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Evaluation of the *in Vivo* Anti-inflammatory Effects of Extracts from *Pyrus bretschneideri* Rehd.

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Pyrus bretschneideri Rehd., as a pharmaceutical supplement, is widely used in northern China to treat respiratory diseases. Our previous studies showed the ethanol extract of *P. bretschneideri* had significant anti-inflammatory activity. To isolate and identify the active ingredients, the ethanol extract was separated into petroleum ether, ethyl acetate, *n*-butanol, and aqueous fractions. The bioactivity of each fraction was investigated using an *in vivo* model. Results showed that the ethyl acetate fraction exhibited the strongest anti-inflammatory effect. Subsequently, this fraction was subjected to separation and purification using silica gel column chromatography, C_{18}−ODS, and recrystallization, leading to two sterols and two triterpenes, which were identified as β-sitosterol, daucosterol, oleanolic acid, and ursolic acid. Moreover, all of the isolated compounds could significantly inhibit the ear edema induced by xylene. These results indicated that *P. bretschneideri* had good anti-inflammatory effects and the constituents β-sitosterol, daucosterol, oleanolic acid, and ursolic acid might well account for it.

KEYWORDS: ***Pyrus bretschneideri* Rehd.; anti-inflammatory activity; sterols; triterpenes**

INTRODUCTION

Pyrus bretschneideri Rehd. (xuehua pear) belongs to the genus *Pyrus* and has been planted in China, with a more than 3000 year history. It was named as "ancestor for numerous fruits" for its unique flavor, such as subtle aroma, sweetness, and crispness. Furthermore, with the effects on clearing the lungs, dissipating phlegm, and promoting fluid production to quench thirst, it has been commonly used to treat with respiratory diseases in traditional Chinese medicine for centuries (*1*).

Phenolics are considered to be the main active ingredients in pears, and the anti-ulcer effect of phenolics in pears has also been reported (*2*). Early studies showed that the main phenolics found in pear are leucocyanidin, catechin, epicatechin, chlorogenic acid, quercitrin, and quercetin (*3*). With more sophisticated equipment, Herrmann (*4*) indicated that the phenolics found in pear and apple are chlorogenic acid, catechin, epicatechin, and procyanidin. These results are supported by the findings of Spanos and Wrolstad (*5*), who claim that the phenolics in pear juice are chlorogenic acid, epicatechin, catechin, caffeic acid, and coumaroylquinic acid. Pear contains ursolic acid in its wax-like coating as well (*6*). Tanriöven and Ekşi (*7*) reported that the amounts of chlorogenic acid, epicatechin, and caffeic and *p*-coumaric acids in juice pear are 73.1−249, 11.9−81.3, 2.4−11.4, and 0.0−3.0 mg/L, respectively.

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Wildanger and Herrmann (*8*) found that the major flavonol in apple and pear is quercetin and there is also kaempferol in small quantities. Oleszek et al. (*9*) isolated four hydroxycinnamic acid esters and eight flavonol glucosides in pear. Three of the glucosides were quercetin, and the remaining five were isorhamnetin glucosides. However, there is no experimental report on the screening of bioactive fractions and components from this fruit.

The ethnomedicinal importance of *P. bretschneideri* prompted us to investigate the chemical constituents of this fruit and its association with anti-inflammation. Therefore, the present study focused on the evaluation of the anti-inflammatory activity of different fractions and subfractions of *P. bretschneideri* by *in vivo* inflammation models. For this purpose, a bioassay-guided fractionation and purification process was carried out. The analysis of the obtained results will define the correlation, if any, between the anti-inflammatory activity and specific components of this fruit.

