5-Lipoxygenase Inhibition of the Fructus of *Foeniculum vulgare* and Its Constituents

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Abstract

The fruits of *Foeniculum vulgare* (Foeniculi Fructus) have been widely used in Chinese medicine as an antiemetic, ameliorating stomach ailments and as an analgesic. In order to establish its potential for anti-allergic use, inhibitory actions of the fruit on 5-lipoxygenase (5-LOX) and β-hexosaminidase release were evaluated. The 70% ethanol extract of this plant material (FR) considerably inhibited 5-LOX-catalyzed leukotriene production from A23187-induced rat basophilic leukemia (RBL)-1 cells. The IC₅₀ was 3.2 µg/ml. From this extract, 12 major compounds including sabinene, fenchone, γ-terpinene, α-pinene, limonene, p-anisylacetone, p-anisylaldehyde, estragole (4-allylanisole), trans-anethole, scopoletin, bergapten and umbelliferone were isolated. And it was found that several terpene derivatives including γ-terpinene and fenchone as well as phenylpropanoid, trans-anethole, showed considerable inhibitory action of 5-LOX. In particular, the IC₅₀ of trans-anethole was 51.6 µM. In contrast, FR and the isolated compounds did not show considerable inhibitory activity on the degranulation reaction of β-hexosaminidase release from antigen-treated RBL-2H3 cells. Against arachidonic acid-induced ear edema in mice, FR and trans-anethole showed significant inhibition by oral administration at doses of 100-400 mg/kg. In conclusion, FR and several major constituents are 5-LOX inhibitors and they may have potential for treating 5-LOX-related disorders.

Key Words: *Foeniculum vulgare*, trans-anethole, 5-lipoxygenase, Ear edema, Allergy

INTRODUCTION

Leukotrienes (LTs) are mediators of inflammation and allergy. LTs, especially cysteinyl-LTs, are known to be involved in several allergic disorders including bronchial asthma and atopic dermatitis (Rubin and Mollison, 2007). Arachidonic acid (AA) released by phospholipase A₂ from membrane lipids is converted to LTs by 5-lipoxygenase (5-LOX). Thus, 5-LOX inhibitors have potential to inhibit inflammatory/allergic response. In this regard, many synthetic small molecules and natural products are evaluated for their capacity to inhibit 5-LOX.

The fructus of *Foeniculum vulgare* (Foeniculi Fructus) is a well known Chinese traditional medicine. This plant has been widely used as an antiemetics, ameliorating stomach conditions and as an analgesic (Him et al., 2008). To present, many compounds have been isolated from this plant material. They include essential oils including trans-anethole, limonene, fenchone and cymene, fatty acids and coumarins such as scopoletin and bergapten (Ozcan and Chalchat, 2010). Previously, the fruits of *F. vulgare* extract were found to possess anti-inflammatory and analgesic activities (Choi and Hwang, 2004). The extract of the same plant material also showed anti-bacterial and anti-fungal activities (Cetin et al., 2010; Pai et al., 2010). As the constituents, essential oils such as fenchone and anethole showed antimicrobial and insecticidal activities (Kwon et al., 2002; Cetin et al., 2010). Trans-anethole and limonene also inhibit nitric oxide (NO) production from RAW 264.7 macrophages (Conforti et al., 2010). In particular, essential oil fractions showed 5-LOX inhibitory activity (Miguel et al., 2010), but the active principles were not identified. In this study, 5-LOX inhibitory activity of *F. vulgare* and its major constituents were examined in order to clearly establish the pharmacological action of *F. vulgare* and its major constituents as well as establish the potential of anti-allergic use.

MATERIALS AND METHODS

Chemicals  
A23187 was obtained from Biomol (Plymouth Meeting, PA, USA).

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PA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), p-nitrophenyl-N-acetyl-b-D-glucosaminide (NDA), quercetin, anti-dinitrophenol (DNP) mouse IgE, siraganian buffer, DNP-BSA, quercetin and arachidonic acid (AA) were purchased from Sigma Chem. (St Louis, MO). DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). A protein assay kit was purchased from Bio-Rad (Hercules, CA).

