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Phytochemical Constituents from the Aerial Part of *Ducrosia ismaelis* Asch.

Abubaker M. A. Morgan[1,2], Jang Hoon Kim[1], Hyun Woo Lee[1], Sang-Hyun Lee[3], Chi-Hwan Lim[2], Hae-Dong Jang[3,*], and Young Ho Kim[1,*]

[1]*College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea*
[2]*College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea*
[3]*Department of Food and Nutrition, Hannam University, Daejeon 305-811, Korea*

Abstract – Phytochemical investigation of the aerial components of *Ducrosia ismaelis* Asch. led to the isolation of six known compounds, psoralen (**1**), isopsoralen (**2**), cnidioside A (**3**), (–)-syringaresinol-*O*-β-D-glucopyranoside (**4**), (*E*)-plicatin B (**5**), trilinolein (**6**). The chemical structures of these compounds were elucidated from spectroscopic data and by comparison of these data with previously published results. The antioxidant, anti-osteoporotic and cardiovascular related activities of the isolated compounds were assessed using oxygen radical absorbance capacity (ORAC), reducing capacity, tartrate-resistant acid phosphatase (TRAP), and soluble epoxide hydrolase (sEH) inhibitory activity assays. Compounds (**3-5**) showed potent peroxyl radical-scavenging capacities with ORAC values of 11.06 ± 0.39, 7.98 ± 0.10, and 13.99 ± 0.06 Trolox equivalent (TE) at concentrations of 10 μM, respectively. Only compounds **4** and **5** was able to significantly reduce Cu^{2+} ions, with a reduction value of 9.06 ± 0.32 and 4.61 ± 0.00 μM Trolox Equivalent (TE) at a concentration of 10 μM. Compound **5** at 10 μM exhibited a potent inhibitory effect on osteoclastic TRAP activity with a TRAP value of 86.05 ± 6.55% of the control. Compounds **1**, **3** and **5** potently inhibited sEH activity with IC_{50} values of 41.6 4.9, 16.0 1.1, and 49.0 5.7 μM, respectively.
Keywords- Ducrosia ismaelis, Apiaceae, ORAC, Reducing capacity, TRAP, Soluble epoxide hydrolase.

Introduction

Oxidative stress arises when the production of reactive oxygen species (ROS) overwhelms the intrinsic anti-oxidant defences. Reactive oxygen species play important roles as second messengers in many intracellular signalling cascades aimed at maintaining the cell in homeostasis with its immediate environment.[1] Severe oxidative stress has been implicated in many chronic and degenerative diseases, including osteoporosis, cancer, ageing, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.[2] Oxidative stress is enhanced in hypertension, atherosclerosis, and other forms of cardiovascular disease and participates in the mechanisms of vascular injury.[3] There are several methods that have been developed to estimate the antioxidant capacity of phytochemicals in foods. The most popular and most well characterised method is the peroxyl radical scavenging assay.[4,5] In addition, reducing capacity is also a popular assay, frequently used for measuring the electron-donating capacity of food extracts or antioxidants.[4,6]

Osteoporosis, one of the most frequently encountered degenerative diseases in ageing communities, is characteristic by a decrease in bone mass and density, causing bones to become fragile and more prone to fracture.[2] Bone formation is related to osteoblastic proliferation, alkaline phosphatase (ALP) activity, and osteocalcin and collage synthesis. Bone resorption is associated with osteoclast formation and differentiation, and tartrate-resistant acid phosphatase activity (TRAP).[7]

In mammals, epoxides of arachidonic acids (called epoxyeicosatrienoic acids or EETs) and of other fatty acids are important lipid mediators that have key roles in the regulation of hypertension, inflammation, and other cardiovascular-related diseases as well as in modulating both inflammatory and neuropathic pain.[8] However, endogenous metabolism of these epoxy-fatty acids to their corresponding hydrated products by soluble epoxide hydrolase (sEH EC 3.3.2.10) generally reduces these biological activities.[9,10] Both in vitro and in vivo studies have demonstrated that the anti-hypertensive and cardio protective effects mediated by the EETs are inversely

*Author for correspondence
Young Ho Kim, College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea.
Tel: +82-42-821-5933; E-mail: yhk@cnu.ac.kr

Hae-Dong Jang, Department of Food and Nutrition, Hannam University, Daejeon 305-811, Republic of Korea.
Tel: +82-42-629-8795; E-mail: haedong@hnu.kr

