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Bioassay-guided isolation and identification of anti-obesity phytochemicals from fruits of *Amomum tsao-ko*

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Abstract

Amomum tsao-ko (Zingiberaceae), an important traditional medicinal herb, possesses many biological activities, including anti-inflammatory effects. Though the anti-obesity properties of the crude ethanol extract of *A. tsao-ko* fruits have been reported, the anti-adipogenic properties of its phytochemical constituents have not been reported. Therefore, in the present study, we isolated the active constituents of *A. tsao-ko* and investigated their anti-adipogenic effects. The bioassay-guided isolation of the phytochemicals from the ethanol extract of *A. tsao-ko* fruits identified four bioactive compounds, comprising one fatty acid (**1**), one sesquiterpene alcohol (**2**), and two phenolic compounds (**3** and **4**). Their structures were elucidated by a combination of 1D and/or 2D nuclear magnetic resonance and mass spectrometry. The anti-adipogenic activities of the four compounds evaluated by Oil Red O staining in 3T3-L1 cells revealed that the treatment with the isolated compounds **1** and **3** reduced the lipid accumulation in 3T3-L1 adipocytes more strongly than the compounds **2** and **4**, in a dose-dependent manner.

Keywords: *Amomum tsao-ko*, Adipocyte differentiation, Oil red O staining, Phytochemicals, 3T3-L1 cells

Introduction

Obesity, the most common metabolic disease, is defined as a state of excess body fat accumulation that results from energy imbalance [1]. It is a serious public health problem implicated in various pathological disorders such as hypertension, type II diabetes, cardiovascular diseases, and certain cancers [2]. Generally, obesity involves the abnormal growth of adipocytes characterized either by hypertrophy (increase in cell size) or hyperplasia (increase in cell number) of fat-storing cells, and both are considered as therapeutic targets for the treatment of obesity [3]. The preadipocytes are the precursors of adipose depots that undergo complete differentiation into mature adipocytes. Therefore, it was hypothesized that the suppression of adipogenesis, which is the development of mature adipocytes from fibroblastic preadipocytes, could reduce the accumulation of adipose tissue mass. Supporting this, Yun, using the 3T3-L1 cell lines, has shown that a reduction in the proliferation and differentiation of preadipocytes and an increase in triglyceride lipolysis in adipose tissue reduced the adipose lumps [4]. The 3T3-L1 cell lines are the preadipocyte cell lines derived from mouse embryonic fibroblasts and are the most commonly used in the study of adipogenesis and adipocyte differentiation in assay models in vitro [4]. Therefore, inhibition of fat accumulation by suppressing adipocyte differentiation is considered a key factor determining the potential of the anti-obesity agents.

Herbal extracts from plants, such as *Moringa oleifera* [5], *Melissa officinalis* [6], *Vitis thunbergii* var. *taiwaniana* [7], *Pleurotus citrinopileatus* [8], *Nelumbo nucifera* [9], and *Melandrium firmum* [10] have been reported to possess anti-obesity effects. In addition, several studies

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using the 3T3-L1 adipocyte cell lines have reported the anti-obesity and/or anti-adipogenic effects of different bioactive compounds, including isoflavone glycosides [11], phytosterols [12], oleane-type saponins [13], and cycloartane triterpene glycosides [14]. These studies indicated that crude drugs and natural products having the potential to reduce lipid accumulation would help solve the obesity problem worldwide.

Amomum tsao-ko Crevost et Lemaire (Zingiberaceae) is a perennial herb known for its use as a food additive (traditional Chinese spice) and in medicine [15, 16]. Besides being an important spice, its dried fruit is commonly used to treat abdominal pain, dyspepsia, malaria, nausea, throat infections, stomach disorders, vomiting, and diarrhea in traditional medicine systems [17]. Recently, the fruits of *A. tsao-ko* have attracted attention as a functional food because of their various pharmacological properties [18–22]. In addition, diarylheptanoids and flavonoids, the major constituents of *A. tsao-ko,* have also demonstrated antioxidant, antitumor, anti-inflammatory, α-glucosidase inhibitory activities, neuroprotective effects, and nitric oxide (NO) inhibitory effects [23–25].

