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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

A new triterpene diglycoside from the roots of *Bupleurum chinense* DC. and its inhibitory effect on adipogensis in 3T3-L1 cells

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Abstract

A new triterpene diglycoside, tibesaikosaponin V (TKV), was isolated from the methanol extract of the roots of *Bupleurum chinense* DC. by normal and reversed phase column chromatography. The chemical structure of this new compound was elucidated based on extensive spectroscopic analysis, including 1D and 2D NMR, HR-ESI-MS, and chemical degradation method. Our results indicated that TKV inhibited lipid accumulation and triacylglycerol content occurred without cytotoxicity to 3T3-L1 adipocytes. Furthermore, TKV significantly suppressed the *m*RNA expression of nuclear transcription factors such as, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα). These results suggest that TKV shows the ability to inhibit 3T3-L1 preadipocyte differentiation of and may have therapeutic potential for obesity and its associated metabolic disorders.

Keywords *Bupleurum chinense* DC. · Triterpene diglycoside · Tibesaikosaponin V

Introduction

Obesity, which is rapidly increasing due to high calorie intake and insufficient exercise, increases the risk of type 2 diabetes, hypertension, cerebrovascular diseases, and various cardiovascular diseases (Lee et al. 2017). Previous investigations have characterized obesity by an increase in the number of fat cells and the size of adipocytes differentiated from preadipocytes (Patel et al. 2016; Ohashi et al. 2014). A critical avenue for obesity treatment may be control of excessive proliferation and differentiation of preadipocytes (Fasshauer and Blüher 2015; Collins et al. 2018). Additionally, more and more attentions have been paid to phytochemicals for the development of anti-obesity agents with low side-effects (Choi et al. 2017; Chyau et al. 2018; Nishina et al. 2017).

About 120 species of *Bupleurum* (Umbelliferae) exist throughout the world, primarily in subtropical regions of the Northern Hemisphere. Of these, 37 species and seven varieties are distributed in China, mainly in the western plateau region (Liang et al. 1998). *Bupleurum chinense* DC., a perennial herb native to China, has been used to treat various diseases for thousands of years (Li et al. 2015). Previous phytochemical and pharmacological investigations of this genus revealed the presence of large amounts of bioactive triterpene glycosides called saikosaponins (Liang

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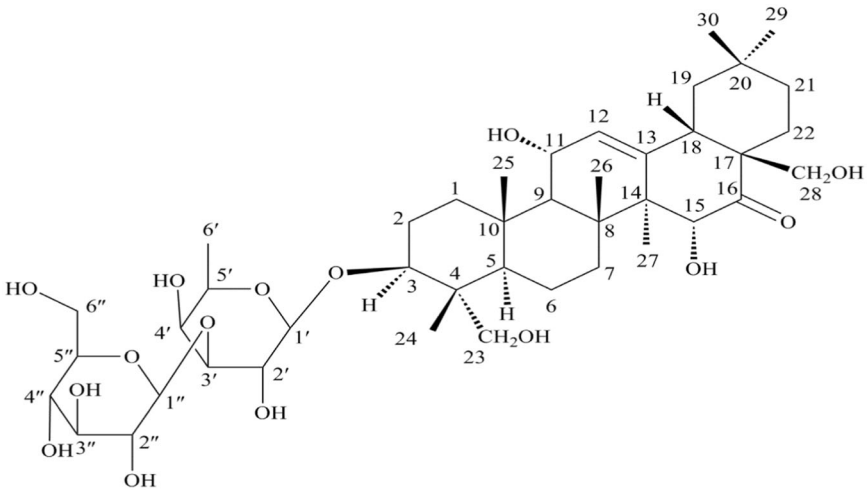

