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Evaluation of Antiinflammatory Activity of the Total Flavonoids of *Laggera pterodonta* on Acute and Chronic Inflammation Models

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The antiinflammatory effect of the total flavonoids of *Laggera pterodonta* (TFLP) was evaluated with various *in vivo* models of both acute and chronic inflammation. In the acute inflammation tests, TFLP significantly inhibited xylene-induced mouse ear oedema, carrageenan-induced rat paw oedema and acetic acid-induced mouse vascular permeability. In the carrageenan-induced rat pleurisy model, TFLP efficiently suppressed inflammatory exudate and leukocyte migration, reduced the serum levels of lysozyme (LZM) and malondialdehyde (MDA), increased the activity of serum superoxide dismutase (SOD), and also decreased the contents of total protein, nitric oxide (NO) and prostaglandin E_2 (PGE_2) in the pleural exudates. No marked effect of TFLP on the activity of serum glutathione peroxidase (GSH-PX) was observed. In the chronic inflammation experiment, TFLP inhibited cotton pellet-induced rat granuloma. The antiinflammatory mechanisms of TFLP are probably associated with the inhibition of prostaglandin formation, influence on the antioxidant systems and the suppression of LZM release. The acute toxicity study revealed that TFLP was nontoxic in mice up to an oral dose of 7.5 g/kg body weight.

Keywords: Laggera pterodonta; phytomedicine; antiinflammation; total flavonoids.

INTRODUCTION

Laggera pterodonta (Asteraceae) is widely distributed in southwestern China, especially in Yunnan and Sichuan provinces. It has been used traditionally as folk medicine for the treatment of various diseases related to inflammation for over 300 years (Jiangsu New Medical College, 1977). Some detailed phytochemical investigations on the plant have been performed by several groups, while flavonoids and eudesmane derivatives were found to be the main secondary metabolites of the herb (Li and Ding, 1994; Wei *et al.*, 1995; Xiao *et al.*, 2003; Zhao *et al.*, 1997a; 1997b; 1997c). However, pharmacological studies of *L. pterodonta* have been reported rarely. There are only two reports involving the defervescent, removing phlegm and antiviral activities of an aqueous extract from *L. pterodonta* (Deng, 1963; Li *et al.*, 2004).

To further clarify the potent type of components existing in the plant, the whole EtOH extract of *L. pterodonta* was separated into a terpenoids part and a crude flavonoids part. An *in vivo* test indicated that the terpenoids part was nearly inactive while the total flavonoids part possessed potent antiinflammatory activity. The inhibitory effects on inflammation of the total flavonoids from *L. pterondonta* (TFLP) were then investigated in more detail with the use of various acute and chronic inflammation models, i.e. xylene-induced ear oedema, carrageenan-induced paw oedema, acetic acid-induced vascular permeability, carrageenan-induced pleurisy and cotton pellet-induced granuloma. The results suggested that TFLP possesses potent anti-inflammatory activity in the above-mentioned acute and chronic inflammation models.

MATERIALS AND METHODS

Materials. The whole herb of *Laggera pterodonta*, a traditional herbal medicine with the Chinese name Chou Ling Dan, was collected from Dali, Yunnan province of China in July 2002. The plant was identified by Professor Liurong Chen of the Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University, China. Voucher specimens (ZY200207LP) are deposited in the herbarium of the College of Pharmaceutical Sciences, Zhejiang University, China.

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Extraction. The whole herb of *L. pterondonta* (10 kg) was dried in shade, then cut into segments 0.5–2.0 cm long and extracted three times with 95% ethanol. The solvent was combined and condensed under reduced pressure to remove ethanol. The aqueous extract remaining was partitioned with ethyl acetate after disposed by 5% $NaHCO_3$. The ethyl acetate fraction was extracted repeatedly using 1% NaOH. The water fraction obtained was acidified to pH 4.0 with 1 N HCl and then extracted three times by ethyl acetate. The ethyl acetate fraction was washed with water to pH 7.0 and concentrated to dryness under reduced pressure. The ethyl acetate extract was concentrated, checked by TLC and $FeCl_3$-Mg reaction, and found to contain mainly flavonoids in a yield of 2.5%, and thus named as the total flavonoids of *L. pterondonta* (TFLP). TFLP was dissolved in normal saline for drug administration in the animal tests.

