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Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis☆

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Abstract

Propolis is a polyphenol-rich resinous substance extensively used to improve health and prevent diseases. The effects of polyphenols from different sources of propolis on atherosclerotic lesions and inflammatory and angiogenic factors were investigated in LDL receptor gene (LDLr−/−) knockout mice. The animals received a cholesterol-enriched diet to induce the initial atherosclerotic lesions (IALs) or advanced atherosclerotic lesions (AALs). The IAL or AAL animals were divided into three groups, each receiving polyphenols from either the green, red or brown propolis (250 mg/kg per day) by gavage. After 4 weeks of polyphenol treatment, the animals were sacrificed and their blood was collected for lipid profile analysis. The atheromatous lesions at the aortic root were also analyzed for gene expression of inflammatory and angiogenic factors by quantitative real-time polymerase chain reaction and immunohistochemistry. All three polyphenol extracts improved the lipid profile and decreased the atherosclerotic lesion area in IAL animals. However, only polyphenols from the red propolis induced favorable changes in the lipid profiles and reduced the lesion areas in AAL mice. In IAL groups, VCAM, MCP-1, FGF, PDGF, VEGF, PECAM and MMP-9 gene expression was down-regulated, while the metalloproteinase inhibitor TIMP-1 gene was up-regulated by all polyphenol extracts. In contrast, for advanced lesions, only the polyphenols from red propolis induced the down-regulation of CD36 and the up-regulation of HO-1 and TIMP-1 when compared to polyphenols from the other two types of propolis. In conclusion, polyphenols from propolis, particularly red propolis, are able to reduce atherosclerotic lesions through mechanisms including the modulation of inflammatory and angiogenic factors.

Keywords: Propolis; Polyphenols; Dietary supplementation; Atherosclerosis; Angiogenesis; Nutrigenomics

1. Introduction

In the past decades, the growing knowledge provided by scientific research on pathophysiology has provided us with new insights about the importance of disease connections. For instance, atherosclerosis, which has a high mortality rate in most populations, is better defined as an immune–inflammatory disease [1]. The atherosclerotic process initiates when macrophage cholesterol influx is greater than efflux, leading to disturbed cholesterol homeostasis and cholesteryl ester accumulation in cytoplasmic droplets [2]. The resulting macrophage-derived foam cells secrete pro-inflammatory factors, which amplify the local inflammatory reaction and produce reactive oxygen species, which, in turn, modify lipoproteins [2,3].

Additionally, the angiogenic process was shown to be increased in human and experimental atherosclerosis [4,5]. The development of new blood vessels from the vasa vasorum is correlated with the severity of the atherosclerotic plaques and possibly with complications such as intraplaque hemorrhage and plaque rupture. Therefore, modulators of angiogenic process might be important in the progression of atherosclerosis [6–8].

Propolis is a polyphenol-rich resinous substance collected by honeybees (*Apis mellifera* L.) from a variety of plant sources. Its chemical composition is very complex, varies according to the geographic origin and depends on the local flora and phenology of the source plants [9]. Propolis has been widely used in folk medicine in various parts of the world for several applications including as an anti-inflammatory, cariostatic and antimicrobial agent [10–12]. In past times, in Eastern Europe propolis was used as a therapeutic product with satisfactory results. In Western European countries, in North and South America and in Japan, propolis did not acquire popularity until the 1980s. Nowadays, Japan is the leading importer of propolis, with a manifest preference for propolis from Brazil [10].

Although brown propolis is the most common, used and studied propolis worldwide [13–16], recent studies have also demonstrated the effect of green propolis in biological systems [17–19]. Postulated as a new type of propolis by Alencar et al. [20], red propolis has

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Table 1
Forward and reverse primers used for real-time PCR experiments

Gene	Primer forward	Primer reverse
18S	5′-TGTGCTAACCGTTACCTGGCT-3′	5′-CAGTGCCACATACCAACTG-3′
INFδ	5′-ATCTTCAAGCCATCCTGTGTGC-3′	5′-CAAGGCCCACAGGGATTTTC-3′
TGFβ	5′-TGTGCTAACCGTTACCTGGCT-3′	5′-CAGTGCCACATACCAACTG-3′
IL-6	5′-TCGCCAGAGTGGTTATCTTTT-3′	5′-TAGTGAACCCGTTGATGTCC-3′
CD36	5′-TGTGTGAAGGTGCAGTTTTG-3′	5′-ATTTCTGTGTTGGCGCAGT-3′
HO-1	5′-CCCAAAACGGACAAAGAGTT-3′	5′-TGGTCTCGATGGTATTCTGG-3′
MCP1	5′-GAACTTTGACAGCGACAAGAAG-3′	5′-CAGTGAAGCGGTACATAGGG-3′
VCAM	5′-ATCTTCAAGCCATCCTGTGTGC-3′	5′-CAAGGCCCACAGGGATTTTC-3′
ABCA1	5′-CAAACATGTCAGCTGTTACTGG-3′	5′-CATTAAGGACATGCACAAGGTCC-3′
ANG I	5′-TGTGCTAACCGTTACCTGGCT-3′	5′-CAGTGCCACATACCAACTG-3′
ANG II	5′-TCGCCAGAGTGGTTATCTTTT-3′	5′-TAGTGAACCCGTTGATGTCC-3′
VEGF	5′-TGTGTGAAGGTGCAGTTTTG-3′	5′-ATTTCTGTGTTGGCGCAGT-3′
MMP2	5′-CCCAAAACGGACAAAGAGTT-3′	5′-TGGTCTCGATGGTATTCTGG-3′
MMP9	5′-GAACTTTGACAGCGACAAGAAG-3′	5′-CAGTGAAGCGGTACATAGGG-3′
FGF	5′-GCTGGCCCCAGCCGCTGGAG-3′	5′-GAGTGCAGGGTCAGCACTAC-3′
PDGF	5′-TGTGCTAACCGTTACCTGGCT-3′	5′-CAGTGCCACATACCAACTG-3′
TIMP-1	5′-TCGCCAGAGTGGTTATCTTTT-3′	5′-TAGTGAACCCGTTGATGTCC-3′
PECAM	5′-TTCCAGACCGTCCAGAAGAACTCC-3′	5′-CACCGAAGCACCATTTCATCTCC-3′

biologically active compounds never reported in other types of Brazilian propolis. The consistent literature data demonstrate that propolis may be beneficial for human health. In previous studies, the effect of powered propolis extract in humans was investigated. The consumption of propolis as a nutritional supplement showed a decrease in free radical-induced lipid peroxidation, as well as an increase in activity of superoxide dismutase [21]. In addition, propolis exhibited significant anti-inflammatory effects in experimental models with respect to thoracic capillary vessel leakage in mice, as well as in carrageenan-induced oedema, carrageenan-induced pleurisy and acute lung damage in rats [22].

