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In vitro and in vivo anti-inflammatory properties of Mayan propolis

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Abstract

Introduction Propolis has been used traditionally for different human diseases and even recently as dental biomaterials because of its antibacterial, antimycotic, and anti-inflammatory properties. However, a proper correlation between in vitro and in vivo anti-inflammatory properties has not been clearly established.

Methods The composition of propolis was determined by high-performance liquid chromatography–ultraviolet mass spectrometry (HPLC-UV-MS). Viability of ethanolic propolis solution was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay on murine macrophages. The anti-inflammatory properties were assessed both in vitro through the enzyme-linked immunosorbent assay (ELISA) quantification of various cytokines and in vivo by induced edemas.

Results Chemical analysis showed pinocembrin, pinobanksin-3-O-acetate, and pinobanksin-3-O-propionate as the main components of propolis. Macrophage viability was high (106%) when propolis was used up to 50 µg/mL. ELISA studies showed a reduction in the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) up to 145 pg/mL, 350 pg/mL, and 210 pg/mL, respectively, while the anti-inflammatory cytokines (IL-10 and IL-4) were increased up to 833 pg/mL and 446 pg/mL. Finally, edema was reduced on paw and ear mice by 9% and 22%, respectively.

Conclusion Mayan propolis has strong in vitro anti-inflammatory properties without compromising macrophage viability, resulting in a low-to-mild in vivo anti-inflammatory response.

Keywords

Anti-inflammatory, cytokines, macrophages, propolis

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Introduction

The mixture known as "iits yik'el kaab" in Mayan language is a "bee resin" better known as propolis with well-documented antibacterial, antimycotic, and anti-inflammatory properties. This is a resinous mixture that honey bees produce by mixing their saliva containing enzymes and beeswax with exudates gathered mainly from leaf and flower buds, stems, and bark cracks of numerous species of trees.[1,2] Chemical composition of propolis is complex, and so far more than 300 compounds

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have been identified.[3] However, many studies showed that the effects of propolis might be the result of synergistic action of its complex constituents.[4,5] In addition, this chemical heterogeneity can be related to the geographic diversity of plant sources and bee species.[6,7] For example, European propolis contains flavonoid aglycones, phenolic acids, and their esters. The main components of Cuban propolis are polyisoprenylated benzophenones. From Chilean propolis were identified phenylpropane, benzaldehyde, dehydrobenzofuran, or benzopyran compounds. In Brazilian propolis, prenylated derivatives of p-coumaric acid, acetophenone, diterpenes, lignins, and flavonoids were found.[8] Mexican propolis showed hypoglycaemic, anti-oxidant, and anti-inflammatory properties, which is attributed to naringin, naringenin, kaempferol, quercetin, acacetin, luteolin, pinocembrin, chrysin, epoxypinocembrin chalcone, and an ε-caprolactone derivative, as well as pinostrobin, izalpinin, cinnamic acid, pinocembrin, and 3,3-dimethylallyl caffeate in a mixture with isopent-3-enyl caffeate, 3,4-dimethoxycinnamic acid, rhamnetin, and caffeic acid.[8,9] Flavonoids such as chrysin and kaempferol have been identified as responsible for the anti-allergic effect of Chinese propolis.[10,11]

From the former argument, it is not clear if a single compound or a synergistic mixture is responsible for the anti-inflammatory properties of propolis. It has been reported that pinostrobin and quercetin, two types of flavanoids, exhibit an anti-inflammatory effect by reducing the presence of important pro-inflammatory cytokines.[12,13] In this context, in vitro inflammatory assay with Chinese propolis has been reported to reduce of IL-1β and IL-6 pro-inflammatory cytokines.[14] In fact, an in vivo inflammatory study with Bulgarian and Chilean propolis has been reported to inhibit ear-edema due to their phenolic and flavonoids content.[15,16]

In traditional medicine, propolis is used for the empirical treatment of diabetes mellitus, gastrointestinal disorder, and infectious diseases, but recently, it has also been used in dental materials because of its antibacterial properties.[10,17] However, in the oral environment, there is a plethora of bacteria, to which propolis showed various degrees of antibacterial activities depending on the source of propolis, concentration, solvent, bacterial strain, type of dental biomaterial, and so forth. Despite this, little evidence has been provided in relation to cell cytotoxicity of propolis and its anti-inflammatory properties. The later is of prime importance, as propolis-modified dental materials can exhibit not only antibacterial behavior but also an accelerated wound healing, as tissue repair is mediated by their macrophage inflammatory response. Therefore, in this study, macrophage viability was assessed by means of a thiazolyl blue tetrazolium bromide (MTT) assay and then cytokine production was estimated by the enzyme-linked immunosorbent assay (ELISA) test in the presence of various propolis concentrations. In addition, the in vivo anti-inflammatory response on induced paw edema and induced ear edema is reported.

