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Biological Activities of Oleanolic Acid Derivatives from *Calendula officinalis* Seeds

Ahmed Zaki,[2,3] Ahmed Ashour,[1,2] Amira Mira,[1,2] Asuka Kishikawa,[1] Toshinori Nakagawa,[1] Qinchang Zhu[1] and Kuniyoshi Shimizu[1]*

[1]Department of Agro-environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[2]Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
[3]National Center for Natural Product Research, University of Mississippi, University, MS 38677, USA

Phytochemical examination of butanol fraction of *Calendula officinalis* seeds led to the isolation of two compounds identified as 28-*O*-β-D-glucopyranosyl-oleanolic acid 3-*O*-β-D–glucopyranosyl (1→3)-β-D-glucopyranosiduronic acid (CS1) and oleanolic acid 3-*O*-β-D–glucopyranosyl (1→3)-β-D-glucopyranosiduronic acid (CS2). Biological evaluation was carried out for these two compounds such as melanin biosynthesis inhibitory, hyaluronic acid production activities, anti obesity using lipase inhibition and adipocyte differentiation as well as evaluation of the protective effect against hydrogen peroxide induced neurotoxicity in neuro-2A cells. The results showed that, compound CS2 has a melanin biosynthesis stimulatory activity; however, compound CS1 has a potent stimulatory effect for the production of hyaluronic acid on normal human dermal fibroblast from adult (NHDF-Ad). Both compounds did not show any inhibitory effect on both lipase and adipocyte differentiation. Compound CS2 could protect neuro-2A cells and increased cell viability against H_2O_2. These activities (melanin biosynthesis stimulatory and protective effect against H_2O_2 of CS2 and hyaluronic acid productive activities of these triterpene derivatives) have been reported for the first time. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Calendula officinalis; triterpene; melanin; hyaluronic acid production; anti obesity.

INTRODUCTION

Calendula officinalis Linn. (Asteraceae) is used medicinally in Europe, China and India amongst several places in the world. It is also known as 'African marigold' and is widely cultivated as an ornamental and medicinal plant (Basch *et al*., 2006). It has been a subject of several chemical and pharmacological studies. It is used in traditional medicine, especially for wound healing, jaundice, blood purification and as an antispasmodic. *Calendula* flowers in European and western Asian folk medicines are used to treat inflammatory conditions of internal organs, gastrointestinal ulcers and dysmenorrhea and as a diuretic and diaphoretic in convulsions. In addition, it is used externally as natural medicine for inflammation of the oral and pharyngeal mucosa, wounds and burns (Tanaka *et al*., 2010). Previously published results indicated that *C. officinalis* posses multiple pharmacological activities including antiinflammatory and antioedematous (Ukiya *et al*., 2006), antioxidant (Matysik *et al*., 2005), immunostimulant (Wagner *et al*., 1985), wound healing (Leach, 2008), hepatoprotective (Lin *et al*., 2002), antibacterial and antifungal (Szakie *et al*., 2005) and antiviral (Silva *et al*., 2007).

Chemical studies have underlined the presence of various classes of compounds, the main being triterpenes, triterpene oligoglycosides, flavonoids, coumarines, quinones, volatile oil, carotenoids and amino acids have been obtained from the flowers (Muley *et al*., 2009). The flower extract of this plant, as well as pure compounds isolated from it, has been demonstrated to possess multiple pharmacological activities such as anti-HIV, cytotoxic, antiinflammatory, hypoglycemic, gastric emptying inhibitory, gastroprotective, hepatoprotective, spasmolytic and spasmogenic, amongst others (Preethi *et al*., 2006 and Preethi and Kuttan, 2009). *C. officinalis* seeds and butanol fraction are selected in order to isolate the more polar compounds as well as evaluation of its biological activities.

* Correspondence to: Kuniyoshi Shimizu, Department of Agro-environmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
E-mail: Japanshimizu@agr.kyushu-u.ac.jp

MATERIALS AND METHODS

Reagents. NaOH and DMSO were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) from Sigma (St. Louis, MO), EMEM from Nissui Chemical Co (Osaka, Japan). Other chemicals are of the highest grade commercially available.