Animals
Male ICR mice (5 weeks old, specific pathogen-free) were obtained from Orient-Bio Co. (Korea). Animals were fed with standard lab. chow and water is freely available. The animals were maintained in the animal facility (KNU) at 20-22°C under 40-60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIAUC-09-0029). The animals were handled according to the guideline described in the KFDA Guide for the Care and Use of Laboratory Animals throughout the experiments.

Preparation of the extracts and isolation of the constituents
The fruits of F. vulgare cultivated in Neimenggu were provided from Prof. Jae-Hyun Lee, College of Oriental Medicine, Dongguk University at Gyeongju, Korea. Air-dried and chopped plant materials (1.0 kg) were extracted with hot 70% ethanol and hot distilled water for 3 h, respectively, to provide both extracts for the pharmacological activity test. Isolation of the constituents, plant materials (5.0 kg) were extracted with hot methanol for 3 h. Evaporation of the solvent yielded crude extracts, which were suspended in distilled water. The resulting solution was consecutively partitioned with hexane, methylene chloride, ethyl acetate and n-butanol to give hexane (221.4 g), methylene chloride (6.1 g), ethyl acetate (5.8 g), n-butanol (26.5 g). The hexane and methylene chloride fractions were subjected to column chromatographic separation. Coumarins (scopoletin, bergapten and umbelliferone) were isolated from the methylene chloride fraction as previously reported (Abdel-Fattah et al., 2003). Other compounds including monoterpenes (sabinene, fenchone, 1,8-pinene, limonene), phenylpropanoids (estragole and trans-anethole) and aromatics (p-anisylacetone and p-anisylaldehyde) (Fig. 1A) were obtained from the hexane fraction by isolation procedures according to the previously published procedure (Akgul, 1986). The spectral analysis of hexane fraction was performed with GC using HP-5MS capillary column (60.0 m×0.250 μm×0.25 μm), and the spectrum was shown in Fig. 1B. The content of trans-anethole in hexane fraction was 23.3% (w/w). The purity of above isolated compounds were scopoletin 99.0%, bergapten 99.4%, umbelliferone 98.0%, sabinene 99.1%, fenchone 99.9%, γ-terpinene 95.0%, α-pinene 98.0%, limonene 97.2%, estragole 97.0%, trans-anethole 99.0%, p-anisylacetone 98.4%, p-anisylaldehyde 98.0%. The spectral data of the most active component, trans-anethole are as follows: 1H-NMR (200 MHz, CDCl3) δ: 1.87 (3H, dd, J=6.7, 1.5 Hz, H-3'), 3.79 (3H, s, H-7), 6.10 (1H, dq, J=15.8, 6.7 Hz, H-2'), 6.35 (1H, dq, J=15.8, 1.5 Hz, H-1'), 6.84 (2H, dt, J=8.7, 3.0, 1.9 Hz, H-2, H-6), 7.25 (2H, dt, J=8.7, 3.0, 1.9 Hz, H-3, H-5); 13C-NMR (50 MHz, CDCl3) δ: 18.2 (C-3'), 55.2 (C-7), 123.5 (C-2'), 130.4 (C-1'), 113.8 (C-2, C-6), 126.8 (C-3, C-5), 158.6 (C-1), 130.8 (C-4); EI-GC/MS m/z 148 [M]+.

Rat basophilic leukemia-1 (RBL-1) cell culture and measurement of leukotriene (LT)
To evaluate the 5-LOX inhibitory activity, RBL-1 cells purchased from the American Type Culture Collection (ATCC, Rockville, VA) were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics under 5% CO2 at 37°C. The 5-LOX activation was carried out by treatment of A-23187 (3 μM) for 15 min according to the previously described (Tries et al., 2002). The test compounds were dissolved in DMSO and were added to the cells simultaneously with A-23187. The cell viability was assessed using an MTT assay as previously described (Mosmann, 1983). The media was then collected and the concentration of the 5-LOX product, cysteinyl leukotrienes (LT_{c}/D/E), was measured using an ELISA kit (Cayman Chem.) as recommended by the manufacturer.

