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Evaluation of human neutrophil elastase inhibitory effect of iridoid glycosides from *Hedyotis diffusa*

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ABSTRACT

Five iridoid glycosides were isolated from the MeOH extract of *Hedyotis diffusa*, and their structures were elucidated as *E*-6-*O*-*p*-methoxycinnamoyl scandoside methyl ester (**1**), *Z*-6-*O*-*p*-methoxycinnamoyl scandoside methyl ester (**2**), *E*-6-*O*-*p*-feruloyl scandoside methyl ester (**3**), *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (**4**), and *Z*-6-*O*-*p*-coumaroyl scandoside methyl ester (**5**) by interpretation of their spectroscopic data. All the isolated compounds were evaluated for human neutrophil elastase inhibitory effect, and compound **1** showed potent activity with an IC_{50} value of 18.0 μM. The molecular docking simulation suggested a structural model for the inhibition of human neutrophil elastase by compound **1**.

Skin aging, a complex biological phenomenon, can be divided into two independent aging processes: intrinsic (chronological) and extrinsic (photo-) aging. Intrinsic aging is the normal, genetic process of physical change over time, and begins the minute we are born. Various expressions of intrinsic aging include smooth, thinning skin with exaggerated expression lines. Extrinsic aging is caused by environmental factors such as sun exposure, air pollution, smoking, alcohol abuse, and poor nutrition. Extrinsic aging is characterized by photo damage as wrinkles, pigmented lesions, patchy hypopigmentations, and actinic keratoses.[1,2] The skin is composed of two tissue layers: epidermis and dermis. Epidermis is a thin protective layer, which consists mainly of keratinocytes, melanocytes, and Langerhans cells. Dermis is a thick complex layer of connective tissue, and the main fibrous tissues in the dermis are collagen and elastic fibers.[3] Collagen is the major structural protein of skin and is responsible for its tensile strength and toughness. Elastin, an important structural protein of extracellular matrix (ECM), is the main component of elastic fiber which provides resilience and elasticity to many tissues such as skin, lungs, ligaments, and arterial walls.[4] Human neutrophil elastase (HNE), a serine protease primarily located in the azurophil granules of polymorphonuclear leukocytes, has a broad substrate specificity being able to break down mainly elastin connective tissue proteins such as fibronectin, collagen, and cartilage tissues.[5,6] Biologically, elastase activity increases with age, fragmenting collagen and elastin leading to a reduction in the elasticity of skin and the appearance of wrinkle and strechmark.[7] Therefore, inhibition of the elastase activity could also be used as a valuable method to protect against skin aging.

The herb of *Hedyotis diffusa* Willd. (synonym *Oldenlandia diffusa* Willd., family Rubiaceae), an annual herb distributed in northeastern Asia, has been known as a traditional oriental medicine for the treatment of hepatitis, tonsillitis, sore throat, appendicitis, urethral infection, and malignant tumors of the liver, lung, and stomach. *H. diffusa* is reported to possess various pharmacological activities such as anticancerous, anti-oxidative, anti-inflammatory, hepatoprotective, and neuroprotective activities.[8–11] Previous phytochemical investigations revealed the presence of terpenoids, flavonids, anthraquinones, and steroids.[12–15] However, no report on HNE inhibitory effect of this plant has been documented. In the course of our screening program for HNE inhibitors, methanol (MeOH) extract of *H. diffusa* was found to exhibit potent HNE inhibitory activity. In the present study, we describe the isolation and structure elucidation of five iridoid glycosides from *H. diffusa*, and their HNE inhibitory activities were evaluated.

