Food Sci. Biotechnol. Vol. 15, No. 5, pp. 763 ~ 767 (2006)

Food Science and Biotechnology

Anticoagulant Properties of Compounds Derived from Fennel (*Foeniculum vulgare* Gaertner) Fruits

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Abstract The anticoagulant properties of compounds derived from fennel (*Foeniculum vulgare* Gaertner) fruits were evaluated using a platelet aggregometer and compared with aspirin. The active constituents of fennel fruits were isolated and identified as (+)-fenchone and estragole by various spectral analysis techniques. With regard to the 50% inhibitory concentration (IC_{50}), (+)-fenchone effectively inhibited platelet aggregation induced by treatment with collagen (IC_{50}, 3.9 μM) and arachidonic acid (AA) (IC_{50}, 27.1 μM), and estragole inhibited collagen-induced platelet aggregation (IC_{50}, 4.7 μM). By way of comparison, (+)-fenchone and estragole proved to be significantly more potent than aspirin at inhibiting platelet aggregation induced by collagen. The inhibitory activity of (+)-fenchone toward platelet aggregation induced by AA was 1.3 times stronger than that of aspirin. These results indicate that (+)-fenchone and estragole may be useful as lead compounds for inhibiting platelet aggregation induced by arachidonic acid and collagen.

Keywords: antiplatelet, estragole, fennel, *Foeniculum vulgare* Gaertner, (+)-fenchone, collagen, arachidonic acid

Introduction

Platelet aggregation is a complex phenomenon which probably involves several intracellular biochemical pathways. Platelets activated by a variety of physiological agonists, including arachidonic acid (AA), collagen, platelet activating factor (PAF), and thrombin, undergo a complex cascade of events which result in morphological changes, the formation of AA metabolites, and aggregation (1). Since platelets readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, they can initiate thrombus formation and precipitate thromboembolism, leading to ischemic diseases (1). In addition, the interactions between platelets and blood vessel walls are relevant to the development of thromboses and cardiovascular diseases (2-4). When blood vessels are damaged, platelet aggregation occurs rapidly, resulting in the formation of haemostatic plugs or arterial thrombi at the sites of vessel injury, or in regions in which blood flow is disturbed. These thrombi are the source of thromboembolic complications, including atherosclerosis, heart attacks, stroke, and peripheral vascular disease (5). Therefore, the inhibition of platelet aggregation represents a promising approach to the prevention of thrombosis.

Plant extracts or their constituents may be used as alternatives to the current anticoagulant, as they constitute a rich source of bioactive chemicals (6-9). As many of these compounds are largely free from adverse effects and exhibit desirable pharmacological activities, research into these plant extracts might lead to the development of new classes of safer anticoagulants (6-8). Additionally, some flavonoids and polyphenols have been shown to effectively inhibit platelet aggregation induced by collagen (10, 11). Therefore, a great deal of effort has been focused on the use of plant materials as commercial anticoagulants or lead compounds. In East Asia, fennel has long been considered to possess medicinal properties attributable to the terpenoids produced by the plant, which include *trans*-anethole, estragole, *d*-limonene, fenchone, α-pinene, terpinene, and *p*-cymene (12, 13). Little work has been done regarding the inhibition of platelet aggregation by fennel fruits. This paper describes a laboratory study conducted in order to evaluate the relevant compounds in fennel fruits with regard to their utility as possible anticoagulants. The anticoagulant properties of fennel fruit-derived compounds were compared with those of the commonly used anticoagulant, aspirin.

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Received July 3, 2006; accepted July 30, 2006

Materials and Methods

Chemicals The fruits of fennel were purchased from a local market in Jeonju, Korea. Anethole, β-caryophyllene, *p*-cymene, estragole, α-pinene, γ-terpinene, and thymol were obtained from Sigma (St. Louis, MO, USA). Collagen, AA, and thrombin were obtained from Chrono-Log Co. (Havertown, PA, USA). All other chemicals used were of analytical grade.

Extraction and identification The fruits (5 kg) of fennel (*Foeniculum vulgare* Gaertner, Family Apiaceae) were washed three times with 2 L of distilled water, then dried at 40°C in an oven for 2 days, and finely powdered. The essential oil (yield 5.8%, 290 g) of fennel fruits was extracted by steam distillation, as described previously (12, 13).

