www.elsevier.com/locate/jep

Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages

Michelle Cristiane Bufalo, Isabel Ferreira, Gustavo Costa, Vera Francisco, Joana Liberal, Maria Teresa Cruz, Maria Celeste Lopes, Maria Teresa Batista, José Maurício Sforcin

PII: S0378-8741(13)00407-8
DOI: http://dx.doi.org/10.1016/j.jep.2013.06.004
Reference: JEP8161

To appear in: *Journal of Ethnopharmacology*

Received date: 28 March 2013
Revised date: 4 June 2013
Accepted date: 5 June 2013

Cite this article as: Michelle Cristiane Bufalo, Isabel Ferreira, Gustavo Costa, Vera Francisco, Joana Liberal, Maria Teresa Cruz, Maria Celeste Lopes, Maria Teresa Batista, José Maurício Sforcin, Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages, *Journal of Ethnopharmacology,* http://dx.doi.org/10.1016/j.jep.2013.06.004

Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages

Michelle Cristiane Bufalo[a,c], Isabel Ferreira[b], Gustavo Costa[c,d], Vera Francisco[b,c,d], Joana Liberal[b,c,d], Maria Teresa Cruz[b,c], Maria Celeste Lopes [b,c], Maria Teresa Batista[c,d], José Maurício Sforcin[a*]

[a] *Departamento de Microbiologia e Imunologia, Instituto de Biociências, UNESP,18618-970, Botucatu, SP, Brasil*

[b] *Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Azinhaga de Santa Comba, 3004-517, Coimbra, Portugal*

[c] *Faculdade de Farmácia, Universidade de Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548, Coimbra, Portugal*

[d] *Centro de Estudos Farmacêuticos – Faculdade de Farmácia, Universidade de Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548, Coimbra, Portugal*

*Corresponding author. Tel.: +55 14 38116058; fax: +55 14 3811 6058236.

E-mail adress: sforcin@ibb.unesp.br (J.M. Sforcin).

Abstract

Ethnopharmacological relevance: Propolis is a bee product with numerous biological and pharmacological properties, such as immunomodulatory and anti-inflammatory activities. It has been used in folk medicine as a healthy drink and in food to improve health and prevent inflammatory diseases. However, little is known about its mechanism of action. Thus, the goal of this study was to verify the antioxidant activity and to explore the anti-inflammatory properties of propolis by addressing its intracellular mechanism of action. Caffeic acid was investigated as a possible compound responsible for propolis action.

Materials and Methods: The antioxidant properties of propolis and caffeic acid were evaluated by using the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging method. To analyze the anti-inflammatory activity, Raw 264.7 macrophages were treated with different concentrations of propolis or caffeic acid, and nitric oxide (NO) production, a strong pro-inflammatory mediator, was evaluated by the Griess reaction. The concentrations of propolis and caffeic acid that inhibited NO production were evaluated on intracellular signaling pathways triggered during inflammation, namely p38 mitogen-

activated protein kinase (MAPK), c-jun NH_2-terminal kinase (JNK1/2), the transcription nuclear factor (NF)-κB and extracellular signal-regulated kinase (ERK1/2), through Western blot using specific antibodies. A possible effect of propolis on the cytotoxicity of hepatocytes was also evaluated, since this product can be used in human diets.

Results: Caffeic acid showed a higher antioxidant activity than propolis extract. Propolis and caffeic acid inhibited NO production in macrophages, at concentrations without cytotoxicity. Furthermore, both propolis and caffeic acid suppressed LPS-induced signaling pathways, namely p38 MAPK, JNK1/2 and NF-κB. ERK1/2 was not affected by propolis extract and caffeic acid. In addition, propolis and caffeic acid did not induce hepatotoxicity at concentrations with strong anti-inflammatory potential.

Conclusions: Propolis exerted an antioxidant and anti-inflammatory action and caffeic acid may be involved in its inhibitory effects on NO production and intracellular signaling cascades, suggesting its use as a natural source of safe anti-inflammatory drugs.

Keywords: Propolis, Caffeic acid, Anti-inflammatory action, Nitric oxide, Mitogen-activated protein kinases, Nuclear factor-κB.

1. Introduction

Propolis is a resinous product used by bees to seal holes in the hive, smooth out the internal walls and protect the entrance against intruders, and its chemical composition depends on the collection site and local flora (Bankova, 2005). This product has attracted researchers' interest in the last decade because of its numerous biological and pharmacological properties, such as antimicrobial (Sforcin et al., 2000; Freitas et al., 2006; Búfalo et al., 2009), antitumoral (Bazo et al., 2002), immunomodulatory (Sforcin, 2007), anti-inflammatory (Silva et al., 2012), antioxidant (Kumazawa et al., 2010), among others.

Propolis has been extensively used as a healthy drink, and in food, to improve health and prevent inflammatory diseases. It is also used in folk medicine due to its hepatoprotective activity; however, little is known about its mechanisms of action (Kolankaya et al., 2012). The knowledge of phytochemicals molecular mechanisms became a good strategy in the search for new anti-inflammatory compounds.

Inflammation is the central feature of many physiopathological conditions in response to tissue injury and as part of host defense against microorganisms (Brodsky et al., 2010). In inflammatory processes, macrophages have a key role in providing an

immediate defense against foreign agents. Upon activation with an inflammatory stimulus, such as lipopolysaccharyde (LPS), macrophages produce pro-inflammatory mediators, including nitric oxide (NO). NO is produced through inducible nitric oxide synthase (iNOS) by certain activated cells and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities; however, excessive NO production may be pathogenic for host tissues because it acts as a reactive radical directly damaging normal cells. Thus, natural products inhibiting NO production induced by inflammatory stimuli may represent a beneficial therapeutic strategy (Nagaoka et al., 2003).

The expression of pro-inflammatory molecules is regulated by several transcription factors and signalling pathways. The functional nuclear transcription factor-κB (NF-κB) protein is a heterodimer composed mainly for p65 and p50 subunits. Under normal conditions, NF-κB is present in the cytoplasm in an inactive state, bound to inhibitory protein κB (IκB). Activation with pro-inflammatory stimulus initiates an intracellular signalling cascade, resulting in the phosphorylation and subsequent degradation of IκB by the 26S-proteasome (Tanaka et al., 2001). IκB phosphorylation is mediated by the inhibitory protein kappaB kinase (IKK), complex that contains two catalytic subunits named IKKα (IKK1) and IKKβ (IKK2) and a non-catalytic regulatory subunit named NF-κB essential modulator (NEMO) or IKKγ (Tang et al., 2003). The degradation of IκBα releases NF-κB, allowing it to translocate into the nucleus and to induce the expression of cytokines, chemokines, anti-apoptotic and cell growth factors that are essential mediators of immune and inflammatory responses and tumorigenesis (Li and Verma, 2002). Thus, inhibition of NF-κB activation has attracted attention as a therapeutic approach for intervention in immune and inflammatory events (Lee et al., 2010).

Furthermore, LPS activates not only NF-κB, which is a pivotal transcription factor for inflammatory gene expression, but also induces phosphorylation of mitogen-activated protein kinases (MAPKs), which in turn leads to the activation of NF-κB in macrophages (Jung et al., 2008; Ha et al., 2010). MAPKs are signalling molecules that play critical roles in the regulation of cell growth, differentiation, cell survival/apoptosis, cellular response to cytokines, stress and inflammation. Three major groups of MAP kinases are identified: p38 MAP kinase family, extracellular signal-regulated kinase (ERK) family and c-Jun NH_2-terminal kinase (JNK) family (Platanias, 2003). MAPKs activity is also required for the expression of several macrophage functions, including NO production and can control the

synthesis and release of pro-inflammatory mediators by LPS-activated Raw 264.7 cells (Cho et al., 2003).

The modulatory effect of natural products on macrophages/monocytes-triggered inflammatory processes could contribute to the establishment of new therapeutic alternatives for the treatment of pathologies with a strong inflammatory component. This study aimed to explore the antioxidant and anti-inflammatory properties of propolis by addressing its molecular mechanism of action, through evaluation of its effect on NO production, activation of MAPKs and NF-κB signalling pathways *in vitro*. A possible effect of propolis on human hepatic carcinoma cell line (HepG-2) was investigated, since it is a useful model *in vitro* to determine hepatotoxicity, considering that this product can be used in human diets. Caffeic acid was studied as possible compound responsible for propolis action. Propolis composition was investigated as well.