Fig. 1 The chemical structure of TKV

et al. 2001a, 2001b, 2001c). The roots of *B. chinense* are rich in saikosaponin A and D, which were used for quality control of the plant materials in the 2015 edition of Chinese Pharmacopoeia (Committee of National Pharmacopoeia 2015). *B. chinense* also contains lots of other saikosaponins, such as saikosaponin B, C, F, B1, and B2, as well as acetylated saikosaponins (Liang et al. 1998; Liang et al. 2001a, 2001b, 2001c; Liu et al. 2001). In the past 5 years, phytochemical investigations of this medicinal plant further revealed the presence of lots of active minor saikosaponins (Li et al. 2015; Wang et al. 2017; Yu et al. 2013). Different from the roots, the aerial part of *B. chinense* was reported to secrete flavonoids, sesquiterpenes and alkaloid glycosides (Zhang et al. 2007; Kuang et al. 2009). Recently, pharmacologists paid more and more attention to the major saikosaponins in *B. chinense* and found saikosaponins A and D possessed extensive biological activities, which include anti-inflammatory, anti-depressant, anti-apoptosis, anti-oxidant, and anti-addictive properties (Li et al. 2017b; Wang et al. 2015; Chen et al. 2018a, 2018b; Li et al. 2017c; Tsuyoshi et al. 2017; Lorrai et al. 2017). To better understand the chemical composition and biological properties of this medicinal resource, our research focuses on the searching of novel saikosaponins and evaluating their medicinal use. As a result, one new minor saikosaponin (see Fig. 1) was isolated and its inhibitory effect on adipogensis in 3T3-L1 cells was investigated.

Materials and methods

Materials and chemicals

The roots of *B. chinense* were purchased in February 2016 from Lotus Pond Chinese Herbal Medicine Market, Sichuan province, People's Republic of China. The plant material was identified by Professor Weikai Bao, Chengdu Institute of Biology, Chinese Academy of Sciences. A voucher specimen (CH-2016-2) was deposited at the Laboratory of Natural Product Research Center, Chengdu Institute of Biology, Chinese Academy of Sciences. 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), oil red O, insulin, 3-isobutyl- 1-methylxanthine (IBMX), and dexamethasone (DEX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Revert-Aid first-strand cDNA synthesis kit and was purchased from Thermo Science (Vilnius, Lithuania). Dulbecco's modified Eagle's medium (DMEM) and antibiotics were obtained from Gibco BRL (Life Technologies, Shanghai, China). Fetal bovine serum (FBS) was obtained from Corning (Mediatech, Inc., Manassas, VA, USA).

General experimental procedures

Optical rotations were determined in methanol on a Perkin-Elmer 341 polarimeter (Perkin-Elmer Corporation, Wellesley, MA, USA). IR spectra were carried out on a Perkin-Elmer 1725X-FT spectrometer with KBr disks. High resolution electrospray ionization mass spectrometry (HRESIMS) was measured on a Vion IMS QTof (Waters Corp., Milford, Massachusetts, USA) in negative ion mode. NMR spectra, including ^{1}H-NMR, ^{13}C-NMR, H-H COSY, HSQC, HMBC, and NOESY experiments, were recorded with a Bruker Avance-600 spectrometer (Bruker Group, Fallanden, Switzerland) operating at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR. HPLC analysis of the sugar residues was performed on an Alltech series III apparatus with an evaporative light-scattering detector, equipped with a 250 × 4.6 mm i.d. COSMOSIL Sugar-D packed column (Nacalai Tesque, Co., Ltd., Tokyo, Japan) using a mobile phase of 80% CH_3CN at a flow rate of 0.8 mL/min. HPLC purifications were performed on a CXTH system, equipped

Table 1 ^{1}H and ^{13}C NMR data for compound **1** (C_5D_5N, δ in ppm, *J* in Hz)