Plant material. *C. officinalis* flowers were collected in April, 2013 from crops grown at Faculty of Pharmacy fields. The plant was identified by Prof. Ibrahim Mashaly, Systematic Botany Department, Faculty of Sciences, Mansoura University. The seeds were separated and dried. A voucher specimen (No. 1865) is kept in Pharmacognosy Department, Faculty of Pharmacy, Mansoura University.

Extraction and isolation procedures. Dried powdered seed (0.6 kg) was percolated with MeOH at room

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temperature. The combined extracts were collected and evaporated to dryness under reduced pressure at 40 °C. The residue, 90 g, was suspended in distilled water and extracted successively with methylene chloride, EtOAc and *n*-butanol. The different extracts were evaporated under reduced pressure to obtain methylene chloride fraction (fraction A, 25 g) and EtOAc fraction (fraction B, 20 g) and *n*-butanol fraction (fraction C, 15 g); the *n*-butanol fraction was selected for further study.

Isolation of compounds. Three grams of fraction **C** was dissolved totally in the smallest volume of methanol (1–2 mL) and then mixed well with about 3-g RP_{18} silica gel and left at room temperature to dry and applied onto the top of a silica gel packed (RP_{18}) glass column (120 g, 33 × 3.7 cm) pre-packed in water. Isocratic elution was carried out with MeOH – H_2O (6.5 : 3.5). The effluent was collected in 10-mL fractions monitored by TLC using MeOH – H_2O (8 : 2) as solvent system, and the developed TLCs were heated after spraying with vanillin/sulfuric acid spray reagent. Similar fractions were pooled together. After further purification through re-crystallization from hot methanol (60 °C), two main compounds were isolated as yellow plates; CS1 (310 mg) and CS2 (220 mg).

Cell line. A mouse melanoma cell line, B16 and mouse neuroblastoma cells neuro-2A were obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 0.09 mg/mL theophylline. Adult normal human dermal fibroblasts (NHDF-Ad, Lonza, Tokyo, Japan) were routinely maintained in 10% FBS in Dulbecco's modified Eagle's medium (DMEM). All of cultivation was conducted at 37 °C in a humidified atmosphere of 5% CO_2.

Melanin assay in B16 melanoma cell line. This assay was determined as described previously (Ashour *et al.*, 2013). The cells (1 mL) were placed in two plates of 24-well plastic culture plates (one plate for determining melanin and the other for cell viability) at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 µL of fresh media and 2 µL of the test sample at their maximum solubility ($n = 3$). At the same time, negative control (2-µL DMSO) and positive control, and arbutin at concentration 50 mg/mL in DMSO were tested. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed. To determine the melanin content (for one plate) after removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1 N NaOH. After overnight keeping in dark, the crude cell extracts were assayed by using a microplate reader at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture. On the other hand, cell viability was determined by using MTT assay which provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. So, for the other well plate, 50 µL of MTT reagent in PBS (250 µg/mL) was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37 °C for 4 h. After the medium was removed, 1.0-mL isopropyl alcohol (containing 0.04 N HCl) was added, and the absorbance was measured at 570 nm after overnight keeping in dark.

Hyaluronic acid (HA) ELISA assay on (NHDF-Ad). The isolated compounds were tested for the effect of the production of HA by the use of HA ELISA assay using Biotech Trading Partners (Encinitas, California) according to the manufacturer's instruction.

This kit is an enzyme-linked binding protein assay that uses a capture molecule known as HA binding protein (HABP). After growing of the fibroblast cells, they are incubated in HABP-coated micro well plate. Properly samples and HA reference solution are added to this plate, allowing HA present to react with the immobilized binding protein. After removal of unbound molecules by washing, HABP conjugated with horseradish peroxidase (HRP) solution is added to the microwells to form complexes with bound HA. Following another washing step, a chromogenic substrate of tetramethylbenzidine and hydrogen peroxide is added to develop a colored reaction. The intensity of the color is measured in optical density units with a spectrophotometer at 450 nm. The higher the intensity of the color, the higher ability of the sample to produce HA.

