Molecular Physiology and Pathology[1], School of Pharma-Sciences, Teikyo University; Japan Bio Science Laboratory Co. Ltd.[2]; Division of Pharmacognosy[3], Osaka University of Pharmaceutical Sciences, Japan

Anti-platelet effects of chalcones from *Angelica keiskei* Koidzumi (Ashitaba) *in vivo*

N. Ohkura[1], K. Ohnishi[2], M. Taniguchi[3], A. Nakayama[1], Y. Usuba[1], M. Fujita[1], A. Fujii[1], K. Ishibashi[1], K. Baba[3], G. Atsumi[1]

Received May 16, 2016, accepted June 17, 2016

Naoki Ohkura Ph.D., Molecular Physiology and Pathology, School of Pharma-Sciences, Teikyo University, Itabashi, Tokyo 173-8605, Japan
n-ohkura@pharm.teikyo-u.ac.jp

Pharmazie 71: 651–654 (2016) *doi: 10.1691/ph.2016.6678*

Angelica keiskei Koidzumi (Ashitaba) is a traditional folk medicine that is also regarded in Japan as a health food with potential antithrombotic properties. The ability of the major chalcones, xanthoangelol (XA) and 4-hydroxyderricin (4-HD) extracted from Ashitaba roots to inhibit platelet aggregation activity *in vitro* was recently determined. However, the anti-platelet activities of Ashitaba chalcones *in vivo* have remained unclear. The present study examines the anti-platelet effects of Ashitaba exudate and its constituent chalcones using mouse tail-bleeding models that reflect platelet aggregation *in vivo*. Ashitaba exudate and the major chalcone subtype XA, suppressed the lipopolysaccharide (LPS)-induced shortening of mouse tail bleeding. However, trace amounts of other Ashitaba chalcone subtypes including xanthoangelols B (XB), D (XD), E (XE) and F (XF) did not affect tail bleeding. These results suggest that the major chalcone subtype in Ashitaba, XA, has anti-platelet-activities *in vivo*.

1. Introduction

Angelica keiskei Koidzumi (Ashitaba) is consumed as a traditional folk medicine and health food in Japan. The two major chalcones isolated from Ashitaba, xanthoangelol and 4-hydroxyderricin, have anti-bacterial, anti-cancer, anti-diabetic and other activities (Inamori et al. 1991; Kimura et al. 2003; Enoki et al. 2007). We previously showed that Ashitaba exudate inhibits lipopolysaccharide (LPS)-induced increases in plasma PAI-1, the main physiological inhibitor of the fibrinolytic system, in mice (Ohkura et al. 2011). This indicated that Ashitaba exerts anti-thrombotic activity by decreasing PAI-1 levels.

The chalcone fraction consists essentially of ~50% each of xanthoangelol (XA) and 4-hydroxyderricin (4-HD) and these major constituents are responsible for exerting the biological activities associated with Ashitaba. However, trace amounts of other chalcones, namely, xanthoangelols B (XB), D (XD), E (XE), and F (XF) (Baba et al., 1990; Nakata et al., 1999) are also biologically active. For example, XD inhibits endothelin-1 production through of nuclear factor-kB (NF-kB) (Sugii et al., 2005) and XE inhibits the formation of thromboxane B2 in platelets (Fujita et al. 1992).

Son et al. 2014 isolated compounds with anti-platelet-activities from Ashitaba by bioassay-guided isolation. The major chalcones, XA and 4-HD from Ashitaba inhibited platelet aggregation *in vitro* . However, whether or not these chalcones exert anti-platelet activities *in vivo* is unclear. The duration of bleeding mainly reflects platelet function and thus the effects of anti-platelet reagents *in vivo* have historically been evaluated as bleeding duration in a tail bleeding model (Harker et al. 1972). Here, we analysed the effects of Ashitaba chalcones on the decreased duration of tail bleeding induced by LPS.

