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Propolis increases Foxp3 expression and lymphocyte proliferation in HIV-infected people: A randomized, double blind, parallel-group and placebo-controlled study

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ABSTRACT

HIV infection and the prolonged use of antiretroviral therapy (ART) contribute to persistent inflammation and immune deregulation in people living with HIV/AIDS (PLWHA). Propolis is a bee product with plenty of biological properties, including immunomodulatory and anti-inflammatory action. This work aimed to evaluate possible changes in the immune/inflammatory response in PLWHA under ART after propolis intake. Asymptomatic PLWHA were double-blindly randomized into parallel groups receiving propolis (500 mg/day, n = 20) for 3 months or placebo (n = 20). Plasma cytokines (TNF-α, IL-2, IL-4, IL-6, IL-10 and IL17) were evaluated by cytometric bead array; cytokine production by PBMC (IFN-γ, IL-5, IL-17, IL-10, IL-1β, IL-18, and IL-33) was assessed by ELISA; gene expression (T-bet, GATA-3, RORγt and Foxp3) was determined by RT-qPCR, and cell proliferation was analysed by flow cytometry using CFSE staining. The average of gender, age, $CD4^+$/$CD8^+$ T cell count, time of diagnosis and treatment were similar in both groups. No differences were observed in cytokine levels nor in inflammasome activation. However, Pearson's correlation showed that IL-10 was directly correlated to $CD4^+$ T cell count and inversely to IFN-γ after treatment with propolis. Foxp3 expression and lymphocyte proliferation increased in the propolis group. Data suggested that daily propolis consumption may improve the immune response and decrease the inflammatory status in asymptomatic PLWHA under ART.

1. Introduction

HIV infection represents one of the biggest public health problems in the world, with a large number of cases and new infections every year [1]. Despite the numerous benefits of the antiretroviral therapy (ART), there is only a partial control of immune activation and inflammation in people living with HIV/AIDS (PLWHA). This is even observed in PLWHA with adequate viral suppression and $CD4^+$ T count > 500 cells/mm^3 compared to uninfected individuals, representing a risk factor for morbidity and mortality in this population [2–7].

The persistent immune activation and chronic inflammation in PLWHA can be related to many factors, including direct pathogenic virus effects, the immune response to the virus, residual HIV replication, microbial translocation imbalance, stimulation by other pathogens, deregulated cytokine/chemokine production, loss of regulatory T cells and cytotoxic effects of ART [5,8,9]. These factors contribute to the

Abbreviations: ART, Antiretroviral therapy; CBA, Cytometric bead array; CFSE, Carboxyfluorescein succinimidyl ester; ELISA, Enzyme-linked immunosorbent assay; ICAM-1, Intercellular adhesion molecule-1; IFN-γ, Interferon gamma; IL, Interleukin; INI, Integrase inhibitor; IP, Protease inhibitor; LPS, Lipopolysaccharide; NNRTI, Non-nucleoside reverse transcriptase inhibitor; PBMC, Peripheral blood mononuclear cells; PHA, Phytohemagglutinin; PLWHA, People living with HIV/AIDS; Th, T helper; TNF-α, Tumor necrosis factor alpha; Treg, T regulatory.

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development of non-AIDS comorbidities [9] and the main causes of death in PLWHA are associated with cardiovascular disease, diabetes mellitus, dyslipidemia, neurologic disorders, liver and kidney disease, bone disorders and cancers not related to AIDS [5,10–12].

Therefore, PLWHA present a systemic inflammation, T cell activation and loss of effector function including the proliferative capacity (Gutiérrez et al., 2019). This process deserves a special attention and its modulation is a great challenge, being an alternative to postpone comorbidities in PLWHA. Thus, it is imperative to seek interventions to improve the quality of life and health of PLWHA, to mitigate the impact of the infection and the adverse effects of ART for these individuals.

Propolis - a bee product without side effects - exhibits several biological properties that can promote human health, justifying the study of its intake by PLWHA. Propolis is a resinous and balsamic product from different parts of plants [13], presenting a complex chemical composition which varies according to the botanical source [14]. Hundreds of constituents have already been identified in propolis, including phenolic acids, flavonoids, esters, diterpenes, sesquiterpenes, lignans, aromatic aldehydes, alcohols, amino acids, fatty acids, vitamins and minerals [15]. Our sample contains artepillin C, isosakuranetin, *p*-coumaric acid, aromadendrin, caffeic acid, and *trans*-cinnamic acid [16].

Propolis stands out for its several biological properties, especially immunomodulatory, anti-inflammatory and antimicrobial action, including anti-HIV activity [17–19]. Regarding its anti-HIV action, propolis inhibited p24 protein expression by $CD4^+$ T and microglial cell, what was potentiated in combination with zidovudine – a reverse transcriptase inhibitor. A possible mechanism involved in propolis anti-HIV activity is the inhibition of viral entry in $CD4^+$ T cells [18]. Silva et al. [20] evaluated the HIV-1 reverse transcriptase inhibition of isolated compounds from Brazilian propolis, observing that isorhamnetin was the most active constituent, followed by naringenin, quercetin and diprenylcinnamic acid. Studies addressing propolis and HIV are mainly related to the antiviral action of propolis in vitro, and little is known about its anti-inflammatory and immunomodulatory effects on HIV infection, motivating us to evaluate its effects on PLWHA under ART.