Anti-obesity activity. The pancreatic lipase inhibition activity of both compounds was measured by an *in vitro* enzyme reaction, which use 4-methylumbelliferyl oleate (4-MUO) as the substrate (Mizutani *et al.*, 2010). Each sample was dissolved in DMSO (400 and 800 µg/mL) and diluted in 25 µL of buffer solution consisting of 13 mM Tris–HCl, 150 mM NaCl and 1.3 mM $CaCl_2$ (pH = 8). These samples were applied to a 96-well plate. DMSO and Orlistat (final concentration 1 µM) were used as a negative control and positive control, respectively. Then, 50 µL of 0.25 mM 4-MUO (dissolved in buffer) was applied, followed by the addition of 25 µL of lipase enzyme (50 U/mL) to all wells except blank wells as well as sample blank wells. After plates were incubated for 30 min at 25 °C, 100 µL of 0.1 M sodium citrate buffer (pH 4.2) was added to each well to stop the reaction. The amount of 4-MUO released by the action of the lipase was measured with a fluorescence spectrophotometer (FlexStation 3 Microplate Reader, Molecular Devices, CA, USA) at excitation and emission wavelengths of 355 and 460 nm, respectively.

Adipocyte differentiation inhibition assay. The effects of the two isolated compounds on adipocyte differentiation were examined as previously described (Zhang *et al.*, 2012 and Wu *et al.*, 2013). Briefly, 3T3-L1 cells were seeded into collagen-coated 24-well plates (IWAKI, Tokyo, Japan) at a density of 0.8×10^5 cells/well. Two days after confluence, the medium was changed to differentiation medium containing 10% FBS, 10 µg/mL insulin (Wako, Tokyo Japan), 1-µM dexamethasone (Wako, Tokyo, Japan), and 0.5 mM 3-isobutyl-1-methylxanhine

(IBMX, Galbiochem, San Diego, CA, USA). At the same time, compounds or solvent control (DMSO) were added to the cells. Berberine was used as a positive control for differentiation inhibition (Huang *et al*., 2006). After 2 days incubation, the medium was replaced with post differentiation medium containing 10% FBS, 10 µg/mL insulin and corresponding samples. The medium was refreshed every 2 days until the 5th day after post differentiation medium was used.

Oil Red-O was used to stain the lipid droplet in the differentiated 3T3-L1 cells. The cells were washed with PBS twice, fixed with 10% formalin for 1 h and stained with 0.36% Oil Red-O (Sigma, St. Louis, MO, USA) in isopropanol–water solution at room temperature for 1 h. After washing with water for 4 times, the cells were photographed using a Nikon Microscope (TE200) at 10× magnification. Finally, the dye retained in the cells were dissolved with isopropanol containing 4% Nonidet P-40 and quantified by reading the absorbance at 570 nm with a microplate reader (Biotek-ELX800, BioTek, Winooski, Vermont, USA).

The toxicity of the samples to the 3T3-L1 cells was measured with the MTT method as described previously (Tanaka *et al*., 2014). Confluent 3T3-L1 cells in collagen coated 24-well plates were treated with samples for 72 h. And then 50 µL of MTT (5 mg/mL) was added to each well. After incubation for 4 h, the formazan crystals in the cells were dissolved with isopropanol, and the absorbance was read at 570 nm.

Protection against H_2O_2-induced neurotoxicity in neuro-2A cells. The assay was performed as we previously described (Mira *et al*., 2013). The concentration of H_2O_2 that could decrease cell viability of neuro-2A cells was first determined and then the ability of isolated compounds at non cytotoxic concentration (2.5, 5 and 10 µg/mL) to protect against H_2O_2-induced oxidative damage (150 µM) (Zhao *et al*., 2011) was determined. Catechin at 100 µM was used as a positive control.

Statistical analysis. The results are expressed as means ± standard deviation ($n = 3$). Data were analyzed for statistical significance using one-way ANOVA, followed by Dunnett's test as a post-hoc test with GraphPad Prism 5.0 software for Windows (Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Identification of isolated compounds

Compound CS1 (Fig. 1) was isolated as faint yellow plates, m.p. 270–272 °C. IR (KBr, υ_{max}) spectrum showed characteristic absorption bands for triterpene glycoside at 3445, 2927, 1634, 1457, 1379, 1071, 1043 cm^{-1} and absorption band at 1732 cm^{-1} ascribable to a carbonyl function (Pavia *et al*., 2001). The ^{1}H-NMR spectrum revealed singlet resonances at δ 0.79, 0.82, 0.90, 0.92, 0.94, 1.04 and 1.1, an olefin proton at δ 5.24 (*s*) and an oxygenated methine at δ 3.19 (*dd*, $J = 4.2, 11.4$); these signals are characteristic to triterpenes of olea-12-ene skeleton.

