Inhibitory Effects of Brazilian Propolis on Lipid Accumulation in 3T3-L1 Cells

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Abstract: We here investigated the inhibitory effect of Brazilian propolis on intracellular lipid accumulation. Seven ethanolic extracts of Brazilian propolis were tested for their inhibitory effect on intracellular lipid accumulation in 3T3-L1 cells. Among the seven Brazilian propolis, the extracts AF-08 and AF-18 had inhibitory effects on intracellular lipid accumulation. In particular, the Brazilian propolis AF-08 exhibited a potential inhibitory effect on intracellular lipid accumulation. Thus, these results suggest that the Brazilian propolis AF-08 contributes to the prevention of metabolic syndrome. In addition, five known triterpenoids were isolated from the Brazilian propolis AF-08: betulonic acid, moronic acid, 3-oxo oleanolic acid, masticadienonic acid, and anwnweizonic acid. Four of the triterpenoids exhibited an inhibitory effect on intracellular lipid accumulation. Thus, these triterpenoids may be important as antiobesity agents that inhibit the metabolic syndrome.

Keywords: Brazilian propolis, 3T3-L1 cells, lipid accumulation, triterpenoids.

INTRODUCTION

Propolis is a resinous material collected by honeybees from various buds and exudates of plants, which is used to protect the beehive from external enemies. Propolis has been widely used in folk medicine since 300 BC because of its varied chemical composition [1, 2]. It is well known that the constituents of propolis depend on its plant origin and the time of collection [3]. Numerous biological properties of propolis have been reported, including antibacterial [4], antiviral [5], anti-inflammatory [6], antitumor [7], and antioxidant activities [8-10]. In Japan, Brazilian propolis extracted by ethanol has been extensively used in food to improve health and prevent diseases such as gingivitis, rheumatism, cold, and cancer.

Obesity is a serious health problem in industrialized countries, and it is a major risk factor for metabolic disorders such as coronary heart disease, hypertension, diabetes, cancer, respiratory diseases, and osteoarthritis [11-13]. Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in the adipose tissue [14]. Adipocyte differentiation has been extensively examined in cultured preadipocytes such as 3T3-L1 cells. Therefore, 3T3-L1 cells are a suitable model system for obesity related research [15-17].

In this study, we describe the inhibitory effect of Brazilian propolis on intracellular lipid accumulation in 3T3-L1 cells. In particular, the Brazilian propolis AF-08 (BP-AF-08) exhibited a potent inhibitory effect on intracellular lipid accumulation. Moreover, the inhibitory effects of the isolated compounds on intracellular lipid accumulation are also reported.

EXPERIMENTAL METHODS

Materials

3T3-L1 fibroblast cells were purchased from the Human Science Research Resources Bank (Osaka, Japan). Isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, adenine, transferrin, glutamine, and berberine chloride were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Triiodothyronine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Calf serum (CS) was purchased from Cell Culture Technologies. Fetal bovine serum (FBS) and Oil Red O were purchased from Cosmo Bio. Co.,

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Ltd. (Tokyo, Japan). Penicillin-streptomycin-neomycin (PSN) and penicillin-streptomycin-glutamine (PSG) were purchased from Life Technologies (Burlington, ON). MTT Cell Count Kit (MTT) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Brazilian Propolis Samples

Various Brazilian propolis samples were obtained from Amazonfood Ltd. (Tokyo, Japan). Voucher specimens of proplis (AF-05, AF-06, AFG-06, AF-08, AF-18, AF-19, and AF-20) were deposited at the Laboratory of Self Medication, School of Pharmacy, Nihon University (Table **1**).

Table 1: Features of Brazilian Propolis

Sample	Species[a]	Family[a]	Area[b]
AF-05	*Baccharis dracunculifolia*	Compositae	Central
AF-06	*Baccharis erioclada*	Compositae	Southan
AFG-06	*Baccharis erioclada*	Compositae	Southan
AF-08	*Myrceugenia euosma*	Myrtaceae	Southan
AF-18	*Baccharis dracunulifolia*	Compositae	Central
AF-19	*Baccharis caprarifolia*	Compositae	Southan
AF-20	*Hyptis divaricate*	Labiatae	Southan

Note: [a]Major botanical origins in areas where propolis was collected. [b]Brazilian area where propolis was collected.

Cell Culture

3T3-L1 preadipocytes were plated in 24-well plates and 6-cm dishes and maintained in DMEM supplemented with 10% CS and 1% PSG at 37°C in a humidified 5% CO_2 incubator. For induction of adipose differentiation, the cells were grown to confluence. The cells were then fed with differentiation medium (2.7:1 mixture of DMEM and Ham's F12 containing 10% FBS, 1% PSN, 1.6 μM insulin, 0.0005% transferrin, 180 μM adenine, 20 pM triiodothyronine, 0.25 μM dexamethasone, and 500 μM IBMX. The cells were treated with fresh differentiation medium (for samples) on day 2 and day 4 without dexamethasone and IBMX after induction of differentiation. After 7 days, the cells were fixed on plates with 4% formalin in phosphate-buffered saline (PBS) for 24 h. The intracellular lipid accumulation was measured 8 days after induction of differentiation. Cell viability was confirmed by microscopic observation and was measured by the MTT assay.