2. Material and Methods

2.1. Propolis sample and caffeic acid

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. Propolis was ground and ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature, with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (110 mg/mL) (Sforcin et al., 2005).

Propolis was diluted in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Química, Madrid, Spain), supplemented with 10% non-inactivated fetal bovine serum (Gibco, Paisley, UK), 100 U/mL penicillin and 100 µg/mL streptomycin, and specific dilutions from this solution were prepared for each assay, in order to achieve different propolis concentrations: 5, 10, 25, 50 and 100 µg/mL.

The chemical analysis of propolis samples revealed that its main components were phenolic compounds. Caffeic acid, the major compound of hydroxycinnamic and phenolic acids in general (Touaibia et al., 2011), was purchased from Acros Organics (Morris Plains, NJ, USA) and diluted in 100 µL of 70% ethanol and DMEM media (1 mg/mL), preparing different concentrations: 5, 10, 25, 50 and 100 µg/mL. The same procedure was carried out with 70% ethanol (propolis and caffeic acid solvent) in order to obtain 0.03,

0.06, 0.15, 0.29 and 0.59% ethanol, which are the respective concentrations of alcohol found in propolis and caffeic acid concentrations.

2.2. Analysis of phenolic compounds composition in propolis by HPLC-PDA-ESI/MS^3

Propolis phenolic composition was assessed on a Surveyor high performance liquid chromatography coupled to photodiode-array detector - PDA (Surveyor) and interfaced with a Finnigan LCQ Advantage Ion Max tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an API-ES ionization chamber (HPLC-PDA-ESI/MS^n). Separation was performed on a Spherisorb ODS-2 column (150 x 2.1 mm i.d.; particle size, 3 μm; Waters Corp., Milford, MA, USA) and a Spherisorb ODS-2 guard cartridge (10 x 4.6 mm i.d.; particle size, 5 μm; Waters Corp., Milford, MA, USA) at 25 ºC. A mobile phase constituted by 2% aqueous formic acid (v/v) (A) and methanol (B) was used with a discontinuous gradient of 5% B (0-2 min), 5–15% B (2–10 min), 15–25% B (10–15 min), 25–50% B (15–50 min), 50–80% B (50–60 min), 80–100% B (60–70 min) followed by an isocratic elution (70–75 minutes), at a flow rate of 200 μL/min. The first detection was done with a PDA detector in a wavelength range 200–450 nm, followed by a second detection in the mass spectrometer. Mass analyses were obtained in the negative ion mode. The mass spectrometer was programmed to perform three consecutive scans: full mass (*m/z* 50–1500), MS^2 of the most abundant ion in the full mass and MS^3 of the most abundant ion in the MS^2. Source voltage was 4.7 kV and the capillary voltage and temperature were -7 V and 275 ºC, respectively. Nitrogen was used as sheath and auxiliary gas at 20 and 7 Finnigan arbitrary units, respectively. The normalized energy of collision was 41%, using helium as collision gas. Data treatment was carried out with XCALIBUR software (Thermo Scientific, Waltham, MA, USA).

The extract of propolis (5 mg/mL) was solubilized in 25% methanol (1 mL) and analyzed by comparison retention times and spectra with chemical standards: caffeic, *p*-coumaric, chlorogenic and ferulic acids (Sigma Chemical Co., St. Louis, USA).

2.3. Cell lines

Raw 264.7 cells, a mouse leukaemic monocyte macrophage cell line (ATCC TIB-71), were cultured in DMEM media supplemented with 10% inactivated fetal bovine

serum, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2.

Human hepatic carcinoma cell line (HepG-2 – ATCC HB-8065) cells were grown in DMEM supplemented with 1 g/L of glucose and pyruvate, 10% inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.5 g/L of sodium bicarbonate, at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. Along the experiments, both cells were monitored by microscopic observation in order to detect any morphological change.

2.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) – Antioxidant assay

DPPH was purchased from Sigma-Aldrich (St Louis, USA). Free radical-scavenging activity was evaluated according to Blois (1958). Aliquots of 100 µL were assessed by their reactivity with methanolic solution of 500 µM DPPH (500 µL) in the presence of 0.2 M acetate buffer, pH 6.0 (1 mL). Reaction mixtures (3 mL) were kept for 30 min at room temperature and in the dark. The decreases in the absorbance were measured at 517 nm, in a Cintra 101 GBC UV-Vis spectrophotometer. The $DPPH^{\bullet}$ concentration (µg/mL) in the reaction medium was calculated from the calibration curve determined by linear regression. Butylhydroxytoluene (BHT – Sigma Chemical Co., St. Louis, USA), a synthetic antioxidant, was used as reference standard.

2.5. Cell viability by MTT assay

Assessment of metabolically active cells was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich, St. Louis, USA) reduction colorimetric assay, as previously reported (Francisco et al., 2011). Raw 264.7 cells (0.3×10^6 cells/well) or HepG-2 cells (0.2×10^6 cells/well) were plated and allowed to stabilize for 12 h. Afterwards, cells were either maintained in culture medium (control) or pre-incubated with different concentrations of propolis, caffeic acid or the vehicle 70% ethanol for 1 h, and later activated with 1 µg/mL LPS (from *E. coli* - serotype 026:B6, Sigma Chemical Co., St. Louis, USA) for 24 h. After treatments, a solution of MTT (5 mg/mL in phosphate buffered saline) was added and cells were incubated at 37 ºC for 15 min (Francisco et al., 2011). Supernatants were removed and dark blue crystals of formazan solubilized with 300 µL acid isopropanol (0.04N HCl in isopropanol). Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

2.6. Nitrite production by the Griess assay

NO production was measured by nitrite accumulation in the culture supernatants, using a colorimetric reaction with the Griess reagent (Green et al., 1982). Briefly, 170 μL of culture supernatants were mixed with an equal volume of Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H_3PO_4] and maintained during 30 min in the dark. Nitrite concentration was determined by measuring the absorbance at 550 nm with a micro-plate reader (SLT, Austria). All measurements were performed in duplicate and expressed as NO micromolar concentrations.

2.7. Preparation of total protein extracts and Western blot analysis

Raw 264.7 cells (1.2 x 10^6 cells/well) were plated and, after stabilizing for 12 h, cells were either maintained in culture medium (control), or pre-incubated with 10 μg/mL of propolis, or 10 μg/mL of caffeic acid – concentrations that inhibited NO generation and were noncytotoxic, for 1 h and then 1 μg/mL LPS was added for 30, 15 or 10 minutes, to verify the exact period of the phosphorylation of proteins. Cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mM ethylenediamine tetraacetic acid) freshly supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails and sonicated (four times for 4 s at 40 μm peak to peak) in Vibra Cell sonicator (Sonics & Material INC.) to decrease viscosity. The nuclei and the insoluble cell debris were removed by centrifugation at 4 ºC, at 12.000 x *g* for 10 min. The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined by the bicinchoninic acid protein assay and cell lysates were denaturated in sample buffer (0.125 mM Tris pH 6.8, 2% (w/v) sodium dodecyl sulfate, 100 mM dithiothreitol, 10% glycerol and bromophenol blue). Western blot analysis was performed to evaluate the levels the activation of MAPKs and NF-κB signaling pathways. Briefly, equivalent amounts of protein (30 μg) were separated by 10% (v/v) SDS-PAGE followed by Western blotting. To examine the different proteins, the blots were incubated overnight at 4ºC with the respective primary antibodies: phospho-p38 MAPK (1:1000), phospho-JNK1/2 (1:1000), phospho-ERK1/2 (1:1000), phospho-IκB and total IκB (1:1000), all obtained from Cell Signaling Technologies (Danvers, MA, USA). Protein detection was performed using the

enhanced chemifluorescence system and the membranes were scanned for blue excited fluorescence on the Storm 860 (GE Healthcare). The generated signals were analyzed using the software TotalLab®. To demonstrate equivalent protein loading, membranes were stripped and reprobed with antibodies against the total form of MAPKs or with anti-*β*-tubulin antibody (Sigma Aldrich, St. Louis, USA).

