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Effects of Three Different Brazilian Green Propolis Extract Formulations on Pro- and Anti-Inflammatory Cytokine Secretion by Macrophages

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Abstract: Propolis is known for its immunomodulatory properties. We investigated the effects of three recently developed propolis extract formulations: polar propolis fraction (PPF), soluble propolis dry extract (PSDE), and microencapsulated propolis extract (MPE), and some of their components, on pro- and anti-inflammatory cytokine production in a macrophage model. Bone marrow cell-derived macrophages (BMDM) in cell culture were *E. coli* lipopolysaccharide (500 ng/mL) stimulated for two hours and subsequently incubated for 20 hours with one of the three propolis extract formulations (1, 10, 25, 50, 100 and 300 µg/mL) or with isolated propolis components (caffeic acid, *p*-coumaric acid, artepillin C, or baccharin) (10, 25, 50 and 100 µg/mL) to determine how they affected secretion of the pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokine, IL-10. PPF increased IL-6 and IL-10 levels. PSDE increased IL-6 and IL-10 at lower concentrations, while at higher concentrations it increased TNF-α and decreased IL-10. MPE increased IL-10. Caffeic acid and PPF increased both IL-6 and IL-10. Artepillin C and PSDE decreased IL-10. Baccharin and MPE increased IL-10. Baccharin also decreased IL-6. *p*-coumaric acid did not affect secretion of these cytokines. Pro- and anti-inflammatory cytokine production by the different propolis extracts differed; however, all three propolis extract formulations have potential as immunomodulatory agents in food supplement and pharmaceutical products.

Keywords: EPP-AF®; microencapsulated extract; caffeic acid; *p*-coumaric acid; artepillin C; baccharin; anti-inflammatory; IL-6; IL-10; TNF-α

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1. Introduction

Chronic inflammatory diseases are among the leading causes of death worldwide [1,2]. Chronic inflammation is intimately associated with various difficult-to-treat diseases, including diabetes mellitus, inflammatory bowel disease, rheumatoid arthritis, psoriasis, amyotrophic lateral sclerosis, and cardiovascular pathologies [3–7]. It can also affect the central nervous

system, in which microglia activation increases the secretion of pro-inflammatory cytokines in pathologies such as Parkinson's disease and depression [8–10].

Inflammation is a complex mechanism of defense and repair, usually prompted by immune system cell detection of tissue damage. These cells release chemical mediators, initiating a complex cascade of events, culminating in vascular cell migration to inflammatory sites. Acute inflammation is usually resolved quickly. However, persistence of this condition can result in a chronic condition, in which immune cells develop a pro-inflammatory profile, releasing substances that exert toxic effects in the intercellular environment, culminating in tissue damage and debris accumulation, which contribute to an on-going inflammatory process [8].

Chronic inflammation is involved in various chronic disease etiologies. Investigation of immune cell activity and chemical mediator levels is essential to determine adequate treatment for these diseases. Macrophages are immune cells that mediate the initiation and conclusion of persistent inflammation. These cells can acquire contrasting activation phenotypes, resulting in pro- or anti-inflammatory profiles, mediated by cytokines such as TNF-α, IL-1β, IL-6, IL-17, and IL-10 [11,12]. Pro-inflammatory cytokines (TNF-α, IL-6) contribute to the maintenance of the inflammatory process [13]. On the other hand, anti-inflammatory cytokines, such as IL-10, can put an end to this process [14].

A thorough understanding of immune cell activities, including their production of chemical mediators, is essential for the development and testing of new anti-inflammatory drugs. Thus, the immunomodulatory properties of potential new drugs that can decrease the plasmatic levels of the pro-inflammatory cytokines interleukin (IL)-6 and tumoral necrosis factor (TNF) and increase the plasmatic levels of anti-inflammatory cytokines, such as IL-10, could result in useful treatment options [15–17]. In vitro studies of the effects of drug candidates on immune cell activities and their secretion of chemical mediators are essential for the development of new anti-inflammatory drugs. Among immune cells, macrophages are particularly relevant because they are involved in the detection, phagocytosis, and destruction of bacteria and other harmful microorganisms [18]. Beyond their ability to activate adaptive immunity through their interaction with T cells [19], macrophages also exert a pivotal role in modulating the immune system through the secretion of pro- and anti-inflammatory mediators, including IL-6, TNF-α, and IL-10.

European propolis is rich in caffeic acid phenethyl ester (CAPE), while Brazilian green propolis is rich in artepillin C; both types of propolis have anti-inflammatory potential [20]. Brazilian green propolis is mainly composed of *Baccharis dracunculifolia* exudates; it has anti-inflammatory and immunomodulatory properties [21–24]. This plant species contributes with compounds exclusively found in green propolis, including drupanin, artepillin C and baccharin, as well as flavonoids and caffeic and *p*-coumaric acids and their derivatives. These components have pronounced anti-inflammatory effects [25,26].

The anti-inflammatory potential of Brazilian green propolis extract has been extensively investigated in in vitro and in vivo studies [27–30]. In a macrophage model, propolis reduced the levels of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β, while increasing the anti-inflammatory cytokine IL-10 [28,31,32]. The broad range of immunomodulatory effects of green propolis makes it potentially suitable for various purposes, including its anti-inflammatory action applied to inflammasome inhibition [32] and to kidney protection [33,34], as well as its use as an antioxidant agent [35]. Its potentiation of immune mechanisms and its antimicrobial properties also make propolis useful for wound healing [36,37] and as an anti-candida agent [38,39].

The immunomodulatory potential of propolis products depends on the type of propolis and the methods used to prepare them [40,41]. Although hydroethanolic extracts are the most common type of propolis extract and the most widely studied, there is a demand for alternative alcohol-free formulations that are safe and effective [42], such as stable powder forms [43,44]. Unfortunately, most currently available dry propolis products are expensive to produce and difficult to maintain dry. Lower-cost methods for obtaining dry propolis extract products with improved stability and reduced hygroscopicity

would have many potential applications. However, the different preparation technologies can affect the appearance, physical–chemical properties, and biological activity of the resulting propolis extracts [45–48], including effects on the production of pro- and anti-inflammatory cytokines [49–52]. This study was designed to evaluate how three recently developed alcohol-free propolis extract formulations affect the production of pro- and anti-inflammatory cytokines in a murine macrophage cell model, in comparison with the effects of some key propolis components analyzed individually.