Chemicals. Carrageenan and Evan's blue were obtained from Sigma Chemical Co., (St Louis, MO, USA). Nitric oxide (NO), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), lysozyme (LZM) and total protein kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). All other reagents were of analytical grade.

Animals. Male standard ICR strain mice weighing 20–25 g and male standard Sprague-Dawley strain rats weighing 180–220 g were bred in the Center of Animal Laboratory, College of Medicine, Zhejang University. The animals were kept in a room maintained at 22 ± 2 °C and at relative humidity between 40% and 70%. The experimental protocol was approved by the Animal Ethics Committee of Zhejiang University, in compliance with the 'Principles of Laboratory Animal Care and Use in Research' (Ministry of Health, Beijing, China).

Xylene-induced ear oedema in mice. The method described by Young and De Young (1989) was employed. Male ICR mice were divided into six groups. Group A: normal saline; group B: dexamethasone (2.5 mg/kg); group C: TFLP (25 mg/kg); group D: TFLP (50 mg/kg); group E: TFLP (100 mg/kg); group F: TFLP (200 mg/kg). The vehicle and drugs were administered orally to individual groups of mice, once a day for 3 days. Group A received the same volume of normal saline orally as a vehicle control. One hour after the last administration of drugs, 0.03 mL xylene was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as the control. One hour after xylene application, the mice were killed by dislocating the neck and 9 mm punches were made in the right and the left ear by a borer. Each ear disc was weighed and the differences in weight of the right and left ear discs of mice were recorded as the oedema level.

Acetic acid-induced vascular permeability in mice. This test was performed by the method described by Whittle (1964). Male ICR mice were divided into three groups. TFLP at 100 mg/kg was administered orally to the test group of mice. The positive and negative control groups of mice were given prednisone (10 mg/kg) and the same volume of normal saline, respectively. The mice received drugs orally once a day for 7 days. One hour after the last treatment of drug, 0.2% Evan's blue in normal saline was injected intravenously into the tail vein at a dose of 0.1 mL/10 g body weight. Thirty minutes later, each mouse was injected intraperitoneally with 0.2 mL of 0.6% acetic acid in normal saline. One hour after intraperitoneal injection, the mice were killed by dislocating the neck and the abdominal wall was cut to expose the intestine. The abdominal cavity was washed using 5 mL of normal saline to collect pigments in a test tube. After centrifuging the contents of the tube to eliminate contaminants, the solution was subjected to colorimetry using a spectrophotometer at a wavelength of 590 nm. Control mice were treated similarly. The vascular permeability effects were expressed by the absorbance (A) of the total dye amount that leaked into the intraperitoneal cavity.

Carrageenan-induced paw oedema in rats. The method was assayed according to Winter *et al.* (1962). The rats were divided into three groups. The drug control group received dexamethasone orally at a dose of 2.5 mg/kg. The same volume of normal saline was administered orally to the vehicle control group of rats, while TFLP at a dose of 100 mg/kg was given orally to the test group of animals. The drugs or vehicle were given to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw oedema was induced by subplantar injection of 0.1 mL of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The left hind paw was injected with 0.1mL of normal saline. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5 and 6 h after carrageenan injection using a plethysmometer.

Carrageenan-induced pleurisy in rats. The method of Mikami and Miyasaka (1983) was employed in this experiment. The rats were divided into four groups. The experimental and positive drug groups of animals were treated with TFLP at a dose of 100 mg/kg and dexamethasone at a dose of 2.5 mg/kg, respectively. The same volume of normal saline was administered to the vehicle and test groups of rats. The drugs were given orally once a day for 7 days. One hour after the last administration of drugs, the rats were lightly anaesthetized under ether and then 0.2 mL of normal saline alone or containing 1% carrageenan was injected into the pleural cavity of each animal. Four hours after the injection of carrageenan, the rats were lightly anaesthetized and blood samples were taken from the eyepit. The serum was separated and stored at –20 °C for the measurement of LZM, MDA, SOD and GSH-PX. The animals were then killed under an overdose of ether and the chest was carefully opened. The exudate was removed and its volume was measured. Exudates contaminated with blood were discarded. The pleural cavity was then washed with 2 mL of phosphate-buffered saline (PBS, pH 7.2, ice cold,) with heparin (5 U/mL). The exudate and washing were combined as the pleural exudates for measurement of NO, PGE_2, total protein and total leukocyte number. The total leukocyte number was counted in a Neubauer chamber.

