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Inhibition of Angiogenesis by Propolis

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Propolis, obtained from honeybee hives, has been used in Oriental folk medicine as an anti-inflammatory, anti-carcinogenic, and immunomodulatory agent. There is considerable evidence suggesting that angiogenesis and chronic inflammation are codependent. Blockage of angiogenesis results in an anti-inflammatory effect. Ethanol (EEP) and ether extracts of propolis (REP), and caffeic acid phenethyl ester (CAPE), an active component of propolis, were examined for their anti-angiogenic activities using the chick embryo chorioallantoic membrane (CAM), and the calf pulmonary arterial endothelial (CPAE) cell proliferation, assays. The presence of EEP, REP and CAPE inhibited angiogenesis in the CAM assay and the proliferation of CPAE cells. The results suggest that anti-angiogenic activities of EEP, REP and CAPE are also responsible for their anti-inflammatory effect.

Key words: Propolis, Caffeic acid phenethyl ester, Angiogenesis, Chorioallantoic membrane, Calf pulmonary arterial endothelial cells

INTRODUCTION

Ethanol and water extracts of propolis, a natural beehive product, have been widely used in Oriental folk medicine. Propolis, and its phenolic constituents, have been shown to exhibit a variety of pharmacological properties including antiproliferative activity in human tumor cells (Grunberger *et al.*, 1988; Guarini *et al.*, 1992), anti-inflammatory (Dobrowolski *et al.*, 1991), antibacterial (Grange and Davey, 1990; Steinberg *et al.*, 1996; Eley *et al.*, 1999), antiviral (Serkedjieva *et al.*, 1992), immunomodulatory (Dimov *et al.*, 1992), antioxidant (Krol *et al.*, 1990; Scheller *et al.*, 1990) and antiprotozoan (Starzyk *et al.*, 1977) activities. We also reported that an ethanol extract of propolis (EEP) showed anti-inflammatory activity in chronic inflammation models with significant improvement of pain symptoms (Park and Khang, 1999). Propolis contains more than 300 constituents, including cinnamic acids, benzoic acids and their esters, substituted phenolic acids and esters, flavonoids, amino acids, and bee wax (Greenaway *et al.*, 1991; Bankova *et al.*, 2000; Marcucci, 1995). Some of the observed biological activities might be attributed to the identified chemical constituents, such as caffeic acid (Cizmarik *et al.*, 1970). The structure of caffeic acid phenethyl ester (CAPE), an active component of propolis, is shown in Fig. 1. CAPE has been shown to inhibit inducible nitric oxide synthase (Song *et al.*, 2002) and NF-κ activation (Natarajan *et al.*, 1996), and to modulate cell proliferation and apoptosis through its antilipoxygenase activity (Mirzoeva *et al.*, 1996). Angiogenesis is the growth of new blood vessels from parent microvessels, which is essential for normal placental, embryonic and fetal growth, but almost never occurs physiologically in adulthood, except in ovarian follicles and postmenstrual endometria (Christenson and Stouffer, 1996). It plays a central role in a variety of pathological processes, such as tumor growth, arteriosclerosis, psoriasis, inflammatory reactions, and rheumatoid arthritis. There is considerable evidence suggesting that angiogenesis and chronic inflammation are mutually codependent (Jeffrey *et al.*, 1997). Inflammatory

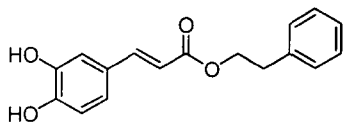

Fig. 1. Chemical structure of caffeic acid phenethyl ester (CAPE), an active compound of propolis.

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mediators can also promote angiogenesis either directly or indirectly. Angiogenesis, in turn, contributes to an inflammatory pathology. New blood vessels can maintain the chronic inflammatory state by transporting inflammatory cells to the site of inflammation, and supplying nutrients and oxygen to the proliferating inflamed tissue. The increased endothelial surface area also creates an enormous capacity for the production of cytokines, adhesion molecules, and other inflammatory stimuli. There are several potential mechanisms whereby suppression of blood vessel growth could provide benefit in arthritis (Firestein *et al.*, 1997; Firestein, 1999).