The cardioprotective effects of propolis extracts have been reported, but the mechanism of action of its polyphenols is not well defined [22,23]. Despite extensive research on the effects of propolis, there are no data concerning the *in vivo* action of propolis on atherogenic and angiogenic processes in atherosclerosis. To better understand the impact of polyphenols from propolis in atherosclerotic lesion formation and angiogenic factors, LDL receptor gene knockout mice (LDLr−/−) were fed with diets rich in saturated fat and cholesterol to investigate the effects of propolis polyphenols on serum lipid profile, atherogenesis, atherosclerosis progression, and inflammatory and angiogenic gene expression in atherosclerotic lesions.

2. Materials and methods

2.1. Propolis samples and extraction of polyphenols

The three following types of propolis were used in this study: the green Brazilian propolis, collected at Minas Gerais State, Brazil, where *Baccharis dracunculifolia* DC is the main botanical source [24]; the red Brazilian propolis, collected at Alagoas State, Brazil, where *Dalbergia ecastaphyllum* is the main botanical source [25]; and the brown Chilean propolis, collected in the Araucanía region, Cunco, Chile, where *Lotus uliginosus* is the main botanical source of the propolis.

The propolis samples (100 g) were extracted with 80% ethanol (v/v) (450 ml), incubated in a water bath at 70°C for 30 min and then filtered to obtain the ethanolic extracts of propolis (EEP) [26]. The EEP was passed through an OASIS column (Waters, WAT106202, Ireland), and the polyphenols were eluted with methanol to obtain the polyphenol concentrated solution (PCS). The PCS filtrate was taken to dryness and redissolved in 70% ethanol, resulting in the polyphenol extract of propolis (PEP).

2.2. Total polyphenol content

Total polyphenol content in PEP was determined by the Folin–Ciocalteu colorimetric method according to Ainsworth and Gillespie [27] with minor modifications. Extract aliquots (50 μl) were mixed with 50 μl of the Folin–Ciocalteu reagent (1:10) and 100 μl of 4% Na_2CO_3. The absorbance was measured at 740 nm after 1 h of incubation at room temperature and in the dark. PEP was evaluated at a final concentration of 500 μg/ml. The total polyphenol contents were expressed as percentages (%) or milligrams per gram (gallic acid equivalents).

2.3. LC-MS Polyphenol analysis

The polyphenols isolated from propolis were characterized by LC-MS analysis (Esquire HCT, Bruker Daltonics, Billerica, MA, USA). The polyphenols from the green, red and brown propolis were separated using a 150×4.6-mm stainless-steel Synergi 4 Fusion- RP (C18) 80A column. The eluents were (A) 0.25% formic acid and (B) methanol. The separations were performed at room temperature by solvent gradient elution from 0 min using 50% A/50% B to 60 min using 100% B at a flow rate of 0.5 ml/min.

2.4. Animals, chow and experimental design

Homozygous LDL receptor-deficient mice (LDLr−/−, C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were maintained in individual plastic cages at 22°C on a 12-h light–dark cycle. A total of 150 LDLr−/− mice (n=15 per group, 8 weeks old) were divided into two protocols, with the same five groups in each protocol: control (water), vehicle (5% ethanol), green (250 mg/kg body weight/day of polyphenols from green propolis), red (250 mg/kg body weight per day of polyphenols from red propolis) and brown (250 mg/kg body weight per day of polyphenols from brown propolis). The doses of polyphenols from propolis were normalized for each sample of propolis according to its total polyphenol content. The polyphenols were administrated in a single dose per day at a volume of 0.5 ml by gavage. The dose was chosen according to previously described acute toxicity studies [28–31].

The experiments were performed in two protocols with a semisynthetic chow based on a Western-type diet containing 20% fat, 0.5% (w/w) cholesterol (CF, Sigma), 0.5% (w/w) colic acid (RV, Sigma), 16.5% casein, vitamins and minerals, according to the recommendations of AIN-93 [32]. This chow did not contain polyphenols.

In Protocol I, termed initial atherosclerotic lesion (IAL), the animals received the chow for 5 weeks and polyphenol supplementation for 4 weeks before euthanasia (1 week with chow and 4 weeks with supplementation plus chow). In Protocol II, termed advanced atherosclerotic lesion (AAL), the animals received the same chow as IAL for 16 weeks and polyphenol supplementation for the last 4 weeks before euthanasia (12 weeks with chow and 4 weeks with supplementation plus chow). The animals received water and chow *ad libitum* throughout the experiment. The study protocols were approved by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences, University of São Paulo (protocol number 170/2009) and followed the rules of the Guide for the Care and the Use of Laboratory Animals published by the US National Institutes of Health (NHI Publication N 85-23, revised in 1996). At the end of the experiment and after overnight fasting, the animals were sedated under ketamine (1.0 g/10.0 ml; Vetaset, Fort. Dodge Saúde Animal Ltda, Brazil) and xylazine (2.0 g/100 ml; Bayer do Brasil, Brazil; 2:1) anesthesia before euthanasia.

2.5. Biochemical analysis

Blood was drawn by cardiac puncture in dry tubes, and the serum was separated for further biochemical analyses. Triacylglycerols (TAG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were determined using commercial kits (LABTEST, Rio de Janeiro, RJ, Brazil). Non–HDL cholesterol was calculated as the difference between TC and HDL-C [33]. Non-HDL-cholesterol provides a single index of all the atherogenic apo B-containing lipoproteins, i.e., LDL, VLDL, IDL and lipoprotein (a).

2.6. Preparation of histological sections and measurement of atherosclerotic lesion area

The heart was perfused with phosphate buffered saline (PBS) and then with 10% PBS-buffered formaldehyde. The heart and aorta were excised, fixed in 10% formaldehyde for at least 2 days and then embedded in 5%, 10% and 25% gelatin and

Table 2
Total polyphenol content and major components of PEP

Samples	Polyphenol content (%)	Major components
Green	31.5±2.6	Artepellin C, pinocembrin, kampferol
Red	30.6±2.9	3-Hydroxy-8,9-dimethoxypterocarpan, medicarpin, daidzein
Brown	34.4±1.7	Pinocembrin, caffeic acid phenyl ester, quercetin, galangin

The EEP samples were eluted from an OASIS column (Waters, WAT106202, Ireland) with methanol. The polyphenol concentrated solution filtrate was taken to dryness and redissolved in ethanol 70% resulting in the polyphenol extract of propolis. The polyphenols isolated from propolis were characterized by LC-MS analysis. The eluents were (A) 0.25% formic acid and (B) methanol. The separations were performed at room temperature by solvent gradient elution from 0 min using 50% A/50% B to 60 min using 100% B at a flow rate of 0.5 ml/min.