No.	^{1}H-NMR (δ_H[a])	^{13}C-NMR (δ_C[b])	HMBC ($^{1}H\rightarrow^{13}C$)	No.	^{1}H-NMR (δ_H[a])	^{13}C-NMR (δ_C[b])	HMBC ($^{1}H\rightarrow^{13}C$)
1	1.77 (m), 2.77 (m)	41.7	–	23	3.71 (m)	64.5	–
2	2.11 (m), 2.35 (m)	26.7	–	24	0.97 (s)	13.9	–
3	4.32 (m)	82.1	C-1′	25	1.26 (s)	17.8	C-5
4		43.9	–	26	1.31 (s)	19.2	C-7
5	1.87 (m)	47.9	–	27	1.42 (s)	21.7	C-13
6	1.79 (m)	18.6	–	28	3.85 (m), 4.44 (m)	70.7	C-16,C-22
7	1.86 (m), 2.14 (m)	36.7	–	29	0.75 (s)	23.4	–
8		45.7	–	30	0.84 (s)	33.1	–
9	2.02 (1H, d, $J = 8.7$)	55.5	–	Fuc-			–
10		38.5	–	1′	4.95 (1H, d, $J = 7.7$)	106.0	C-3
11	4.55 (m)	66.7	C-9,C-10,C-12,C-13	2′	4.50 (m)	72.2	–
12	5.81 (1H, d, $J = 3.3$)	131.0	–	3′	4.04 (m)	85.3	–
13		143.0	–	4′	4.14 (m)	73.7	–
14		54.5	–	5′	3.67 (m), 3.85 (m)	71.0	–
15	5.05 (s)	73.7	C-8,C-16,C-17,C-27	6′	1.40 (3H, d, $J = 6.4$)	17.3	–
16		215.8	–	Glc-			–
17		53.3	–	1′′	5.30 (1H, d, $J = 7.8$)	106.5	C-3′
18	2.77 (m)	47.5	C-12,C-16	2′′	4.25 (m)	75.9	–
19	1.16 (m), 1.45 (m)	47.3	–	3′′	4.24 (m)	78.4	–
20		30.9	–	4′′	4.49 (m)	71.9	–
21	1.26 (m), 1.74 (m)	35.7	–	5′′	3.99 (m)	78.8	–
22	1.51 (m), 2.87 (m)	27.3	C-16	6′′	4.34 (m), 4.53 (m)	62.8	–

[a]Measured at 600 MHz

[b]Measured at 150 MHz

with a UV3000 detector at 203 nm (Beijing Chuangxin-tongheng Instruments Co. Ltd., Beijing, People's Republic of China). The preparative HPLC column used was a 50 × 250 mm i.d., 10 μm, YMC-pack ODS-AM (YMC Co. Ltd., Kyoto, Japan). The flow rate was 90 mL/min. Silica gel (100–200 mesh) for column chromatography and silica gel GF254 (10–40 μm) for TLC were purchased from Qingdao Haiyang Chemical Group Co. Ltd. (Qingdao, People's Republic of China). Chemical reagents for isolation were of analytical grade and purchased from Chengdu Kelong Chemical Reagent Co. Ltd. (Chengdu, People's Republic of China).

Extraction and isolation

The roots of *B. chinense* (60.0 kg) were pulverized and heated to 60 °C to extract with 600 L of industrial methanol for three times. The extract (11.6 kg) was concentrated under reduced pressure and the residue was then suspended in H_2O, and successively partitioned with dichloromethane and *n*-butanol. The *n*-butanol soluble fraction (2.1 kg) was subjected to silica-gel column chromatography (12.0 kg) with a gradient solvent system of H_2O saturated MeOH/CH_2Cl_2 (12:1 → 1:1), affording nine fractions based on TLC analyses, i.e., Fr.1 (114 g), Fr.2 (100 g), Fr.3 (460 g), Fr.4 (170 g), Fr.5 (145 g), Fr.6 (110 g), Fr.7 (222 g), Fr.8 (50 g), and Fr.9 (60 g). Fr.7 was firstly separated by preparative HPLC (solvent system: MeOH/H_2O (75/25)) to yield six subfractions: Fr.7-1 → Fr.7-6. Fr.7-1 was further purified by preparative HPLC (solvent system: CH_3CN/H_2O (38/62)) to give compound **1** (65.0 mg).

Tibesaikosaponin V (1)

White amorphous powder; $[\alpha]_D^{20}$ −37.7 (c 0.16, MeOH); IR (KBr) νmax: 3461, 2939, 1705, 1388, 1038 cm^{-1}; ^{1}H-NMR (C_5D_5N, 600 MHz) and ^{13}C-NMR (C_5D_5N, 150 MHz) data, see Table 1; HR-ESI-MS (positive): *m/z* 835.4454 $[M+Na]^+$ (calcd. for $C_{42}H_{68}O_{15}Na$, 835.4450).

