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Suppressive Effects of Propolis in Rat Adjuvant Arthritis

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The effects of ethanolic extract (EEP) of propolis on chronic inflammation were evaluated using rat adjuvant arthritis. In the chronic inflammatory animal model, the arthritis index was suppressed by EEP treatments (50 mg/kg/day and 100 mg/kg/day, p.o.). Moreover, physical weakness, induced by the chronic disease state, was dose-dependently improved in the EEP-treated groups. Its analgesic effect, assessed using the tail-flick test, was comparable to prednisolone (2.5 mg/kg/day, p.o.) and acetyl salicylic acid (100 mg/kg/day, p.o.). In carrageenan rat hind paw edema, which was conducted to test the effects of subfractions of EEP, the petroleum ether sub-fraction (100mg/kg, p.o.) showed an inhibitory effect on the paw edema whereas EEP (200 mg/kg, p.o.) showed a significant anti-inflammatory effect at 3 and 4 hrs after carrageenan injection. From these results, we conclude that the ethanolic extract of propolis had a profound anti-inflammatory effects on both chronic and acute inflammations.

Key words: Rat adjuvant arthritis, Physical weakness, Tail-flick test, Carrageenan edema

INTRODUCTION

Rat adjuvant arthritis was used as a relevant animal model of rheumatoid arthritis in this study. Rheumatoid arthritis is a representative chronic inflammatory disease of the joints resulting in the destruction of cartilage and bone by the proliferation of synovial tissue. This experimental model shares many features with arthritic patients such as the presence of a proliferating synovitis, swelling of the extremities and ultimately cartilage and bone erosion (Pearson *et al.*, 1959).

In recent years, the biological properties of propolis, a resinous bee glue, have become the focus of particular interest because it is believed to exhibit a broad spectrum of activities, which include antibacterial, antifungal, cytostatic and anti-inflammatory properties. Some of the observed activities of propolis may due to its chemical constituents such as cinnamic acid, benzoic acid and its ester, substituted phenolic acids and their esters, flavonoid glycones and bee wax (Greenway *et al.*, 1987). Its antibacterial, antiviral and antiseptic activities have in particular warranted the use of its extracts in dermatology. Rao *et al.* (1992) have shown that propolis also has cytostatic activities on mutagenicity and adenocarcinoma primarily attributed to caffeic acid esters. Inhibitory effects on lipid peroxidation and the radical scavenging activities of propolis (Volpert *et al.*, 1993; Scheller *et al.*, 1990) demonstrate that it possesses anti-inflammatory activity. We previously reported that an ethanolic extract of propolis significantly inhibited the development of paw edema in the carrageenan model and increased vascular permeability coupled with an excellent analgesic effect (Park *et al.*, 1996). It also showed a significant inhibitory effect upon granuloma and exudate formation (Park *et al.*, 1996). In this study, we investigated the effects of an ethanolic extract of propolis on chronic inflammatory process using the rat adjuvant-induced arthritis models.

MATERIALS AND METHODS

Materials

Complete Freund's adjuvant, mineral oil, and carrageenan lambda were purchased from Sigma Chemical Co., USA. Killed, dried *Mycobacterium butyricum* was obtained from Difco Laboratories, USA. Hydrocortisone was purchased from Hanwool Pharm. Ltd., Korea; acetyl salicylic acid from Janssen Pharm. Co., Japan; Tween 80, absolute ethanol, petroleum ether, diethyl ether and ethyl acetate from Duksan Pharm. Co., Korea; Prednisolone from the Tokyo Chemical Co.

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Animals

Female SD rats, 160-180 gm and male ICR mice, 25-30 gm, were used in these experiments. All animals were housed in an animal room under normal conditions of 24 ± 1°C, 12-h light-dark cycle and humidity (55±5%). The animals were provided a commercially certified rodent ration (Samyang Co.) and tap water *ad libitum*.

Preparation of propolis extract and solvent fractionation

Propolis was purchased from the Korean Apiculture Society. Ground propolis was extracted with 10-fold absolute ethanol for two weeks. The ethanolic extract was evaporated using a rotary evaporator under vacuum and dried with a freeze dryer. The dried residual powder (EEP) was kept at -20°C, to minimize bacterial contamination and was dispersed immediately prior to use in a 10% solution of Tween 80 in saline, to produce the injectable preparation. An aqueous solution of EEP in water-ethanol (7:3 by volume) was prepared. Petroleum ether, diethyl ether and ethyl acetate were used sequentially for solvent fractionation. The ethanol extract of propolis was obtained as powder. The yield of the petroleum ether fraction was 22.25%, the diethyl ether fraction 74.09%, and the ethyl acetate fraction 0.47%, and 3.19% remained in the aqueous layer.