Materials and methods

Natural and chemical products

Yucamiel propolis paste was used for all experiments. HPLC-MS grade methanol (MeOH), acetonitrile (ACN), and water (H_2O) from J. T. Baker and reagent grade formic acid (95%) were employed to acidify the mobile phase for chromatography experiments. Ethanol (95%), dexamethasone, trypan-blue dye, dimethyl sulfoxide (DMSO), lipopolysaccharides (LPS) from *Escherichia coli* (0111: B4), MTT, carrageenan, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), acetone, and indomethacin were purchased from Sigma Aldrich® (MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Invitrogen Gibco-BRL® (NY, USA). Murine IL-1β ELISA development kit (900-K47), murine IL-4 ELISA development kit (900-K49), murine IL-6 ELISA development kit (900-K50), murine IL-10 ELISA development kit (900-K53), and murine TNF-α ELISA development kit (900-K54) were obtained from Peprotech® (London, UK).

Animals

Balb/c male mice (6–8 weeks of age and 20 ± 5 g weight) were obtained from Centro de Investigaciones Regionales (CIR) "Dr. Hideyo Noguchi" from Universidad Autónoma de Yucatán (UADY). The animals were maintained according to the principles and guidelines of National

Institutes of Health (NIH) Guide for Treatment and Care for Laboratory Animals and by Official Mexican Standard (NOM-062-ZOO-1999). The animals were housed in standard polypropylene cages under standard laboratory conditions with access to special food and purified water ad libitum, pathogen- and stress-free environment, a temperature of 22 ± 2°C, and a controlled room with a 12 h light/dark cycle.

High-performance liquid chromatography–ultraviolet mass spectrometry analysis

The ethanolic extract of propolis 1 mg/mL was studied by high-performance liquid chromatography–ultraviolet mass spectrometry (HPLC–UV-MS) in order to identify some of its components. For analysis, a quaternary pump (Agilent Technologies 1290-series, Agilent, San Jose, CA, USA) coupled to a UV diode array detector (DAD) and a QqQ mass spectrometer (Agilent Technologies 6470, Agilent, San Jose, CA, USA) equipped with an JetStream-ESI source (operated in negative mode) was used. The QqQ mass spectrometry parameters were set as follows: Nebulizer to 40 psi; drying gas flow to 13 L/min; temperature to 350 °C; and capillary voltage to 3,000 V. Spectra were recorded in negative-ion mode between m/z 100 and 3,000, and UV-DAD spectral data were plotted at 290 nm. The LC–MS system was equipped with a Zorbax Poroshell 120 XDB-C18 column (150 mm × 4.6 mm, internal diameter 5.0 μm; Agilent, USA). Chromatography was performed under gradient conditions with H_20 (0.1 % v/v formic acid) and MeOH: ACN (1:1, v/v) with a flow rate of 600 uL/min and injecting 10 uL of the sample. All signals have the maximum absorption at 280 nm, and the relative concentration from each signal was calculated using naringenin at 100 μg/mL as internal standard (ISTD).

Isolation of peritoneal murine macrophages

The isolation of peritoneal murine macrophages was carried out according to the work by Arana-Argaez et al.[18] Briefly, Balb/c mice were sacrificed by the method of cervical dislocation, and then 10 mL of cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity. The macrophages were collected by aspiration of the peritoneal fluid and centrifugation for 10 min at 3,000 rpm and 4°C. The cells were washed two times with cold PBS for 5 min (2,000 rpm and 4°C) and re-suspended in supplemented DMEM media with 10% FBS and 1% penicillin–streptomycin. The number of macrophages was determined in a hemocytometer by the trypan-blue dye exclusion method ($\geqslant 95\%$), and 2×10^4 cells (200 μL/well) were seeded in each well of a 96-well plate for cell viability assay, while 1×10^5 cells (500 μL/well) were placed in each well of a 24-well plate for cytokines assay. The plates were placed in the humidified incubator for 72 h (37°C and 5% CO_2).

Viability assay

After removing the non-adherent cells, peritoneal murine macrophages from the 96-well plates were treated with a propolis solution at 10, 25, 50, and 100 μg/mL dissolved in 0.1% (v/v) of ethanol and supplemented with culture media DMEM. These cells were treated with 100% DMSO as cytotoxicity control and without treatment as viability control. Then, the 96-well plates were incubated for 24 h (37°C and 5% CO_2).

MTT assay was determined by the method reported by Mosmann.[19] The supernatants were removed by aspiration on the cells isolated previously, and then, cells were treated with 200 μL of culture media with 0.5 mg/mL of MTT or without cells as blank. The plates were incubated for 4 h (37°C and 5% CO_2), the supernatants were removed, and 100 μL of 100% DMSO was added to dissolve formazan crystals. Absorbance at 492 nm was measured using a Bio-Rad® iMark microplate reader. The cell viability percentage (%CV) was calculated as reported by[20]

$$\%CV = [(Abs_x - Abs_b) / (Abs_c - Abs_b)] \times 100$$

where Abs_x is the absorbance of treatments, Abs_c is the absorbance of viability control, and Abs_b is the absorbance of blank.

