JPP Journal of Pharmacy and Pharmacology

JPP 2007, 59: 1405–1412

Received April 7, 2007
Accepted June 28, 2007
DOI 10.1211/jpp.59.10.0011
ISSN 0022-3573

Effect of orally administered *Eriobotrya japonica* seed extract on allergic contact dermatitis in rats

Guangchen Sun, Yixiu Zhang, Daisuke Takuma, Masahide Onogawa, Junko Yokota, Atsuhide Hamada, Saburo Yoshioka, Masahiko Kusunose, Mitsuhiko Miyamura, Shojiro Kyotani and Yutaka Nishioka

Abstract

The anti-allergic activity of *Eriobotrya japonica* seeds extract (ESE) was investigated. Oral administration of ESE dramatically inhibited ear swelling due to allergic contact dermatitis caused by repeated application of two antigens, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) and dinitrofluorobenzene (DNFB), respectively. The increase of histamine content in inflamed ear tissue induced by oxazolone and DNFB was significantly antagonized by orally administered ESE. Eosinophil peroxidase and myeloperoxidase activity in both models was suppressed by orally administered ESE. Tumour necrosis factor-α in the inflamed region caused by repeated application of DNFB was also significantly suppressed. The findings suggest that ESE may be effective for treating allergic contact dermatitis.

Introduction

Traditional Chinese herbal medicines are widely used for the treatment of many types of acute and chronic diseases. The loquat, *Eriobotrya japonica* Lindl. (Rosaceae), is a small tree native to Japan and China that is widely cultivated for its succulent fruit. Its leaves have been used as a folk medicine for the treatment of chronic bronchitis, coughs, phlegm, high fever and ulcers in Japan and other Asian countries (Perry 1980). A traditional therapy using the leaves in a compress has also been used to treat cancers in Japan. Terpenoids (Shimizu et al 1986; Liang et al 1990; De Tommasi et al 1992; Nozato et al 1994; Shimizu et al 1996) and flavonoids (Jung et al 1999) have been found in the leaves and some of these compounds have been reported to be biologically active, exhibiting anti-inflammatory, anti-HIV, or hypoglycaemic properties. *E. japonica* seeds, as with those of apricots and peaches of the same family (Rosaceae), contain amygdalin, β-sitosterol, wax alcohol, amino acids, fatty acids, starch and free hydrocyanic acid. The active components in *E. japonica* seeds extract (ESE) have being studied in our laboratory; polyphenols, solvent fractions and amino acids were analysed and the structure of two types of polyphenols, namely caffeic acid and chlorogenic acid, were determined (Yokota et al 2006). In traditional Chinese medicine, *E. japonica* seeds are used as dispersing stagnated hepato qi, antitussive and expectorant agents and are used to treat oedema, hernia and scrofula.

It has been reported that ESE has effects on rat intestinal flora and its immunologic function (Li et al 2005). We are interested in identifying and developing clinically useful and safe products from herbal medicines. In the present study, we examined the anti-inflammatory and immunosuppressive effects of ESE on allergic contact dermatitis.

Materials and Methods

Chemicals

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) and *o*-phenylenediamine were purchased from Sigma Chemical (St Louis, MO, USA). *o*-Dianisidine chloride was obtained from MP Biomedicals Inc., Solon, Ohio, UAS. Hexadecyl-trimethyl ammonium

Department of Biomedical Science, Kochi Medical Graduate School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

Guangchen Sun, Daisuke Takuma, Yutaka Nishioka

Department of Pharmacy, Kochi Medical School Hospital, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

Masahide Onogawa, Atsuhide Hamada, Saburo Yoshioka, Masahiko Kusunose, Mitsuhiko Miyamura, Shojiro Kyotani, Yutaka Nishioka

Japan Science and Technology Agency, Miyanokuti Tosayamada Kamishi, Kochi, Japan

Junko Yokota

Graduate School, Jiamusi University, China

Yixiu Zhang

Correspondence: Guangchen Sun, Department of Biomedical Science, Kochi Medical Graduate School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan. E-mail: sungc2004@yahoo.com.cn

chloride was purchased from Nacalai Tesque Inc (Kyoto, Japan). 2,4-Dinitrofluorobenzene (DNFB), *o*-phthaldialdehyde, β-sitosterol, histamine diphosphate salt and tumour necrosis factor-α (TNF-α) kit were obtained from Wako Chemical, Ltd (Osaka, Japan).

Preparation of extract

E. japonica seeds were collected in Wagayama and Kochi prefectures, and dried well in the sun. *E. japonica* seeds (1 kg) were powdered in a blender equipped with a refrigerator at 1000 rev min^{-1} and extracted by stirring with a mixer at 300 rev min^{-1} for 7 days after being dissolved in 70% ethanol. After filtration, the supernatant was collected and evaporated under vacuum to afford 130 g of dried extract. The dried extract was diluted in 1 L water and the diluted solution was stored in a refrigerator until being administered to animals.