The structure of the active isolate was then determined by instrumental analyses. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer at 600 and 150 MHz (tetramethylsilane as an internal standard), respectively, and the chemical shifts

were expressed in δ (ppm). Unambiguous ^{1}H and ^{13}C NMR chemical shifts were acquired using a ^{1}H-^{1}H correlation spectrum, as well as a ^{13}C-^{1}H correlation spectrum. UV spectra were obtained in methanol using a Uvikon 922 spectrometer (Kontron, Eching, Germany), and mass spectra using a JEOL GSX 400 spectrometer (JEOL, Tokyo, Japan). Optical rotation was measured using an Autopol III polarimeter (Rudolph, Newburgh, NY, USA).

Preparation of washed rabbit platelets Platelet rich plasma (PRP) was obtained from healthy male white rabbit blood treated with a one-tenth volume of 1% EDTA followed by 10 min of centrifugation at 230×g at room temperature. The platelets were sedimented by 15 min of centrifugation of the PRP at 800×g, and then washed twice with Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM Hepes, pH 6.5) containing 0.35% bovine serum albumin and 0.4 mM EDTA. The washed platelets were then resuspended in Hepes buffer (pH 7.4). Platelet numbers were counted with a Coulter Counter (Coulter Electronics, Hialeah, FL, USA), and adjusted to a concentration of 3×10^{8} platelets/mL.

Aggregation of washed rabbit platelets Platelet aggregation was measured with an aggregometer (470-vs; Chrono-log Co., Havertown, PA, USA) as described previously (10). In brief, the washed platelets (3×10^{8} platelets/mL) were incubated for 3 min at 37°C in the aggregometer with various concentrations of samples in the presence of 1 mM $CaCl_2$, after which platelet aggregation was induced by the addition of collagen (2 μg/mL), AA (100 μM), or thrombin (0.1 unit/mL). The resulting aggregation, which was measured as the change in light transmission, was recorded for 10 min. Each inhibition rate was obtained on the basis of the maximal aggregation induced by the respective agonists. The degree to which platelet aggregation was inhibited was expressed as % inhibition (X) in accordance with the following equation: $X = [(A-B)/A] \times 100$. The maximal aggregation of the control and the maximal aggregation of the sample-treated washed platelets were A and B, respectively.

Results and Discussion

When the oil derived from fennel fruits was bioassayed, the essential oil showed strong inhibition of platelet aggregation induced by collagen (2 μg/mL) in an experiment using washed rabbit platelets (data not shown). Due to this strong activity, the biologically active components from the essential oil were purified by both silica gel column chromatography and HPLC (Fig. 1). The oil (10 g) was then chromatographed on a silica gel column (Merck 70-230 mesh, 720 g, 6.0 cm i.d. × 80 cm) and successively eluted using a stepwise gradient of hexane/ethyl acetate (100:0, 90:10, 80:20, 50:50, and 0:100). The bioactive fraction (2.9 g) was then successively rechromatographed on a silica gel column using hexane-ethyl acetate (80:20). The column fractions were analyzed by thin-layer chromatography (TLC) (Silicagel 60 F_{254}), and fractions exhibiting similar streaking patterns on TLC

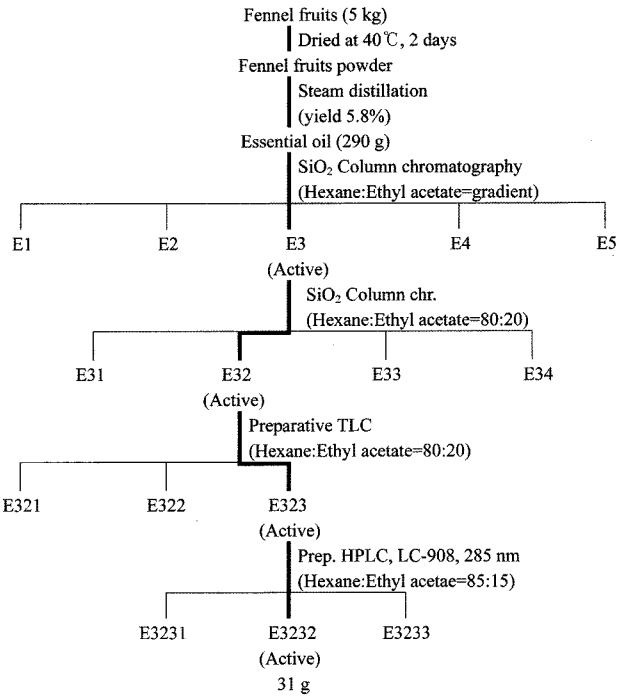


Fig. 1. Isolation procedure for (+)-fenchone from fennel fruits.

plates were pooled. The active fraction was then purified by JAI LC-908 preparative high-pressure liquid chromatography (Japan Analytical Industry, Tokyo, Japan) in order to separate out the biologically active constituent(s) for further biological examination. A JAIGEL GS-310 column was used for this procedure (20×500 mm i.d.; Japan Analytical Industry, Tokyo, Japan) with hexane-ethyl acetate (85:15) at a flow rate of 3.5 mL/min and detection at 285 nm. In this manner, 31 g of the bioactive compound was isolated.

