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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Nitro derivatives and other compounds from sugar apple (*Annona squamosa L.*) leaves exhibit soluble epoxide hydrolase inhibitory activity

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Abstract

A phytochemical study for the MeOH extract of *Annona squamosa* leaves led to the isolation of seven compounds. Among them there were two nitro derivative compounds (**1** and **2**), two phenolic glucoside compounds (**3** and **4**), and three sesquiterpenes (**5–7**). The structures of these natural compounds were determined by the spectroscopic methods including 1D and 2D NMR methods. All the isolated compounds were assessed for the effects on the sEH inhibitory activity; of all the compounds, thalictoside (**1**) and arabinothalictoside (**2**) displayed strong inhibition of sEH activities with IC_{50} value of 20.2 μM and 47.1 μM, respectively. A kinetic analysis of compounds **1** and **2** revealed that both of them were competitive inhibitory action. The molecular docking increases our understanding of receptor–ligand binding of all compounds. These results suggested that nitro derivatives from *Annona squamosa* leaves are potential sEH inhibitors.

Keywords *Annona squamosa* L. · Molecular docking · Nitro derivative · Soluble epoxide hydrolase (sEH)

Introduction

Annona squamosa L. (Annonaceae) is commonly known as sugar apple, custard apple, or sweetsop, with a long history of traditional usage, which in recent years has been cultivated in tropical and subtropical regions worldwide. Different portions of *A. squamosa* revealed various phytochemicals and constituents including alkaloids, terpenes, flavonoids, annonaceous acetogenins, cyclopeptides, and essential oils (Ma et al. 2017; Soni et al. 2012). The long history of usage with *A. squamosa* L. could suggest a vast natural product source of active phytochemical constituents. Many studies of extractions and fractions revealed significant potential of anti-cancer (Kim et al. 1998; Mazahery et al. 2009), anti-diabetic and hypolipidemic (Kaleem et al. 2006; Kaleem et al. 2008), antioxidant (Panda and Kar 2015), and anti-hypertensive (Morita et al. 2006) properties. Nonetheless, previous literatures reported ~150 compounds from different portions of *A. squamosa*; most of them were isolated from the seeds and ~14 compounds were isolated from the stem and leaves, the majority of which were aporphine alkaloids. Therefore, the phytochemical investigation for the leaves of *A. squamosa* needs to be studied further.

Soluble epoxide hydrolase (sEH) is widely distributed in mammalian tissues, with potent biological activities on cardiovascular and urinary systems (Campbell et al. 1996). It is responsible for the hydrolysis of epoxyeicosatrienoic acids (EETs), which are endothelium-derived hyperpolarizing factors (EDHFs) that act as regulators of vascular function (Imig and Hammock 2009). The sEH can convert EETs to its corresponding diols (dihydroxyeicosatrienoic acids) and reduce EET effects on cardiovascular systems

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through vasodilation, anti-migration of vascular smooth muscle cells, and anti-inflammatory actions. Therefore, sEH was considered a potential therapeutic target for vascular disease (Bai et al. 2015). However, the components from *A. squamosa* exhibited vasorelaxant effect (Morita et al. 2006); nevertheless, sEH inhibitory activities for the components of *A. squamosa* have not been reported.

Materials and methods

Plant material

Dried leaves of *A. squamosa* were kindly provided from Chung Kim, Danang, Vietnam, in 2015. Its scientific name was identified by one author (Professor Young Ho Kim). A voucher specimen (CNU 15003) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Republic of Korea.

