Brazilian propolis on *Leishmania amazonensis*. *Mem Inst Oswaldo Cruz* 2007; 102: 215–220.
37. Machado GMC *et al*. Activity of Brazilian and Bulgarian propolis against different species of *Leishmania*. *Mem Inst Oswaldo Cruz* 2007; 102: 73–77.
38. Duran G *et al*. In vitro antileishmanial activity of Adana propolis samples on *Leishmania tropica*: a preliminary study. *Parasitol Res* 2008; 102: 1217–1225.
39. Pontin K *et al*. In vitro and in vivo antileishmanial activities of a Brazilian green propolis extract. *Parasitol Res* 2008; 103: 487–492.