As a part of an ongoing effort to discover naturally occurring anti-obesity agents from medicinal herbal extracts, our preliminary experiment revealed the potential of the ethanol extract of the fruits of *A. tsao-ko* in suppressing lipid accumulation in 3T3-L1 adipocytes [26]. However, its bioactive constituents responsible for the anti-obesity effects have not been investigated. Therefore, in this study, we aimed to isolate the active phytochemical constituents from the ethanol extract of *A. tsao-ko* fruit through bioassay-guided fractionation. In addition, we also aimed to elucidate the structures of the isolated compounds and evaluate their anti-adipogenic properties.

Materials and methods

Sample materials

The fruits of *A. tsao-ko* were purchased from the Gyeongdong Oriental Medicine Market, Seoul, South Korea in February 2012, and identified by Professor Joa Sub Oh, College of Pharmacy, Dankook University, Cheonan, South Korea. A voucher specimen (G47) was deposited at the Natural Product Chemistry Laboratory, Bio-center, Gyeonggido Business & Science Accelerator, Suwon, Korea.

Reagents and instruments

Optical rotations were measured using a JASCO P-2000 polarimeter (Tokyo, Japan). 1D (^{1}H, ^{13}C, and DEPT) and 2D (^{1}H-^{1}H COSY, HSQC, and HMBC) nuclear magnetic resonance (NMR) spectra were measured on a Bruker Ascend III 700 spectrometer (Bruker BioSpin GmbH., Rheinstetten, Germany) with tetramethylsilane as an internal standard, and chemical shifts were expressed as δ values. Electrospray ionization mass spectra (ESI–MS) were obtained on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc., MA, USA). Gas chromatography–mass spectrometry (GC–MS) was carried out using a GCMS-QP2010 Ultra gas chromatograph interfaced with a single quadrupole QP-5000 mass spectrometer (Shimadzu Scientific Instruments Pty. Ltd., Tokyo, Japan). Preparative high-performance liquid chromatography (HPLC) was performed on a Shimadzu Prominence HPLC system with an LC-8A pump, SPD-20A UV/VIS detector, and YMC-Pack ODS A column (250 × 20 mm I.D.), using a mixed solvent system of MeCN: Water at a flow rate of 12 mL/min. Medium-pressure liquid chromatography (MPLC) was conducted using a CombiFlash Rf flash chromatography system (Teledyne ISCO Inc., NE, USA), and the separations were performed on a Redi*Sep*® Rf C_{18} and silica gold column. Open column chromatography was performed using silica gel (Kieselgel 60, 70–230 mesh, Merck KGaA, Darmstadt, Germany), and thin-layer chromatography (TLC) was performed using a pre-coated silica gel 60 F254 (0.25 mm, Merck KGaA, Darmstadt, Germany). All chemicals and solvents were of analytical grade and were used without further purification.

Extraction and isolation

The dried fruits of *A. tsao-ko* (5 kg) were extracted with 80% aqueous ethanol (3 × 18 L) at room temperature for 2 days and filtered. The extracts were combined and concentrated under reduced pressure to obtain a brown residue (219 g). The concentrate was suspended in H_2O (5 L) and partitioned with EtOAc (3 × 5 L). The EtOAc-partitioned sections (30 g) was separated by silica gel column chromatography and eluted with a gradient solvent system of *n*-hexane–acetone. The eluent was combined based on the TLC analysis to obtain nine fractions ($ATF_1 - ATF_9$). The solvent extracts and subfractions ($ATF_1 - ATF_9$) of *A. tsao-ko* fruits were screened for the adipogenesis inhibition assay at 12.5, 25, and 50 μg/

