Available online at www.sciencedirect.com
SCIENCE DIRECT®
Life Sciences 73 (2003) 73–80
Life Sciences
www.elsevier.com/locate/lifescie

Vascular effects of caffeic acid phenethyl ester (CAPE) on isolated rat thoracic aorta

Carla Cicala[a,*], Silvana Morello[a], Carolina Iorio[a], Raffaele Capasso[a,b,*], Francesca Borrelli[a], Nicola Mascolo[a]

[a] *Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli "Federico II", via D.Montesano 49, 80131, Napoli, Italy*

[b] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084, Fisciano (SA), Italy*

Received 16 September 2002; accepted 6 December 2002

Abstract

This study was aimed to investigate the vascular activity of caffeic acid phenethylester (CAPE), one of the major components of honeybee propolis. Experiments were performed on rat thoracic aortic rings, mounted in an isolated organ bath and connected to an isometric force transducer. The effect of CAPE (0.1–300 μM) was evaluated on tissue pre-contracted with phenylephrine (PE, 1 μM) or with KCl (100 mM). In another set of experiments, tissue was incubated with CAPE (1–100 μM) and responses to PE (0.01–3 μM) or KCl (60 mM) were evaluated. The effect of CAPE on cytosolic Ca^{2+} concentration in aortic smooth muscle cells stimulated with PE or KCl was also evaluated. CAPE (0.1–300 μM) caused a concentration-dependent relaxation (pEC_{50} 4.99 ± 0.19; Emax 100.75 ± 1.65%; n = 4) of tissue pre-contracted with PE that was reduced by endothelium removal or by incubation with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM). CAPE also relaxed KCl-precontracted tissue (pEC_{50} 4.40 ± 0.08; n = 4). CAPE inhibited contractile responses to PE or to KCl, and also inhibited the contractile response to PE obtained in a Ca^{2+}-free medium. In addition, CAPE inhibited the increase in cytosolic Ca^{2+} concentration triggered by stimulation of aortic smooth muscle cells with PE or KCl. Our results demonstrate a vascular activity for CAPE, that is only partially dependent on nitric oxide. Indeed, at high concentrations, CAPE vasorelaxant effect occurs also in absence of endothelium and it is likely due to an inhibitory effect on calcium movements through cell membranes.

Keywords: Propolis; CAPE; Thoracic aorta; Vasorelaxation; Phenylephrine; Contraction; Calcium

* Corresponding authors. Tel.: +39-081678437; fax: +39-081678403.
E-mail address: cicala@unina.it (C. Cicala).

0024-3205/03/$ - see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0024-3205(03)00235-2

Introduction

CAPE is a structural relative of flavonoids and it is one of the major components of honeybee propolis, a product that for its broad spectrum of activities has been largely used as a folk medicine. Similar to flavonoids, CAPE has demonstrated several biological and pharmacological properties, such as anti-inflammatory [7,10], anticarcinogenic [1,8,15], immunomodulatory [13] and antioxidant activities [14,16]. Furthermore, CAPE has recently been shown to prevent ischemia reperfusion injury and the effect correlates with an increased eNOS activity [5,6]. The molecular mechanism of CAPE beneficial effects is still unknown. CAPE antinflammatory properties have been attributed to suppression of eicosanoid synthesis, through inhibition of arachidonic acid release from cell membrane and the expression of COX 2 gene, and by inhibiting COX 1 and COX 2 activity [10,11]. In addition, Natarajan et al. [12] have demonstrated that CAPE completely and specifically blocks the activation of NFkB induced by a wide variety of inflammatory agents; this inhibitory effect has been recently shown to account for the beneficial effect of this compound in a rat model of vascular injury [9]. The purpose of the present study was an in vitro characterisation of the vascular activity of CAPE.

Materials and methods

Chemicals and drugs

Acetylcholine (ACh), bovine serum albumin, caffeic acid phenethyl ester (CAPE), collagenase, dithiothreitol, EGTA, Fura-2/AM, indomethacin, ionomycin, L-NAME, papain, polyethylene glycol (PEG 400), phenylephrine (PE), soybean trypsin inhibitor, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine) (SQ22,536), taurine, were purchased from SIGMA-ALDRICH (Milan, Italy). All salts were purchased from Carlo Erba (Milan, Italy).