Acid hydrolysis of Compound 1 and determination of absolute configuration of sugars

The absolute configurations of the sugar moieties were determined by the method previously described (Li et al. 2017a). Compound **1** (10 mg) were heated with 5% H_2SO_4 (2 mL) under reflux for 8 h. Then, the reaction mixture was exhaustedly extracted with EtOAc. The H_2O layer was neutralized with $Ba(OH)_2$, filtered and subject to HPLC analysis with authentic samples. The optical rotation of the acid hydrolysis solution was measured as $[\alpha]_D^{20}$ + 58.3° (c 0.064, H_2O). Hence, the absolute configuration of the fucose and glucose in the new compounds should be both in D-form.

Cell culture and adipocyte differentiation

3T3-L1 pre-adipocyte cells were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured in DMEM, 10% inactivated FBS and 1% antibiotics (Biological Industries, Bet Haemek, Israel). After the cultures reached confluence (day 0), 3T3-L1 cells were cultured in a differentiation-inducing medium that contained full DMEM medium with a cocktail (0.5 mM IBMX, 1 µM DEX, and 1 µg/mL insulin, MDI) for 2 days. Cells were then cultured for an additional 4 days in DMEM medium containing 5 µg/mL insulin.

Cell viability assay

3T3-L1 cells (4×10^4 cells/well) were seeded into 96-well plates and cultured overnight. The culture medium was then replaced with fresh medium along with TKV at various concentrations for 48 h. Cytotoxicity was investigated by MTT assay as previously described (Kim et al. 2018). Cell viability was expressed as the percentage of control cells.

Oil red O staining

After treatment, cells were washed twice with PBS and fixed in 5% formalin for 10 min at room temperature. Adipocytes containing lipid droplets were incubated with ORO dye (3 mg/ml in 60% isopropanol) for 15 min. After 20 min, cells were washed with deionized water three times, after which stained lipid droplets were extracted using 100% isopropanol, and absorbance was measured at 500 nm.

Determination of triacylglycerol (TG) content

After treatment, cells were washed twice with PBS, suspended in PBS and homogenized under a Fast Prep-24 homogenizer (MP, Solon, OH) with glass beads. The homogenate was then centrifuged at $6000 \times g$ for 10 min, and the supernatant was collected for future experiments. The TG content was determined following the manufacturer's instructions (Biovison, Mountain View, CA, USA).

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIZOL reagent (Ambion, Life Technologies, CA, USA). RNA pellets were dissolved in diethyl pyrocarbonate-treated water. For RT-PCR, 5 µg of total RNA was incubated at 65 °C for 5 min with 1 µL of oligo (dT) 18 primer (0.5 µg/µL) and deionized water (up to 12 µL). The reverse transcription reaction was performed using 4 µL of reverse transcription 5 × reaction buffer, 1 µL RiboLock RNase inhibitor, 1 µL of RevertAid reverse transcriptase, and 2 µL of 10 mM dNTP mixture at 42 °C for 60 min. The reaction was terminated by heating to 70 °C for 5 min. Primers used in this experiment were as follows: PPARγ, forward: 5′-GGAAGACCACTCGCATTCCTT-3′, reverse: 5′-GTAATCAGCAACCATTGGGTCA-3′; C/EBPα, forward: 5′-CAAGAACAGCAACGAGTACCG-3′, reverse: 5′-GTCACTGGTCAACTCCAGCAC-3′; and GAPDH, forward: 5′-CACTCACGGCAAATTCAACGGCACA-3′, reverse: 5′-GACTCCACGACATACTCAGCAC-3′. *m*RNA was quantified in real-time RT-PCR using a SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Data analysis

The values are presented as the mean ± standard deviation (SD). The experimental and control groups were compared using one-way analysis of variance (ANOVA) followed by a Student's *t*-test. *P*-values <0.05 were considered significant.