Propolis exerts an immunomodulatory effect, acting on different cells involved in innate and adaptive immune response [21]. This natural product is able to increase the concentration of anti-inflammatory mediators like IL-10 [22,23] and to decrease the concentration of many inflammatory markers, such as IFN-γ, IL-1β [24], TNF-α, IL-6 [25], the intercellular adhesion molecule-1 (ICAM-1), leukotrienes D4, prostaglandins E2 and F2α [22]. Propolis also inhibits the expression of induced nitric oxide synthase [26], the inflammasome activation [27] and the production of the chemokines CXCL1/KC and CXCL2/MIP-2 [28]. Data from literature suggested that propolis may have an important role in controlling immunological disorders [21] and inflammatory diseases [27]. Therefore, clinical trials are essential in considering the routine use of propolis as a therapeutic agent [29], especially in infections and chronic diseases, such as HIV/AIDS.

In this scenario, an intervention with anti-inflammatory and immunomodulatory agents is necessary for PLWHA, especially with the increasingly early start of ART. Thus, this work aimed to investigate whether propolis intake (500 mg/day for 3 months) could modulate the immunological response and decrease the inflammatory status of PLWHA under ART, in order to postpone the development of comorbidities and improve the quality of life and survival of these individuals. Propolis effect was evaluated on plasma cytokines (TNF-α, IL-6, IL-10, IL-17, IL-2 and IL-4), on markers of the immune response Th1 (IFN-γ and T-bet), Th2 (IL-5 and GATA-3), Th17 (IL-17 and RORγt) and T regulatory (IL-10 and Foxp3), on inflammasome activation (IL-1β, IL-18 and IL-33) and on lymphocyte proliferation.

2. Material and methods

2.1. Study design

A randomized, double blind, parallel-group, placebo-controlled trial was conducted in the Specialized Outpatient Service for Infectious Diseases "Domingos Alves Meira" (SAEI-DAM) - Botucatu Medical School Complex (FMB)-UNESP, in São Paulo State, Brazil.

The sample size of the study was determined according to the variables: glucose, cholesterol, IL-1β, IL-10 and reactive oxygen species [30]. A sample of 20 individuals per group of intervention was defined, assuming errors of type I and II (5% and 20%, respectively) and a loss of follow-up of 30%, as determined by the statisticians from the institution's Research Support Office (EAP - FMB/UNESP).

Inclusion criteria were HIV^+ patients, 20–55 years old, regular ART use for at least two years, with verified adherence of pharmacy discharge history, and sustained immunovirological response during the same period ($CD4^+$ T lymphocyte count $\geq$ 500 cells/mm^3 and plasma HIV-1 viremia <40 copies of RNA/ml).

Exclusion criteria for participants included registration in potentially conflicting research protocols, cancer history, diabetes mellitus, cardiovascular or autoimmune diseases, pregnancy, co-infections, transplants, patients who perform intense physical exercises regularly, genetic diseases, treatment with anxiolytics or antidepressants, vitamin or nutritional supplementation, use of illicit drugs or excessive alcohol consumption.

A questionnaire was applied to assess the inclusion and exclusion criteria, as well as the sociodemographic, clinical and therapeutic data (Supplementary Fig. 1.), which were confirmed by the electronic medical record.

PLWHA (n = 800) were assessed for eligibility and 738 were excluded (480 not meeting inclusion criteria and 258 declined to participate). The eligible PLWHA were randomized by random-numbers table (blocking randomization) and equally allocated in a group receiving propolis (500 mg/day) or placebo, for 3 months. Placebo group lost 4 PLWHA to follow-up and 7 discontinued the intervention. Propolis group lost 3 PLWHA to follow-up and 8 discontinued the intervention, finally totaling 20 patients in each group.

Blood samples were collected twice: before (moment 0 = M0) and after 3 months of intervention (moment 1 = M1). The propolis dose was based on pre-clinical studies of safety and efficacy, and on the findings of a previous clinical trial assessing patients with chronic kidney disease using the same propolis sample (500 mg/day) [31].

Tablets containing a standardized propolis extract (EPP-AF®) were kindly provided by Apis Flora Company, Ribeirão Preto, SP, Brazil (Patent Letter nº 0405483–0, approved by Industrial Property Magazine on July 23th, 2019). The chemical composition of propolis was previously analyzed by Berretta et al. [16] and contained artepillin C, isosakuranetin, *p*-coumaric acid, aromadendrin, caffeic acid, and *trans*-cinnamic acid.

This work was approved by the Research Ethics Committees of the Botucatu Medical School (FMB), UNESP (CAAE nº 58694816.6.0000.5411) and by the Brazilian Clinical Trials Registry (ReBec; nº RBR-33mjbg). The research was carried out according to the ethical principles of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization. An informed consent was obtained from all participants.

2.2. Socio-demographic, clinical and therapeutic data

Socio-demographic, clinical and therapeutic characteristics of PLWHA were obtained from electronic medical records and by interviews (Supplementary Fig. 1.). The following variables were analysed: gender, age, skin color, time of HIV infection (considering the diagnosis), time of ART, current ART type and $CD4^+$ and $CD8^+$ T lymphocyte count.