Functional studies

Male Wistar rats (200–250 g) were sacrificed by decapitation and exanguinated. Thoracic aorta was rapidly removed, gently cleaned taking care not to damage the endothelium and cut in rings of about 3 mm each. Aortic rings were placed in a 2.5 ml organ bath containing Krebs solution composed of (nM) NaCl, 115.3; KCl, 4.9; $CaCl_2$, 1.46; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 and glucose 11.1; warmed at 37 °C and oxygenated (95% O_2 and 5% CO_2) and connected to an isometric force transducer (model 7004, Ugo Basile) under a resting tension of 0.5 g. Changes in tension were recorded continuously by a polygraph linear recorder (WR 3310 Graphtec). After about 60 minutes period of equilibration, the tissue was pre-contracted with PE (1 μM) and the presence of a functional endothelium was verified by evaluating the relaxation to a single concentration of ACh (1 μM). Tissues were then washed, pre-contracted with PE (1 μM) and a cumulative concentration-response curve to CAPE (0.1 to 300 μM) was performed. The relaxant effect of CAPE was also evaluated on aortic rings pre-contracted with KCl (100 mM). In some cases, endothelium was mechanically removed by gently rubbing the lumen and response to CAPE was also evaluated.

The effect of CAPE on rings with endothelium was also tested on tissue previously incubated with the cyclooxygenase inhibitor, indomethacin (10 μM, 20 minutes incubation), or the nitric oxide synthase inhibitor, L-NAME (100 μM, 15 minutes incubation). The effect of CAPE on rings without endothelium was also tested in presence of the adenylate cyclase inhibitor, SQ 22,536 (100 μM, 30 minutes incubation).

In another set of experiments, rat aortic rings, prepared and mounted as described above, were incubated with CAPE at concentration ranging between 1 and 100 μM for 15 min and the contractile tissue response to PE (0.01–3 μM) or to KCl (60 mM) was evaluated on rings with or without endothelium. We also evaluated the effect of CAPE (100 μM) on PE (10 μM)-induced contraction in a Ca^{2+}-free medium. All drugs used were dissolved in Krebs solution, apart from CAPE that was dissolved in PEG 400; in this case, all appropriate controls with PEG 400 were performed.

Measurement of cytosolic Ca^{2+} concentration in aortic smooth muscle cells

Vascular smooth muscle cells (VSMC) were isolated from rat thoracic aorta according to the method described by Tostes et al. [17]. Briefly, the thoracic aorta was rapidly removed, placed in 0.1 mM Ca^{2+} Hank's solution at 22 °C, cleaned of fat and connective tissue. The composition of Hank's solution was NaCl, 140 mM; KCl, 5.4 mM; KH_2PO_4, 0.44 mM; $NaHCO_3$, 4.17 mM; NaH_2PO_4, 0.42 mM; $CaNa_2$-EDTA, 26 μM; $CaCl_2 \cdot H_2O$, 0.1 mM; HEPES, 5 mM and dextrose, 5.5 mM, pH 7.35. Vessels were cut in rings of about 3 mm in width and placed into 5 ml of 100 μM Ca^{2+} Hank's solution containing 10 mg/ml bovine serum albumin, 1 mg/ml soybean trypsin inhibitor, 5 mM taurine, 0.4 mg/ml dithiothreitol, 0.6 mg/ml type I collagenase and 0.6 mg/ml papain. Tissue was incubated at 37 °C for 40 minutes. After incubation period and centrifugation at 1500 rpm for 5 minutes, cells were re-suspended in 100 μM Ca^{2+} Hank's solution, containing albumin, trypsin inhibitor and taurine as described above, and incubated with 5 μM Fura-2 acetoxy methylester (Fura-2/AM) for 45 minutes at room temperature. After loading with Fura-2/AM, cells were washed twice to remove excess of enzymes and dye, and then were centrifuged and resuspended in Ca^{2+}-free Krebs buffer at a concentration of 20×10^3 cells/ml. Aliquots of 400 μl (8×10^3 cells) were used for $[Ca^{2+}]i$ measurements in a Perkin–Elmer LS 5B spectrofluorometer. Cell fluorescence emissions were obtained by rapidly alternating the excitation wavelength between 340 and 380 nm and recording the 515 nm emission intensity. The fluorescence of Fura 2/AM was calibrated by releasing dye from the cells at the end of each experiment with ionomycin (1 μM) to obtain the maximal fluorescence (Fmax) and then by quenching with EGTA (15 mM) to obtain the minimum signal (Fmin). The 340/380 nm emission ratios were converted to $[Ca^{2+}]i$ using the standard formula described by Grynkiewicz [3]. The effect of CAPE (0.1–100 μM) on PE (1–10 μM) and KCl (60 mM)-induced fluorescence emission was evaluated in absence of extracellular calcium and after the addition of $CaCl_2$ 1.5 mM.