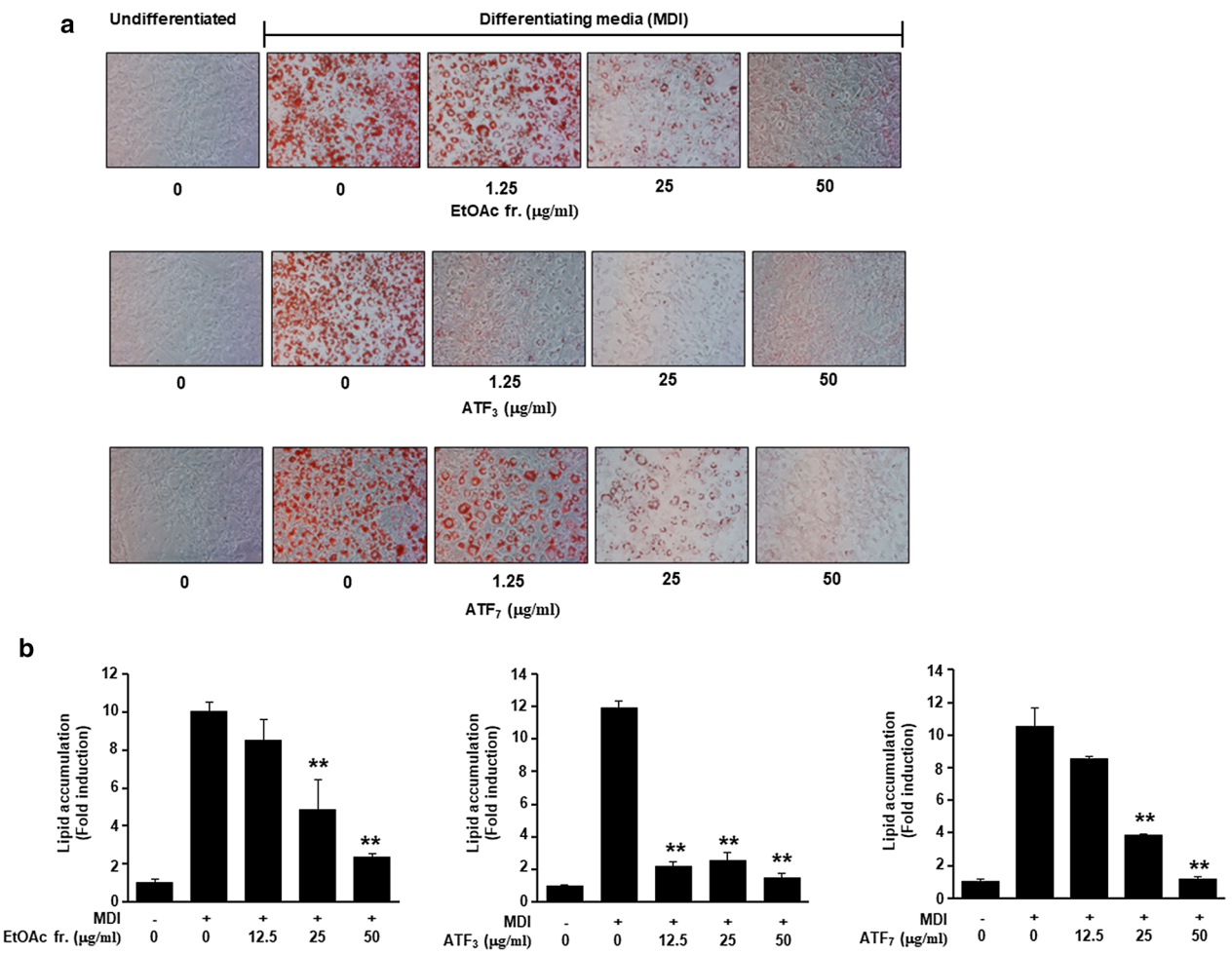

Fig. 1 Anti-adipogenic effect of *A. tsao-ko* EtOAc extract and subfraction (ATF_3 and ATF_7). 3T3-L1 cells were treated with various concentrations of EtOAc extract and subfraction from start of adipocyte differentiation (day 0) until eight day. **a** The representative Oil Red O staining images of differentiated adipocytes on day 8. **b** Quantification of Oil Red O staining levels. Values are means ± SD of 3 experiments. **$p < 0.01$, (compared with MDI-treated cells)