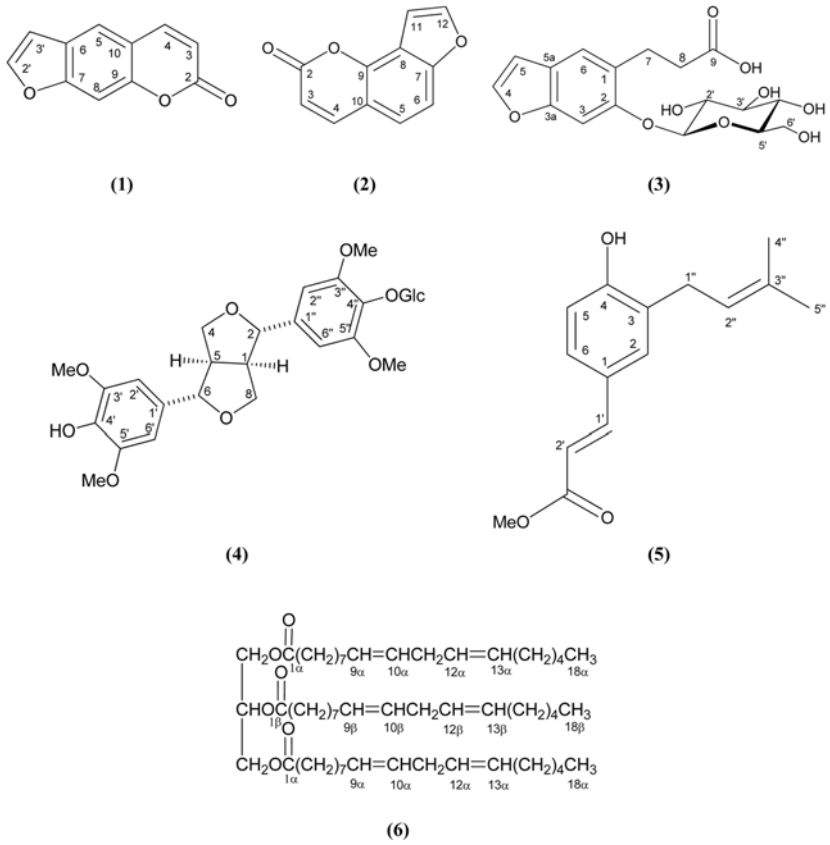

Fig. 1. Chemical structures of isolated compounds (**1 - 6**).

dependent on the extent of sEH hydrolysis of the EETs.[10,11] Previous report explained for the first time that EETs increase antioxidant enzyme expression and activity in cancer cells. This effect enables tumor cells to resist the oxidative damage and the subsequent apoptosis induced by arsenic trioxide (ATO).[12] Thus, maintaining the in vivo concentration of EETs through sEH inhibition is a promising therapeutic pathway to treat cardiovascular and other diseases.[8]

Ducrosia ismaelis (commonly known as Haza or Geshea) belong to the Apiaceae family, is a perennial, herbaceous, and branched plant with a characteristic aromatic odor.[7] The whole herb, especially its aerial parts, has been used in traditional medicines to treat skin infections and to repel insects and reptiles.[13] Previous phytochemical investigation on *D. ismaelis* led to the isolation of flavonoids, lignans, and phenolic compounds from the methanol extract of the aerial parts.[7] Pharmacological studies on some of these compounds showed anti-osteoporotic and antioxidant activities.[7] In a continuation of our phytochemical study on *D. ismaelis* to provide promising lead compounds for the treatment of osteoporosis and cardiovascular diseases, we inves-tigated the chemical constituents of the *D. ismaelis* plant and evaluated their biological activities. Compounds **1 - 6** were isolated from the aerial parts of *D. ismaelis* (Fig. 1). This report details the isolation, structural determination, antioxidant, anti-osteoporotic, and inhibitory activity on sEH of these compounds using ORAC, reducing capacity, TRAP and sEH inhibitory activity assays. Dried aerial parts of *D. ismaelis* were extracted with 100% methanol at room temperature. The crude extract was suspended in water and then successively partitioned with dichloromethane and ethyl acetate to give CH_2Cl_2, EtOAc, and water fractions, respectively. Using various types of column chromatography, psoralen (**1**),[14] isopsoralen (**2**),[15] cnidioside A (**3**),[16] (–)-syringaresinol-*O*-β-D-glucopyranoside (**4**),[17] (*E*)-plicatin B (**5**),[18] trilinolein (**6**),[19] were isolated for the first time from *D. ismaelis*. Their chemical structures (Fig. 1) were elucidated based on 1D and 2D NMR spectra, MS data, and by comparisons with previously published data acquired from similar compounds.

Experimental

Plant material – Dried aerial parts of *D. aismaelis*

were purchased from a folk medicine market, in Wad-Medani, Gezira state, Central Sudan, in December 2012. The plant material was identified by Professor A. M. Hamdoun, Faculty of Agricultural Sciences, University of Gezira, Sudan. A voucher specimen (CNU12113) was deposited at the herbarium, College of Pharmacy, Chungnam National University.