MATERIALS AND METHODS

Plant Materials and Reagents. *P. bretschneideri* Rehd. (xuehua pear) was collected from Yantai, Shandong Province, China, in September 2009, a region that is exclusively rich in *P. bretschneideri*. The material was identified by Professor Wenyuan Gao (School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China) and stored at 4 °C in the laboratory of Tianjin University. Xylene and glacial acetic acid were purchased from Guangfu Fine Chemical Research Institute (Tianjin, China). Evan's blue was purchased from the Sigma Company (St. Louis, MO). Dexamethasone (DEX) was purchased from the Lisheng Pharmaceutical Company, Ltd. (Tianjin, China).

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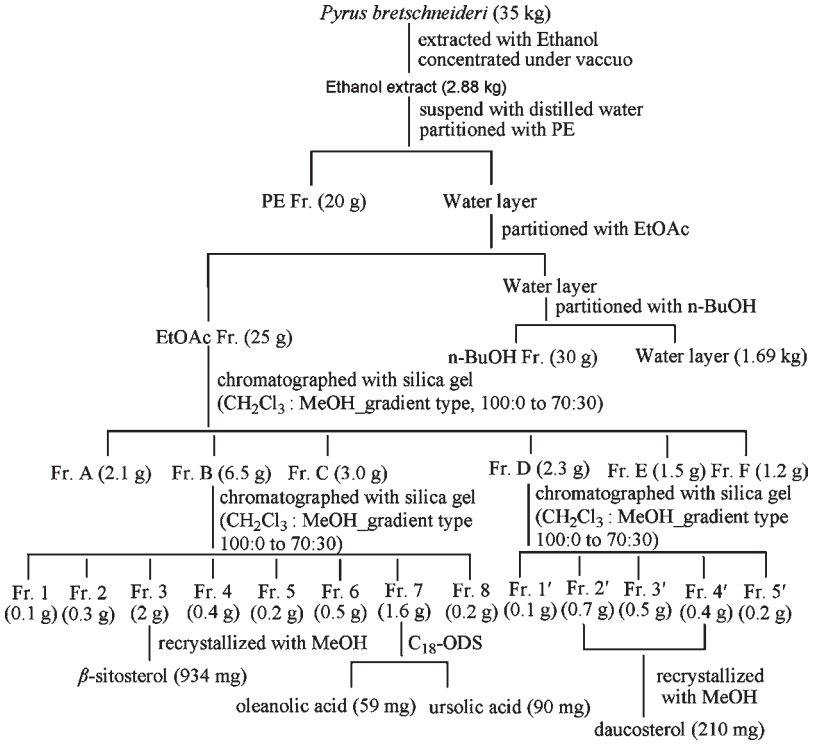

Figure 1. Isolation procedure of β-sitosterol, daucosterol, oleanolic acid, and ursolic acid from *P. bretschneideri*.

Chemicals. β-Sitosterol, daucosterol, oleanolic acid, and ursolic acid were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Peking, China).

Preparation of Fruit Extracts. *P. bretschneideri* Rehd. (35 kg) was extracted with 95% ethanol (1:8, w/v) for 3 times, 2 h each, and the extract obtained was evaporated under reduced pressure to remove ethanol and concentrated to obtain the crude extract (CE, 2.88 kg) at a temperature below 45 °C. The CE was fractionated by liquid−liquid extraction using solvents in growing order of polarity, resulting in petroleum ether (PE, 20 g), ethyl acetate (EtOAc, 25 g), *n*-butanol (*n*-BuOH, 30 g), and aqueous (AQ, 1.69 kg) fractions.

