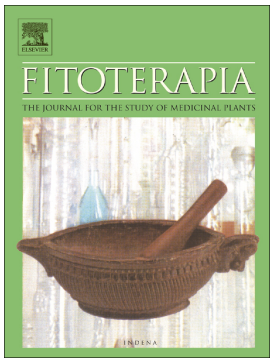

Constituents of the leaves and twigs of Elaeagnus umbellata and their proliferative effects on human keratinocyte HaCaT cells

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Constituents of the leaves and twigs of *Elaeagnus umbellata* and their proliferative effects on human keratinocyte HaCaT cells

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ABSTRACT

Bioassay-guided fractionation of an extract of leaves and twigs of *Elaeagnus umbellata* led to the isolation of a serotonin derivative, *N*-[2-(5-hydroxyl-1*H*-indol-3-yl)ethyl]-butanamide (**1**), along with six flavonoid glycosides, kaempferol-3-*O*-β-D-xylopyranosyl(1→2)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**2**), kaempferol-3-*O*-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**3**), kaempferol-3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**4**), kaempferol-3-*O*-β-D-xylopyranosyl(1→2)-β-D-galactopyranoside (**5**), kaempferol-3-*O*-rutinoside (**6**), and kaempferol-3-*O*-β-D-glucopyranosyl(1→2)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**7**). Their structures were elucidated using 1D/2D nuclear magnetic resonance spectroscopy and mass spectrometry. Compounds **1**–**6** were evaluated for their proliferative effects on HaCaT keratinocytes; **1**–**5** promoted keratinocyte proliferation dose dependently. Compounds **3** and **4** showed potent activities. These results suggest that the leaves and twigs of *E. umbellata* have wound healing and skin cell regeneration potentials.

Keywords:

Elaeagnus umbellata; Elaeagnaceae; serotonin derivative; flavonoid glycoside; cell proliferation; HaCaT keratinocyte

1. Introduction

The genus *Elaeagnus* (Elaeagnaceae) consists of approximately 90 species, which can be found at altitudes ranging from 1,200 to 2,100 m and are mostly distributed in subtropical regions of Asia [1, 2]. Various species of *Elaeagnus*, such as *E. umbellata*, *E. pungens*, *E. angustifolia*, and *E. multiflora*, have been used as medicinal plants [1]. *E. umbellata* Thunb. is well known for the lycopene contents in its berries, with antioxidant and anti-inflammatory activities [2, 3]. In this study, we investigated the phytochemistry of the twigs and leaves, waste products of this plant. *E. umbellata* has shown antiplasmodial [4], antioxidant [5, 6], and antihypertensive [7] activities. Several secondary metabolites including β-carboline alkaloids [8], coumarins [4], anthraquinone glycosides [9], sterols [4], and flavonoids [10] have been reported for this species. In the present study to uncover skin-protective components through bioassay-guided fractionation, a serotonin derivative and six flavonoid glycosides were isolated from the leaves and twigs of *E. umbellata* and identified as *N*-[2-(5-hydroxyl-1*H*-indol-3-yl)ethyl]-butanamide (**1**), kaempferol-3-*O*-β-D-xylopyranosyl(1→2)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**2**) [11], kaempferol-3-*O*-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**3**) [12], kaempferol-3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**4**) [13], kaempferol-3-*O*-β-D-xylopyranosyl(1→2)-β-D-galactopyranoside (**5**) [14], kaempferol-3-*O*-rutinoside (**6**) [15], and kaempferol-3-*O*-β-glucopyranosyl(1→2)-β-galactopyranoside-7-*O*-α-rhamnopyranoside (**7**) [16] through spectroscopic data analysis involving 1D and 2D nuclear magnetic resonance (NMR) (Fig. 1) and by comparing their data with reported values. Various bioactivities have been reported for these isolated compounds, such as the melatonin-inducing activity of **1**, antioxidant activities of **2** and **4**, melanogenesis inhibitory activities of **3** and **6**, and antimalarial activity of **5** [17-21].