2. Materials and Methods

2.1. Preparation of the Three Propolis Extracts

The three extracts were produced from the same blend of raw propolis used to produce EPP-AF® by the Brazilian company Apis Flora (Ribeirão Preto, SP, Brazil), according to Berretta et al. [53]. Propolis raw material was evaluated and was found to be within the correct parameters according to Brazilian Normative Instruction no. 3/2001 [54].

First, the raw propolis blend was maintained at −20 °C for 12 h. Subsequently, it was powdered and extracted in a hydroethanolic solution (ethanol and water; 7:3) by dynamic maceration for 72 h at room temperature and posteriorly filtered.

The resulting hydroethanolic EPP-AF® was used to produce MPE, according to Marquiafável et al. [48], with some modifications. Arabic gum was dispersed in purified water; the hydroethanolic EPP-AF® was then added (40:60) and dispersed under intense agitation. The final emulsion was submitted to a spray dryer process, resulting in a microencapsulated powder.

An alkaline hydrolysis step was added to the hydroethanolic EPP-AF® extraction procedure to produce PSDE [55]. Maltodextrin (20:80) was added to the resulting product, followed by a spray dryer process [53], resulting in a concentrated water-soluble fine powder.

To obtain the PPF, the blend of propolis EPP-AF® raw material was subjected to extraction by maceration in an aqueous solution of NaOH (0.5 M) for one hour at room temperature. This solution was acidified with HCl until pH 1.0. Then, the extract was vacuum-filtered and partitioned with ethyl acetate, followed by ethyl acetate evaporation.

2.2. Chemical Marker (Propolis Component) Sources

The caffeic acid (Sigma-Aldrich, lot number: SLBZ6416, St. Louis, MO, USA), *p*-coumaric acid (Sigma-Aldrich, lot number: 091M119V), and artepillin C (PhytoLab, lot number: 111674647, Vestenbergsgreuth, Germany) were purchased from the indicated suppliers.

Baccharin was isolated according to De Sousa et al. [56] and Silva et al. [57]. Brazilian green propolis was obtained from Apis Flora Commercial Ltda, Ribeirão Preto, SP, Brazil (lot number: 65,400,918). The crude Brazilian green propolis was powdered in a blender. A total of 350.0 g of propolis powder was macerated during 24 h with 4 L of a mixture containing ethanol-water 7:3 at room temperature, thus furnishing the hydroalcoholic crude extract (187.4 g) after lyophilization [57]. The hydroalcoholic crude extract was partitioned with hexane, ethyl acetate and butanol.

Fifteen grams of ethyl acetate extract were chromatographed on vacuum liquid chromatography (500 g; silica gel 60 H) using increasing amounts of ethyl acetate in hexane. In this procedure, seven new fractions were collected (600 mL each; Fr1 to Fr7). Fr3 (3.9 g) was also chromatographed on vacuum liquid chromatography over silica gel 60 H (120.0 g) with increasing amounts of 4% EtOAc (hexanes to hexanes/EtOAc 4:1; 200 mL each fraction), resulting in six additional fractions (Fr3.1–Fr3.6). Baccharin (201.0 mg) was purified from Fr3.5 (847.0 mg) after separation by classic chromatography (100.0 g silica gel 60; isocratic mobile phase: hexane/CHCl3 9:1).

2.3. Investigation of Propolis Extracts Composition

2.3.1. Chemical Characterization by HPLC

Three samples of each extract (PPF, PSDE, and MPE) were submitted to high-performance liquid chromatography (HPLC) in Shimadzu equipment with a CBM-20A controller, a LC-20AT quaternary pump, an SPD-M 20A diode array matrix detector and Shimadzu LC software (version 1.21 SP1). The mobile phase consisted of methanol and aqueous formic acid solution (0.1% *v/v*), pH 2.7. The method consisted of a 20–95% gradient for 77 min at a flow rate of 0.8 mL/min in a CLC-ODS column (4.6 mm × 250 mm, particle diameter 5 µm, pore diameter 100 A). Detection was set at 275 nm. The chemical markers caffeic acid, *p*-coumaric acid, 3,5-dicapheoylquinic acid, 4,5-dicapheoylquinic acid, aromadendrin-4-*O*′-methyl-ether, drupanin, chrysin, galangin, artepillin C and baccharin were identified and quantified, according to Berretta et al. [53].

2.3.2. Determination of Total Phenolic Content

The total phenolic contents of the samples were estimated using a colorimetric assay according to the Waterman and Mole [58] procedure, with some modifications. PSDE and MPE were diluted in a 50 mL volumetric flask with 30 mL of water or a 30 mL water: methanol (3:2) solution, respectively, and were homogenized in an ultra-sound bath. Subsequently, the flask volume was completed with the corresponding solvent and filtered with an analytical paper filter. PPF was diluted in 5 mL of methanol in a 10 mL volumetric flask, homogenized, and the flask volume was completed with methanol. Then, 1.0 mL aliquots of the samples were transferred to 50 mL volumetric flasks containing 30 mL of water. Subsequently, 2.5 mL of Folin–Denis reagent and 5.0 mL of 35% w/v sodium carbonate were added; the volume of the 50 mL volumetric flasks was completed with purified water, and they were protected from light for 30 min. The samples were read in a spectrophotometer at 760 nm and each reagent alone was used as a blank. Gallic acid (GAE) was considered as the standard to determine the total phenolic content in the samples [58]. A 0.4 mg/mL solution was prepared by dissolving 4 mg of dry GAE in 10 mL of distilled water, in a volumetric flask. A sequence of GAE standard solutions with concentrations of 3.20, 3.60, 4.00, 4.40 and 4.80 µg/mL was used to prepare the standard calibration curve. The means of three absorbance measurements were calculated, and the total phenolic content of the samples was presented in mg of GAE equivalents/g of sample.

2.3.3. Determination of Total Flavonoid Content

To determine the total flavonoid content, we used an aluminum chloride colorimetric assay, according to Funari and Ferro [59], with some modifications. PPF and PSDE were diluted in 5 mL of methanol, and MPE in 5 mL of water: methanol (1:1) solution, all of them in 10 mL volumetric flasks, and homogenized in an ultrasound bath. The flask volume was completed with the same solvent used for each sample and filtered with an analytical paper filter. Then, 1.0 mL aliquots of each sample were transferred to 25 mL volumetric flasks containing 15 mL of methanol. The reaction was run with 0.5 mL of 5% w/v aluminum chloride; the volume was completed with methanol and the flasks were protected from light for 30 min. The samples were then read in a spectrophotometer at 425 nm, using a solution of 24.5 mL of methanol and 0.5 mL of 5% w/v aluminum chloride as a blank. The total flavonoid content was expressed as quercetin (QUE) equivalent/g of the sample. A 0.3 mg/mL solution was prepared by dissolving 3 mg of dry QUE in 10 mL of methanol in a volumetric flask. The standard calibration curve of QUE was established for the range of 4.80, 5.40, 6.00, 6.60 and 7.20 µg/mL, and the values were calculated as mg of QUE equivalents/g of propolis.

