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Soluble Epoxide Hydrolase Inhibitory Activity from *Euphorbia supina* Rafin

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Abstract – In our search for natural soluble epoxide hydrolase (sEH) inhibitors from plants, an extract of the dried whole plants of *Euphorbia supina* Rafin was found to significantly inhibit sEH activity *in vitro*. Phytochemical investigation of *E. supina* resulted in isolation of 17 compounds (**1 - 17**), including triterpenes (**1 - 4**), phenolic compounds (**5 - 8**), and flavonoid derivatives (**9 - 17**). The structures of the isolated compounds were established mainly by extensive analysis of the 1D and 2D NMR, and MS data. All of the isolated compounds were evaluated for their sEH inhibitory activity. Among the isolated phenolic compounds, **8** was identified as a significant inhibitor of sEH, with an IC_{50} value of 15.4 ± 1.3 μM. Additionally, a kinetic analysis of isolated compounds (**2**, **5**, **8 - 11**, **13**, and **17**) indicated that the inhibitory effects of flavonoid derivatives **10** and **11** were of mixed-type, with inhibitory constants (K_i) ranging from 3.6 ± 0.8 to 21.8 ± 1.0 μM, whereas compounds **2**, **5**, **8**, **9**, **13**, and **17** were non-competitive inhibitors with inhibition K_i values ranging from 3.3 ± 0.2 to 39.5 ± 0.0 μM.
Keywords – *Euphorbia supina*, Euphorbiaceae, Soluble epoxide hydrolase

Introduction

The genus *Euphorbia* is a member of the Euphorbiaceae family, which consists of about 2000 species ranging from annuals to trees, with worldwide distribution. The genus *Euphorbia* has been the subject of intense phytochemical examination because of its medicinal use in the treatment of numerous diseases including skin diseases, gonorrhea, migraine, intestinal parasites, and wart.[1]

Euphorbia supina Rafin is an annual summer broadleaf weed and spotted spurge belonging to the Euphorbiaceae family, which is native to North America. It is also quite common along roadsides and in fields throughout Korea. In traditional Korean medicine, *E. supina* is used to treat bronchitis, jaundice, hemorrhage, and gastrointestinal diseases including gastritis or gastritis, gastric ulcers, peptic ulcers, diarrhea, and hemorrhoids.[2] It has also been used in folk medicines for wounds in Mexico and China. Previous studies showed that *E. supina* contains as non-polar, triterpenoid derivatives,[3] hydrolyzable tannins, flavonoids,[4] megastigmane glucosides, and hydroxynitrile glucosides.[5] Some of these compounds exhibit various pharmacological actions, including peroxynitrite-scavenging,[2] antioxidant[6] activities. Additionally, *E. supina* fractions and extracts were showed to have antibacterial activity against both gram-positive and gram-negative bacteria.[7]

Epoxide hydrolases are a family of enzymes that catalyze the hydrolysis of epoxides or arene oxides to their corresponding diols by the addition of water. The epoxide hydrolase family consists of five main subtypes: sEH, microsomal epoxide hydrolase (mEH), leukotriene A4 hydrolase, hepoxilin A3 hydrolase, and cholesterol 5,6-oxide hydrolase. sEH is a xenobiotic metabolizing enzyme that has crucial roles in the metabolism of epoxyeicosatrienoic acids (EETs) and leukotoxins (LTX) to their less active metabolites, dihydroxyeicosatrienoic acids (DHETs), and leukotoxin diol (LTX diol), respectively.[8] EETs are P450-derived metabolites of arachidonic acid with a wide range of biologic activities, including modulation of potassium channels, antiapoptotic effects in