Extraction and isolation – Dried aerial parts of *D. ismaelis*. (3.0 kg) were extracted with 100% methanol at room temperature three times. After removal of the solvent under reduced pressure, the crude extract (258.0 g) was dissolved in 1.0 L of H_2O to form a suspension that was successively partitioned with dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc), to give CH_2Cl_2 (72.9 g), EtOAc (8.5 g), and aqueous extract (177.0 g), respectively. CH_2Cl_2 extract was chromatographed over silica gel (70 - 230 mesh), using n-hexane/EtOAc/MeOH (20 : 1 : 0.1 - 1 : 1 : 0.1 v/v/v) gradually as eluents, to afford fractions (1a - 1f). Repeated silica gel column chromatography of fractions 1b, 1c, 1d, and 1e with n-hexane/EtOAc (5 : 1 - 1 : 1 v/v), to give **6** (70.0 mg), **2** (50.0 mg), **1** (20.0 mg), and **5** (30.0 mg), respectively. EtOAc extract was subjected to column chromatography using silica gel (70 - 230 mesh), eluting with CH_2Cl_2/MeOH/H_2O (20 : 1 : 0.2 - 5 : 1 : 0.1v/v/v) to give five fractions (2a - 2e). Repeated silica gel column chromatography of fraction 2d with CH_2Cl_2/MeOH/H_2O (8 : 1 : 0.1v/v/v), further by YMC column and eluting with MeOH/H_2O (1 : 1), and (1.5 : 1 v/v) to obtain **3** (25.0 mg). Finally aqueous extract was chromatographed on a column of highly porous polymer (Diaion HP-20) and eluted with H_2O and MeOH, successively to give four fractions (3a-3d). Fraction 3c was chromatographed over silica gel eluting gradually with $CHCl_3$/MeOH/H_2O (12 : 1 : 0.1 - 1 : 1 : 0.1 v/v/v), further by YMC column and eluting with MeOH/Me_2CO/H_2O (0.3 : 0.3 : 1 v/v/v), to obtain **4** (15.0 mg).

Psoralen (**1**) – White amorphous powder, mp 158 - 161 ºC; UV λ_{max} (MeOH) nm: 328.0, 290.0, 244.0; FT-IR (KBr): ν_{max} 2925.6, 2359.4, 1722.8, 1575.7, 1284.4, 823.9, 748.9 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[14] ESI-MS *m/z* 187 $[M + H]^+$.

Isopsoralen (2) – Yellow amorphous powder, mp 137 - 140 ºC; UV λ_{max} (MeOH) nm: 297.0, 246.0; FT-IR (KBr): ν_{max} 2921.0, 2364.6, 1736.4, 1613.6, 1113.4, 829.5, 746.3 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[15] ESI-MS *m/z* 187 $[M + H]^+$.

Cnidioside A (3) – White amorphous powder, $[\alpha]_D^{25}$ = –29.4 (*c* 0.05, MeOH); UV λ_{max} (MeOH) nm: 285.0, 244.0; FT-IR (KBr): ν_{max} 3396.1, 2925.0, 1711.6, 1467.9, 1294.8, 1073.4 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[16] ESI-MS *m/z* 369 $[M + H]^+$.

(–)-Syringaresinol-*O*-*β*-D-glucopyranoside (4) – Colorless needles, $[\alpha]_D^{25}$ = –20.0 (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm: 269.0, 243.0; FT-IR (KBr): ν_{max} 3448.4, 2924.3, 1560.5, 1119.1 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[17] EI-MS *m/z* 603 $[M + Na]^+$.

(*E*)-Plicatin B (5) – White amorphous powder; mp 83 - 88 ºC $[\alpha]_D^{20}$ = +4.84 (*c* 0.05 MeOH); FT-IR (KBr): ν_{max} 3404.2, 2924.0, 1700.4, 1624.8, 1198.5, 831.5 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[18] HR-ESI-Q-TOF MS *m/z* 269.1164 $[M + Na]^+$ (calcd for $C_{15}H_{18}O_3Na$, 269.1148).

Trilinolein (6) – Yellow pale; mp 248 - 250 ºC; FT-IR (KBr): ν_{max} 1747.5, 1461.7, 1377.8, 1159.1 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previous reported data;[19] EI-MS *m/z* 879.8 $[M]^+$.

