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The inhibitory effect of propolis and caffeic acid phenethyl ester on cyclooxygenase activity in J774 macrophages

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Summary

The effect of an ethanolic extract of propolis, with and without CAPE, and some of its components on cyclooxygenase (COX-1 and COX-2) activity in J774 macrophages has been investigated. COX-1 and COX-2 activity, measaured as prostaglandin E_2 (PGE_2) production, were concentration-dependently inhibited by propolis (3×10^{-3}–3×10^{2} µgml^{-1}) with an IC_{50} of 2.7 µgml^{-1} and 4.8×10^{-2} µgml^{-1}, respectively. Among the compounds tested pinocembrin and caffeic, ferulic, cinnamic and chlorogenic acids did not affect the activity of COX isoforms. Conversely, CAPE (2.8×10^{-4}–28 µgml^{-1}; 10^{-9}–10^{-4} M) and galangin (2.7×10^{-4}–27 µgml^{-1}; 10^{-9}–10^{-4} M) were effective, the last being about ten-twenty times less potent. In fact the IC_{50} of CAPE for COX-1 and COX-2 were 4.4×10^{-1} µgml^{-1} (1.5×10^{-6} M) and 2×10^{-3} µgml^{-1} (6.3×10^{-9} M), respectively. The IC_{50} of galangin were 3.7 µgml^{-1} (15×10^{-6} M) and 3×10^{-2} µgml^{-1} (120×10^{-9} M), for COX-1 and COX-2 respectively. To better investigate the role of CAPE, we tested the action of the ethanolic extract of propolis deprived of CAPE, which resulted about ten times less potent than the extract with CAPE in the inhibition of both COX-1 and COX-2, with an IC_{50} of 30 µgml^{-1} and 5.3×10^{-1} µgml^{-1}, respectively. Moreover the comparison of the inhibition curves showed a significant difference ($p < 0.001$).These results suggest that both CAPE and galangin contribute to the overall activity of propolis, CAPE being more effective.

Key words: cyclooxygenase, CAPE, galangin, J774 macrophages, prostaglandins, propolis

Introduction

Propolis is a resinous material elaborated by honey bees (*Apis mellifera*) well known for its medical effects, including antinflammatory, antiviral, immunostimulatory and carcinostatic activities (Dobrovolski et al., 1991). The most used formulation in the folk medicine is the ethanol extract (Mezenes et al., 1999). Standardization of propolis preparations is indeed difficult because of changes in chemical composition and pharmacological activities, resulting from variation in geographical and botanical origin (Ghisalberti, 1997). The wide spectrum of propolis activities was mainly attributed to the large number of flavonoids (Vennat et al., 1995). In addition to flavonoids, propolis contains cinnamic derivatives such as caffeic, ferulic, cinnamic, chlorogenic acids and its esters (Mirzoeva and Calder, 1996). Recently it has been reported that the caffeic acid phenethyl ester (CAPE), a component of propolis, exherts a potent antinflammatory activity. The antinflammatory properties of CAPE have been attributed to the suppression of prostaglandin (PG) and leukotrienes synthesis (Mirzoeva and Calder, 1996). It has also been reported that CAPE inhibits in vitro and in vivo histamine release in the rat (Scheller et al., 2000).

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PG, produced by cyclooxygenase (E. C. 1.14.991; COX), are important antinflammatory mediators (Vane, 1971). The enzyme is present in constitutive (COX-1) and inducible (COX-2) isoform. The constitutively expressed isoform release PG involved in physiological events in various organs of the body. The inducible isoform, which is expressed by several cell types in response to inflammatory and immunological stimuli, is responsible for the increased PG generation at the inflammation site (Smith and Dewitt, 1995).

Unstimulated J774 macrophages possess a significant COX-1 activity, whereas the immunostimulation by endotoxin, such as lipopolysaccharides, results in a significant induction of COX-2 and a down regulation of COX-1 (Zingarelli et al., 1997).

The present study was designed to investigate the role of CAPE and other components in the overall antinflammatory activity of propolis. To this purpose we have investigated the effect of an ethanolic extract of propolis, with and without CAPE, and some of its components on COX-1 and COX-2 activity, measured as PGE_2 production, in J774 macrophages.

Materials and Methods

Drugs tested

• ***Propolis:*** The dried ethanol extract of propolis, with and without CAPE, was obtained from Carlo Sessa, Milan, Italy.