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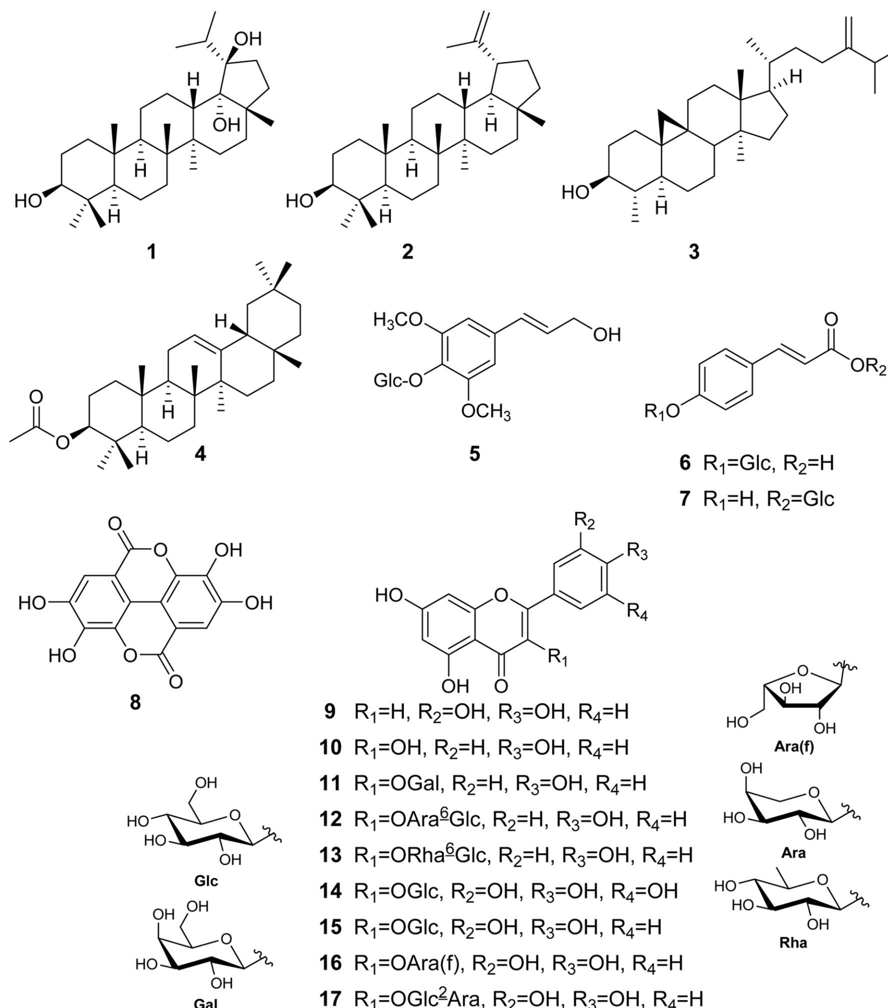

Fig. 1. Chemical structure of isolated compounds (**1 - 17**) from *E. supina.*

endothelial cells, anti-inflammatory properties, cardiovascular effect, and inhibitory effects on the nuclear factor kappa-B (NF-κB) signaling pathway. Various reports have suggested that EETs may function as an endothelium-derived hyperpolarizing factor that plays an integral role in smooth musclehyperpolarization.[9] EETs and sEH inhibitors have been extensively characterized in a variety of cellular assays and disease models.

In our search for natural sEH inhibitors from plants, the extract of *E. supina* was previously been tested *in vitro* for its potential sEH inhibitory properties. We found that a methanolic extract of this plant showed significant *in vitro* sEH inhibitory activity. The present study details the sEH inhibitory activities of compounds (**1 - 17**, see Fig. 1) isolated from *E. supine* was determined using the fluorescent substrate, 3-phenyl-cyano(6-methoxy-2-naphthalenyl) methyl ester-2-oxiraneacetic acid (PHOME).

Experimental

Plant materials – Dried whole plants of *Euphorbia supina* Rafin were purchased from Wonkwang Herb Co., Ltd. (Jinan, Korea) and taxonomically identified by Prof. Young Ho Kim, College of Pharmacy, Chungnam National University. A voucher specimen (CNU 13111) was deposited at Herbarium of the College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea.

General experimental procedures – The UV spectra were acquired using a JASCO V-550 UV/VIS spectrometer. FT-IR spectra were recorded on a JASCO Report 100 infrared spectrophotometer. The NMR spectra were recorded on a JEOL ECA 600 MHz and JEOL JNM-AL 400 MHz spectrometer and TMS was used as an internal standard, chemical shift (δ) are expressed in ppm with reference to the TMS signals. The electronspray ionization

(ESI) mass spectra were performed on an AGILENT 1100 LC-MSD trap spectrometer (Agilent Technologies, Palo Alto, CA, USA). Silica gel (70 - 230, 230 - 400 mesh, Merck, Whitehouse Station, NJ), YMC RP-18 resins (75 µm, Fuji Silysia Chemical Ltd., Kasugai, Japan) were used as absorbents in the column chromatography. Thin layer chromatography (TLC) plates (silica gel 60 F_{254} and RP-18 F_{254}, 0.25 µm, Merck) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation (254 and 365 nm) and by spraying the plates with 10% H_2SO_4 followed by heating with a heat gun. 3-Phenyl-cyano(6-methoxy-2-naphthalenyl)methyl ester-2-oxiraneacetic acid (PHOME), 12-[[(tricyclo[3.3.1.13,7] dec-1-ylamino)carbonyl]amino]-dodecanoic acid, purified recombinant sEH, 6-methoxy-2-naphtaldehyde (internal standard for fluorometric assays) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemical reagents and standard compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction and isolation – The dried whole plants of *E. supina* (2.5 kg) were extracted with methanol (7L × 3 times) under reflux condition. Evaporation of the solvent under reduced pressure gave MeOH extract (250 g). The MeOH extract was suspended in H_2O and successively separated with CH_2Cl_2 and EtOAc to yield CH_2Cl_2 fraction (128 g), EtOAc fraction (48 g), and water layer, respectively.