The present study investigated whether EEP, ether extract of propolis (REP), and CAPE affected angiogenesis using *in vivo* CAM and *in vitro* endothelial cell proliferation assays.

MATERIALS AND METHODS

Materials

Propolis, collected in Namyangju city (Korea), was purchased from the Korean Apiculture Society, and the voucher specimen stored in the herbarium at the College of Pharmacy, Sookmyung Women's University. Coarsely powered propolis (100 g) was extracted with 10 volumes of 95% ethanol for two weeks. The ethanol extract was filtered, evaporated and lyophilized to give the dried ethanol extract (33.3 g). The REP was fractionated successively, with diethyl ether, from the EEP suspended in 30% ethanol solution at room temperature. The CAPE was synthesized as described by Grunberger *et al.* (1988). Fertilized chick eggs were obtained from Pulmuwon Farm (Eumsung, Korea). Fat emulsion (10%) was purchased from Green Cross Pharm. Co (Seoul, Korea). Thermanox cover slips were purchased from Nunc Inc. (Naperville, IL, USA). The retinoic acid and methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO. USA). Each of these chemicals was dissolved in 70% ethanol solution in each assay. The final concentration of ethanol in the culture medium was adjusted to less than 0.5%.

Chick embryo chorioallantoic membrane (CAM) assay

The fertilized chicken eggs were kept in a humidified egg incubator at 37°C. After 3 days of incubation, approximately 3 ml of albumin was aspirated from the eggs with an 18-gauge hypodermic needle through a small hole drilled in the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. On day 4, the shell covering the air sac was punched out and removed by forceps, and the shell membrane, on the floor of air sac, peeled away. Embryos with chorioallantois, 3 to 5 mm in diameter, were used for the assay for anti-angiogenic activity. Samples of each solution (5 μl) were applied to 15 mm sterile disks, and allowed to dry under laminar flow conditions. The loaded-disks were inverted and applied to the CAM surface of 4.5-day-old embryos through the windows. The air ends of the embryo shells were covered with scotch tape. Two days later, an appropriate volume of 10% fat emulsion was injected into the 6.5-day-old embryo chorioallantois, using a 33-gauge needle, so that the vascular network of CAM stood out against the white background of the lipid. The anti-angiogenic response was assessed by measuring the avascular zone of the CAM beneath the disk. When the CAM showed an avascular zone of 3 mm, or larger, in diameter, the response was scored positive, according to the method previously described by Crum *et al.* (1985). Only frequencies were monitored, therefore it was not indicated whether a higher dose also yielded a larger avascular zone. At least 20 eggs were used for each dose of the agents. Finally, the chorioallantois was photographed.

Calf pulmonary arterial endothelial (CPAE) cell proliferation assay

Calf pulmonary arterial endothelial (CPAE) cells were cultured in RPMI 1640 containing 2.5 ng/ml basic-fibroblast growth factor (bFGF), 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO_2 at 37°C. Endothelial cells were plated in 24-well plates at a cell density of 10^5 cells/well, which was repeated in triplicate. After a 24-h incubation, the cells were treated with various doses of EEP, REP, and CAPE in 2% FBS RPMI 1640 containing 2.5 ng/ml bFGF. After a 2-day incubation, the endothelial cells were kept in fresh medium, without EEP, REP, or CAPE, for another 24-h. The numbers of cells were measured by the MTT assay, and monitored by Microscopy (CK30, Olympus Optical Co., Japan).