General experimental procedures

The nuclear magnetic resonance (NMR) spectra were recorded using a BRUKER AVANCE III 600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) (Bruker Biospin GmbH, Karlsruhe, Germany), with tetramethylsilane as an internal standard. Heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation, rotating frame nuclear overhauser effect spectroscopy, and ^{1}H–^{1}H correlation spectroscopy spectra were recorded using a pulsed field gradient. The high-resolution electrospray ionization mass spectrometry (MS) spectra were obtained by using an Aglient 1200 LC-MSD Trap spectrometer (Agilent, Santa Clara, CA, USA). Preparative high-performance liquid chromatography (HPLC) was performed using a GILSON 321 pump, 151 UV/VIS detector (Gilson, VILLIERS-LE-BEL, France), and RStech HECTOR-M C_{18} column (5 µm, 250 × 21.2 mm) (RS Tech Crop, Chungju, South Korea). Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany) and YMC C-18 resins, and thin layer chromatography was performed using pre-coated silica-gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm, Merck, Darmstadt, Germany); the spots were detected under UV light using 10% H_2SO_4.

Extraction and isolation

The dry leaves of *A. squamosa* (2.0 kg) was reflux extracted with MeOH (5 L × 3 times). The total extraction (300 g) of MeOH was suspended in deionized water and partitioned with Hexane and water fraction. Then the water fraction was partitioned sequentially with EtOAc and BuOH, yielding EtOAc (1A, 12.2 g) and BuOH (1B, 60.5 g). The BuOH fraction was subjected to a silica gel column chromatography with a gradient of $CHCl_3$-MeOH-H_2O (10:1:0, 9:1:0, 8:1:0, 6:1:0.1, 5:1:0.1, 4:1:0.1, 3:1:0.1, and 2:1:0.1, MeOH, 5 L for each step) to give 11 fractions (Fr. 1B-1–1B-11). Fraction 1B-2 was isolated with a gradient of MeOH-H_2O (1:4, 1:3, 1:2, and 1:1 MeOH) by middle-pressure liquid chromatography using YMC C_{18} column to give eight fractions (Fr. 1C-1–1C-8). The fraction 1C-2 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound **1** (40.0 mg) and compound **3** (13.3 mg). The fraction 1C-3 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfractions were isolated by prep-HPLC to give compound **2** (23.2 mg). The fraction. 1C-4 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound **5** (24.0 mg). The fraction 1C-5 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound **4** (13.5 mg). The fraction 1C-6 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound **6** (9.1 mg). The fraction 1C-7 was separated by a Sephadex LH-20 column and eluted by MeOH, and its subfraction was isolated by prep-HPLC to give compound **7** (4.6 mg).

sEH assay

sEH activity assay

In sEH inhibitory assay, Bis-Tris (B9754) and albumin (A8806) were purchased from Sigma Aldrich (St. Louis, MO,USA). Human recombinant sEHs (10011669) and 3-phenyl-cyano (6-methoxy-2-naphthalenyl) methyl ester-2-oxiraneacetic acid (PHOME) (10009134) were purchased from Cayman Chemical (Cayman, MI, USA). Ninety-six-well white plate was purchased from Costar (Corning, NY, USA). Fluorescence intensity measurement was conducted on using Tecan infinite F200 microplate reader (Tecan, Mannedorf, Switzerland).

The enzymatic assays were performed according to methods modified from previous study (Bai et al. 2015). The 130 µL aliquot of recombinant human sEH (12.15 ng/mL) was diluted with buffer (25 mM Bis-Tris-HCl, pH 7.0 containing 0.1 mg/mL bovine serum albumin (BSA)) and mixed with 20 µL of MeOH, and then 50 µL of PHOME (10 µM) was added. The amount of product converted from substrate by the enzyme was measured by fluorescence photometry (excitation filter 330 nm and emission filter 465 nm) as follows: enzyme activity (%) = $[S_{40} - S_0/C_{40} - C_0] \times 100$. Where C_{40} and S_{40} are the fluorescence of control

and inhibitor after 40 min, and S_0 and C_0 are the fluorescence of inhibitor and control at 0 min. In this testing, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) was used as a positive control.

sEH kinetic assay

Kinetic assays were determined under steady-state condition as described. Buffer, incubation times, and enzyme concentrations were specific for each single nucleotide polymorphism to achieve detectable product formation. The enzyme inhibition properties of these components were modeled using double-reciprocal plots (Lineweaver–Burk and Dixon analyses). In brief, 50 µL of sEH and 20 µL of various concentrations of the compounds dissolved in MeOH were added in 96-well plate containing 80 µL of 25 mM bis-Tris–HCl buffer (pH 7.0) containing 0.1% BSA and then mixed with 50 µL of range of 5–80 µM PHOME as a substrate. After starting the enzyme reaction at 37 °C, products by hydrolysis of the substrate were monitored at excitation and emission of 330 and 465 nm during 30 min (Kim et al. 2014).

