Antiplatelet effects of *Cyperus rotundus* and its component (+)-nootkatone

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**Abstract**

**Ethnopharmacological relevance:** *Cyperus rotundus*, a well-known oriental traditional medicine, has been reported to exhibit wide spectrum activity in biological systems including the circulatory system, however, little information is available on its antiplatelet activity. This study was undertaken to investigate the antiplatelet effects of *Cyperus rotundus* EtOH extract (CRE) and its constituent compounds.

**Materials and methods:** The antiplatelet activities of CRE and its eight constituent compounds were evaluated by examining their effects on rat platelet aggregations in vitro and ex vivo, and on mice tail bleeding times.

**Results:** During the *in vitro* platelet aggregation study, CRE showed significant and concentration-dependent inhibitory effects on collagen-, thrombin-, and/or AA-induced platelet aggregation. Of its eight components, (+)-nootkatone was found to have the most potent inhibitory effect on collagen-, thrombin-, and AA-induced platelet aggregation. In addition, CRE- and (+)-nootkatone-treated mice exhibited significantly prolonged bleeding times. Furthermore, (+)-nootkatone had a significant inhibitory effect on rat platelet aggregation ex vivo.

**Conclusions:** This study demonstrates the antiplatelet effects of CRE and its active component (+)-nootkatone, and suggests that these agents might be of therapeutic benefit for the prevention of platelet-associated cardiovascular diseases.

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1. Introduction

Arterial thrombosis is an acute complication that develops in association with the lesions of atherosclerosis and causes cardiovascular diseases, such as, ischemic heart disease and stroke (Ruggeri, 2002; Davi and Patrono, 2007). Platelets are key components of thrombosis, and may also participate in the progression of atherosclerotic plaque (Ruggeri, 2002; Jennings, 2009). Hence, the inhibition of platelet aggregation is important for preventing the progression of atherosclerosis and arterial thrombosis, and a suitable antiplatelet therapy could be a cornerstone for the management of atherosclerotic cardiovascular diseases (Ruggeri, 2002; Gross and Weitz, 2009).

Antiplatelet agents, such as, aspirin and clopidogrel, are widely used clinically and directly inhibit platelet aggregation (Gaglia et al., 2010). However, the continuous use of these agents is limited because they can induce resistance or adverse effects, such as, headache, abdominal cramps, vomiting, and gastric ulceration (Angiolillo et al., 2007; Cannon et al., 2007). For these reasons, a number of studies have been conducted to search for naturally derived platelet aggregation inhibitors with fewer adverse effects. In particular, plant extracts offer a rich potential source of novel antiplatelet agents (Tsai et al., 2000; Ballabeni et al., 2007), and since many of these extracts are free from adverse effects and have excellent pharmacological actions, their use could lead to the development of new classes of safe antiplatelet agents (Tognolini et al., 2006; Ryu et al., 2009). Furthermore, some flavonoids and polyphenols have been found to effectively inhibit platelet aggregation (Bucki et al., 2003), and therefore, much effort has focused on the identification of plant-based materials with commercial potential.

*Cyperus rotundus* is a sedge of the Cyperaceae family, and grows naturally in tropical, subtropical, and temperate regions. It is one of the earliest known and most important edible herbs, and has been reported to exhibit wide spectrum activity in biological systems, including antioxidant and anti-inflammatory effects.

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(Ardestani and Yazdanparast, 2007; Kilani et al., 2008; Kilani-Jaziri et al., 2009). Cyperus rotundus continues to be used as a traditional medicine to improve blood circulation, particularly, in gynecological diseases caused by blood stagnation (Yang, 1997; Elizabeth, 2002). The constituents of Cyperus rotundus have been previously examined for monoterpenes and sesquiterpenes (Kilani et al., 2008). It has been reported that terpenoids from Annona squamosa have inhibitory effects on platelet aggregation (Yang et al., 2002).

In order to evaluate the effects of Cyperus rotundus on platelet-related cardiovascular diseases, we investigated whether Cyperus rotundus EtOH extract (CRE) has antiplatelet effects in vitro and in vivo. Furthermore, we examined the effects of compounds of Cyperus rotundus to identify the constituent which is associated with its bioactivities.