Adjuvant-induced arthritis

Freund's complete adjuvant was prepared at a concentration of 10 mg/ml. 0.1 ml of adjuvant was injected subcutaneously into the right hind foot-pad (Breliere *et al.*, 1986; Pearson *et al.*, 1959). The first day of adjuvant injection was designated day 0. The animals of the EEP-treated groups received EEP in 10% Tween 80 solution orally every day from day 0 to day 28 at doses of 50 mg/kg and 100 mg/kg, respectively. Control animals received only the vehicle, 10% Tween 80.

Arthritis Index (AI)**:** AI was scored for each rat on days 10, 13, 16, 19, 22, 25, 28 by the same person. Non-injected left hind paw and both forelegs were graded separately from 0 to 4, depending on the severity. Based on a scale of 0 to 4 for each paw the assessment was made as follows : 0, no response; 1, slight edema of the digital joints; 2, edema of the digital joints and footpad; 3, gross edema of the entire footpad below the joint; 4, edema of the entire foot including the joint. In the case of the more severe responses, swellings of the tail and ears also were generally noted, but no additional score was ascribed for these clinical signs. The right rear footpad, a primary inflammatory lesion, was not included in the arthritic response assessment. Therefore, the highest score achievable was 12, since only the three non-injected paws were assessed. The sum of the scores for three limbs was defined as the arthritis index.

***Analgesia test*:** In the same manner as Tortorici and Vanegas (1994) and Bickel *et al.* (1994) an analgesia test was performed on the same days as the hind paw swelling assessment with a tail-flick unit (Ugo Basile, Italy). The test was performed 1 hr after oral administration. To make the rats accustomed to the experimental conditions, they were placed on the tail-flick unit at least twice, before the test was performed. The base-line measurement was taken one day before adjuvant injection. To examine the level of the analgesic effect of EEP, prednisolone 2.5 mg/kg/day-and acetyl salicylic acid 100 mg/kg-treated groups were established as positive controls.

***Physical weakness assessment*:** According to the method used by Breliere *et al.* (1986) and Capacio *et al.* (1992) physical weakness was measured by the ability of rats to remain on a rotating rod. Tests were performed on day 19 and day 28, after training twice a week throughout the duration of the experiment. In a given trial on the accelerating rotarod, up to 4 rats were placed on the stationary rod for about ten seconds to accustom them to the experimental environment. The animals were placed facing in the opposite direction to the rod rotation. During this time animals that fell off were placed back on the rod. The rod was accelerated to a maximum rate of 10 RPM in 30 seconds. An upper limit time on the rod was 30 seconds. The results were expressed as the mean time.

Carrageenan rat hind paw edema

Acute paw edema in rats was induced by injecting 0.1 ml of 1% sterile(w/v) carrageenan in saline into the subplantar region of the right hind paw (Winter *et al.*, 1962). Each experimental group consisted of eight rats. EEP, its subfractions and aspirin as a positive control material in 10% Tween 80, were administered orally (200 mg/kg for EEP, 100 mg/kg for subfractions of EEP and 200 mg/kg for aspirin) 1 hr before carrageenan injection. The control group received only the vehicle, 10% Tween 80.

Statistics

Unless otherwise indicated, results are presented as the mean ± S.E. Statistical comparisons were performed for all parameters. Comparisons between groups were made using Student's *t*-test. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The effects of the ethanolic extract of propolis on rat adjuvant arthritis

Preventative effect on polyarthritis onset: The disease symptoms appeared between the 9th and 13th day after adjuvant injection, reaching a sustained maximum 10 days later. Oral administration of EEP, from day 0 to day 28 at a dose of 100 mg/kg/day, moderated the clinical course of adjuvant-induced arthritis. The mean arthritic indices for the EEP 100 mg/kg/day treated rats were dramatically inhibited, compared to vehicle only treated rats during the overall observation period, especially day 25 ($p<0.05$) and day 28 ($p<0.01$). Furthermore, the lower dose of 50 mg/kg/day, showed a slight inhibitory effect, which resulted in a dose response relationship from day 10 to day 28 (Fig. 1). These results indicate that EEP has a suppressive effect in the chronic inflammatory animal model.