2. Investigations, results and discussion

2.1. Effect of Ashitaba exudate and chalcones

An injection of LPS significantly shortened the duration of tail bleeding. Orally administered Ashitaba exudate and intraperitoneally injected ethylacetate fraction of the exudate inhibited this response to LPS (Fig. 1A and B). The duration of bleeding mainly reflects platelet function and the effects of anti-platelet reagents *in vivo* have histori-

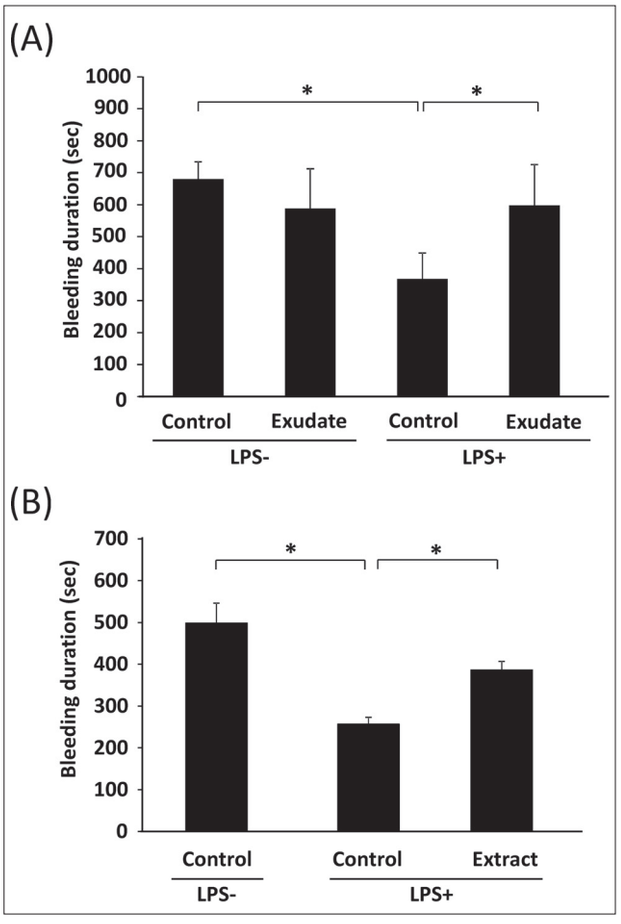

Fig. 1: Effect of orally administered Ashitaba exudate (A) and an intraperitoneal injection of ethylacetate fraction of Ashitaba exudate (B) on shortened duration of tail bleeding in mice induced by LPS.