Oxygen radical absorbance capacity (ORAC) assay – ORAC assay, was carried out using a Tecan GENios multifunctional plate reader (Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm, emission filter: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with AAPH (20 mM) as a peroxyl radical generator in the peroxyl radical-scavenging capacity assay. The analyzer was programmed to record fluorescein fluorescence every 2 min after AAPH had been added. All fluorescence measurements were expressed relative to the initial reading. Final values were calculated based on the difference in the area under the fluorescence decay curve between the blank and test sample. All data are expressed as net protection area (net area). Trolox (1 μM) was used as the positive control to scavenge peroxyl radicals.[20]

Reducing capacity assay – The electron-donating capacities of extracted compounds (**1 - 6**) to reduce Cu^{2+} to Cu^+ were assessed according to the method of Aruoma et al.[2] Forty microliters of different concentrations of compounds dissolved in ethanol were mixed with 160 μL of a mixture containing 0.5 mM $CuCl_2$ and 0.75 mM neocuproine, a Cu^+ specific chelator, in10 mM phosphate buffer. Absorbance was measured using a microplate reader at 454 nm for 1 h. Increased absorbance of the reaction mixture indicated greater reducing power.[20]

Tartrate-resistant acid phosphatase (TRAP) Assay – RAW 264.7 cells were seeded in 96-well plates (1×10^4 cells/well) containing DMEM medium plus 10% FBS, and the medium was replaced with test samples in differentiation medium containing 50 ng/mL RANKL. The differentiation medium was changed every 2 days. After 5 days, the medium was removed, and the cell

monolayer was gently washed twice using ice-cold PBS. The cells were fixed in 3.5% formaldehyde for 10 min and ethanol-acetone (1 : 1) for 1 min. Subsequently, the dried cells were incubated in 50 mM citrate buffer (pH 4.5) containing 10 mM sodium tartrate and 6 mM PNPP. After 1 h incubation, the reaction mixtures were transferred to new well plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm using an enzyme-linked immunoassay reader, and TRAP activity was expressed as the percent of the untreated control.[20]

sEH inhibitory activity assay – The sEH inhibitory activity was determined using a hydrolysis reaction of PHOME in the presence or absence of the Inhibitor. The final reaction volume was 200 μL, and contained 25 mM bis-Tris HCl buffer (including 0.1% BSA (bovine serum albumin), pH 7.0), 20 μL of various concentrations of samples, 50 μL sEH enzyme, and 50 μL of 40 μM PHOME, AUDA (IC_{50}: 7.2 ± 2.7 nM) was used as the positive control. Reaction systems were incubated at 37 ºC for 1 h, and fluorescence intensity was then monitored every 2 min (during 1 h) using a Genios microplate reader (Tecan, Mannedorf, Switzerland) at excitation and emission wavelengths of 330 and 465 nm, respectively. sEH inhibitory activity for each sample was calculated as follows:

$$\text{Enzyme inhibition (\%)} = 100 - [(S - S_0) / (C - C_0)] \times 100$$

where C is the fluorescence of the control (enzyme, buffer, MeOH, and substrate) after 60 min of incubation, C_0 is the fluorescence of the control at zero time, S is the fluorescence of the tested samples (enzyme, buffer, sample solution, and substrate) after incubation, and S_0 is the fluorescence of the tested samples at zero time.[21,22]

sEH kinetic assay – To study the kinetics of sEH inhibition by isolated compounds **1 - 3**, various concentrations of compounds **1 - 3** were added in 96 well plate containing 80 μl of 25 m-M bis-Tris HCl buffer (including 0.1% BSA (bovine serum albumin), pH 7.0) with a series of substrate concentrations range of 10 - 80 μM, and fluorescence intensity was monitored at 37 ºC e-very 2 mins for 20 mins. The *Ki* value was also derived by plotting slopes obtained from Lineweaver-Burk plots and Dixon plots using SigmaPlot (SPSs Inc., Chicago, IL).[22,23]

Statistical analysis – All data are presented as means ± standard deviations (SD). Statistical analysis was carried out using the IBM SPSS statistical package (Version 21.0, IBM, New York, USA) program, and the significance of each group was verified with a one-way analysis of variance (ANOVA) followed by Duncan's test. A p value < 0.05 was considered significant.

Results and Discussion

A methanolic extract of the dried aerial parts of *D. ismaelis* was partitioned with dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc), successively. Repeated column chromatography on silica gel, C-18, and Diaion HP-20 of the CH_2Cl_2, EtOAc, and aqueous soluble fractions led to the isolation of six compounds (**1 - 6**) (Fig. 1). To determine the structures of compounds, the combined analyses with a series of 1D and 2D NMR, infrared, and mass spectra were accomplished. In addition, all physical and spectroscopic data obtained in this present study were compared with those of previously published manuscripts.