Molecular docking

The three-dimensional (3D) structure of the ligand was modeled and minimized by MM2 using Chem 3D Pro. (version: 17.0). The flexible bonds of ligands were assigned with Autodock Tools. The sEH structure (PDB ID: 3ANS) was downloaded from research collaboratory for structural bioinformatics (RCSB) (protein data bank), after ligands were removed from the original sEH using Chimera. All hydrogen atoms and gasteiger charges in the protein structures were added using Autodock Tools. Simulation studies were performed using the Autodock 4.2 version program according to the instructions on the Autodock Homepage (http://autodock.scripps.edu/). In brief, to perform docking in Autodock 4.2, the grid dimensions were established to contain all the enzyme (for the blind docking) with packing (0.375 Å). Docking simulation of protein structures and building new ligands were performed using the Lamarckian Genetic Algorithm and runs 30 number. Finally, the maximum number of evals was set as long and then the docking scores were calculated.

Results and discussion

Seven compounds were isolated from the MeOH extract of *A. squamosa* (Fig. 1). Their structures were identified as thalictoside (**1**) (Wang et al. 2017), arabinothalictoside (**2**) (Yoshikawa et al. 1996), benzyl-*O*-β-D-glucopyranoside (**3**) (Zeng et al. 2011), icariside F_2 (**4**) (Bai et al. 2015), austroside A (**5**) (Wang et al. 2005), staphylionoside D (**6**) (Hisamoto et al. 2004), and icariside B_1 (**7**) (Hisamoto et al. 2004). Their structures were elucidated by one- and two-dimensional NMR and MS. In this study, to the best of our knowledge, for the first time **2–6** were isolated from the Annonaceae family, and **1** and **7** were isolated from the species of *A. squamosa* L.

We investigated the sEH inhibitory effect of the isolated compounds (**1–7**) from *A. squamosa* L. The sEH inhibitory activities were determined using recombinant human sEH incubated with PHOME, an artificial substrate for fluorescence detection (Table 1). All the isolated compounds were tested against 100 µM sEH. Compounds **1** and **2** exhibited sEH inhibitory effect with 78.6% and 55.8%, respectively, whereas other compounds revealed no inhibitory activity. From the previous reports, these two nitro derivatives were rarely studied. However, thalictoside (**1**) was considered as an active component for anti-osteoporosis (Xu et al. 2016). To our surprise, the monosaccharide and disaccharide of nitro derivatives displayed with dramatic difference of IC_{50} values according to the various glycon numbers for onefold for **1** with 20.2 µM and **2** with 41.9 µM. Such results indicated that not merely the nitro moiety has strong inhibition effects for sEH but also the numbers of glycon could influence the inhibitory activity, which indicated that the more glycons the less inhibitory activity. However, **3** and **4** were also phenolic compounds connected with a