RBL-2H3 cell culture and antigen-induced degranulation of β-hexosaminidase
RBL-2H3 cells (ATCC) were cultured in 24-well plates (2×10^5 cells/well) using DMEM with 10% FCS. According to

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Fig. 1. Chemical structures of the active constituents of F. vulgare. (A) Chemical structures of the selected compounds isolated, (B) GC spectrum of the hexane fraction. The retention times were 15.15 (α-pinene), 16.78 (sabinene), 16.93 (β-pinene), 17.52 (myrcene), 19.28 (p-cymene), 19.51 (limonene), 19.65 (1,8-cineole), 19.95 (E-cimene), 21.25 (γ-terpinene), 23.09 (fenchone), 30.33 (estragole), 34.22 (p-anisylaldehyde), 36.58 (trans-anethole) and 42.59 min (anisylacetone).
the previously described procedure (Choi et al., 1996), anti-DNP mouse IgE was added for sensitization and incubated overnight. Twenty four hours later, the cells were washed with siraganian buffer (pH 7.2). DNP-BSA (1 μg/ml) was added for activation and degranulation. The test compounds were simultaneously added. After 10 min incubation, the reaction was stopped by cooling in an ice bath for 10 min. After centrifugation, the supernatant was transferred into 96-well plates. The substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) was added and incubated for 1 h at 37°C. The reaction was stopped by adding 0.1 M Na₂CO₃/NaHCO₃ (200 μl/well) and the absorbance was measured at 405 nm.

**Arachidonic acid (AA)-induced ear edema in mice and measurement of LT concentration**

For establishment of in vivo inhibitory activity against 5-LOX-mediated response, AA-induced ear edema assay were carried out according to the previously reported procedures (Kim et al., 1993). AA (2%) dissolved in acetone (25 μl/ear) was applied topically to mouse ear. One hour later, the ear thickness was measured using engineering gauge (Mitsutoyo, Japan). Test compounds dissolved in DMSO (50 μl/mouse) were orally administered at 1 h prior to AA application.

**Statistical analysis**

All data were represented as arithmetic mean ± SD. One-way analysis of variance (ANOVA), followed by Dunnett’s test was used to determine the statistical significance.

**RESULTS**

A-23187 (ionophore) treatment to RBL-1 cells activates 5-LOX, which produces high concentrations of cysteinyl-LTs. A-23187 treatment increased LT concentrations to 749.5 ± 294.0 pg/ml from a basal level of 44.9 ± 17.5 pg (n=3). The water and 70% ethanol extracts of the fruits of *F. vulgare* inhibited LT production under these conditions (Fig. 2A). Comparing the IC₅₀ values, the ethanol extract of the fruits of *F. vulgare* (FR) possessed a higher inhibitory activity (3.2 mg/ml) against 5-LOX on activated RBL-1 than that of the water extract (25.4 mg/ml). The reference compound, NDGA, showed 92% inhibition at 1 μM.

By antigenic stimulation, mast cells release histamine which produces vasodilation and itching. Along with histamine production, β-hexosaminidase is also released. Thus, β-hexosaminidase release could be used as a biomarker in RBL-2H3 cells. When the anti-allergenic activities of FR and the water extract were evaluated, both extracts, however, showed weak inhibitory activity on degranulation of RBL-2H3 cells.

**Table 1. Inhibition of the constituents of the fruits of *F. vulgare* against 5-LOX-catalyzed LT production**

| Compounds                  | % inhibition at 50 μM* |
|----------------------------|------------------------|
| NDGA                      | 92.4                   |
| Monoterpenes              | 22.3                   |
| Sabinene                  | 40.9 (>50)             |
| Fenchone                  | 48.3 (>50)             |
| γ-Terpinene               | 9.9                    |
| α-Pinene                  | 32.6 (>50)             |
| Limonene                  | 49.1 (51.6)            |
| Phenylpropanoids          |                        |
| Anisylacetone             | 32.6 (>50)             |
| 4-Allylanisole (estragole) |                       |
| Trans-anethole            | 49.1 (51.6)            |
| Phenolic                  |                        |
| p-Anisaldehyde            | -                      |
| Coumarins                 | -                      |
| Scopoletin                | -                      |
| Bergapten                 | -                      |
| Umbelliferone             | -                      |