2. Materials and methods

2.1. Materials and animals

Collagen, ADP, thrombin, and arachidonic acid (AA) were purchased from Chrono-Log Co. (Harvertown, PA, USA). Dimethylsulfoxide (DMSO), polyethylene glycol (PEG), Tween-80, β-nicotinamide adenine dinucleotide (reduced disodium salt hydrate, β-NADH), and pyruvic acid were purchased from Sigma (St. Louis, MO, USA).

Sprague-Dawley (SD) rats and ICR mice were purchased from the Samtako Laboratory Animal Center (Republic of Korea), and housed in a conventional animal facility with free access to food and water in a temperature and relative humidity monitored controlled environment under artificial lighting (12 h of light per day). Animals were allowed to acclimatize for at least 7 days before experiments. All animals related study protocols were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996), and were approved by the Committee on Animal Research at Ajou Medical Center, Ajou University.

2.2. Extraction and isolation

2.2.1. Plant collection

The rhizomes of Cyperus rotundus, which are cultivated in Koryung (Republic of Korea), were provided by Je-Hyun Lee (College of Oriental Medicine, Dongguk University, Gyeongju, Republic of Korea). A voucher specimen has been deposited at the Herbarium of the Traditional Herb Research Center, Korean Food and Drug Administration.

2.2.2. Extraction

Air-dried and chopped rhizomes of Cyperus rotundus (3.0 kg) were extracted twice with hot 70% EtOH (10 L) for 3 h in a water bath. The EtOH extract obtained was evaporated to dryness in a rotary evaporator (Eyela N-1, Japan) under reduced pressure (400 mmHg) using a water aspirator, affording crude extract (342 g, 11.4% yield) of Cyperus rotundus.

2.2.3. Isolation

Dried extract of Cyperus rotundus (342 g) was dissolved in distilled water (1 L), and 800 mL of the resulting solution was consecutively partitioned using hexane, CH₂Cl₂, EtOAc, and n-BuOH (each 800 mL), and obtained hexane, CH₂Cl₂, MeOH (10:10:1, v/v), and subfractions of hexane fraction were then combined according to their TLC (Thin layer chromatography) patterns. Each of these subfractions was further chromatographed on a silica gel column using a gradient hexane–EtOAc mixture to give valencene (1.03 g, 0.3%), β-pinene (0.14 g, 0.04%), limonene (0.20 g, 0.06%), p-cymene (0.07 g, 0.02%) and 1,8-cineole (0.07 g, 0.02%). These five components were identified by comparing their spectral data with published values (Minyard et al., 1965; Majetich et al., 1985; Miyazawa et al., 1989; Guerrini et al., 2006). (+)-Nootkatone (1.6 g, 0.47%) was obtained as yellowish needles by silica gel column chromatography using stepwise elution (hexane:EtOAc = 15:1, 7:1, 3:1, and 1:1, v/v) from another subfraction, and was also identified by comparing its spectral data with previously reported values (Miyazawa et al., 2000). Caryophyllene oxide (1.7 g, 0.5%) was also obtained by silica gel column chromatography from another subfraction using a hexane/EtOAc gradient and 80–90% acetone filtrate (RP-18) (Thebtaranonth et al., 1995). Coumarin (0.03 g, 0.008%) (Chan et al., 1977) was obtained by Sephadex LH-20 (CH₂Cl₂:MeOH = 1:1) column chromatography from the other subfraction. The purity of above isolated compounds was determined by GC–MS (HP 6890 series) to be valencene 90.6%, β-pinene 98.1%, limonene 97.2%, p-cymene 99.0%, and 1,8-cineole 98.0%. (+)-Nootkatone 98.6%, caryophyllene oxide 89.5%, and coumarin 99.0%.