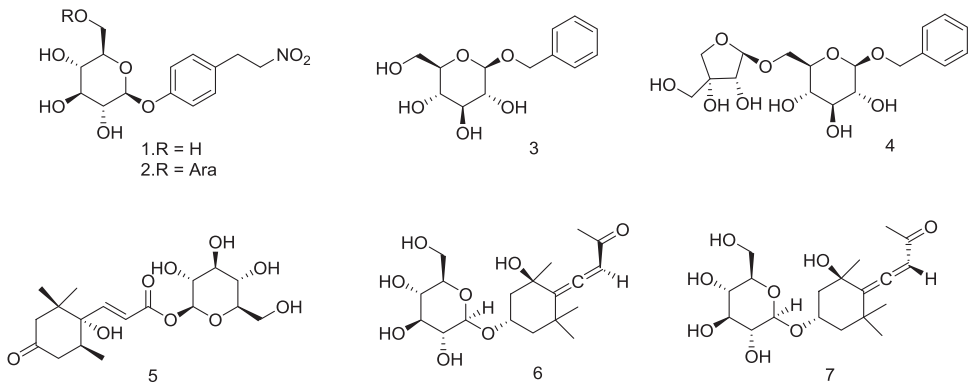

Fig. 1 Structures of compounds **1–7** from *A. squamosa*

monosaccharide and a disaccharide with similar sEH inhibitory activities for 44.4% and 44.9%, respectively. The results above suggested that the nitro moieties were crucial for the sEH inhibitory activities, despite the different glycon numbers substituted with the phenolic compound. Nonetheless, the sesquiterpene-type compounds were different in structures but the sEH inhibitory activity results were similar, with the results of 44.0%, 45.5%, and 40.4% for **5**, **6**, and **7**, respectively. The results suggested such sesquiterpene compounds were trivial for the sEH inhibitory activity.

Table 1 Inhibitory effects of isolated compounds **1–7**

Inhibition of compounds on sEH			
Compounds	100 μM(%)	IC_{50} (μM)	Type (K_i, μM)
1	78.6 ± 0.8	20.2 ± 0.2	Competitive (7.3 ± 0.4)
2	55.8 ± 0.6	41.9 ± 2.1	Competitive (14.1 ± 0.9)
3	44.4 ± 6.0	NT	NT
4	44.9 ± 0.1	NT	NT
5	44.0 ± 0.4	NT	NT
6	45.5 ± 1.5	NT	NT
7	40.4 ± 3.3	NT	NT
AUDA[a]		16.7 ± 0.6	

sEH activity was expressed as the percentage of control activity

Values represent means ± SD ($n = 3$)

NT not tested

[a]Positive control

Since AUDA completed clinical trial for heart failure in 2012, tremendous difficulties have been faced in obtaining Food and Drug Administration approval for sEH inhibitors evaluated in completed clinical trials (Tran et al. 2012). The recent studies for sEH inhibitors have more attention on the nitro derivatives (Wagner et al. 2017; Zarriello et al. 2019). The candidate inhibitory compounds (**1** and **2**) were analyzed in an enzyme kinetic study to access the binding mode between the receptor and ligands. The enzyme inhibition properties of these nitro derivatives were modeled via double-reciprocal plots (Lineweaver–Burk and Dixon analyses). The Lineweaver–Burk plot analysis suggested that increasing the inhibitor concentration increased the