Skin is a self-renewing organ that defends the body against external threats such as heat, infection, and ultraviolet radiation [22]. It also protects against microbes, light, and injury [23]. Skin cell proliferation is important for proper wound healing after skin injury. Skin wound healing is a complex process involving the re-epithelialization of missing cellular structures and the tissue layer through three phases: inflammation, proliferation, and remodeling [24]. The smooth progression of these events leads to successful wound healing and restoration of the disrupted anatomical and functional state of the skin [25]. Of the many cell types required during wound healing, keratinocytes are important for epithelialization in the proliferative phase as they are the predominant cells of the outermost layer of the skin [26]. Skin wound healing can be facilitated by natural products with medicinal properties. Many studies on the wound healing properties of natural products with anti-inflammatory, antioxidant, antibacterial, and pro-collagen synthesis actions have been conducted [27]. For instance, it has been found that saponins can enhance the synthesis of pro-collagen, while flavonoids have antiseptic and antibacterial activities. These natural compounds can regulate one or more phases of skin wound healing. Natural products and their phytochemicals have important roles in skin wound healing and are used in the design of new synthetic compounds for this purpose [28]. To the best of our knowledge, this is the first report on the isolation of these compounds from this species. Selected compounds **1**–**6** were examined for their cell proliferation activities in HaCaT cells.

2. Experimental section

2.1. General experimental procedures

The NMR spectra were acquired on a 600 MHz Varian spectrometer (Varian, Billerica, MA, U.S.A.) and a 250 MHz Bruker AVANCE DPX spectrometer (Bruker, Billerica, MA, USA).

HR-MS was performed on a Waters ACQUITY UPLC system (Milford, MA, USA) coupled to a Micromass Q-Tof Micro mass spectrometer and Agilent 6220 Accurate-Mass TOF LC/MS system. Column chromatography was performed with silica gel 60 (230–400 mesh, Merck KGaA, Darmstadt, Germany) and YMC-GEL ODS-A (12 nm, S-150 μm, YMC, Kyoto). TLC was carried out on silica gel 60 F_{254} and RP-18 $F_{254}S$ aluminum plates (0.2-mm thickness, Merck KGaA, Darmstadt, Germany) by visualization under UV light at 254 nm/365 nm and 10% (v/v) sulfuric acid in EtOH followed by heating. Isolation was performed by HPLC (Waters Corporation. U.S.A.) equipped with YMC-Pack ODS-AQ (10.0 × 250 mm: S-5 μm, 12 nm, YMC, Kyoto) and J'Sphere ODS-M80 (10.0 × 250 mm: S-4 μm, 8 nm) columns.

2.2 Plant material

The twigs and leaves of *E. umbellata* were purchased from the Gongryongnara farm in Goseong-gun, Gyeongsangnam-do, Korea and identified by J.-W. Nam, one of the authors. A voucher specimen (no. YNPC004) has been deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, Yeungnam University.

2.3 Extraction and isolation

The dried twigs and leaves of *E. umbellata* (1.9 Kg) were extracted with 70% acetone (3 × 10 L) for 72 hr. at room temperature. The solvent was evaporated under reduced pressure to yield the concentrated 70% acetone extract (31.0 g). This extract was suspended in deionized H_2O (600 mL) and sequentially fractionated with hexanes (5 × 600 mL), MC (3 × 600 mL), EtOAc (2×600 mL), and saturated *n*-BuOH (2 × 600 mL). A portion of the n-BuOH extract (4.0 g) was separated through NP open column by using MC-MeOH (100:0 to 0:100, a step gradient solvent system) to afford 18 fractions (Frc.1-Frc.18). Compound **1** was isolated from Frc. 6 (10.2