RESULTS AND DISCUSSION

The present study examined whether propolis and CAPE, one of the constituents of propolis, acted as inhibitors of angiogenesis, using the CAM and CPAE cell proliferation assays. The anti-angiogenic effects of retinoic acid, and EEP, on the treated CAM are shown in Fig. 2A. Retinoic acid (1 μg/egg), which is known to have anti-angiogenic activity, was used as a positive control (Oikawa *et al.*, 1989). EEP (5 μg/egg) inhibited angiogenesis in the CAM, causing an avascular zone, whereas the vehicle alone, as a control, did not. Retinoic acid (1 μg/egg), EEP (5 μg/egg), REP (5 μg/egg) and CAPE (5 μg/egg) caused avascular zones, reflecting anti-angiogenic activities of about 94%, 42%, 54% and 68%, respectively (Fig. 2B).

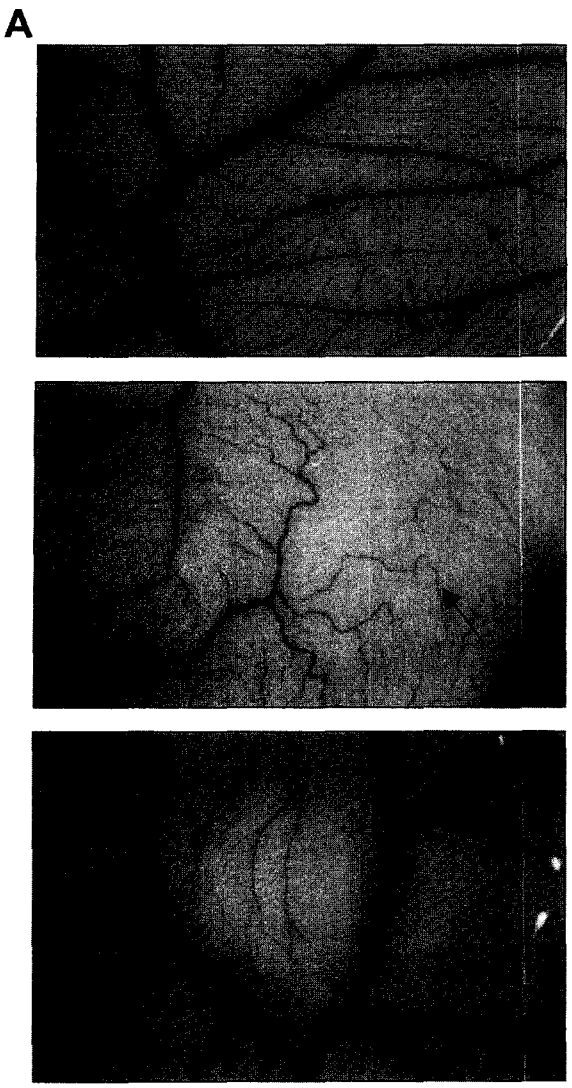

Fig. 2(A). Effects of retinoic acid and EEP on embryonic angiogenesis in the CAM assay after sample implantation. Control (a) CAMs treated with empty cover slip show no disturbance of angiogenesis. CAMs implanted with cover slips loaded with retinoic acid (b) (1 μg/egg), EEP (c) (5 μg/egg).

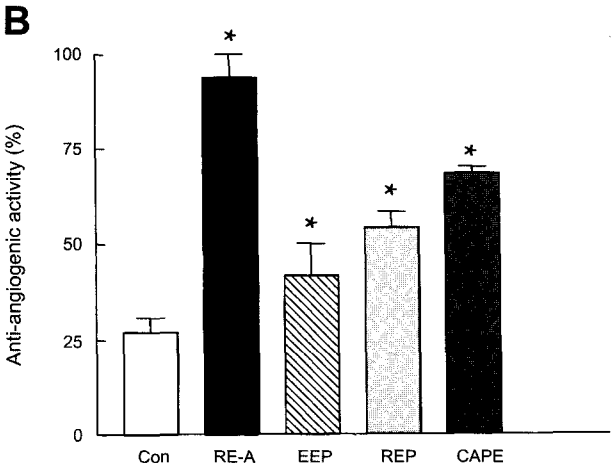

Fig. 2(B). Inhibitory effects of retinoic acid (Re-A, 1 μg/egg), EEP (5 μg/egg), REP (5 μg/egg), and CAPE (5 μg/egg) on embryonic angiogenesis. Anti-angiogenic effects were evaluated by measuring an avascular zone 2 days after implantation of the samples. Retinoic acid was included as a positive control. *$p<0.05$.