Propolis extract is a commercial product consisting of propolis deprived of waxes; it is usually obtained from propolis by alcoolic extraction and normally called "propolis".

• ***Propolis deprived of CAPE:*** Ten g of propolis extract were dissolved in 150 ml of methanol and 30 ml of 2N sulphuric acid were added. The vessel was tightly capped and heated for 1 hour in a boiling water bath. After cooling, the solution was neutralized to pH 7,0 with 25% ammonia solution and the methanol was rotary evaporated under vacuum. The hydrolysis products (caffeic acid and phenethyl alcool) were removed from the residual mass by extracting twice with 200 ml water under reflux and discarding the water solutions. The residue was finally dried under vacuum. The yield was about 95%.

Original propolis, water solutions and residual propolis were subjected to thin layer chromatography (TLC) on silica gel using a fluorescent indicator (Kieselgel 60 WF_{254}). The developing solvent was: toluene/ethyl formiate/formic acid 6/3/0.5. Reference compounds were CAPE, caffeic acid and phenethyl alcool, revealed by UV lights (254 nm). Flavonoids and other components were detected by a methanol solutions of 1% 2-aminoethyl diphenylborate. CAPE and caffeic acid were also revealed by this reagent (see Fig. 1).

• ***Other compounds:*** CAPE, caffeic, ferulic, cinnamic, chlorogenic acids and galangin were obtained from Sigma-Aldrich, Milan, Italy. Pinocembrin was obtained from Trimital, Milan, Italy.

Stock solutions of test compounds were prepared in ethanol, an equivalent amount of ethanol was included in control samples.

• ***Determination of total flavonoids (as galangine) and caffeic acid contents in original and residual propolis:*** Galangine was determined spectrophotometrically (Alì et al., 1997), while caffeic acid was determined by HPLC (Alì et al., 1997).

Materials

Arachidonic acid was obtained from SPIBIO, Paris, France. [^{3}H-PGE_2] was from NEN Du Pont (Milan, Italy). TLC plates were from Merck (Darmstadt, Germany). Bacterial lipopolysaccharide from *Salmonella thyphosa* (LPS) and all other reagents and compounds used were obtained from Sigma-Aldrich, Milan, Italy.

Cell culture

The murine monocyte/macrophage J774 cell line was grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 2mM glutamine, 25 mM Hepes, penicillin (100 µ/ml), streptomycin (100 μgml^{-1}), 10% foetal bovine serum (FBS) and 1.2% Na-pyruvate (Bio Whittaker, Europe). Cells were plated in 24 well culture plates at a density of 2.5×10^5 cells/ml or in 10 cm-diameter culture dishes (1×10^7 cells/10ml/dish) and allowed to adhere at 37 °C in 5% CO_2/95% O_2 for 2h. Immediately before the experiments, culture medium was replaced by fresh medium without FBS in order to avoid interference with radioimmunoassay (Zingarelli et al., 1997) and cells were stimulated as described.

Assessment of COX-1 activity

Cells were pretreated with test coumpounds for 15 min and further incubated for 30 min with arachidonic acid (AA) 15×10^{-6} M (Zingarelli et al., 1997). At the end of the incubation the supernatants were collected for the measurement of PGE_2 by radioimmunoassay (Sautebin et al.,1999). Test coumpounds were the following: propolis, with and without CAPE (3×10^{-3}–3×10^2 μgml^{-1}), CAPE (2.8×10^{-4}–28 μgml^{-1}; 10^{-9}–10^{-4} M), galangin (2.7×10^{-4}–27 μgml^{-1}; 10^{-9}–10^{-4} M), pinocembrin (2.8×10^{-4}–28 μgml^{-1}; 10^{-9}–10^{-4} M) caffeic acid (1.8×10^{-4}–18 μgml^{-1}; 10^{-9}–10^{-4} M), ferulic