Isolation and Identification of the Compounds. The ethyl acetate fraction was subjected to silica gel column chromatography with a CH_2Cl_2−MeOH step gradient (from 100:0 to 70:30: 100:0, 95:5, 90:10, 85:15, 80:20, and 70:30, with 2 L of each solvent) to yield six fractions (fractions A−F). The experiment of the activity-guided separation of fractions on anti-inflammatory action showed that fractions B and D were more potent than others. Using re-chromatography on a silica gel column, fraction B (6.5 g) was separated with a CH_2Cl_2−MeOH step gradient (from 100:0 to 70:30: 100:0, 98:2, 95:5, 90:10, 85:15, 80:20, 75:25, and 70:30, with 200 mL of each solvent) to yield eight fractions (fractions 1−8). Subsequently, 2 g of fraction 3 was submitted to recrystallization with MeOH to obtain β-sitosterol (934 mg), and then fraction 7 (1.6 g) was further subjected to separation and purification using C_{18}−ODS with MeOH−H_2O (85:15) and yielded two pure compounds (59 mg of oleanolic acid and 90 mg of ursolic acid, respectively). Fraction D (2.3 g) was separated by a silica gel column chromatograph with a CH_2Cl_2−MeOH step gradient (from 100:0 to 70:30: 100:0, 95:5, 90:10, 80:20, and 70:30, with 100 mL of each solvent) to yield five fractions (fractions 1′−5′). The white powder (daucosterol, 210 mg) was isolated from fractions 2′−4′ by recrystallization with MeOH (**Figure 1**).

The structures of the known compounds were identified on the basis of their physical characteristics, spectroscopic data [ultraviolet (UV), ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, infrared (IR), and liquid chromatography−mass spectrometry (LC−MS)] measurements, comparison to spectral data obtained from the literature (*10*−*12*), and co-thin-layer chromatography (TLC) with authentic samples.

Animals. Kunming mice at 6−8 weeks of age typically weighing 18−22 g for females and standard Sprague−Dawley rats at 8−10 weeks of age typically weighing 180−220 g for females were obtained from the Institute of Laboratory Animal of Chinese Academy of Medical Science, Peking, China. The animals were fed with a rodent standard diet with free access water *ad libitum* and were housed in rooms maintained at 25 ± 1 °C with a 12 h light/dark cycle following international recommendations. The Animal Ethics Committees of the Faculty of Medicine, Tianjin University, Tianjin, China, approved all experimental protocols, in accordance with "Principles of Laboratory Animal Care and Use in Research" (Ministry of Health, Beijing, China).

Carrageenan-Induced Paw Edema. The experiment was carried out using the method by Winter et al. (*13*). The CE of *P. bretschneideri* ranging from 0.5 to 2 g/kg were administered orally for 5 consecutive days. At the 5th day, before any treatment, the average thickness (the center thickness of the paw, at 0.5 cm below the ankle joint) of the right paw of each animal was determined (basal thickness) using a sliding caliper. Subsequently, test materials were administered orally, and 45 min later, 0.1 mL of freshly prepared carrageenan [1% (w/v), in normal saline] was injected in the subplantar region of the right hind paw. The paw thickness was measured at 0.5, 1, 3, and 5 h after the carrageenan injection. Swelling was assessed in terms of the mean thickness increase of each paw in comparison to the control group.

Acetic-Acid-Induced Capillary Permeability Accentuation. Oral treatments with the CE before inducing capillary permeability accentuation were given for 5 consecutive days. At the 5th day, 1% Evan's blue solution in saline (0.1 mL/10 g) was injected intravenously into the tail vein at 30 min after the last oral treatment. At 10 min later, 0.7% acetic acid in saline (0.1 mL/10 g) was injected into the peritoneal cavity to increase capillary permeability. Mice were killed by cervical dislocation at 20 min after the acetic acid injection, and then the abdominal cavity was washed with saline (1 × 5 mL). The washing solutions were collected and centrifuged (1000*g* for 10 min), and then supernatants were collected. Their absorbance was measured at 590 nm with a spectrophotometer (*14*).

Xylene-Induced Ear Edema. The CE, fractions, and the isolated compounds were administered orally for 5 consecutive days. At 45 min after the last oral treatment of mice, edema was induced in each mouse by applying 0.1 mL of xylene to the inner and outer surfaces of the right ear. At 1.5 h later, the animals were killed and both ears were cut off and weighed. The edematous response was measured as the weight difference between the right and left ears. The anti-inflammatory activity was expressed as a percentage of the inhibition of edema in treated mice in comparison to control mice (*15*).