mg) by RP HPLC (YMC-Pack ODS-AQ column, S-5 μm, 12 nm, 10.0 × 250 mm, 35% MeOH in 65% acidified H_2O with 0.1% FA, 2 mL/min). A small portion (3.5 mg) of compound **1** was obtained from Frc. 4 (7.9 mg) and Frc. 5 (9.2 mg) with the same procedure. Compounds **2-5** were isolated from Frc. 15 by RP HPLC (YMC-Pack ODS-AQ column, S-5 μm, 12 nm, 10.0 × 250 mm, 38% CH_3CN in 62% acidified H_2O with 0.1% FA, 2 mL/min). Compound **6** was isolated from Frc. 14 by RP HPLC (J'Sphere ODS-M80 column, S-4 μm, 8 nm, 10.0 × 250 mm, 38% CH_3CN in 62% acidified H_2O with 0.1% FA, 2 mL/min). For compound **7**, another batch of *E. umbellata* twigs and leaves was extracted with 100% MeOH (3 × 48 L) for 3 days at room temperature. This extract was evaporated under reduced pressure to give 400 g of concentrated methanol extract. The methanol extract was separated by liquid-liquid extraction with deionized H_2O (1.5 L) and hexanes (1.5 L× 9), and the water layer was sequentially separated with MC (1.5 L × 2), EtOAc (1.5 L× 2), and saturated n-BuOH (1.5 L× 3) to yield 39 g of n-BuOH extract. The resulting n-BuOH extract was further fractionated through NP open column chromatography using MC-MeOH (100:0 to 0:100) to yield 23 subfractions. From these 23 fractions, fractions 14-16 were combined for the next step NP-open column chromatography using MC-MeOH (100:0 to 0:100) to give 14 fractions. Among the resulting 14 fractions, fractions 9-11 were combined and subjected to reverse phase open column chromatography by using MeOH- H_2O (20:80 to 100:0) to yield 22 fractions. From these 22 fractions, fraction 7 was injected in RP HPLC (YMC-Pack ODS-AQ column, S-5 μm, 10.0 × 250 mm, 22% CH_3CN in H_2O, 2 mL/min) to give compound **7** (0.8 mg).

*2.3.1. Data for compound **1***

Brownish powder; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; HR-ESIMS: *m/z* 247.1439 $[M+H]^+$ (calcd. 247.1441 for $C_{14}H_{19}N_2O_2$).

*2.3.2. Data for compound **2***

Yellowish powder; ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; HR-ESIMS: *m/z* 727.2079 $[M+H]^+$ (calcd. 727.2080 for $C_{32}H_{39}O_{19}$).

2.4. Acid hydrolysis

To determine the absolute configurations of sugars in compound **2**, a portion (0.5 mg) of compound **2** was hydrolyzed with 1 N HCl (200 μL) at 80°C for 2 hr. The hydrolysates were washed with EtOAc (200 μL × 3) to remove the aglycone. The resulting aqueous layer was dried and treated with p-isothiocyanate to prepare arylthiocarbamoyl thiazolidine derivatives. The reaction mixture was analyzed with RP HPLC system (Agilent Eclipse plus C18 column, 5 μm, 4.6 × 250 mm, 25% CH_3CN in H_2O, 0.8 mL/min, monitoring at 250 mm). Retention times of detected arylthiocarbamoyl thiazolidine derivatives were compared with those of authentic D-glucose, L-glucose, D-galactose, L-galactose, D-xylose, L-xylose and L-rhamnose (Sigma-Aldrich, St. louis, MO, USA). The retention times of hydrolysates and authentic sugar are as follows: D-galactose derivative (13.6 min), D-xylose derivative (18.3 min) and L-rhamnose derivative (28.1 min).

2.5. Cell culture

HaCaT human keratinocytes were from cell Lines Service Gmbh (Eppelheim, Germany). The HaCaT cells were cultured in Dulbecco's modified Eagles's medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/ streptomycin (HyClone), and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

2.6. Evaluation of cell viability by CCK assay

Cell viability was measured by cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay. CCK-8 allows very convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. CCK-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity using CCK-8 is higher than assays using other tetrazolium salts such as MTT, XTT, MTS or WST-1. HaCaT cells were seeded in 96-well plates at a density of 0.2×10^4 cell/well, incubated for 24 h, and subsequently treated with various concentrations of agents for 3 days. The solution was removed from each well and replaced with CCK-8, was added to each well and incubated at 37°C for 1 h. The absorbance wavelength was then measured at 450 nm using spectrophotometer.

2.7. Statistical Analysis

All results were expressed as mean ± s.d. Student's t-test was used for comparisons involving two groups.

3. Results and Discussion

Compound **1** was obtained as an amorphous brownish powder and showed a molecular ion peak at *m/z* 247.1439 $[M + H]^+$ (calcd. 247.1441) in the high-resolution electrospray ionization mass spectrometry (HR-ESIMS) spectrum, consistent with an elemental formula of $C_{14}H_{18}N_2O_2$.