^{13}C-NMR spectrum revealed the presence of 48 resonances, two characteristic signals at δ 123.9 and 144.8 assignable for the aglycone of the oleanone skeleton. Twenty oxygenated carbons (Table 1) were detected; two of them are because of carboxy groups at δ 176.6 and 178.1, respectively (Agrawal, 1992 & Mahato and Kundu, 1994).

The DEPT-135 experiment helped in the differentiation of the 48 carbon resonances into 7 methyl, 12 methylene, 20 methine and 9 quaternary carbons. Within these resonances 7 methyl, 10 methylene, 5 methine and 8 quaternary carbons were attributed to the aglycon moiety with a characteristic olean-12-ene olefin bond at C-12, and a carboxy group at C-28, corresponding to oleanolic acid.

Upon acid hydrolysis of compound CS1 using 10% HCl and co-chromatography of the resulted sugar with the authentic sugar and sugar acid samples, using BuOH–AcOH–H_2O (5:1:4) as a solvent system: glucose and two acids, glucuronic acid and oleanolic acid, were liberated.

^{3}C-NMR spectra showed resonances for three sugar (18 signals) units with anomeric carbons at δ 95.7, 105.3 and 106.4. The up-field shifted anomeric carbon at δ 95.7 indicated the glycosylation of C-28 carboxy group (δ 178.1). This conclusion was confirmed by the correlation between the anomeric proton of this glucose moiety H-1′ at δ 5.4 (*d*, $J = 7.8$) and the C-28 at δ 178.1. The presence of carbon signal at δ 176.6 is assignable for C-6 of sugar acid: β-D-glucopyranosiduronic acid moiety. In the HMBC experiment of **CS1** (Fig. 2), long-range correlations were observed between the H-1′′-proton δ 4.3 (*d*, $J = 7.2$, δ_c 106.4) and the C-3