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD) of 3 similar and independent experiments. For cell viability and NO production, one-way Anova followed by Dunnett's test were performed. For western blot assays, two-sided unpaired t-test was used. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Propolis composition by HPLC-PDA-ESI/MS^n

Propolis phenolic composition was evaluated by HPLC-PDA. Hydroxycinnamic acids and mainly caffeic acid derivatives were detected through chromatographic and UV spectral profiles (Fig. 1; Table 1), and only trace amounts of flavonoids were identified (Table 2). Structural elucidation was performed essentially on the basis of the HPLC-PDA-ESI/MS^n analysis (Tables 1 and 2).

Caffeic and *p*-coumaric acids (Rt 20.04 and 27.16, respectively) were identified by comparison of their Rt and UV spectra with standard compounds.

A derivative of chlorogenic acid (caffeoylquinic acid derivative) with Rt = 21.26 was identified on the basis of its fragments at *m/z* 353 and 191, corresponding to caffeoylquinic acid and quinic acid, respectively, this last fragment obtained in MS^3 by loss of one caffeic acid molecule. With similar UV spectra more three compounds were identified on the basis of their mass spectra.

Compound with Rt = 37.98 presented a pseudomolecular ion at *m/z* 515 and a fragmentation pattern typical of a dicaffeoylquinic acid. MS2 data showed fragments at *m/z* 353 $[(M-H)-162]^-$ and 191 $[(M-H)-324]^-$. Since the fragment at m/z 203 was not detected, the structure proposed for this compound was the 1,3-dicaffeoylquinic acid, according to Clifford et al. (2005).

Compound with Rt = 43.60 also showed a pseudomolecular ion at *m/z* 515. However, MS2 data evidenced the fragment at *m/z* 203 $[(M-H)-312]^-$. Given the relative abundance of the fragments 203 $[(M-H)-312]^-$ and 179 $[(M-H)-336]^-$ the structure proposed for this compound was 4,5-dicaffeoylquinic acid (Clifford et al., 2005).

Compound with Rt = 54.87 presented a pseudomolecular ion at *m/z* 677 and a fragmentation pattern typical of a tricaffeoylquinic acid. MS2 data exhibited fragments at *m/z* 515 $[(M-H)-162]^-$ and 353 $[(M-H)-162-162]^-$, this last signal corresponding to chlorogenic acid, which resulted from the loss of two caffeic acid molecules. The fragments at *m/z* 299, 255 and 203 with a relative intensity less than 7% suggested the presence of 3,4,5-tricaffeoylquinic acid as referred by Clifford et al. (2007).

Compound with Rt = 30.44 showed an UV maximum absorption (λ = 322 nm) slightly lower than that of caffeic acid and similar to the ferulic acid, and a deprotonated ion at *m/z* 461. MS^2 analysis evidenced a base peak at *m/z* 341 $[(M-H)-120]^-$ and a minor at *m/z* 371 $[(M-H)-90]^-$, which are fragments related to one *C*-hexosyl unit. In MS3 the most abundant ion observed at *m/z* 326 involves the loss of 15 a.m.u. $[M-H-120-15]^-$, occurring a minor fragment at *m/z* 149, which may be resulting of ferulate ion decarboxylated.

Flavonoids identification (Table 2) was performed according to the fragmentation pattern referred in Figueirinha et al. (2008).

3.2. Antioxidant activity

The antioxidant activity of propolis and caffeic acid was addressed by the DPPH method. The determination of the effective concentration (EC50) value expresses the amount of sample necessary to decrease the absorbance of DPPH by 50% (Antolovich et al., 2002). The EC_{50} was 4.67 μg/mL for BHT, 18.51 μg/mL for propolis and 2.5 μg/mL for caffeic acid, demonstrating that caffeic acid showed a higher antioxidant activity than propolis extract and BHT. Accordingly, caffeic acid derivatives present in our propolis sample can contribute to the antioxidant activity verified.

3.3. Effect on cell viability

The *in vitro* cytotoxic effect of propolis and caffeic acid on macrophages viability was represented as percentage of control (non-treated cells), as shown in Fig. 2. Viability higher than 80% was observed in concentrations lower than 25 μg/mL. A cytotoxic effect was observed using 25, 50 and 100 μg/mL of propolis and caffeic acid. 70% ethanol, in all

dilutions (0.03, 0.06, 0.15, 0.29 and 0.59% – the respective concentrations of alcohol found in propolis and caffeic acid concentrations, had no effect on cell viability (data not shown).

As to HepG-2 cells, no cytotoxic effects were seen using 5 and 10 μg/mL of propolis and caffeic acid (Fig. 3). These preliminary data suggested that such concentrations exhibited a strong anti-inflammatory potential without affecting cell viability.

3.4. Propolis effect on NO production

As shown in Fig. 4, untreated macrophages Raw 264.7 produced low levels of nitrites (1.3 ± 0.19 μM). After macrophages activation with LPS for 24 h, nitrite production increased to 13.9 ± 0.90 μM. On the other hand, macrophages pre-treatment with propolis or caffeic acid decreased the LPS-induced nitrite production in all non-cytotoxic concentrations (10.4 ± 1.3 and 5.5 ± 1.541 for 5 and 10 μg/mL of propolis, respectively; 10.2 ± 1.90 and 5.2 ± 0.78 for 5 μg/mL and 10 μg/mL of caffeic acid, respectively).

The concentration of propolis and caffeic acid used in the assays of intracellular signalling pathways by Western blot was chosen based on the absence of cytotoxicity, but inhibiting more than 50% of NO production (10 μg/mL of propolis and 10 μg/mL of caffeic acid).

3.5. Effect of propolis and caffeic acid in the NF-κB intracellular signalling pathways

Since propolis and caffeic acid inhibited NO production triggered by the strong pro-inflammatory stimulus LPS, we next evaluated whether these two compounds could block LPS-induced NF-κB translocation, preventing IκBα degradation. Raw 264.7 cells were pretreated with propolis or caffeic acid (10 μg/mL) for 1 h and incubated with LPS for 30, 15 and 10 min. As shown in Fig. 5 and Fig. 6, treatment with LPS for 10 min started the phosphorylation and subsequent degradation of IκBα, and at 15 min there was almost a complete degradation of cytosolic IκBα, indicating an increase in the NF-κB activity and consequently an increase in the production of inflammatory mediators, such as iNOS expression and NO production. After 30 min, this effect on degradation of IκBα remained.

Pre-treatment with propolis (Fig. 5A) and caffeic acid (Fig. 6A) inhibited LPS-induced phosphorylation of IκB and partially prevented IκBα degradation (Fig. 5B and

Fig. 6B, respectively) indicating an inhibitory effect in the LPS-induced NF-κB activity (at 10 min).

3.6. Effect of propolis and caffeic acid in the activation of MAPKs

The effect of propolis and caffeic acid on the activation of ERK1/2, JNK1/2 and p38 MAP kinases pathways was examined by detecting their phosphorylated forms by Western blot using specific antibodies. As shown in the Fig. 7 and Fig. 8, LPS stimulation for 30, 15 and 10 min induced phosphorylation of all MAPKs. On the other hand, pre-treatment with propolis and caffeic acid inhibited the LPS-induced phosphorylation of JNK1/2 (Fig. 7A and Fig. 8A, respectively) and p38 MAPK (Fig. 7B and Fig. 8B, respectively) at a short time (10 min), but had no effect in the ERK1/2 pathways (Fig. 7C and Fig. 8C, respectively). Propolis and caffeic acid alone added to control cells did not stimulate MAPKs signalling pathways. To demonstrate equivalent protein loading, membranes were incubated with antibodies against the total form of MAPKs, and there was no change in the proteins expression in our assays conditions (data not shown).