The ^{1}H NMR spectrum (Table 1) of **1** showed resonances at δ_H 6.65 (dd, *J* = 8.7, 2.2 Hz, 1H, H-6), 6.93 (d, *J* = 2.2 Hz, 1H, H-4), and 7.15 (d, *J* = 8.7 Hz, 1H, H-7) indicating the presence of a trisubstituted benzene ring, which was determined to be part of a 3,5-disubstituted indole moiety based on heteronuclear multiple bond correlation (HMBC) correlations of H-2 (δ_H 7.00, s, 1H) with C-8 and C-9 (Fig. 2). Moreover, two methylene signals at δ_H 2.86 (t, *J* = 7.4 Hz, 2H, H-10) and 3.45 (t, *J* = 7.4 Hz, 2H, H-11) were observed. These were attributed to a 5-hydroxytryptamine (serotonin) moiety, based on the correlated spectroscopy (COSY) cross-peak of H-10/H-11 and the HMBC cross-peak of H-11 with C-3 (Fig. 2). In the ^{1}H and ^{13}C NMR spectra (Table 1), additional signals for an *n*-propyl moiety at δ_H/δ_C 0.91 (t, *J* = 7.3 Hz, 3H, H-16)/13.9 (C-16), 1.61 (h, *J* = 7.3 Hz, 2H, H-15)/20.4 (C-15), and 2.14 (t, *J* = 7.3 Hz, 2H, H-14)/39.1 (C-14) were observed, and were further confirmed by the COSY correlations of H-15 with both H-14 and H-16. A resonance for a carbonyl group appeared at δ_C 176.1 (C-13) in the ^{13}C NMR spectrum. Correlations of the signals at δ_H 3.45 (H-11) and 2.14 (H-14) with that at δ_C 176.1 in the HMBC spectrum revealed that the carbonyl group is located at C-13 to form a butyramide group. Thus, **1** was determined to be a serotonin derivative, *N*-[2-(5-hydroxy-1*H*-indol-3-yl)ethyl]-butanamide, which was isolated from a natural product for the first time in this study. Serotonin derivatives are rarely found in plants. Since they were first identified in safflower seeds, they have been found in various plant species and families. They not only act as phytoalexin in plants but also have various biological activities such as antioxidant, anticancer and skin whitening activities [29].

Compound **2** was isolated as an amorphous yellow powder and its molecular formula was determined to be $C_{32}H_{38}O_{19}$ based on the molecular ion peak $[M + H]^+$ at *m/z* 727.2079 (calcd. 727.2080) in the HR-ESIMS spectrum. In the ^{1}H NMR spectrum of **2**, resonances for a typical

AA'BB' spin system and an AB spin system were observed at δ_H 8.14 (d, J = 8.9 Hz, 2H, H-2', 6')/6.89 (d, J = 8.9 Hz, 2H, H-3', 5') and at δ_H 6.75 (d, J = 2.1 Hz, 1H, H-8)/6.45 (d, J = 2.1 Hz, 1H, H-6), respectively, suggesting that the aglycone moiety of **2** is a kaempferol [30]. The ^{1}H and ^{13}C NMR spectra (Table 1) revealed the presence of three anomeric protons at δ_H 5.57 (d, J = 1.2 Hz, 1H, H-1'')/δ_C 99.8 (C-1''), 5.45 (d, J = 7.6 Hz, 1H, H-1')/101.0 (C-1'), and 4.73 (d, J = 7.0 Hz, 1H, H-1''')/105.7 (C-1'''). Acid hydrolysis of **2** afforded D-galactose, D-xylose, and L-rhamnose, which were identified by comparing their retention times in high-pressure liquid chromatography (HPLC) analysis with those of standard sugars [31]. Furthermore, the anomeric configurations of the sugar units were established to be β-D-galactose, β-D-xylose, and α-L-rhamnose through their ^3J coupling constants (Gal, d, J = 7.6 Hz; Xyl, d, J = 7.0 Hz; Rha, d, J = 1.2 Hz) [32, 33]. The linkage positions of the galactose and rhamnose moieties were confirmed by HMBC cross-peaks between anomeric protons and aglycone carbons [δ_H 5.45 (H-1') and δ_C 135.3 (C-3); δ_H 5.57 (H-1'') and δ_C 163.4 (C-7)]. The 1→2 link between xylose and galactose was confirmed by the HMBC cross-peaks of δ_H 4.73 (H-1''') and δ_C 80.6 (C-2'-galactose) (Fig. 2). Thus, **2** was determined to be kaempferol-3-*O*-β-D-xylopyranosyl(1→2)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside, which had been previously isolated from *Trifolium lupinaster* [11]. However, this is the first report of the absolute configurations of these sugars.