mL concentrations. Among them, EtOAc extract, subfractions ATF3 and ATF7 were found to the decrease in % lipid content was in concentration dependent manner (Fig. 1a, b). Of these, the fraction, ATF_3 (3.5 g) was subjected to MPLC over ODS eluting with a step gradient of 60–100% MeOH in H_2O to give nine subfractions ($ATF_{3\text{-}1} - ATF_{3\text{-}9}$). Purification of the subfraction $ATF_{3\text{-}3}$ by preparative HPLC (MeCN: H_2O, 75:25 to 100:0, 40 min) yielded compound **3** (10.2 mg), and the purification of the subfraction $ATF_{3\text{-}2}$ with 80% MeCN in H_2O yielded compound **4** (7.8 mg). Fraction ATF_7 was separated using MPLC (RediSep® Rf silica gold 120 g, 80 mL/min, a gradient system of CH_2Cl_2 in MeOH, 100 min), and fractionated to 12 subfractions ($ATF_{7\text{-}1} - ATF_{7\text{-}12}$). Purification of the subfractions $ATF_{7\text{-}3}$ (MeCN: H_2O, 25:75 to 70:30) and $ATF_{7\text{-}6}$ (MeCN: H_2O, 20:80 to 70:30) by preparative HPLC yielded compound **1** (17.2 mg) and compound **2** (5.8 mg), respectively. The purity of each compound was verified to be > 95% by HPLC.

Cell culture and induction of differentiation

3T3-L1 preadipocyte cells were purchased from the American Type Culture Collection (CL-173, ATCC, Manassas, VA, USA). Cells were maintained in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Two days after the cells reached confluence, they were induced with initiation media (1 μM M dexamethasone (Calbiochem, Billerica, MA, USA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Calbiochem), and 1 μg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA) in 10% fetal bovine serum (FBS; ATCC)-DMEM. On day 2, the initiation medium was replaced with progression media (1 μg/mL insulin in 10% FBS-DMEM). On days 4 and 6, the progression medium was replaced with the maintenance media (10% FBS-DMEM). From days 0 to 8, the cells were treated with the effective nontoxic concentrations of the isolated compounds, identified through the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell viability assay

The MTT assay was used for in vitro determination of cell proliferation in 3T3-L1 cells. Cells were plated at a density of 5×10^3 cells per well in 100 μL culture medium. One day after plating, a time-zero control plate was prepared. The isolated compounds (**1**–**4**) were applied directly, and the cells were incubated for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C. Cell proliferation was then determined. MTT (5 mg/mL in phosphate-buffered saline [PBS]) was added to each well, followed by incubation for 3 h. The media was removed from the wells by aspiration, and 0.1 mL of buffered dimethyl sulfoxide was added to each well, after which the plates were shaken. Subsequently, the absorbances were measured on a SpectraMax 190PC microplate reader at 540 nm (Molecular Devices, Sunnyvale, CA, USA).

Oil red O staining

On day 8, after induction of differentiation, the cells were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min, and then washed twice with PBS and 70% ethanol. The cells were then stained with 0.35% Oil Red O (Sigma-Aldrich) in isopropyl alcohol/distilled water for 30 min. Excess stain was removed by washing with 70% ethanol and PBS. Stained lipid droplets were dissolved in isopropyl alcohol containing 4% Nonidet P-40 (Sigma-Aldrich) and quantified using an enzyme-linked immunosorbent assay reader (SPECTRAmax 190PC; Molecular Devices, Sunnyvale, CA, USA) at 510 nm.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The results were analyzed for statistical significance using Student's *t*-test and one-way analysis of variance. Values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered statistically significant.