Structural elucidation of the isolated compounds – Compound **1**; ^{1}H-NMR spectrum showed two proton doublets at δ_H 6.35 (J = 9.6 Hz) and δ_H 7.78 (J = 9.6 Hz) characteristic of α-pyrone protons assignable to H-3 and H-4 respectively, and a pair of doublets occurring at δ_H 6.82 (J = 2.0 Hz) and δ_H 7.69 (J = 2.0 Hz) typical of furanic protons assignable to H-3' and H-2' respectively.[24] The spectrum further showed two protons singlet at δ_H 7.68 and 7.45 assignable to C-5 and C-8 of the furanocoumarin structure. The ^{13}C NMR spectrum exhibited 11 carbon resonances including six methines, one carbonyl and four quaternary carbons. This NMR data compared favourably with that published for psoralen (**1**).[14] Compound **2**; Both of the ^{1}H- and ^{13}C-NMR spectrum of **2** were similar to those of **1**, except that the spectra of **2** showed a pair of doublets occurring at δ_H 7.28 (J = 8.5 Hz) and δ_H 7.33 (J = 8.5 Hz) assignable to a H-5 and H-6, typical the angular furanocoumarin structure. This NMR data compared favourably with that published for isopsoralen (**2**).[15] Compound **3**; ^{1}H-NMR and ^{1}H-^{1}H COSY spectrum showed signals due to an anomeric proton at δ_H 4.86 (1H, d, J = 7.8 Hz), methylene protons at δ_H 2.58 (2H, d, J = 7.3 Hz) and 3.00 (2H, d, J = 7.3 Hz) coupled with each other and four aromatic protons at δ_H 6.61(lH, br s), 7.25 (1H, s), 7.28 (1H, br s) and 7.53 (IH, br s), suggesting that **3** is an aromatic glycoside. The ^{13}C NMR spectrum of **3** gave seventeen carbon signals caused by one β-glucopyranosyl unit with an ether linkage, one carbonyl at δ_C 176.48, two methylenes at δ_C 26.13 and 34.59, three oxygen-bearing aromatic carbons at δ_C 144.61, 153.64 and l54.49, two aromatic carbons at δ_C 121.91, 126.08 and three aromatic carbons bearing a proton at δ_C 98.44, 105.82 and 120.85. By the comparison of its spectral data with those of literature values, the structure of **3** was identified as cnidioside A.[16] Compound **4**; ^{1}H-NMR and ^{13}C NMR spectrum, indicated the presence of one glucose, two benzene rings, two propane units and

four methoxyl groups in the molecule. On the basis of the above spectral evidence, compound **4** was concluded to be (−)-syringaresinol-*O*-*β*-D-glucopyranoside. The physio-chemical and spectral data were good agreement with those published.[17] Compound **5**; ^{1}H NMR spectrum showed an ortho-coupled aromatic protons at δ_H 7.35 (1H, d, J = 8.40 Hz) and δ_H 6.69 (1H, d, J = 8.40 Hz) and an isolated proton at δ_H 7.48 (1H, s). The presence of a *trans*-methyl cinnamate moiety was shown by a pair of doublets of olefinic protons at δ_H 6.76 (1H, d, J = 15.92 Hz) and 5.74 (1H, d, J= 15.92 Hz) and methoxyl group at δ_H 3.65 (3H, s). In addition, the spectrum showed the presence of a prenyl group (two methyl group at δ_H 1.71 (6H, s); one proton at δ_H 5.25 (1H, m) and two protons at δ_H 3.27 (2H, d, J = 7.8 Hz). The ^{13}C NMR spectrum determined in the DEPT mode, showed the presence of 15 carbon atoms with three methyl at δ_C 17.96, 25.88 and 51.44, one methylene at δ_C 29.78, six methines at δ_C 115.44, 116.33, 121.63, 130.15, 132.66 and 143.97, four quaternary carbon atoms at δ_C 126.60, 127.32, 134.92 and 156.67), and one carbonyl at δ_C 167.27. By the comparison of its spectral data with those of literature values, the structure of **5** was identified as (*E*)-plicatin B.[18] Compound **6**; ^{1}H NMR spectrum showed methine protons resonated only between δ_H 5.28 and 5.37, the methyl groups at δ_H 0.9 and the rest of the peaks being methylene protons. The deshielded methylene protons at δ_H 4.12 to 4.33 and methine proton at δ_H 5.28 suggests protons attached to oxygen bearing carbons, imblecating a glycerol ester type of structure. The olefinic complex multiplet resonating at δ_H 5.4 was assigned to the aliphatic double bonds (H-9/10 and 12/13), the doublet of doublets between δ_H 4.12 and 4.33 to H-1' (2H) and H-3' (2H) and the deshielded multiplet at δ_H 5.28 to the tertiary oxomethine (H-2', C-68.5 ppm). The ^{13}C NMR spectra showed six double bonds at δ_C 127.80, 128.40, 128.93, 129.51, 129.83 and 130.45 and three carbonyl (two at δ_C 173.07 and one at δ_C 172.71). On the basis of the above spectral evidence, compound **6** was concluded to be trilinolein. The physio-chemical and spectral data were good agreement with those published.[19,25]

***In vitro* Antioxidant activities** – The antioxidant activities of compounds **1 - 6** were investigated in terms of their peroxyl radical-scavenging and reducing capacity. Based on the chemical reactions involved, major antioxidant capacity assays can be roughly divided into hydrogen atom transfer-based and single electron transfer-based assays.[26] ORAC assay is based on hydrogen atom transfer while reducing capacity assay depends upon single electron transfer.[26] The scavenging activities of compounds **1 - 6** in the presence of peroxyl radicals were shown in Fig. 2, at