The air-dried whole plant of *H. diffusa* was extracted with MeOH and then partitioned with *n*-hexane and EtOAc. Repeated column chromatography and preparative reversed-phase HPLC led to the isolation of five compounds (**1**–**5**).[16] Compound **1** was obtained as white powder. The UV spectrum of **1** showed an absorption maximum at 229 and 311 nm. The ^{1}H NMR spectrum of **1** displayed an enol ether proton at δ 7.52 (1H, br s, H-3), which was characteristic of iridoid glycosides possessing a carbonyl group on C-4,[17] the signal at δ 5.85 (1H, t, J = 1.2 Hz) was assigned to acetalic proton. In addition, a pair of *ortho*-coupled A_2B_2 type signals at δ 7.56 and 6.96 (each 2H, d, J = 8.1 Hz) indicated the

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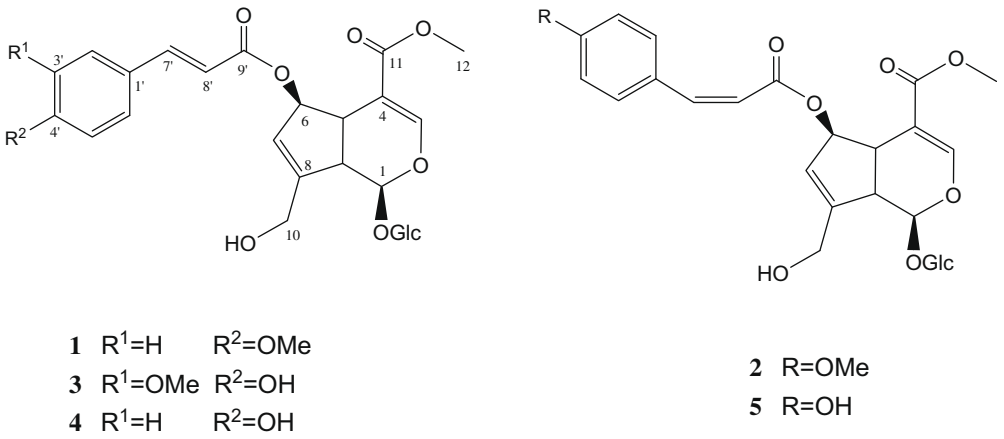

1 R^1=H R^2=OMe
3 R^1=OMe R^2=OH
4 R^1=H R^2=OH

2 R=OMe
5 R=OH

Figure 1. Chemical structures of compounds isolated from *H. diffusa*.

presence of a 1,4-disubstituted benzene ring, the signals at δ 7.66 and 6.39 (each 1H, d, J = 15.3 Hz) were assigned to two *trans* olefinic protons, and the signal at δ 3.83 (3H, s) was attributed to a methoxyl group. These data suggested the presence of *p*-methoxycinnamoyl group in compound **1**. Consistent with the ^{1}H NMR spectrum of **1**, its ^{13}C NMR spectrum exhibited 27 carbon signals including two carbonyl groups, 12 olefinic carbons, one methoxy group and six sugar carbons. Furthermore, the doublet signal at δ 4.70 (d, J = 8.1 Hz) for an anomeric proton of the glucosyl moiety indicated the β-configuration, and the β-*O*-substituted configuration at C-6 was established by $J_{H1,9}$ = 6.8 Hz, $\Delta\delta$ C3–C4 = 44.1 ppm and δ C1 = 98.1 ppm.[18,19] Therefore, the structure of **1** was determined as *E*-6-*O*-*p*-methoxycinnamoyl scandoside methyl ester.[20] Other four compounds were identified as *Z*-6-*O*-*p*-methyoxycinnamoyl scandoside methyl ester (**2**), *E*-6-*O*-*p*-feruloyl scandoside methyl ester (**3**), *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (**4**), and *Z*-6-*O*-*p*-coumaroyl scandoside methyl ester (**5**) by direct comparison of their physical and spectral data with those reported in the literature.[21,22]

All of the isolated compounds were iridoid glycosides possessing acyl moieties but differ in the stereochemistry of the double bond and the existence of a *p*-methoxy substituent in the aromatic ring (Fig. 1). The isolated compounds were evaluated for HNE inhibitory activity[23] at different concentration ranging from 1 to 100 μM, and the result was shown in Table 1. As a result, compound **1** significantly inhibited HNE activity with an IC_{50} value of 18.0 μM, which was comparable to the positive control, EGCG (IC_{50}, 12.8 μM). However, other compounds showed negligible HNE inhibitory activities. This structure–activity relationship demonstrates that the *p*-methoxy group in the aromatic ring and the *trans* double bond (C7′–C8′) in the acyl moiety of acylated iridoid glycosides appear to influence potency.