Statistical analysis

All responses are expressed as mean ± S.E.M. of percentage relaxation from PE- or KCl-pre-contraction levels. Contractile responses are expressed as force developed by the tissue in dine/mg of wet weight tissue. Non-linear regression analysis for all concentration response curves were performed and data were analysed by two ways ANOVA. A value of $p < 0.05$ was considered significant.

Results

Functional studies

At concentrations up to 300 μM, CAPE had no effect on baseline tension in rat aortic rings. On rings with endothelium, pre-contracted with PE (1 μM), CAPE (0.1–300 μM) caused a concentration-dependent slowly developing relaxation (pEC_{50} 4.99 ± 0.19; n = 4) with a maximum relaxation (Emax) of 100.75 ± 1.65% reached at the concentration of 100 μM. Incubation of the tissue with the nitric oxide synthase inhibitor, L-NAME (100 μM), abolished the effect of low concentrations of CAPE, without modifying the maximum effect. Vasorelaxation induced by CAPE was not affected by indomethacin (data not shown). CAPE caused also a concentration-dependent relaxation of KCl (100 mM) pre-contracted tissue (pEC_{50} 4.40 ± 0.08; n = 4). CAPE relaxed endothelium-denuded rat thoracic aortic rings and the effect was comparable to that given in presence of L-NAME or on KCl pre-contracted tissue. The effect was not modified by tissue incubation with the adenylate cyclase inhibitor, SQ 22,536 (Figs. 1 and 2, A and B).

CAPE (1–100 μM) inhibited in a concentration-dependent manner the contractile response to PE (0.01–3 μM). In absence of endothelium, the inhibitory effect of CAPE on PE-induced contraction was only observed at the highest concentration tested (Emax 65.96 ± 16.49 dine/mg wet weight tissue vs. 159.50 ± 17.35 dine/mg wet weight tissue, n = 4; $p < 0.001$). Tissue contractility in response to PE was restored after CAPE washout, ruling out any toxic effect of compound (Fig. 2, C and D). CAPE inhibited significantly the contractile response to KCl (82.37 ± 14.45 dine/mg tissue vs. 243.8 ± 9.90 dine/mg tissue; $p < 0.001$, n = 5) and the effect was not altered by endothelium removal (data not shown).

In Ca^{2+}-free medium, PE (10 μM) gave a contraction reaching the maximum value in about 50 minutes (Fig. 3), presumed to reflect Ca^{2+} mobilisation from intracellular stores. After ring incubation with CAPE (100 μM), PE-induced contraction was significantly reduced (51.90 ± 6.88 vs. 327.71 ± 37.26 dine/mg wet weight tissue; $p < 0.001$ n = 6).

Effect of CAPE on cytosolic Ca^{2+} concentration in aortic smooth muscle cells

Cell incubation with CAPE (100 μM, 15 minutes) abolished the increase in $[Ca^{2+}]i$ triggered by PE (10 μM) or by KCl (60 mM) either in presence and in absence of external Ca^{2+}. CAPE alone did not

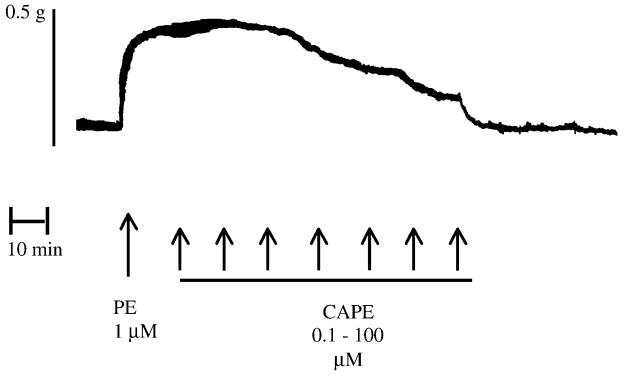

Fig. 1. Typical trace of CAPE-induced relaxation of rat thoracic aortic rings pre-contracted with phenylephrine. Cumulative concentrations (0.1–100 μM) of CAPE were added to the tissue pre-contracted with PE (1 μM).