Results and discussion

Structural elucidation of Compound 1

Compound **1** was isolated as white amorphous powder. The molecular formula of **1** was deduced as $C_{42}H_{68}O_{15}$ based on the quasi-molecular ion peak observed at *m/z* 835.4454 $[M+Na]^+$ (calcd. for $C_{42}H_{68}O_{15}Na^+$ 835.4450) in positive HR-ESI-MS spectrum. The ^1H and ^{13}C-NMR data of **1** (Table 1), together with DEPT and HSQC experiments, suggested the presence of seven characteristic methyl groups at δ_H 0.75 (3H, s, H-29), 0.84 (3H, s, H-30), 0.97 (3H, s, H-24), 1.26 (3H, s, H-25), 1.31 (3H, s, H-25), 1.39 (3H, d, $J = 6.3$ Hz, Fuc-6), and 1.42 (3H, s, H-27), one olefenic proton at δ_H 5.81 (1H, d, $J = 3.3$ Hz), two anomeric protons at δ_H 4.95 (1H, d, $J = 7.7$ Hz, H-1′) and 5.30 (1H, $J = 7.8$ Hz, H-1′′), and one carbonyl group at δ_C 215.8. The NMR data of **1** were similar to those of tibesaikosaponin II except for signals due to the double bonds (Fang et al. 2017). The existence of a hydroxyl group at C-15 was confirmed by HMBC correlations from δ_H 5.05 (H-15) to δ_C 45.7 (C-8), 53.3 (C-17), and 21.7 (C-27). The HMBC correlations between δ_H 5.05 (H-15), 2.77 (H-18), 1.51 (H-22), 3.85 (H-28), and δ_C 215.8 (C-16) indicated the carbonyl group was at C-16. The presence of a hydroxyl group at C-11 was confirmed by HMBC correlations between δ_H 4.55 (H-11) and δ_C 38.5 (C-10), 55.5 (C-9). The double bond was determined to be located between C-12 and C-13 due to HMBC correlations from δ_H 2.77 (H-18) to δ_C 131.0 (C-12)