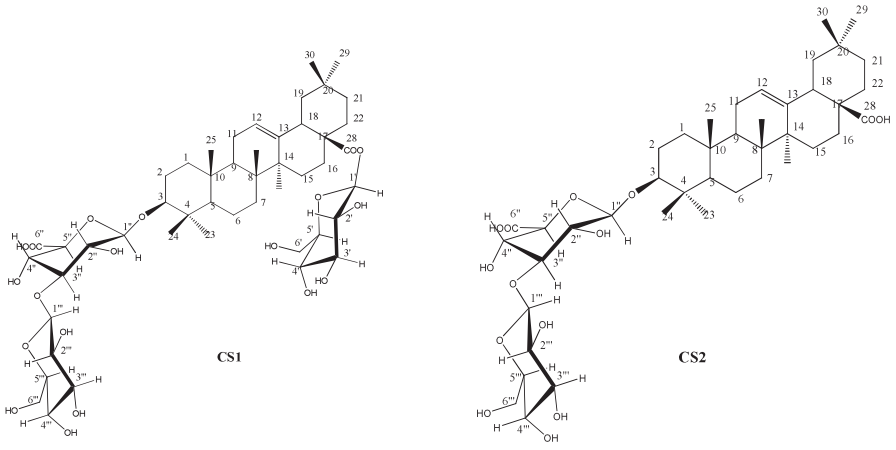

Figure 1. Chemical structure of **CS1** and **CS2.**

Table 1. ^{13}C and ^{1}H-NMR data of CS1 and CS2

cpd #	CS1		CS2	
	$^{13}C^{a}$	$^{1}H^{a}$	$^{13}C^{a}$	$^{1}H^{a}$
1	39.8	1.01, 1.58	39.8	
2	26.9	1.66, 1.88	26.9	
3	90.6	3.19, *dd*, *J* = 4.2, 11.4	90.8	3.18, *dd*, *J* = 4.2, 11.4
4	40.2	—	40.2	—
5	57.0	0.76	57.1	0.76
6	19.3	1.41, 1.52	19.4	
7	33.2	1.59, 1.68	34.2	
8	40.7	—	40.6	—
9	49.2	1.61	ND	
10	37.9	—	37.9	—
11	24.0	1.97, 2.1	24.6	1.97, 2.1
12	123.9	*br s*, 5.24	122.5	*br s*, 5.24
13	144.8	—	146.7	—
14	42.9	—	43.0	—
15	28.9	0.98, 1.07	29.3	
16	24.2	1.2, 1.4	24.6	
17	48.0	—	49.3	—
18	42.6	2.85, *dd*, *J* = 9.6, 13.8	43.5	2.9, *dd*, *J* = 9.6, 13.8
19	47.2	1.19, 1.72	48.2	
20	31.5	—	31.8	—
21	34.9	1.2, 1.37	35.3	
22	34.0	1.149, 1.08	34.4	
23	28.5	*s*, 1.04	28.5	*s*, 1.04
24	17.0	*s*, 0.828	17.0	*s*, 0.831
25	16.0	*s*, 0.942	16.0	*s*, 0.938
26	17.7	*s*, 0.794	18.2	*s*, 0.869
27	26.3	*s*, 1.14	26.5	*s*, 1.13
28	178.1	—	186.0	—
29	33.5	*s*, 0.928	34.0	*s*, 0.946
30	23.9	*s*, 0.901	24.4	*s*, 0.869
1′	95.7	5.4, *d*, *J* = 7.8		
2′	73.9	3.32		
3′	78.7	3.30		
4′	71.1	3.30		
5′	78.3	3.38, *m*		
6′	62.4	3.61, 3.81		
1″	106.4	4.3, *d*, *J* = 7.2	106.4	4.3, *d*, *J* = 7.8
2″	74.8	3.41, *m*	74.8	3.41, *m*
3″	86.1	3.60	86.1	3.60
4″	72.3	3.56	72.3	3.56
5″	77.3	3.58, *m*	77.2	3.58, *m*
6″	176.6	—	176.6	—
1‴	105.3	4.5, *d*, *J* = 7.8	105.3	4.5, *d*, *J* = 7.8
2‴	72.8	3.6	72.9	3.6
3‴	75.1	3.49, *dd*, *J* = 3, 9.6	75.2	3.49, *dd*, *J* = 3, 9.6
4‴	70.6	3.78	70.6	3.78
5‴	77.3	3.58, *m*	77.3	3.58, *m*
6‴	62.7	3.61, 3.81	62.7	3.61, 3.81

[a] ^{13}C and ^{1}H are measured in CD_3OD at 150 MHz and 600 MHz, respectively. N.D.: non detectable.

(δ_c 90.6), between H-1‴ δ 4.5 (*d*, *J* = 7.8, δ_c 105.3) and C-3″ (δ 86.1), which indicated the glycosidation of C-3 with the glucuronic acid with additional glucose linked to its C-3″. This linkage was confirmed through the downfield shift of C-3″ at δ 86.1. The full assignments of ^{1}H- and ^{13}C-NMR (Table 1) spectra were facilitated by comparison with those of oleanolic acid glycosides (Gohar *et al*., 2002) and further confirmed by HSQC, HMBC (Fig. 2) and COSY spectra. Accordingly, compound **CS1**, was concluded to be 28-*O*-β-D-glucopyranosyl-oleanolic acid 3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucopyranosiduronic acid. Further confirmation was done by comparing the data with those in the literature (Liang *et al*., 2010).

Compound CS2 was isolated as faint yellow plates, m. p. 262–264 °C. The ^{1}H- and ^{13}C-NMR data of this compound showed close similarity to **CS1**. The ^{13}C-NMR spectra showed the absence of the anomeric carbon at δ 95.7 and the other oxygenated carbon signals at δ

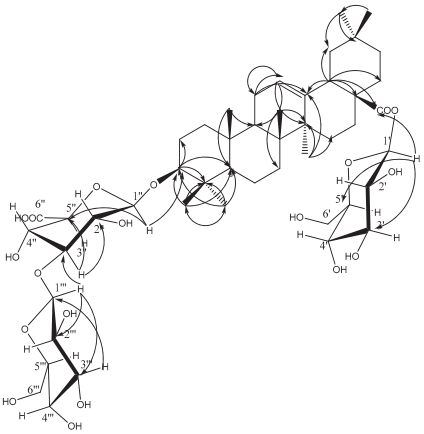

Figure 2. HMBCs of **CS1** and **CS2**.