Qualitative analysis of active components by gas chromatography-mass spectrometry

ESE aqueous solution was shaken with the same volume of ethyl acetate, the extract in the ethyl acetate phase was analysed in a gas chromatograph with a mass selective detector (Model QP5050A, GC/MS Shimadzu), using a capillary column (DB-1 column 60 m × 0.32 mm; film thickness 1.0 μm). The conditions were as follows: GC injection temperature 250°C; column temperature 40°C–280°C; injection volume (10 μL); helium was used as the carrier gas, pressure 100 kPa. MS: I/F temperature 250°C; electronic impact mode 70eV; ionization electric current 60 μA. Identification of compounds was made by comparing mass spectra of standard compounds with those of the samples.

Animals

Male 6-week-old Sprague-Dawley rats were purchased from SLC, Inc., Japan. The animals were kept in a specific pathogen-free animal facility maintained at a temperature of 19–25°C, humidity 30–70% and a 12-h day–night cycle. They were given access to pellet food and water. The experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. The study protocol was approved by the Animal Experimental Committee of Kochi Medical School.

Induction of allergic contact dermatitis

Allergic contact dermatitis was induced by challenging the ear of previously sensitized rats with DNFB or oxazolone (Dhabhar & McEwen 1999). The rats were sensitized (Day 0) on the shaved abdomen with oxazolone and DNFB 7 days before the first challenge (Day 7), and were repeatedly challenged on the right ear with oxazolone and DNFB, respectively, at 3-day intervals until the end of the experiment (Day 22). The immune reaction induced by using this procedure is characterized by swelling at the site of challenge and by an infiltration of monocytes, macrophages and lymphocytes into the epidermis and dermis (Vadas et al 1975; Turk 1980; Malorny et al 1990). A positive correlation between the intensity of the immune reaction and an increase in ear thickness has been reported (Phanuphak et al 1974; Kimber & Dearman 1993). This model for skin delayed-type hypersensitivity reaction has been widely used to monitor cell-mediated immune responses in-vivo (Turk 1980; Thorne et al 1991). Animals were divided into four groups of seven animals each: the normal group received drinking water; the vehicle group had acetone/olive oil (4:1) applied to the right ear; the control group had oxazolone and DNFB applied to the right ear; the ESE-treated group had oxazolone and DNFB applied to the right ear, and ESE was administered. ESE was administered in drinking water from two days before the first challenge with DNFB or oxazolone (Day 5) and throughout the experiment.

Measurement of histamine

The rats were killed under ether anaesthesia and the ears were excised, washed with ice-cold saline, and weighed. The samples were minced and then homogenized in 3 mL perchloric acid (0.4 N) using a Polytron on ice. After centrifugation at 1500 *g* for 10 min at 4°C, the supernatants were used for the measurement of histamine by the OPA spectrofluorometric procedure (Shore et al 1959). Briefly, 2 mL of sample was transferred to tubes containing 0.2 g NaCl, to which 0.25 mL NaOH (5 N) and 5 mL butanol were added. The samples were shaken for 5 min and centrifuged at 1500 *g* for 5 min at 20°C and the upper organic phases were transferred to tubes containing 0.2 g NaCl, 2.5 mL NaOH (0.1 N) and stirred; 2.25 mL HCl (0.1 N) and 6 mL *n*-heptane were added to the organic phase and shaken. The phases were separated and 2 mL of the lower aqueous phase were made alkaline (pH 12.5) with 200 μL NaOH (1 N). The histamine derivative was formed by incubating the samples at room temperature with 100 μL *o*-phthaldialdehyde (10 mg mL^{-1} methanol) for 4 min. The histamine/*o*-phthaldialdehyde reaction was halted at pH 3.5 by the addition of 200 μL HCl (6 N) and the fluorescence was subsequently assessed at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Measurement of eosinophil peroxidase (EPO) activity

EPO activity was measured as described previously (Strath et al 1985; White et al 1991). The skin was homogenized in 2 mL of ice-cold 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100 with a Polytron homogenizer. The homogenized samples were centrifuged at 1500 *g* for 10 min at 4°C and the supernatant was used for the determination of EPO activity.

The assay is based on the oxidation of *o*-phenelenediamine by EPO in the presence of hydrogen peroxide (H_2O_2). Hydrogen peroxide (0.01%) and *o*-phenylenediamine (16 mM) were dissolved in 100 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100, immediately before use. For the assay, 50 μL of sample was combined with 100 μL of substrate in a 96-well microplate and placed in a thermoregulating microplate absorbance spectrophotometer at 37°C. Absorbance at 490 nm was measured every 9 s for 3 min; the maximum velocity of the reaction was calculated by interpolation between three successive points utilizing customized software.

Measurement of myeloperoxidase (MPO) activity

MPO activity was assayed in ears (seven ears from each group) 72 h after the fifth antigen application (Bradley et al 1982). Frozen samples of tissue (approx. 100 mg) were homogenized (Polytron homogenizer) in 2 mL of 50 mmol L^{-1} potassium phosphate buffer, pH 6. The homogenate (1 mL) was centrifuged at 10 000 *g* for 10 min at 4°C, and the pellet was suspended in 1 mL of 50 mmol L^{-1} potassium phosphate buffer, containing 0.5% hexadecyl-trimethyl ammonium chloride to negate the peroxidase activity of haemoglobin and myoglobin, and to solubilize membrane-bound MPO. The suspensions were treated to three cycles of freezing and thawing, sonicated on ice for 10 s, and centrifuged at 12 000 *g* for 10 min. MPO activity was determined in the supernatants: 50 μL of the supernatant was mixed with 150 μL of 50 mmol L^{-1} potassium phosphate buffer, pH 6, containing 0.167 mg mL^{-1} of *o*-dianisidine chloride and 0.0005% H_2O_2 as a substrate for MPO activity. Oxidized *o*-dianisidine forms a stable chromophore, with an absorbance wavelength of 460 nm. The values of tissue MPO activity were obtained by standard concentrations of *o*-dianisidine in the presence of excess H_2O_2. Absorbance at 460 nm was measured every 9 s for 3 min; the maximum velocity of the reaction was calculated by interpolation between three successive points utilizing customized software.