Effect on pain: On day 1, there were no significant differences between the test groups. However, from day 7 to day 22, the control group showed a reduced latency in tail-flick test. During this period fine analgesic effects, especially from day 16 to 22, in the 50 mg/kg/day and 100 mg/kg/day treated groups were observed. The analgesic effects of EEP were comparable to those of ASA 100 mg/kg/day and the prednisolone 2.5 mg/kg/day treated groups (Fig. 2). These results suggest that EEP has a prominent analgesic effect.

Effect on physical weakness: The clinical changes

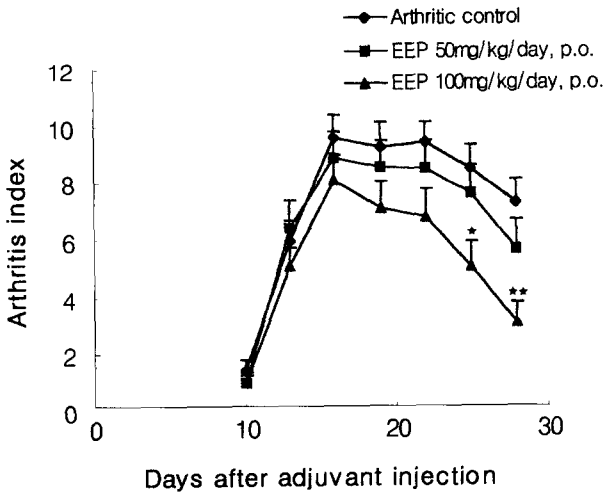

Fig. 1. Effects of the ethanol extract of propolis on the development of adjuvant-induced arthritis in the rat (arthritix index). Each point represents the means ± S.E. for 14 rats. EEP=ethanol extract of propolis. Arthritic contro=10% Tween 80. $^{*}p< 0.05$, $^{**}p<0.01$

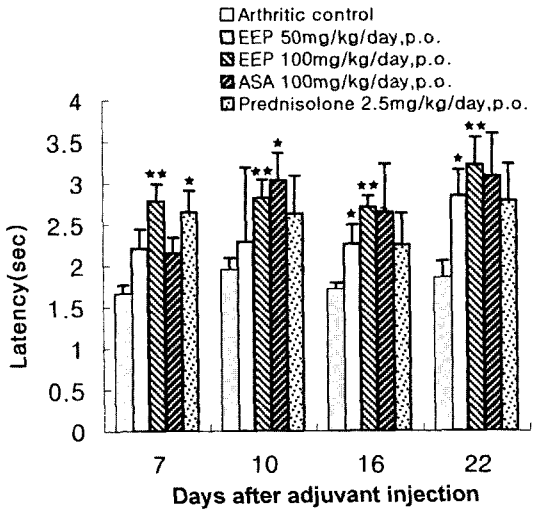

Fig. 2. Effects of ethanol extract of propolis on the tail flick test in adjuvant arthritic rats. Each column represents the mean±S.E. for 11 rats. EEP=ethanol extract of propolis. ASA =acetyl salicylic acid. Arthritic control:10% Tween 80. $^{*}p< 0.05$, $^{**}p< 0.01$

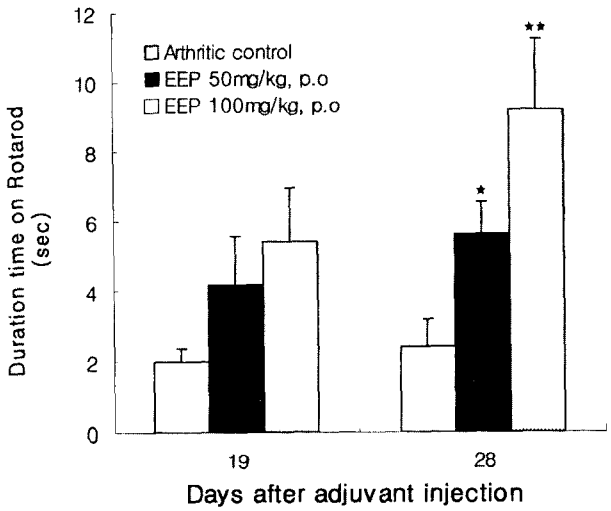

Fig. 3. Effects of the ethanol extract of propolis on the grip strength in adjuvant arthritic rats. Each column represents the mean±S.E. for 14 rats. EEP=ethanol extract of propolis. Arthritic control : 10% Tween 80. $^{*}p< 0.05$, $^{**}p< 0.01$

(arthritis index) mentioned above were reflected in the physical weakness measurements. The improvement in the arthritis index (Fig. 1) of the EEP-treated groups enabled the animals to remain on the rotarod significantly longer than the control group on day 28 (Fig. 3). EEP significantly reduced physical weakness associated with chronic inflammation in these animals.