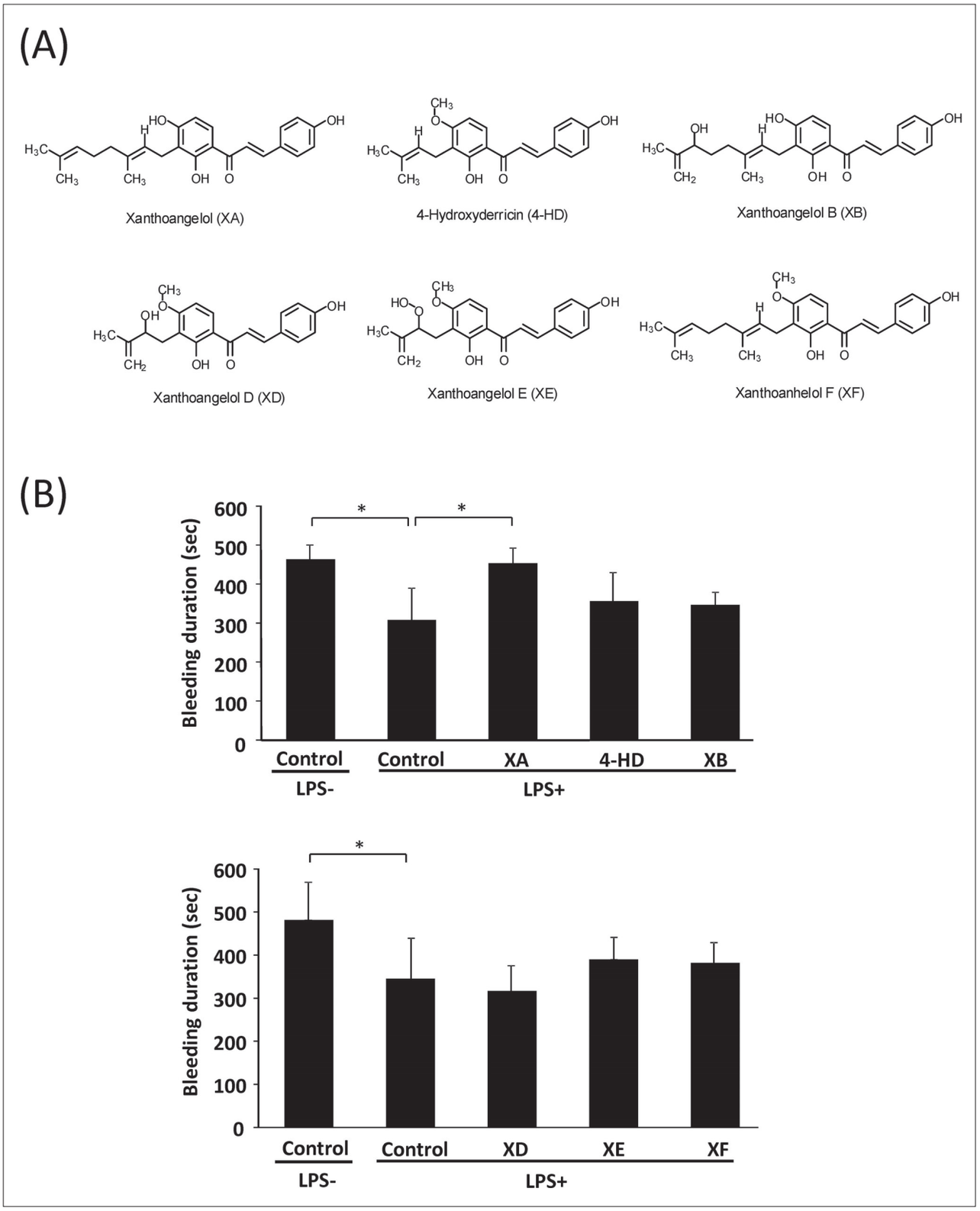

Fig. 2: Effect of orally administered Ashitaba exudate (A) and an intraperitoneal injection of ethylacetate fraction of Ashitaba exudate (B) on shortened duration of tail bleeding in mice induced by LPS.

cally been evaluated using tail bleeding (Harker et al. 1972). Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria that induces prothrombotic conditions when injected. Since LPS induces platelet aggregation (MacIntyre et al. 1977; Ito et al. 1990), we concluded that the effect of Ashitaba exudate on tail bleeding was exested mainly *via* suppressed platelet aggregation. Ashitaba exudate mostly comprises XA and 4-HD accompanied by trace amounts of XB, XD, XE and XF that have the same basic structure as XA or 4-HD plus a slightly modified side chain (Fig. 2A). We then injected XA as well as 4-HD, XB, XD, XE and XF into the mouse peritoneal

