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Anticoagulant Properties of the Active Compound Derived from *Cinnamomum cassia* Bark

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Abstract The anticoagulant properties of *Cinnamomum cassia* bark-derived materials were evaluated against platelet aggregation induced by arachidonic acid (AA), collagen, platelet activating factor (PAF), or thrombin, and these effects were then compared to those of three commercially available compounds (cinnamic acid, cinnamyl alcohol, and aspirin). The active constituent obtained from *C. cassia* barks was isolated by silica gel column chromatography and high pressure liquid chromatography (HPLC), and was characterized as *trans*-cinnamaldehyde by MS, ^{1}H-NMR, ^{13}C-NMR, and IR spectroscopy. With regard to 50% inhibitory concentration (IC_{50}) values, cinnamaldehyde was found to effectively inhibit platelet aggregation induced by AA (IC_{50}, 43.2 μM) and collagen (IC_{50}, 3.1 μM). By way of comparison, cinnamaldehyde proved to be a significantly more potent platelet inhibitor against platelet aggregation induced by collagen than aspirin. The effect exerted by cinnamaldehyde against platelet aggregation induced by AA was 1.2 times less than that of aspirin. These results indicate that cinnamaldehyde may prove useful as a lead compound for the inhibition of platelet aggregation induced by AA and collagen.

Keywords: anticoagulant property, arachidonic acid, cinnamaldehyde, *Cinnamomum cassia*, collagen

Introduction

Platelet aggregation is a complex phenomenon, which appears to involve several intracellular biochemical pathways (1). Platelets activated by arachidonic acid (AA), collagen, platelet activating factor (PAF), or thrombin undergo a complex cascade of events, which ultimately result in morphological alterations, secretion, the formation of AA metabolites, and aggregation (1). As platelets readily aggregate in response to a variety of endogenous substances and secrete various substances that can cause further aggregation, they also initiate thrombus formation and can precipitate thromboembolism, both of which contribute to the development of ischemic disease. The interactions occurring between platelets and blood vessel walls are relevant to the development of thrombosis and certain 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 has been disrupted. These thrombi are the primary source of thromboembolic complications of atherosclerosis, heart attacks, strokes, and peripheral vascular disease (5). Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis.

Plants may be used to derive alternatives to the currently-used anticoagulant agents, as they are known to be a rich source of bioactive chemicals (6-10). As many of these compounds are largely free from adverse effects and have desirable pharmacological activities, the study of these plant-derived materials may eventually lead to the development of new classes of safer anticoagulant agents (6-10). Additionally, arylnaphthalide lignans, polyphenols, and quinoline alkaloids have been shown to exert effective inhibitory activities against platelet aggregation induced by collagen (9, 10). Therefore, a great deal of effort has been focused on the study of plant materials for the derivation of potentially useful products, for the eventual development of commercial anticoagulant agents or lead compounds. In the course of our characterization studies regarding the anticoagulant properties of natural compounds, we have determined that the extracts of natural medicines and medicinal foodstuffs, including *Acorus gramineus, Eugenia caryophyllata*, and Galla Rhois, exhibited potent inhibitory activity against platelet aggregation (2, 6-8). *Cinnamomum cassia*, which is the commercial source of cinnamon, is not only an important and popular spice, but is also considered in East Asia to exert some certain medicinal properties, as a stomachic agent, an anti-microbial agent, an astringent, a carminative agent, and as a nitric oxide inhibitor (11, 12). The plant, which contains cinnamaldehyde, *trans*-cinnamic acid, cinnamyl alcohol, and salicylaldehyde (11, 12), is rich in both essential oils and tannins (13). However, relatively few studies have been thus far been carried out with regards to the anticoagulant activity of *C. cassia* bark, despite its observed excellent pharmacological action in East Asian traditional medicine (11, 12, 14). The anticoagulant agent isolated from the *C. cassia* bark may prove to be an excellent source for medicinal foodstuffs and lead compounds, both of which may be used as alternatives to the anticoagulant agents currently in use. The importance of discovering new effective anticoagulant agents compelled us to further investigate these natural compounds.