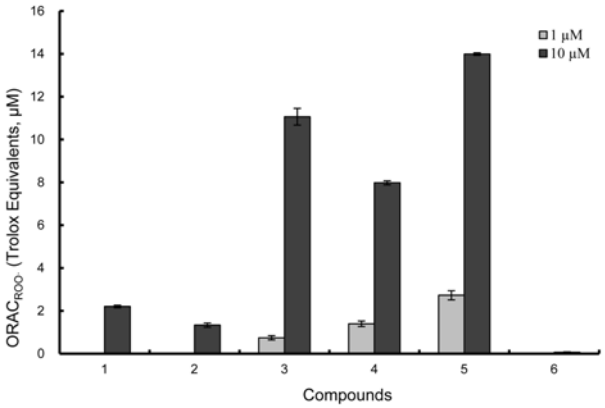

Fig. 2. Peroxyl radical-scavenging activity (Trolox Equivalent, μM) of compounds (**1 - 6**) in ORAC assay.

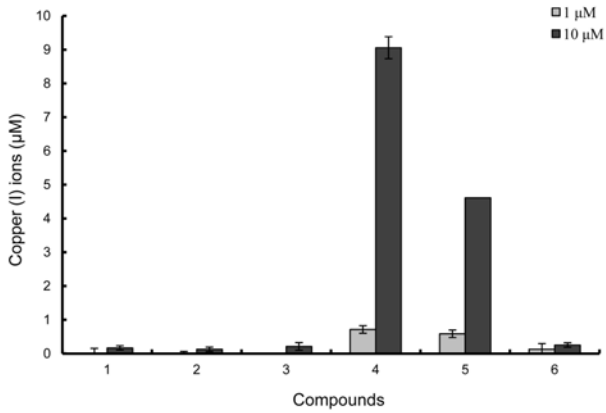

Fig. 3. Reducing capacities of compounds **1 - 6**. The results represent the mean SD of values obtained from three measurements.

dose-dependent manner at 1 - 10 μM. Among them, compounds (**3 - 5**) showed potent peroxyl radical-scavenging activities with 11.06 ± 0.39, 7.98 ± 0.10, and 13.99 ± 0.06 Trolox equivalent (TE) at concentrations of 10 μM, respectively. In particular, at a concentration of 10 μM, **5** showed the strongest peroxyl radical-scavenging activity against peroxyl radicals generated from (AAPH). Based on structural analysis of the active isolates (**3**, **4** and **5**), the hydroxyl groups on the structure seem to be responsible for peroxyl radical-scavenging activity by donating hydrogen atoms to peroxyl radicals.[2] The ability of the isolated compounds **1 - 6** to stimulate the reduction of copper ions (Cu^{2+} to Cu^{+}) by donating electrons was investigated to determine whether their peroxyl radical-scavenging capacities, could be related to their reduction capacities. As shown in Fig. 3, only compounds **4** and **5** was able to significantly reduce Cu^{2+} ions, with a reduction value of 9.06 ± 0.32 and 4.61 ± 0.00 μM TE at a concentration of 10 μM. Present data suggest that the ability of

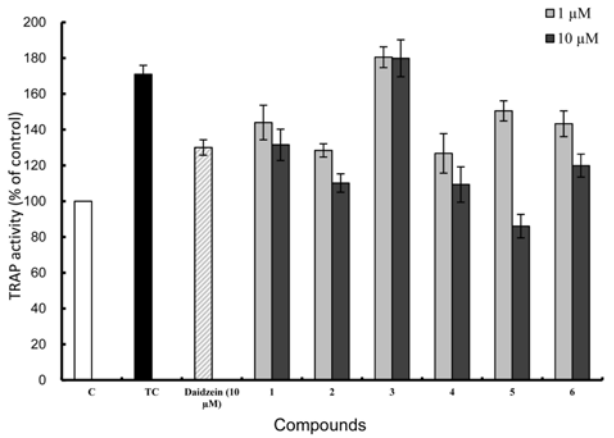

Fig. 4. Tartrate-resistant acid phosphatase (% of control) of compounds (**1 - 6**). C: control, which was not treated; TC: treated control, which was treated with RANKL.