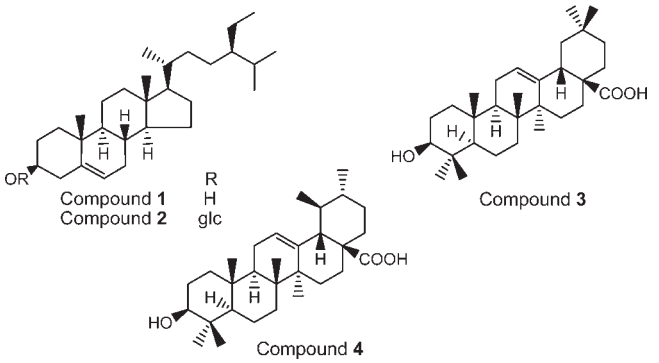

Figure 2. Chemical structures of compound **1** (β-sitosterol), compound **2** (daucosterol), compound **3** (oleanolic acid), and compound **4** (ursolic acid) isolated from the ethyl acetate fraction of *P. bretschneideri*.

Statistical Analysis. Results obtained from animal experiments were expressed as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variation (ANOVA) followed by Scheffe's multiple range tests. The criterion for statistical significance was $p < 0.05$.

RESULTS

Phytochemical Analysis. In this study, according to the Lieberman−Burchard reactions, preliminary phytochemical analysis showed that the CE of *P. bretschneideri* contained a significant amount of steroids and terpenoids. Accordingly, we isolated β-sitosterol (compound **1**), daucosterol (compound **2**), oleanolic acid (compound **3**), and ursolic acid (compound **4**) (**Figure 2**) from the ethyl acetate fraction. Except for ursolic acid, these compounds were all isolated from *Pyrus* for the first time. β-Sitosterol represented 3.74%; daucosterol represented 0.84%; oleanolic acid represented 0.24%; and ursolic acid represented 0.36% of the ethyl acetate fraction.

Effects of the CE of *P. bretschneideri* on Carrageenan-Induced Paw Edema. The reduction of carrageenan-induced paw edema was measured for 5 h (**Figure 3**). The CE of *P. bretschneideri* significantly inhibited the carrageenan-induced paw edema in a dose-dependent manner ($p < 0.01$). The inhibited rates of CE at a dose of 2 g/kg were up to 46.40% (0.5 h), 45.26% (1 h), 53.90% (3 h), and 47.98% (5 h) after injection of carrageenan. The effect at 3 h was comparable to that of DEX (10 mg/kg, 58.38%).

Effects of the CE of *P. bretschneideri* on Acetic-Acid-Induced Capillary Permeability Accentuation. Acetic acid (0.7% (v/v), 0.1 mL/10 g, intraperitoneally) significantly accentuated the capillary permeability by increasing exudation into the peritoneal cavity compared to the vehicle controls. The CE of *P. bretschneideri* (0.5−1 g/kg) significantly decreased the acetic-acid-induced capillary permeability accentuation from 0.341 ± 0.047 to 0.221 ± 0.035 ($p < 0.01$). The inhibited rates were up to 28.04% (1 g/kg) and 35.34% (2 g/kg), respectively (**Figure 4**).

Effects of the CE of *P. bretschneideri* and Its Derived Fractions on Xylene-Induced Ear Edema. As shown in **Table 1**, the CE of *P. bretschneideri* and its different solvent fractions exhibited varying degrees of anti-inflammatory activities. The ethyl acetate fraction administered orally at a dose of 200 and 400 mg/kg showed strong dose-dependent reduction of the xylene-induced ear edema, whereas other solvent fractions had little or no effect at 400 mg/kg. The peak inhibitory effect (44.66%) recorded with the ethyl acetate fraction at a dose of 400 mg/kg was comparable to that of DEX (10 mg/kg, 50.28%).