Measurement of tissue TNF-α

TNF-α in the inflamed region was determined by enzyme linked-immunosorbent assay (ELISA); the biopsies were homogenized vigorously with a 30-fold volume of potassium phosphate buffer containing 0.1% Tween-20. The homogenates were frozen in nitrogen, thawed in a 37°C water bath, sonicated for 10 s, and centrifuged for 10 min at 12 000 *g* (Ferguson et al 1994). After centrifugation, TNF-α in the supernatant was determined by ELISA. Briefly, a solid phase sandwich ELISA kit was used; a monoclonal antibody specific for rat TNF-α was coated onto the wells of the microtitre strips. During the first incubation, standards of known rat TNF-α content and unknown samples were pipetted into the coated wells, followed by the addition of biotinylated second antibody. After washing, streptavidin peroxidase was added. After a second incubation and washing to remove all unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce colour. The absorbance was read at a wavelength of 450 nm.

Histopathological study

Rat ears were excised 72 h after the last application of oxazolone and DNFB and fixed in 10% buffered formalin solution, embedded in paraffin by standard methods, cut into 4-μm sections and stained with Congo red (Grouls & Helpap 1981) and toluidine blue.

Statistical analysis

The data were expressed as the mean ± s.e. Inhibition percentages arise from differences between treated and control tissues. Data were analysed by analysis of variance followed by Newman–Keuls test. A P value less than 0.05 was considered statistically significant.

Results

Gas chromatography-mass spectrometry

The quantitative active components in ESE were analysed by gas chromatography-mass spectrometry. Figure 1 shows the total ion chromatograph of ESE. In addition to previously reported components, β-sitosterol was identified by gas chromatography-mass spectrometry. The peak of β-sitosterol is shown with a retention time of 6.31 min. Mass spectral data showed the molecular ion at m/z 414 and fragmentation at m/z 273, m/z 255 and m/z 131. These data suggest that the component was β-sitosterol.

Effects on the time course of ear thickness and ear swelling

Oxazolone applied to the ear of sensitized mice caused erythema (reddening of the skin), oedema and/or induration and sometimes abrasion. When the ear thickness was measured as an index of skin inflammation, it increased with repeated application, and reached the maximum 22 days after sensitization. Dermatitis persisted for 2 days and then slowly declined. Repeated application of DNFB (another completely different antigen) onto the rat ears caused swelling, thickening of the epidermis, formation of scabs and infiltration of abundant inflammatory cells. The normal ear thickness was 0.58 ± 0.02 mm. The body weights of the treated animals were recorded and no significant difference was found between the ESE groups and the control groups (results not shown). ESE at a concentration 5 times that of the human dose inhibited the ear swelling after oxazolone and DNFB at 16 days after sensitization by 11.3% and 19.2%, respectively (Table 1), compared with the oxazolone control group. On the first challenge, the ear swelling response appeared within 30 min after application of DNFB, reached a peak at 24 h and then decreased gradually to a basal line within 48 h. The time course of the ear swelling response indicated that administration of ESE suppressed ear swelling (Figure 2). We also investigated the effects of various concentrations of ESE on ear oedema and found that

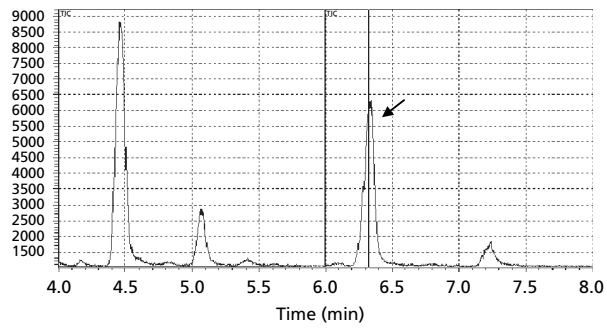

Figure 1 Representative gas chromatograms for β-sitosterol. The peak of β-sitosterol is indicated by an arrow.