2.4. Cytokine Secretion by BMDM Macrophages

2.4.1. Preparation of Donor Animals

Four adult male C57BL/6 wild-type mice were maintained in plastic laboratory cages with free access to water and food. They were maintained in a rearing facility with controlled temperature (20–24 °C), air exclusion, and noise free. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Ribeirão Preto Medical School of the University of São Paulo (031/2017).

2.4.2. Macrophage Collection

The protocol of macrophage extraction was run according to Marim et al. [60], with some modifications. The mice were deeply anesthetized with isoflurane in a controlled flow chamber and then euthanized by cervical dislocation. Their femurs were removed, and BMDM murine bone marrow cells were isolated and cultured in RPMI medium (Sigma, St. Louis, MO, USA) containing 20% FBS (*v/v*; Gibco, Waltham, MA, USA), penicillin (100 U/mL), amphotericin B (2 µg/mL), and 20% of L929 cell culture supernatant (*v*/*v*) for 7 days, at 37 °C in a 5% CO_2 atmosphere. The differentiated macrophages were harvested as described previously [60], and the cells were seeded at a density of 2×10^5 cells per well in 96-well flat-bottom plates. The non-adherent cells were removed and the attached macrophages were stimulated with medium (negative control), or LPS from *Escherichia coli* (500 ng/mL, Sigma-Aldrich) for two hours. The cells were washed and incubated overnight with treatments for 20 h.

2.4.3. Lipopolysaccharide (LPS) Stimulation of Macrophages and Propolis Treatments

The treatment method and the concentrations of propolis extracts and isolated compounds were chosen according to Bachiega [31] and Hori et al. [32], with some modifications. The macrophages were incubated with LPS (500 ng/mL) for two hours. After LPS stimulation, 20 h treatments were carried out with one of the three extracts, PPF, MPE, or PSDE (1, 10, 25, 50, 100, and 300 µg/mL) or isolated propolis components, caffeic acid, p-coumaric acid, artepillin C, and baccharin (10, 25, 50, and 100 µg/mL). All samples were evaluated in triplicate, including positive and negative controls: the macrophages activated with LPS without propolis treatment, and the inactivated macrophages (no LPS or propolis added to the culture medium), respectively.

2.5. Quantification of Cytokine Levels

After the stimulation and treatment procedures, the levels of cytokines IL-6, IL-10 and TNF-α present in the supernatants from BMDM cell culture were quantified by ELISA using antibodies obtained from R&D Systems, according to the manufacturer's instructions. The optical density of the individual samples was measured at 450 nm in triplicate (Spectra Max-250, Molecular Devices, San Jose, CA, USA).

2.6. Cell Viability Assessment

After collecting the supernatant for the ELISA analysis, the plates were filled with RPMI medium (without phenol red). NBT/TB (nitro blue tetrazolium/tetrazolium bromide) was added to all plates and then they were incubated at 37 °C for 24 h. The next day, DMSO with 10% SDS and 162 µL acetic acid were added to the plates for 40 min. Living cells metabolize salt, turning the medium blue. Then, a spectrophotometer absorbance analysis at 610 nm was carried out. The cell viability of the negative control was considered to be 100% (macrophages that did not receive any treatment or stimulation). The percentage cell viability was calculated: % cell viability = sample absorbance × 100/absorbance of the negative control.

2.7. Statistical Analysis

For comparisons of caffeic and *p*-coumaric acid content in the three extracts and for comparisons of cytokine secretion by macrophages, we applied a one-way ANOVA for independent samples and Tukey's test for multiple comparisons using Prisma GraphPad Software, versions 6.0 and 8.0, respectively. For comparisons of chemical marker contents in PSDE and MPE, we applied a t test for independent samples using Prisma GraphPad Software, version 6.0. The differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Chemical Characterization of the Three Propolis Extracts

The three propolis extracts were developed and characterized, and their physicochemical, antimicrobial and antioxidant properties were investigated and described in Berretta et al. [53]. Two of them, PSDE (propolis soluble dry extract) and MPE (microencapsulated propolis extract), were produced from the Brazilian Green propolis EPP-AF® hydroalcoholic extract [53] and standardized and patented by the Brazilian company Apis Flora (Ribeirão Preto, SP, Brazil), while the polar propolis fraction (PPF) was directly obtained from the EPP-AF® propolis raw material blend. A chemical characterization of the three extracts was made according to standard methodologies used for evaluating propolis, including the determination of total flavonoids and phenolics by measuring quercetin and gallic acid [61], respectively, along with HPLC analysis [36]. The results are shown in Table 1 and Figure 1.