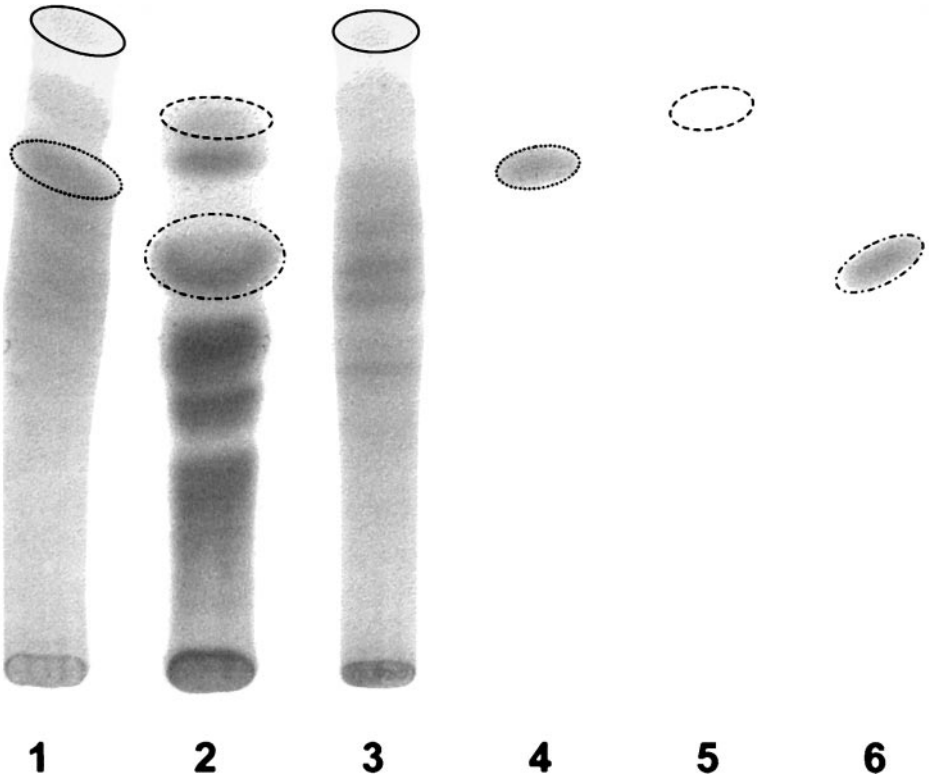

Fig. 1. Thin layer chromatography (TLC) of original propolis (line 1), water solutions (line 2), residual propolis (line 3), CAPE (line 4), phenethyl alcool (line 5) and caffeic acid (line 6).

acid (1.9×10^{-4}–19 μgml^{-1}; 10^{-9}–10^{-4} M), cinnamic acid (1.5×10^{-4}–15 μgml^{-1}; 10^{-9}–10^{-4} M), chlorogenic acids (3.5×10^{-4}–35 μgml^{-1}; 10^{-9}–10^{-4} M). The tested concentrations of CAPE and galangin corresponded to those present in the extract.

Assessment of COX-2 activity

Cells were stimulated, for 24h, with lipopolysaccharide (LPS, 10 μgml^{-1}), to induce COX-2, in the absence or presence of test compounds, at the concentrations previously reported. The supernatants were collected for the measurement of PGE_2 by radioimmunoassay (Sautebin et al., 1999).

Statistical analysis

In each experimental day, triplicate wells were used for the various treatment conditions. Results are expressed as the mean, for 4 experiments, of the % inhibition of PGE_2 production by test compounds in respect to control samples. Data fit was obtained using the sigmoidal dose-response equation (variable slope) (GraphPad software). The IC_{50} and the 95% confidence intervals (reported in bracket) were calculated by GraphPad Instat program (GraphPad software). Inhibition curves were analyzed and compared with two-way analysis of variance (ANOVA). Data (ng of PGE_2 produced) were analysed by using one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p value less than 0.05 was considered to be statistically significant.

Results

Analysis of propolis deprived of CAPE

CAPE was undetectable in the residual propolis, whereas caffeic acid and phenethyl alcool were abundant in water solutions. The amounts of the other detectable components of the original propolis were roughly the same in the residual propolis (Fig.1).

No evident changes in the content of the original total flavonoids and caffeic acid before and after the procedure, have been observed**.** Total flavonoid content of propolis with CAPE, expressed as galangin, was 9.04%; CAPE content was 10.44%. Total acidity, expressed as caffeic acid, was 13%. Total flavonoid content of propolis without CAPE, expressed as galangin, was 8.5%.

Inhibition of COX-1 activity by test compounds

Stimulation of J774 macrophages with arachidonic acid (15×10^{-6}M) for 30 min induced a significant increase ($p < 0.001$) of PGE_2 (5.1 ± 0.3 ng $\times 10^6$ cells) levels in comparison to unstimulated control cells (0.32 ± 0.06 ng $\times 10^6$ cells).