Table 3
Effects of polyphenols from green, red and brown propolis supplementation on body mass and biochemical profile of LDLr−/− mice

	Experimental protocols									
	IAL (groups)					AAL (groups)				
	Control (n=13)	Vehicle (n=12)	Green (n=14)	Red (n=15)	Brown (n=11)	Control (n=13)	Vehicle (n=13)	Green (n=10)	Red (n=11)	Brown (n=12)
Initial BM (g)	26±1.6	25.8±0.9	26.2±1.3	26.1±0.8	25.9±1.5	25.9±0.8	26.1±0.6	26.4±1.3	25.8±0.7	26.2±1.4
Final BM (g)	32±1.7	32.4±1.3	30.9±0.9	30.8±1.8	31±0.6	47.1±2.3	47.4±1.9	45.6±2.7	45.1±1.1	44.9±1.4
Chow intake (g/day)	3.4±0.6	3.2±0.7	3.4±0.5	3.0±0.3	3.4±0.8	3.8±0.4	3.6±0.6	3.9±0.9	3.5±0.2	3.4±0.9
TAG (mg/dl)	478±139	450±48	363±79	266±60[a]	347±51	456±69	377±96	275.5±22	211.1±55[a]	305±29
TC (mg/dl)	1942±139	2048±89	2096±60	1620±81[a]	2084±68	1920±158	2271±253	2030±55	1750±64[a]	2069±119
HDL-C (mg/dl)	25±5	24±3	37±5[b]	42±8[a]	36±2[b]	26±5	26±6	22±3	34±5[a]	20±1.2
Non-HDL-C (mg/dl)	1917±141	2025±201	2072±198	1585±172[a]	2055±201	1873±169	2244±252	1908±56	1816±67	2049±107

BM, Body mass; IAL, animals were feed with a Western-type diet during 5 weeks and they received a respective polyphenol supplementation (green, red and brown; 250 mg/kg per day) for 4 weeks before the euthanasia; AAL, animals were feed with a Western-type diet during 16 weeks and they received a respective polyphenol supplementation (green, red and brown; 250 mg/kg per day) for 4 weeks before the euthanasia.

[a] P<.05.

[b] P<.01 vs. control group.

fixed in mounting medium (Tissue-Tek OTC compound, Sakura Finetek, USA). The aortic root at the heart was sectioned proximally to distally in 10-μm-thick slices starting from the semilunar valves. The area of the atherosclerotic lesion was reported as the sum of the lesions in six equidistant sections (80 μm) along an aortic root length of 400 μm. The results from the five mice/group protocols are reported as mean square micrometer±S.D. To quantify the cross-sectional area of the oil red O-stained lesions in the aortic root, the processing and staining were carried out as described [34] and the images were analyzed using the Image-Pro Plus software (version 3.0; Media Cybernetics, USA). All analyses were double blind and were carried out independently by two observers.

2.7. Quantitative real-time polymerase chain reaction

mRNA was extracted from an atherosclerotic lesion area using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Single-strand cDNA was synthesized from RNA using a high-capacity cDNA reverse transcription kit according to the manufacturer's

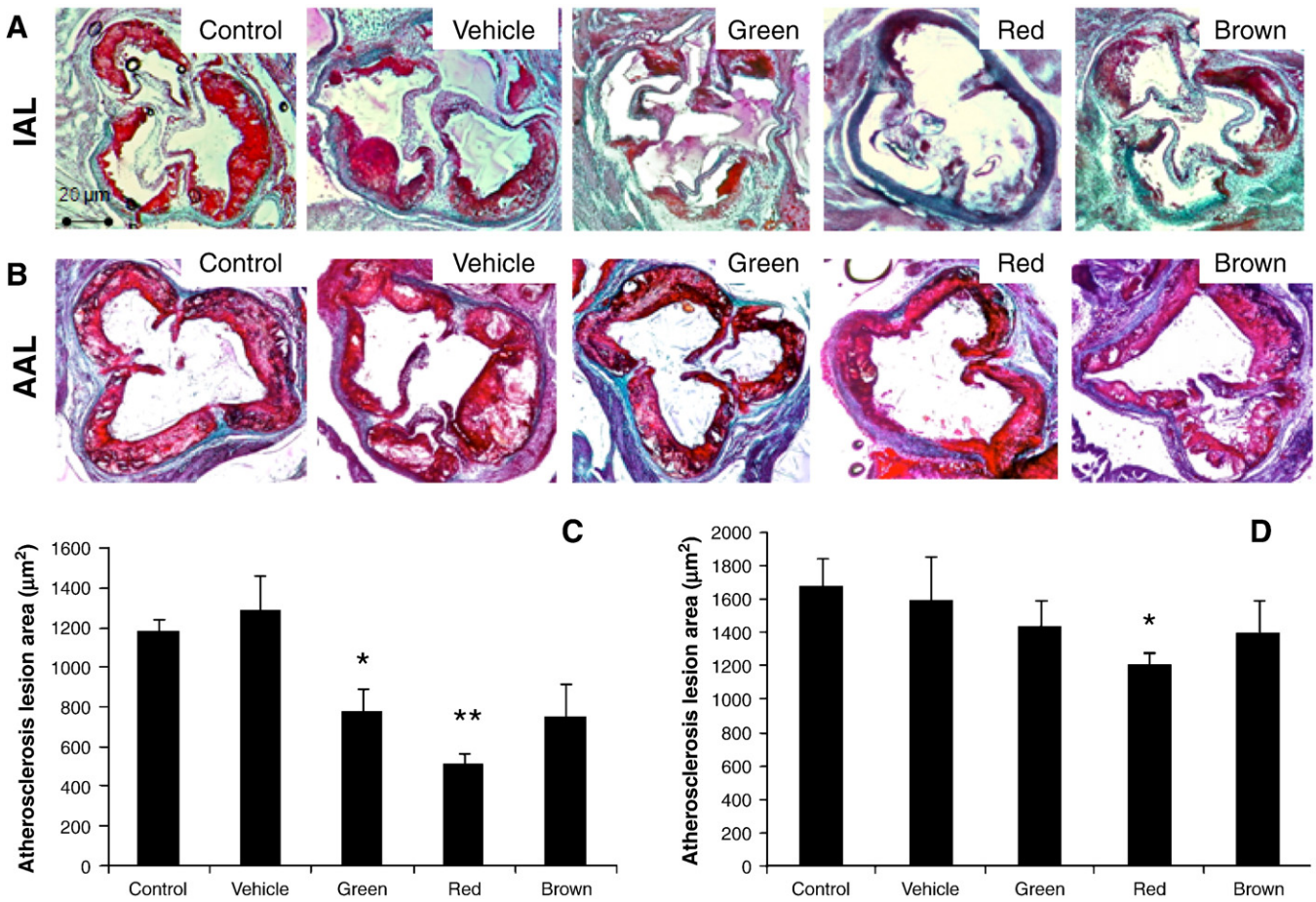

Fig. 1. Impact of polyphenols from propolis oral supplementation (250 mg/kg per day) on the extent of atherosclerosis LDLr−/− mice in (A) initial atherosclerosis lesion (IAL) and (B) advanced atherosclerotic lesion (AAL) in the aortic sinus. Representative sections of the aortic sinus from the control, vehicle, green, red and brown groups are shown. Average±S.D. of lesion area for IAL (C) and AAL (D) are represented.

instructions (Applied Biosystems). Primers for CD36, IL-6, ABCA1, MCP-1, INFδ, TGFβ, HO-1, VCAM, ANG I, ANG II, VEGF, FGF, MMP-2, MMP-9, PDGF, TIMP-1, PECAM and 18S (Table 1) were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA). The data were normalized against the ribosomal protein 18S housekeeping gene. The primer amplification efficiency and specificity were verified for each set of primers. The cDNA levels of the target genes were measured using the power SYBR green master mix in a real-time PCR 7500 machine (Applied Biosystems). The RT-PCR reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min and 1 cycle of dissociation stage. The mRNA fold change was calculated using the 2(-Delta Delta C(T)) method [35].