Results and discussions

Earlier, we have shown that the ethanol extract of *A. tsao-ko* fruits inhibited the adipocyte differentiation in 3T3-L1 preadipocytes in a dose-dependent manner [26]. Therefore, in the present study, we analyzed the ethanol extract of *A. tsao-ko* fruits and isolated the phytochemical constituents. The ethanol extract was suspended in H_2O and successively fractioned into the ethyl acetate fraction. The ethyl acetate-soluble fraction was further subjected to repeated column chromatography to yield four compounds (**1** − **4**) (Fig. 2). The isolated compounds were identified as methyl linolenate (**1**) [27], *trans*-nerolidol (**2**) [28], catechol (**3**) [29], and phloroacetophenone 2′-*O*-glucoside (myrciaphenone A, **4**) [30] by MS analysis and further comparison with previously reported NMR spectroscopic data (Fig. 2 and Additional file 1: pages S3–S5). The quantitative analysis of the isolated compounds (**1**–**4**) by HPLC–PDA estimated the contents of compounds **1**–**4** with values of 1.89 ± 0.04, 1.68 ± 0.02, 0.86 ± 0.01, and 0.73 ± 0.01 g/100 g of the EtOH extract weight, respectively (Table 1). According to our quantitative analysis, compounds **1** and **2** were the predominant component in the fruits of *A. tsao-ko* (see Additional file 1: page S7). In previous studies based on the biological activities of the isolated compounds, it has been shown that **1** reduces the expression of microphthalmia-associated transcription factor and tyrosinase-related proteins [31]. Compound **2**, a sesquiterpene alcohol, has been shown to reduce the adhesion of HT29 cells to collagen and suppress the adhesion of HT29 cells in the presence of TNF-α cytokines [32]. It has also been shown to induce apoptosis by decreasing the phosphorylation of NF-κB and increasing the activity of caspases [32]. In addition, it also exhibits anti-malarial and insecticidal

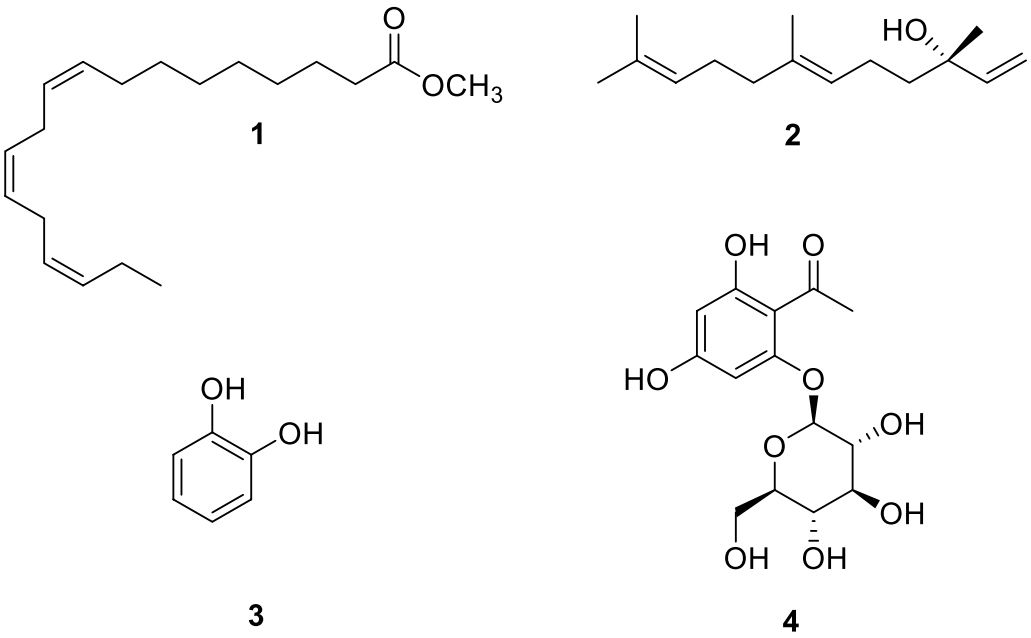

Fig. 2 Chemical structures of compounds **1**–**4** identified from the fruits of *A. tsao-ko*