The ethanolic extract of propolis (3×10^{-1}–3×10^{2} μgml^{-1}) significantly ($p < 0.01$ at the lowest concentration; $p < 0.001$ at all the other concentrations) and concentration-dependently inhibited PGE_2 generation (Fig.2), with an IC_{50} of 2.7 μgml^{-1} (1.3 to 5.8 μgml^{-1}). Same results were obtained with CAPE (28×10^{-3}–28 μgml^{-1}; 10^{-7}–10^{-4} M) which significantly ($p < 0.01$ at the lowest concentration; $p < 0.001$ at all the other concentrations) and concentration-dependently inhibited prostanoid generation (fig. 2). The IC_{50} was 4.4×10^{-1} μgml^{-1} (3.3 to 6×10^{-1} μgml^{-1}), corresponding to 1.5×10^{-6}M. The production of PGE_2 was also significantly inhibited by galangin (27×10^{-3}–27 μgml^{-1}; 10^{-7}–10^{-4} M), but only from the concentration of 27×10^{-2} μgml^{-1} ($p < 0.05$; $p < 0.01$ at 27×10^{-1}; $p < 0.001$ at 27 μgml^{-1}) (fig. 2), with an IC_{50} of 3.7μgml^{-1} (1.4 to 10.2 μgml^{-1}) corresponding to 15×10^{-6}M. The other compounds tested (pinocembrin, caffeic, chlorogenic, ferulic and cinnamic acids) did not inhibit, at all the concentrations tested (10^{-7}–10^{-4} M), prostanoid generation (data not shown).

To investigate the role of CAPE in the overall inhibitory activity of propolis we tested the action of the ethanolic extract of propolis (3×10^{-1}–3×10^{2} μgml^{-1}) deprived of CAPE. The inhibition was significant only

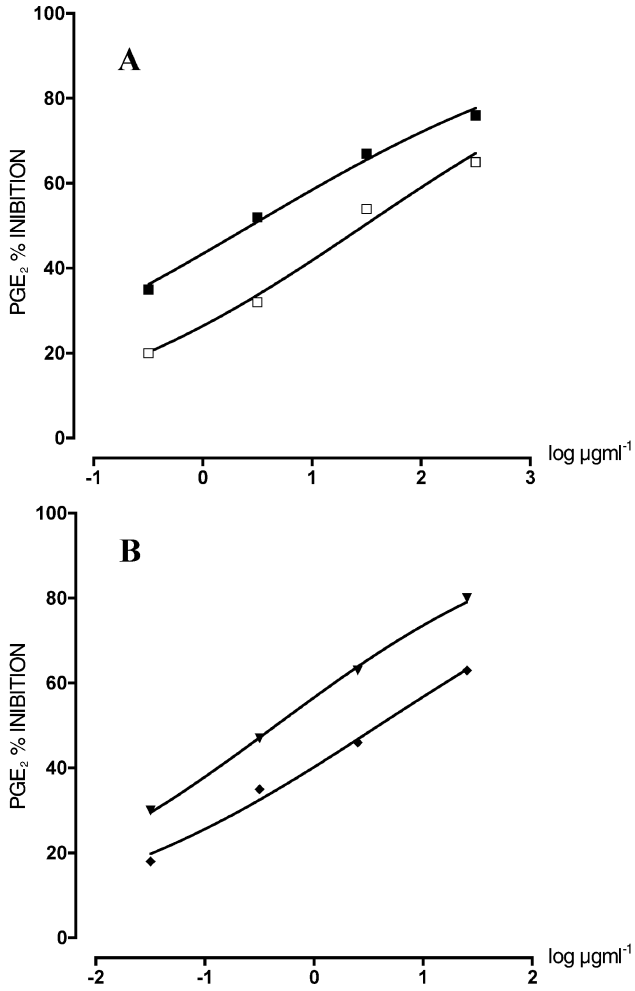

Fig. 2. Inhibitory effect of an ethanolic extract of propolis with (■) and without (□) CAPE (panel **A**), CAPE (▼) and galangin (◆) (panel **B**) on PGE_2 production by J774 macrophages incubated with arachidonic acid (15×10^{-6} M) for 30 min. Cells were pretreated for 15 min with test compounds before the addition of arachidonic acid. The concentrations are expressed as log μgml^{-1}. Each point represents the mean for 4 experiments performed in triplicate.