Effects of the Different Subfractions from the Ethyl Acetate Fraction on Xylene-Induced Ear Edema. All of the different subfractions from the ethyl acetate fraction showed varying degrees of inhibited effects on xylene-induced ear edema, and

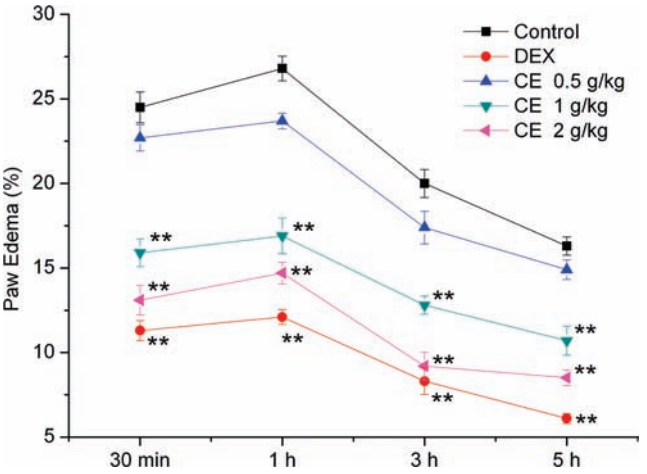

Figure 3. Effects of the CE of *P. bretschneideri* on carrageenan-induced paw edema in rats. DEX, dexamethasone; CE, the ethanol extract of *P. bretschneideri*. Results were expressed as the mean ± SEM ($n = 8$). (**) $p < 0.01$ was compared to the control group.

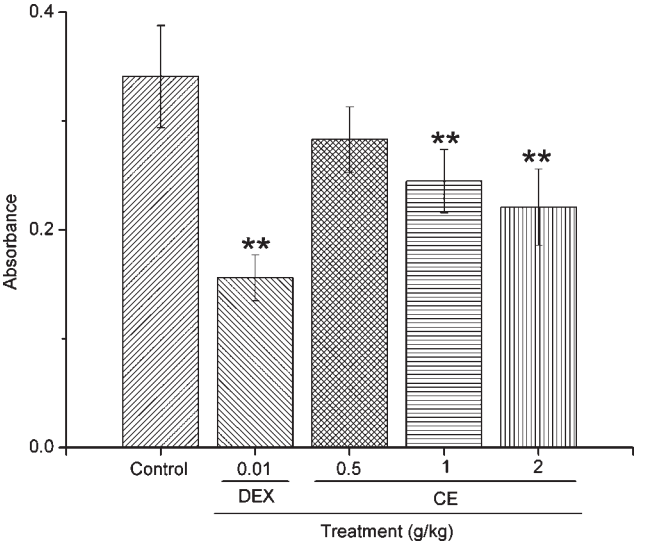

Figure 4. Effects of the CE of *P. bretschneideri* on acetic-acid-induced capillary permeability accentuation in mice. DEX, dexamethasone; CE, the ethanol extract of *P. bretschneideri*. Results were expressed as the mean ± SEM ($n = 8$). (**) $p < 0.01$ was compared to the control group.

fractions B and D showed more significant anti-inflammatory activities in the tested model. The inhibited rates at a dose of 200 mg/kg were up to 49.17% (fraction B) and 48.01% (fraction D), which were comparable to that of DEX (10 mg/kg, 57.77%) (**Table 2**).

Effects of the Isolated Compounds from the Ethyl Acetate Fraction on Xylene-Induced Ear Edema. As shown in **Table 3**, all of the compounds isolated from the ethyl acetate fraction could significantly inhibit the ear edema induced by xylene. The inhibited rates of β-sitosterol, daucosterol, oleanolic acid, and ursolic acid at a dose of 20 mg/kg were up to 41.06, 52.08, 44.45, and 39.42% ($p < 0.01$), respectively.