Materials and Methods

Chemicals and Reagents The bark of *C. cassia* was purchased from a local market in Jeonju, Korea. Aspirin,

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cinnamic acid, cinnamyl alcohol, and PAF were obtained from Sigma (St. Louis, MO, USA). AA, collagen, and thrombin used in these experiments were obtained from Chrono-Log Co. (Havertown, PA, USA).

Isolation and Identification *C. cassia* bark (3.1 kg) was dried at 60°C in an oven for 2 days, finely powdered, extracted twice with methanol (10 L) at room temperature, and then filtered (Toyo filter paper No. 2, Japan). The combined filtrate was then concentrated *in vacuo* at 35°C, to yield about 9.9% (based on the weight of the bark: gum). The extract (306 g) was then partitioned sequentially into hexane (59.8 g), chloroform (68.5 g), ethyl acetate (30.1 g), and water-soluble (147.6 g) portions for subsequent bioassays against collagen, using washed rabbit platelets. The organic solvent portions were then concentrated to dryness by rotary evaporation at 35°C, and the water portion was freeze-dried.

The hexane portion (10 g) was separated on a silica gel column (70-230 mesh, 600 g, 6.5 i.d. × 70 cm; Merck, Germany), and successively eluted with a stepwise gradient of hexane-ethyl acetate (0, 10, 30, 50, and 80%). The active 50% fraction (3.7 g) was separated on a silica gel column, and then eluted with hexane-ethyl acetate (2:1). Twenty-three column fractions were collected and analyzed by thin layer chromatography (TLC) (hexane-ethyl acetate, 3:1). Fractions exhibiting similar TLC patterns were pooled. The active fraction (2.4 g) was then separated on a silica gel column, and eluted with hexane-ethyl acetate (8:2). For the further separation of the bioactive substance, preparative HPLC (Waters Delta Prep 4000) was conducted. The column used was a 29 i.d. × 300 mm Bondapak C_{18} (Waters, Millipore, MA, USA), using methanol-water (3:7) as the elution buffer at a 10 mL/min flow rate, with UV detection at a wavelength of 260 nm. Finally, a potent active principle compound was isolated. The structure of the active isolate was then determined by instrumental analyses. The ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer, at 600 and 150 MHz (*tetra*-methylsilance was used as an internal standard), respectively, and the chemical shifts are given 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 anticoagulated with a 1/10 volume of 1% ethylenediamine tetra acetic acid (EDTA) by 10 min of centrifugation at 230×g at room temperature (6-8). The