To understand the structural basis for inhibition of HNE by compound **1**, we generated a structural model for HNE in complex with compound **1** by molecular docking.[24] Figure 2A shows the structure of compound **1** bound to active site of HNE. The binding site and overall binding mode of compound **1** is very similar to those previously observed for peptide chloromethyl ketone inhibitor.[25] The major interactions of compound **1** with HNE include four hydrogen bonds with Cys42, His57, Ser195, and Arg177 and van der Waals contacts with Leu99B, Phe215, and Val216 (Fig. 2A). In particular, *p*-methoxy group in the aromatic ring of compound **1** forms a hydrogen bond with side-chain nitrogen atom of Arg177. Since this contrasts with non-active compounds with the negligible HNE inhibitory activities, the hydrogen bond mediated by *p*-methoxy group seems to be important for binding the enzyme. For non-active compounds **1** and **5**, the introduction of the *cis* double bond (C7′–C8′) in the acyl moiety may convert the orientation of the aromatic ring, disrupting the two hydrogen bonds mediated by His57 and Arg177 in HNE (Fig. 2B). For example, the distances between oxygen atoms of the compounds **1** and **5** and the nitrogen atom of Arg177 are over 13 Å. For non-active compounds **3** and **4**, the hydroxyl groups in place of *p*-methoxy group in the aromatic ring do not appear to form the hydrogen bond with Arg177.

In summary, five *O*-acylated iridoid glycosides were isolated from *H. diffusa* and their HNE inhibitory effects were evaluated for the first time. Compound **1** exhibited the most potent inhibitory activity with an IC_{50} value of 18.0 μM. The molecular docking experiment provided a structural model for the HNE in complex with compound **1**.

Table 1
Human neutrophil elastase inhibitory activity of compounds **1**–**5**

	Compounds					
	1	**2**	**3**	**4**	**5**	EGCG[a]
1 μM	19.1 ± 6.2	0.4 ± 7.4	24.7 ± 2.8	1.0 ± 1.1	0.8 ± 16.3	26.6 ± 7.3
3 μM	36.6 ± 3.7	15.9 ± 6.8	23.3 ± 0.6	0.8 ± 6.3	2.5 ± 5.6	41.3 ± 1.2
10 μM	46.3 ± 10.5	13.0 ± 2.6	22.9 ± 3.6	13.1 ± 0.3	6.0 ± 3.0	63.6 ± 2.8
30 μM	63.0 ± 8.5	15.7 ± 3.3	23.6 ± 1.0	16.8 ± 3.5	13.6 ± 1.2	71.4 ± 3.8
100 μM	81.2 ± 6.4	40.3 ± 10.4	25.1 ± 3.3	28.6 ± 3.0	20.2 ± 12.9	78.4 ± 1.5
IC_{50}[b]	18.0	>100	>100	>100	>100	12.8

[a] EGCG [(−)-epigallocatechin-3-gallate] was used as a positive control.
[b] IC_{50} values were determined by concentration-dependent experiments.