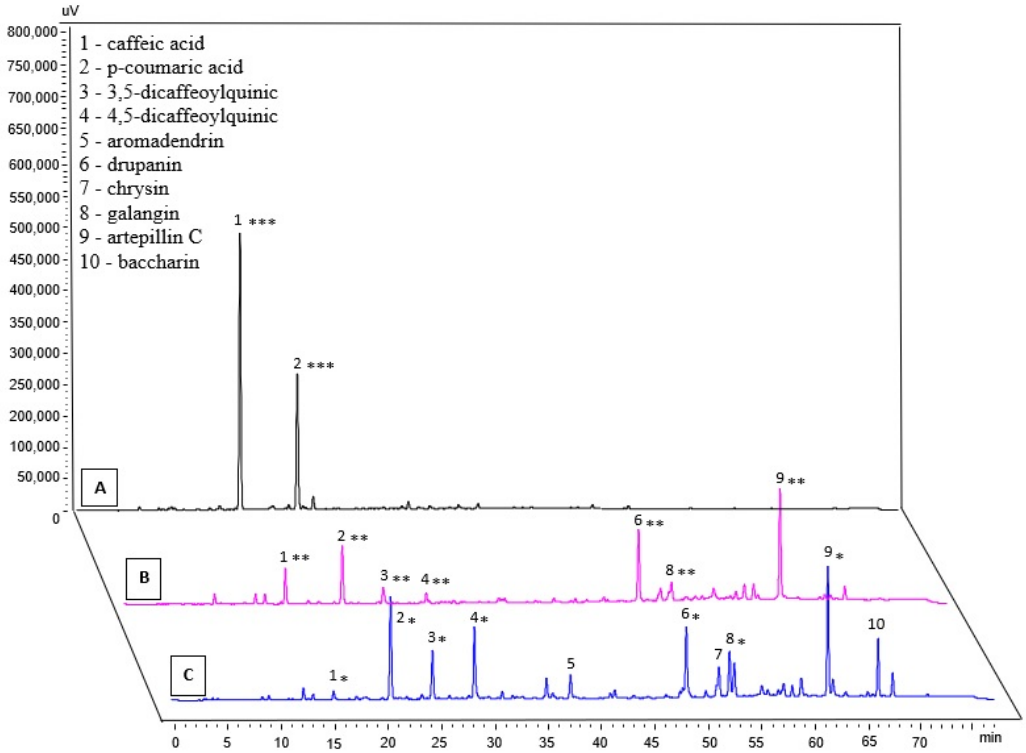

Figure 1. Chromatographic profile of the three propolis extracts: (**A**) Polar propolis fraction (PPF), (**B**) Propolis soluble dry extract (PSDE), (**C**) Microencapsulated propolis extract (MPE). Different numbers of asterisks (*, ** or ***) indicate significant differences in compound content between the extracts ($p \leq 0.05$).

Table 1. Chemical profile of polar propolis fraction (PPF), propolis soluble dry extract (PSDE), and microencapsulated propolis extract (MPE). Data obtained based on HPLC/DAD methodology [36] (n = 3 samples analyzed for each product).

Chemical Marker	PPF	PSDE	MPE
	Mean (mg/g) ± SD	**Mean (mg/g) ± SD**	**Mean (mg/g) ± SD**
Caffeic acid	168.647 ± 17.4 ***(a)	7.045 ± 0.0274 **(a)	0.795 ± 0.0044 *(a)
p-Coumaric acid	50.989 ± 3.9 ***(a)	6.982 ± 0.0132 **(a)	4.667 ± 0.0317 *(a)
3,5 Dicaffeoylquinic acid	ND	7.289 ± 0.030 **(b)	6.623 ± 0.035 *(b)
4,5 Dicaffeoylquinic acid	ND	4.741 ± 0.0429 **(b)	11.844 ± 0.2153 *(b)
Aromadendrin-4-*O*′-methyl-ether	ND	ND	2.619 ± 0.038 *(b)
Drupanin	ND	18.945 ± 0.134 **(b)	7.715 ± 0.040 *(b)
Chrysin	ND	1.963 ± 0.035 **(b)	1.225 ± 0.029 *(b)
Galangin	ND	ND	3.613 ± 0.089 *(b)
Artepillin C	ND	44.970 ± 1.078 **(b)	18.850 ± 0.373 *(b)
Baccharin	ND	ND	2.581 ± 0.071 *(b)

(a) Statistical analysis with ANOVA (and Tukey's test); (b) Statistical analysis with *t* test for independent samples; different numbers of asterisks (*, ** or ***) indicate significant differences in compound content between extracts ($p \leq 0.05$); SD: Standard Deviation; ND: Not detected.

3.1.1. Chemical Characterization of Propolis Extracts by HPLC

The PPF, PSDE and MPE extracts contained 94, 80 and 40% w/w of propolis dry matter, respectively. In the PPF, only caffeic and *p*-coumaric acids were detected (Table 1, Figure 1). These two biomarkers were also detected in PSDE and MPE; however, in PPF these compounds were detected at much higher concentrations ($p \leq 0.05$) (Table 1, Figure 1).

PSDE and MPE extract constitutions were more complex than that of PPF. PSDE was found to contain seven, while MPE contained all of the 10 chemical markers of propolis that were assayed. Both of these two extracts contained the components 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, drupanin, galangin, and artepillin C; this latter was the predominant compound in both extracts. All compounds were in significantly different concentrations in PSDE versus MPE ($p \leq 0.05$). Aromadendrin, chrysin and baccharin were detected only in MPE (Table 1, Figure 1). Among the four propolis components tested for immunomodulation in the macrophage model, caffeic and *p*-coumaric acid were detected in all three extracts, artepillin C was found in PSDE and MPE, and baccharin was detected only in MPE. All four compounds were in different concentrations in each extract ($p \leq 0.05$) (Table 1, Figure 1). These compounds were selected to include two from the more polar fraction (caffeic and *p*-coumaric acids) and two that are prenylated compounds (artepillin and baccharin); the latter are characteristic of green propolis and more apolar than the phenolic acids. Caffeic and *p*-coumaric acids were present in all four extracts, and were reported to have antioxidant activity in a previous analysis Berretta et al. [53]. Additionally, considering that artepillin C, drupanin, and baccharin are characteristic of Brazilian green propolis [56], along with artepillin C, baccharin was selected because this substance differs structurally from artepillin C and drupanin, as it has an additional cinnamic acid group in its composition, and because it was detected only in MPE.

3.1.2. Total Phenol and Flavonoid Content of the Propolis Extracts

The GAE and QUE curves were found to be linear (Figure S2, Supplementary Materials). All absorbance determinations were made thrice at 765 nm for GAE and 425 nm for QUE. The mean values of the three measurements were used for constructing the calibration plot. Correlation coefficient (r^2) values of 0.99 and 0.99 were found for GAE and QUE, respectively. The linearity data of each isolated compound are presented in Table 2.

Table 2. Linearity, quantitation, and detection limits obtained for the isolated propolis compounds investigated in this study. Data obtained based on HPLC/DAD methodology [36] (n = 3 samples analyzed for each product).