(+)-Nootkatone: C₁₅H₂₂O; Colorless oil; [α]D + 195.5 (CHCl₃, c = 0.01); 1H NMR (500 MHz, CDCl₃) δ = 0.97 (3H, d, J = 6.4 Hz, H-14), 1.12 (3H, s, H-15), 1.20–2.60 (methylene + methine), 1.74 (3H, s, H-13), 4.73 (2H, br s, H-12), 5.77 (1H, s, H-1); 13C NMR (125 MHz, CDCl₃) δ = 39.3 (C-5), 40.3 (C-4), 40.4 (C-7), 42.1 (C-3), 43.9 (C-11), 109.2 (C-12), 124.7 (C-1), 149.1 (C-11), 170.5 (C-10), 199.6 (C-2); EI-GC/MS m/z: 218 [M]+ (18), 147 (base peak).

2.3. Preparations of samples

For in vitro experiments, CRE was dissolved in 10% Tween 80-saline. The eight components isolated from Cyperus rotundus were dissolved in DMSO. The final concentration of DMSO in platelet suspension never exceeded 1%. For ex vivo and in vivo experiments, (+)-nootkatone was suspended in 70% PEG. All agents were prepared immediately prior to use.

2.4. Preparation of platelets

Platelet-rich plasma was prepared as described previously (Jung et al., 2002). Briefly, SD rats, weighing 200–250 g, were lightly anesthetized with diethyl ether. Blood was collected in a syringe containing 3.8% sodium citrate (1:9, v/v) from the abdominal aorta, and then centrifuged at 150 × g for 10 min at room temperature. The supernatant (platelet-rich plasma, PRP) obtained was used in the aggregation study. The platelet count in PRP was finally adjusted to about 2 × 10⁸ cells/mL with Tyrode solution (pH 7.4, NaCl 11.9 mM, KCl 2.7 mM, MgCl₂ 2.1 mM, NaH₂PO₄ 0.4 mM, NaHCO₃ 11.9 mM, glucose 11.1 mM) containing bovine serum albumin (3.5 mg/mL).

2.5. In vitro platelet aggregation study

Platelet aggregation studies were performed using the turbidimetric method described by Mustard et al. (1972). PRP was stimulated with different aggregating agents at the following final concentrations: collagen 2 µg/mL, thrombin 0.4 U/mL, or AA 100 µM. Platelet aggregation was recorded for 5 min after platelet stimulation. Aggregation was measured and expressed as a percent change in light transmission, with the value for the blank sample
Dose-dependent inhibitory effects of *Cyperus rotundus* EtOH extract (CRE) on platelet aggregation in vitro. Platelet-rich plasma (PRP) were preincubated for 5 min with various concentrations of CRE at 37 °C before being induced to aggregate by collagen 2 μg/mL (A), thrombin 0.4 U/mL (B), or arachidonic acid 100 μM (C). Data are expressed as means ± SEM. *P < 0.05 versus vehicle.

2.6. Determination of cytotoxicity

The cytotoxic effects of samples on platelets were determined by measuring lactate dehydrogenase (LDH) leakage from platelets, as described previously (Lee et al., 2009; Ryu et al., 2009). After incubating rat PRP at 37 °C for 5 min with vehicle or samples, it was centrifuged at room temperature for 1 min at 10,000 × g. To measure LDH release, 25 μL aliquots of supernatant were collected from each group to 96-well, and mixed with 100 μL of NADH solution (0.03% β-NADH in phosphate buffer) and 25 μL of pyruvate solution (22.7 mM pyruvic acid in phosphate buffer) at room temperature. Reductions in absorbance at 340 nm due to the conversion of NADH to NAD⁺ were measured to determine LDH activity in supernatant. LDH leakages were expressed as percentages of total enzyme activity measured in platelets completely lysed with 0.2% Triton X-100.

2.7. Ex vivo platelet aggregation study

(+)-Nootkatone (3, 10, or 30 mg/kg) or aspirin (30 mg/kg) was administered once by oral gavage in 0.5 mL of 70% PEG (vehicle) to each rat 2 h before experiments. Control animals received vehicle only. Blood samples were collected and platelet aggregation was performed as described above in 2.4. and 2.5.