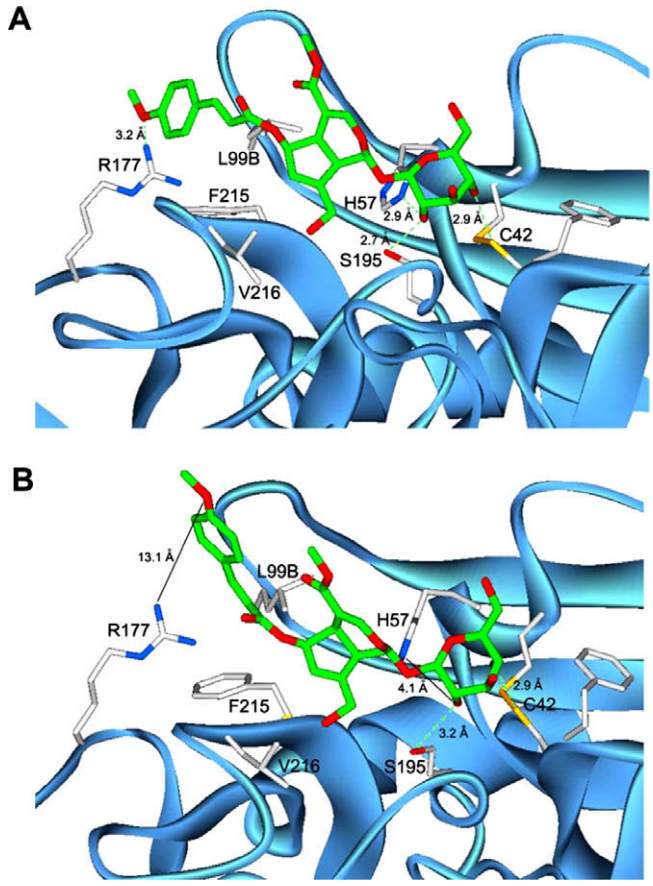

Figure 2. A structural model of HNE in complex with the compounds **1** (A) and **2** (B). The HNE is shown in gray side chains and blue ribbon. The compounds **1** and **2** are colored green. The dashed green lines indicate the hydrogen bonds. The solid black line in the compound **2** indicates the distance between the atoms that form the hydrogen bond in the compound **1**.