Table 1 Effects of *Eriobotrya japonica* seeds extract (ESE) on ear swelling induced by repeated application of oxazolone and dinitrofluorobenzene (DNFB), respectively

Treatment	Group	Ear thickness (10^{-2} mm)					
		Day 7	Day 10	Day 13	Day 16	Day 19	Day 22
Oxazolone	Control	57.0 ± 0.5	68.0 ± 2.4[c]	81.3 ± 4.7[c]	93.0 ± 6.9[c]	99.1 ± 5.9[c]	97.2 ± 2.8[c]
	ESE	57.8 ± 0.4[a]	65.7 ± 1.7[c]	70.7 ± 2.3[a,c]	81.4 ± 3.7[a,c]	85.7 ± 4.5[a,c]	86.3 ± 3.7[a,c]
	Vehicle	57.3 ± 0.5	56.6 ± 0.9	56.7 ± 0.8	55.9 ± 1.3	56.3 ± 0.7	57.3 ± 0.6
DNFB	Control	58.2 ± 0.6	79.5 ± 2.2[c]	89.0 ± 3.4[c]	101.7 ± 4.5[c]	117.0 ± 4.9[c]	125.2 ± 5.2[c]
	ESE	57.6 ± 1.0	67.6 ± 2.2[b,c]	80.3 ± 2.4[a,c]	85.6 ± 4.7[a,c]	97.0 ± 6.9[a,c]	101.0 ± 9.5[a,c]
	Vehicle	54.1 ± 1.65	57.0 ± 1.1	55.9 ± 1.0	59.1 ± 1.0	58.6 ± 1.1	59.3 ± 1.0

Values represent the mean ± s.e.m. of seven animals. The rats were sensitized on the shaved abdomen with oxazolone and DNFB 7 days before the first challenge, and were repeatedly challenged on the right ear with oxazolone and DNFB, respectively, at 3-day intervals until the end of the experiment. [a]$P < 0.05$, [b]$P < 0.01$ significantly different compared with the control group. [c]$P < 0.01$ significantly different compared with the vehicle-treated group (Newman-Keuls test).

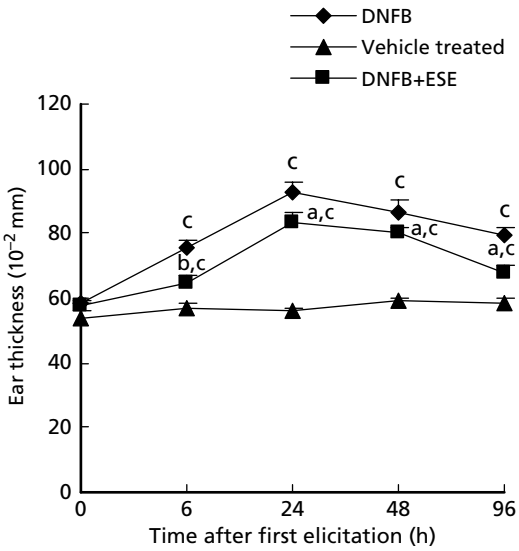

Figure 2 Effect of *Eriobotrya japonica* seeds extract (ESE) on ear oedema induced by dinitrofluorobenzene (DNFB). The rats were sensitized on the shaved abdomen with DNFB. Ears were challenged 7 days later by application of DNFB. Data represent the mean ± s.e. of seven animals. [a]$P < 0.05$, [b]$P < 0.01$, compared with the DNFB group. [c]$P < 0.01$ compared with vehicle-treated group (Newman–Keuls test).

the concentration used was effective against the ear oedema (data not shown).

Effects of ESE on ear TNF-α production

The effects of ESE on TNF-α content in ears after DNFB challenge (5 times) are shown in Figure 3; ESE significantly suppressed the increase in TNF-α.

Changes in ear mediators

After treatment for 17 days by ESE, the histamine content and the EPO and MPO activity in the ears of rats was determined. The results are show in Table 2. Oral administration of ESE significantly inhibited the histamine content and EPO and MPO activity in ear swelling induced by oxazolone and DNFB.

Histopathological observation

For histopathological analysis, we stained ear sections with Congo red for eosinophil and toluidine blue for mast cells (Figure 4). The ears sensitized with oxazolone swelled markedly and sometimes showed abrasion. The ears with oxazolone and DNFB applied (Figure 4B) swelled so dramatically that the entire section could not be shown. Also, the ear showed prominent epidermal hyperplasia and marked infiltration of inflammatory cells, mainly into the dermis and some into the epidermis, whereas only a thin epidermal layer and sparse cells were observed in the vehicle-treated ear. Congo red stain showed eosinophil infiltration into the dermis; toluidine blue stain showed marked mast cell infiltration into the dermis. However, ESE reduced the thickness of swollen ears after the application of oxazolone (Figure 4C). The infiltration of eosinophils was less in the ESE group compared with the oxazolone and DNFB control groups (Figure 4C). At the same time, the infiltration of mast cells was alleviated by the administration of ESE in both models.