2.8. In vivo mice tail bleeding times

Bleeding times were determined as previously described (Cho et al., 2008). Male ICR mice, weighing 35–40 g, were used in this experiment. Mice were fasted overnight before experiments. Two hours after administering CRE (3, 10, or 30 mg/kg), (+)-nootkatone (3, 10, or 30 mg/kg), or aspirin (50 mg/kg) orally, mice were then anesthetized with sodium pentobarbital (75 mg/kg), and individually placed on a hotplate to control body temperature at 37 °C. In each case, the tail was transected 3 mm from its tip with a razor blade, and then immersed in a 15-mL clear conical tube containing normal saline prewarmed to 37 °C. Times to blood flow cessation (defined as no bleeding for 15 s) were measured.

2.9. Statistical analysis

All results are expressed as means ± SEM of at least four different experiments. One way ANOVA followed by Dunnett’s test and/or the paired t-test were used for the analysis. P-values of <0.05 were considered statistically significant.

3. Results

3.1. In vitro antiplatelet effect of CRE

We evaluated the antiplatelet effect of CRE using rat PRP. Our results showed that CRE at 300 μg/mL had a maximum
Fig. 3. Effects of EtOH extract (CRE) and the eight compounds of *Cyperus rotundus* on LDH release from platelets. LDH release was measured after incubating rat PRP (2 × 10^8 cells/mL) for 5 min with vehicle or samples. Data represent means ± SEM. *P < 0.05 versus vehicle.

Table 1
IC₅₀ values of *Cyperus rotundus* EtOH extract (CRE) on *in vitro* platelet aggregation induced by collagen, thrombin, and arachidonic acid (AA).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Collagen (µM)</th>
<th>Thrombin (µM)</th>
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<tbody>
<tr>
<td>CRE</td>
<td>86.7 ± 0.7</td>
<td>208.4 ± 0.9</td>
</tr>
<tr>
<td>Values are presented as mean ± SEM.</td>
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inhibitory effect on collagen (2 µg/mL)-, thrombin (0.4 U/mL)-, and AA (100 µM)-induced platelet aggregation (100 ± 0.0%, 89.0 ± 6.7%, and 84.9 ± 15.1%, % of inhibition, respectively; Fig. 1). IC₅₀ (half inhibitory concentration) values of CRE for collagen-, thrombin-, and AA-induced platelet aggregation are shown in Table 1.

3.2. *Effect of CRE on bleeding times*

Bleeding times provide a useful means of estimating platelet function and studying the *in vivo* effects of compounds that interfere with platelet aggregation (Praga et al., 1972). Therefore, we determined bleeding times in ICR mice administered with CRE (3, 10, or 30 mg/kg) to evaluate its *in vivo* antiplatelet effect. CRE significantly prolonged bleeding times as compared with vehicle alone.

Furthermore, this effect of CRE was greater than that of aspirin, a well-known antiplatelet drug used as a positive control (Fig. 2).

3.3. *In vitro antiplatelet effects of the eight compounds of Cyperus rotundus*

The present study primarily focused on hexane fraction for further isolation because hexane fraction was the major fraction (over 50%) among four fractions from CRE, and obtained eight compounds, which included three monoterpenes and three sesquiterpenes. The antiplatelet effects of eight compounds were examined using rat PRP. IC₅₀ values of compounds with respect to collagen (2 µg/mL)- and thrombin (0.4 U/mL)-induced aggregation are shown in Table 2. Of the compounds tested, (+)-nootkatone inhibited platelet aggregation with greater potency than that of aspirin. Furthermore, (+)-nootkatone dose-dependently inhibited AA (100 µM)-induced platelet aggregation (Fig. 4).

To determine whether the eight compounds have cytotoxicity that may affect to the antiplatelet effect, we examined their effects on LDH release (an index of cellular injury) from platelets. However, the amount of LDH released was not significantly altered by CRE or the eight compounds for 5 min at the highest concentrations examined (Fig. 3), whereas digitonin (40 µM; a positive control) significantly increased LDH release. These findings show

![Fig. 4. Dose-dependent inhibitory effects of (+)-nootkatone on *in vitro* platelet aggregation. (A) The chemical structure of (+)-nootkatone. PRP was preincubated for 5 min with various concentrations of (+)-nootkatone at 37 °C before aggregation induction with collagen 2 µg/mL (B), thrombin 0.4 U/mL (C), or arachidonic acid 100 µM (D). Data represent means ± SEM. *P < 0.05 versus vehicle.](image-url)
that neither CRE nor the eight compounds affected cell membrane integrity.