Chemical Marker	Regression Curve	r	LOD	LOQ
			µg/mL	µg/mL
Caffeic acid	y = 32,558x – 11,096	1.00	0.59	1.79
p-Coumaric acid	y = 52,979x – 2033	0.99	1.18	3.57
3,5 Dicaffeoylquinic acid	y = 18,942x – 44,655	0.99	6.41	19.43
4,5 Dicaffeoylquinic acid	y = 15,827x – 13,446	0.99	7.15	21.68
Aromadendrin-4-O′-methyl-ether	y = 23,304x – 4193	0.99	0.49	1.47
Drupanin	y = 26,870x – 36,385	0.99	1.40	4.24
Chrysin	y = 66,663x – 3433.7	0.99	1.35	4.08
Galangin	y = 34,798x – 17,489	0.99	3.04	9.20
Artepillin C	y = 19,313x – 725,866	0.99	8.03	24.34
Baccharin	y = 49,810x – 39,607	0.99	0.85	2.58

An evaluation of the phenolic and flavonoid contents of the three extracts is presented in Table 3. PPF, PSDE and MPE contain 94, 80 and 40% w/w of propolis dry matter, respectively. The three extracts all had a greater concentration of phenolics than flavonoids. PSDE is a more concentrated extract, with higher contents of both compound classes, compared with PPF and MPE; these other two extracts contained around one third of the total phenolics of PSDE and approximately one half of the flavonoids. The quantities of total flavonoids were almost proportional to the propolis dry matter content of each extract; higher concentrations of propolis resulted in larger amounts of flavonoids. In the evaluation of MPE and PSDE, when normalized for the same concentration of propolis, the quantity of flavonoids was almost equivalent, different from the total phenolics, which apparently were impacted by the different processes used to produce MPE and PPF. An extensive evaluation and discussion of chemical differences between these three extracts is available in Berretta et al. [53].

Table 3. Total amounts of phenolic and flavonoid compounds, expressed as amounts of gallic acid (GAE) and quercetin (QUE), respectively, in the three propolis extracts investigated using spectrophotometric methods. See Table 1 for definitions of the propolis formulation abbreviations.

Propolis Extracts	Total Phenolic Compounds		Total Flavonoid Compounds	
	Mean (mg GAE/g) ± SD	RSD (%)	Mean (mg QUE/g) ± SD	RSD (%)
PPF	53.05 ± 1.30	2.37	18.11 ± 1.3	3.53
PSDE	123.24 ± 1.90	1.53	45.48 ± 0.5	1.17
MPE	49.45 ± 1.30	2.60	23.17 ± 0.6	2.42

SD: Standard Deviation; RSD: Relative Standard Deviation.

3.2. Effect of the Propolis Extracts on IL-6, IL-10, and TNF-α Secretion by BMDM Macrophages

Since the three propolis extraction methods resulted in three extracts with distinct compositions, the next step was to determine whether these three extracts differ in their effects on cytokine production by LPS-stimulated macrophages. BMDM in cell culture were activated with LPS for two hours, followed by treatment with one of the three extracts, PPF, MPE, or PSDE, at concentrations of 1, 10, 25, 50, 100, or 300 µg/mL for 20 h.

As expected, LPS stimulation of BMDM significantly increased the secretion of IL-6, IL-10, and TNF-α compared to cytokine levels observed in the negative control (Figure 2). When we compared the effects of propolis extracts on the LPS-stimulated macrophages with the control (no propolis), we observed that the PPF extract increased IL-6 and IL-10 secretion at almost all concentrations tested, though with no significant effect on TNF-α (Figure 2A–C).

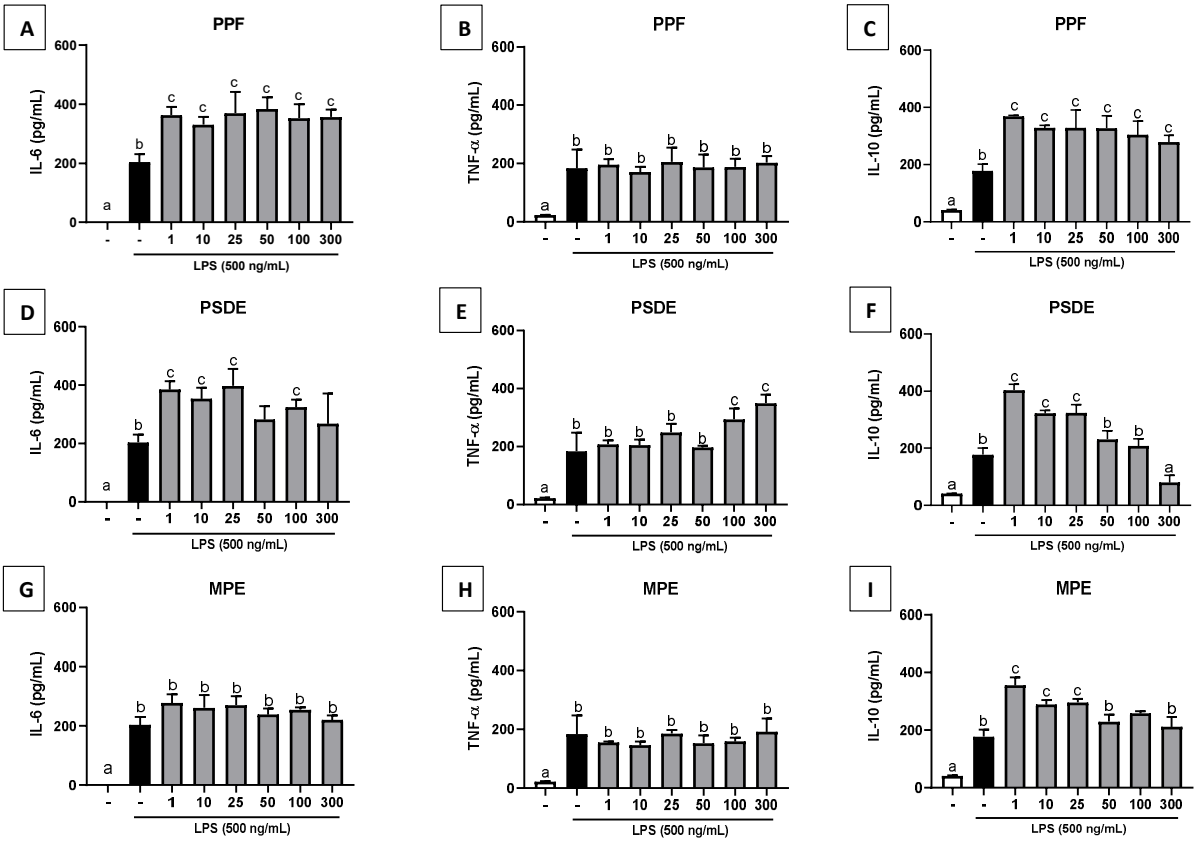

Figure 2. Effects of the three propolis extract treatments: PPF (**A**–**C**), PSDE (**D**–**F**) and MPE (**G**–**I**) at various concentrations (1, 10, 25, 50, 100 and 300 µg/mL) on cytokine secretion (pg/mL): IL-6 (**A**,**D**,**G**), TNF-α (**B**,**E**,**H**) and IL-10 (**C**,**F**,**I**) by macrophages previously stimulated with LPS (500 ng/mL) (gray bar). The white bars represent cytokine secretion by macrophages that were not stimulated. The black bars represent macrophages that were stimulated with LPS but were not treated with propolis. Significant differences ($p \leq 0.05$) are represented by the different letters "a", "b" and "c".