Discussion

Oxazolone was applied to the ears of Sprague-Dawley rats once every 3 days, starting from 7 days after sensitization with oxazolone (applied onto the shaved abdomen). After repeated application of oxazolone the ears exhibited erythema, oedema and/or induration and sometimes abrasion. Ear thickness was measured as an index of skin inflammation and it increased as the application was repeated, reaching a maximum 16 days after sensitization. DNFB was applied to the ears of Sprague-Dawley rats every 3 days, starting from 7 days after sensitization with DNFB (applied onto the shaved dorsal back). After repeated application of DNFB the ears

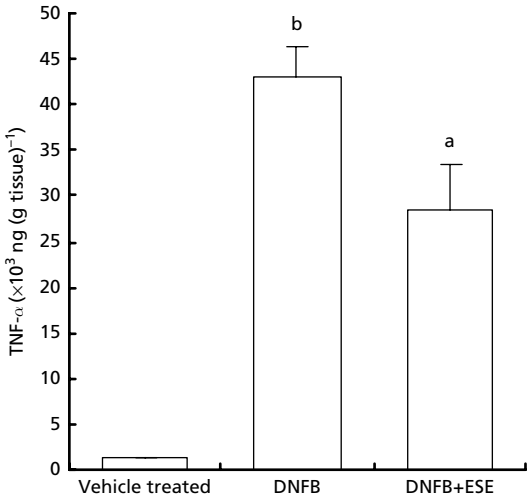

Figure 3 Protective effect of *Eriobotrya japonica* seeds extract (ESE) on tumour necrosis factor-α (TNF-α) in ear oedema induced by dinitrofluorobenzene (DNFB). The rats were sensitized on the shaved abdomen with DNFB 7 days before the first challenge, and the right ears were repeatedly challenged with DNFB, respectively, at 3-day intervals until the end of the experiment. Data represent the mean ± s.e. of seven animals. $^{a}P < 0.05$ compared with the DNFB group. $^{b}P < 0.01$ compared with the vehicle-treated group (Newman–Keuls test).

exhibited erythema, oedema and incrustation. In the model of allergic contact dermatitis, ear swelling started at 30 min after challenge, became marked between 24 and 48 h, and then declined gradually up to 96 h. Microabscesses were observed from 24 to 48 h, which consisted of eosinophils and mast cells. We have shown that orally administered ESE suppresses the ear swelling response in chronic contact dermatitis caused by repeated application of oxazolone and DNFB. In this study, ESE was administered to rats at a concentration that is 5-fold the clinical dose applied in humans. In a previous report, we clarified that oral administration of ESE was useful for the improvement of nephropathy in rats with adriamycin-induced nephropathy (Hamada et al 2004), and we also identified the components of ESE and investigated their antioxidative action (Yokota et al 2006). Given the free radical scavenging activity and lipid peroxidation inhibition activity of ESE, we believe that drinking ESE can improve inflammatory and allergic conditions; however, there is no report that directly confirms such an effect. We therefore examined whether oral administration can also inhibit inflammatory and allergic contact dermatitis.

The assay for the peroxidase activity of eosinophils has been utilized both as an assay of eosinophil function (Kroegel et al 1988) and number (Strath et al 1985) in biological media. Human eosinophils contain large quantities of eosinophil peroxidaes, which are different in structure from the myeloperoxidase of neutrophils (Wever et al 1982). EPO activity was measured as an index of eosinophil sequestration into the challenged skin. This parameter has been shown to be an easy, reproducible and quantitative method for the detection of eosinophils, without interference from neutrophils. Oxazolone and DNFB caused eosinophil accumulation, suggesting that eosinophil infiltration may be one of the causes of the dermatitis induced by repeated application of oxazolone and DNFB, and the improvement of allergic contact dermatitis by ESE may be as a result of the inhibition of eosinophil infiltration. In both models, ESE also reduced MPO activity. MPO is an enzyme present in neutrophils and, at a much lower level, in monocytes and macrophages. It is well known that the level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue (Bradley et al 1982), and measurement of MPO enzyme activity has been considered as a quantitative and sensitive marker of chemotaxis and neutrophil infiltration in the inflammatory process (Smith 1994). These results together with previous reports suggest that the anti-allergic effects of ESE may be as a result of its potent antioxidant activity.

Histamine has been shown to affect chronic inflammation and regulate several essential events in the immune response in addition to its effects in acute and allergic responses. Histamine can induce chronic inflammation by selectively recruiting the major effector cells into tissue sites and affecting their maturation, activation, polarization and effector functions. In the present study, ESE potently inhibited histamine release in oxazolone- and DNFB-induced contact hypersensitivity, suggesting the possibility that ESE may be active in the inhibition of mast cell mediator release and thus in the treatment of allergic disorders.

Table 2 Inhibitory effects of *Eriobotrya japonica* seeds extract (ESE) on histamine content and eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activity in ears after repeated application of oxazolone and DNFB, respectively

	Oxazolone-induced ear oedema			**DNFB-induced ear oedema**		
	Control	ESE	Inhibition (%)	Control	ESE	Inhibition (%)
Histamine (μg (g tissue)$^{-1}$)	5.7 ± 0.5	4.0 ± 0.5[a]	30.4	5.7 ± 0.2	3.8 ± 0.41[a]	32.8
EPO (OD min^{-1} (g tissue)$^{-1}$)	1258.1 ± 115.8	929.8 ± 141.4[a]	26.2	1107.9 ± 64.3	912.3 ± 44.4[a]	42.8
MPO (OD min^{-1} (g tissue)$^{-1}$)	131.7 ± 19.8	80.8 ± 5.9[a]	38.6	146.2 ± 11.0	95.4 ± 13.4[a]	34.8

Values represent the mean ± s.e.m. of seven animals. The rats were sensitized on the shaved abdomen with oxazolone and DNFB 7 days before the first challenge, and were repeatedly challenged on the right ear with oxazolone and DNFB, respectively, at 3-day intervals until the end of the experiment. $^{a}P < 0.05$ significantly different compared with the control group (Newman–Keuls test).