2.8. Immunohistochemical analysis

To evaluate the expression of vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule (PECAM-1), the remaining aortic arch atheromatous lesion sections were mounted with Tissue-Tek OTC and embedded in paraffin. VCAM-1 and PECAM were detected with the respective anti-mouse antibodies H-276 (1:400) and ER-MP12 (1:400) purchased from Santa Cruz Biotechnology (USA). The VCAM-1 and PECAM-1 staining was quantified using image analysis software (KS300, Kontron, Germany). Histochemical analysis of the atheromatous lesion was performed in 5-μm-thick tissue sections stained with Harris hematoxylin.

2.9. Statistical analysis

Statistical analysis was performed with the use of SPSS software (version 12.0; SPSS, Chicago, IL, USA) for the Mac. Results are reported as means±S.D. Shapiro–Wilk test showed non-normal distribution of data for all parameters. The statistical significance of experimental observations was determined by one-way analysis of variance followed by the Kruskal–Wallis test. With five animals per group, our study had >95% power to detect a 50% decrease in aortic lesion from five animals per group. Statistical significance was accepted at a value of $P<.05$.

3. Results

3.1. Total polyphenol content of propolis extracts and LC-MS analysis

The total polyphenol content in PEP for all types of propolis is shown in Table 2. The brown propolis presented a slightly higher but not significantly different concentration of polyphenols when compared to the green and red propolis. The average content of total polyphenols in all types of propolis was 32%. Although the samples contained a similar amount of total polyphenols, the variety of polyphenol types differed from one propolis to another. As described in Table 2, the major components found in the extract from the red propolis were characterized as isoflavonoids and pterocarpans. The polyphenols from the brown propolis were constituted by many types of flavonoids and phenolic acid esters. In contrast, the major components in the green propolis of Brazilian origin were terpenoids and prenylated derivatives of *p*-coumaric acids.

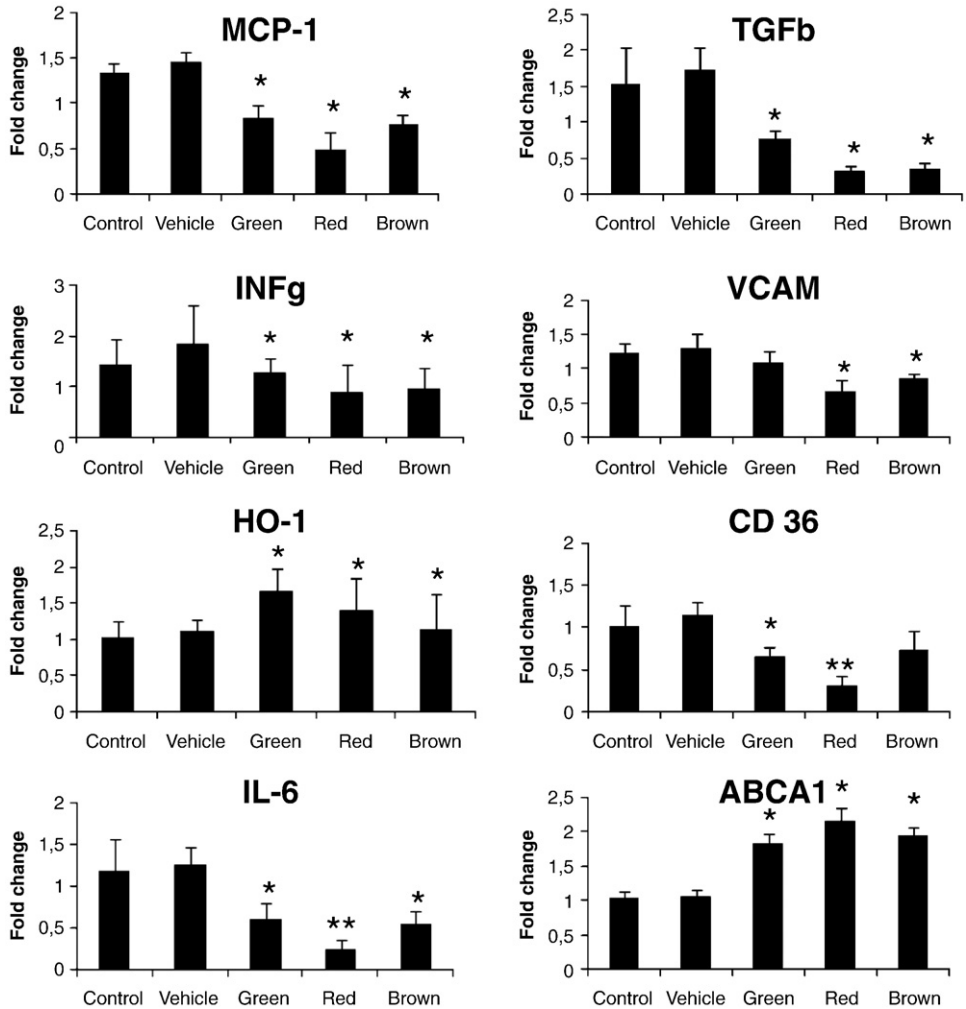

Fig. 2. Effect of propolis polyphenol supplementation on atherogenic gene expression by quantitative real-time RT-PCR in the IAL. The relative mRNA expression of CD36, IL-6, ABCA1, MCP-1, INFδ, TGFβ, HO-1 and VCAM was monitored. LDLr−/− mice were fed a Western-type diet chow without polyphenols during 1 week; after this period, polyphenols were supplemented (250 mg/kg per day) with the respective propolis types (green, red or brown) for 4 weeks. The control and placebo-treated mice received only water and water with 5% ethanol, respectively. The real-time PCR results are shown (fold induction vs. control animals, corrected for the 18S expression, mean±S.D.; *$P<.05$ vs. control).

3.2. Chow intake, body weight and lipid profile

The daily food intake did not differ among the groups (green, red and brown) in the IAL and AAL protocols. At the end of the experiment, no significant differences in weight gain were observed among the five groups in both protocols (Table 3).