In the case of PSDE, the cytokine behavior varied with the concentration used. Low concentrations (1, 10, and 25 µg/mL) increased IL-6 and IL-10 production, while TNF-α was not significantly affected. On the other hand, at the highest concentration (300 µg/mL), this extract increased TNF-α and reduced IL-10 (Figure 2D–F).

MPE did not significantly affect the secretion of the pro-inflammatory cytokines IL-6 and TNF-α. However, at low concentrations (1, 10, and 25 µg/mL), this extract increased IL-10 secretion (Figure 2G–I).

3.3. Effects of Isolated Propolis Compounds on IL-6, IL-10, and TNF-α Secretion by Macrophages

We also investigated cytokine production induced by selected propolis components present in different proportions in the extracts. Caffeic acid significantly increased IL-6 and IL-10 at the highest concentrations (100 µg/mL), while TNF-α levels were not significantly affected (Figure 3A–C). In the case of *p*-coumaric acid, there were no significant alterations in any of the cytokines (Figure 3D–F). Artepillin C did not significantly alter IL-6 or TNF-α secretion; however, it significantly decreased IL-10 at 100 µg/mL (Figure 3G–I). Baccharin (10, 25, and 100 µg/mL) increased IL-10 levels, and at 50 µg/mL, it decreased IL-6, although with no significant effects on TNF-α (Figure 3J–L).

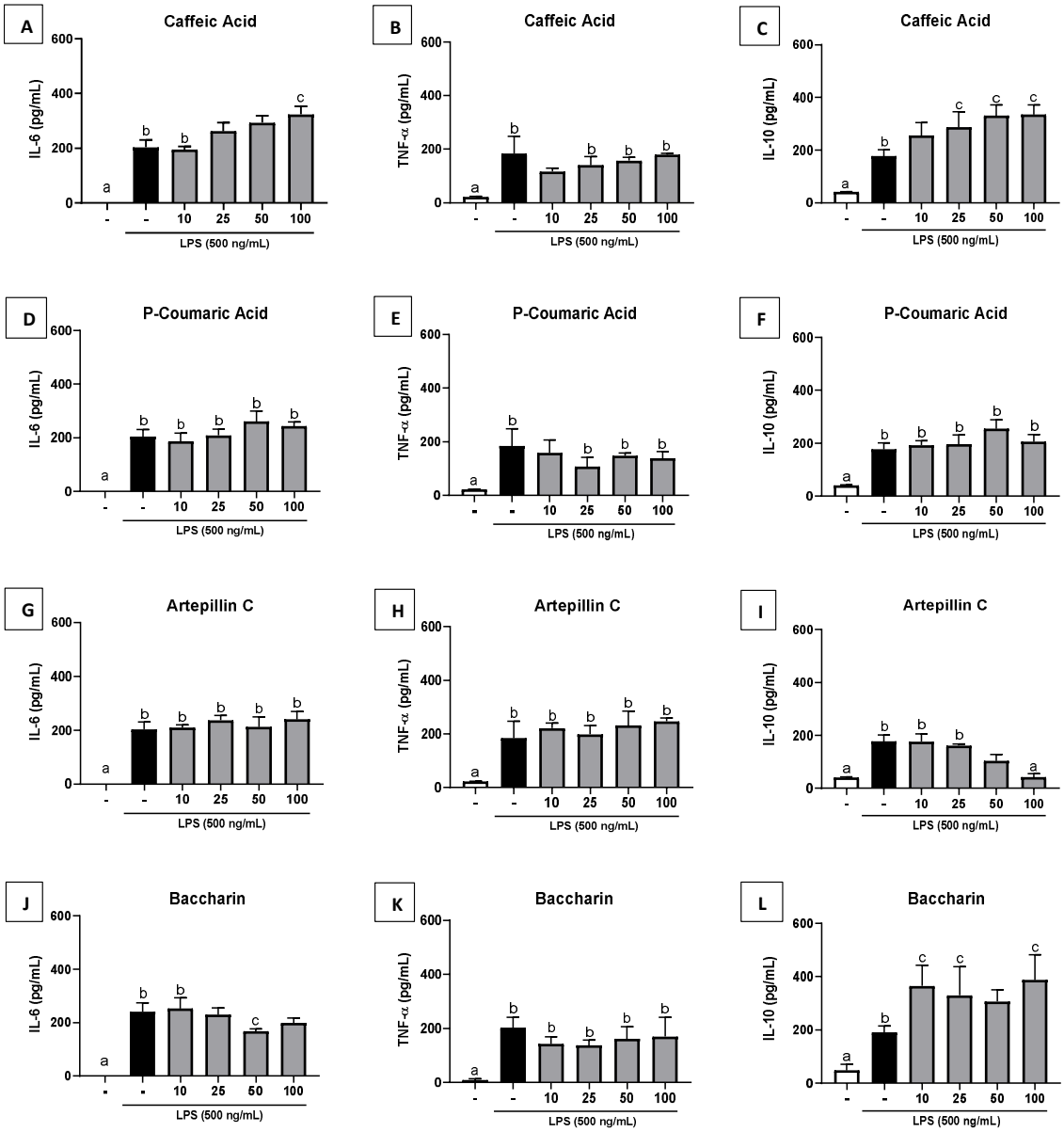

Figure 3. Effects of treatment with isolated propolis compounds: caffeic acid (**A**–**C**), *p*-coumaric acid (**D**–**F**), artepillin C (**G**–**I**) and baccharin (**J**–**L**) at different concentrations (10, 25, 50 and 100 µg/mL) on cytokine secretion (pg/mL): IL-6 (**A**,**D**,**G**,**J**), TNF-α (**B**,**E**,**H**,**K**) and IL-10 (**C**,**F**,**I**,**L**) by macrophages previously stimulated with LPS (500 ng/mL) (gray bars). The white bars represent cytokine secretion by macrophages that were not stimulated or treated with propolis, and the black bars represent macrophages that were stimulated with LPS and not treated with propolis. Significant differences ($p \leq 0.05$) are indicated by the different letters "a", "b" and "c".