The levels of LZM, MDA, SOD and GSH-PX in the pleural rat serum were assayed with LZM, MDA, SOD and GSH-PX kits, respectively. The production

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Table 1. Effects of total flavonoids of *Laggera pterodonta* (TFLP) and dexamethasone (DEX) on xylene-induced ear oedema in mice

Group	Dose (mg/kg, p.o.)	Oedema degree (mg)	Inhibition (%)
Vehicle	—	29.45 ± 0.57	—
DEX	2.5	8.90 ± 0.26[a]	69.8
TFLP	25	18.46 ± 0.52[a]	37.3
	50	15.89 ± 0.48[a]	46.0
	100	13.33 ± 0.73[a]	54.7
	200	10.85 ± 0.42[a]	63.2

Values are mean ± SEM of differences in weight between right and left ear of animals (n). $n = 10$, [a] $p < 0.001$, compared with vehicle control group (one-way ANOVA, Dunnett's t-test as the post hoc test).

of NO and total protein in the pleural exudate was measured with NO and total protein kits, respectively. The measurement of PGE_2 in the pleural exudates was performed according to the method described by Wu (1991).

Cotton pellet-induced granuloma in rats. The experiment was carried out by the method of Winter and Porter (1957). The rats were divided into five groups. Under ether anesthesia, sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously in both axilla regions of each rat through a single needle incision, one on each side. The second group served as a drug control and received dexamethasone daily at a dose of 2.5 mg/kg orally for 7 days. The same volume of normal saline was given orally to the first group of animals as a vehicle control. TFLP at doses of 50, 100 and 200 mg/kg was administered orally to the other three groups of rats, respectively, for 7 days from the day of cotton pellet implantation. On day 8, the animals were killed under an overdose of ether. The granuloma tissue was dissected out and dried at 60 °C to a constant weight. The increase in dry weight of the pellets was taken as the measure of granuloma formation.

Acute toxicity. Different doses of TFLP were given orally to groups of ten mice each. The number of deaths was recorded within 7 days after treatment.

Statistical analysis. Values of the measured index were expressed as the mean ± standard error of the mean (SEM), while the statistical model of one-way analysis of variance (ANOVA) followed by Dunnett's t-test or Tamhane's T_2 was applied to test the differences among the study groups. A value of $p < 0.05$ was chosen as the criterion of statistical significance.

RESULTS

Effect of TFLP on xylene-induced ear oedema in mice

The oral administration of TFLP suppressed significantly xylene-induced ear oedema in mice (Table 1). The oedema inhibitory rates of TFLP were 37.3%, 46.0%, 54.7% and 63.2% at doses of 25, 50, 100 and 200 mg/kg, respectively, whereas dexamethasone (2.5 mg/kg), used as a reference drug, exhibited a 69.8% inhibitory rate compared with the control.

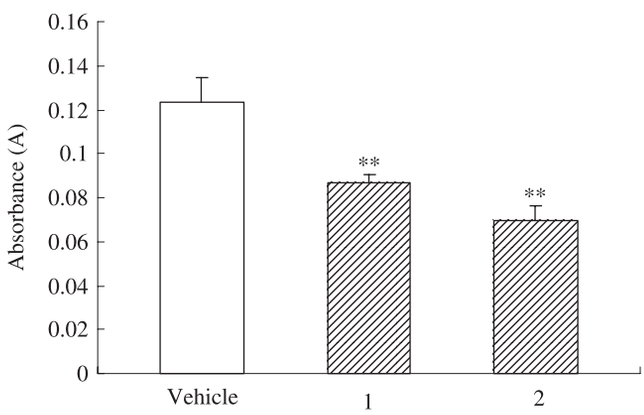

Figure 1. Effect of total flavonoids of *Laggera pterodonta* (TFLP) and prednisone on acetic acid-induced increased vascular permeability in mice. Drugs were administered p.o. once a day for 7 days. 1, prednisone 10 mg/kg; 2, TFLP 100 mg/kg. Values are mean ± SEM, $n = 7$, ** $p < 0.01$ compared with vehicle control group (one-way ANOVA, Dunnett's t-test as the post hoc test).