3.3. Effects of polyphenols from propolis on lipid profile

In the IAL protocol, the plasma TAG, TC, HDL-C and non-HDL-C levels were significantly different among the groups (Table 3). Compared to the controls, the TAG, TC and non-HDL-C levels were lower in mice treated with the red propolis polyphenols. In fact, the levels of non-HDL-C were lower in all groups supplemented with polyphenols when compared to control, although mice supplemented with the red propolis polyphenols showed lower non-HDL-C than those that received polyphenols from the green and brown propolis. In the AAL protocol, only the polyphenols from the red propolis promoted a significant decrease in TAG (45%) and TC (22%) and increased HDL-C (30%) levels compared to control.

3.4. Effect of polyphenols on atherosclerosis progression

Morphometric analysis of atherosclerotic lesions in the IAL protocol (Fig. 1A) showed that the lesion area in the aortic sinus was reduced ($P<.05$) following treatment with green, brown and red propolis polyphenols by 34%, 37% and 57%, respectively, compared to control animals. The most effective lesion area reduction was observed in the red propolis group ($P<.01$) (Fig. 1C). In the AAL protocol (Fig. 1B), no difference was observed either in the green or in the brown propolis polyphenol-treated groups when compared to the control. However, the red propolis group showed a significant reduction (30%, $P<.05$) of the atherosclerotic lesion area when compared to control (Fig. 1D).

3.5. Gene expression in atherosclerotic lesion

To investigate how the polyphenols, especially those from the red propolis, attenuate the progression of atherosclerotic lesions, the expression of the genes involved in the atherosclerotic process was evaluated. Moreover, we also investigated whether polyphenols from

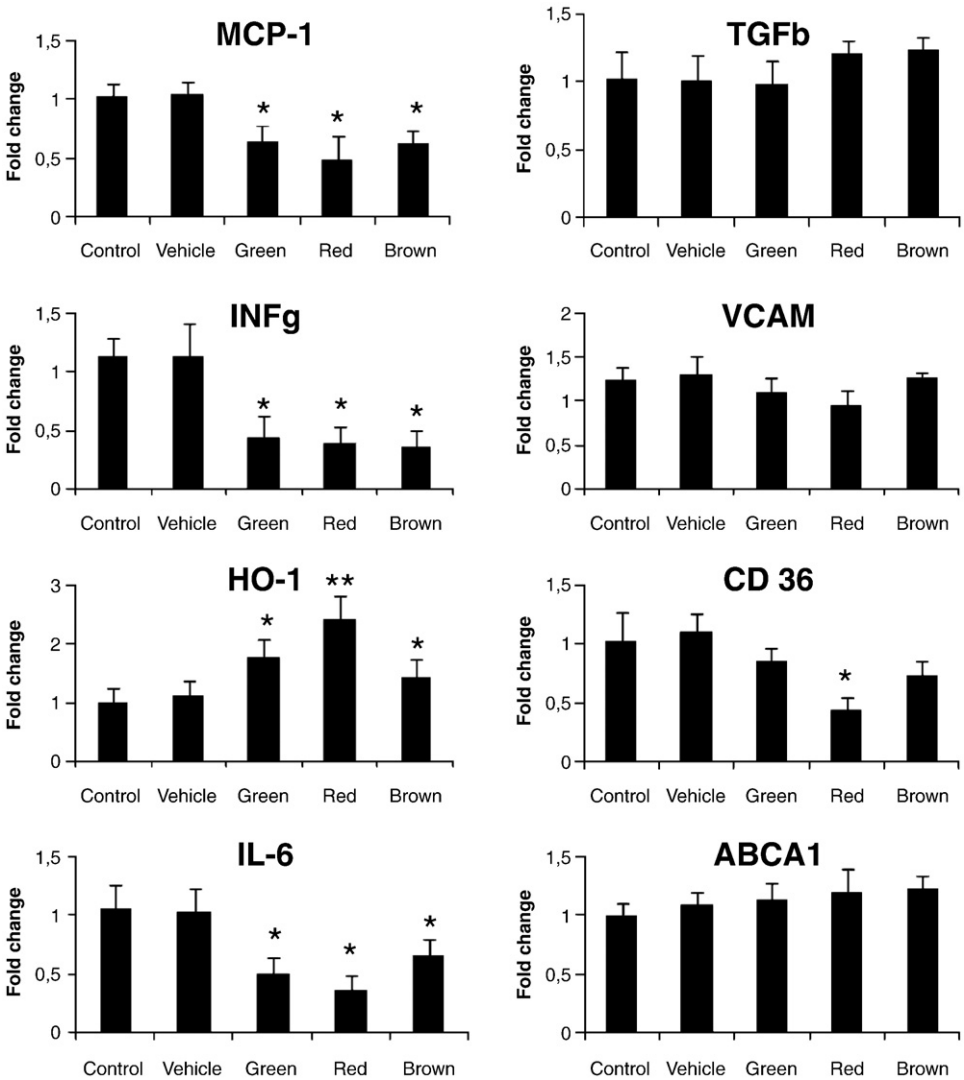

Fig. 3. Effect of propolis polyphenol supplementation on atherogenic gene expression by quantitative real-time RT-PCR in AAL. The relative mRNA expression of CD36, IL-6, ABCA1, MCP-1, INFδ, TGFβ, HO-1 and VCAM was monitored. LDLr−/− mice were fed a Western-type diet chow without polyphenols during 12 weeks; after this period, polyphenols were supplemented (250 mg/kg per day) with the respective propolis types (green, red or brown) for 4 weeks. The control and placebo-treated mice received only water and water with 5% ethanol, respectively. The real-time PCR results are shown (fold induction vs. control animals, corrected for the 18S expression, mean±S.D.; *$P<.05$ vs. control).

propolis could modulate the expression of genes involved in angiogenesis in the atherosclerotic lesions. In the IAL protocol, polyphenols from green, red and brown propolis decreased MCP-1, INFg, IL6, CD36 and TGFβ mRNA expression (Fig. 2) compared to the expression in the control group ($P<.05$). Only the polyphenols from red and brown propolis decreased VCAM mRNA expression ($P<.05$). Additionally, the group treated with polyphenols from red propolis showed the lowest expression ($P<.01$) of IL-6 and CD36 mRNA compared to the other groups. In all the experimental groups (green, red and brown), the expression of HO-1 and ABCA1 ($P<.05$) was higher compared to the control group. As for the IAL protocol, in the AAL studies, all the experimental groups induced the down-regulation of MCP-1, INFδ and IL-6 gene expression (mRNA) compared to the control (Fig. 3). However, a down-regulation of CD36 gene expression was observed only for the group supplemented with polyphenols from the red propolis.

We further investigated the expression of angiogenesis regulator genes, angiopoietin I, angiopoietin II, VEGF, FGF, MMP-2, MMP-9, PDGF, TIMP-1 and PECAM. In the IAL protocol, angiopoietin I, angiopoietin II, VEGF, FGF, MMP-9, PDGF and PECAM gene expression was significantly down-regulated upon supplementation with polyphenols from the green, red and brown propolis (Fig. 4) compared to control; in addition, the animals in this protocol displayed an up-regulation of TIMP1 and did exhibit MMP2 expression compared to the control group (Fig. 5). In the AAL protocol, VEGF, MMP9, PDGF and PECAM expression was negatively modulated by polyphenols from the green, red and brown propolis in comparison to controls. However, only the polyphenols from the red propolis were able to increase TIMP1 gene expression (Fig. 5).