2.3. Determination of plasma cytokines

Blood samples of PLWHA were collected and centrifuged for 10 min at 200 x *g* to obtain the plasma. TNF-α, IL-6, IL-10, IL-17, IL-2, IL-4 levels were determined using the Human Th1/Th2/Th17 cytokine kit BD™ Cytometric Bead Array (CBA), according to the manufacturer's guidelines. Cytokine levels were assessed using a FACS Calibur TM cytometer (Becton Dickinson, USA).

2.4. Peripheral blood mononuclear cells (PBMC)

After plasma collection, cells of PLWHA were centrifuged at 400 x *g* for 30 min at room temperature in the presence of Ficoll-Paque (GE Healthcare Bio-Sciences, Sweden – density = 1.077) to obtain the peripheral blood mononuclear cells (PBMC). The interface layer of PBMC was aspirated and washed twice with RPMI at 200 x *g* for 10 min

2.5. Cytokine production by PBMC

PBMC (1×10^6 cells/ml) were incubated in the presence of LPS (10 μg/ml; Sigma-Aldrich, USA), for 18 h at 37°C and 5% CO_2. The supernatant was collected and cytokine production was measured by enzyme-linked immunosorbent assay (ELISA).

Cytokines involved in inflammasome activation (IL-1β, IL-18 and IL-33) were measured using DuoSet ELISA kit (R&D Systems, USA); IFN-γ, IL-17, IL-10 were measured by ELISA Max Deluxe kit (BioLegend, USA) and IL-5 by BD OptEIA™ Set Kit (BD Biosciences, USA), according to manufacturer's guidelines.

The plates were read at 450 nm using an ELISA plate reader (BioTek, UK).

2.6. Gene expression of T cell transcription factors

T-bet, GATA-3, RORγt and Foxp3 expression was carried out using the real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR).

After PBMC separation, total RNA was extracted using the "*Total RNA Purification*" kit (Norgen Biotek, Canada), according to the manufacturer's instruction. RNA concentration was evaluated spectrophotometrically using a NanoDrop 2000c (Thermo Scientific, USA). RNA integrity was verified using the "*Agilent RNA 6000 Nano Kit*" (Agilent Technologies, Germany). Total RNA was incubated with RNase-free DNase (Promega, USA) to remove the genomic DNA. Subsequently, the synthesis of complementary DNA (cDNA) was performed, using *ImProm-IITM Reverse Transcription System* (Promega, USA). Gene expression was evaluated by RT-qPCR, using SYBR™ Green Master Mix (Promega, USA) and the primers listed in Table 1. The reaction was carried out on 7300 fast real-time PCR Systems (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize gene expression. The differential expression was performed using a standard curve [32].

Table 1
Genes and primer sequences.

Gene	Primer Sequence (5′–3′)	GeneBank
T-bet	Forward: (906) GGATGCGCCAGGAAGTTTCA (925) Reverse: (993) TGGAGCACAATCATCTGGGT (974)	NM_013351
GATA-3	Forward: (174) CTCTTCGCTACCCAGGTGAC (193) Reverse: (269) ACGACTCTGCAATTCTGCGA (250)	NM_001002295.1
RORγt	Forward: (363) CATGTCCCGAGATGCTGTCA (382) Reverse: (473) GGTTCCTGTTGCTGCTGTTG (454)	NM_005060.3
Foxp3	Forward: (614) AGGAAGGACAGCACCCTTT (633) Reverse: (726) GGAAGTCCTCTGGCTCTTCG (707)	NM_014009
GAPDH	Forward: (684)CGTGGAAGGACTCATGACCA (703) Reverse: (801)GGCAGGGATGATGTTCTGGA (782)	NM_002046.4

2.7. Lymphocyte proliferation

Lymphocyte proliferation assay was performed using the CellTrace™ Cell Proliferation kit (Life Technologies, USA). PBMC (1×10^6 cells/ml) were labeled with 0.2 μl/ml CFSE and incubated with the mythogen phytohemagglutinin (PHA) (3 μg/ml) for 120 h. Proliferating cells were phenotyped using the monoclonal anti-CD4-PerCP/Cy5.5 and anti-CD3-APC (BioLegend, USA) antibodies. In each sample, 10.000 events were analyzed by flow cytometry, using FACSCanto II™ (BD Biosciences, USA). Data was analysed using *Flowjo software (Tree Star)*.

2.8. Statistical analysis

The analysis study population comprised only patients who completed the treatment originally allocated (Per-protocol analysis). Analyses were performed using SAS for Windows (version 9.2) software, with the assistance of the institution's Research Support Office (EAP - FMB/UNESP). Data was analysed before and after 3 months of intervention using Poisson Distribution for count variables, Gamma distribution for asymmetric data and Analysis of Variance (ANOVA) followed by the Tukey-Kramer test for data with normal distribution. Significant differences were considered when $p \leq 0.05$.