*All values are arithmetic mean of % inhibition at 50 μM except NDGA (1 μM). n=3. "The values of the parenthesis are IC₅₀ values in μM. "-": not active.*

![Fig. 2. Effects of the extracts of *F. vulgare* on 5-LOX and degranulation reaction. (A) Inhibition of 5-LOX catalyzed LT production from A23187-treated RBL-1 cells. (B) Inhibition of β-hexosaminidase release from antigen-treated RBL-2H3 cells. The water extract (○), 70% ethanol extract (●). All points and bars represent arithmetic mean ± SD (n=3), *p<0.01, significantly different from the control group.](image-url)
Active Components from Oriental Herbal Medicines from Korea

**DISCUSSION**

The present investigation demonstrated that FR and some of its constituents possess 5-LOX inhibitory activity. It is also suggested that trans-anethole may contribute, at least in part, to the pharmacological activity of FR. Our study is significant since in vivo activity of FR and trans-anethole was demonstrated and several constituents such as trans-anethole and γ-pinene were found to be the active components in FR as 5-LOX inhibitors, for the first time.

Some constituents of the fruits of *F. vulgare* were previously reported to possess several pharmacological activities. For example, T-lymphocyte proliferation and IL-2 production were inhibited by anethole (Yea et al., 2006). Anethole also showed a preventive effect against thrombosis (Tognolini et al., 2007). Recently, anethole and limonene inhibited NO production from RAW 264.7 cells (Conforti et al., 2010). In our recent study, monoterpenes such as pinene, cineole and limonene did not considerably inhibit 5-LOX from mast cells (Jin et al., 2011). On the other hand, the present study demonstrated that several monoterpenic derivatives such as fenchone and γ-terpinene are 5-LOX inhibitors.

AA topically applied to the ears of mice produces acute inflammation characterized by inflammatory cell recruitment and edema, peaking at 1 h (Inoue et al., 1988; Kim et al., 1993). In this model, AA topically applied to mouse ear is converted to LTs via 5-LOX, which evokes edema in 1 h. Thus, this model is sensitive to 5-LOX inhibitors. Indeed, NDGA (5-LOX inhibitor) used as a reference drug showed significant inhibition in this model. Therefore, it is reasonably suggested that FR and trans-anethole might inhibit 5-LOX in ears of mice, leading to the reduction of edema in vivo.

In conclusion, FR and several of its constituents such as trans-anethole, fenchone and γ-terpinene were found to be 5-LOX inhibitors. In particular, FR and trans-anethole showed in vivo inhibitory activity against AA-induced ear edema in mice, possibly via 5-LOX inhibition. These results suggest that FR and trans-anethole may be beneficial for treating 5-LOX-related disorders and trans-anethole may certainly contribute to the pharmacological action of FR.

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**Fig. 3.** Inhibition of trans-anethole on 5-LOX-catalyzed LT production. LT production (LT/C/D/E) was expressed as a percentage of control. All data are arithmetic means ± SD (n=5), *p<0.01, significantly different from the control group.

**Table 2.** Inhibition of arachidonic acid (AA)-induced ear edema in mice by FR and trans-anethole

| Compounds       | Dose (mg/kg)* | Ear thickness increased (mm) | % inhibition |
|-----------------|--------------|-----------------------------|-------------|
| AA-treated      | –            | 0.080 ± 0.016               | –           |
| NDGA            | 2*           | 0.044 ± 0.013*              | 45.0        |
| FR              | 100*         | 0.044 ± 0.013*              | 45.0        |
| Trans-anethole  | 50           | 0.038 ± 0.017*              | 52.5        |
|                 | 200          | 0.036 ± 0.015*              | 72.5        |

*All compounds were orally treated one hour prior to AA application, except NDGA. *NDGA (2 mg/ear) was treated topically to ears of mice 30 min prior to AA application. +Higher dose than 100 mg/kg could not be tested due to the insolvibility of the extract in DMSO. All data are arithmetic means ± SD (n=5). *p<0.01, significantly different from the AA-treated group.
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