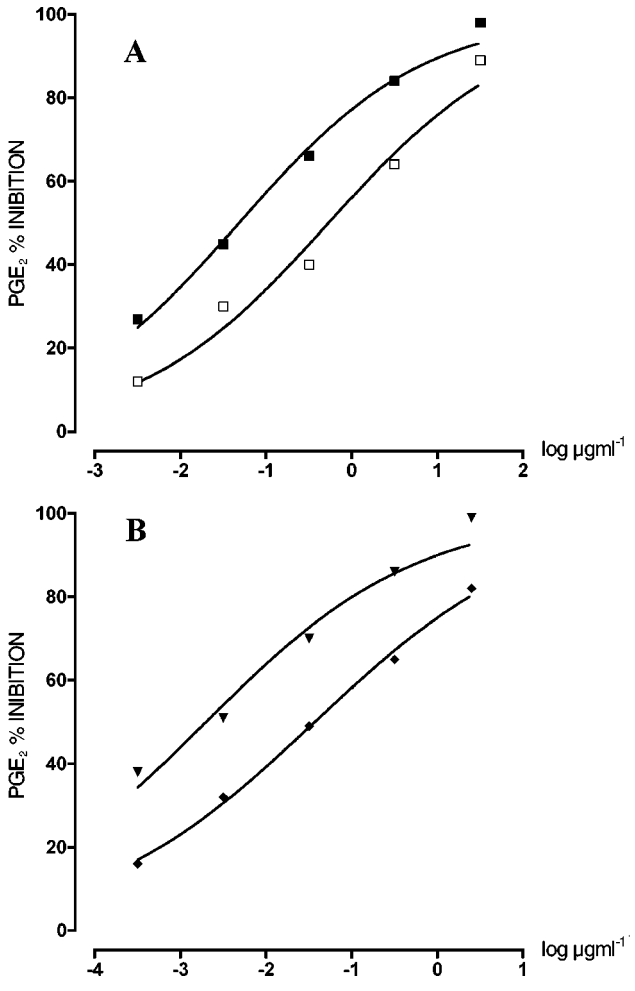

Fig. 3. Inhibitory effect of an ethanolic extract of propolis with (■) and without (□) CAPE (panel **A**), CAPE (▼) and galangin (◆) (panel **B**) on PGE_2 production by J774 macrophages stimulated with LPS (10 $\mu gml^{-1)}$ in presence or absence of test compounds. The concentrations are expressed as log μgml^{-1}. Each point represents the mean for 4 experiments performed in triplicate.

from the concentration of 3 μgml^{-1} ($p < 0.05$; $p < 0.01$ at 30, $p < 0.001$ at 300 μgml^{-1}). This extract was about ten times less potent than the extract containing CAPE (Fig. 2), the IC_{50} being 30 μgml^{-1} (10 to 87 μgml^{-1}). Moreover the comparison of the inhibition curves showed a significant difference ($p < 0.001$) between propolis with and without CAPE.

Inhibition of COX-2 activity by test compounds

Stimulation with LPS (10 μgml^{-1}) for 24h induced a significant ($p < 0.001$) increase of PGE_2 generation (28 ± 3.4 ng × 10^6 cells) in comparison to unstimulated control cells (1.32 ± 0.1 ng × 10^6 cells).

In the presence of increasing concentrations of the ethanolic extract of propolis (3×10^{-3}–30 μgml^{-1}) a concentration-dependent and significant ($p < 0.01$ at 3×10^{-2} μgml^{-1}; $p < 0.001$ at all the other concentrations) inhibition of PGE_2 production was observed (Fig. 3) with an IC_{50} of 4.8×10^{-2} μgml^{-1} (2.5 to 9.2×10^{-2} μgml^{-1}). CAPE (2.8×10^{-4}–2.8 μgml^{-1}; 10^{-9}–10^{-5} M), exerted a similar significant ($p < 0.01$ at 2.8×10^{-4} μgml^{-1}: $p < 0.001$ at all the other concentrations) and concentration-dependent inhibition (Fig.3), with an IC_{50} of 2×10^{-3} μgml^{-1} (6×10^{-4} to 6×10^{-3} μgml^{-1}) (6.3×10^{-9} M). Galangin (2.7×10^{-4}–2.7 μgml^{-1}; 10^{-9}–10^{-5} M) was also effective (Fig. 3), but the inhibition was significant only from the concentration of 2.7×10^{-2} μgml^{-1} ($p < 0.01$; $p < 0.001$ at all the other concentrations**)** . The IC_{50} was 3×10^{-2} μgml^{-1} (2 to 4×10^{-2} μgml^{-1}) (120×10^{-9} M). The other compounds tested pinocembrin, caffeic, chlorogenic, ferulic and cinnamic acids did not inhibit, at all the concentrations tested (10^{-9}–10^{-5} (M) prostanoid generation (data not shown).