Cytokine quantification

Treatment and activation of macrophages. Macrophages were treated with various propolis concentrations including 5, 10, 25, 50, 100, 150, and 250 μg/mL on 24-well plates, with 0.25% (v/v) of ethanol as final concentration in the experiments. Cells treated

with LPS (1 μg/mL) and dexamethasone (10 μM) were used as pro-inflammatory and anti-inflammatory controls, respectively, while non-treated cells were used as negative control. The 24-well plates were incubated for 24 h (37°C and 5% CO_2). Then, macrophages were activated with LPS at 1 μg/mL in supplemented DMEM media and incubated for 48 h (37°C and 5% CO_2). Finally, the supernatants were collected and stored at –20°C.

Determination of cytokines. The measurement of cytokines production (IL-1β, IL-4, IL-6, IL-10, and TNF-α) was conducted following the manufacturer's instructions using commercial ELISA kits (Peprotech®, London, UK). A capture antibody was employed at 1 μg/mL for IL-4 or TNF-α and 2 μg/mL for IL-1β, IL-6, or IL-10. Serial dilutions of recombinant cytokines were used as the standard curve (0–2,000 pg/mL for IL-4 or TNF-α, 0–3,000 pg/mL for IL-10, and 0–4,000 pg/mL for IL-1β or IL-6). For Cytokines quantification, 100 μL of macrophage supernatants were incubated with antibody detection at 0.25 μg/mL for TNF-α, 0.5 μg/mL for IL-1β, IL-6, or IL-10, and 1 μg/mL for IL-4, as well as avidin-peroxidase and 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS). The absorbance was measured at 490 nm using the microplate reader, as mentioned above. The concentrations (x) of cytokines were calculated by interpolation of absorbance (Y) on linear regression of the corresponding standard curve.

$$x = (Y - b) / m$$

where Y is the absorbance of treatments, b is the intercept of linear regression, and m is the slope of the line in linear regression.

Edema induction

Preparation of samples. The calculation of the sample size (five animals per group) in the study was based on the expected attrition or death of animals and was calculated according to the formula[21]

$$\text{Corrected sample size} = \text{Sample size} / 0.9$$

where Sample size is the number of animals per group, 0.9 is the result obtained from the operation (1 − (% attrition/100)), while % attrition in previous operation is the expecting of 10% attrition in the sample size.

The experimental group was treated with 100 μL of the propolis solution at 50 mg/kg dissolved in 3% of ethanol and 0.9% physiological saline solution (PSS). The positive anti-inflammatory control group received 100 μL of indomethacin (10 mg/kg) in 0.9% PSS, while the negative anti-inflammatory control group received only 100 μL of 0.9% PSS. The samples were administered intra-gastrically for 5 days to all groups.

Carrageenan-induced paw edema. The paw edema was induced according to the work by Villa-de la Torre et al.[22] After 1 h on the last day of treatment, the subplantar tissue of the right hind paw of each animal was injected with 50 μL of 10 mg/mL carrageenan solution in 0.9% PSS. The right hind paw thickness was measured at 1, 2, and 3 h after induction. The inhibition percentage of edema (%IPE) was determined as follows[23]

$$\%IPE = [(1 - ET_x) / ET_C] \times 100$$

where ET_x is the edema thickness of treatments and ET_C is the edema thickness of negative anti-inflammatory control.

TPA-induced ear edema. The ear edema was induced following the method proposed by Villa-de la Torre et al.[22] After 1 h on the last day of administration, the inner and outer surfaces of the right ear of each mouse were treated by the topical application of a TPA solution (0.1 μg/μL in acetone), 10 μL per side, after ear edema induction thickness was measured at 1, 2, and 3 h. The %IPE was calculated as mentioned above.

Statistical analysis

The MTT and cytokine results were expressed as mean ± standard deviation (SD) of three independent experiments of every treatment to document a variation between data. The in vitro values were compared by one-way analysis of variance (ANOVA) with Dunnett's post hoc test. The in vivo results were expressed as mean ± standard error (SEM) of five independent assay per group to report of data variation on representative samples. The values were compared by two-way ANOVA with Dunnett's post hoc test. Levels of $p < 0.05$