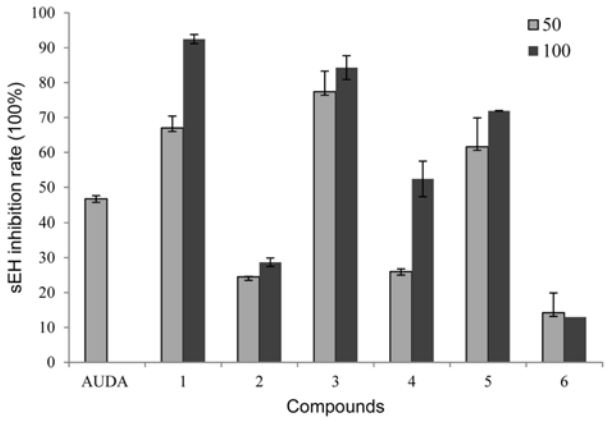

Fig. 5. Inhibitory effect of the isolated compounds **1 - 6** on sEH activity determined using the uorometric method. The sEH activities expressed as the percentage of control activity. Values represent means ± SD from triplicate experiments. (n = 3). (AUDA (12.5 nM): positive control).

compounds **4** and **5** to donate hydrogen atoms or electrons to peroxyl radicals and to convert them into relatively stable compounds may contribute to their peroxyl radical-scavenging capacity. Although compound **3** showed potent peroxyl radical-scavenging capacities, it appeared to have lower capacities for reducing Cu^{2+} to Cu^{+} compared to compounds **4** and **5**. These data suggest that the hydrogen donating ability of compounds **3** is strong, but that its single electron transferring activity is weak. This may be due to the absence of hydroxyl substituent on the aromatic ring, which results in unfavourable electron transfer to the Cu^{2+} ion. This data agrees with the conclusions of previous studies.[2,20]

***In vitro* anti-osteoporotic activities** – The anti-osteoporotic activities of compounds **1 - 6** were determined using TRAP assay. TRAP activity was expressed as the percent of the untreated control (mean ± one standard deviation, n = 3). RANKL treatment induced osteoclast formation from RAW 264.7 pre-osteoclast cells and dramatically enhanced TRAP activity up to 170.9%, as shown in Fig. 3. Compounds **5**, **4**, and **2** suppressed osteoclast formation in a dose-dependent manner with TRAP values of 86.05 ± 6.55, 109.30 ± 9.37, and 110.12 ± 5.15 (% of control, daidzein used as a positive control) at concentrations of 10 μM, respectively. Compound **5** exhibited the highest anti-osteoporotic activity among tested compounds, as indicated by the relatively low TRAP value (Fig. 2). This may be due to the high cell membrane permeability of **5**, resulting from the absence of a glucose unit and the presence of two hydrophobic side chains.[7] When compared both anti-osteoporotic and antioxidant activities of **1 - 6**, the potent TRAP, peroxyl radical scavenging and reducing capacity activities were

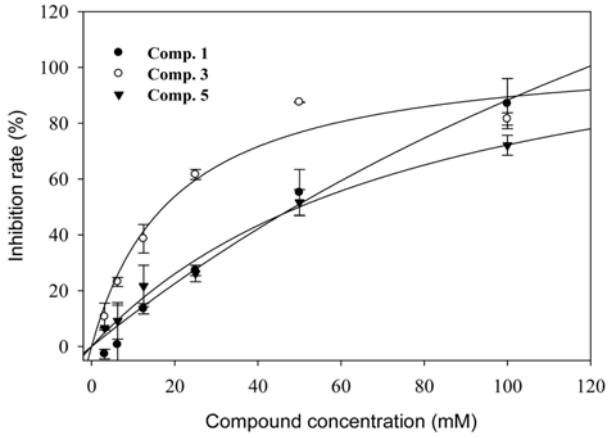

Fig. 6. Effects of compounds **1**, **3** and **5** on the activity of sEH for hydroysis of substrate.

observed in **4** and **5** among 6 isolated compounds. Therefore, these results imply that compound **4** and **5** may augment antiosteoporotic activity by directly scavenging intracellular reactive oxygen species produced during RANKL-mediated osteoclast differentiation through donating hydrogen atom or electron.[20]

***In vitro* sEH inhibitory activity** – sEH inhibitory activity of isolated compounds **1 - 6** was evaluated *in vitro* using a fluorescent method based on hydrolysis of the specific substrate PHOME in the presence of sEH. AUDA [12-(3-adamantan-1-yl-ureido) dodecanoic acid] one of the most effective sEH inhibitors, was used as a positive control (IC_{50}: 7.2 ± 2.7 nM). The sEH inhibitory activity was assessed at doses of 50 and 100 μM. At a concentration of 50 μM, isolated compounds showed sEH Inhibition values ranging from 14.2% (**6**) to 77.4% (**3**)