Next we tested the action of the ethanolic extract of propolis without CAPE. This extract (3×10^{-3}–30 μgml^{-1}) significantly and concentration-dependently inhibited PGE_2 production but only from the concentration of 3–10^{-1} μgml^{-1} ($p < 0.05$; $p < 0.001$ at the other concentrations), resulting ten times less potent than the extract contents CAPE (Fig. 3). The IC_{50} was 5.4×10^{-1} μgml^{-1} (1.8×10^{-1} to 1.6 μgml^{-1}). Moreover the comparison of the inhibition curves showed a significant difference ($p < 0.001$) between propolis containing CAPE and propolis without CAPE.

Discussion

The antinflammatory activity of propolis is well known although the exact mechanism of action is still under debate. The inhibition of PG generation seems to be, at least in part, the mechanism mainly responsible for this property (Mirzoeva and Calder, 1996; Mezenes et al., 1999). Moreover the complicated chemical compositions of crude extract of propolis make very difficult to establish which compounds are primarily involved. Flavonoids and related compounds, which inhibit arachidonic acid metabolism (Baumann et al., 1980), are certainly involved, but other compounds may act synergistically and contribute to the overall antinflammatory activity (Ghisalberti, 1997). Recently it has been reported that CAPE, the caffeic acid phenethyl ester, which is present in the ethanolic extract of propolis, inhibits AA metabolism in mouse peritoneal macrophages stimulated with LPS or A23187 ionophore (Mirzoeva and Calder, 1996). In human oral epithelial cells and in a rat model of inflammation CAPE exherts antinflammatory activity by inhibiting the activity and expression of cyclooxygenase-2 (Michaluart et al., 1999). Moreover CAPE seems to inhibit the activation by tumor necrosis factor and other antinflammatoy agents, such as phorbol ester, ceramide, hydrogen peroxide and okadaic acid, of the transcription factor NF-κB (Natarajan et al**,** 1996) which regulates the expression of many genes involved in inflammation (Wulczyn et al., 1996). The inhibition of NF-κB and TNF_{α} has also been reported in a macrophage cell line (NR 8383), in a classic epithelial cell line (SW 620) and in a model of colitis in the rat (Fitzpatrick et al., 2001). The antinflammatory activity of CAPE through the modulation of NF-κB has also been described in a rat model of carrageenin-induced subcutaneous inflammation (Orban et al., 2000).

In this study we have studied the effect of an ethanolic extract of propolis, with and without CAPE, and some ot its components on COX-1 and COX-2 activity, measured as PGE_2 production, in J774macrophages. Our results show that propolis concentration–dependently inhibits COX-1 and COX-2 activity and link this effect mainly to CAPE and galangin. In fact among the compounds tested only CAPE and galangin were active**.** Cinnamic, caffeic, chlorogenic and ferulic acids were inactive on both COX-1 and COX-2 activity. It has been previously reported (Huang et al., 1991) that these compounds were unable to inhibit arachidonic acid conversion to PGE_2 and PGD_2 by epidermal microsomes. The comparison of the inhibition curves and the analysis of the IC_{50} indicate that, in our experimental model, CAPE is more potent than galangin. The central role of CAPE is further supported by the evaluation of the effect of the extract of propolis without CAPE. In fact this extract was a less potent (about ten times) inhibitor of COX isoforms when compared to the extract containing CAPE. In our experimental conditions the IC_{50} ratio COX-1/COX-2 for CAPE is about 200–300. Recently it has been reported that CAPE inhibits COX-1 (IC_{50} 58×10^{-6} M) and COX-2 (IC_{50} 82×10^{-6} M) activity in a cell free assay, using baculovirus-expressed human recombinant enzymes (Michaluart et al., 1999), whithout any apparent selectivity.

In conclusion our results suggest that although both CAPE and galangin contribute to the overall inhibitory activity of propolis, CAPE gives a greater contribution. Thus CAPE could be a usefull tool in the control of pathologies characterized by an elevated PG biosynthesis.

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