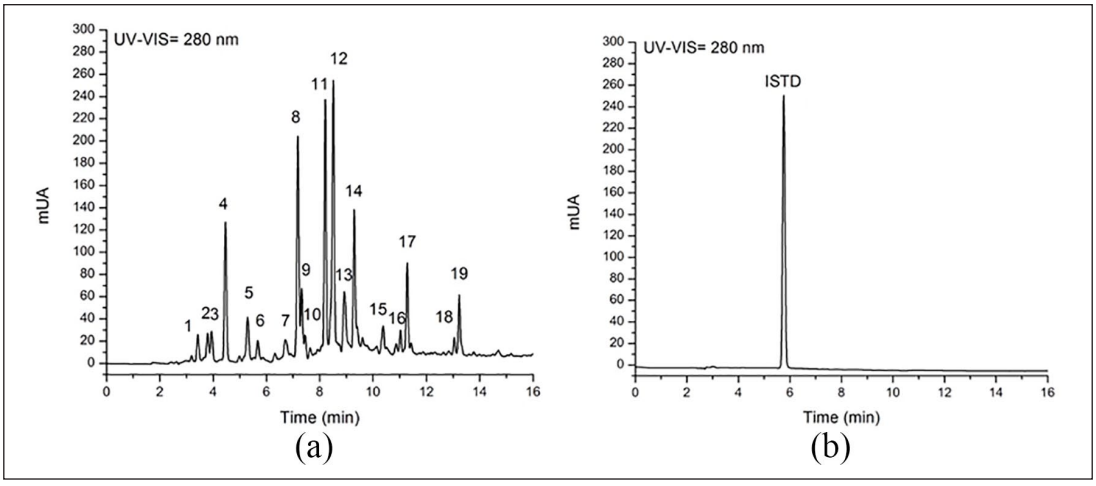

Figure 1. Fingerprint signals of propolis ethanolic extract (a) naringenin (b) as Internal Standard, at detection wavelength to 280 nm.

were used as a criterion of statistical significance in contrast with its respective control group in all assays. The calculations were done using GraphPad Prism® V7.00 software (GraphPad Software Inc., CA, USA).

Results

Propolis properties and chromatographic profile

Yucamiel propolis paste showed a flavonoid total content of 25.94 ± 2.06 mg quercetin/g propolis, phenol total content of 49.68 ± 0.29 mg galic acid/g propolis, 2.5 μg/mL of average inhibitory concentration (IC_{50}), and antiradical power of 0.40.

The fingerprint analysis of propolis showed 19 signals (Figure 1) plotted at 280 nm. The first three signals were attributed to a derivate of the chlorogenic acid and caffeic acid. Signal four suggests that this analyte is coumaric acid. The following signals (5 to 10) showed as apigenin, pinocembrim, galangin, hesperetin, kaempferol, and derivate flavonoid compounds. Signals from 11 to 15 corresponded to molecules such as acetylated flavonoids including pinobanksin-3-O-acetate and their derivatives. Finally, the last signals (16 to 19) were assigned to artepillin C, which has a significant antioxidant and anti-inflammatory potential.[24,25] All product ion m/z values are documented in Table 1. In fact, the signals with more relative concentrations were signal 8 (pinocembrin), 11 (pinobanksin-3-O-acetate), and 12 (pinobanksin-3-O-propionate).

Macrophage viability

Viability of macrophages did not change between 10 and 25 μg/mL, but slightly increased to 106% and 108% for 50 and 100 μg/mL of propolis, respectively. Although maximum macrophage viability (108%) was achieved with 100 μg/mL of propolis, this was not statistically different regarding the control (Figure 2). Overall, macrophage viability suggests that a concentration higher than 50 μg/mL of propolis allows cell culture and the quantification of other parameters such as cytokine production. A previous work has reported that between 2 and 20 μg/mL of propolis did not change monocyte viability.[24]

In vitro inflammatory response

The effect of propolis on the secretion of pro-inflammatory IL-1β, IL-6, and TNF-α cytokines is shown in Figure 3(a–c). For propolis concentrations from 5 to 250 μg/mL, these treatments yielded 1335 to 145 pg/mL of IL-1β (Figure 3(a)). The highest IL-1β production (1335 pg/mL) was observed at 10 μg/mL of resin, while between 25 and 100 μg/mL of propolis, IL-1β secretion was reduced by more than 50%, with the lowest secretion (145 pg/mL) at 100 μg/mL of propolis (Figure 3(a)). In this case, LPS induced 1270 pg/mL, while dexamethasone stimulus yielded 295 pg/mL. In all cases, IL-1β had statistical significance regarding control (LPS) except for 5 and 10 μg/mL of propolis.

Table 1. The results of relative retention time and relative concentration in fingerprint analyses of ethanolic extract of propolis.