4. Discussion

Propolis chemical composition is very complex and dependent upon the source plant and local flora, so different biological activities are expected. Cinnamic acid derivatives and flavonoids were frequently reported in Brazilian propolis, which may be associated to antiulcer activity in green propolis (Lemos et al., 2007), while flavonoids such as C-glycosylflavones have been rarely identified. According to Righi et al. (2011), a C-glycosylflavone was referred, for the first time, in propolis, by studying the Brazilian red propolis. In Brazil, green propolis is obtained mainly in the southeast region and derived from apices of *Baccharis dracunculifolia* (Asteraceae), containing predominantly prenylated phenylpropanoids (artepillin C), chlorogenic and benzoic acids and triterpenoids. Brazilian red propolis may be found in the northeast region (Alagoas State) and has been chemically characterized as containing pterocarpans, isoflavonoids, chalcones, prenylated benzophenones and phenylpropanoids (Salatino et al., 2005; Awale et al., 2008). The phenolic profile of our propolis sample by HPLC-PDA-MS^n showed that its main compounds were hydroxycinnamic acids (di- and tricaffeoylquinic acids and *p*-coumaric acid, among others). Curiously, three apigenin *C*-glycosides were also detected, but at very low concentration.

Caffeic acid and its conjugates such as chlorogenic and caftaric acids demonstrated to be powerful antioxidants (Fukumoto and Mazza, 2000). Cinnamic acid derivatives from Brazilian propolis showed a potent antioxidant activity as well (Banskota et al., 2000). Besides, caffeic acid showed antiproliferative properties and cytotoxic activity against different human cancer cell lines (Bufalo et al., 2010; Touaibia, et al., 2011). Since there are few data on the anti-inflammatory activities of caffeic acid, this work investigated its involvement in propolis action. Propolis extract has shown a cytotoxic action towards a variety of tumor cell lines, but not to normal cells (Kouidhi et al., 2010). Our results demonstrated that only higher concentrations (25, 50 and 100 μg/mL) of our propolis sample and caffeic acid exerted a cytotoxic action on Raw 264.7 cells and on HepG-2 cells.

NO production was evaluated using noncytotoxic concentrations of propolis and its isolated compound, and in an attempt to identify a possible mechanism for their inhibitory effect on NO production we further analyzed signaling pathways by Western blot, such as the activation of MAPKs and the transcription factor NF-κB. Data showed that propolis and caffeic acid inhibited LPS-induced NO production by Raw 264.7 macrophages in a concentration-dependent manner, acting at the transcriptional level and suggesting that their anti-inflammatory effect were mediated by down-regulating NF-κB, p38 MAP kinase and JNK1/2, but not ERK1/2.

Ansorge et al. (2003) reported the effect of isolated constituents from propolis on ERK-2 mRNA in human peripheral blood mononuclear cells (PBMC), observing a decreased ERK-2 expression when phythemagglutinin (PHA)-activated PBMC were exposed to quercetin, hesperidin, or caffeic acid phenethyl ester (CAPE). These data suggested that propolis constituents modulated the MAP kinase ERK-2; however, this finding did not exclude additional signal pathways and transcription factors targeted by caffeic acid derivatives or plant flavonoids on immunomodulatory activities of propolis. No significant differences were found in the ERK1/2 signalling pathway after Raw 264.7 cells incubation with propolis or caffeic acid and subsequently stimulated by LPS in different periods of time. Thus, a different chemical composition of propolis can support these results.

NF-κB activation may be responsible, in part, for increased inflammatory mediators production, including NO (Wang et al., 2010). Activation of MAPKs can regulate NO production by controlling the activation of NF-κB (Jung et al., 2008), and various

intracellular signaling pathways are involved in the modulation of NF-κB and inflammatory mediators' expression. MAPKs are a group of signaling molecules that play an important role in inflammatory processes (Jung et al., 2008) and it has been previously demonstrated that LPS regulates NO production through MAPK signaling pathways activation. LPS treatment results in the phosphorylation of p38 MAPK, ERK1/2 and JNK1/2, leading to activation of NF-κB in macrophages (Cario et al., 2000). Interestingly, only the phosphorylation of p38 MAPK and JNK1/2 in response to LPS was decreased by propolis and caffeic acid treatments, whereas no significant changes were observed in LPS-induced phosphorylation of ERK1/2 in response to both treatments. These results suggested that p38 MAPK and JNK1/2 but not ERK1/2 were involved in the inhibitory effect of propolis and caffeic acid on LPS-induced NF-κB activation and NO production. Accordingly, previous studies demonstrated that JNK1/2 (Zhou et al., 2008) and p38 MAPK, but not ERK1/2 (Chen and Wang, 1999) modulated NO production in LPS-stimulated Raw 264.7 macrophages.

Among propolis constituents, anti-inflammatory properties have also been demonstrated for hydroxycinnamic acids, namely *p*-coumaric and ferulic acids, in LPS-stimulated Raw 267.4 macrophages, showing that the inhibition of NO production was mainly due to down-regulation of the expression of NF-κB-mediated iNOS gene (Kim et al., 2012). Moreover, caffeic acid derivatives also showed inhibitory effects on LPS-induced NO production in Raw 264.7 cells (Uwai et al., 2008). CAPE is the most extensively studied caffeic acid derivative of propolis, which has been reported as an anti-inflammatory compound and also as a potent and specific inhibitor of NF-κB activation and protein kinase B (Aκt) phosphorylation in T cells, with no effect on p38 MAP kinase phosphorylation (Wang et al., 2010).

Caffeoylquinic acid derivatives inhibited LPS-induced Raw 264.7 macrophage inflammation by suppressing NO/iNOS and prostaglandin E2/cyclooxygenase-2 pathways through inhibiting nucleus translocation of NF-κB subunits, p50 and p65 (Puangpraphant et al., 2011). Tricaffeoylquinic acid seems to attenuate TNF-α-stimulated inflammatory mediators production by keratinocytes, suppressing the activation of Akt and NF-κB pathways which may be mediated by reactive oxygen species, suggesting that this compound may exert inhibitory effects against the pro-inflammatory mediator-induced skin disease (Lee et al., 2011). Another active component present in the propolis (*p*-

coumaric acid) showed anti-inflammatory effects in adjuvant-induced arthritic rats by decreasing TNF-α expression (Pragasam et al., 2013).

Taken together, our data are in agreement with those found in literature, since propolis and caffeic acid exerted a potent *in vitro* anti-inflammatory effect by inhibiting NO accumulation, what could be mediated by down-regulating NF-κB pathways, through preventing IκBα degradation. Therefore, it is likely that propolis and caffeic acid attenuated LPS-induced NO production possibly by inhibiting phosphorylation of p38 MAPK and JNK1/2, suggesting that both compounds selectively inhibit different LPS-induced pro-inflammatory signalling cascades. Caffeic acid highly contributed to the anti-inflammatory properties of propolis extract, since it seemed to be involved in the inhibition of LPS-induced NF-κB, p38 MAPK and JNK1/2 activation.

In addition, propolis and caffeic acid showed no hepatotoxicity using the same anti-inflammatory concentrations. This is a crucial information, since propolis is extensively used in food, beverage and in folk medicine for treating various ailments, and may be a potential product for the development of new anti-inflammatory drugs (Sforcin and Bankova, 2011).

Acknowledgements

The authors wish to thank the Programa Institucional de Bolsas de Doutorado Sanduíche no Exterior da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PDSE-CAPES, 573711-7) for the grant and Foundation for Science and Technology (FCT) for financial support by the project PEst-OE/SAU/UI0177/2011.

References

Ansorge, S., Reinhold, D., Lendeckel, U., 2003. Propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGF-beta1 production of human immune cells. Zeitschrift fur Naturforschung 58c, 580-589.

Antolovich, M., Prenzler, P.D., Patsalides, E., Mcdonald, S., Robards, K., 2002. Methods for testing antioxidant activity. The Analyst 127, 183-198.

Awale S., Li F., Onozuka H., Esurni H., Tezuka Y., Kadota S., 2008. Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. Bioorganic & Medical Chemistry Letters 16, 181-189.

Bankova, V.S., 2005. Recent trends and important developments in propolis research. Evidence-based Complementary and Alternative Medicine 2, 29-32.

Banskota, A.H., Tezuka, Y., Adnyana, I.K., Midorikawa, K., Matsushige, K., Message, D., Huertas, A.A., Kadota, S., 2000. Cytotoxic, hepatoprotective and free radical scavenging effects of propolis from Brazil, Peru, the Netherlands and Chine. Journal of Ethnopharmacology 72, 239-246.