Table 1 Contents (g/100 g of EtOH extract weight) of the isolated compounds (1–4) in the EtOH extract of *A. tsao-ko*

Compound	Content[a]
1	1.89 ± 0.04
2	1.68 ± 0.02
3	0.86 ± 0.01
4	0.73 ± 0.01

[a] Data shown are the mean of three replicated measurements

activities [33, 34]. Compound **4**, a phenolic glycoside, exhibited anti-leishmanial activity against promastigotes of *Leishmania donovani* [35]. However, the regulatory effects of the isolated compounds on adipocyte differentiation have not been reported to date.

In the present study, the cell viability was estimated by the MTT assay; the assay was used to determine the non-toxic concentrations of the compounds **1**–**4** by treating 3T3-L1 cells with various concentrations (12.5–200 μM) of these compounds. The results revealed that none of the compounds were cytotoxic at their effective concentrations (see Additional file 1: Figure S3); therefore, we treated the cells with compounds at this concentration for further analysis. The anti-adipogenic activities of the isolated compounds **1**–**4** were evaluated by assessing the fat accumulation in 3T3-L1 cells treated with the compounds. NAC (*N*-Acetyl-L-cysteine) was used as a positive control [36, 37]. During differentiation (days 0–8), the preadipocyte cells were differentiated into mature adipocytes with cytoplasmic lipid vesicles. Furthermore, the Oil Red O staining on day 8 of differentiation and the quantification of the lipid contents revealed that the levels of Oil Red O were significantly lower in cells treated with compounds **1** and **3** than in cells treated with compounds **2** and **4** (Fig. 3a). The isolated compounds inhibited the differentiation of the 3T3-L1 cells in a concentration-dependent manner with a lower percentage of differentiated cells at a higher concentration (Fig. 3b). In addition, the inhibitory effect of compounds **1** and **3** was relatively higher than the compounds **2** and **4**, and at a concentration of 50 μM, the compounds **1** and **3** exerted 20–50% inhibitory effect compared to control (MDI-treated cells). These findings indicated that the bioactive compounds **1** and **3** had a stronger potential to exert anti-adipogenic effects. The active constituents can be further studied for their exact mechanism of action as