The CH_2Cl_2 fraction was fractionated on a silica gel column chromatography (CC) eluting with gradient solvent systems of *n*-hexane-acetone (0 - 100% acetone, step-wise) to obtain six fractions (B.1 to B.6). Compounds **1** (15 mg), **2** (10 mg), **3** (10 mg), and **4** (15 mg) were isolated from fraction B.3 by silica gel CC using *n*-hexane-acetone (4/1, v/v) as eluent, and further purified by silica gel CC eluting with EtOAc-acetone (4/1, v/v).

The EtOAc extract was fractionated on a silica gel CC eluting with gradient solvent systems of CH_2Cl_2-MeOH (0-100% MeOH, step-wise) to obtain five fractions (C.1 to C.5). Compound **10** (18 mg) was isolated from fraction C.2 by silica gel CC using CH_2Cl_2-MeOH (3/1, v/v) as eluent, and further purified by YMC reverse-phase (RP) CC eluting with MeOH-H_2O (1/2, v/v). Fraction C.3 was separated on YMC RP-18 CC eluting with MeOH-H_2O (1/2, v/v) and further purified by silica gel CC using EtOAc-MeOH (4/1, v/v) as eluent to give **9** (25 mg). Fraction C.4 was fractionated on a silica gel CC eluting with gradient solvent systems of CH_2Cl_2-MeOH (0-100% MeOH, step by step) to obtain four fractions (C.4.1 to C.4.4). Compounds **8** (40 mg) and **13** (10 mg) were obtained from fraction C.4.2 by a silica gel CC eluting with CH_2Cl_2-MeOH (4/1, v/v) and further purified by an YMC RP-18CC eluting with MeOH-H_2O (1/2, v/v). Similary, fraction C.4.3 were isolated on YMC RP-18CC eluting with MeOH-H_2O (1/2, v/v) to afford **12** (20 mg), **14** (10 mg), **15** (40 mg), and **16** (20 mg).

The water layer were fractionated on a Diaion HP-20 CC eluting with gradient solvent systems of MeOH-H_2O (0-100% MeOH, step-wise) to obtain four fractions (D.1 through D.4). Fraction D.2 were separated by CC over silica gel, eluting with gradient solvent systems of CH_2Cl_2-MeOH (0-100%, step by step) to obtain seven fractions (D.2.1-D.2.7). Next, fraction D.2.2 were further chromatographed on a Sephadex CC and eluted with MeOH-H_2O (1/2, v/v) and further purified by silica gel CC eluting with EtOAc-MeOH (2/1, v/v) to provide **5** (12 mg) and **11** (100 mg). Compound **6** (17 mg) was isolated from fraction D.2.5 using YMC RP-18 CC eluting with MeOH-H_2O (1/1, v/v). Finally, fraction D.2.6 was subjected to a silica gel CC eluting with EtOAc-MeOH (5/1, v/v) to obtain compounds **7** (10 mg) and **17** (50 mg).

Cycloeucalenol (2): White amorphous powder; ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 4.72 (1H, br s, H-28a), 4.67 (1H, br s, H-28b), 3.23 (1H, m, H-3), 1.05 (3H, d, J = 7.0 Hz, H-27), 1.03 (3H, d, J = 7.0 Hz, H-26), 0.99 (3H, br s, H-29), 0.98 (3H, br s, H-18), 0.90 (6H, br s, H-21 and H-30), 0.40 (1H, d, J = 4.0 Hz, H-19b), and 0.15 (1H, d, J = 4.0 Hz, H-19a); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 157.0 (C-24), 106.0 (C-28), 76.6 (C-3), 52.17 (C-17), 48.9 (C-14), 46.8 (C-8), 45.3 (C-13), 44.5 (C-4), 43.3 (C-5), 36.1 (C-20), 35.3 (C-15), 35.0 (C-22), 34.7 (C-2), 33.8 (C-25), 32.8 (C-12), 31.3 (C-23), 30.7 (C-1), 29.6 (C-10), 28.0 (C-16), 27.2 (C-19), 26.9 (C-11), 25.1 (C-7), 24.6 (C-6), 23.5 (C-9),21.9 (C-26),21.8 (C-27), 19.1 (C-30), 18.3 (C-21), 17.7 (C-18), and 14.3 (C-29).

Syringin (5): White powder; ^{1}H-NMR (400 MHz, CD_3OD) δ_H: 6.74 (2H, s, H-2 and H-6), 6.53 (1H, d, J = 15.8 Hz, H-7), 6.31 (1H, dt, J = 15.8, 5.5 Hz, H-8), 4.85 (1H, d, J = 7.8 Hz, H-1'), 4.20 (2H, dd, J = 5.5, 1.4Hz, H-9), 3.81 (6H, s, 3,5-OCH_3), 3.75 (1H, dd, J = 2.0, 12.0 Hz, H-6'b), 3.63 (1H, dd, J = 4.0, 12.0 Hz, H-6'a), 3.44 (1H, m, H-2'), 3.38 (2H, m, H-4' and H-5'), and 3.17 (1H, m, H-3'); ^{13}C-NMR (100 MHz, CD_3OD) δ_C:154.6 (C-3, 5), 136.1 (C-1), 135.5 (C-4), 131.5 (C-7), 130.3 (C-8), 105.6 (C-2 and C-6), 105.5 (C-1'), 78.6 (C-3'), 75.9 (C-2'), 78.0 (C-5'), 71.5 (C-4'), 63.7 (C-9), 62.7 (C-6'), and 57.2 (3,5-OCH_3).