Sig.	Rt (min)	Relative Rt	Area (mUA)	Relative area	Relative concentration (ug/mL)	Precursor ion $(m/z)^-$	Product ion $(m/z)^-$
1	3.422	0.594	161.9	0.096	9.616	353	161/135
2	3.791	0.658	160.3	0.095	9.521	179	161/135
3	3.94	0.684	179.9	0.107	10.685	179	161/135
4	4.461	0.774	719.2	0.427	42.718	163	119
5	5.293	0.919	307.3	0.183	18.253	270	151
6	5.676	0.985	135.1	0.080	8.024	301	151
ISTD	5.761	1.000	1683.6	1.000	100.000	271	151
7	6.709	1.165	234.7	0.139	13.940	285	151
8	7.178	1.246	1147.3	0.681	68.146	255	151
9	7.324	1.271	361.2	0.215	21.454	269	151
10	7.45	1.293	130.6	0.078	7.757	301	151
11	8.205	1.424	1260.3	0.749	74.857	313	253
12	8.506	1.476	1630.2	0.968	96.828	328	253
13	8.924	1.549	662.1	0.393	39.326	341	253
14	9.295	1.613	775.7	0.461	46.074	356	253
15	10.379	1.802	273.6	0.163	16.251	370	253
16	11.029	1.914	177.4	0.105	10.537	299	255
17	11.28	1.958	535.7	0.318	31.819	321	255
18	13.052	2.266	141.3	0.084	8.393	327	255
19	13.236	2.298	421.6	0.250	25.042	339	255

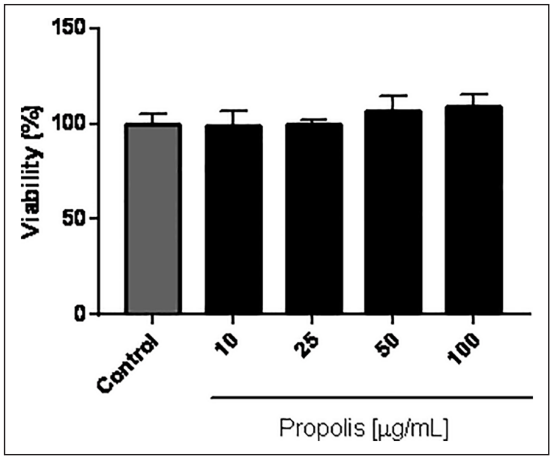

Figure 2. Viability dependence on propolis concentration for macrophages. Control: no treatments. Values were expressed as means ± SD (n = 3). The *$p < 0.05$ represent statistically significant data.

For the same propolis concentrations, IL-6 secretion ranged from 930 to 350 pg/mL, as shown in Figure 3(b). In fact, IL-6 production was lower than that of IL-1β, being 930 pg/mL, the maximum detected at 50 μg/mL, while the lowest secretion, 350 pg/mL, quantified at 250 μg/mL of resin, as shown in Figure 3(b). For IL-6, LPS induced 1100 pg/mL, while dexamethasone stimulus yielded 480 pg/mL. In all cases, IL-6 had statistical significance regarding control.

Finally, propolis reduced TNF-α secretion from 691 to 210 pg/mL for concentrations of 5–250 μg/mL (Figure 3(c)). The highest TNF-α production was 691 pg/mL, achieved at 10 μg/mL, while the lowest, 210 pg/mL, was observed at 150 μg/mL of propolis (Figure 3(c)). TNF-α was statistically significant regarding control, except for the 5–25 μg/mL range. For TNF-α, LPS induced 741 pg/mL, while dexamethasone stimulus yielded 266 pg/mL.

According to these results, it was observed that between 100 and 250 μg/mL is the optimal concentration to decrease cytokines IL-1β, IL-6, and TNF-α. In the case of IL-1β and TNF-α, after its optimal concentration, propolis did not have additional benefits as shown by these in vitro inflammation tests. These results suggest that propolis exhibits reduced pro-inflammatory cytokine production, and this was demonstrated by anti-inflammatory cytokine production including IL-10 and IL-4.

Propolis at concentrations between 5 and 250 μg/mL induced 266 to 833 pg/mL of IL-10 secretion, respectively (Figure 4(a)). In this case, the highest IL-10 production (833 pg/mL) was observed at 50 μg/mL of propolis. Dexamethasone, a well-known anti-inflammatory, induced 1183 pg/mL of IL-10, whereas LPS stimulated only 166 pg/mL (Figure 4(a)).

In the same manner, propolis induced 215 to 446 pg/mL of IL-4 secretion for the previously