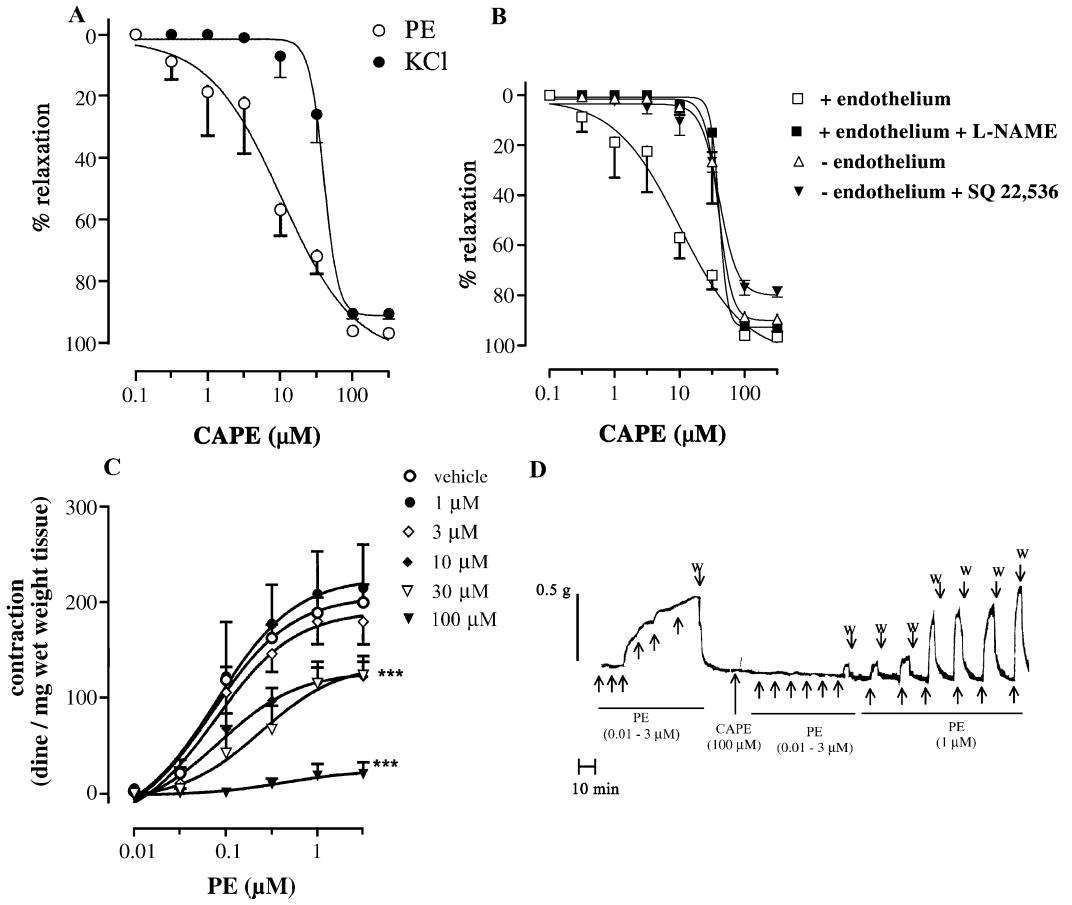

Fig. 2. (A) and (B) Vasorelaxation induced by CAPE (0.1–300 μM) on rat thoracic aortic rings. (A) Effect of CAPE on PE (1 μM) or KCl (100 mM) pre-contracted tissue. (B) Effect of L-NAME (100 μM) on CAPE-induced relaxation and effect of SQ 22,536 (10 μM) on relaxation induced by CAPE in endothelium-denuded rings. (C) Effect of CAPE (1–100 μM) on contractile response to PE (0.01–3 μM) of rings with endothelium. *** $p < 0.001$ (two ways ANOVA); n = 4–6. (D) Typical trace showing the effect of CAPE (100 μM) on contractile response to PE (0.01–3 μM) of rings without endothelium.

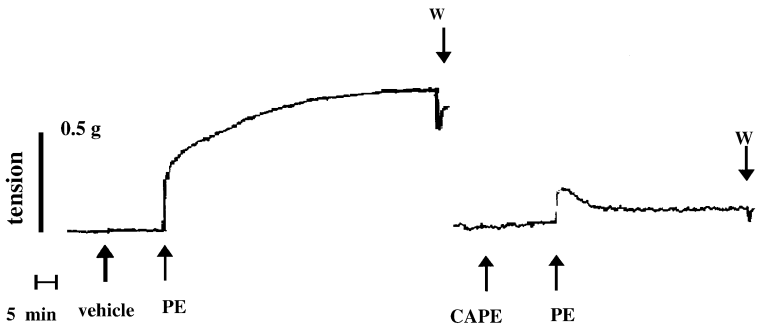

Fig. 3. Typical trace representing the effect of CAPE (100 μM) on contraction induced by PE (10 μM) in a Ca^{2+}-free medium.