3.6. PECAM and VCAM protein expression in atherosclerotic lesion

The atherosclerotic lesions of the aortic valve sinus from LDLr−/− mice treated with the polyphenol extracts from the three types of propolis were utilized to evaluate the expression of VCAM-1 and PECAM-1 by immunohistochemistry (Fig. 6). The results showed that, in the IAL protocol, a decreased expression ($P<.05$) of PECAM-1 and VCAM-1 protein occurred in all groups supplemented with polyphenols. Furthermore, the lowest PECAM-1 expression was found in the groups supplemented with polyphenols from the red and brown propolis compared to other groups ($P<.01$); the lowest VCAM-1 expression ($P<.01$) was observed in the group supplemented with polyphenols from the red propolis (Fig. 6). In the AAL protocol, supplementation with polyphenols from the three types of propolis reduced ($P<.05$) the expression of PECAM-1 and VCAM in comparison to controls. Additionally, lower VCAM-1 expression was seen in the groups treated with polyphenols from the red and brown propolis ($P<.01$) than in those treated with the green propolis (Fig. 6).

4. Discussion

Propolis is a natural compound rich in polyphenols that is commonly used in alternative medicine. This study demonstrates that polyphenols from propolis inhibited atherosclerosis progression

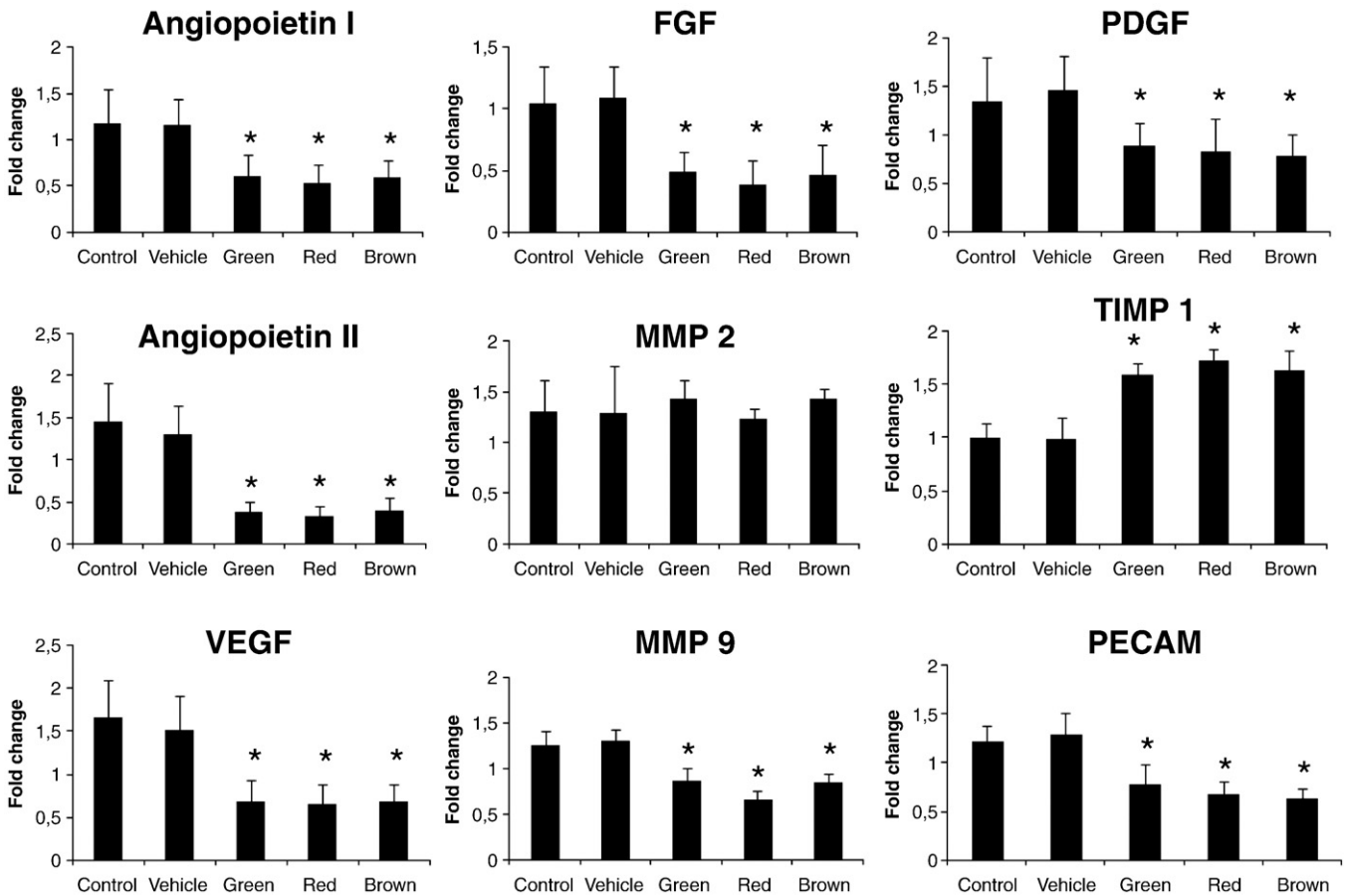

Fig. 4. Impact of propolis polyphenol supplementation on angiogenic gene expression by quantitative real-time RT-PCR in IAL. The relative mRNA expression of ANG I, ANG II, VEGF, FGF, MMP-2, MMP-9, PDGF, TIMP-1 and PECAM was monitored. LDLr−/− mice were fed a Western-type diet chow without polyphenols during 1 week; after this period, polyphenols were supplemented (250 mg/kg per day) with the respective propolis types (green, red or brown) for 4 weeks. The control and placebo-treated mice received only water and water with 5% ethanol, respectively. The real-time PCR results are shown (fold induction vs. control animals, corrected for the 18S expression, mean±S.D.; *$P<.05$ vs. control).