3.4. Ex vivo platelet response to (+)-nootkatone administration

The inhibitory effect of (+)-nootkatone on ex vivo platelet aggregation is shown in Fig. 5. Isolated platelets from rats administered with (+)-nootkatone (3, 10, or 30 mg/kg; p.o.) showed a great tendency to inhibit collagen (2 μg/mL)-induced platelet aggregation, which concurred with the results obtained when PRP was directly incubated with (+)-nootkatone. Aspirin (30 mg/kg) also significantly inhibited ex vivo platelet aggregation.

3.5. Effect of (+)-nootkatone on bleeding times

We also determined bleeding times in ICR mice administered with (+)-nootkatone to evaluate its in vivo antiplatelet effect. (+)-Nootkatone significantly prolonged bleeding times as compared with vehicle alone, and mice administered with (+)-nootkatone at 30 mg/kg had bleeding times similar to those of mice administered with aspirin at 50 mg/kg (Fig. 6).

4. Discussion

Platelets are responsible for the formation of pathogenic thrombi that cause the acute manifestations of atherothrombotic cardiovascular diseases. Increased platelet aggregation and atherosclerosis are two essential contributors to the onset and development of the cardiovascular diseases, which remain leading causes of death (Davi and Patrono, 2007). Therefore, much research has been carried out to develop antiplatelet agents with improved efficacy to prevent and/or treat atherothrombotic cardiovascular diseases (Gross and Weitz, 2009). The present study demonstrates the antiplatelet effects of CRE and its active component (+)-nootkatone, and suggests that CRE and (+)-nootkatone have therapeutic potential for the prevention of platelet-related cardiovascular diseases.

Several studies reported that uncontrolled blood pressure is associated with thrombotic and atherosclerotic complications (Lip et al., 1997; Felmeden and Lip, 2005), and that the pathophysiological changes in high blood pressure are contributed to an intravascular microenvironment which promotes platelet aggregation and thrombus formation (Lip et al., 2001). Moreover, the increased platelet activity was found in patients suffering from vascular injury and hypertension (Felmeden and Lip, 2005). Hence, antiplatelet therapy has been supposed to be beneficial for patients with hypertension. Indeed, it has been reported that several antihypertensive agents, including amlodipine, have antiplatelet activity (Wallen et al., 1995; Chou et al., 1999). Cyperus rotundus, a widely used oriental medicine, has been known to elicit various beneficial effects including anti-inflammatory effect, and it continues to be used to improve blood circulation. Furthermore, its blood pressure-lowering effect has been reported in animals and humans (Singh et al., 1970; Elizabeth, 2002). In the present study, we found that Cyperus rotundus has an antiplatelet effect based on the results that CRE inhibited the platelet aggregations induced by collagen, thrombin and AA in vitro. The antiplatelet activity of CRE was further confirmed by its prolongation effect on bleeding time in vivo.

Of the eight components of the hexane fraction of Cyperus rotundus examined, (+)-nootkatone was found to be the most potent, in terms of inhibiting platelet aggregation. (+)-Nootkatone is a well-known sesquiterpenoid, and the sesquiterpenoids are the largest subclass of terpenoids (Sowden et al., 2005). Terpenoids are found in almost every plant and have diverse bioactivities that range from anti-inflammatory, antioxidant and antiplatelet effects (Wagner and Elmadfa, 2003). Although previous studies on sesquiterpenoids, such as ergolide and parthenolide, have mainly focused on anti-inflammatory activity (Salminen et al., 2008), the present study demonstrates for the first time that (+)-nootkatone has considerable antiplatelet activity; inhibition of platelet aggregation and prolongation of bleeding time.