73.9, 78.7, 71.1, 78.3 and 62.4, which indicated the liberation of C-28 glycosidation. Therefore, the downfield shift of C-28 from δ 178.1 to δ 186.1. Furthermore, ^{1}H-NMR spectrum revealed the disappearance of corresponding resonances anomeric proton at δ 5.4.

Compound **CS2** was further confirmed by HSQC, HMBC and COSY spectra. Accordingly, compound **CS2** was concluded to be oleanolic acid 3-O-β-D-glucopyranosyl(1→3)-β-D-glucopyranosiduronic acid.

B16 melanoma cell line assay

Compound CS2 showed melanin biosynthesis stimulatory effect based on cell viability as shown in Fig. 3. Compound CS1 did not show any activity at any tested concentration. Results are shown in Fig. 3.

HA production assay in fibroblast

Scientific studies have shown that HA improves skin hydration, stimulates production of collagen in skin, works as an antioxidant and free radical scavenger, maintains skin elasticity, cushions joints and nerve tissues, has an antibacterial and antiinflammatory activity and maintains the fluid in the eye tissues, which may help to protect against numerous possible eye concerns (Necas *et al.*, 2005).

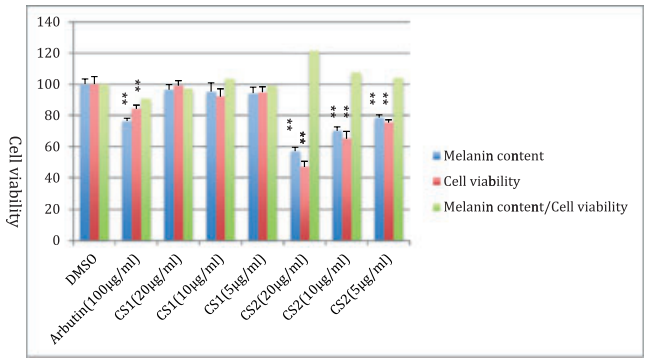

Figure 3. Inhibitory activity of compounds (CS1 and CS2) on melanin synthesis in B16 melanoma cell. The values are represented as the mean ± standard deviation (SD), $n = 3$. Significant difference from the control value and each compound was determined by Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

First, sample concentration that showed no cell toxicity to NHDF-Ad (normal-human dermal fibroblast from adult skin) after 48-h incubation with sample was determined by MTT assay. HA production-promoting effect on NHDF-Ad was evaluated at concentration of the samples that showed no cell toxicity. The amount of HA produced by NHDF-Ad was measured using an ELISA kit. Previously, NHDF-Ad cells (2.0×10^4 cells/well) were cultured in a 96-well plate with 100 μL of DMEM supplemented with 10% FBS. After 24-h incubation, test samples [0.5 μL/well, DMSO as a control, ascorbic acid (17.6 μg/mL) as a positive control] were added to the cells soon after the medium was replaced with 100 μL of DMEM supplemented with 0.5% FBS. After 48-h incubation, the HA amount produced by NHDF-Ad in the supernatant was quantified by using a human HA ELISA kit (ACEL, Japan). A standard curve was made in the same ELISA plate ($n = 3$). On the other hand, the cell viability of the remaining cells was measured by MTT assay ($n = 3$). The statistical difference between the control and each sample was determined by Student's *t*-test.

It was found that compound CS1 at both tested concentration has significant stimulating effect for the production of HA and at the same time it showed no toxicity to the cell. However, compound CS2 did not show any stimulating effect on the production of HA at both tested concentration. Results are shown in Fig. 4.