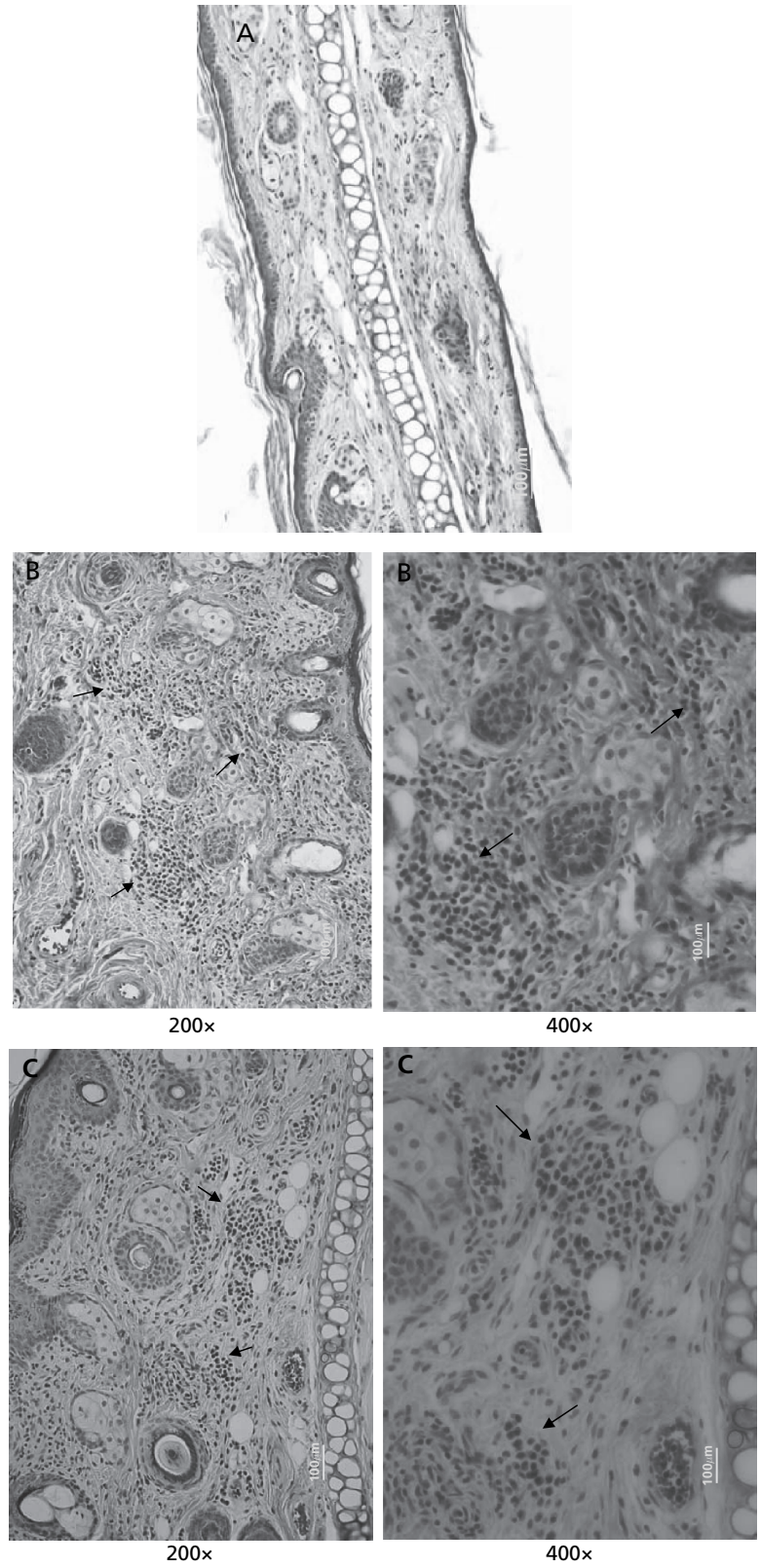

Figure 4 Light microscopy of Congo red (A–C) stained ear sections from rats 72 h after antigen challenge (5 times). A. Vehicle treated; infiltration of inflammatory cells was hardly observed. B. Oxazolone elicitation control; infiltration of inflammatory cells, mainly eosinophils (pink: indicated by an arrow), was observed. C. *Eriobotrya japonica* seeds extract (ESE) treated ear elicited by repeated application of oxazolone; ESE inhibited eosinophil infiltration when orally administered from 2 days before the antigen challenge.

TNF-α is a pro-inflammatory cytokine that plays a critical role in both acute and chronic inflammation (Holtmann et al 2002). In the early phase of elicitation of contact hypersensitivity (3–24 h after challenge), the release of serotonin and TNF-α from mast cells and platelets results in increased vascular permeability and tissue swelling (Askenase et al 1980; Van Loveren et al 1983; Askenase et al 1995). When TNF-α is specifically blocked, the severity of inflammation is reduced. In the present study, ESE inhibited the production of TNF-α, suggesting that the anti-allergic effect of ESE results from its reduction of TNF-α, thus reducing the permeability of endothelial cell monolayers to macromolecules and lower molecular weight solutes.