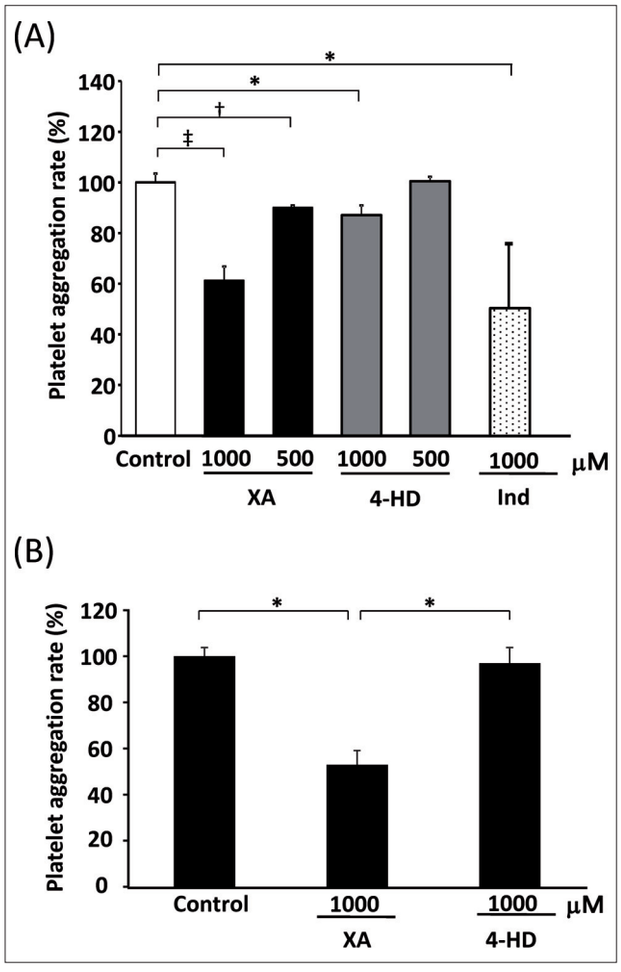

Fig. 3: Effect of XA and 4-HD on collagen induced platelet aggregation in blood.
(A) Bovine whole blood was incubated at room temperature with XA, 4-HD or indomethacin (Ind) for 30 min. Thereafter, platelet aggregation was induced by adding collagen solution. Platelet aggregation rates (%) were calculated according as described in the Methods section. Values are shown as means ± SD. (n = 3; *P < 0.05, †P < 0.01, ‡P < 0.005)
(B) Mouse blood (0.2 mL) was incubated at room temperature with test samples for 60 min and then platelet aggregation was induced by adding collagen solution. Platelet aggregation rates (%) were calculated as described in Methods section. Values are shown as means ± SD. (n = 3; *p < 0.005).

cavity and found that XA significantly recovered tail bleeding (Fig. 2B). However, this effect was not elicited by XB, which is structurally identical to XA except for a hydroxyl group in the long hydrocarbon chain. Xanthoangelol F that has the same side chain as XA but an additional methoxy group on the A ring of XA also did not affect tail bleeding. None of 4-HD, XD and XE that has a short side chain in the A ring affect tail bleeding. These findings suggest that slight modifications to the side hydrocarbon chain or a small functional group added to the A ring play important roles in chalcone activities.

2.2. Effects of XA and 4-HD on platelet aggregation

Both XA and 4-HD inhibit the aggregation of isolated platelets (Son et al. 2014). We confirmed these inhibitory effects in assays of whole-blood platelet aggregation (Fig. 3A). Our findings agreed with those of Son et al. (2014) who described that XA is slightly more inhibitory than 4-HD . However, only XA recovered mice tail bleeding *in vivo* (Fig. 2B). We then found that XA with a geranyl group significantly inhibited mouse platelet aggregation *in vitro* (Fig. 3B) whereas 4-HD with a dimethylallyl group did not. Prenylation increases hydrophobicity and thus might increase the translocation of or interaction with mice platelets. Xanthoangelol (XA) suppresses LPS-induced TNFα production from macrophages more effectively than 4-HD (Yasuda et al. 2014) and TNFα induces a prothrombotic state including indirect platelet activation (Joseph et al. 2002). These pathways might also influence the effect of XA.

The main xanthoangelol subtype in Ashitaba is XA, which was the most potent inhibitor of shortened mouse tail bleeding induced by LPS. However, the total content of XB, XD XE and XF in Ashitaba exudate comprises < 1% of total chalcones (Baba et al. 1990). Therefore, the presented findings suggest that the shortened tail bleeding inhibited by oral or intraperitoneal Ashitaba exudate is mainly due to XA.

The main chalcone subtype, XA, in Ashitaba was the most potent inhibitor of LPS-induced shortened tail-bleeding duration in mice, which reflects platelet function *in vivo*. The side hydrocarbon chain played an important role in this process and small modifications to the hydrocarbon chain, or the addition of a small functional group to the A ring of XA also influenced its activity. Ashitaba might serve as an antithrombotic herbal medicine or health supplement to control platelet function.

3. Experimental

3.1. Materials

Lyophilised yellow exudate from cut ends of *A. keiskei* stems was a gift from the Japan Bioscience Laboratory Co. (Osaka, Japan). Citrated bovine whole blood was purchased from Tokyo Shibaura Zoki Co. (Tokyo, Japan) and horse tendinous collagen was sourced from LMS Co. (Tokyo Japan). Lipopolysaccharides from *Escherichia coli* 0111:B4 were obtained from Sigma Chemical Co. (St. Louis MO, USA). All other materials were commercial products of the highest grade available.