Acknowledgments

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16. *Procedure for extraction and isolation*: The plant of *Hedyotis diffusa* was purchased in Kwangyang, Chonnan, October 2008. The voucher specimen (CNU 2102) was identified by Professor Ki-Hwan Bae and deposited at the herbarium of Chungnam National University, Daejeon, Korea. The air-dried whole plant of *H. diffusa* (450 g) was extracted three times with MeOH at room temperature for 3 days each. The combined MeOH solution was evaporated in vacuo to give a residue (40 g), which was then suspended in H_2O, and successively partitioned with *n*-hexane and EtOAc. The EtOAc layer (4.4 g), which showed strong HNE inhibitory effect, was then loaded on a silica gel column eluting with CH_2Cl_2–MeOH in a gradient mode (20:1→1:1) to give six fractions (HD1–6). The fraction HD4 (800 mg) was subjected to Sephadex LH-20 column chromatography and eluted with CH_2Cl_2–MeOH (1:1) to give three fractions (HD41–43), then the fraction HD41 (200 mg) was loaded on a RP-18 column and eluted with MeOH–H_2O (1:4→4:1) to give two fractions (HD411 and 412). The fraction HD412 was further separated by preparative HPLC (YMC-Pack ODS column, 20 × 250 mm, MeOH–H_2O = 1:1, flow rate, 4.0 mL/min) to afford **1** (R_t, 33 min, 12 mg) and **2** (R_t, 26 min, 2.5 mg), respectively. The fraction HD5 (1.2 g) was subjected to Sephadex LH-20 column chromatography and eluted with CH_2Cl_2–MeOH (1:1) to give two fractions (HD51 and 52). The fraction HD51 (500 mg) was applied to a RP-18 column and eluted with MeOH–H_2O (1:4→4:1) to give three fractions (HD511–513). The fraction HD512 was subjected to Sephadex LH-20 column chromatography and eluted with 70% MeOH to afford **3** (5 mg), and the fraction HD513 was further separated by preparative HPLC (MeOH–H_2O = 2:3) to afford **4** (t_R, 25 min, 50 mg) and **5** (t_R, 33 min, 40 mg), respectively.
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20. *Physicochemical and spectroscopic data of E-6-O-p-methoxycinnamoyl scandoside methyl ester* (**1**): White powder; $[\alpha]_D^{20}$ − 164.0 (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm: 229, 311; ^{1}H NMR (900 MHz, CD_3OD): δ 7.66 (1H, d, *J* = 15.3 Hz, H-7′), 7.56 (2H, d, *J* = 8.1 Hz, H-2′,6′), 7.52 (1H, br s, H-3), 6.96 (1H, d, *J* = 8.1 Hz, H-3′,5′), 6.39 (1H, d, *J* = 15.3 Hz, H-8′), 5.85 (1H, t, *J* = 1.2 Hz, H-7), 5.69 (1H, m, H-6), 5.32 (1H, d, *J* = 6.3 Hz, H-1), 4.70 (1H, d, *J* = 8.1 Hz, H-1″), 4.39 (1H, d, *J* = 16.2 Hz, H-10a), 4.22 (1H, d, *J* = 16.2 Hz, H-10b), 3.89 (1H, dd, *J* = 11.7, 1.8 Hz, H-6″a), 3.83 (3H, s, 4′-OMe), 3.65 (4H, m, H-12,6″b), 3.39 (1H, t, *J* = 9.0 Hz, H-5), 3.36 (1H, dd, *J* = 7.2, 5.4 Hz, H-5″), 3.32–3.27 (2H, m, overlapped with solvent peak, H-3″,4″), 3.22 (1H, dd, *J* = 9.0, 8.2 Hz, H-2″), 3.09 (1H, t, *J* = 7.2 Hz, H-9); ^{13}C NMR (225 MHz, CD_3OD): δ 169.2 (C-11), 168.8 (C-9′), 163.3 (C-4′), 154.2 (C-3), 150.5 (C-8), 146.3 (C-7′), 131.1 (C-2′,6′), 128.5 (C-1′), 127.3 (C-7), 116.6 (C-8′), 115.6 (C-3′,5′), 110.1 (C-4), 100.4 (C-1″), 98.1 (C-1), 83.8 (C-6), 78.6 (C-3″), 78.0 (C-5″), 74.9 (C-2″), 71.7 (C-4″), 62.8 (C-6″), 61.1 (C-10), 56.0 (C4′-OMe), 52.1 (C-12), 47.0 (C-9), 42.4 (C-5); positive ESI-MS *m/z* 587 $[M+Na]^+$, negative ESI-MS *m/z* 563 $[M-H]^-$.
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23. HNE inhibitory activity was evaluated according to previously described procedure (Benedek, B.; Kopp, B.; Melzig, M. F. *J. Ethnopharmacol.* **2007**, *113*, 312.). Briefly, 125 μL of substrate solution [1.4 mM *N*-methoxysuccinyl-ala-ala-pro-val-*p*-nitroanilide, in Tris–HCl buffer (pH 7.5)], 60 μL of test solution [stock solutions of the test compounds were dissolved in 500 μL DMSO and diluted with Tris–HCl buffer to give the final sample concentrations] and 390 μL of Tris–HCl buffer were mixed and vortexed. After addition of 25 μL enzyme solution (approx. 0.18 U elastase from human neutrophils, EC 3.4.21.37, from Serva, Heidelberg, Germany) the samples were incubated for 1 h at 37 °C. After the reaction was stopped by addition of 500 μL soybean trypsin inhibitor [Sigma, Germany; 0.2 mg/mL Tris–HCl buffer (pH 7.5)], absorbance was immediately measured at 405 nm. Epigallocatechin gallate (EGCG) (Sigma, Germany) was used as a positive control.
24. To build a structural model for the HNE in complex with compound **1**, compound **1** was docked into the HNE (PDB code: 1HNE) by CDOCKER program in the software Discovery Studio 2.1 (Accelrys). Ten initial random conformations were taken and 10 initial poses were chosen for final refinement. CHARMm forcefield was employed for molecular dynamics and dynamics steps and target temperature were set to be 1000 and 1000 K, respectively. Figures were drawn by using the software Discovery Studio 2.1.
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