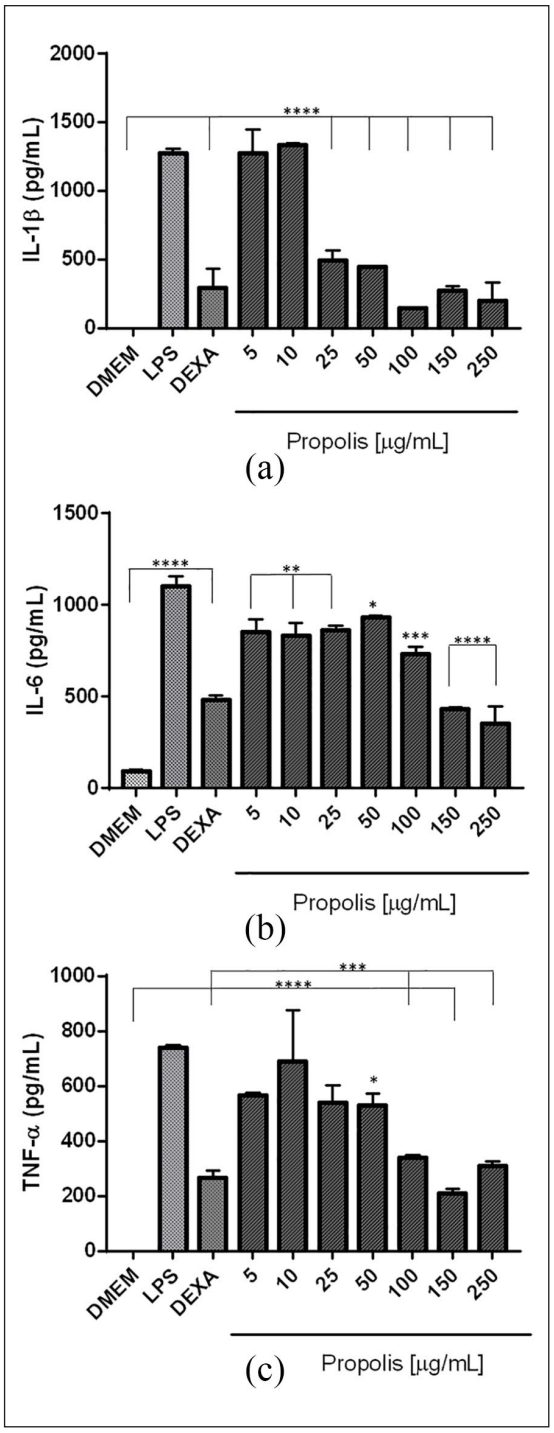

Figure 3. Pro-inflammatory secretion of cytokines in response to different concentrations of propolis. Secretion of IL-1β (a), IL-6 (b), and TNF-α (c).
DMEM: Dulbecco's modified Eagle's medium. LPS: Lipopolysaccharides. DEXA: Dexamethasone. Results were expressed as means ± SD (n = 3 biological repetitions). According to the ANOVA test followed by Dunnett post hoc tests, (*$p < 0.05$), (**$p < 0.01$), (***$p < 0.001$), and (****$p < 0.0001$).

mentioned concentrations (Figure 4(b)). The data showed that IL-4 behaves similarly to IL-10, as the maximum amount (446 pg/mL) was produced at 50 μg/mL of propolis. In this case, dexamethasone induced 683 pg/mL of IL-4, whereas LPS stimulated only 227 pg/mL (Figure 4(b)). All cases had statistical significance regarding control (DEXA).

This part of the study showed that 50 μg/mL of propolis is the optimal concentration to secrete anti-inflammatory cytokines (IL-10 and IL-4), and that after 50 μg/mL of resin, the production of both cytokines tends to diminish. Interestingly, the in vitro pro-inflammatory and anti-inflammatory studies correlate well after treatment with 50 μg/mL of resin (Figures 3 and 4) and correlate well with high macrophage viability (Figure 2). Previous works have reported that between 2 and 20 μg/mL of propolis decreased pro-inflammatory cytokines, while increasing anti-inflammatory cytokine production. This behavior was also confirmed with in vivo studies.[26,27]

In vivo inflammatory assay with propolis

The percentage of swelling inhibition is shown in Figure 5. Indomethacin (C +) inhibited between 9% and 26% of paw edema in the 1 h to3 h range. These results showed that indomethacin showed high inhibition activity (26% of paw edema) in the first 2 hours and that this effect was reduced at 3 h (Figure 5(a)). For the same period, propolis inhibited 6% to 9% of paw edema with better activity during the first hour (Figure 5(a)).

For TPA experiments, indomethacin inhibited 84% to 91% of ear edema in the same time but showed improved activity after 2 hours (Figure 5(b)). In contrast, the bee resin inhibited 4% to 22% of ear edema; the maximum of 22% was observed during the first 2 hours, and then, this effect decreased at 3 h.

This study showed the inhibition of edemas at short times and confirmed the anti-inflammatory effect of propolis when using 50 mg/kg doses. Both studies in vitro and in vivo showed anti-inflammatory properties of propolis when used at a concentration of 50 μg/mL or 50 mg/kg. This is different with previous works in rats where a dose of 500 mg/kg of propolis reduced 65% paw swelling at 2 h, while the topical propolis model on TPA-induced edema reduced 50% of swelling at the same conditions.[24]

Discussion

At first glance, anti-inflammatory properties of Yucamiel propolis can be attributed to their antioxidant potential as shown by their flavonoid and