3.2. Preparation of chalcones and crude chalcone fraction

We isolated the Ashitaba chalcone subtypes, xanthoangelol (XA), xanthoangelols B (XB), D (XD), E (XE) and F (XF) as well as 4-hydroxyderricin (4-HD) from *Angelica keiskei* Koidzumi (Umbelliferae) as described (Baba et al. 1990) and from Ashitaba root and exudate. The amounts of Ashitaba chalcones in the exudate and extract were quantified using HPLC as described (Ohta et al. 2015). The chalcone content in the exudate was 8.52 ± 0.46 % (n = 8). Chalcones were recovered at a rate of > 90% from an ethyl acetate fraction prepared as described.

3.3. Animal and experimental protocols

Seven-week-old male Kwl ICR mice (specific-pathogen free grade) purchased from Tokyo Laboratory Animals Science Co. (Tokyo, Japan) were housed at 24 ± 2 °C and provided with food and water *ad libitum*. Lyophilized yellow exudate suspended in corn oil (100 mg/mice) was orally administered (p.o.) every day for seven days. Ethylacetate fraction (100 mg/kg) or purified chalcones (10 mg/kg) were injected once into the peritoneum and then the mice were injected i.p. with LPS (0.05 mg/kg) in saline or with saline alone (vehicle) one hour later. Lyophilized yellow exudates (100 mg) contained 8.7 mg of chalcones (XA, 5.7 mg; 4-HD, 2.9 mg). The ethyl acetate fraction (100 mg) contained about 27.1 and 13.8 mg of XA and 4-HD, respectively. Three hours thereafter, the mice were anesthetized with pentobarbital (30 mg/kg) and the tails were cut 2 mm from the tip. Blood from the wounded tail was spotted onto filter paper every ten seconds. Bleeding duration was determined in a blinded manner as the amount of time that elapsed from LPS or vehicle injection until the moment when blood flow stopped. Blood specimens collected from the inferior vena cava with using a plastic syringe and needle under pentobarbital (40 mg/kg i.p.) and ether anesthesia were immediately mixed with 0.2 volumes of 3.2 % sodium citrate. Platelet-poor plasma was separated from citrated blood by centrifugation at 3800 × g for 15 min. Plasma samples were collected and stored at -80 °C for later measurements of plasma factors. All experiments proceeded in accordance with the Guide for the Care and Use of Laboratory Animals at Teikyo University and were approved by the Animal Care and Use Committee at Teikyo University (approval numbers, 12-014 and 12-041).

3.4. Platelet aggregation assay

Platelet aggregation was measured by counting platelets in whole bovine and mouse blood using a KX-21 blood cell counter (Sysmex, Kobe, Japan) as described (Saniabadi et al. 1983; Cheeseman et al. 1984). In brief, bovine (1 mL) or mouse (0.2 mL) whole blood samples were incubated in polypropylene tubes at room temperature (24 ±2 °C) with test samples (10 and 1.1 μL, respectively) for 30 min and then platelet aggregation was induced by adding collagen solution (20 and 4.4 μL, respectively). The tubes were then gently and continuously mixed at room temperature for 20 s and platelets were counted three times at intervals of 70 s. Platelet counts are expressed as ratios (%) of the initial counts in the presence of vehicle only. Platelet aggregation rates (%) were calculated as:
[100 - remaining platelet rate (%) (sample)/100 - remaining platelet rate (%) (Control)] × 100.

3.5. Statistics

All values are expressed as means ± SD. The significance of differences was evaluated using Dunnett's test and Student's t-test. A p-value of < 0.05 was considered to represent a significant difference.

Disclosure statement: KO is employed at the Japan Bio Science Laboratory Co. Ltd. None of the other authors have any conflicts of interest to declare.
This study was supported in part by a Grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) 25350144 (to N Ohkura).

References

Baba K, Nakata K, Taniguchi M, Kido T, Kozawa K (1990) Chalcones from *Angelica keiskei*. Phytochemistry 29: 3907-3910.