Ellagic acid (8): Pale yellow amorphous powder; ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H: 7.44 (2H, s, H-5, 5'); ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C: 159.7 (C-7and C-7'), 148.6 (C-4, 4'), 140.1 (C-3 and C-3'), 136.9 (C-2 and C-

2'), 112.8 (C-1 and C-1'), 110.8 (C-5 and C-5'), and 108.2 (C-6 and C-6').

Luteolin (9): Yellow powder; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H: 7.30 (3H, overlapped, H-2' and H-6'), 6.83 (1H, d, J = 8.0 Hz, H-5'), 6.47 (1H, s, H-3), 6.42 (1H, s, H-6), and 6.19 (1H, s, H-8); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C: 182.4 (C-4), 164.9 (C-7), 164.7 (C-2), 162.3 (C-5), 158.1 (C-9), 150.4 (C-4'), 146.5 (C-3'), 122.3 (C-6'), 119.7 (C-1'), 116.8 (C-5'), 114.1 (C-2'), 104.4 (C-10), 103.6 (C-3), 99.5 (C-6), and 94.5 (C-8).

Kaempferol (10): Yellow powder; ^{1}H-NMR (400 MHz, CD$_3$OD) δ_H: 8.09 (2H, d, J = 8.0, H-2' and H-6'), 6.92 (2H, d, J = 8.0 Hz, H-3' and H-5'), 6.40 (1H, d, J = 2.4 Hz, H-8), and 6.20 (1H, d, J = 2.4 Hz, H-6); ^{13}C-NMR (100 MHz, CD$_3$OD) δ_C: 177.5 (C-4), 165.7 (C-7), 162.6 (C-5), 160.6 (C-4'), 158.4 (C-9), 148.1 (C-2), 137.2 (C-3), 130.8 (C-2' and C-6'), 123.8 (C-1'), 116.4 (C-3' and C-5'), 104.6 (C-10), 99.3 (C-6), and 94.5 (C-8).

Trifolin (11): Yellow powder; ^{1}H-NMR (600 MHz, DMSO-d_6) δ_H: 8.04 (2H, dd, J = 1.8, 8.0 Hz, H-2' and H-6'), 6.88 (2H, dd, J = 1.8, 8.0 Hz, H-3' and H-5'), 6.43 (1H, d, J = 1.8 Hz, H-8), 6.20 (1H, d, J = 1.8 Hz, H-6), 5.46 (1H, d, J = 7.2 Hz, H-1''); ^{13}C-NMR (150 MHz, DMSO-d_6) δ_C: 177.0 (C-4), 163.6 (C-7), 160.7 (C-5), 159.5 (C-4'), 155.9 (C-2), 155.8 (C-9), 132.7 (C-3), 130.4 (C-2' and C-6'), 120.4 (C-1'), 114.6 (C-3' and C-5'), 103.5 (C-10), 100.3 (C-1''), 98.2 (C-6), 93.2 (C-8), 77.0 (C-5''), 75.9 (C-3''), 73.7 (C-2''), 69.4 (C-4''), and 60.3 (C-6'').

Nicotiflorin (13): Yellow needles; ^{1}H-NMR (600 MHz, CD$_3$OD) δ_H: 6.11 (1H, d, J = 2.4 Hz, H-6), 6.31 (1H, d, J = 2.4 Hz, H-8), 7.96 (2H, dd, J = 2.4, 8.4 Hz, H-2' and H-6'), 6.88 (2H, d, J = 8.4 Hz, H-3' and H-5'), *Glc*: 5.12 (1H, d, J = 7.2 Hz, H-1''), *Rha*: 4.51 (1H, d, J = 1.2 Hz, H-1'''), and 1.12 (3H, d, J = 6 Hz, H-6'''). ^{13}C-NMR (150 MHz, CD$_3$OD) δ_C:179.4 (C-4), 166.0 (C-7), 163.0 (C-5), 161.5 (C-4'), 159.4 (C-9), 158.6 (C-2), 135.5 (C-3), 132.4 (C-2' and C-6'), 122.7 (C-1'), 116.1 (C-3' and C-5'), 105.7 (C-10), 100.0 (C-6), 94.9 (C-8), *Glc*: 104.6 (C-1''), 75.8 (C-2''), 78.2 (C-3''), 71.4 (C-4''), 77.2 (C-5''), 68.6 (C-6''), *Rha*: 102.4 (C-1'''), 72.1 (C-2'''), 72.3 (C-3'''), 73.9 (C-4'''), 69.7 (C-5'''), and 17.9 (C-6''').