Bazo, A.P., Rodrigues, M.A., Sforcin, J.M., Camargo, J.L.V., Ribeiro, L.R., Salvadori, D.M.F., 2002. Protective action of propolis on the rat colon carcinogenesis. Teratogenesis, Carcinogenesis and Mutagenesis 22, 183-194.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181, 1199-1200.

Brodsky, M., Halpert, G., Albeck, M., Sredni, B., 2010. The anti-inflammatory effects of the tellurium redox modulating compound, AS101, are associated with regulation of NFκB signaling pathway and nitric oxide induction in macrophages. Journal of Inflammation 7, 1-8.

Búfalo, M.C., Figueiredo, A.S., De Sousa, J.P.B., Candeias, J.M., Bastos, J.K., Sforcin, J.M., 2009. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. Journal of Applied Microbiology 107, 1669-1680.

Búfalo, M.C., Candeias, J.M.G., Sousa, J.P.B., Bastos, J.K., Sforcin, J.M., 2010. *In vitro* cytotoxic activity of *Baccharis dracunculifolia* and propolis against HEp-2 cells. Natural Product Research 24, 1710-1718.

Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C., Podolsky, D.K., 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. Journal of Immunology 164, 966-972.

Chen, C.C., Wang, J.K., 1999. p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in Raw 264.7 macrophages. Molecular Pharmacology 55, 481-488.

Cho, S.Y., Park, S.J., Kwon, M.J., Jeong, T.S., Bok, S.H., Choi, W.Y., Jeong, W.I., Ryu, S.Y., Do, S.H., Lee, C.S., Song, J.C., Jeong, K.S., 2003. Quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in lipopolysaccharide-stimulated macrophage. Molecular and Cellular Biochemistry 243, 153-160.

Clifford, M.N., Knight, S., Kuhnert, N., 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. Journal of Agricultural and Food Chemistry 53, 3821-3832.

Clifford, M.N., Wu, W., Kirkpatrick, J., Kuhnert, N., 2007. Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. Journal of Agricultural and Food Chemistry 55, 929-936.

Figueirinha, A., Paranhos, A., Péres-Alonso, J.J., Santos-Buelga, C., Batista, M.T., 2008. *Cymbopogon citratus* leaves: characterisation of flavonoids by HPLC-PDA-ESI/MS/MS and an approach to their potential as a source of bioactive polyphenols. Food Chemistry 110, 718-728.

Francisco, V., Figueirinha, A., Neves, B.M., García-Rodríguez, C., Lopes, M.C., Cruz, M.T., Bastista, M.T., 2011. *Cymbopogon citratus* as source of new and safe anti-

inflammatory drugs: bio-guided assay using lipopolysaccharide-stimulated macrophages. Journal of Ethnopharmacology 133, 818-827.

Freitas, S.F., Shinohara, L., Sforcin, J.M., Guimarães, S., 2006. In vitro effects of propolis on *Giardia duodenalis* trophozoites. Phytomedicine 13, 170-175.

Fukumoto, L.R., Mazza, G., 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. Journal of Agricultural Food and Chemistry 48, 3597-3604.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analytical Biochemistry 126, 131-138.

Ha, S.K., Moon, E., Kim, S.Y., 2010. Crysin suppresses LPS-stimulated proinflammatory responses by blocking NF-κB and JNK activations in microglia cells. Neuroscience Letters 485, 143-147.

Jung, W.K., Choi, I., Lee, D.Y., Yea, S.S., Choi, Y.H., Kim, M.M., Park, S.G., Seo, S.K., Lee, S.W., Lee, C.M., Park, Y.M., Choi, I.W., 2008. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in Raw 264.7 macrophages via the p38/ERK and NF-kappaB pathways. The International Journal of Biochemistry & Cell Biology 40, 2572-2582.

Kim, E.O., Min, K.J., Kwon, T.K., Um, B.H., Moreau, R.A., Choi, S.W., 2012. Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated Raw 264.7 macrophages. Food and Chemical Toxicology 50, 1309-1316.

Kolankaya, D., Selmanoglu, G., Sorkun, K., Salih, B., 2012. Protective effects of Turkish propolis on alcohol-induced serum lipid changes and liver injury in male rats. Food Chemical 78, 213-217.

Kouidhi, B., Zmantar, T., Bakhrouf, A., 2010. Anti-cariogenic and anti-biofilms activity of Tunisian propolis extract and its potential protective effect against cancer cells proliferation. Anaerobe 16, 566-571.

Kumazawa, S., Ahn, M.R., Fujimoto, T., Kato, M., 2010. Radical-scavenging activity and phenolic constituents of propolis from different regions of Argentina. Natural Product Research 24, 804-812.

Lee, Y., Shin, D.H., Kim, J.H., Hong, S., Choi, D., Kim, Y.J., Kwak, M.K., Jung, Y., 2010. Caffeic acid phenethyl ester-mediated Nrf2 activation and IκB kinase inhibition are involved in NFκB inhibitory effect: structural analysis for NFκB inhibition. European Journal of Pharmacology 643, 21-28.

Lee, C.S., Lee, S.A., Kim, Y.J., Seo, S.J., Lee, M.W., 2011. 3,4,5-Tricaffeoylquinic acid inhibits tumor necrosis factor-α-stimulated production of inflammatory mediators in keratinocytes via suppression of Akt- and NF-κB-pathways. International Immunopharmacology 11, 1715-1723.

Lemos M., de Barros M.P., Sousa J.P., da Silva Filho A.A., Bastos J.K., de Andrade S.F. 2007. *Baccharis dracunculifolia*, the main botanical source of Brazilian green propolis, displays antiulcer activity. Journal of Pharmacy and Pharmacology 59, 603-608.

Li, Q., Verma, I.M., 2002. NF-kappa B regulation in the immune system. Nature Reviews Immunology 2, 725-734.

Nagaoka, T., Banskota, A.H., Tezuka, Y., Midorikawa, K., Matsushige, K., Kadota, S., 2003. Caffeic acid phenethyl ester (CAPE) analogues: potent nitric oxide inhibitors from the Netherlands propolis. Biological & Pharmaceutical Bulletin 26, 487-491.

Platanias, L.C., 2003. Map kinase signaling pathways and hematologic malignancies. Blood 101, 4667-4679.

Pragasam, S.J., Venkatesan, V., Rassol, M., 2013. Immunomodulatory and anti-inflammatory effect of *p*-coumaric acid, a common dietary polyphenol on experimental inflammation in rats. Inflammation 36, 169-176.

Puangpraphant, S., Berhow, M.A., Vermillion, K., Potts, G., Mejia, E.G., 2011. Dicaffeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and -3 in human colon cancer cells. Molecular Nutrition & Food Research 55, 1509-1522.

Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A. 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. Journal Science of Food and Agriculture 91, 2363-2370.

Salatino A., Teixeira E.W., Negri G., Message D., 2005. Origin and chemical variation of Brazilian propolis. Evidence Based Complementary and Alternative Medicine 2, 33-38.

Sforcin, J.M., 2007. Propolis and the immune system: a review. Journal of Ethnopharmacology 113, 1-14.

Sforcin, J.M., Bankova, V., 2011. Propolis: is there a potential for the development of new drugs? Journal of Ethnopharmacology 133, 253-260.

Sforcin, J.M., Fernandes Jr, A., Lopes, C.A.M., Bankova, V., Funari, S.R.C., 2000. Seasonal effect on Brazilian propolis antibacterial activity. Journal of Ethnopharmacology 73, 243-249.

Sforcin, J.M., Orsi, R.O., Bankova, V., 2005. Effect of propolis, some isolated compounds and its source plant on antibody production. Journal of Ethnopharmacology 98, 301-305.

Silva, J.C., Rodrigues, S., Feas, X., Estevinho, L.M., 2012. Antimicrobial activity, phenolic profile and role in the inflammation of propolis. Food and Chemical Toxicology 50, 1790-1795.

Tanaka, K., Kawakami, T., Tateishi, K., Yashiroda, H., Chiba, T., 2001. Control of IκBα proteolysis by the ubiquitin-proteasome pathway. Biochimie 83, 351-356.