It has been reported that repeated application of contact agents to the same skin site results in a shift in the time course of antigen-specific hypersensitivity responses from a typical delayed-type hypersensitivity to an immediate time response followed by a late reaction (Kitagaki et al 1995). In patients with atopic dermatitis, repeated exposure to antigens through the skin is thought to contribute to the development of eczematous skin lesions, histologically indistinguishable from those in allergic contact hypersensitivity (Kitagaki et al 1997). Clinically, immediate reactions to contact allergens such as fragrance mix, balsam of Peru, and paraben mix have been reported (Forsbeck & Skog 1977; Safford et al 1990; Dikeakou et al 1988) and some of the patients had combined responses of both immediate and delayed reactions. In our study, the ear repeatedly challenged with DNFB and oxazolone showed a biphasic response that comprised an immediate reaction, appearing in 1 h, and a late phase. ESE may alleviate the late phase reaction induced by repeated application of oxazolone and DNFB.

The model of allergic contact dermatitis used in this study is significantly different compared with human tests, especially regarding the protocol of the repeated insult patch test in humans. Humans have a broad range of individual characteristics in terms of skin type, and there are individual studies that demonstrate population differences in skin properties or in responses to chemicals (Robinson 1999). The present results with ESE on allergic contact dermatitis should be further validated in clinical experiments.

In the present study, we used the whole 70% ethanol extract of *E. japonica* seeds and therefore the active components responsible for the biological effects are not clear at this time. The extract contains β-sitosterol, polyphenols and various amino acids, and it has been reported that in addition to their cholesterol-lowering effect, β-sitosterol and β-sitosterol glycoside have anti-inflammatory, antipyretic (Bouic 2002), antineoplastic and immune-modulating effects (Bouic & Lamprecht 1999). The results suggest that the improvement of allergic dermatitis by ESE may be due to its β-sitosterol and β-sitosterol glycoside components. The effort to identify other active components from *E. japonica* seeds is ongoing in our laboratory.

References

Askenase, P. W., Bursztajn, S., Gershon, M. D., Gershon, R. K. (1980) T cell-dependent mast cell degranulation and release of serotonin in murine delayed-type hypersensitivity. *J. Exp. Med.* **152**: 1358

Askenase, P. W., Geba, G. P., Levin, J., Ratzlaff, R. E., Anderson, G. M., Ushio, H., Ptak, W., Matsuda, H. (1995) A role for platelet release of serotonin in the initiation of contact sensitivity. *Int. Arch. Allergy Immunol.* **107**: 145

Bouic, P. J. (2002) Sterols and sterolins: new drugs for the immune system? *Drug Discov. Today* **7**: 775–778

Bouic, P. J., Lamprecht, J. H. (1999) Plant sterols and sterolins: a review of their immune-modulating properties. *Altern. Med. Rev.* **4**: 170–177

Bradley, P. P., Priebat, D. A., Christensen, R. O., Rothstein, G. (1982) Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* **78**: 206–209

De Tommasi, N., De Simone, F., Pizza, C., Mahmood, N., Orsi, N., Stein, M. L. (1992) Constituents of *Eriobotrya japonica*. A study of their antiviral properties. *J. Nat. Prod.* **55**: 1067–1073

Dhabhar, S. D., McEwen, B. S. (1999) Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc. Natl Acad. Sci. USA* **96**: 1059–1064

Dikeakou, T., Katsarou-Katsari, A., Hatziolou, E., Katsambas, A. (1988) Relative frequency of immediate and delayed patch test reactions. *Contact Dermatitis* **19**: 151–152

Ferguson, T. A., Dube, P., Griffith, T. S. (1994) Regulation of contact hypersensitivity by interleukin 10. *J. Exp. Med.* **179**: 1597–1604

Forsbeck, M., Skog, E. (1977) Immediate reactions to patch tests with balsam of Peru. *Contact Dermatitis* **3**: 201–205

Grouls, V., Helpap, B. (1981) Selective staining of eosinophils and their immature precursors in tissue sections and autoradiography with Congo Red. *Stain Technol.* **56**: 323

Hamada, A., Yoshioka, S., Takuma, D., Yokota, J., Cui, T., Kusunose, M., Miyamura, M., Kyotani, S., Nishioka, Y. (2004) The effect of *Eriobotrya japonica* seed extract on oxidative stress in adriamycin-induced nephropathy in rats. *Biol. Pharm. Bull.* **29**: 1961–1964

Holtmann, M. H., Schuchmann, M., Zeller, G., Galle, P. R., Neurath, M. F. (2002) The emerging distinct role of TNF-receptor 2 (p80) signaling in chronic inflammatory disorders. *Arch. Immunol. Ther. Exp.* **50**: 279–288

Jung, H. A., Park, J. C., Chung, H. Y., Kim, J., Choi, J. S. (1999) Antioxidant flavonoids and chlorogenic acid from the leaves of *Eriobotrya japonica. Arch. Pharm. Res.* **22**: 213–218

Kimber, I., Dearman, R. J. (1993) Approaches to the identification and classification of chemical allergens in mice. *J. Pharm. Toxicol. Methods* **29**: 11–16