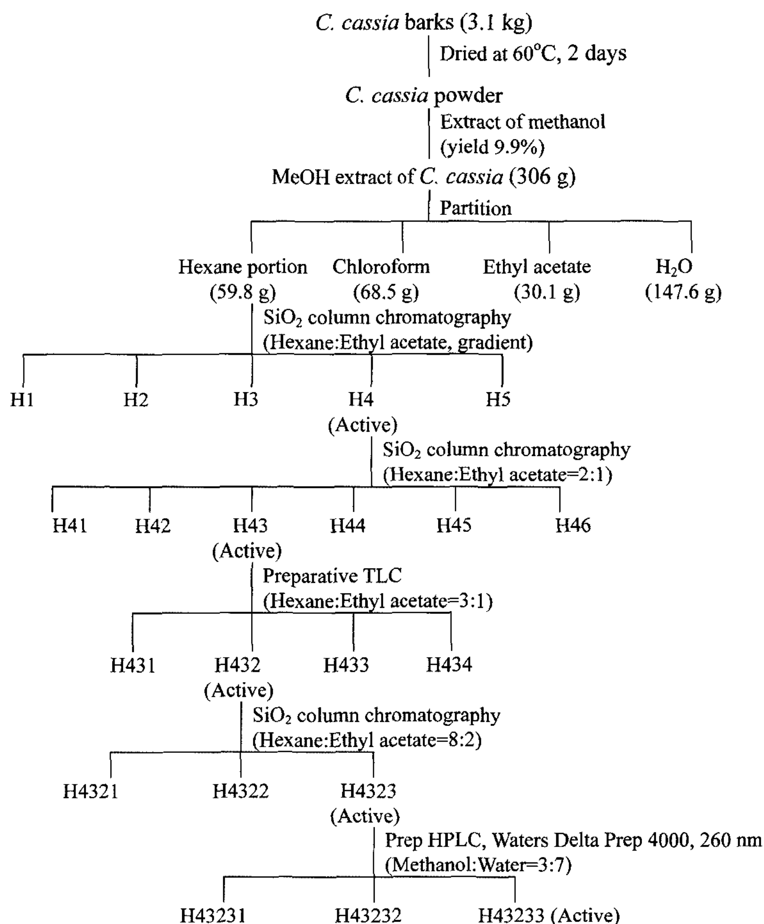

Fig. 1. Isolation procedure of *trans*-cinnamaldehyde from *C. cassia* bark.

platelets were sedimented by centrifugation of the PRP at 800×g for 15 min, and then washed twice in 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). The number of platelets was 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 evaluated with an aggregometer (470-vs; Chrono-Log Co.) as described previously (9). Washed platelets (3×10^8 platelets/mL) were incubated at 37°C for 3 min in the aggregometer with various concentrations of samples for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by AA (100 μM), collagen (2 μg/mL), PAF (10 nM), 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 determined from the maximal aggregation induced by the respective agonists. The percentage of aggregation is presented as mean±SE (n=3) at the probability of $^*p<0.05$, $^{**}p<0.01$ as compared with the respective control. The degree of inhibition of platelet aggregation is expressed as % inhibition (X), in accordance with the following equation: $X = [(A-B)/A]\times100$. The maximal aggregation of the control and the maximal aggregation of the sample-treated washed platelets are listed as A and B, respectively.

Results and Discussion

Four fractions obtained from methanolic extracts of the *C. cassia* bark were evaluated with regard to their *in vitro* anticoagulant effects against platelet aggregation induced by collagen (2 μg/mL), using washed rabbit platelets (data not shown). The anticoagulant activity at a concentration of 100 μg/mL was determined for each of the four prepared fractions. The hexane fraction obtained from the methanolic extract of the *C. cassia* barks showed strong inhibition (100%) against collagen, whereas the other fractions exhibited no or weak inhibitory effects. Owing to the potent activity of the hexane fraction, the biologically active component was purified by silica gel column chromatography and by HPLC (Fig. 1). Finally, one potent active compound was isolated. The structure of this isolate was characterized by 1H- NMR, ^{13}C-NMR, UV, and IR (Fig. 2-5), as well as by direct comparison with an authentic reference compound, and the isolate was ultimately identified as *trans*-cinnamaldehyde. This compound was identified on the basis of the following evidence: C_9H_8O (molecular weight, 132); EI-MS (70 eV) *m/z* (% rel. int.): M^+ 132 (3), 103 (2), 74 (83), 59 (100), 58 (75); IR (neat) max/cm: 2920, 1680, 1630, 1130; 1H-NMR (CD_3OD, 400 MHz): 6.60 (dd, J = 8 and 18 Hz), 7.35 (d, J = 18 Hz), 7.1-7.7 (m), 9.52 (d, J = 8 Hz); ^{13}C-NMR (CD_3OD, 100 MHz): 195.6, 154.4, 135.0, 132.1, 129.9 129.7, 129.5, 129.0, 128.9. The spectroscopic data of *trans*-cinnamaldehyde was determined to be identical to the data obtained with cinnamaldehyde isolated from *C. cassia* (11, 12).

The anticoagulant activities of cinnamaldehyde, cinnamic acid, and cinnamyl alcohol derived from *C. cassia* bark (11, 12) against platelet aggregation induced by collagen were compared to those of aspirin, a naturally occurring flavonoid which has been shown to be a potent *in vitro* anticoagulant agent (Table 1). At concentrations of 50, 10, and 5 μg/mL, cinnamaldehyde showed strong anticoagulant effects with 100, 100, and 61.7% values against platelet aggregation induced by collagen. However, cinnamyl acid and cinnamyl alcohol exhibited moderate and weak inhibitory effects. These results clearly show that the anticoagulant effect of the *C. cassia* extract against platelet aggregation induced by collagen is caused primarily by cinnamaldehyde. Based on known activities, the anticoagulant effects of cinnamaldehyde were evaluated against platelet aggregation induced by collagen (2 μg/mL), AA (100 μM), PAF (10 nM), or thrombin (0.1 unit/mL), and were compared with aspirin with regard to its efficacy as an anticoagulant agent (Table 2). Based on the IC_{50} values, cinnamaldehyde inhibited platelet aggregation induced by collagen followed by AA, with IC_{50} values of 3.1 and 43.2 μM, respectively, but evidenced weak or no inhibitory effects against thrombin or PAF. The commonly used anticoagulant agent, aspirin, served as a standard of