Tang, E.D., Wang, C.Y., Xiong, Y., Guan, K.L., 2003. A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. Journal of Biological Chemistry 278, 37297-37305.

Touaibia, M., Jean-François, J., Doiron, J. 2011. Caffeic acid, a versatile pharmacophore: an overview. Mini-Reviews in Medicinal Chemistry, 11, 695-713.

Uwai, K., Osanai, Y., Imaizumi, T., Kanno, S., Takeshita, M., Ishikawa, M., 2008. Inhibitory effect of the alkyl side chain of caffeic acid analogues on

lipopolysaccharide-induced nitric oxide production in Raw 264.7 macrophages. Bioorganic & Medicinal Chemistry 16, 7795-7803.

Wang, L.C., Chu, K.H., Liang, Y.C., Lin, Y.L., Chiang, B.L., 2010. Caffeic acid phenethyl ester inhibits nuclear factor-κB and protein kinase B signaling pathways and induces caspase-3 expression in primary human $CD4^+$ T cells. Clinical and Experimental Immunology 160, 223-232.

Zhou, H.Y., Shin, E.M., Guo, L.Y., Youn, U.J., Bae, K., Kang, S.S., Zou, L.B., Kim, Y.S., 2008. Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in Raw 264.7 macrophages via NF-kappaB, JNK and p38 MAPK inactivation. European Journal of Pharmacology 586, 340-349.

Table 1. UV-vis and MS spectra and proposal for the main hydroxycinnamic acids identification in propolis.

Rt (min)	HPLC-PDA λ max (nm)	HPLC-ESI/MS^n (*m/z*)			
		[M-H]$^-$	MS^2	MS^3	Proposed compound
20.04	256, 295sh, 326	nd	-	-	caffeic acid
21.26	252, 295sh, 325	467	353(100)	191(100)	caffeoylquinic acid derivative
27.16	244, 291sh, 306	nd	-	-	*p*-coumaric acid
30.44	265, 295sh, 322	461	446(2), 371(2), 341(100), 326(2), 313(2)	326(100), 313(70), 298(55), 254(2), 149(2)	ferulic acid derivative
37.98	253, 295sh, 328	515	379(10), 353(100), 179(2)	191(100), 179(80)	1,3-dicaffeoylquinic acid
43.60	253, 295sh, 328	515	379(10), 353(100), 335(15), 203(20)	173(100), 179(95), 191(18), 135(9)	4,5-dicaffeoylquinic acid
54.87	256, 295sh, 327	677	582(2), 515(100), 353(5)	469(2), 353(100), 299(5), 255(3), 203(4),173(10)	3,4,5-tricaffeoylquinic acid

sh: sholder; nd: not detected

Table 2. UV-vis and MS spectra and proposed flavonoids identification in propolis.

Rt (min)	HPLC-PDA λ max (nm)	HPLC-ESI/MS^n (*m/z*)			
		[M-H]$^-$	MS^2	MS^3	Proposed compound
27.61	273, 295sh, 326	593	575(10), 503(35), 473(100), 353(45), 325(2)	455(2), 383(20), 353(100), 325(2)	6,8 Di-*C*-hexosyl apigenin
31.06	271, 331	563	545(20), 503(65), 473(100), 353(60), 311(5), 171(2)	455(2), 383(5), 353(100), 326(2)	6-*C*-Pentosyl-8-*C*-hexosyl apigenin
37.49	272, 329	563	545(5), 473(60), 443(100), 353(30), 311(2), 251(2)	383(10), 353(100), 337(2)	6-*C*-Hexosyl-8-*C*-pentosyl apigenin

sh: sholder

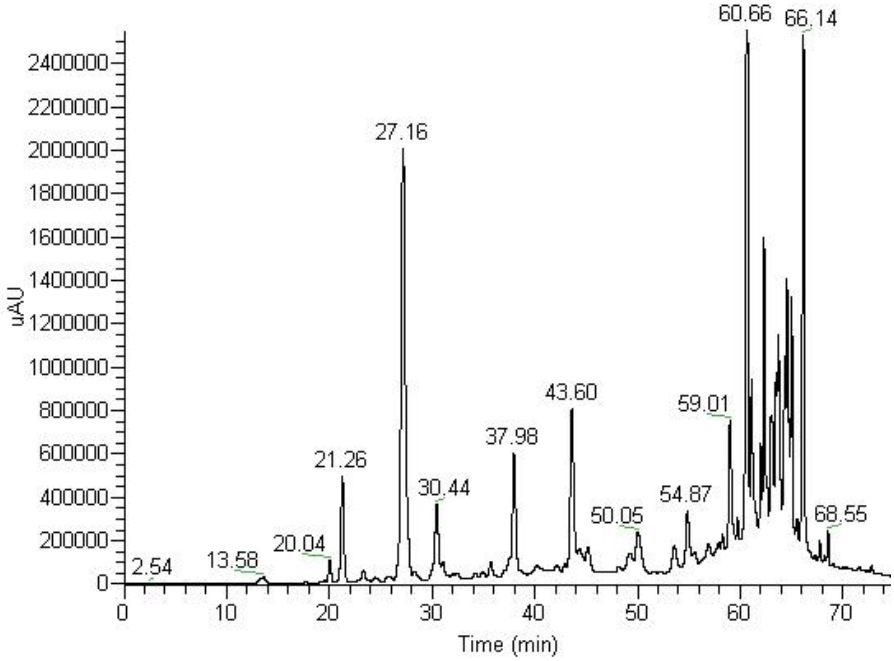

Fig. 1. HPLC profile of a propolis methanol extract, recorded at 320 nm, providing two major fractions: hydroxycinnamic acids and flavonoids. Identification of caffeic acid derivatives is proposed in Table 1.

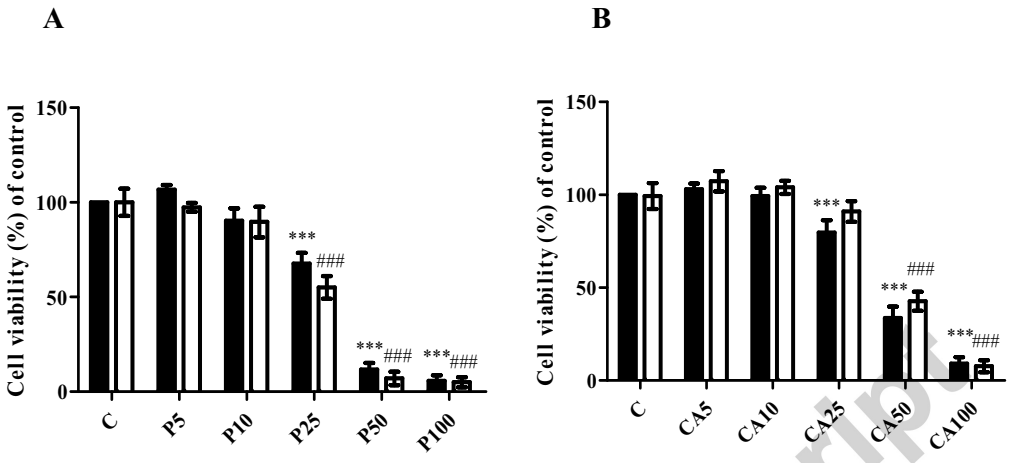

Fig. 2. Cell viability (% of the control) of Raw 264.7 cells after incubation with (A) propolis (P) or (B) caffeic acid (CA) (5, 10, 25, 50 and 100 µg/mL) for 1 h, followed incubation with LPS (white bar) or without LPS (black bar) for 24 h. Data represent mean ± SD of 3 independent assays. $^{***}P < 0.001$, compared to control without LPS; $^{###}P < 0.001$, compared to control with LPS.