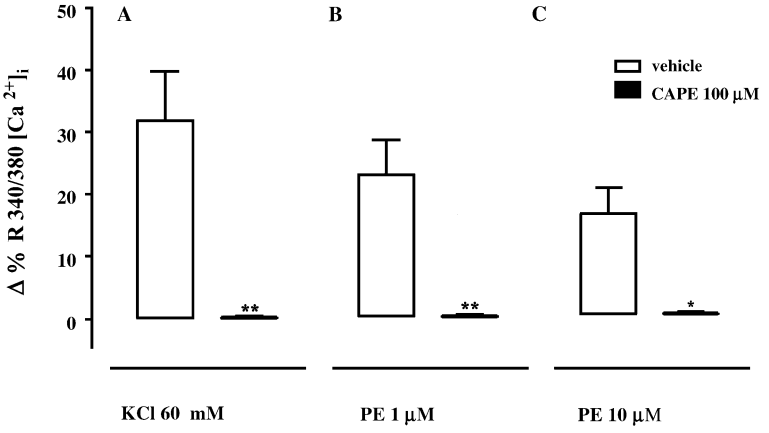

Fig. 4. Inhibitory effect of CAPE on intracellular Ca^{2+} concentration increase in aortic smooth muscle cells triggered by KCl (A) or by PE in a medium with Ca^{2+} (B) or in a Ca^{2+}-free medium (C). * $p < 0.05$ and ** $p < 0.01$ ($n = 6–8$).

modify basal cell fluorescence (Fig. 4). Concentrations of CAPE lower than 100 µM were inactive (data not shown).

Discussion

Here, for the first time we show a vascular activity of CAPE, in vitro. Our results demonstrate that CAPE causes both concentration- and endothelium-dependent relaxation of rat thoracic aortic rings pre-contracted with PE or with KCl. Furthermore, CAPE reduces the contractile response to PE or to KCl. In the end, CAPE reduces PE-induced contraction in a Ca^{2+}-free medium and abolishes PE- and KCl-induced intracellular Ca^{2+} increase.

When tissue was incubated with L-NAME, or deprived of endothelium, the relaxant effect of low concentrations of CAPE was abolished, while the effect triggered by high concentrations was unaltered. Furthermore, under these conditions, the effect of CAPE was comparable to that obtained on KCl-pre-contracted tissue, suggesting that CAPE acts via an NO-dependent and an NO-independent mechanism, the latter is irrespective of the contractile agent used. In addition, our experiments show that on endothelium-denuded vessels relaxation induced by CAPE was not affected by inhibition of adenylate cyclase, ruling out any involvement of cAMP.

CAPE was also able to inhibit both PE and KCl contractile effect. The inhibitory effect of PE-induced contraction was concentration-dependent on rings with endothelium, while it was evident only at highest concentration (100 µM) in absence of endothelium and on KCl pre-contracted tissue, suggesting that nitric oxide release is partly involved and modulate PE-induced contraction, but does not affect KCl-induced contraction. Nonetheless, even at the highest concentration tested, it was not due to a toxic effect of compound on the tissue, since after washout tissue was responsive to further PE stimulation. PE contractile effect, in a medium with Ca^{2+}, is due to a first component reflecting the increased Ca^{2+} influx through receptor-operated channels and a second component reflecting Ca^{2+} release from intracellular store. High KCl concentration-induced contraction is due to membrane depolarisation leading to an increased Ca^{2+} influx through voltage stimulated Ca^{2+} channels [2]. The ability of CAPE