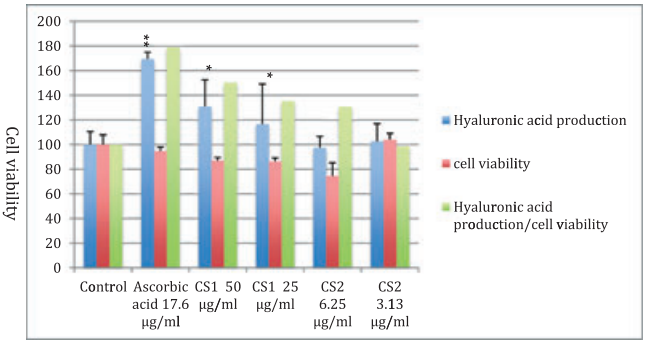

Figure 4. Effect of isolated compounds (CS1 and CS2) on the production of hyaluronic acid. The values are represented as the mean ± standard deviation (SD), $n = 3$. Significant difference between 1% DMSO and each concentration was determined by Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

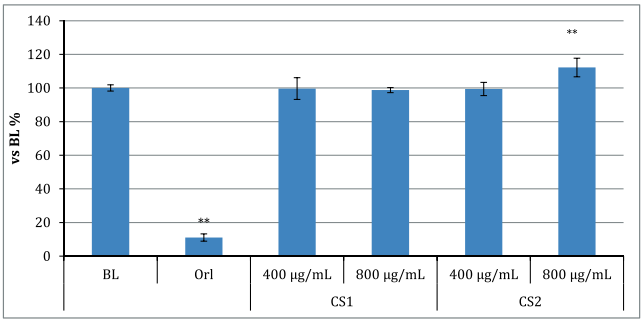

Figure 5. Inhibition effect of isolated compounds (CS1 and CS2) on pancreatic lipase. The values are represented as the mean ± standard deviation (SD), $n = 3$. Significant difference between 1% DMSO and each concentration was determined by Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

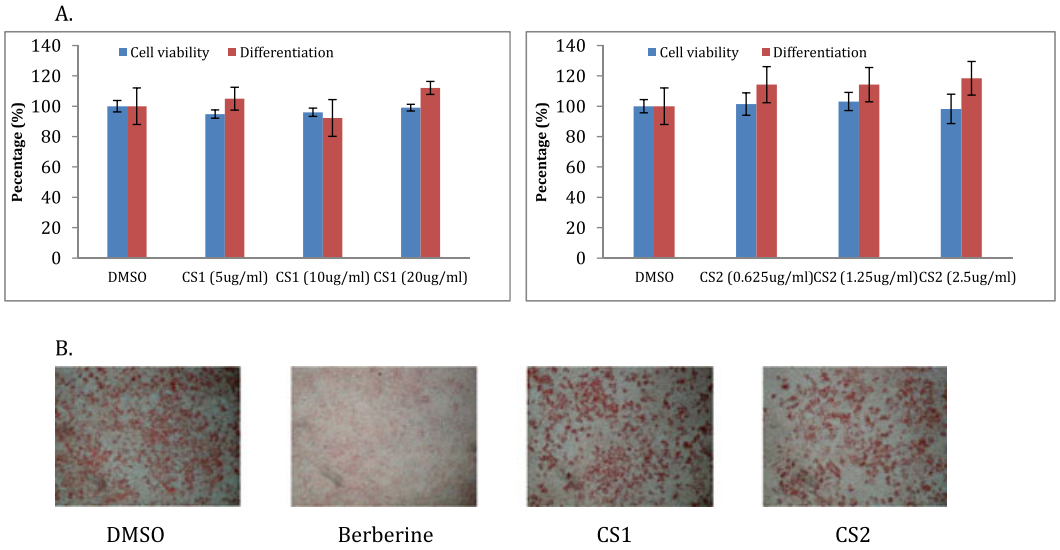

Figure 6. Inhibition effect of isolated compounds (CS1 and CS2) on 3T3-L1 adipocyte differentiation. A, the lipid droplet formation in 3T3-L1 cells were stained with oil Red-O. B, Relative 3T3-L1 differentiation was calculated from the quantification of stained lipid content by measuring absorbance at 570 nm. Values are represented as the mean ± standard deviation (SD), $n = 3$. Significant difference between 1% DMSO and each concentration was determined by Student's *t*-test: $*P < 0.05$, $**P < 0.01$.