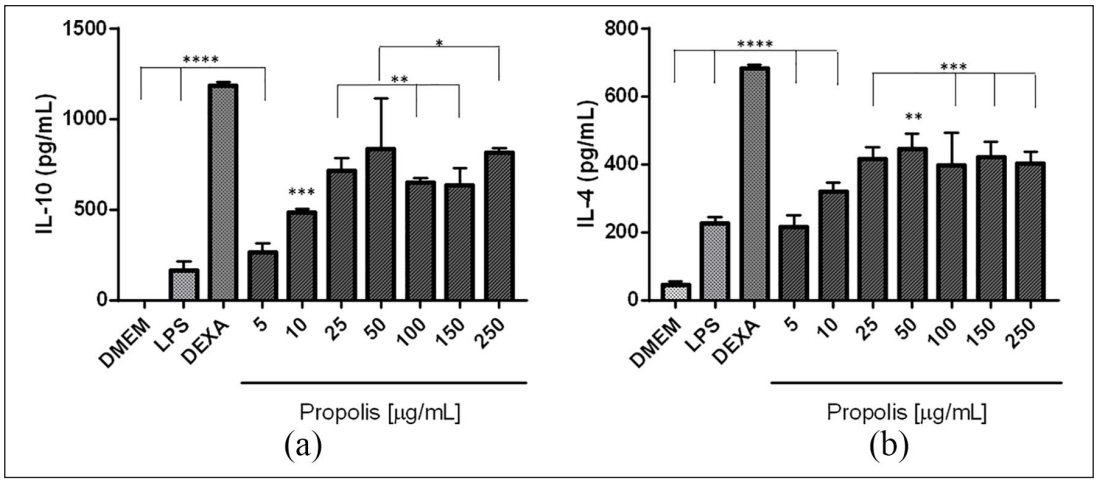

Figure 4. Anti-inflammatory secretion of cytokines in response to different concentrations of propolis. Secretion of IL-10 (a) and IL-4 (b).
DMEM: Dulbecco's modified Eagle's medium. LPS: Lipopolysaccharides DEXA: Dexamethasone. Data were expressed as means ± SD (n = 3 independent studies). According to ANOVA test followed by Dunnett post hoc tests (*$p < 0.05$), (**$p < 0.01$), (***$p < 0.001$) and (****$p < 0.0001$).

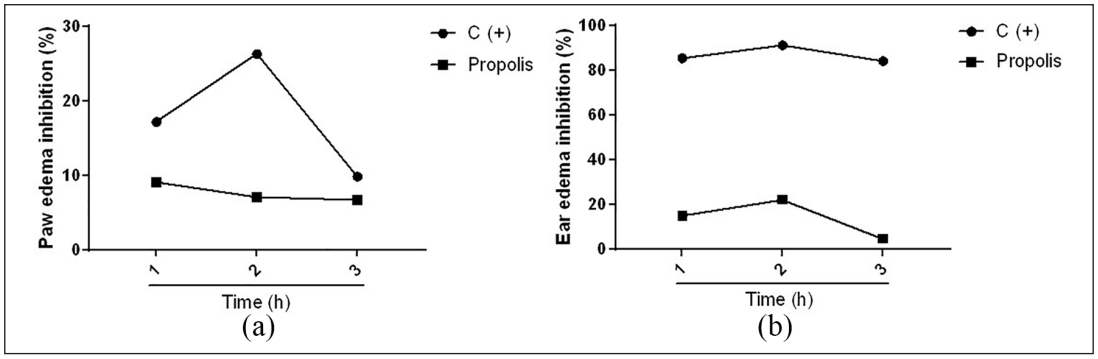

Figure 5. Percentage of propolis inhibition on edema induction. Paw edema inhibition (a) and ear edema inhibition (b) at different times after propolis treatment. C (+): Indomethacin. Data were expressed as means ± SD (n = 5 biological repetitions).

phenol content in addition to is radical scavenging activity. However, it is not clear if the effect is due to a single compound or due to the synergic effect of various chemicals found in their composition. For example, pinostrobin, quercetin (flavonoids or flavanones), and artepillin C, pinocembrin (a phenolic compound), isolated from bee resin, exhibited an anti-inflammatory effect.[12,13,28,29] In contrast, some works have reported synergistic action of the chemical compounds found in propolis.[4,5] In this regard, it has been suggested that caffeic acid and particularly artepillin C play a synergistic role in anti-inflammatory action with propolis flavonoids.[30] The chromatographic profile of the propolis used in our study showed different compounds, and according to their relative amount, it is hypothesized that coumaric acid, pinocembrin, pinobanksin-3-O-acetate, pinobanksin-3-O-propionate, and artepillin C derivates are responsible for this effect (see Figure 1 and Table 1). In this regard, the present study is limited, as it was not able to demonstrate which compound and which concentration are suitable for a proper anti-inflammatory response. Future works are also recommended to isolate each compound from Yucamiel propolis and establish its synergistic effect.