DISCUSSION

Data from this study indicated that the CE of *P. bretschneideri* and its derived fractions had an important anti-inflammatory effect in the tested models. Moreover, two sterols and two triterpenes were isolated from the best active site of *P. bretschneideri* for the anti-inflammatory activity. Accordingly, our present

Table 1. Effects of the CE of *P. bretschneideri* and Its Derived Fractions on Xylene-Induced Ear Edema[a]

group	dose (g/kg)	swelling (mg)	inhibition (%)
control	10 mL	20.17 ± 6.82	
DEX	0.01	10.03 ± 3.66[b]	50.28[b]
	0.5	15.91 ± 6.43[c]	21.11[c]
CE	1	13.38 ± 4.65[b]	33.69[b]
	2	12.21 ± 3.78[b]	39.46[b]
	0.2	17.50 ± 4.63	13.24
PE	0.4	16.33 ± 6.01	19.03
	0.2	12.50 ± 7.80[b]	38.03[b]
EtOAc	0.4	11.16 ± 5.44[b]	44.66[b]
	0.2	16.41 ± 4.90	18.63
n-BuOH	0.4	15.04 ± 2.64[c]	25.45[c]
	0.2	17.55 ± 4.18	12.30
AQ	0.4	16.55 ± 8.72	17.95

[a] Results were expressed as the mean ± SEM ($n = 8$). [b] $p < 0.01$ was compared to the control group. [c] $p < 0.05$ was compared to the control group.

Table 2. Effects of the Different Subfractions from the Ethyl Acetate Fraction on Xylene-Induced Ear Edema[a]

group	dose (mg/kg)	swelling (mg)	inhibition (%)
control	10 mL	19.76 ± 2.65	
DEX	10	8.34 ± 3.50[b]	57.77[b]
	100	15.97 ± 5.97	19.16
fraction A	200	15.13 ± 4.68[c]	23.40[c]
	100	12.07 ± 3.84[b]	38.93[b]
fraction B	200	10.04 ± 3.86[b]	49.17[b]
	100	14.39 ± 4.63[c]	27.19[c]
fraction C	200	13.59 ± 4.31[b]	31.24[b]
	100	12.07 ± 3.71[b]	38.90[b]
fraction D	200	10.27 ± 4.86[b]	48.01[b]
	100	14.39 ± 3.82[c]	27.19[c]
fraction E	200	12.14 ± 3.01[c]	38.54[b]
	100	15.03 ± 4.02[c]	23.93[c]
fraction F	200	14.46 ± 5.18[c]	26.83[c]

[a] Results were expressed as the mean ± SEM ($n = 8$). [b] $p < 0.01$ was compared to the control group. [c] $p < 0.05$ was compared to the control group.

Table 3. Effects of the Isolated Compounds from the Ethyl Acetate Fraction on Xylene-Induced Ear Edema[a]

group	dose (mg/kg)	swelling (mg)	inhibition (%)
control	10 mL	17.91 ± 3.18	
DEX	10	6.09 ± 4.33[b]	66.02[b]
	10	12.55 ± 3.80[c]	29.93[c]
β-sitosterol	20	10.56 ± 5.25[b]	41.06[b]
	10	10.84 ± 5.73[b]	39.46[b]
daucosterol	20	8.58 ± 3.65[b]	52.08[b]
	10	11.95 ± 3.86[b]	33.28[b]
oleanolic acid	20	9.95 ± 5.34[b]	44.45[b]
	10	13.35 ± 3.66[c]	25.47[c]
ursolic acid	20	10.85 ± 4.98[b]	39.42[b]

[a] Results were expressed as the mean ± SEM ($n = 8$). [b] $p < 0.01$ was compared to the control group. [c] $p < 0.05$ was compared to the control group.

investigation verified for the first time that the sterols and triterpenes were the main chemical components of *P. bretschneideri* with its anti-inflammatory potential.