3. Results

3.1. Data from medical records and interviews

The groups were homogeneous regarding the epidemiological, therapeutic and clinical variables. PLWHA enrolled in the study were predominantly male (65.5% in both groups), with a mean age of 38.75 ± 7.93 years in placebo and 41.6 ± 7.24 in propolis group, white skin color (85% in placebo and 70% in propolis group) and heterosexuals (57.9% and 61.1% in placebo and propolis group, respectively). The mean time since diagnosis was 8.65 ± 5.25 years in placebo and 10.2 ± 5.91 years in propolis group, and the use of ART was 7.6 ± 4.37 years (placebo) and 8.32 ± 5.27 years (propolis). ART containing Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) was used by most patients (40% and 75% for placebo and propolis, respectively), followed by a Protease Inhibitor (IP, 50% and 15% for placebo and propolis, respectively) and Integrase Inhibitor (INI, 10% in both groups). Viral load remained undetectable (<40 copies/ml) and no changes were seen in $CD4^+$ and $CD8^+$ T cells count after the intervention. Additionally, no adverse events or complaints were reported during propolis intervention, and the patients showed no significant changes in their biochemical, metabolic, nutritional and dietary profiles during the intervention period, as previously reported by Tasca et al. [33].

3.2. Plasma cytokines

Since PLWHA present an immune dysregulation and persistent inflammation, we evaluated plasma cytokine levels to determine propolis action at a systemic level. The results before and after the intervention are present in Table 2. No significant differences were observed in plasma cytokine. High frequencies of undetectable cytokines were obtained pre- and post-treatment, what made it difficult to detect any difference. In addition, we observed a large difference in the results due to the great variability among individuals, usually found in clinical trials.

3.3. Cytokine production by PBMC

Cytokine production by PBMCs was evaluated in order to observe the influence of propolis on Th1 (IFN-γ), Th2 (IL-5), Th17 (IL-17), Treg (IL-10) profiles and on the inflammasome complex activation (IL-18, IL-1β and IL-33), since HIV infection strongly influence these parameters.

There was no significant difference in cytokine production by PBMC

Table 2
Concentration of plasma cytokines (fg/ml) in the placebo or propolis group, before (M0) and after (M1) the intervention. Data represent mean ± standard deviation of 20 individuals per group.

	PLACEBO					PROPOLIS					
Cytokines (fg/ml)	M0		M1		*p*	M0		M1		*p*	
TNF-α	38.90	±152.93	26.26	±117.42	NSD	46.11	±201.01	95.77	±376.52	NSD	*Gamma*
IL-6	797.70	±1374.10	626.99	±843.75	NSD	534.73	±837.88	1085.52	±1712.28	NSD	*Gamma*
IL-10	126.05	±548.78	44.28	±107.53	NSD	106.48	±351.12	3.91	±13.926	NSD	*Gamma*
IL-17	0.00	±0.00	0.00	±0.00	NSD	0.00	±0.00	0.00	±0.00	NSD	*Gamma*
IL-2	1.42	±6.37	0.00	±0.00	NSD	22.99	±100.22	58.65	±226.474	NSD	*Gamma*
IL-4	8.79	±39.30	0.00	±0.00	NSD	0.00	±0.00	0.00	±0.00	NSD	*Gamma*

SD: standard deviation; TNF-α: *Tumor necrosis fator alpha*; IL-6: *Interleukin 6*; IL-10: *Interleukin 10*; IL-17: *Interleukin 17*; IL-2: *Interleukin 2*; IL-4: *Interleukin 4*. NSD: not significantly different.

Table 3
Cytokine production (pg/ml) by PBMC (1 $\times 10^6$ cells/ml) incubated with LPS (10 μg/ml) for 18 h, before (M0) and after (M1) treatment with placebo or propolis. Data represent mean ± standard deviation of 20 individuals per group.

	PLACEBO					PROPOLIS					
Cytokines (pg/ml)	M0		M1		*p*	M0		M1		*p*	Test
IFN-γ	116.48	±153.83	144.16	±228.78	NSD	198.35	±227.44	233.32	±268.99	NSD	*Gamma*
IL-5	17.18	±21.75	10.83	±14.39	NSD	9.18	±8.58	8.25	±9.28	NSD	*Gamma*
IL-17	1.70	±0.87	1.66	±1.10	NSD	2.05	±1.00	2.05	±2.02	NSD	*Gamma*
IL-10	5411.76	±2447.56	4985.99	±2853.01	NSD	5595.18	±2893.33	5605.81	±3212.26	NSD	*Anova*
IL-18	99.96	±36.74	139.12	±144.82	NSD	92.63	±70.52	111.87	±93.33	NSD	*Gamma*
IL-1β	8334.16	±3341.41	9600.20	±5215.10	NSD	7664.57	±5258.88	9545.82	±7755.81	NSD	*Gamma*

SD: standard deviation; IFN-γ: *Interferon-gamma*; IL-5: *Interleukin-5*; IL-17: *Interleukin-17*; IL-10: *Interleukin-10*; IL-18: *Interleukin-18*; IL-1β: *Interleukin-1 beta*. NSD: not significantly different.

(Table 3). No detectable IL-33 levels were registered (data not shown).