3.4. Comparative Effects of Propolis Extracts and Their Isolated Compounds on IL-6, IL-10, and TNF-α Secretion by Macrophages

The overall effects of PPF did not vary with the concentrations used. These effects were similar to the effects of caffeic acid at 100 µg/mL; both PPF and caffeic acid increased IL-6 and IL-10 without significantly affecting TNF-α. The PSDE effects varied according to the extract concentrations. At the lowest concentrations (1, 10, and 25 µg/mL), its effects were similar to those produced by PPF at 1 to 300 µg/mL, and to caffeic acid at 100 µg/mL, while at higher concentrations (100 and 300 µg/mL), PSDE increased TNF-α secretion and at 300 µg/mL it decreased IL-10. The decrease in IL-10 levels was similar to that found for artepillin C at 100 µg/mL, its predominant component (Table 1, Figure 1B). The MPE effects

at 1 to 25 μg/mL were similar to those of baccharin, increasing IL-10, without affecting IL-6 and TNF-α. Baccharin was found only in this extract (Table 1, Figure 1C). Baccharin additionally decreased IL-6 at 50 μg/mL. Figure 4 gives an overview of this information through a heat map graphic presentation.

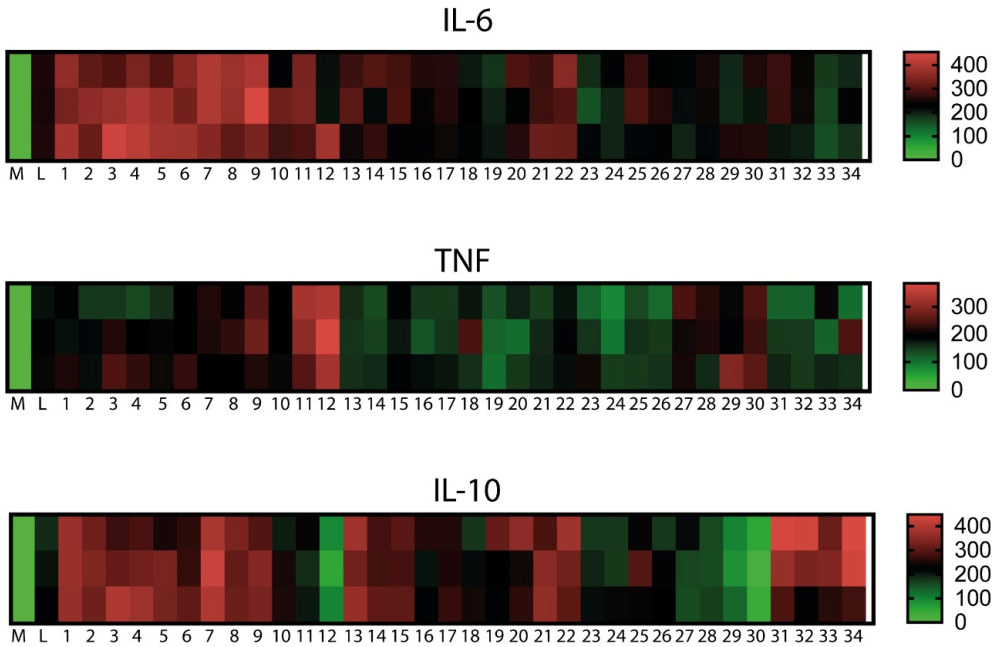

Figure 4. Cytokine concentrations (0–400 pg/mL) in triplicate (three lines) for each treatment: **M:** medium alone; **L:** after LPS (500 ng/mL) stimulation of bone marrow cell-derived macrophages; **1–6**: Polar propolis fraction (PPF) (1, 10, 25, 50, 100 and 300 μg/mL); **7–12**: Propolis soluble dry extract (PSDE) (1, 10, 25, 50, 100 and 300 μg/mL); **13–18**: Microencapsulated propolis extract (MPE) (1, 10, 25, 50, 100 e 300 μg/mL); **19–22**: Caffeic Acid (10, 25, 50 and 100 μg/mL); **23–26**: *p*-coumaric acid (10, 25, 50 and 100 μg/mL); **27–30**: Artepillin C (10, 25, 50 and 100 μg/mL) and **31–34**: Baccharin (10, 25, 50 and 100 μg/mL).

3.5. Evaluation of Cell Viability

We tested the three propolis extracts (PPF, PSDE and MPE) and several isolated propolis compounds (caffeic acid, *p*-coumaric acid, artepillin C and baccharin) for their toxicity. To do this, we performed an MTT assay on the BMDM with the same treatments used for evaluating the effects on cytokine secretion. The results showed no cytotoxicity, thus demonstrating that the cytokine results were not biased by possible toxic effects (Figure S5). Additionally, higher dosages of PSDE (100 and 300 μg/mL), MPE (300 μg/mL), caffeic acid (50 and 100 μg/mL) and *p*-coumaric acid (50 and 100 μg/mL) resulted in a significant increase in cellular viability when compared with the negative control (Figure S5, Supplementary Materials).

An increase in cellular viability with high concentrations of propolis and propolis components was also observed in several other studies [62,63], although some studies have reported cytotoxicity of propolis extracts and of isolated compounds at high concentrations [64,65]. An objective comparison between these studies concerning cytotoxicity is not possible because of differences between the methodologies employed, in the types of propolis extracts, when they were applied, and other variables. These dissimilarities in the effects of increasing concentrations of propolis and its constituents deserve further investigation.

4. Discussion

The PPF extract, which contains high levels of caffeic and *p*-coumaric acids, induced an increase in IL-10 and IL-6, without modulating TNF-α secretion by the macrophages, giving effects similar to those produced by caffeic acid and by PSDE at low concentrations (1–25 μg/mL). In some studies, propolis extracts also affected both IL-6 and IL-10, but in an opposite direction, decreasing the concentrations of both cytokines, also without affecting TNF-α [20,41], while caffeic acid caused an increase in IL-6 and IL-10 at lower concentrations (5 and 10 μg/mL) and a decrease in these cytokines at higher concentrations (50 and 100 μg/mL) [31]. IL-6 is an important pro-inflammatory cytokine that contributes to an organism's defense when tissue injury is detected by the immune system. Increases in the levels of this cytokine are a consequence of an immune system response to aggression [8,11,12]. However, persistent secretion of IL-6 by macrophages can contribute to the maintenance of an inflammatory process and eventually to chronic inflammatory disease [13]. On the other hand, IL-10 plays a role in suppressing exacerbated local inflammation, thus helping avoid chronic inflammation [14]. The simultaneous increase in IL-6 and IL-10 secretion by macrophages may be part of an organism's defense process. In this response, IL-6 participates in the stimulation of an inflammatory defense mechanism, and IL-10 prevents an exacerbated inflammatory process, resulting in a controlled inflammatory mechanism of defense [66].