to inhibit, at high concentration, in a no-selective manner a receptor mediated contraction and the contraction mediated by KCl suggests that it might interact through a receptor independent mechanism, likely interfering with Ca^{2+} channels to cause vasorelaxation. Interestingly, CAPE also inhibited the contraction triggered by PE in a Ca^{2+}-free medium, resulting from Ca^{2+} mobilisation from intracellular stores [4]. These results, together with the observation that the vasorelaxant effect of CAPE is only partially due to the presence of a functional endothelium and does not involve adenylate cyclase activation, strongly suggest that vascular effects of CAPE on rat aorta might be due to a dual mechanism, one secondary to nitric oxide release and activated by low concentrations, and one activated only by high concentrations and due to inhibition of both Ca^{2+} entry and Ca^{2+} release from intracellular stores. This hypothesis is further supported by the observation that CAPE at high but not at low concentrations was able to abolish intracellular Ca^{2+} increase triggered by PE or by KCl in aortic smooth muscle cells, and this effect was preserved after removal of extracellular Ca^{2+}, confirming an effect of CAPE on both Ca^{2+} influx and Ca^{2+} release from intracellular stores. In conclusion, to our knowledge this is the first report showing a vascular activity of CAPE that is dependent upon the presence of a functional endothelium and, at high concentrations, upon an inhibitory effect on Ca^{2+} movements through the cell membrane and within the vascular smooth muscle cell.

References

[1] Chen YJ, Shiao MS, Wang SY. The antioxidant caffeic acid phenethyl ester induces apoptosis associated with selective scavenging of hydrogen peroxide in human leukemic HL-60 cells. Anticancer. Drugs 2001;12:143–9.

[2] Godfraind M, Miller R, Wibo M. Calcium antagonism and calcium entry blockade. Pharmacological Reviews 1986; 38:324–416.

[3] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. Journal of Biological Chemistry 1985;260:3440–50.

[4] Hudgins PM, Weiss G. Differential effects of calcium removal upon vascular smooth muscle contraction induced by norepinephrine, histamine and potassium. Journal of Pharmcological and Experimental Therapeutics 1968;150:91–7.

[5] Ilhan A, Koltuksuz U, Ozen S, Uz E, Ciralik H, Akyol O. The effects of caffeic acid phenethyl ester (CAPE) on spinal cord ischemia/reperfusion injury in rabbits. European Journal of Cardiothoracic Surgery 1999;16:458–63.

[6] Koltuksuz U, Ozen S, Uz E, Aydinc M, Karaman A, Gultek A, Akyol O, Gursoy MH, Aydin E. Caffeic acid phenethyl ester prevents intestinal reperfusion injury in rats. Journal of Pediatric Surgery 1999;34:1458–62.

[7] Krol W, Scheller S, Czuba Z, Matsuno T, Zydowicz G, Shani J, Mos M. Inhibition of neutrophils' chemiluminescence by ethanol extract of propolis (EEP) and its phenolic components. Journal of Ethnopharmacology 1996;55:19–25.

[8] Lee YJ, Liao WK, Chen WK, Yang CC. Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. Cancer Letters 2000;153:51–6.

[9] Maffia P, Ianaro A, Pisano B, Borrelli F, Capasso F, Pinto A, Ialenti A. Beneficial effect of caffeic acid phenethyl ester in a rat model of vascular injury. British Journal of Pharmacology 2002;136:353–60.

[10] Michaluart P, Masferrer JL, Carothrs AM, Subbaramaiah K, Zweifeil BS, Koboldt C, Mestre JR, Grunberger D, Sacks PG, Tanabe T, Dannenberg AJ. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclo-oxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Research 1999;59:2347–52.

[11] Mirzoeva OK, Calder PC. The effect of propolis and its components on eicosanoid production during the inflammatory response. Prostaglandins Leukotrienes and Essential Fatty Acids 1996;55:441–9.

[12] Natarajan K, Singh S, Burke TR, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent specific inhibitor of activation of nuclear transcription factor NF-kB. Immunology 1996;93:9090–5.

[13] Park EH, Kahng JH. Suppressive effects of propolis in rat adjuvant arthritis. Archives of Pharmacological Research 1999;22:554–8.

[14] Pascual C, Gonzales R, Torricella RG. Scavenging action of propolis extract agents oxygen radicals. Journal of Ethnopharmacology 1994;41:9–13.

[15] Rao C, Desai D, Kaul B, Amin S, Reddy BS. Effect of caffeic acid esters on carcinogen-induced mutagenicity and human colon adenocarcinoma cell growth. Chemical Biological International 1992;84:277–90.
[16] Sud'ina GF, Mirzoeva OK, Pushkareva GA, Korshunova GA, Sumbatyan NV, Varfolomeev SD. Caffeic acid phenetyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS Letters 1993;329:21–4.
[17] Tostes RCA, Wilde DW, Bendack LM, Webb RC. The effects of cyclopiazonic acid on intracellular Ca^{2+} in aortic smooth muscle cells from DOCA-hypertensive rats. Brazilian Medical Biological Research 1997;30:257–67.