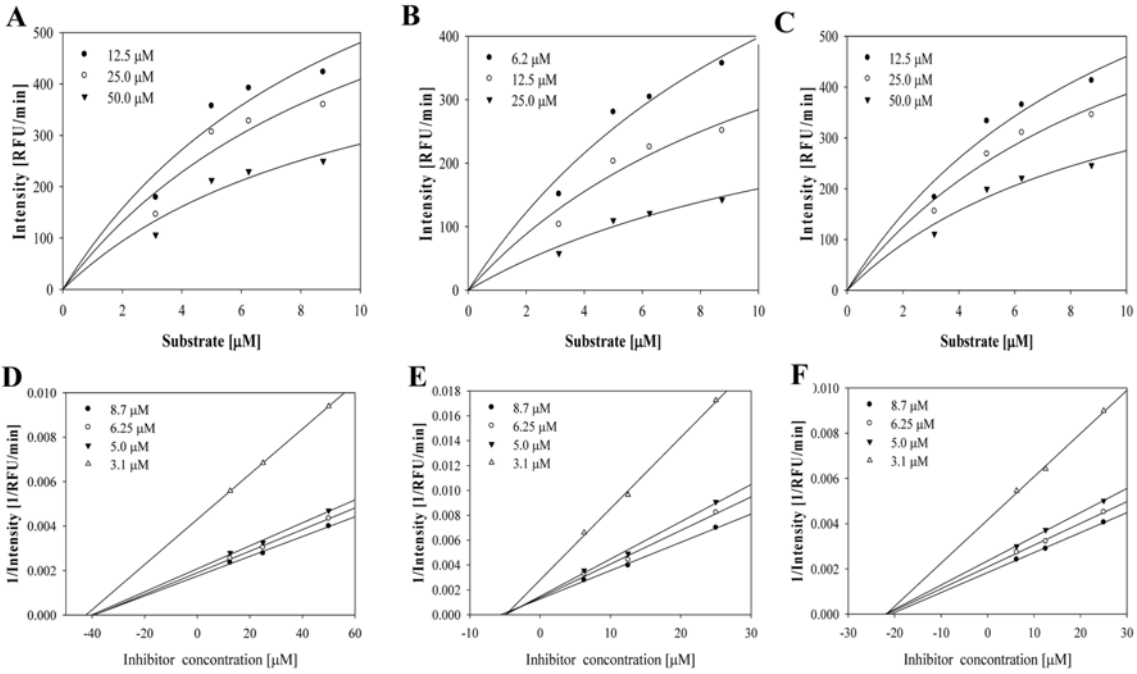

Fig. 7. Graphical determination of the inhibition type for isolated compounds **1**, **3** and **5**. (**A - C**) Lineweaver-Burk plots for the inhibition of compounds **1**, **3**, and **5** on sEH for the hydrolysis of substrate. (**D - F**) Dixon plots for the inhibition of compounds **1**, **3**, and **5** to determined *ki* value.

Table 1. *In vitro* sEH inhibitory activity and kinetic study of compounds **1**, **3** and **5**

Compound	IC_{50}[a] (μM)	Binding mode (*Ki*[b], μM)
1	41.6 ± 4.9	Noncompetitive (40.1 ± 1.8)
3	16.0 ± 1.1	Noncompetitive (4.9 ± 0.2)
5	49.0 ± 5.7	Noncompetitive (21.2 ± 0.4)
AUDA[c]	7.2 ± 2.7 nM	

[a]All compounds were examined in a set of experiments three times
[b]Inhibition constant
[c]Positive control

(Fig. 5). Compounds **1**, **3** and **5** inhibited sEH more than 50% and were assessed for further investigation. Effects of those compounds were examined at concentrations from 3.1 to 100 μM, and the 50% inhibitory concentration (IC_{50}) was calculated using a dose-dependent response curve (Fig. 6). Among the 6 isolated compounds, compounds **1**, **3** and **5** exhibited high sEH inhibitory activity; their IC_{50} values were 41.6 ± 4.9, 16.0 ± 1.1, and 49.0 ± 5.7 μM, respectively. The enzyme kinetic was measured with a series of substrate concentrations and various inhibitor concentrations. The enzyme inhibition mechanisms of **1**, **3** and **5** were modeled using double-reciprocal plots (Lineweaver-Burk and Dixon plots). The Lineweaver-Burk plot (1/V vs 1/[S]) resulted in a series of straight lines passing through the same point on the *x*-axis (Fig. 7A-C). These ndings indicate that compounds **1**, **3** and **5** exhibit non-competitive inhibition characteristics. The *Ki* values were calculated from the Dixon plots (Fig. 7D-F), which are useful for determining the *Ki* value of the inhibitor. From these kinetic plots, the sEH inhibitors **1**, **3** and **5**, had inhibition constants (*Ki*) of 40.1 ± 1.8, 4.9 ± 0.2, and 21.2 ± 0.4 M, respectively (Table 1). Also when compared antioxidant and sEH inhibitory activities of **1-6,** the potent peroxyl radical scavenging reducing capacity and sEH inhibitory activities were observed in **5** among 6 isolated compounds. Therefore, these results imply that potential activity of sEH inhibitors may be enhance the antioxidant defense mechanisms.[12] Further study using an *in vivo* animal model may be required to elucidate the correlation between antioxidant and sEH inhibitory activities of the phytochemicals from *D. ismaelis.*

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