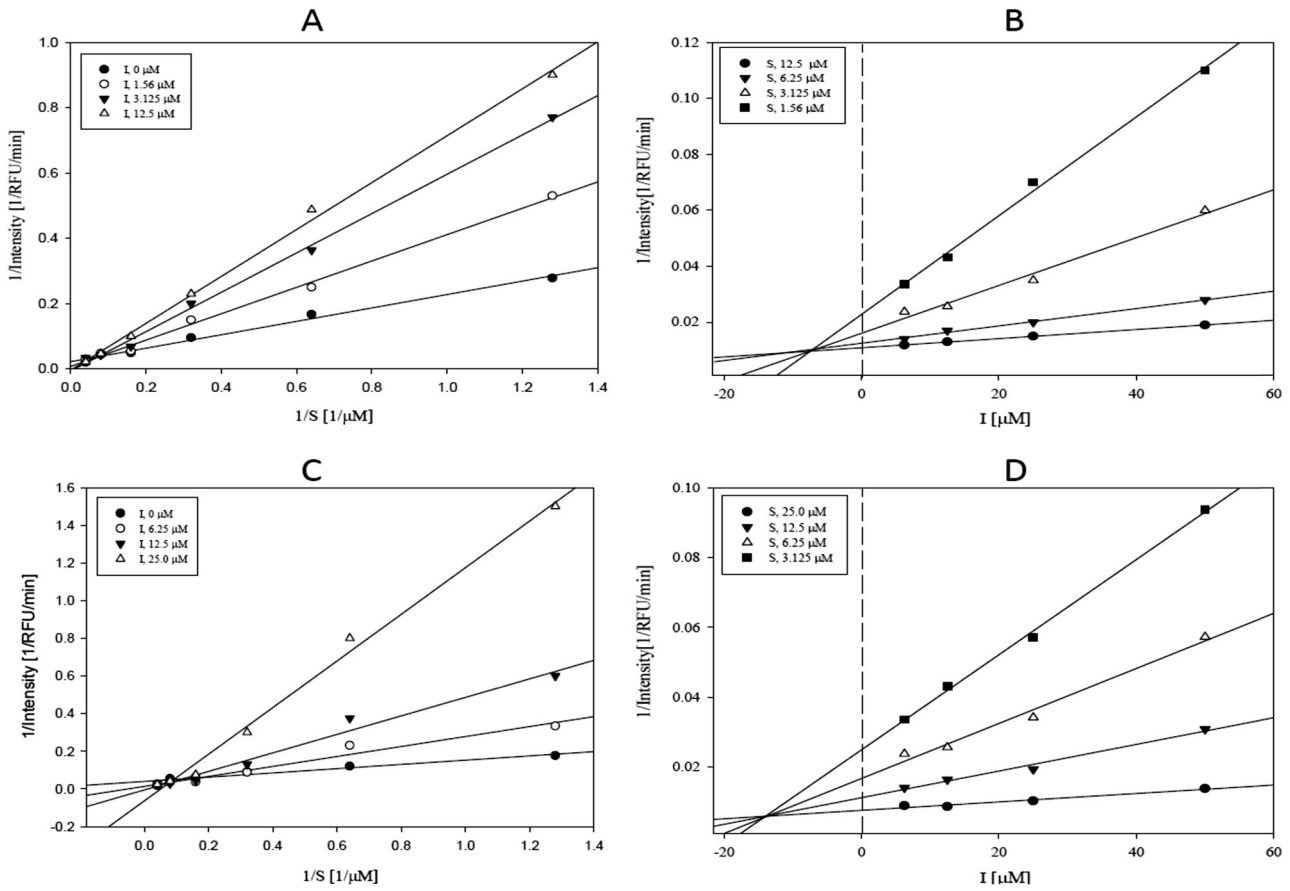

Fig. 2 Study of binding mechanism between compounds **1** and **2**, and sEH: **a**, **c** Lineweaver−Burk plots for the inhibition of compounds **1** and **2**, respectively; **b**, **d** Dixon plots for compound **1** and **2**, respectively. Data are the mean of three experiments carried out in triplicate and were determined by one-way analysis of variance followed by Dunnett's multiple comparison test, $P < 0.05$ vs. control