Effect of TFLP on acetic acid-induced vascular permeability in mice

The TFLP showed significant activity against acute inflammation induced by acetic acid (Fig. 1). At a dose of 100 mg/kg, the effect of TFLP on vascular permeability was much stronger than that of 10 mg/kg of prednisone. TFLP and the standard drug used for comparison produced 44.1% and 29.9% inhibition of dye leakage, respectively.

Effect of TFLP on carrageenan-induced paw oedema in rats

The oral treatment with TFLP inhibited carrageenan-induced paw oedema in rats (Fig. 2). At a dose of 100 mg/kg of TFLP, oedema formation was suppressed and inhibitory rates were 6.4%–65.2% at 1–6 h after carrageenan treatment. As a standard control, dexamethasone produced a greater inhibition of oedema development by 51.8%–76.8% at 1–6 h after carrageenan injection at a dose of 2.5 mg/kg.

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Table 2. Effect of total flavonoids of *Laggera pterodonta* (TFLP) and dexamethasone (DEX) on exudate volume, leukocyte migration, total protein content, NO and PGE_2 levels in pleurisy in rats induced by carrageenan

Group	Dose (mg/kg, p.o.)	Exudate (mL)	Total leukocyte ($\times 10^7$/mL)	Total protein (g/L)	NO (μmol/L)	PGE_2 (A)
Vehicle	—	0.03 ± 0.009[a]	1.10 ± 0.153[a]	25.20 ± 1.39[a]	40.17 ± 1.20[a]	0.189 ± 0.022[a]
Model	—	1.44 ± 0.090	3.13 ± 0.127	36.54 ± 1.09	130.47 ± 3.49	0.754 ± 0.036
DEX	2.5	0.43 ± 0.084[a]	1.20 ± 0.124[a]	18.84 ± 1.26[a]	77.17 ± 2.50[a]	0.446 ± 0.031[a]
TFLP	100	0.78 ± 0.076[a]	2.07 ± 0.145[a]	24.36 ± 1.10[a]	88.95 ± 2.42[a]	0.339 ± 0.038[a]

Values are mean ± SEM, $n = 8$, [a] $p < 0.001$ with respect to model control group; Exudate volume was analysed statistically by Tamhane's T_2, while index for leukocyte counts, total protein, NO and PGE_2 were done by Dunnett's *t*-test, one-way ANOVA.

Table 3. Effects of total flavonoids of *Laggera pterodonta* (TFLP) and dexamethasone (DEX) on the serum level of lysozyme, MDA, SOD and GSH-PX in pleurisy in rats induced by carrageenan

Group	Dose (mg/kg, p.o.)	Lysozyme (μg/mL)	MDA (nmol/mL)	SOD (U/mL)	GSH-PX (μmol/L)
Vehicle	—	31.95 ± 1.40[c]	9.11 ± 0.25[a]	249.24 ± 2.23[c]	379.62 ± 3.68[b]
Model	—	47.31 ± 1.73	12.45 ± 0.80	215.40 ± 2.11	346.20 ± 5.76
DEX	2.5	35.40 ± 2.29[c]	7.67 ± 0.19[b]	238.35 ± 3.86[c]	372.41 ± 1.90[a]
TFLP	100	40.42 ± 1.44[a]	8.77 ± 0.20[a]	248.09 ± 1.86[c]	366.63 ± 5.22

Values are mean ± SEM, $n = 8$, [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ compared with model control group; MDA was analysed statistically by Tamhane's T_2, while LZM, SOD and GSH-PX were done by Dunnett's *t*-test, one-way ANOVA.

Table 4. Effect of oral treatment with total flavonoids of *Laggera pterodonta* (TFLP) and dexamethasone (DEX) on cotton pellet-induced granuloma in rats

Group	Dose (mg/kg, p.o.)	Animals (*n*)	Granuloma weight (mg/100 g body weight)	Inhibition (%)
Vehicle	—	8	10.24 ± 0.30	—
DEX	2.5	8	3.14 ± 0.12[a]	69.2
TFLP	50	8	7.12 ± 0.21[a]	30.5
	100	8	6.01 ± 0.26[a]	41.3
	200	8	4.87 ± 0.24[a]	52.4

Values are mean ± SEM, [a] $p < 0.001$, compared with vehicle control group (one-way ANOVA, Dunnett's *t*-test as the post hoc test).