Anti-obesity activity

Excessive gastrointestinal absorption of fats and adipogenesis are two major causes of obesity. To evaluate the potential anti-obesity effect of both isolated compounds, both the lipase and adipocyte differentiation inhibition activity were investigated for them.

For the *in vitro* lipase assay, the clinically used anti-obesity drug orlistat (0.5 µg/mL) was used as positive control and was found to inhibit the activity of pancreatic lipase by 88.9% (Fig. 5); however, it was found that both compounds have no effect on lipase inhibition. These results are not consistent with De Meloa *et al.*, 2010 which showed a significant effect for oleanolic acid for decreasing the obesity. These results suggest that the anti obesity effect may need the presence of free hydroxyl and carboxyl group of the oleanolic acid moiety.

The mouse 3T3-L1 cell line is a well characterized cell line for adipogenic assay. 3T3-L1 preadipocyte cells show fibroblastic phenotype in normal condition and adipocyte phenotype after treatment with differentiation media. This assay was run parallel with anti-lipase assay as both compounds were subjected to the adipocyte differentiation assay at the concentration without significant cytotoxicity. Results showed that both compounds did not show any inhibitory effect, but it showed that compound CS2 has stimulating effect on lipase at concentration 800 µg/mL. Results are shown in Fig. 6. The results of this assay comply with the result of anti-lipase.

Protection against H_2O_2-induced neurotoxicity in neuro-2A cells

H_2O_2 (150 µM) induced neurotoxicity in neuro-2A cells and caused 47.5 ± 2.3% decrease in cell viability (Fig. 7). While compound CS1 did not show any neuroprotection, compound CS2 showed dose-dependent increase in cell viability. CS2 could protect neuro-2A cells and increased cell viability by 9.4 ± 7.6 and 20.4 ± 3.4% at 5 and 10-µM concentration. These results confirm that glycosidation at C-28 decreases the activity, and the presence of free carboxylic group is necessary for the activity.

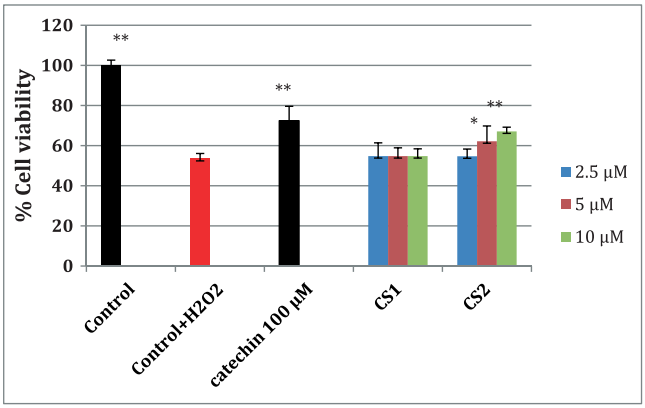

Figure 7. Neuroprotection against H_2O_2-induced neurotoxicity in neuro-2A cells by isolated compounds. Values are represented as means ± standard deviations (SD), $n = 5$. * and ** Significant difference from control in (A) and from cell viability of H_2O_2 (150 µM) treatment in (B, C, D) at $P < 0.05$ and $P < 0.01$, respectively.

CONCLUSION

In our ongoing study to find biologically active compounds from medicinal plants, two compounds were isolated from the butanol faction of *C. officinalis* seeds identified as 28-*O*-β-D-glucopyranosyl-oleanolic acid 3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucopyranosiduronic acid (**CS1**) and oleanolic acid 3-*O*-β-D-glucopyranosyl (1→3)-β-D-glucopyranosiduronic acid (**CS2**). Biological evaluation was carried out for these two compounds. It was found that compound **CS2** has a melanin biosynthesis stimulatory activity and neuroprotection against H_2O_2 induced-neurotoxicity; however, compound **CS1** has a potent stimulatory effect for the production of HA on

normal human dermal fibroblast from adult (NHDF-Ad). Both compounds did not show any inhibitory effect on both lipase and adipocyte differentiation that are not consistent with the published result which showed a significant effect for oleanolic acid for decreasing the obesity. These results suggest that the anti obesity effect may need the presence of free hydroxyl and carboxyl group of the oleanolic acid moiety. It is worth to mention that these activities were done for the first time for these triterpene derivatives.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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