Quercetin 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*α*-L-arabinopyranoside (17): Yellow amorphous powders; ^{1}H-NMR (400 MHz, CD$_3$OD) δ_H: 7.66 (1H, d, J = 2.0 Hz, H-2'), 7.62 (1H, dd, J = 2.0, 8.0 Hz, H-6'), 6.87(1H, d, J = 8.0 Hz, H-5'), 6.33 (d, J = 2.0 Hz, H-8), 6.15 (1H, d, J = 2.0 Hz, H-6), 5.50 (1H, d, J = 8.0 Hz, Ara-1''), and 4.80 (2H, overlapped, Glc-1''', Ara-2''); ^{13}C-NMR (100 MHz, CD$_3$OD) δ_C: 179.5 (C-4), 165.6 (C-7), 163.0(C-5), 158.4 (C-9), 158.3 (C-2), 149.6 (C-4'), 145.9 (C-3'), 135.2 (C-3), 123.4 (C-6'), 123.3 (C-1'), 117.4 (C-5'), 116.1 (C-2'), 105.8 (Ara-1''), 105.1 (C-10), 100.9 (C-6), 99.7 (Glc-1'''), 94.6 (C-8), 82.0 (Ara-2''), 78.1 (Glc-3'''), 78.0 (Glc-5'''), 76.9 (Glc-2'''), 74.7 (Ara-3''), 71.0 (Ara-4''), 70.9 (Glc-4'''), 66.5 (Ara-5''), and 62.4 (Glc-6''').

sEH inhibitory activity – sEH inhibitory activity was determined using a hydrolysis reaction of PHOME in the presence of the sEH enzyme. The final reaction volume was 200 µL, and contained 25.0 mM Bis-Tris buffer (including 0.1% bovine serum albumin, pH 7.0), 1.0 µM PHOME, 3 nM sEH enzyme, and various concentrations of samples or AUDA (150 nM) as a positive control. Reaction systems were incubated at 30 °C for 1 h, and fluorescence intensity was then monitored every 3 min (during 1 h) using a Genios microplate reader (Tecan, Mannedorf, Switzerland) at excitation and emission wavelengths of 320 and 465 nm, respectively. sEH inhibitory activity for each sample was calculated as follows:

$$\text{sEH inhibitory activity (\%)} = \times 100) - \left(\frac{\int SA}{\int CA} - 100 \right)$$

Where, $\int SA$ and $\int CA$ are the integrated areas under the curve from the sample and control reactions, respectively. A sEH inhibitory activity value of 0 ($\int SA$ / $\int CA$ = 1) corresponds to a sample lacking both inhibition of sEH enzyme and PHOME hydrolysis. Nevertheless, a maximum theoretical sEH inhibitory activity value of 100 would indicate complete inhibition of PHOME hydrolysis throughout the assay ($\int SA = 0$).

sEH kinetic assay – Kinetic assay were determined under steady-state condition as described. The enzyme inhibition properties of these derivatives were modeled using double-reciprocal plots (Lineweaver-Burk and Dixon analyses). Briefly, 50.0 µL of sEH and 20.0 µL of various concentrations of the compounds dissolved in MeOH were added in 96-well plate containing 80.0 µL of 25.0 mM Bis-Tris-HCl buffer (pH 7.0) containing 0.1% BSA and then mixed with 50.0 µL of range of 5.0 to 160 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 minutes.

Statistical analysis – All experiment was performed repeatedly at least three times. Values are expressed as mean ± SD. Statistical analyses were performed by one-way ANOVA analysis using Graph Pad software(San Diego, CA, USA), $P < 0.05$ and $P < 0.005$ vs. control.