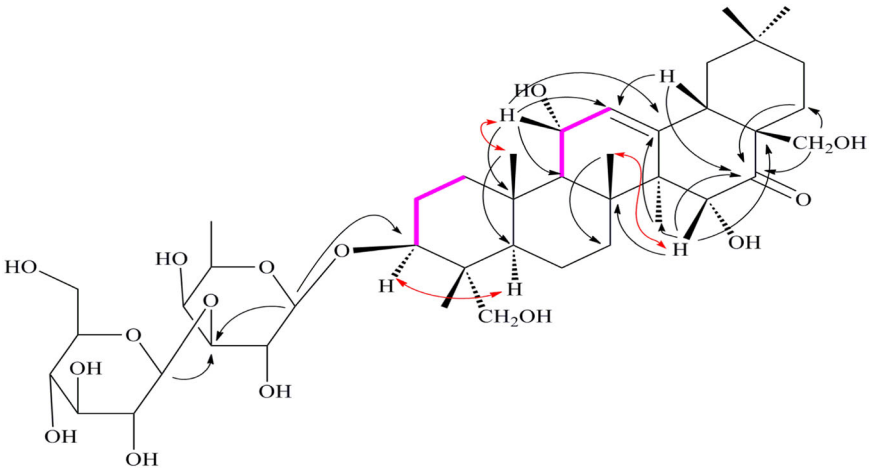

Fig. 2 Key HMBC and 1H–1H COSY correlations of TKV

and 143.0 (C-13), from δ_H 1.42 (H-27) to δ_C 143.0 (C-13), and from δ_H 4.55 (H-11) to δ_C 131.0 (C-12) and 143.0 (C-13) (Fig. 2). The hydroxyl groups at C-11 and C-15 were both α-oriented according to the NOE correlations between H-11 and H-25, and between H-15 and H-26 (Fig. 2). The sugar moieties in **1** were assigned as D-fucose (Fuc) and D-glucose (Glu) from its NMR spectroscopic data and by determing the optical rotation value of the water solution after acid hydrolysis (Li et al. 2017a). The large *J* values ($J > 7.5$ Hz) of the anomeric proton signals demonstrated both β configurations for the sugar residues (Jiang et al. 2018). The Fuc was connected to 3-OH of the aglycone and the Glu was linked to 3′-OH of Fuc on the basis of the HMBC correlations of δ_H 4.95 (H-1′) with δ_C 82.1 (C-3) and δ_H 5.30 (H-1′′) with δ_C 85.3 (C-3′). The NOE correlation of δ_H 4.32 (H-3) with 1.87 (H-5) indicated the 3-OH was in β orientation (Fig. 2). Based on the above data analyses, compound **1** was identified and named as tibesaikosaponin V (TKV).

Effects of TKV on 3T3-L1 viability

The 3T3-L1 preadipocytes were differentiated with optimized conditions using differentiation media and the preliminary results showed that the cocktail containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone (DEX), and 1 µg/mL insulin resulted in a high degree of adipocyte differentiation with ~80% oil red O staining in each field (data not shown). To examine whether TKV had cytotoxic effects on 3T3-L1 adipocyte cells, we examined cell viability using a 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay following treatment with various concentrations of TKV (25, 50, 100, 200, 400, and 600 µM) for 48 h. TKV showed no significant cytotoxicity toward to 3T3-L1 cells at concentrations up to 200 µM, but inhibited cell viability by 17.77% and 23.55% at 400 and 600 µM TKV, respectively (Fig. 3). Based on the cell viability assay, concentrations of TKV up to a maximum of 400 µM were used in the following experiments to evaluate TKV's anti-adipogenic activity.

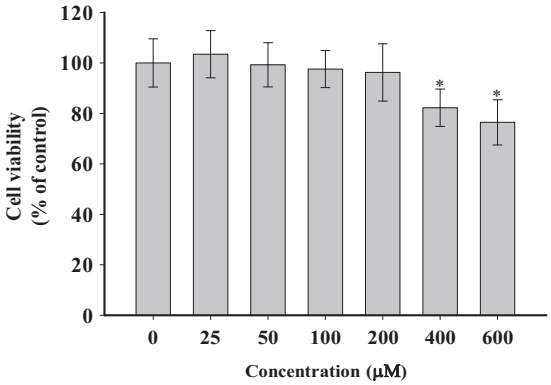

Fig. 3 Cytotoxicity of TKV on 3T3-L1 preadipocytes. Cells were treated with various concentration of TKV (25, 50, 100, 200, 400, or 600 µM) for 48 h. Cell viability was measured by MTT assay. The data are presented as mean ± SD ($n = 3$). *$p < 0.05$ vs control group

TKV attenuates lipid accumulation in 3T3-L1 adipocytes

To investigate the effect of TKV on lipid accumulation during differentiation, preadipocytes were differentiated into adipocytes in the presence of TKV. Lipid accumulations in the adipocytes were stained with oil red O dye and then extracted using isopropanol. The results showed that treatment with MDI resulted in a 5.3-fold increase in lipid accumulation compared to untreated cells. In contrast, TKV treatment prevented lipid accumulation by 21.5, 35.11, 43.16, and 52.28% at 25, 50, 100, and 200 µM, respectively, with an IC_{50} value of 170.47 µM (Fig. 4a). We further tested intracellular triacylglycerol (TG) content. As expected, cellular triglyceride was significantly increased by