The bioassay-guided fractionation of the fennel oil yielded an active compound which was then identified by spectroscopic analyses, including EI-MS, ^{13}C NMR, and ^{1}H NMR, and by direct comparison with an authentic reference compound (Fig. 2-4). The biologically active compound obtained was characterized as (+)-fenchone (Fig. 5). This compound was identified on the basis of the following evidence: (+)-fenchone, $C_{10}H_{16}O$: $[\alpha]_D^{20}$ + 67; UV (MeOH) λ_{max} nm (ε) 203 (17478); EI-MS (70 eV), *m/z* (% rel int) M^+ 152 (16), 137 (20), 109 (27), 81 (100), 69 (49). ^{1}H-NMR (CD_3OD, 600 MHz): δ 2.14 (1H, br, s), 1.77-1.81 (2H, m), 1.69-1.75 (2H, m), 1.52-1.58 (2H, m), 1.36-1.41 (2H, m), 1.14 (3H, s), 1.04 (6H, s); ^{13}C-NMR (CD_3OD, 150 MHz): δ 223.52, 54.15, 47.39, 45.31, 41.65, 31.83, 24.94, 23.35, 21.71, 14.63. The spectroscopic analyses of (+)-fenchone isolated from the essential oil was identical to the corresponding data of fenchone isolated from funnel (13).

In order to determine the anticoagulant effects of other components derived from the oil of funnel fruits (13), anethole, β-caryophyllene, *p*-cymene, estragole, (+)-fenchone, α-pinene, γ-terpinene, and thymol were all also assessed with regard to their possible inhibitory effects toward collagen-induced platelet aggregation (Table 1). At

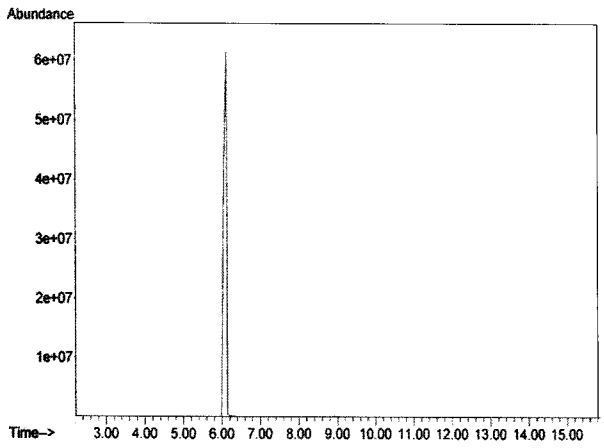


Fig. 2. Mass spectra of (+)-fenchone isolated from fennel fruits.

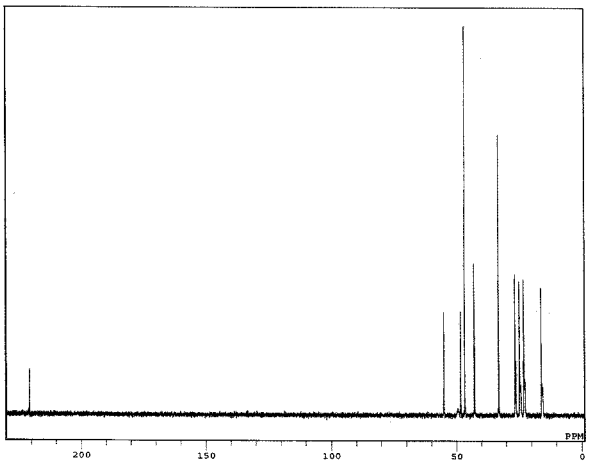


Fig. 3. ^{13}C NMR spectra of (+)-fenchone isolated from fennel fruits.