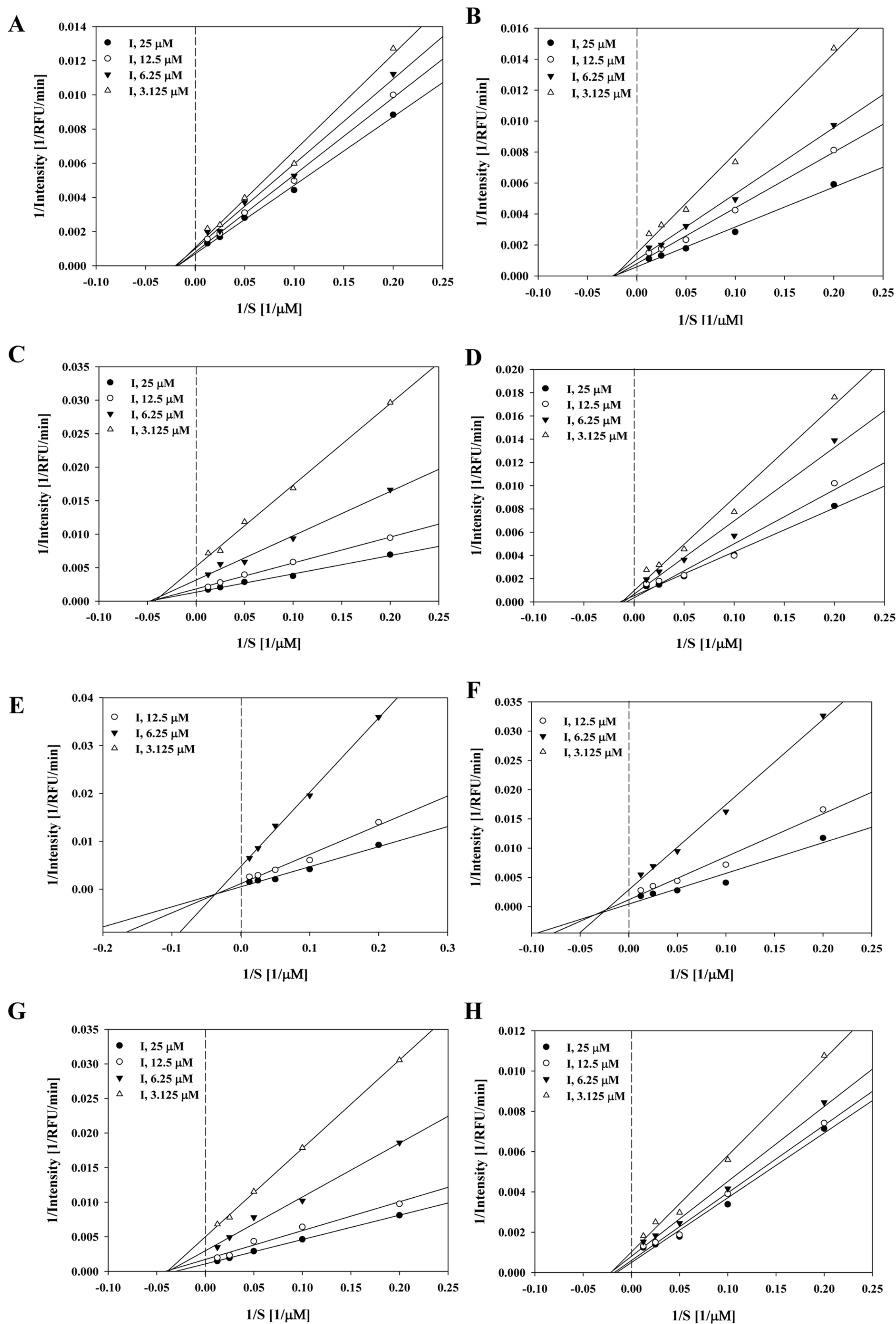

Fig. 2. Lineweaver-Burkplots (**A-H**) for the inhibition of compounds **2**(**A**), **5** (**B**), **8** (**C**), **9** (**D**), **10** (**E**), **11** (**F**), **13** (**G**), and **17** (**H**).

Results and Discussion

Phytochemical analysis – The MeOH extract of *E. supina* Rafin was suspended in H_2O and successively extracted with CH_2Cl_2, EtOAc fractions, and water layer. The CH_2Cl_2-, EtOAc-soluble fractions, and water layer were subjected to multiple chromatographic steps on silica gel, Sephadex™ LH-20, and RP-18 CC yielding compounds **1 - 17** (see Materials and methods section). The structures of isolated compounds were identied based on direct comparison of their NMR and MS data with those reported in previous studies, as followes 3*β*,18,19*β*-trihydroxylupane (**1**),[10] 5*α*-lup-20(29)-en-3*β*-ol (**2**),[11] 4*α*,14*α*-dimethyl-9,19-cyclo-5*α*,9*β*-ergost-24(24')-en-3*β*-ol (**3**),[12] *β*-amyrin acetate (**4**)[13] syringin (**5**),[14] *trans*-*p*-coumaric acid 4-*O*-*β*-D-glucopyranoside (**6**),[15] (*E*)-*p*-coumaroyl glucose ester (**7**),[16] ellagic acid (**8**),[17] luteolin (**9**),[18] kaempferol (**10**),[19] trifolin (**11**),[20] kemferol 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*α*-L-arabinopyranoside (**12**), nicotiflorin (**13**), myricetin 3-*O*-*β*-D-glucopyranoside (**14**), quercetin 3-*O*-*β*-D-glucopyranoside(**15**), quercetin 3-*O*-L-arabinofuranoside (**16**),and quercetin 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*α*-L-arabinopyranoside (**17**).[21]

Biological activity – The extracts of the rhizomes of *E. supina* and its constituents were shown to exhibit several pharmacological activities. However, sEH inhibitory activities of extracts and/or isolated compounds of *E.* supina have not yet been reported. Therefore, the inhibitory activities of constituents isolated from *E. supina* against sEH were evaluated *in vitro* using a fluorescent method based on specific PHOME hydrolysis in the presence of sEH. Specifically, the amount of 6-methoxy-2-naphthaldehyde produced from the substrate (PHOME) was quantified in the presence or absence of compounds **1 - 17** using a fluorescence photometer at wavelengths of 330 and 465 nm.[22] The well-known sEH inhibitor, 12-(3-adamantan-1-yl-ureido) dodecanoic acid,[23] which is a small urea-type molecule and likely acts forming a salt bridge between the active site of sEH and urea, was used as a positive control (IC_{50} = 7.99 ± 0.5 nM). The inhibitory activities of all of the isolated compounds were evaluated against 100 μM sEH.