Kitagaki, H. S., Fujisawa, K., Watanabe, Hayakdwa, K., Shiohara, T. (1995) Immediate-type hypersensitivity response followed by a late reaction is induced by repeated epicutaneous application of contact sensitizing agents in mice. *J. Invest. Dermatol.* **105**: 749–755

Kitagaki, H., Ono, N., Hayakawa, K., Kitazawa, T., Watanabe, K., Shiohara, T. (1997) Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. *J. Immunol.* **159**: 2484–2491

Kroegel, C., Yukawa, T., Dent, G., Chanez, P., Chung, K. F., Barnes, P. J. (1988) Platelet-activating factor induces eosinophil peroxidase from purified human eosinophils. *Immunology* **64**: 559

Li, Y., Shi, X., Tang, X. (2005) Effect of the loquat core extracts on rat's intestinal flora and its immunologic function. *Chin. J. Microecol.* **4**: 247–248

Liang, Z. Z., Aquino, R., De Feo, V., De Simone, F., Pizza, C. (1990) Polyhydroxylated triterpenes from *Eriobotrya japonica. Planta Medica* **56**: 330–332

Malorny, U., Goebeler, M., Gutwald, J., Roth, J., Sorg, C. (1990) Difference in migration inhibitory factor production by C57B1/6 and BALB/c mice in allergic and irritant contact dermatitis. *Int. Arch. Allergy Appl. Immunol.* **92**: 356–360

Nozato, N., Matsumoto, K., Uemitsu, N. (1994) Triterpenes from the leaves of *Eriobotrya japonica. Nat. Med*. **48**: 336

Perry, L. M. (1980) *Medicinal plants of east and southeast Asia*. MIT Press, Cambridge, MA

Phanuphak, P., Moorhead, J. W., Claman, H. N. (1974) Tolerance and contact sensitivity to DNFB in mice I. In vivo detection by ear swelling and correlation with in vitro cell stimulation. *J. Immunol*. **112**: 115–123

Robinson, M. K. (1999) Population differences in skin structure and physiology and the susceptibility to irritant and allergic contact dermatitis: implications for skin safety testing and risk assessment. *Contact Dermatitis* **41**: 65–79

Safford, R. J., Basketter, D. A., Allenby, C. F., Goodwin, B. F. J. (1990) Immediate contact reactions to chemicals in the fragrance mix and a study of the quenching action of eugenol. *Br. J. Dermatol*. **123**: 595–606

Shimizu, M., Fukumura, H., Tsuji, H., Tanaami, S., Hayashi, T., Morita, N. (1986) Anti-inflammatory constituents of topically applied crude drugs. I. Constituents and anti-inflammatory effect of *Eriobotrya japonica* Lindl. *Chem. Pharm. Bull*. **34**: 2614–2617

Shimizu, M., Uemitsu, N., Shirota, M., Matsumoto, K., Tezuka, Y. (1996) A new triterpene ester from *Eriobotrya japonica. Chem. Pharm. Bull*. **44**: 2181–2182

Shore, P. A., Burkhalter, A., Cohn, V. H. (1959) A method for the fluorometric assay of histamine in tissues. *J. Pharm. Exp. Ther*. **127**: 182–186

Smith, J. A. (1994) Neutrophils, host defense, and inflammation: a double-edged sword. *J. Leukocyte Biol*. **56**: 672–686

Strath, M., Warren, D. J., Sanderson, C. J. (1985) Detection of eosinophils using an eosinophil peroxidase: its uses as an assay for eosinophil differentiation factors. *J. Immunol. Methods* **83**: 209–215

Thorne, P. S., Hawk, C., Kaliszweski, S. D., Guiney, P. D. (1991) The noninvasive mouse ear swelling assay. I. Refinements for detecting weak contact sensitizers. *Fund. Appl. Toxicol*. **17**: 790–806

Turk, J. L. (1980) *The role of delayed hypersensitivity in granuloma Formation*. Elsevier, Amsterdam

Vadas, M. A., Miller, J. F. A. P., Gamble, J., Whitelaw, A. (1975) A radioisotopic method to measure delayed type hypersensitivity in the mouse. I. Studies in sensitized and normal mice. *Int. Arch. Allergy Appl. Immunol*. **49**: 670–692

Van Loveren, H., Meade, R., Askenase, P. W. (1983) An early component of delayed-type hypersensitivity mediated by T cells and mast cells. *J. Exp. Med*. **157**: 1604

Wever, R., Hamers, M. N., De Graaf, C. J., Weening, R. S., Roos, D. (1982) Characterization of the peroxidase in human eosinophils. *Adv. Exp. Med. Biol*. **141**: 501

White, S. R., Kulp, G. V. P., Spaethe, S. M., Alstyne, E. V., Leff, A. R. (1991) A kinetic assay for eosinophil peroxidase activity in eosinophils and eosinophil conditioned media. *J. Immunol. Methods* **44**: 257–263

Yokota, J., Takuma, D., Hamada, A., Onogawa, A., Yoshioka, S., Kusunose, M., Miyamura, M., Kyotani, S., Nishioka Y. (2006) Scavenging of reactive oxygen species by *Eriobotrya japonica* seed extract. *Biol. Pharm. Bull*. **29**: 467–471