Pearson's analysis was applied to observe a possible positive or negative correlation between the variables. All variables were crossed and only two correlations were observed in the propolis group after treatment: IL-10 was directly associated with $CD4^+$ T cell count (p = 0.0459) and inversely correlated with IFN-γ (p = 0.0046) in propolis-treated group (Fig. 1).

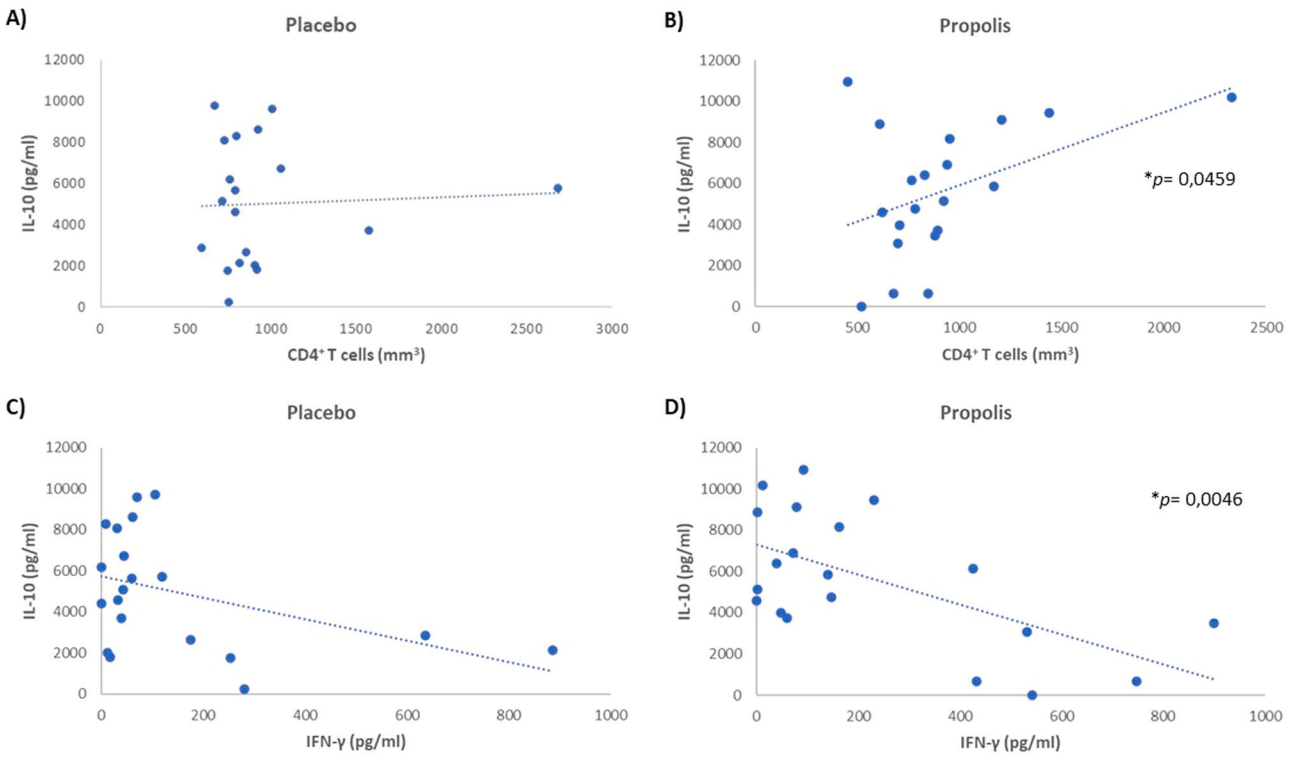

Fig. 1. Pearson's correlation analysis in placebo and propolis group after treatment. A) IL-10 (pg/ml) production and $TCD4^+$ cells count (mm^3) in placebo group (not significantly different). B) IL-10 (pg/ml) production and $TCD4^+$ cells count (mm^3) correlation in propolis group (* $p < 0.05$). C) IL-10 (pg/ml) and IFN-γ (pg/ml) production in placebo group (not significantly different). D) IL-10 (pg/ml) and IFN-γ (pg/ml) correlation in propolis group (* $p < 0.05$).

3.4. Gene expression of transcription factors

In order to assess whether propolis intake for 3 months could modulate gene expression of transcription factors, the gene expression of T-bet (Th1), GATA-3 (Th2), RORγt (Th17) and Foxp3 (Treg) was evaluated.

An increased Foxp3 expression was observed in the propolis group ($p = 0.0406$), as shown in Fig. 2. No differences were seen with respect to T-bet, GATA-3 and RORγt relative expression.

3.5. Proliferation assay

The intense immune dysregulation leads to a suppression of T cell proliferation. These facts prompted us to evaluate whether propolis could restore the cellular function of PLWHA, evaluating the lymphocyte proliferation after stimulation with the mythogen PHA.

Data from cell proliferation revealed that the propolis group presented a higher lymphocyte proliferation after treatment ($p = 0.0058$) (Fig. 3). Gate strategies for samples analysis are shown in the Supplementary Fig. 2.

4. Discussion

The control of the persistent immune activation and chronic inflammation is a current challenge for PLWHA, reflecting on the development of non-AIDS comorbidities. Thus, interventions with anti-inflammatory and immunomodulatory drugs are necessary, making propolis an important candidate to improve the quality of life and survival of PLWHA.