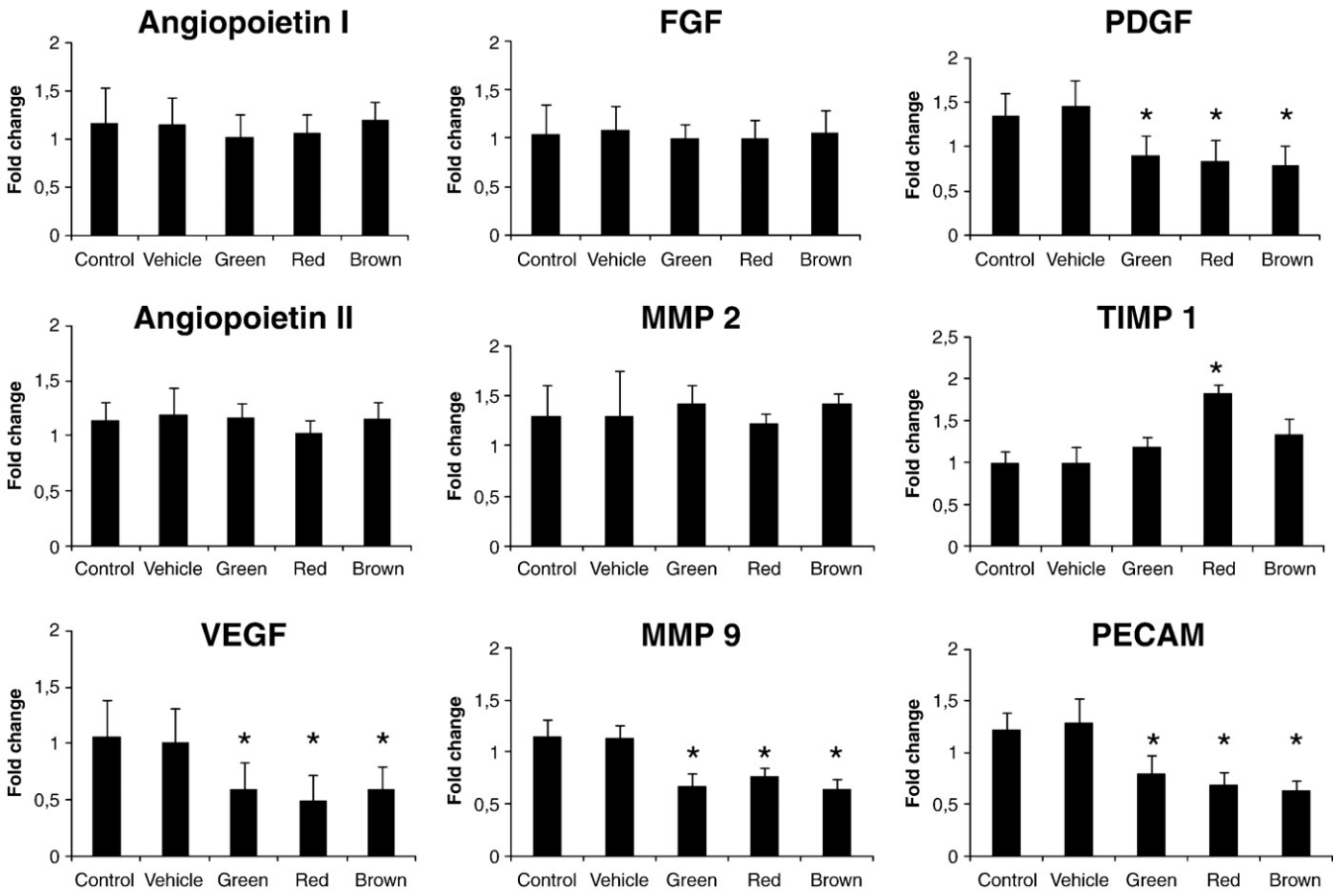

Fig. 5. Impact of propolis polyphenol supplementation on angiogenic gene expression by quantitative real-time RT-PCR in AAL. The relative mRNA expression of ANG I, ANG II, VEGF, FGF, MMP-2, MMP-9, PDGF, TIMP-1 and PECAM was monitored. LDLr−/− mice were fed a Western-type diet chow without polyphenols during 12 weeks; after this period, polyphenols were supplemented (250 mg/kg per day) with the respective propolis types (green, red or brown) for 4 weeks. The control and placebo-treated mice received only water and water with 5% ethanol, respectively. The real-time PCR results are shown (fold induction vs. control animals, corrected for the 18S expression, mean±S.D.; *P<.05 vs. control).

in LDLr−/− mice by improving the lipid profile and down-regulating pro-inflammatory cytokines, chemokines and angiogenic factors.

The biological effects of three different types of propolis with distinct polyphenol composition were evaluated in this study. The Brazilian red propolis was characterized by a high content of isoflavones such as medicarpin, homoterocarpin and pterocarpans. A previous study demonstrated that this type of propolis presented a composition similar to a specific type of Cuban red propolis in which no benzophenones were detected [26]. In contrast, several studies have established that artepillin C is the major constituent of green propolis [36,37]. In this study, we found that the major components of green propolis were terpenoids and prenylated derivatives of *p*-coumaric acids. Finally, the Chilean brown propolis has been characterized mainly by the presence of phenolic compounds, as previously reported by Russo et al. [38] and Munoz et al. [39]. Although the three types of propolis presented a similar amount of total polyphenol, their impact on the AALs or IALs was more associated with their specific polyphenol composition.

The dose of polyphenols chosen for this study is supported by previous reports [29,40] demonstrating that 250 mg of polyphenols from propolis per kilogram per day has no toxic effects and is an effective dose for studies with mice. In the present study, for both protocols, IAL or AAL, the LDLr−/− mice received polyphenol supplementation during 4 weeks. Recent reports using polyphenols from different sources have shown that supplementation during 4 to 6 weeks is able to induce protective effects in several pathological conditions in mice [29,41,42].

It is well known that modification of the lipid profile is highly associated with cardiovascular diseases [43–45]. Analysis of blood plasma lipids revealed that all EEPs diminished total cholesterol and elevated HDL-cholesterol concentrations in LDLr−/− mice of the IAL protocol. Our results corroborated previous studies that demonstrated the regulation of lipid metabolism by propolis from different sources [46–48]. Because increased ABCA1 expression is associated with increased HDL levels [49], the ABCA1 up-regulation observed in this study might be one of the mechanisms by which the three EPPs studied here improved the lipid profile. Interestingly, regarding the other biochemical analyses, our study did not show any significant differences among the control, IAL and AAL groups. However, the AAL group showed either higher body weight or larger atherosclerotic lesion area than controls. According to Schreyer et al. [50], the loss of LDLr induces hyperleptinemia that increases the sites of lipid deposition, mainly as visceral and subcutaneous fat, in detriment of high levels of lipids in blood.

Considering the association between atherosclerosis and lipid profile abnormalities, it is reasonable to assume that if polyphenols from propolis could affect blood lipids, they might have effects on the prevention and development of atherosclerotic lesions. In fact, our results demonstrated that treatment of mice with EEP from all three types of propolis promoted a decrease in atherosclerotic lesion areas in the IAL group compared to the controls. Conversely, only polyphenols from the red propolis attenuated the progression of atherosclerosis in mice of the AAL group.