The common pro-inflammatory response of macrophages is associated with IL-1β, TNF-α, IL-6, IL-15, IL-17, and IL-18, while the anti-inflammatory response is associated with IL-4, IL-10, and IL-13.[31] However, their role is more complex, as they are involved in different biochemical processes. It is well known that macrophages secreted IL-1β as an enhanced inflammatory response and defense mechanism, but they also contribute to pro-inflammatory angiogenesis and regeneration of vascular

tissue.[32] On the other hand, IL-6 has been involved in the defense mechanism, hematopoiesis, chronic inflammation, and autoimmunity.[33] In fact, TNF-α has the ability to induce apoptosis, cachexia, defense mechanism against pathogens, and pro-inflammatory angiogenesis.[34] However, IL-4 induces proliferation, differentiation, apoptosis, and defense mechanism and has also been associated with allergy, autoimmunity, and cancer.[35] Finally, IL-10 exhibits activity on natural killer cells to destroy pathogens, regeneration of vascular tissue, and inhibition of pro-inflammatory cytokines.[36]

Our results showed the in vitro anti-inflammatory activity of propolis, as the levels of pro-inflammatory IL-1β, IL-6, and TNF-α were low, while the levels of IL-10 and IL-4 were high but still below that of dexamethasone. This is in agreement with recent studies which showed the ability of propolis to reduce pro-inflammatory cytokines or to increment the anti-inflammatory cytokines.[25,29,37] However, it was also clear that this response was dose-dependent for the pro-inflammatory cytokines, as concentrations higher than 100 μg/mL of propolis were needed to inhibit cytokine production. In contrast, a concentration of 50 μg/mL of Yucamiel propolis was enough for high levels of IL-10 and IL-4 production.

It was also demonstrated that macrophage viability was maintained at 50 μg/mL of propolis without sacrifying cytokine production. However, it should be noted that the same macrophages were not used for MTT and cytokine assay, limiting the scope of the study. In agreement with high macrophage viability, propolis also showed 76% viability with peripheral blood mononuclear cells (data not shown). In fact, this is similar to previous studies where it was reported that between 2 and 50 μg/mL of resin maintained monocytes' and macrophages' survival around 100%.[25,26] In this regard, a previous work showed that propolis can increase the production of hydrogen peroxide, suggesting that this product modulated the activation of macrophages and their mediators such as cytokines.[27] Besides this, in an in vivo model of chronic inflammation, it was also demonstrated that propolis did not compromise collagen deposition.[27]

The in vivo results, however, showed a low to moderate anti-inflammatory response at short times (2 h). This suggests that the route or mechanism of action of propolis followed using an animal model (murine in this case) is not necessarily the same for the in vitro experiments. Therefore, it is recommended to use higher concentrations of Yucamiel for the in vivo experiments not only for an improved anti-inflammatory response but also to assess other biochemical clues related to tissue repair.

Conclusion

These studies showed that propolis can be considered as a non-cytotoxic natural material, as it did not change macrophage viability. At 50 μg/mL of propolis, macrophage viability was 106%, which is higher than that recommended by ISO 10993-5 standard. Furthermore, at this concentration, two well-known anti-inflammatory cytokines, IL-10 (833 pg/mL) and IL-4 (446 pg/mL), reached a maximum, confirming their anti-inflammatory properties. However, the in vivo experiments showed a low to moderate inhibition of both paw (9%) and ear (22%) edemas before 2 h. The chemical compounds responsible for this behavior are coumaric acid, pinocembrin, pinobanksin-3-O-acetate, pinobanksin-3-O-propionate, pinobanksin derivatives, and artepillin C derivatives, as they were the main components of Mayan propolis. In conclusion, a low propolis dose correlates well with both high macrophage viability and in vitro production of IL-10 and IL-4 but a low to moderate in vivo anti-inflammatory response.

Animal welfare

The animals were maintained according the principles and guidelines of the National Institute of Health (NIH) Guide for Treatment and Care for Laboratory Animals and by Official Mexican Standard (NOM-062-ZOO-1999). The animals were housed in standard polypropylene cages under standard laboratory conditions with access to special food and purified water ad libitum, pathogen- and stress-free environment, temperature of 22 ± 2°C and controlled room with 12 h light/dark cycle.

Authors' contribution

JX-T performed the experiments, viability assay, and in vitro and in vivo inflammatory assay with different propolis concentrations; IC-Z participated in implementation of the ELISA protocol and anti-inflammatory in vivo models; VEA-A participated in the conception and design of in vitro and in vivo experiments, data analysis, writing and review of the paper; FV-T participated in the design of viability experiments, analysis, interpretation, and critical revision; JCT-R participated in data analysis and interpretation, drafting of the manuscript and critical revision;

JAA-L participated in the fingerprint profile; FJA-A, MER-P, and NCC-L participated in idea generation; RFV-C participated in the experimental work; JVC-R participated in idea generation and writing the manuscript and coordinated the team.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

This study was approved by the Ethics Review Committee for research involving animal subjects (CB-CCBA-M-2016-005) at Campus de Ciencias Biológicas y Agropecuarias-Universidad Autónoma de Yucatán. Human subjects were not included in the study.

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