In this study, PLWHA in both groups were homogeneous regarding the epidemiological, clinical and therapeutic variables, minimizing the influences of possible distorting effects in our findings. HIV viral load remained undetectable (<40 copies of RNA/ml) and there was no change in the $CD4^+/CD8^+$ T lymphocyte count, demonstrating the absence of propolis interference in antiretroviral response. Additionally, no adverse events or changes in the biochemical and metabolic profile were seen, signalling propolis as a safe intervention for asymptomatic PLWHA [33].

PLWHA present high levels of inflammatory cytokines that favours the development of non-AIDS comorbidities [34]. HIV also leads to inflammasome activation, a multiprotein cytoplasmic complex that contributes to the inflammatory process [35]. Propolis is able to modulate cytokine production [21], thus we evaluated whether propolis treatment could affect cytokine levels in PLWHA under ART.

A low frequency of plasma cytokines was observed. In contrast, evaluating the cytokines in PBMC supernatant by ELISA, detectable values were seen in almost all cell cultures, only without producing IL-33. Furthermore, low levels of IL-17 were observed - a cytokine that seemed to be less prominent in HIV-infected individuals [36]. Regarding IL-4, Pina et al. [37] found no detectable concentration of this cytokine in PLWHA and Gorenec et al. [38] reported that IL-4 levels were increased in the acute HIV infection. This may be a possible explanation for the difficulty in detecting IL-4 in the samples of our patients, who were HIV-positive for at least 2 years.

It is worth mentioning that no change was seen in the concentration of cytokines related to the inflammasome activation (IL-1β, IL-18 and IL-33), whereas the same propolis sample exerted an inhibitory effect on IL-1β secretion by murine macrophages [27]. Since IL-1β exerts a pro-inflammatory action, the fact that propolis decreases or maintains its levels reinforces the anti-inflammatory effect of this bee product.

Although propolis consumption did not change the cytokine levels, the propolis-treated group presented an important correlation related to IL-10, a cytokine that exerts an anti-inflammatory activity and is able to regulate the immune response, suppressing the synthesis of pro-inflammatory cytokines [39]. PLWHA exhibit a persistent inflammatory status and high levels of biomarkers associated with inflammation [3,9]. Even in PLWHA with a regular $CD4^+$ T cell count, inflammation is still an important risk factor for mortality [40]. In the propolis group, $CD4^+$ T cell count was positively correlated with IL-10 production, and higher levels of IL-10 were associated with lower production of the inflammatory cytokine IFN-γ. Evaluating subcutaneous injection of IL-10 in HIV-1 infected individuals for 4 weeks, Pott et al. [41] observed a lower production of IFN-γ and IL-1β compared to the placebo, demonstrating its anti-inflammatory and immunomodulatory features.

Our findings indicated that propolis use may be associated with suitable immunity and decreased inflammatory status, as is in agreement with other studies [22–24,27,42,43]. Conti et al. [23] observed that Brazilian green propolis stimulated IL-10 production by human

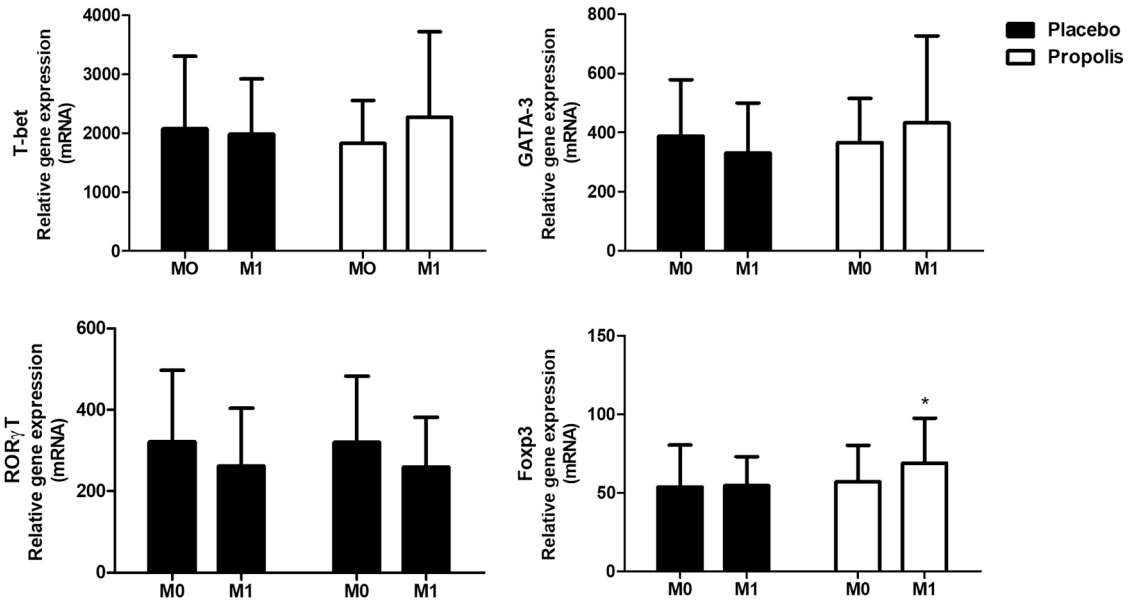

Fig. 2. Relative gene expression of T-bet, GATA-3, RORγt and Foxp3 by PBMC (1×10^6 cells/ml), before (M0) and after (M1) treatment with placebo or propolis. T-bet and GATA-3 were analyzed by Gamma test; RORγT and Foxp3 were analyzed by Anova followed by the Tukey-Kramer test. Data represent mean ± standard deviation of 20 individuals per group. * Significantly different from propolis M0 ($p < 0.05$).