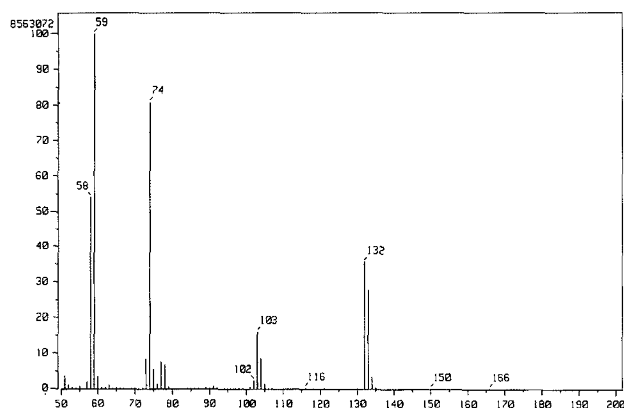

Fig. 2. Mass spectrum of *trans*-cinnamaldehyde from *C. cassia* bark.

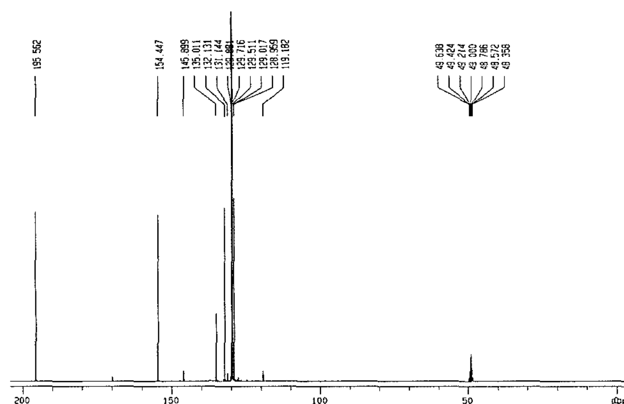

Fig. 3. ^{13}C NMR spectrum of trans-cinnamaldehyde from *C. cassia* bark.

comparison for anticoagulant effects. Aspirin inhibited platelet aggregation induced by AA with an IC_{50} value of 35.3 μM. However, aspirin evidenced weak or no inhibitory effects for collagen, PAF, or thrombin. The activity of cinnamaldehyde against platelet aggregation induced by collagen was approximately 65 times stronger than aspirin, but the effect of aspirin against platelet aggregation induced by AA was 1.2 times stronger than that of cinnamaldehyde. The findings from this study support the stronger effect of cinnamaldehyde against collagen-induced platelet aggregation as compared with aspirin. In this regard, cinnamaldehyde is clearly worthy of additional study as a potential anticoagulant agent or lead compound. This study is, to the best of our knowledge, the first to describe the anticoagulant functions of the components derived from *C. cassia* bark.

Bioactive chemicals derived from plants with anticoagulant effects can prove to be useful therapeutic agents. Aspirin, dipyridamole, sulfinpyrazone, and propranolol have already been identified as anticoagulant agents (15, 16). However, these agents are ineffective under long-term use conditions, due to their side effects. Recently, gallic acid, methyl gallate (Galla Rhois), eugenol, and isoeugenol (*E. caryophyllata*) have been shown to inhibit platelet aggregation *in vitro* in human blood (6, 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 (8). Eugenol effectively inhibited the platelet aggregation induced by AA (IC_{50}, 0.05 μM) and collagen (IC_{50}, 0.7 μM), and isoeugenol was most efficient in the inhibition of platelet aggregation induced by AA (IC_{50}, 0.3 μM), collagen (IC_{50}, 0.9 μM), or PAF (IC_{50}, 12.2 μM) (6). In this study, *C. cassia* bark-derived cinnamaldehyde appears to exert at least one pharmacological effect which inhibits platelet aggregation induced by AA and collagen.