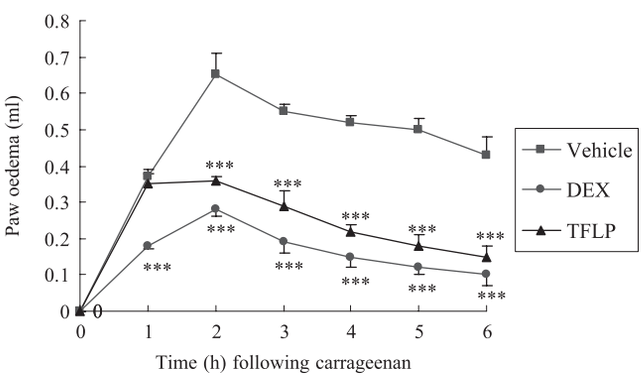

Figure 2. Effect of total flavonoids of *Laggera pterodonta* (TFLP) and dexamethasone (DEX) on carrageenan-induced paw oedema in rats. ■ Vehicle control; ▲ TFLP 100 mg/kg; • dexamethasone 2.5 mg/kg. Values are mean ± SEM, $n = 8$, *** $p < 0.001$ compared with vehicle control group (one-way ANOVA, Dunnett's *t*-test as the post hoc test).

Effect of TFLP on carrageenan-induced pleurisy in rats

At a dose of 100 mg/kg, the oral pre-treatment of TFLP significantly reduced the pleural exudate volume and the total leukocyte migration, inhibited the production of total protein and two inflammatory mediators (NO and PGE_2) in the pleural exudates (Table 2), increased the activity of serum SOD, and also decreased the serum levels of LZM and MDA. But no marked effect of TFLP on the activity of serum GSH-PX was observed (Table 3). Dexamethasone used as the standard drug indicated a similar effect.

Effect of TFLP on cotton pellet-induced granuloma in rats

The effects of TFLP and dexamethasone on cotton pellet-induced granuloma in rats are shown in Table 4.

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At doses of 50, 100 and 200 mg/kg, TFLP as well as dexamethasone (2.5 mg/kg) inhibited markedly the granuloma formation compared with the vehicle control group; 200 mg/kg of TFLP exhibited a maximum inhibition of 52.4%, while 50 mg/kg and 100 mg/kg of TFLP showed 30.5% and 41.3% inhibition in granuloma weight, respectively, when compared with 69.2% for dexamethasone.

Acute toxicity

No dead animal was found in the course of the acute toxicity test. The results revealed that TFLP was nontoxic in mice up to an oral dose of 7.5 g/kg body weight.

DISCUSSION

The antiinflammatory activity of large amounts of extracts from *L. pterondonta* was evaluated by the xylene-induced mouse ear oedema model. The results of prescreening indicated that TFLP had a strong inhibitory effect on xylene-induced ear oedema (data not shown). To confirm whether the components of TFLP from the original EtOH extract of *L. pterondonta* changed during acidic and basic treatment procedures, TFLP was compared with the original EtOH extract by HPLC/TLC. The results suggested that the components of TFLP from the original EtOH extract were unaltered.

Increased vascular permeability is one of the essential features of the acute inflammatory response. Acetic acid-induced increased vascular permeability in mice, a typical capillary permeability assay, was employed to investigate the effect of TFLP on the increase in vascular permeability. The study indicated that TFLP produced a significant inhibition on increased vascular permeability.

Carrageenan-induced paw oedema, a classical model of acute inflammation, has been widely used in the study of steroid and non-steroid antiinflammatory drugs (Vinegar *et al.*, 1987). This test showed that TFLP inhibited significantly rat paw oedema at 2–6 h after carrageenan injection.

Cotton pellet-induced granuloma in rats, a chronic model of inflammation, has been utilized to assess the activity of antiinflammatory drugs on the proliferative phase of inflammation (Selye, 1953). The results showed that TFLP possessed marked antiproliferative activity.

Carrageenan-induced pleurisy is a well-characterized experimental model of inflammation that permits the quantification and correlation of both exudates and cellular migration with changes of other inflammatory parameters (Vinegar *et al.*, 1982). The effect of TFLP on carrageenan-induced pleurisy in rats further confirmed its antiinflammatory activity, which included a reduction in the volume of pleural exudates and the content of total protein as well as inhibition of leukocyte migration.