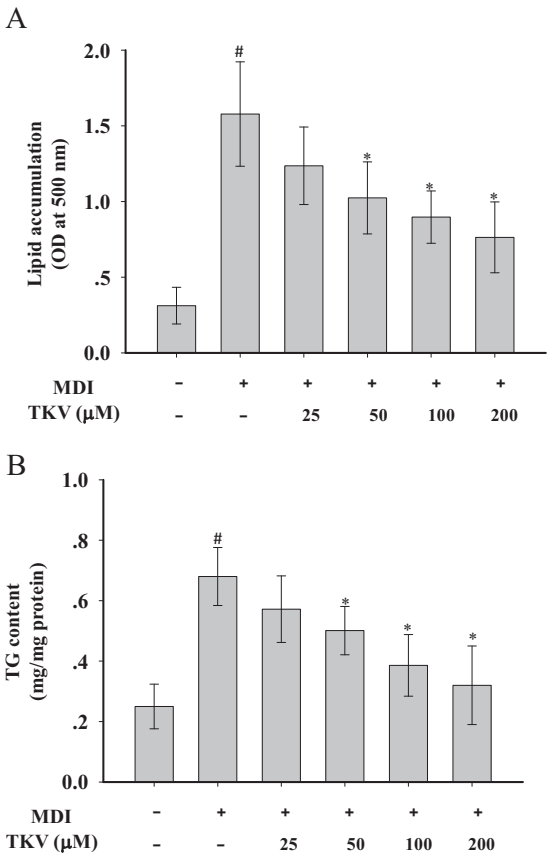

Fig. 4 Inhibitory effect of TKV on lipid accumulation **a** and TG content **b**. MDI-induced 3T3-L1 cells were treated with various concentration of TKV (25, 50, 100, or 200 μM). **a** Lipid droplets were extracted using 100% isopropanol after staining with oil red O dye, and absorbance was measured at 500 nm. **b** TG content was determined using a commercial kit according to the manufacturer's instructions. The data are presented as mean ± SD ($n = 3$). $^{\#}p < 0.05$ vs control group; $^{*}p < 0.05$ vs MDI group

treatment with MDI, and TKV treatment inhibited triglyceride accumulation in a dose dependent manner (Fig. 4b).

TKV inhibited lipid accumulation by regulating adipogenic genes

Adipogenesis is a physiological process in which the differentiation from preadipocytes to mature adipocytes occurs (Rosen and MacDougald 2006). PPARγ and C/EBPα are the two critical transcription factors, regulating the terminal process of adipocyte differentiation (Guo et al. 2017; Moseti Regassa et al. 2016). To investigate whether TKV inhibits the expression of PPARγ and C/EBPα in MDI-induced 3T3-L1 cells, we treated 3T3-L1 cells with different concentrations of TKV. As shown in Fig. 5, PPARγ and C/EBPα *m*RNA expression was significantly inhibited by TKV. At 200 μM, TKV significantly decreased the expression levels of PPARγ and C/EBPα by 62.35% and 57.86%, respectively, compared to the control. Our result

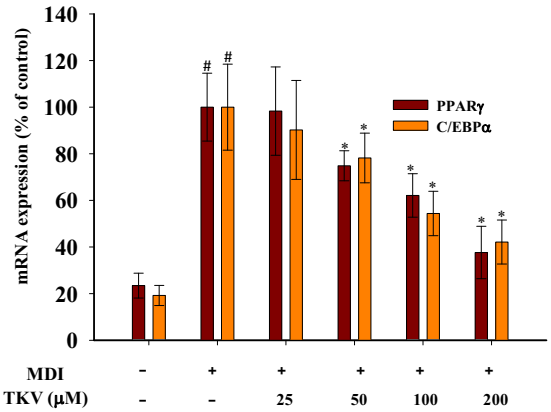

Fig. 5 Effects of TKV on the expression of PPARγ and C/EBPα in differentiated adipocytes. MDI-induced 3T3-L1 cells were treated with various concentrations of TKV (25, 50, 100, or 200 μM). *m*RNA expression levels of PPARγ and C/EBPα were measured by quantitative real-time PCR. The data are presented as mean ± SD ($n = 3$). $^{\#}p < 0.05$ vs control group; $^{*}p < 0.05$ vs MDI group

indicate that TKV inhibited lipid accumulation by down-regulating the *m*RNA expression levels of PPARγ and C/EBP*α*.

Conclusions

A new triterpene diglycoside (**1**) was isolated from the roots of *B. chinense* DC. The chemical structure of this new compound was elucidated based on the extensive spectroscopic data and by comparison with previously reported compounds. TKV inhibited lipid accumulation in 3T3-L1 cells by regulating adipogenesis-related genes. Further in-depth studies can pave the way to determine the mechanism underlying its effect.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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