Methanol extract, dichloromethane, ethyl acetate fractions, and the water layer exhibited sEH inhibitory rates of 49.5 ± 1.2, 81.1 ± 2.4, 29.4 ± 1.7, and 27.3 ± 0.5 μg/mL, respectively (data not shown). As listed in Table 1, isolated compounds (**1 - 17**) exhibited inhibitory ratios values ranging from 19.4 ± 1.4% to 99.5 ± 0.2% of the

Table 1. Effect of isolated compounds (**1 - 17**) from *E. supina* on sEH inhibition

Compounds	Inhibitory activity on sEH[a]			Binding mode
	100 μM (%)	IC_{50} (μM)	K_i, (μM)	
1	24.5 ± 0.8	NT[b]	NT	NT
2	85.2 ± 4.0	27.4 ± 1.8	39.5 ± 0.0	Non-competitive
3	19.4 ± 1.4	NT	NT	NT
4	27.3 ± 0.5	NT	NT	NT
5	99.5 ± 0.2	29.0 ± 1.6	15.1 ± 0.2	Non-competitive
6	69.8 ± 1.9	62.4 ± 3.7	NT	NT
7	63.5 ± 3.2	NT	NT	NT
8	94.9 ± 1.1	15.4 ± 1.3	3.3 ± 0.2	Non-competitive
9	87.1 ± 1.0	20.4 ± 1.0	15.1 ± 1.4	Non-competitive
10	87.8 ± 0.4	28.8 ± 0.5	21.8 ± 1.0	Mixed-competitive
11	93.5 ± 2.3	22.9 ± 1.1	3.6 ± 0.8	Mixed-competitive
12	61.0 ± 0.4	81.2 ± 0.4	NT	NT
13	91.7 ± 2.6	22.5 ± 0.1	4.0 ± 0.1	Non-competitive
14	75.7 ± 3.5	47.0 ± 1.5	NT	NT
15	81.7 ± 0.3	37.6 ± 2.9	NT	NT
16	71.8 ± 2.6	35.9 ± 5.5	NT	NT
17	96.5 ± 0.8	29.0 ± 1.0	31.4 ± 1.4	Non-competitive
AUDA[c]	56.2 ± 4.1	7.99 ± 0.5 (nM)	–	–

[a]The sEH activity was expressed as the percentage of control activity. Values represent means ± SD from triplicate experiments.
[b] NT: Not tested.
[c] AUDA was used as a positive control (12.5 nM).