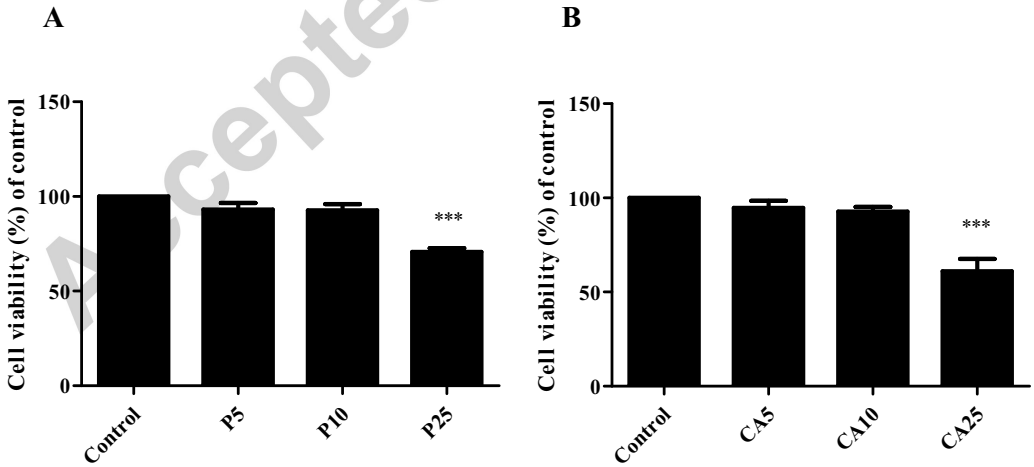

Fig. 3. Cell viability (% of the control) of HepG-2 cells after incubation with (A) propolis (P) or (B) caffeic acid (CA) (5, 10, 25 µg/mL) for 24 h. Data represent mean ± SD of 3 independent assays. $^{***}P < 0.001$, compared to control.

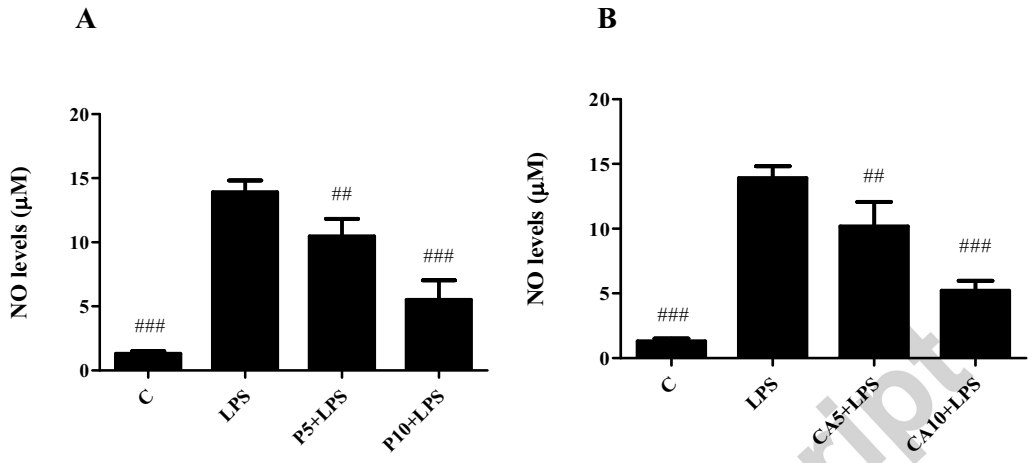

Fig. 4. Effect of (A) propolis (P) or (B) caffeic acid (CA) on NO levels (μM). Raw 264.7 cells were maintained in culture medium (control) or pre-incubated with propolis or caffeic acid (5 and 10 μg/mL) for 1 h, and then stimulated with 1 μg/mL LPS for 24 h. Data represent mean ± SD of 3 experiments. $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, compared to LPS.

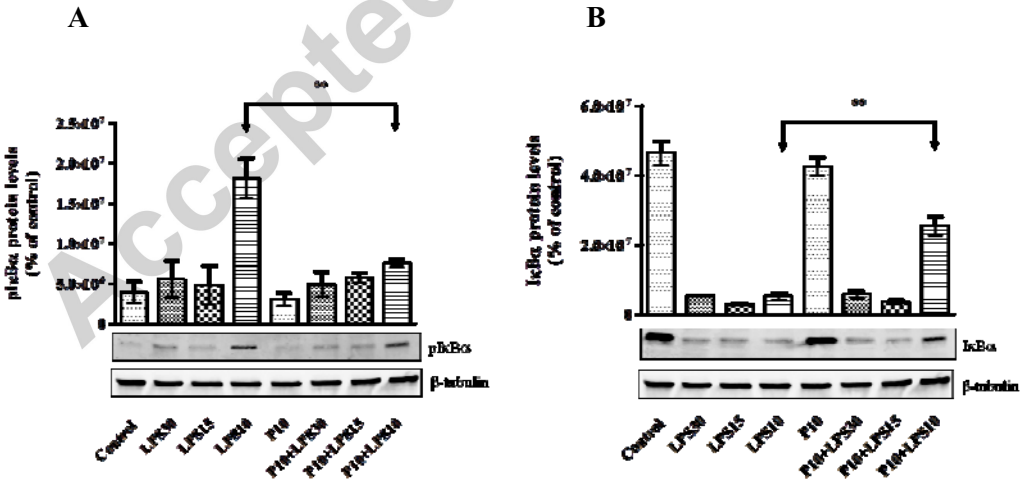

Fig. 5. Effect of propolis (P) on the phosphorylation of IκB (A) and total IκB (B) in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with propolis (P-10 μg/mL) for 1 h and then incubated with LPS (1 μg/mL) for 30, 15 and 10 min. An anti-β tubulin

antibody was used to confirm equal protein loading. The blot is representative of 3 similar blots. $^{**}P < 0.01$, compared to LPS10; pIκBα= 40 kDa and IκBα= 39 kDa.

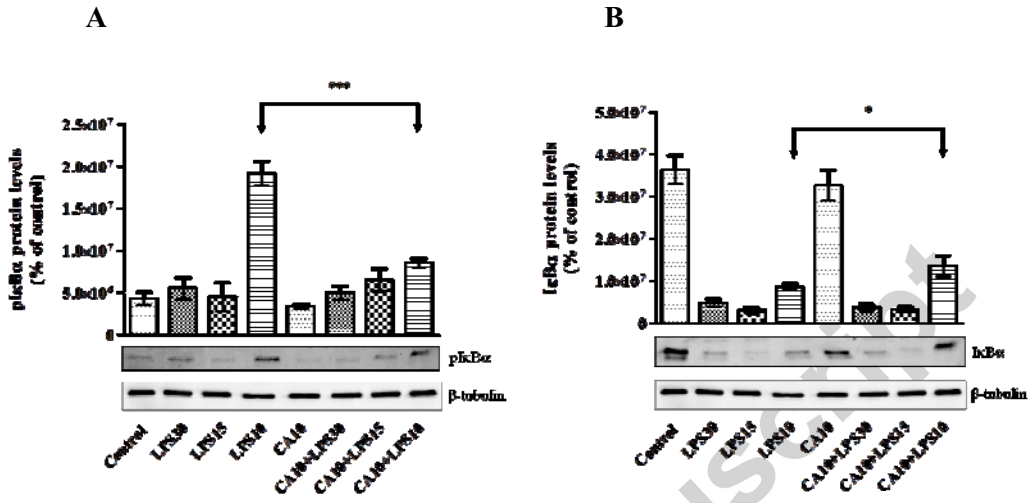

Fig. 6. Effect of caffeic acid (CA) on the phosphorylation of IκB (A) and total IκB (B) in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with caffeic acid (P-10 μg/mL) for 1 h and then incubated with LPS (1 μg/mL) for 30, 15 and 10 min. An anti-β tubulin antibody was used to confirm equal protein loading. The blot is representative of 3 similar blots. $^{***}P < 0.001$; $^{*}P < 0.05$, compared to LPS10; pIκBα= 40 kDa and IκBα= 39 kDa.