Cheeseman JE, Mills SP, Hardisty RM (1984) Platelet aggregometry on whole blood: the use of the ELT 8/ds blood cell counter in the investigation of bleeding disorders. Clin Lab Haematol 6: 265-272.

Enoki T, Ohnogi H, Nagamine K, Kudo Y, Sugiyama K, Tanabe M, Kobayashi E, Sagawa H, Kato I (2007) Antidiabetic activities of chalcones isolated from a Japanese herb, *Angelica keiskei*. J Agric Food Chem 55: 6013-6017

Fujita T, Sakuma S, Sumiya T, Nishida H, Fujimoto Y, Baba K, Kozawa M (1992) The effects of xanthoangelol E on arachidonic acid metabolism in the gastric antral mucosa and platelet of the rabbit. Res Commun Chem Pathol Pharmacol 77: 227-240.

Harker LA, Slichter SJ (1972) The bleeding time as a screening test for evaluation of platelet function. N Engl J Med 287: 155-159.

Inamori Y, Baba K, Tsujibo H, Taniguchi M, Nakata K, Kozawa M (1991) Antibacterial activity of two chalcones, xanthoangelol and 4-hydroxyderricin, isolated from the root of *Angelica keiskei* KOIDZUMI. Chem Pharm Bull *(Tokyo)* 39: 1604-1605.

Ito T, Asai F, Oshima T, Kobayashi S (1990) Role of activated platelets in endotoxin-induced DIC in rats. Thromb Res 59:735-747.

Joseph L, Fink LM, Hauer-Jensen M (2002) Cytokines in coagulation and thrombosis: a preclinical and clinical review. Blood Coagul Fibrinolysis 13:105-116.

Kimura Y, Baba K (2003) Antitumor and antimetastatic activities of *Angelica keiskei* roots, part 1: Isolation of an active substance, xanthoangelol. Int J Cancer 106: 429-437.

MacIntyre DE, Allen AP, Thorne KJ, Glauert AM, Gordon JL (1977) Endotoxin-induced platelet aggregation and secretion. I. Morphological changes and pharmacological effects. J Cell Sci 28: 211-223.

Nakata K, Taniguchi M, Baba K (1999) Three chalcones from *Angelica keiskei*. Natural Med 53: 329-332.

Ohkura N, Nakakuki Y, Taniguchi M, Kanai S, Nakayama A, Ohnishi K, Sakata T, Nohira T, Matsuda J, Baba K, Atsumi G (2011) Xanthoangelols isolated from *Angelica keiskei* inhibit inflammatory-induced plasminogen activator inhibitor 1 (PAI-1) production. Biofactors 37: 455-461.

Ohta M, Fujinami A, Kobayashi N, Amano A, Ishigami A, Tokuda H, Suzuki N, Ito F, Mori T, Sawada M, Iwasa K, Kitawaki J, Ohnishi K, Tsujikawa M, Obayashi H (2015) Two chalcones, 4-hydroxyderricin and xanthoangelol, stimulate GLUT4-dependent glucose uptake through the LKB1/AMP-activated protein kinase signaling pathway in 3T3-L1 adipocytes. Nutr Res 35: 618-625.

Saniabadi AR, Lowe GD, Forbes CD, Prentice CR, Barbenel JC (1983) Platelet aggregation studies in whole human blood. Thromb Res 30: 625-632.

Son DJ, Park YO, Yu C, Lee SE, Park YH (2014) Bioassay-guided isolation and identification of anti-platelet-active compounds from the root of Ashitaba (*Angelica keiskei* Koidzumi). Nat Prod Res 28: 2312-2316.

Sugii M, Ohkita M, Taniguchi M, Baba K, Kawai Y, Tahara C, Takaoka M, Matsumura Y (2005) Xanthoangelol D isolated from the roots of *Angelica keiskei* inhibits endothelin-1 production through the suppression of nuclear factor-kappaB. Biol Pharm Bull 28: 607-610.

Yasuda M, Kawabata K, Miyashita M, Okumura M, Yamamoto N, Takahashi M, Ashida H, Ohigashi H (2014) Inhibitory effects of 4-hydroxyderricin and xanthoangelol on lipopolysaccharide-induced inflammatory responses in RAW264 macrophages. J Agric Food Chem 62:462-467.