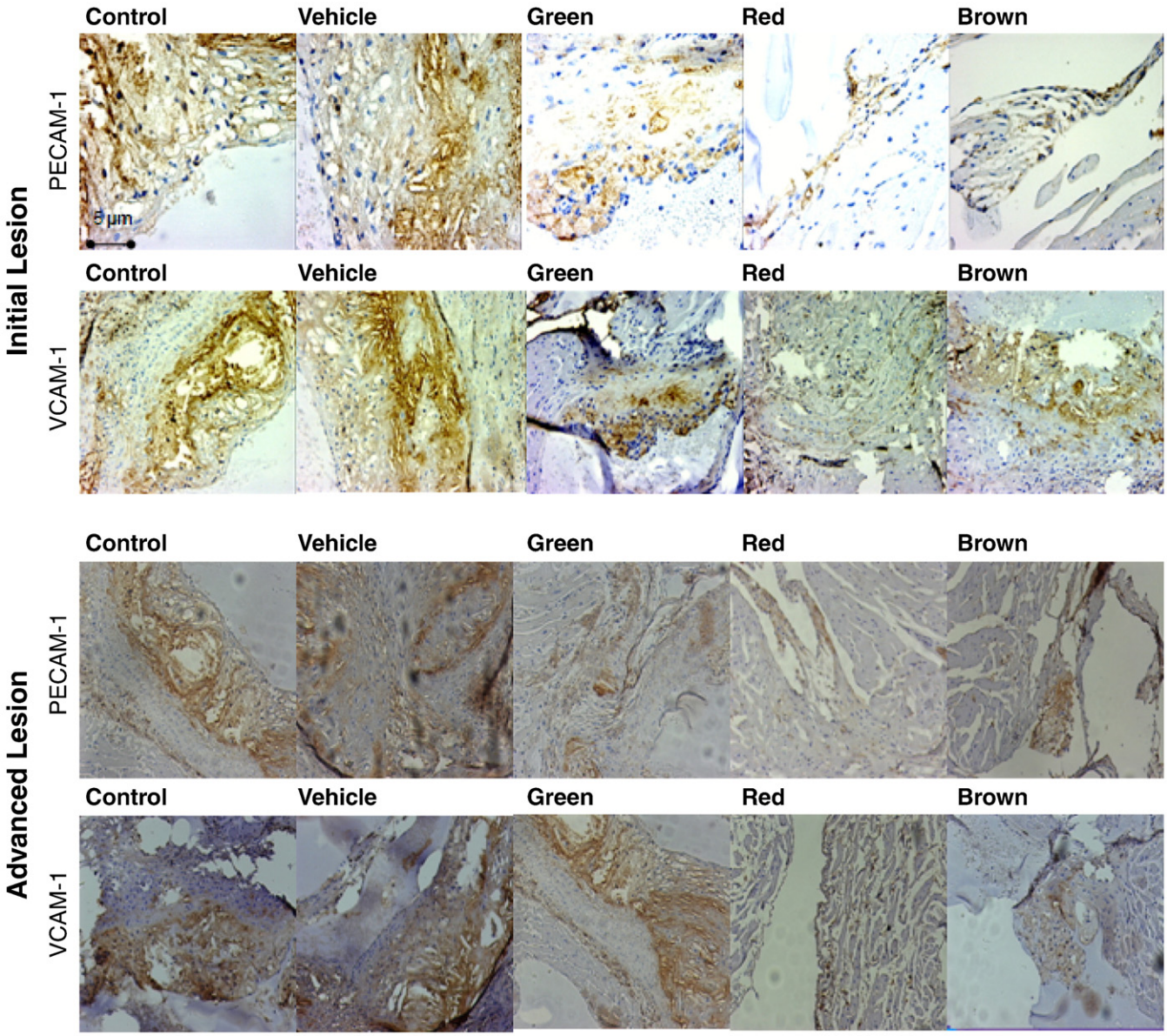

Fig. 6. The vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule (PECAM-1) proteins were evaluated in aortic arch atheromatous lesion sections of IALs and AALs. The animals received oral polyphenol supplementation (250 mg/kg per day) with the respective propolis types (green, red or brown) for 4 weeks. The control and placebo-treated mice received only water and water with 5% ethanol, respectively.

The development of atherosclerotic lesions has been related to the expression of CD36, a multiligand scavenger receptor responsible for the recognition and internalization of oxidatively modified LDL, which leads to foam cell formation and atherosclerosis [51]. Thus, CD36 and foam cells are well-known targets for therapeutic interventions in atherosclerosis [52]. Interestingly, only the polyphenols from red propolis were able to down-regulate CD36 expression in animals with advanced lesions. In contrast, all EEPs induced a decrease in atherosclerotic lesion area which was associated with diminished CD36 expression in the IAL group.

As atherosclerosis is defined as a chronic inflammatory disease [53], cytokines play a key role in amplifying the local inflammatory response and favoring the progression of atherosclerotic lesions. In this study, we analyzed whether the atheroprotective effects of EEPs were related to the inflammatory response. Evaluation of the pro-inflammatory profile demonstrated that all EEPs studied here were capable of modulating the expression of pro-inflammatory cytokines such as MCP-1, IFN-γ and IL-6 in both IAL and AAL groups. In addition, the expression of HO-1, which is an adaptive molecule in the inflammatory repair process [54], was up-regulated by EEPs. However, the down-regulation of TGF-β and VCAM-1 expression was only observed by the EEP treatment in the IAL group. These data are of particular interest because VCAM-deficient mice show at present fewer early atherosclerotic lesions compared to the wild-type mice, indicating a key role of VCAM-1 in the initiation of plaque development [55]. Together with TGF-β, which has a strong profibrogenic effect [56], the down-regulation of VCAM expression may be the key factor for the inhibition of the IALs observed in this study.

The angiogenic process is a critical feature of atherosclerotic plaque development. Moreover, atherosclerotic lesions are highly vascularized when compared to normal vessel tissues [57]. In fact, our findings demonstrated that PECAM, a marker of endothelial cells, was down-regulated by EEPs in both models, suggesting a diminished presence of endothelial cells in atherosclerotic lesions. Similarly, the two major angiogenic biomarkers, VEGF and PDGF, which are

responsible for the migration and proliferation of endothelial cells [58,59], were also down-regulated by EEPs in the initial and advanced lesions. In contrast, EEP treatment was able to modulate the expression of angiopoietin and FGF only in mice of the IAL group compared to controls.

Other crucial angiogenic biomarkers in atherosclerosis are the metalloproteases (MMPs), which degrade most of the extracellular matrix and also induce an increase in arterial stiffness and angiogenesis [60]. In fact, MMP9 expression was significantly down-regulated by EEP treatment in both IAL and AAL groups in comparison to controls. The expression of TIMP-1, an MMP inhibitor, was also evaluated in this study. Although TIMP-1 expression was elevated by EEP treatment in lesions of mice from the IAL group, only the polyphenols from red propolis had this same effect in the AAL group. Therefore, we can speculate that the stronger effect of red propolis polyphenols on angiogenic biomarkers can be reflected in the decreased atherosclerotic lesion area promoted by this type of propolis.

Taken together, our findings demonstrated the potential of polyphenols from propolis not only as preventive agents but also as nutritional therapeutic components against the progression of atherosclerosis. Polyphenols from propolis, particularly those from red propolis, may have beneficial actions for preventing or reducing atherosclerotic lesions through modulation of inflammatory and angiogenic factors.

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