To compare the potency between CRE and (+)-nootkatone, we recalculated the μM concentration of (+)-nootkatone into μg/mL, and got IC50 values of (+)-nootkatone for collagen- and thrombin-induced platelet aggregation to be 11 μg/mL and 34.3 μg/mL, respectively, and those of CRE were 86.7 μg/mL and 208.4 μg/mL, respectively, as shown in Tables 1 and 2. As per this comparison, (+)-nootkatone was likely to be more potent than CRE in in vitro platelet aggregation. However, in in vivo experiments, as shown in Figs. 2 and 6, CRE showed greater potency than (+)-nootkatone in prolongation of bleeding time. This discrepancy of the results might be due to the other constituents rather than (+)-nootkatone present in the CRE, such as valencene and Caryophyllene oxide, which might contribute to the antiplatelet activity of CRE. In particular, (+)-valencene, the one of eight compounds from Cyperus rotundus, is known as the biosynthetic precursor of (+)-nootkatone, and the biosynthesis and biotransformation of the (+)-valencene to (+)-nootkatone has also been demonstrated (Fraatz et al., 2009). These reports suggest that (+)-valencene may contribute to the antiplatelet activity of CRE through metabolism in vivo. Taken together, CRE may be more effective than (+)-nootkatone in terms of antiplatelet activity in vivo, while (+)-nootkatone alone appears to be more potent than CRE in vitro.
Under pathologic conditions, platelet aggregation involves multiple agonists, and in particular, thrombin has great activity and binds to protease-activated receptor-1 (PAR-1) on the surfaces of platelets, which causes platelet aggregation (Jennings, 2009). Collagen, which also induces platelet activation, is a strong thrombogenic agent, and in damaged endothelium promotes platelet adhesion to subendothelium by binding to several receptors, such as, integrin α2β1 and glycoprotein VI (GPVI), on platelet surfaces, thus, inducing platelet aggregation (Varga-Szabo et al., 2008). AA also induces platelet aggregation. In fact, upon exposure to activating agonists, platelets release AA stored as phospholipid in the platelet plasma membrane, and this AA is then converted by cyclooxygenase and TXA2 synthase into thromboxane A2 (TXA2), which acts as a positive feedback mediator for the activation and recruitment of more platelets (Jennings, 2009). Accordingly, platelets are activated by multiple physiological agonists that interact with specific platelet receptors, and thus, antiplatelet agents that act only at one site are limited in terms of preventing the formation of pathogenic thrombi. Indeed, it has been reported that antiplatelet therapy with aspirin, which inhibits both the AA/cyclooxygenase pathway, is not sufficient to prevent ischemic events in patients with atherothrombotic cardiovascular disease (Yusuf et al., 2001). Furthermore, dual antiplatelet therapy with aspirin plus clopidogrel, a P2Y12-receptor antagonist, has been shown to be more effective at reducing ischemic events in patients with atherothrombotic cardiovascular diseases than aspirin alone (Chen et al., 2005). Thus, available evidence suggests that antiplatelet agents acting at multiple sites are likely to provide more effective arterial thrombosis treatments. Hence, it appears that CRE and (+)-nootkatone, which can inhibit multiple sites of platelet aggregation, may be useful for the prevention of atherothrombotic diseases.

Although we did not include a mechanistic study to identify the signaling pathways responsible for the inhibition of platelet aggregation by (+)-nootkatone, recent studies have reported that (+)-nootkatone is an activator of adenosine monophosphate-activated protein kinase (AMPK), which was suggested to be linked to the inhibition of platelet aggregation (Fleming et al., 2003; Murase et al., 2010). AMPK is also known to promote cardiovascular homeostasis by ensuring an optimal redox balance in the cardiovascular system, and AMPK dysfunction is believed to underlie several cardiovascular pathologies (Shirwany and Zou, 2010). It is evident that further study is needed to determine whether AMPK-activating activity of (+)-nootkatone is associated with its antiplatelet activity.

In conclusion, the present study suggests that the antiplatelet activities of CRE and its constituent (+)-nootkatone may be therapeutically useful for the treatment of atherothrombotic diseases.

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