Effects of EEP and its subfractions on carrageenan rat hind paw edema

EEP was previously shown to inhibit the development

Table I. Effects of oral administration of ethanol extract of propolis (EEP) and the fractions of EEP on carrageenan-induced rat hind paw edema

Group	Dose	Swelling Percentage (%)				
	(mg/kg,p.o)	1hr	2hr	3hr	4hr	5hr
Control	-	32.71±2.87	71.06±4.2	88.48±4.46	90.55±4.03	78.68±4.71
EEP	200	21.74±5.26	54.92±6.51	63.41±6.77*	68.37±5.1*	66.35±5.16
Petroleum ether Fr.	100	26.98±2.59	64.91±4.20	63.41±6.77	80.49±3.54	72.37±2.59
Diethyl Ether Fr.	100	30.15±3.29	73.65±4.87	85.19±3.85	87.82±4.12	80.10±4.23
Ethyl acetate Fr.	100	31.02±3.02	72.65±4.71	87.29±5.58	88.29±4.26	76.12±5.53
Water Fr.	100	27.05±3.02	63.08±4.69	79.02±3.52	85.35±4.27	80.60±4.85
Aspirin	200	12.54±1.58**	25.76±2.21	42.38±2.78**	50.62±3.19**	53.79±3.17**

10% Tween 80 was administered to the control group. Each value represents the mean±S.E. *$p< 0.05$, **$p< 0.01$.

of paw edema in the carrageenan model (Park *et al.*, 1996). As a preliminary step directed towards identifying the active principle in EEP, it was sequentially fractionated with petroleum ether, diethyl ether and ethyl acetate. EEP and its subfractions were then used in the carrageenan rat hind paw edema test. EEP showed an inhibitory effect on the hind paw edema 3 and 4 h after carrageenan injection ($p < 0.05$), whereas subfractions of EEP inhibited the inflammatory response only slightly (Table I). However, the petroleum ether subfraction was shown to have a relatively potent effect when swelling percentages at 2 and 3 h after carrageenan injection were compared. These results suggest that propolis contains two or more components with different solubility properties, which synergistically are responsible for its anti-inflammatory effect.

Propolis is a multifunctional material used by bees for the construction and maintenanace of their hives. The use of propolis by man has a long history, which has resulted in extensive dermal contact, and it is now being increasingly used a dietary suplement. It has many antibiotic, antifungal (Doborowolski *et al.* 1991), antiviral (Serkedjieva *et al.*, 1992, Amoros *et al.*, 1992), anti-tumour (Hladon *et al.*, 1980) and anti-inflammatory properties. Volpert *et al.* (1993, 1996) reported that the propolis extract had antioxidative activity and the inhibitotory effects of propolis extract on leukocytic myeloperoxidase and NADH oxidase activities were assigned to its excellent radical scavenging properties. Khayyal *et al.* (1993) showed the subtle inhibitory properties of propolis extract by in vitro studies upon platelet aggregation, eicosanoid synthesis, the anti-inflammatory effects of the aqueous extract on carrageenan rat paw edema and adjuvant-induced arthritis models. Ledon *et al.* (1997) also suggested that extracts of propolis had anti-psoriatic, anti-inflammatory, and analgesic effects. Doborowolski *et al.* (1991) reported that the propolis bee preparation had a significant anti-inflammatory effect upon formaldehyde-induced arthritis, a chronic inflammation model. The purpose of the present study was to investigate the anti-inflammatory and anti-angiogenic effects of ethanolic extract of propolis.

In conclusion, the ethanolic extract of propolis had a profound anti-inflammatory effect upon chronic inflammation, and produced a significant improvement in disease symptoms, especially pain in the chronic state. The mechanism of action of propolis on adjuvant arthritis is presumed to be due to its anti-inflammatory and immunosuppressive effects. We suggest that the anti-inflammatory effects of propolis might be due to its inhibitory effect on prostaglandin production, which has to be investigated in more detail.

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