Carrageenan-induced paw edema and xylene-induced ear edema are correlated with the early exudative phase of inflammatory pathology (*16*) and involve the action of vasoactive amines, such as histamine, serotonin, and kinins, on vascular permeability (*17*, *18*). Carrageenan-induced paw edema in rats is a biphasic response, and the early phase (1−2 h) of the inflammation is due to the release of vasoactive amines, such as histamine and serotonin. The later phase (3−5 h) is due to the activation of kinin-like substances, such as prostaglandins, proteases, and lysosome (*19*). Xylene-induced ear edema is another acute inflammation model, which may involve inflammatory mediators, such as histamine, serotonin, bradykinin, and prostaglandins. These mediators can induce ear edema by promoting vasodilation and increasing vascular permeability (*20*). The inflammatory response is also a physiological characteristic of vascularized tissues, and an acetic-acid-induced capillary permeability increase in mice is a commonly used vascular permeability assay. Moreover, the increase of capillary permeability is related to an increase in prostaglandins (e.g., $PGE_{2\alpha}$ and $PGF_{2\alpha}$) in the peritoneal fluid during the first 30 min after induction of acute inflammation by acetic acid (*21*). These results indicated that the ethanol extract of *P. bretschneideri* could inhibit the exudation on the process of acute inflammation and the early phase inflammatory responses were related to the release of pro-inflammatory mediators, such as histamine, serotonin, kinins, and amino acid metabolites (e.g., prostaglandins, leukotrienes, and thromboxanes).

To isolate and identify the active ingredients of *P. bretschneideri*, the ethanol extract was separated into PE, EtOAc, *n*-BuOH, and aqueous fractions and the anti-inflammatory activity of each fraction was evaluated in the mouse model of ear edema induced by xylene. Results showed that the ethyl acetate fraction at a dose of 400 mg/kg exhibited a similar suppressive effect to DEX, and this fraction appeared to be the most effective against inflammatory. Therefore, the ethyl acetate fraction was employed for the following bioassay-guided fractionation and isolation procedures.

The ethyl acetate fraction was submitted to silica gel column chromatography, and six subfractions were obtained. The effects of these subfractions were also investigated in the ear edema model. Considering the higher activities of fractions B and D, further fractionation procedures were applied on these fractions. Subsequently, fraction B was subjected to silica gel column chromatography, C_{18}−ODS, and recrystallization, leading to three compounds, which were identified as β-sitosterol, oleanolic acid, and ursolic acid. Fraction D was separated and purified using silica gel column chromatography and recrystallization and yielded daucosterol.

Moreover, the anti-inflammatory activities of the isolated compounds were evaluated using the ear edema model. Our results showed that all of these compounds could significantly inhibit the ear edema induced by xylene.

For β-sitosterol and daucosterol, their anti-inflammatory properties have been widely reported in the literature (*22*, *23*). β-Sitosterol was reported to be the anti-inflammatory principle of *Opuntia ficus-indica* Mill. (cactus) (*24*) and *Culcasia scandens* P. Beauv (*25*). We also noted that β-sitosterol glucoside was more active than its aglycone (41.06 versus 52.08% of inhibition, respectively) in our test, which was inconsistent with the findings by Mavar-Manga et al. (*26*).

The anti-inflammatory effect is a common property of many triterpenoids. In relation to oleanolic acid and ursolic acid, studies from the literature have demonstrated the anti-inflammatory effect some years ago (*27*, *28*). Their anti-inflammatory activities have also been confirmed in later studies, and the mechanisms for this activity have been certified gradually (*29*, *30*).

This paper reported for the first time the presence of β-sitosterol, daucosterol, oleanolic acid, and ursolic acid in *P. bretschneideri* and their topical anti-inflammatory activities. Furthermore, in CEs used in traditional medicine, these compounds might also act additionally or synergistically with the polyphenols (tannins and flavonoids).

Our results indicated that *P. bretschneideri* had good anti-inflammatory effects and the constituents β-sitosterol, daucosterol, oleanolic acid, and ursolic acid might well account for it, which may support and supplement the use of *P. bretschneideri* in folk medicines. These compounds have potential as novel lead compounds for the future development of therapeutic interventions in the treatment of patients with inflammatory disorders.

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