The data from this study indicated that several factors may contribute to the antiinflammatory action of TFLP. Firstly, one possible mechanism involves the inhibition of prostaglandin formation at the site of inflammation. Prostaglandins (PGs), arachidonate metabolites, are important mediators in inflammation. The increase in the formation of PGs corresponds to the release of arachidonic acid from membrane phospholipids and the up-regulation of cyclooxygense-2 (Subbaramaiah *et al.*, 1997). Among the different prostaglandins, PGE_2 plays an important role in plasma exudation of carrageenan-induced rat pleurisy (Harada *et al.*, 1982). The result showed that TFLP significantly decreased the content of PGE_2 in the pleural exudates, thereby suggesting that TFLP interferes with the cyclooxygenase pathways of arachidonate metabolism.

Secondly, the antiinflammatory mechanism of TFLP is partially associated with its influence on the antioxidant systems. It has been reported that in the process of carrageenan-induced inflammation, the early phase is related to the production of histamine, leukotrienes, platelet-activating factor and possibly cyclooxygenase products, while the delayed phase is linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as to the release of other neutrophil-derived mediators (Oh-ishi *et al.*, 1989; Dawson *et al.*, 1991). In addition to oxyradicals, overproduction of NO also plays an important role in various models of inflammation (Cuzzocrea *et al.*, 1998; Moncada *et al.*, 1991). The reaction of NO with superoxide anion forms peroxynitrite (Beckman *et al.*, 1990), a potent cytotoxic oxidant eliciting lipid peroxidation and cellular damage (Rubbo *et al.*, 1994). As an indicator of lipid peroxidation, the serum level of MDA was measured in the current study. Furthermore, the serum levels of SOD and GSH-PX, both of which are antioxidant enzymes, were also measured to evaluate the ability to scavenge radicals. The results from the experimental group in carrageenan-induced rat pleurisy confirmed the overproduction of NO in the pleural exudates, an increase in the serum level of MDA, and a decrease in the serum levels of SOD and GSH-PX during the course of inflammation. Meanwhile, TFLP was found to be able to reduce NO production, to lower the serum level of MDA and to increase the serum levels of SOD and GSH-PX, implying that TFLP could not only inhibit lipid peroxidation but also scavenge radicals by enhancing the activities of the antioxidant enzymes. However, the effect of TFLP on serum GSH-PX was rather weak. Therefore, the antioxidative properties of TFLP may be attributed to an alleviation of the inflammatory response.

Finally, another antiinflammatory mechanism of TFLP is due, at least in part, to the inhibition of lysozyme release. It is known that degranulation occurs after neutrophils reach the injured tissue by margination, adhesion and emigration. As a result, LZM is discharged from lysosomes of neutrophils. Released LZM destroys not only phagosomes but also the tissue itself, which aggravates the responses to inflammation (Heiman *et al.*, 1989). In our study, TFLP apparently attenuated the serum level of LZM. The inhibitory effect of TFLP on LZM release hence protects the tissue from damage induced by inflammation.

A large number of plants containing flavonoids are used in folk medicine, in many cases as antiinflammatory agents. Pharmacological studies indicated that flavonoids exert antiinflammatory, antimicrobial, antiviral and antiplatelet activities and possess inhibitory

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effects on protein exudation, leucocyte migration and capillary permeability (Pelzer *et al.*, 1998). The ability of flavonoids to inhibit both the cyclooxygenase and 5-lipoxygenase pathways of the arachidonate metabolism may contribute to the antiinflammatory properties (Williams *et al.*, 1995). Flavonoids also display many antioxidant properties including scavenging free radicals and preventing lipid peroxidation (Torel *et al.*, 1986). Our findings on the effect of TFLP agree with the description of the antiinflammatory action and mechanism of flavonoids in the aforementioned references.

According to the studies described above, the conclusion could be drawn that TFLP possesses potent antiinflammatory activity. The antiinflammatory mechanisms of TFLP are probably associated with an inhibition of prostaglandin formation, an influence on the antioxidant systems and a suppression of LZM release. Based on the acute toxicity study, TFLP could be considered as almost non-toxic to tested animals. Taking into account its potent bioactivity and relatively very low toxicity, TFLP is worthy of further investigation to explore the bioactive components of *L. pterondonta* for the discovery of anti inflammation natural drugs.

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