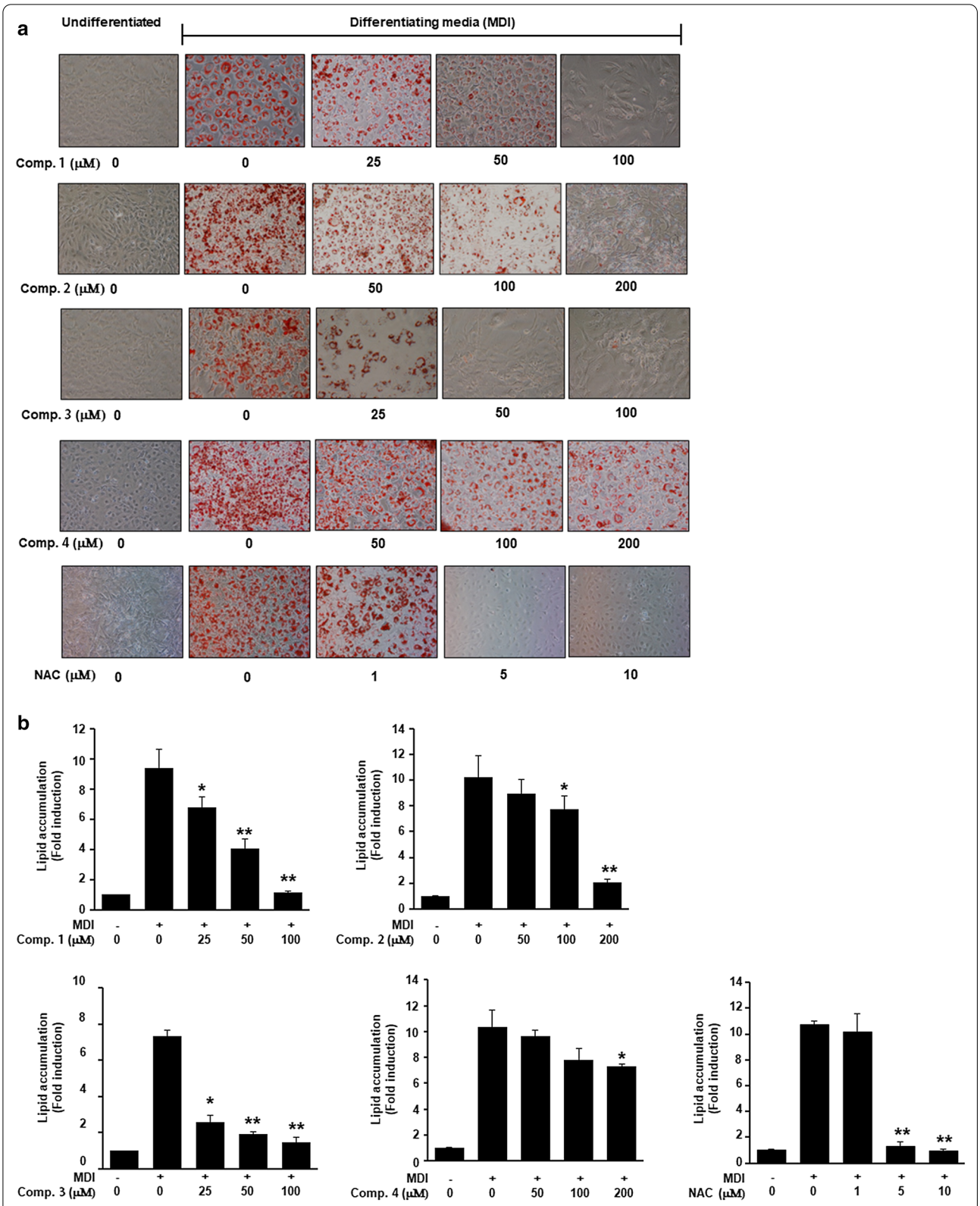

Fig. 3 Effect of isolated compounds from the fruits of *A. tsao-ko* on lipid accumulation during adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were treated with various concentrations of compounds **1**–**4** from start of adipocyte differentiation (day 0) until eight day. NAC (N-Acetyl-L-cysteine, Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control. **a** The representative Oil Red O staining images of differentiated adipocytes on day 8. **b** Quantification of Oil Red O staining levels. Values are means ± SD of 3 experiments. *$p < 0.05$, **$p < 0.01$, (compared with MDI-treated cells)

well as they can be tested in the in vivo obesity models. These biological results provide credible evidence supporting the role of the phytochemicals isolated from A. tsao-ko in regulating adipocyte differentiation, rendering A. tsao-ko as a potential source for the development of functional ingredients to treat obesity-related ailments.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-020-00576-0.

Additional file 1: Additional figures.

Abbreviations

COSY: Correlated spectroscopy; DEPT: Distortions enhancement by polarization transfer; DMEM: Dulbecco's modified Eagle's medium; HMBC: Heteronuclear multiple bond correlation; HPLC: High-performance liquid chromatography; HSQC: Heteronuclear single quantum coherence spectroscopy; MPLC: Medium-pressure liquid chromatography; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC: *N*-Acetyl-L-cysteine; NMR: Nuclear magnetic resonance; PBS: Phosphate-buffered saline; SD: Standard deviation; TLC: Thin-layer chromatography; MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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Authors' contributions

Performed the experiments: CWC, JEL, YWJ, JAL, Data analysis: SSH, WJ, YHC, HC, Wrote the manuscript: SSH, EKA, Designed and supervised the study: SSH, JSO. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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