In other studies, propolis decreased the levels of IL-6 and TNF-α, promoting an anti-inflammatory effect through other mediators, such as NF-kB and IL-1β [32,63–65]. A possible explanation for these differences among studies could be the parameters of the protocols used, such as timing of LPS stimulation and whether the treatment with propolis was included before or after the inflammatory stimulus. In an in vivo model, various other immunological cells and stimuli could be interacting, affecting macrophage response. Other parameters that could affect the response include propolis extract composition and concentration.

The PSDE formulation presented the same immunomodulating profile as PPF and caffeic acid, when used at low concentrations. However, at the highest concentration of PSDE (300 μg/mL), there was an increase in the TNF-α levels and a decrease in IL-10. This effect was similar to that observed for artepillin C, its main component, under the same conditions. The increase in TNF-α secretion by macrophages may be interpreted as an inflammatory effect participating in the progression to autoimmune and inflammatory chronic diseases [67]; however, this pattern is also essential for the control of infection in organisms through oxidative activation [68]. Thus, this effect may be considered an antimicrobial defense mechanism. Concerning this aspect, future investigations with PSDE and artepillin C in other models may contribute to more comprehensive conclusions. On the other hand, in another study using a macrophage model, artepillin C did not affect IL-6 or IL-10 secretion, but it decreased TNF-α levels [69]. The authors incubated the cells with artepillin C for 24 h before stimulation with LPS and IFN-γ [69]. Additionally, in this other investigation, the macrophages used were RAW264.7, an immortalized macrophage type, in which some signaling pathways can be altered due to the immortalization process. Of note is the fact that our results are based on a model of primary and non-immortalized cells, which guarantees greater proximity of the results with those found in an in vivo model. These comparisons show that differences between methods can result in different profiles of effects, such as the lack of effect of *p*-coumaric acid on the secretion of cytokines that we found. In other studies, *p*-coumaric affected macrophage IL-6 secretion only when it was applied after stimulation with LPS [31].

In our investigation, microencapsulated EPP-AF® (MPE) increased IL-10 levels without affecting the pro-inflammatory cytokines IL-6 or TNF-α. These activities were not observed for caffeic acid, *p*-coumaric acid or artepillin C. This profile was similar to that observed for baccharin. Baccharin produced effects similar to those of MPE, also decreasing IL-6 levels. This effect is considered characteristic of an anti-inflammatory profile. In an animal model of acute inflammation, Ferreira et al. [26] reported anti-inflammatory effects of baccharin due to decreases in pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β) and increased

IL-10, in addition to decreasing neutrophil and lymphocyte recruitment to the inflammation site. Baccharin had anti-mutagenic, antioxidant, and neurotrophic effects in vitro [70,71]. It also had a strong hypoglycemic effect in animal models of type 2 diabetes [72].

Brazilian propolis extracts can have anti-inflammatory, pro-inflammatory, or immunoregulatory effects on the immune system. The effects which predominate generally depend on the extract concentration [73–79]. In a study by Hori et al. [32], Brazilian green propolis EPP-AF® produced an anti-inflammatory effect at lower concentrations (30 and 100 μg/mL) and a cytotoxic effect at a higher concentration (300 μg/mL). Búfalo et al. [80] reported that in human monocytes, propolis extract produced an anti-inflammatory effect, with increased IL-10 secretion at lower concentrations (5 and 10 μg/mL); however, at higher concentrations (50 and 100 μg/mL), the extract decreased IL-10 secretion and had a fungicidal effect.

Our study had some limitations, since it evaluated only three cytokines, with only a single treatment protocol (twenty hours of treatment after two hours of LPS stimulation). Further investigations should be carried out with other models and protocols to better understand how propolis impacts the immune response. However, the data obtained was sufficient to demonstrate that different extract compositions, concentrations, and presentations can affect their effects on immune cells.

These three different propolis extract presentations that were produced from the same Brazilian green propolis blend have different physical–chemical characteristics, chemical content and biological properties, which have the potential for use in various types of products, including foods, food supplements, and medicines, as well as hygiene and cosmetic products, with differences and advantages depending on the type of use, physical aspect and formulation compatibilities. Detailed physical–chemical, physical, macro and microscopic aspects of these three presentations are available [53]; this type of information can be useful for selecting the best option for each kind of formulation.

Despite the limitations of our study, our results contribute to knowledge about the anti-inflammatory and immunomodulation properties of propolis, confirming its potential for helping to treat chronic inflammatory diseases. Our findings emphasize the importance of an adequate characterization and testing of each type of extract to better understand their immunomodulatory properties and to determine the most appropriate options for specific applications.

5. Conclusions

We investigated the effects of three different recently developed propolis extracts (PPF, PSDE, and MPE) and several propolis components on pro- and anti-inflammatory cytokine production in a macrophage model. Each extract formulation produced a distinct response profile. PPF produced effects similar to those of caffeic acid, which could be interpreted as a controlled pro-inflammatory response. PSDE had a similar effect when used at low concentrations; however, at higher concentrations, this extract produced a pro-inflammatory effect similar to that of artepillin C. MPE, similar to baccharin, had an anti-inflammatory effect. The results show that cytokine production stimulated by propolis extracts can vary according to the manufacturing process and the concentration of propolis extracts that are tested. The diverse range of effects on cytokine production exhibited by these propolis extracts has the potential for products that are tailored to different specific health conditions.

6. Patents

Patent requests for PPF, PSDE and MPE are under preparation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app13106247/s1, Figure S1: Chromatographic profile of the three samples of propolis raw material used to produce the three extracts: (A) Propolis raw material sample used to produce the polar propolis fraction (PPF), (B) Alcoholic extract used to produce propolis soluble dry extract (PSDE), (C) Alcoholic extract used to produce microencapsulated propolis extract (MPE);

Figure S2: Calibration curve for A) gallic acid (GAE) and B) quercetin (QUE); Figure S3: (A) ^{1}H NMR spectrum (400 MHz, CDCl3) of baccharin and (B) ^{13}C NMR spectrum (400 MHz, CDCl3) of baccharin; Figure S4: Chromatographic profile of Baccharin; Figure S5: Effects of the propolis extracts on cellular viability of macrophages previously stimulated with LPS.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Ribeirão Preto Medical School of the University of São Paulo (031/2017).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Sample Availability: Samples of the compounds were adequately stored and are available from the authors.

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