The cell proliferative effects of each compound on human keratinocyte HaCaT cells were evaluated (Table 2) [34]. Because of its chemical similarity with the tested compounds, kaempferol (Sigma-Aldrich) was used for comparison in the present biological study. Cells were treated with the indicated concentrations of the selected compounds (**1**–**6**) or dimethyl sulfoxide (DMSO) alone (vehicle) and cell viabilities were compared after three days of incubation using cell counting kit-8 (CCK-8). Upon daily microscopic examination of the cells, no differences in

cell attachment or morphology between the compound-treated and control cells during incubation were observed. Therefore, compounds **1**–**5** promoted keratinocyte cell growth dose dependently. Among the six compounds, **3** and **4** showed relatively high activities at concentrations of 1, 5, and 10 μM and their proliferation activities were significantly greater than that of kaempferol. In particular, incubation with low doses (1 and 5 μM) of compounds **3** and **4** promoted HaCaT cell proliferation. The viabilities of HaCaT cells following incubation with **3** at concentrations of 1, 5, and 10 μM were 174.14%, 181.48%, and 193.94%, respectively. Similar dose-dependent growth promotion by **4** was also observed; the proliferative effects increased to 181.82%, 180.47%, and 197.98%, respectively. Growth promotion rates in HaCaT cells caused by compounds **1**, **2**, and **5** also showed significant increases at 10 μM (194.61%, 162.63%, and 194.70%, respectively).

In conclusion, compounds **1**–**5** showed proliferation activities for human keratinocyte HaCaT cells; in particular, **3** and **4**, at low doses, appeared to have the most potent proliferation activities compared to those of kaempferol and the other compounds. Therefore, these isolated compounds may be helpful in skin regeneration and wound healing in human skin and are also potential cosmetic materials.

Declaration of Competing Interest

The authors declare no conflict of Interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://.

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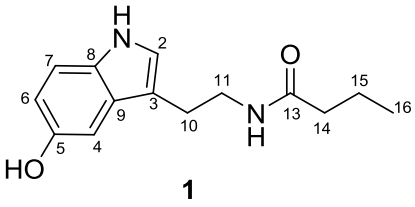
Figures and tables

Fig. 1. Chemical structures of compounds **1**–**7** **(**Rha = rhamnopyranoside; Gal = galactopyranoside; Xyl = xylopyranoside; Glc = glucopyranoside).



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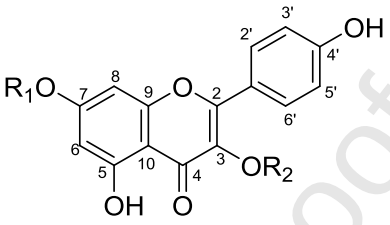


	R_1	R_2
2	Rha	$Gal^2 \rightarrow Xyl$
3	Rha	Gal
4	Rha	$Gal^6 \rightarrow Rha$
5	H	$Gal^2 \rightarrow Xyl$
6	H	$Glc^6 \rightarrow Rha$
7	Rha	$Gal^2 \rightarrow Glc$

Fig. 2. Key ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC correlations of compounds **1** and **2**.

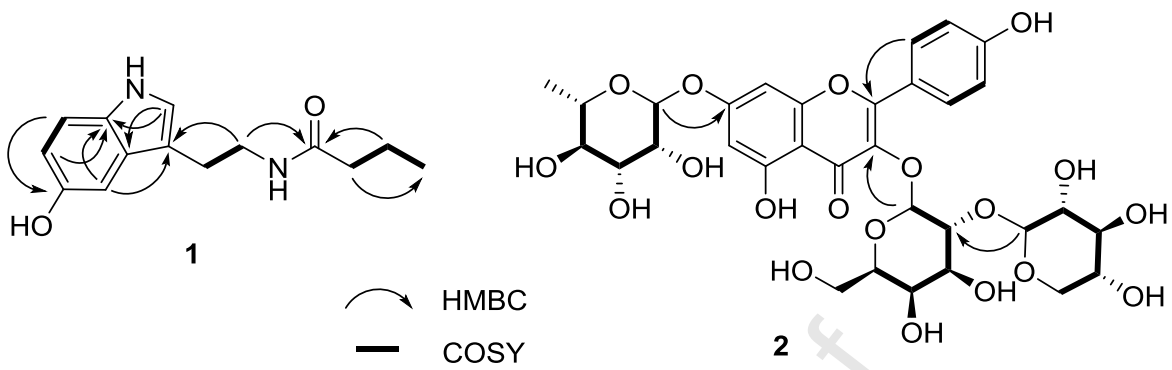


Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of compounds **1** and **2**.