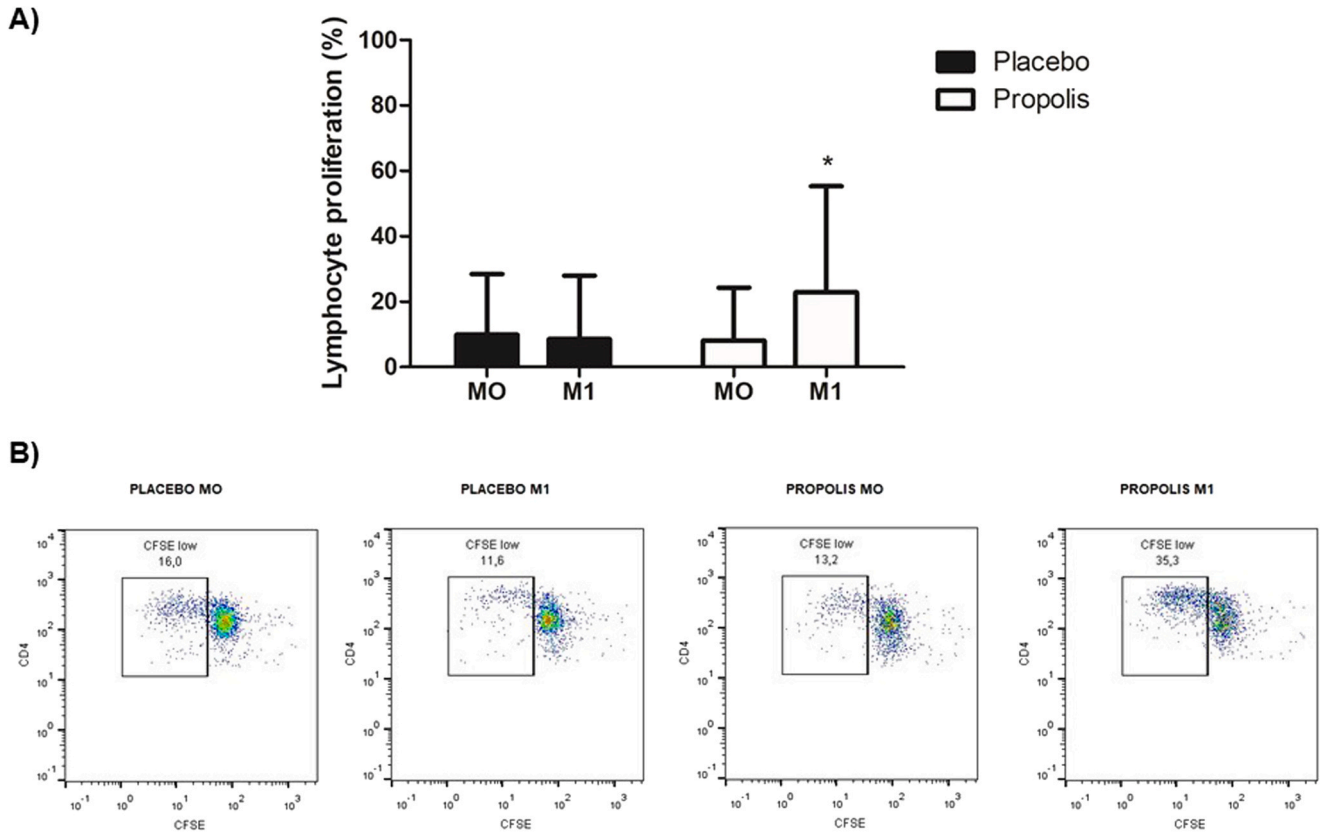

Fig. 3. A) Proliferation (%) of T $CD3^+CD4^+$ lymphocytes (1×10^6 cells/ml) after 120 h of culture with PHA (3 μg/ml), before (M0) and after (M1) treatment with placebo or propolis. Data were analyzed by Poisson distribution. Data represent mean ± standard deviation of 20 individuals per group. *Significantly different from propolis M0 ($p < 0.05$). B) Representative dot plots of lymphocytes proliferation in placebo and propolis groups, before (M0) and after (M1) the treatments.

monocytes. Increased IL-10 levels were seen in asthmatic patients treated with propolis, as well as a significant reduction in the pro-inflammatory cytokines TNF-α, IL-8 and IL-6 [22]. Propolis treatment of murine macrophages pre-treated with LPS attenuated IFN-γ and IL-1β production, among other inflammatory mediators [24]. Hori et al. [27] observed that propolis reduced IL-1β production by murine macrophages stimulated with LPS. Propolis treatment of diabetic rats reduced IL-1β and increased IL-10 levels, compared to the control group [42]. Isolated compounds, such as caffeic acid phenethyl ester (CAPE), administered to LPS-treated rats decreased pro-inflammatory cytokines production and increased the anti-inflammatory ones [43]. Thus, data from literature demonstrated the immunomodulatory action of propolis, generally favouring anti-inflammatory responses.