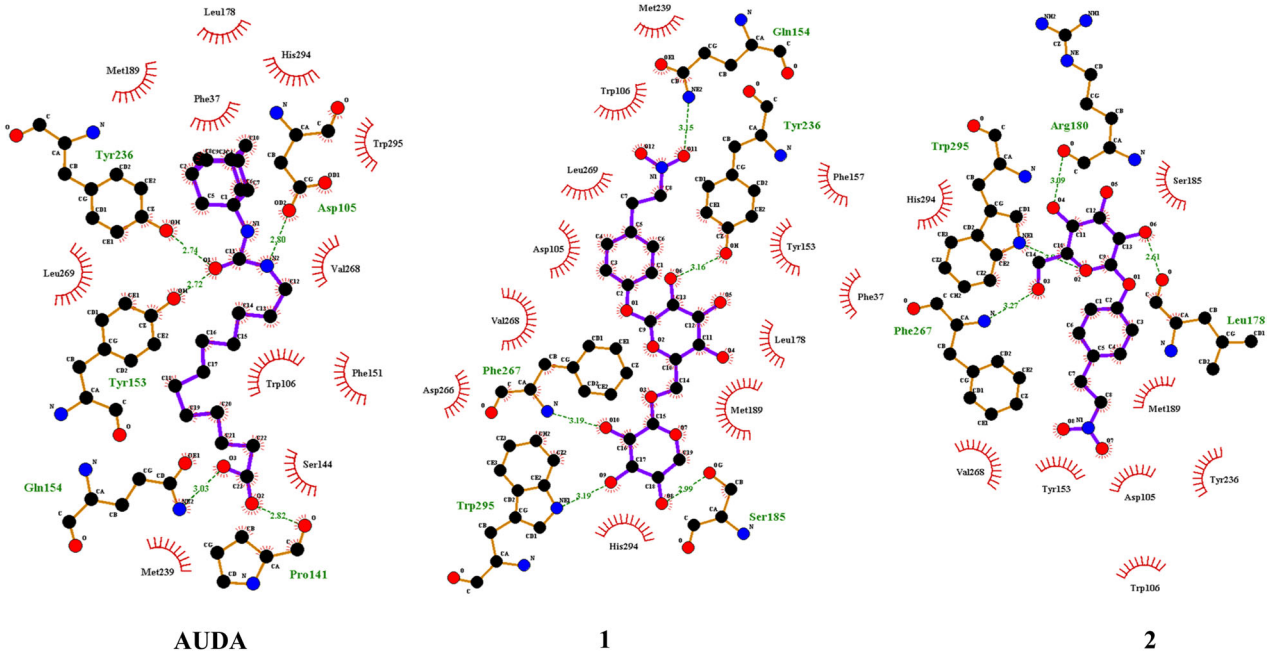

Fig. 3 Docking pose of compounds **1** and **2** into the predicted binding site of sEH

Table 2 Hydrogen bonding between sEH compounds **1**, **2**, and AUDA

Compounds	Receptor[a] (Å)	Autodock score (kcal/mol)
AUDA[b]	Asp105(2.80), Pro141(2.82), Tyr153(2.72), Gln154(3.03), Tyr236(2.74),	−8.8
1	Gln154(3.15), Ser185(2.99), Phe267(3.19), Trp295(3.19), Tyr236(3.16)	−8.4
2	Leu178(2.61), Arg180(3.09), Phe267(3.27), Trp295(3.04)	−8.2

[a]Amino acid sequence number of receptors

[b]Positive control

K_m values without changing the V_m values (Cleland 1963). Both the intercepts on the positive axis indicated that compounds **1** and **2** were competitive inhibitors. The K_i values for compounds **1** and **2** were calculated by Dixon plots, given the K_i values for 7.3 and 14.1 μM, respectively (Fig. 2).

A blind docking system was used to determine the enzyme sequences using the program Autodock 4.2. The results obtained from 30 docking runs suggested a reliable complex with a low Autodock score. Compounds **1** and **2** bounded to their predicted binding sites, which were considered allosteric sites (Fig. 3). The Autodock scores for AUDA, **1**, and **2** were −8.8, −8.4, and −8.2 kcal/mol (Table 2), respectively. Compounds **1** and **2** bounded the same target sites and participated in hydrogen bond with Phe267 and Trp295, whereas compound **1** and AUDA participated in hydrogen bond with Gln154 and Tyr236. Such bounding sites indicated that these amino acid sequences might be crucial receptors for nitro derivatives to inhibited sEH enzyme activities. In other sites, there were Asp105, Pro141, Tyr153, Ser185, Leu178, and Arg180 between AUDA, **1**, and **2**.

Conclusions

In this study, seven compounds (**1–7**) were isolated from the MeOH extract of *A. squamosa*. The sEH inhibitory effect suggested that the nitro derivative components have potential biological activities on cardiovascular and urinary system, with **1** and **2** exhibiting 78.6% and 55.8% sEH inhibitory effect, respectively. However, the phytochemistry research was limited with n-BuOH fraction. For further study with *A. squamosa*, the nitro derivative components could serve as target compounds to investigate both phytochemistry and sEH inhibitory mechanism on cardiovascular and urinary system.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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