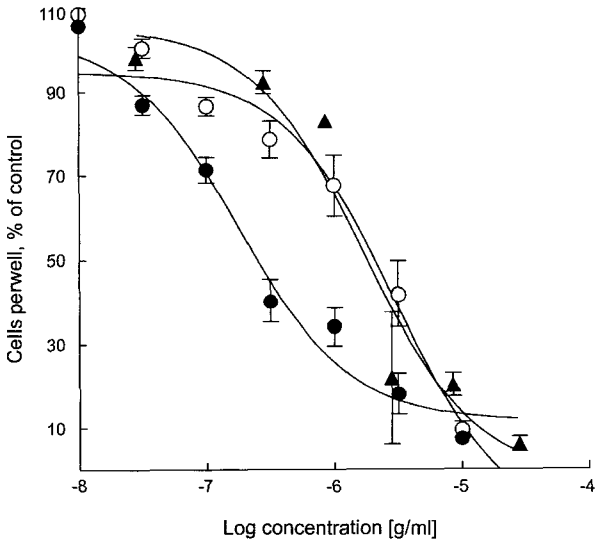

Fig. 3. Effects of EEP (●), REP (○), and CAPE (▲) on the proliferation of CPAE cells. They inhibited the proliferation of CPAE cells in a concentration-dependent manner. The viable cell number was determined by the metylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay.

The anti-angiogenic effects of the EEP, REP, and CAPE, on the CAM assay, were weaker than that of the retinoic acid. To determine this inhibition was due to the inhibition of the vascular endothelial cell proliferation, CPAE cells were cultured in the presence of various concentrations of EEP, REP, and CAPE. These compounds were found to inhibit the proliferation of CPAE cells in a concentration-dependent manner. The EC_{50} values of EEP, REP, and CAPE were determined to be 0.18, 2.97, and 1.71 μg/ml, respectively (Fig. 3). As propolis is composed of many compounds (Greenaway *et al.*, 1991), each compounds in propolis may have additive effects on the inhibition of CPAE cell proliferation. The inhibitory effect of EEP, on CPAE cell proliferation, was more potent than that of CAPE. These results demonstrated that EEP, REP, and CAPE have anti-angiogenic activities via the inhibition of the proliferation of endothelial cells.

There is much evidence suggesting angiogenesis to be an important factor in inflammatory processes (Jeffrey *et al.*, 1997). Angiogenesis factors, like vascular endothelial growth factor, bFGF, and tumor necrosis facror-α, are produced in rheumatoid joints, and contribute to vascular proliferation. The inhibition of angiogenesis may lead to an anti-inflammatory effect. Therefore, inhibitors of angiogenesis

could be inhibitors of inflammation. The benefit of agents that suppress neovascularization in arthritis was first demonstrated by Brahn and colleagues (Peacock *et al.*, 1992), when they reported the remarkable efficacy of the fumagillin derivative, AGM-1470 (TNP-470), which prevented arthritis in both adjuvant, and collagen-induced, arthritis in rats. Taxol, which can induce endothelial cell apoptosis, was also effective in an animal model of arthritis (Arsenault *et al.*, 1998). The present results demonstrated that EEP, REP, and CAPE have anti-angiogenic activities, via the inhibition of embryonic capillary formation in CAM, and the inhibition of CPAE cell proliferation. Previously, we reported the suppression of the arthritis index by EEP treatments (50 mg/kg/day and 100 mg/kg/day, p.o.) in the chronic inflammatory animal model (Park and Khang, 1999). In conclusion, the anti-angiogenic activities of EEP, REP and CAPE may contribute to their anti-inflammatory effects. Our results will help in the development of useful therapeutic agents for angiogenic disorders, such as rheumatoid arthritis, cancer, and diabetic retinopathy.

ACKNOWLEDGEMENTS

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