HIV infection also promotes a profound imbalance in the immune response of patients [44–46], leading to a reduction of Treg cells [47]. We observed in an unprecedented way that propolis increased Foxp3 expression, a marker of Treg lymphocytes. Furthermore, propolis was also able to increase the number of Treg cells in asthmatic mice, reducing lung inflammation [48]. Since Treg cells are responsible for regulating immune responses, secreting anti-inflammatory cytokines, controlling exacerbated inflammatory responses and maintaining immune homeostasis [39], propolis may be a beneficial alternative in controlling the immune activation and inflammation in PLWHA. Data from literature indicated that propolis can also prevent Th17 [49] and Th1 differentiation [50], what might be useful for controlling inflammatory diseases.

Our data demonstrated that propolis influenced the gene expression of transcription factors without interfering in cytokine production, increasing speculation for a longer-term intervention or a higher propolis dosage. Additionally, it is important to note that our study included only young PLWHA, with good immune status and without comorbidities, what could make it difficult to observe other effects of propolis.

HIV infection induces an intense immune deregulation with loss of cell function. The observed immune exhaustion in PLWHA is characterized by an increase in a given activated T cell phenotype, with a decreased proliferative and effector capacity of immune cells [51]. Thus, immune cells become increasingly dysfunctional during chronic HIV infection [52].

Korencak et al. [52] reported that the use of integrase inhibitors (elvitegravir and dolutegravir) is associated with slow proliferation, impaired cell respiration and mitochondrial dysfunction, resulting in decreased $CD4^+$ T cell functions. This data demonstrates that the immune function impaired by HIV remains even after ART.

Our results indicated for the first time that daily propolis intake is able to restore cell activity in PLWHA under ART, inducing the proliferation of $CD4^+$ T cells and favouring an anti-inflammatory profile. Thus, propolis can be useful in complementary medicine to restore the effector cell ability, the immunity of PLWHA and to prevent inflammatory response.

Other nonclinical studies have already demonstrated the ability of propolis and its constituents to modulate $CD4^+$ T cell activities. Kimoto et al. [53] described that artepillin C increased the ratio of $CD4^+/CD8^+$ T cells and the total number of T helper cells. Park et al. [54] observed an increased $CD4^+$ T cell count in mice treated with CAPE. Assessing the effects of irradiation after propolis administration in mice, Takagi et al. [55] verified the protective action of the bee product on $CD4^+$ T cells. Al Ghamd et al. [30] observed an increased T and B lymphocyte proliferation in diabetic mice treated with propolis, demonstrating its potential to improve the function of immune cells in this disease, which is characterized by impaired immunity. Regarding HIV infection, propolis inhibited cell lysis mediated by the HIV nef protein and increased $CD4^+$

T cell proliferation [56]. Investigating the action of propolis as an adjuvant in vaccine formulations containing HIV proteins (tat/env/-pol/gag), Mojarab et al. [57] verified that aqueous and ethanolic extracts of propolis induced lymphocyte proliferation in mice.

Several experimental studies conducted by our group have documented the immunomodulatory action of Brazilian propolis in animal models, such as rats and mice [58–60], and in human immune cells, like monocytes and dendritic cells [23,61–64]. In addition, propolis is able to improve humoral and cellular immunity, being considered a promising candidate to strengthen the immune system in various pathological conditions [21].

5. Conclusions

The daily intake of propolis (500 mg) can be an alternative therapy to improve the immune response of PLWHA due to its ability to induce T cell proliferation, mitigating the inflammatory status in PLWHA through the activation of Treg cells. However, further investigation is still needed to indicate propolis as an effective intervention for these patients in the long-term, especially for immunological non-responders, symptomatic patients and those with concomitant non-AIDS illnesses.

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CRediT authorship contribution statement

Fernanda Lopes Conte, Karen Ingrid Tasca and José Maurício Sforcin designed the study Karina Basso Santiago and Eliza de Oliveira Cardoso randomized the patients Fernanda Lopes Conte and Karen Ingrid Tasca included and followed the patients Fernanda Lopes Conte, Karen Ingrid Tasca, Karina Basso Santiago and Eliza de Oliveira Cardoso developed the protocols Graziela Gorete Romagnoli, Marjorie de Assis Golim and Aline Márcia Marques Braz assisted the flow cytometry analysis Fernanda Lopes Conte and Karen Ingrid Tasca performed all the experiments Andresa Aparecida Berretta, Lenice do Rosário de Souza and José Maurício Sforcin contributed to the reagents/materials Fernanda Lopes Conte, Karen Ingrid Tasca, Andresa Aparecida Berretta, Lenice do Rosário de Souza and José Maurício Sforcin interpreted and discussed the results Fernanda Lopes Conte, Karen Ingrid Tasca and José Maurício Sforcin structured and wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interests to disclose.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111984.

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