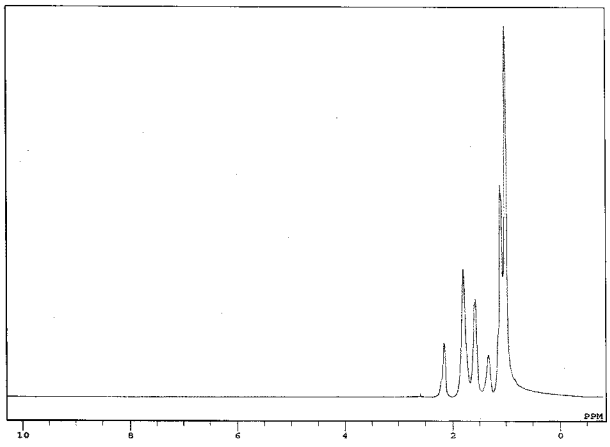
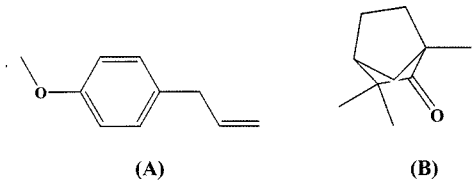


Fig. 4. ^{1}H NMR spectra of (+)-fenchone isolated from fennel fruits.



(A) (B)

Fig. 5. Structure of estragole (A) and (+)-fenchone (B).

Table 1. Effects of constituents derived from fennel fruits on platelet activation induced by collagen

Compound	Conc.[1] (μg/mL)	Aggregation[2] (%)	Inhibition[3] (%)
Control		77±2.0	
(+)-Fenchone	50	0±0.0**	100
	10	5±0.7**	93.5
	5	32±1.6**	58.4
	1	57±2.8*	26.0
Estragole	50	0±0.0**	100
	10	1±0.4**	98.7
	5	35±2.0**	54.6
	1	61.6±2.4*	20.0
Anethole	100	4±0.4**	100
	10	31±1.7**	59.7
	5	65±2.5**	15.6
β-Caryophyllene	100	77±2.1**	0
	10	77±2.7**	0
p-Cymene	100	77±2.8**	0
	10	76±2.5**	0
α-Pinene	100	0±0.0**	100
	10	42±2.4**	54.5
	5	72±2.7**	6.5
γ-Terpinene	100	76±2.4**	0
	10	77±2.6**	0
Thymol	100	76±2.3**	0
	10	77±2.9**	0

[1]Washed rabbit platelets were preincubated with compound and DMSO (0.5% control) at 37°C for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by the addition of collagen (2 μg/mL).
[2]Percentage of aggregation is presented as the mean±SE (n=3). *p<0.05, **p<0.01 as compared with the respective control.
[3]Inhibition(%)=[(A-B)/A] × 100. A, Control aggregation %; B, Sample aggregation %.

concentrations of 10 and 5 μg/mL, (+)-fenchone showed strong inhibition of collagen-induced (2 μg/mL) platelet aggregation, with 93.5 and 58.4% inhibition, respectively. Estragole also exhibited strong inhibition of platelet aggregation induced by collagen, with values of 98.7 and 54.6% at concentrations of 10 and 5 μg/mL, respectively, and also showed weak inhibitory activity at a concentration

of 1 μg/mL, with a value of 20%. Furthermore, at concentrations of 10 and 5 μg/mL, both anethole and α-pinene showed moderate inhibitory activity at 10 μg/mL, with values of 59.7 and 54.5%, respectively, and weaker activity at 5 μg/mL, with values of 15.6 and 6.5%, respectively. β-Caryophyllene, *p*-cymene, γ-terpinene, and thymol exhibited no inhibitory activity. With regard to these results, estragole and (+)-fenchone showed stronger inhibitory effects than anethole and α-pinene. Thus, the inhibitory activities of estragole and (+)-fenchone toward platelet aggregation induced by collagen (2 μg/mL), AA (100 μM), PAF (10 nM), and thrombin (0.1 unit/mL) were evaluated.

These utility of these compounds as anticoagulants were then compared to that of aspirin (Table 2). The IC_{50} values of (+)-fenchone and estragole with regard to the inhibition of platelet aggregation induced by collagen were 3.9 and 4.7 μM, respectively. Furthermore, (+)-fenchone inhibited platelet aggregation induced by AA followed by collagen with an IC_{50} value of 27.1 μM. However, (+)-fenchone and estragole exhibited weak or no inhibitory effects toward PAF or thrombin-induced platelet aggregation. In this regard, fennel fruit-derived (+)-fenchone and estragole both appear to exhibit pharmacological inhibitory effects against platelet aggregation induced by AA and collagen.

In this investigation, the commonly-used anticoagulant aspirin was utilized as a standard for comparison. Aspirin inhibited platelet aggregation induced by AA with an IC_{50} value of 34.9 μM. However, aspirin showed weak or no inhibitory effects toward platelet aggregation induced by collagen, PAF, or thrombin. The inhibitory activity of (+)-fenchone and estragole toward platelet aggregation induced by collagen was stronger than that of aspirin. Furthermore, (+)-fenchone inhibited platelet aggregation induced by AA 1.3 times greater than aspirin. (+)-fenchone also proved to be significantly stronger than aspirin with regard to inhibiting platelet aggregation induced by AA and collagen. Thus, both (+)-fenchone and estragole appear worthy of further study as potential anticoagulants or as lead compounds.