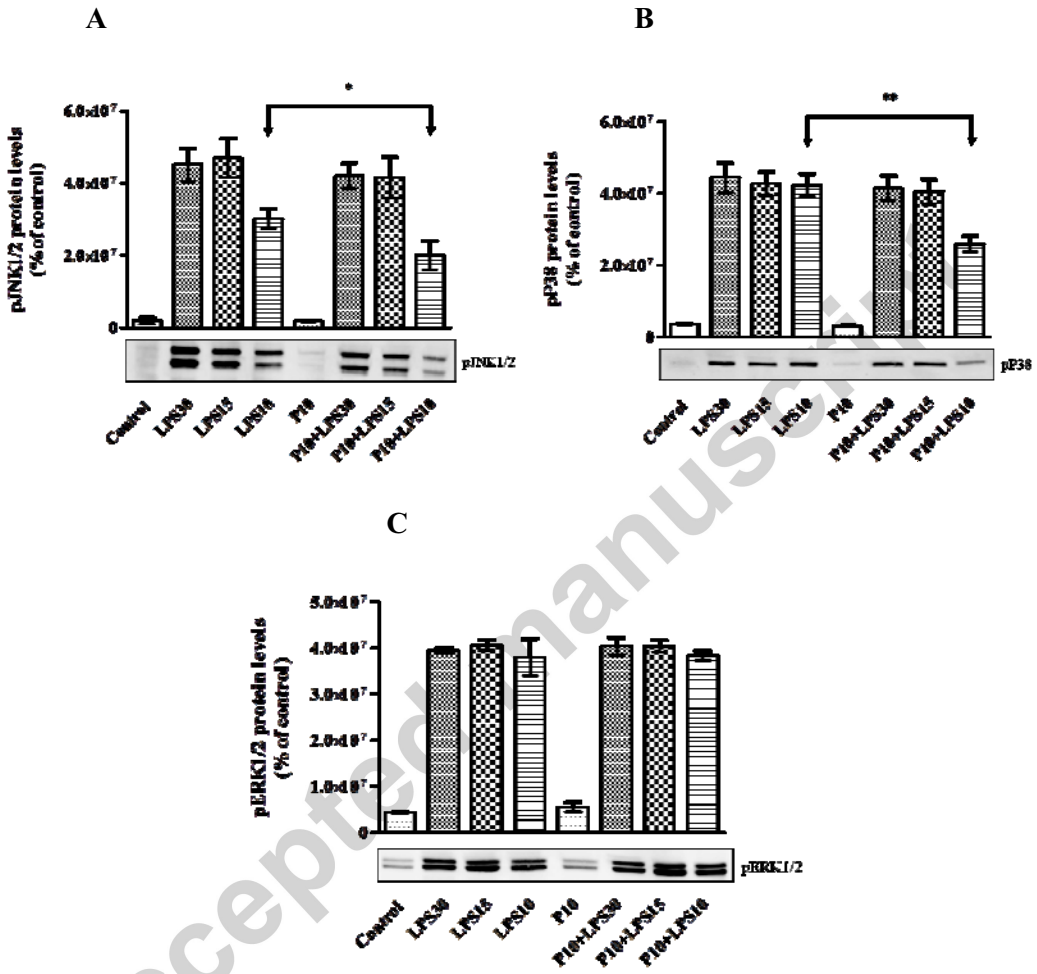

Fig. 7. Effect of propolis on pJNK1/2 (A), pP38 MAPkinase (B) and pERK1/2 (C) activation in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with propolis (P-10 μg/mL) for 1 h and then incubated with LPS (1 μg/mL) for three time points (30, 15 and 10 min). The blot is representative of 3 similar blots. $^{*}P < 0.05$; $^{**}P < 0.01$, compared to LPS10; pJNK= 46, 54 kDa; pP38= 43 kDa; pERK= 42, 44 kDa.

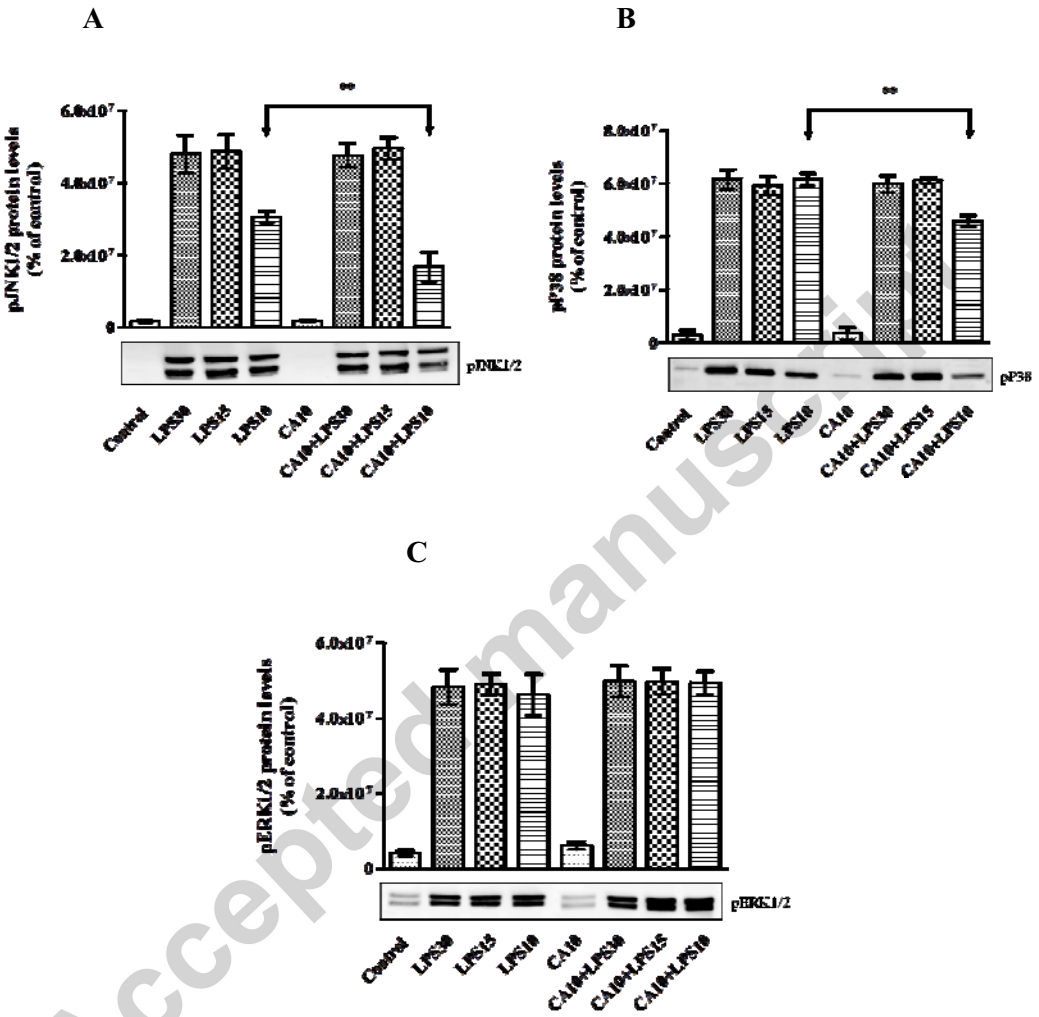

Fig. 8. Effect of caffeic acid on pJNK1/2 (A), pP38 MAPkinase (B) and pERK1/2 (C) activation in LPS-stimulated Raw 264.7 cells. Cells were pre-treated with caffeic acid (CA-10 μg/mL) for 1 h and then incubated with LPS (1 μg/mL) for three time points (30, 15 and 10 min). The blot is representative of 3 similar blots. $^{**}P < 0.01$, compared to LPS10; pJNK= 46, 54 kDa; pP38= 43 kDa; pERK= 42, 44 kDa.

The antioxidant and anti-inflammatory properties of propolis (P) and caffeic acid (CA) were investigated, by addressing its intracellular mechanism of action

Raw 264.7 cells

culture supernatants

P, CA ⊣ ↓

NO levels

Cell viability (MTT)

↓

> 80% viable cells using [5 and 10] of P or CA

HepG-2cells

Cell viability (MTT)

↓

> 80% viable cells using [5 and 10] of P or CA

Raw 264.7 cells

↓

[10] of P or CA

↓

LPS

(10, 15, 30 min)

P, CA ⊣ → pIκB

P, CA ⊣ → pJNK1/2

P, CA ⊣ → p38 MAP kinase

→ pERK1/2

Propolis exerted antioxidant and anti-inflammatory activities and caffeic acid may be involved in its inhibitory effects on NO production and intracellular signaling cascades, suggesting its use as a natural source of safe anti-inflammatory drugs