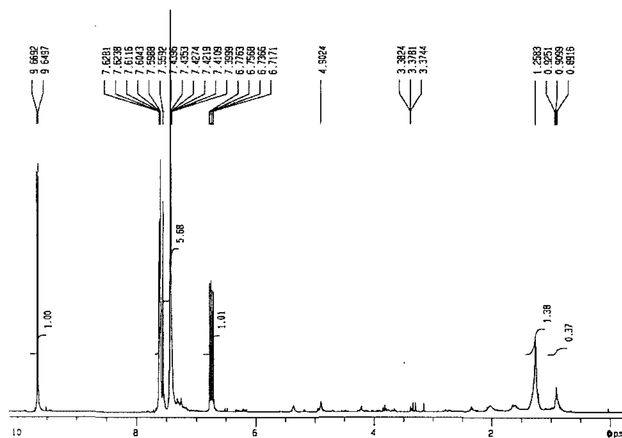

Fig. 4. ^{1}H NMR spectrum of *trans*-cinnamaldehyde from *C. cassia* bark.

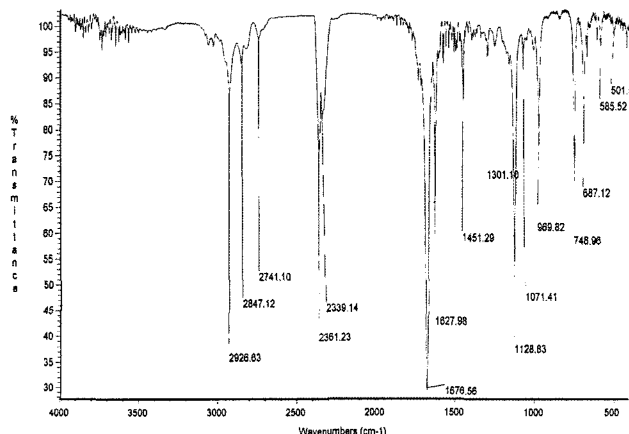

Fig. 5. IR spectrum of *trans*-cinnamaldehyde from *C. cassia* bark.

Table 1. Effects of cinnamaldehyde, cinnamyl acid, and cinnamyl alcohol against platelet activation induced by collagen

Compound	Conc.[1] (μg/mL)	Aggregation[2] (%)	Inhibition[3] (%)
Control		78±1.9	
Cinnamaldehyde	100	0±0.0**	100.0
	50	0±0.0**	100.0
	10	0±0.0**	100.0
	5	29.9±2.5**	61.7
	1	54.7±2.1*	29.9
Cinnamyl acid	100	2.2±0.3**	97.2
	50	37.9±1.9**	51.4
	10	64.3±2.5*	17.6
Cinnamyl alcohol	100	2.1±0.2**	97.3
	50	43.4±2.2**	44.4
	10	71.7±2.7*	8.1

[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 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 %.

Table 2. IC_{50} (μM) of aspirin and cinnamaldehyde against platelet aggregation induced by various agonists in washed rabbit platelets

Agonist[1]	Cinnamaldehyde	Aspirin
Collagen	3.1±0.9[2]	> 200
AA	43.2±2.3	35.3±1.9
Thrombin	> 200	> 200
PAF	> 200	> 200

[1]Washed rabbit platelets were preincubated with aspirin, DMSO (0.5% control), and cinnamaldehyde at 37°C for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by 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.

In conclusion, the results of this study show that *C. cassia* bark-derived cinnamaldehyde exerts an anticoagulant effect *in vitro* against platelet aggregation induced by AA and collagen. On the basis of our limited data, as well as some earlier findings, it appears that cinnamaldehyde may prove useful as lead compound for the development of anticoagulant agents and medicinal foodstuffs, although the *in vivo* efficacy and clinical utility of these compounds remains to be thoroughly evaluated.

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