This study is, to the best of our knowledge, the first to describe the anticoagulant effects of the active compounds obtained from the fruits of fennel. The oil of fennel fruits is considered to be important in the diet and in medical therapies for the treatment of biological tissue deterioration attributable to free radicals. It is also an essential ingredient in certain anti-inflammatory, analgesic, antioxidant, and hepatoprotective agents (14, 15). The use of fennel fruits for antibacterial and fungicidal effects has been fairly well established (7, 17). Among the components derived from fennel fruits, anethole, estragole, and (+)-fenchone constitute a primary group of secondary metabolites (13, 18). Estragole has been shown to exhibit antifungal effects toward *Blastoschizomyces capitatus*, repellent properties toward *Aedes aegypti* and *Anopheles braziliensis*, and insecticidal effects toward *Sitophilus oryzae*, *Callosobruchus chinensis*, and *Lasioderma serricorne* (12, 16, 17). (+)-Fenchone also exhibited potent acaricidal activity toward *Dermatophagoides farinae* and *D. pteronyssinus*, antifungal activity toward *Aspergillus niger*, *Fusarium tricinctum*, *Penicillium ochrochloron*, *Trichoderma viride*, and *Phomopsis helianthi*, insecticidal activity toward *S. oryzae*, *C. chinensis*, and *L. serricorne*, and repellent activity toward *A. aegypti* (12, 13, 19, 20).

When blood vessels are damaged, platelet aggregation rapidly occurs resulting in the formation of haemostatic plugs or arterial thrombi at the sites of vessel injury, or in regions in which blood flow has been disrupted. These thrombi constitute the primary sources of the thromboembolic complications of atherosclerosis, heart attacks, and peripheral vascular disease (5). The inhibition of platelet aggregation represents a promising approach to the prevention of thrombosis. Recently, the active components derived from plant extracts have been shown to exhibit a number of biological activities (6-8). In previous reports, it has been shown that gallic acid, methyl gallate (Galla Rhois), eugenol, and isoeugenol (*Eugenia caryophyllata*) inhibit the aggregation of platelets in human blood *in vitro* (7, 8). Gallic acid inhibited platelet aggregation induced by collagen and AA with IC_{50} values of 5 and 94 μM, and methyl gallate inhibited platelet aggregation induced by collagen and AA with IC_{50} values of 33 and 11 μM (7). Eugenol proved effective in the inhibition of platelet aggregation induced by AA (IC_{50}, 0.05 μM) and collagen (IC_{50}, 0.7 μM), and isoeugenol was most efficient with regard to its inhibitory activity toward platelet aggregation induced by AA (IC_{50}, 0.3 μM), collagen (IC_{50}, 0.9 μM), and PAF (IC_{50}, 12.2 μM) (8). In this study, fennel fruit-derived estragole and (+)-fenchone appeared to have pharmacological activity against platelet aggregation induced by collagen and AA.

In conclusion, the results of this study clearly show that fennel fruit-derived estragole and (+)-fenchone exert *in vitro* anticoagulant effects. In a previous study, the oral LD_{50} values of estragole and (+)-fenchone in rats was reported to be 1.23 and 6.16 g/kg, respectively, indicating a low degree of acute toxicity in mammals (21, 22). On the basis of our limited data and some earlier findings, fennel fruit-derived estragole and (+)-fenchone may prove useful as lead compounds for the development of new anticoagulants and medicinal foodstuffs, although the *in vivo* efficacy and clinical utility exhibited by this compound remain to be evaluated.

Table 2. IC_{50} (μM) of estragole and (+)-fenchone with regard to platelet aggregation induced by various agonists

Agonists[1)]	Estragole	(+)-Fenchone	Aspirin
Collagen	4.7±2.8[2)]	3.9±2.5	> 200
Arachidonic acid	> 200	27.1±3.1	34.9±2.5
Thrombin	> 200	> 200	> 200
PAF	> 200	> 200	> 200

[1)]Washed rabbit platelets were preincubated with aspirin, estragole, (+)-fenchone, and DMSO (0.5% control) at 37°C for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by the addition of collagen (2 μg/mL), AA (100 μM/mL), thrombin (0.1 unit/mL), or PAF (10 nM). [2)]The 50% inhibitory concentration (IC_{50}) values were calculated from at least three separate experiments. Values are presented as means±SD.

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