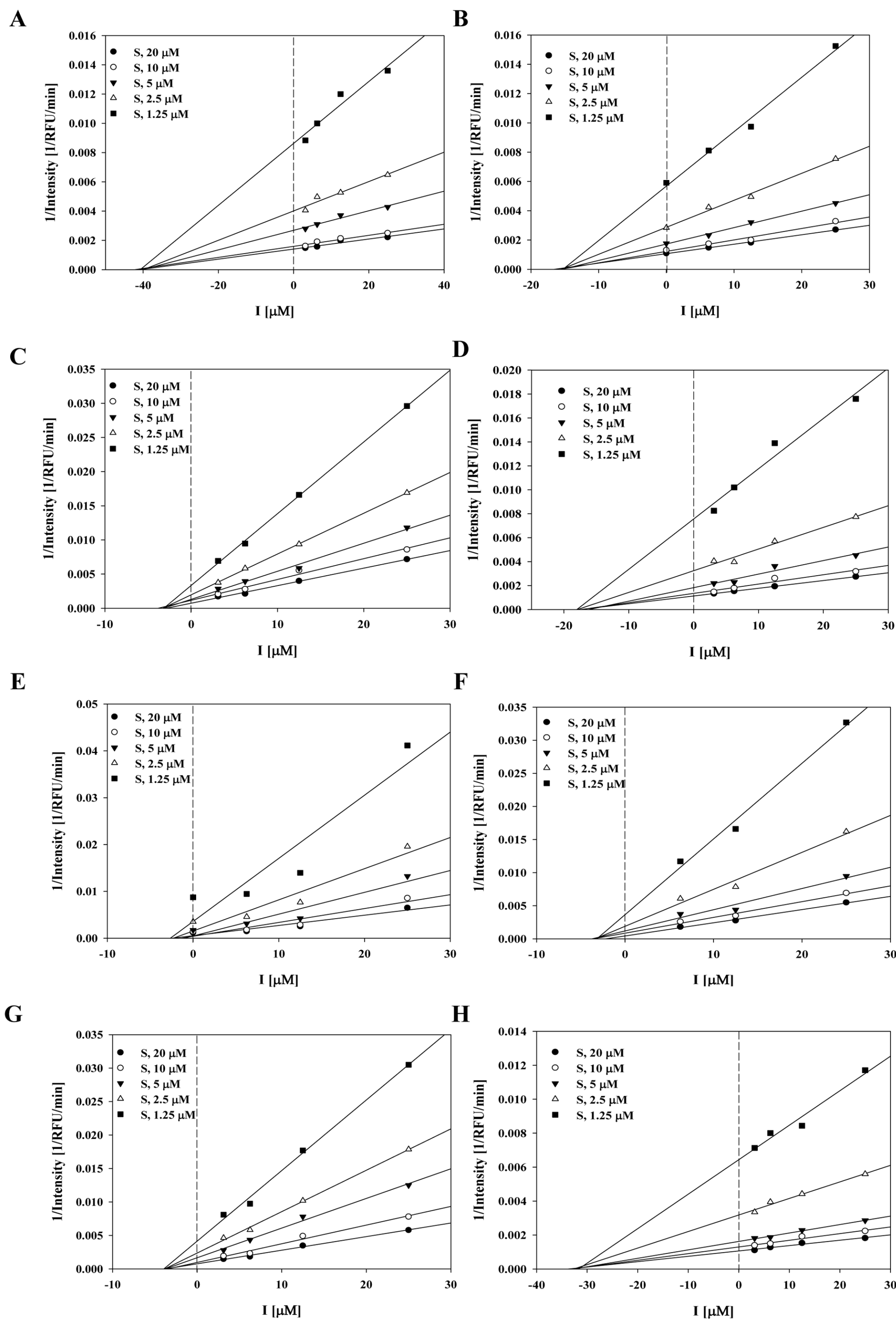

Fig. 3. Dixon plots (**AH**) for the inhibition of compounds **2** (**A**), **5** (**B**), **8** (**C**), **9** (**D**), **10** (**E**), **11** (**F**), **13** (**G**), and **17** (**H**).

control value at 100 μM. Of the compounds tested, **2**, **5**, **8 - 11**, and **13 - 17** showed > 70% inhibition in a dose-dependent manner, with IC_{50} values ranging from 15.4 ± 1.3 to 47.0 ± 1.5 μM. Ellagic acid (**8**) was the most potent

inhibitor, with an IC_{50} value of 15.4 ± 1.3 μM (see Table 1).

To investigate the binding mechanisms of these eight inhibitors (**2**, **5**, **8 - 11**, **13**, and **17**) to the enzyme, kinetic analyses were carried out in the presence the inhibitor (3.3 to 39.5 μM) at various substrate concentrations (3.12 to 25.0 μM). The results were used to construct Lineweaver-Burk plots for each inhibitor, which yielded a family of straight lines with different slopes and a common intercept above the abscissa; in this case independent binding of the substrate and inhibitor with the enzyme is designated as mixed inhibition. Furthermore, the secondary plot of the slopes and ordinate intercepts of the respective Lineweaver-Burk data showed straight lines with the abscissa intercepts K_ic (binding constant of the inhibitor with free enzyme)and K_iu (binding constant of an inhibitor with enzyme-substrate complex), respectively.

The candidate inhibitory compounds were analyzed in an enzyme kinetic study to assess that the mode of binding between the receptor and ligands. Lineweaver-Burk plot analysis showed that increasing the concentration of inhibitors decreased the $\surd_{max}$ without changing K_m. As shown in Fig. 3, the x-intercept ($-1 / K_m$) was unaffected by the concentration of inhibitor, whereas 1 / $\surd_{max}$ increased.[24] This behavior suggested that **2**, **5**, **8**, **9**, **13**, and **17** were a non-competitive inhibitors. The data plotted a series of lines that intersected to the left of the vertical axis and above the horizontal axis (see Fig. 2). This suggests that increasing inhibitor concentration led to a decrease in $\surd_{max}$ and an increase in K_m,[25] indicating that compounds **10** and **11** were mixed-type inhibitors (interaction with the free enzyme or thee nzyme-substrate complex at allosteric sites). The K_ic values of **2**, **5**, **8 - 11**, **13**, and **17**were also measured using Dixon plots (see Table 1 and Fig. 3).

These findings indicated that flavonoid derivatives isolated from *E. supina* significantly exhibit sEH activity *in vitro*, with IC_{50} values ranging from 20.4 ± 1.0 to 81.2 ± 0.4 μM. Kinetic analysis confirmed that kaempferol (**10**) and trifolin (**11**) were mixed inhibitors with K_i ranging from 3.6 ± 0.8 to 21.8 ± 1.0 μM, whereas compounds **2**, **5**, **8**, **9**, **13**, and **17** were non-competitive inhibitors with inhibition constants (K_i) values ranging from 3.3 ± 0.2 to 39.5 ± 0.0 μM (see Table 1 and Fig. 3).The results were not sufficient for discussions regarding the structure-activity relationships of flavonoid derivatives and/or other components. However, the selective inhibition of sEH indicated that the active compounds have an on-general mechanism of sEH action. This study represents the first report of isolation of sEH inhibitors from *E. supina*.

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