Position	**1**		Position	**2**	
	δ_C	δ_H mult (*J* in Hz)	aglycone	δ_C	δ_H mult (*J* in Hz)
2	124.2	7.00 s	2	158.7	
3	112.5		3	135.3	
4	103.5	6.93 d (2.2)	4	179.9	
5	151.1		5	162.9	
6	112.4	6.65 dd (8.7, 2.2)	6	100.5	6.45 d (2.1)
7	112.6	7.15 d (8.7)	7	163.4	
8	133.1		8	95.4	6.75 d (2.1)
9	129.5		9	157.9	
10	26.4	2.86 t (7.4)	10	107.4	
11	41.3	3.45 t (7.4)	1'	122.6	
13	176.1		2'	132.5	8.14 d (8.9)
14	39.1	2.14 t (7.3)	3'	116.3	6.89 d (8.9)
15	20.4	1.61 h (7.3)	4'	161.7	
16	13.9	0.91 t (7.3)	5'	116.3	6.89 d (8.9)
			6'	132.5	8.14 d (8.9)
			Gal		
			1"	101.0	5.45 d (7.6)
			2"	80.6	3.97 dd (9.7, 7.6)
			3"	75.2	3.72 dd (9.7, 3.4)
			4"	70.3	3.82 dd (3.4, 1.0)
			5"	77.1	3.45 td (6.0, 1.0)
			6"	62.0	3.61 dd (11.4, 6.0) 3.56 dd (11.4, 6.4)
			Xyl		
			1"'	105.7	4.73 d (7.0)
			2"'	75.1	3.37 m
			3"'	77.3	3.38 m
			4"'	71.1	3.51 m
			5"'	66.8	3.24 dd (11.5, 9.7) /3.94 dd (11.5, 5.3)
			Rha		
			1""	99.8	5.57 d (1.2)
			2""	71.7	4.02 dd (3.3, 1.2)
			3""	72.1	3.84 dd (9.4, 3.3)
			4""	73.6	3.48 t (9.4)
			5""	71.3	3.59 dd (9.4, 6.1)
			6""	18.1	1.25 d (6.1)

Chemical shifts (δ) are expressed in ppm; *J* values are given in parentheses. Data were measured in CD_3OD.

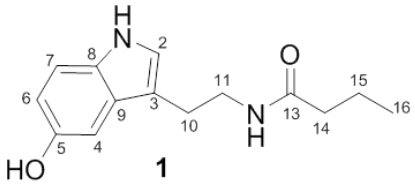
Table 2. Cell proliferative effects of **1-6** on HaCaT cells.

	Viable cells, %				
	DAY 0	DAY 3			
		Concentration of compound (μM)			
Compound		vehicle	1	5	10
Kaempferol[a]			158.25±1.07*	157.58±1.76*	193.27±2.36*
1			160.94±1.35*	161.62±1.67*	194.61±1.02*
2			153.87±0.84*	155.22±1.50*	162.63±0.67*
3	100.00±1.20	155.22±1.35	174.14±0.81*[#]	181.48±0.51*[#]	193.94±0.88*
4			181.82±1.00*[#]	180.47±1.17*[#]	197.98±2.19*[#]
5			144.11±1.02*	145.79±1.35*	194.70±0.79*
6			146.46±1.67*	142.69±0.40*	146.92±1.06*

[a] Positive control

* $P<0.01$ compared to 0 μM of each compound, [#] $P<0.01$ compared to kaempferol.

Leaves and Twigs of *Elaeagnus umbellata*



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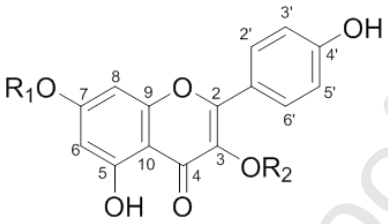

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Graphical abstract