Oil-Red O Staining

For Oil-red O staining, cultured cells were fixed on plates with 4% formalin in PBS for 24 h, rinsed once with H_2O, and then air dried. The fixed cells were stained with Oil-red O staining solution (0.5% Oil-red O in isopropanol, diluted 3:2 in H_2O, and then filtered using a 1.0-μm filter) for 15 min. The cells were then rinsed thrice with H_2O before visualization and documentation.

The density of lipid content was evaluated using AK09F extraction liquid after Oil-red O staining. The absorbance at 540 nm was measured using a microplate reader. The value of dimethyl sulfoxide (DMSO)-treated cells was normalized to 100%.

Statistical Analysis

When applicable, results are presented as mean ± SD. Student's *t* test was used to calculate *p* values. Results were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

General Experimental Procedure

The nuclear magnetic resonance (NMR) spectra were measured using a JEOL ECA-600 spectrometer (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) in $CDCl_3$ containing tetramethylsilane (TMS) as the internal standard. The mass spectra were recorded using a JEOL CG mate instrument. High-performance liquid chromatography (HPLC) was performed using a JASCO PU-2089 apparatus equipped with JASCO UV-2075.

Extraction and Isolation

The ethanol extract of the BP-AF-08 (2.50 g) was fractionated to *n*-hexane-MeOH-H_2O (19:19:2), which produced *n*-hexane (0.55 g) and MeOH-H_2O (1.85 g) fractions. The *n*-hexane fraction was purified by HPLC using an InertSustain C18 column (10 × 250 mm, 5 μm) (GL Sciences Inc., Japan) with 0.1% trifluoroacetic acid-CH_3CN (12:88 v/v) at a flow rate of 4.0 ml/min, to yield **1** (6.58 mg), **2** (7.02 mg), **3** (5.66 mg), **4** (8.02 mg), and **5** (7.86 mg).

RESULTS AND DISCUSSION

The seven Brazilian propolis samples were extracted by ethanol, and then the extracts were dried.

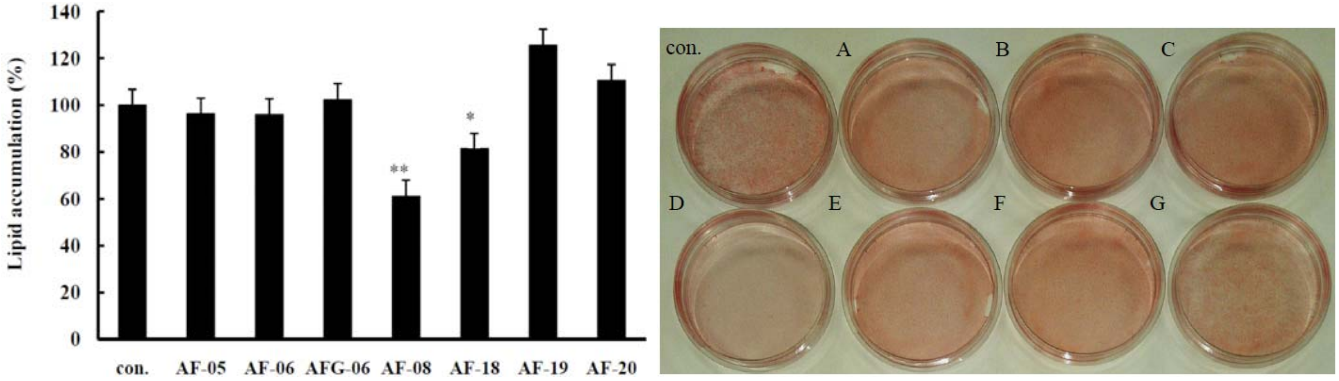

Figure 1: A: Effect of Brazilian propolis on lipid accumulation in 3T3-L1 cells. Con, control; A, AF-05; B, AF-06; C, AFG-06; D, AF-08; E, AF-18; F, AF-19; and G, AF-20. Results are expressed as mean ± SD of three individual experiments. *$p < 0.05$ and **$p < 0.01$ vs. control. The value of DMSO-treated cells was normalized to 100%.

B: Oil-red O staining of Brazilian propolis. Con: control; A, AF-05; B, AF-06; C, AFG-06; D, AF-08; E, AF-18; F, AF-19; and G: AF-20.Cells were treated with the five fractions (50 μg/ml) on day 2 and day 4. The intracellular lipid content was measured 8 days after induction of differentiation.

The cytotoxicity of the seven extracts was evaluated by the MTT assay in 3T3-L1 cells and all the extracts were found to be nontoxic at 50 μg/ml (data not shown). The seven Brazilian propolis extracts were tested to verify inhibitory effects on intracellular lipid accumulation (Figure **1A**). Lipid accumulation assay results demonstrated that the BP-AF-08 and AF-18 had an inhibitory effect on intracellular lipid accumulation. In particular, the BP-AF-08 exhibited a potent inhibitory effect on intracellular lipid accumulation. As presented in Figure **1B**, the differentiation of the BP-AF-08 was inhibited compared to the other Brazilian propolis, as determined oil-red O staining. These results indicate that the BP-AF-08 contributes to the prevention of metabolic syndrome. Therefore, we focused our efforts on investigating the chemical constituents of the BP-AF-08.

Five known triterpenoids were isolated from the BP-AF-08 (Figure **2**). Identification of the five triterpenoids, betulonic acid (**1**) [18], moronic acid (**2**) [18, 19], 3-oxo oleanolic acid (**3**) [20], masticadienonic acid (**4**) [21],

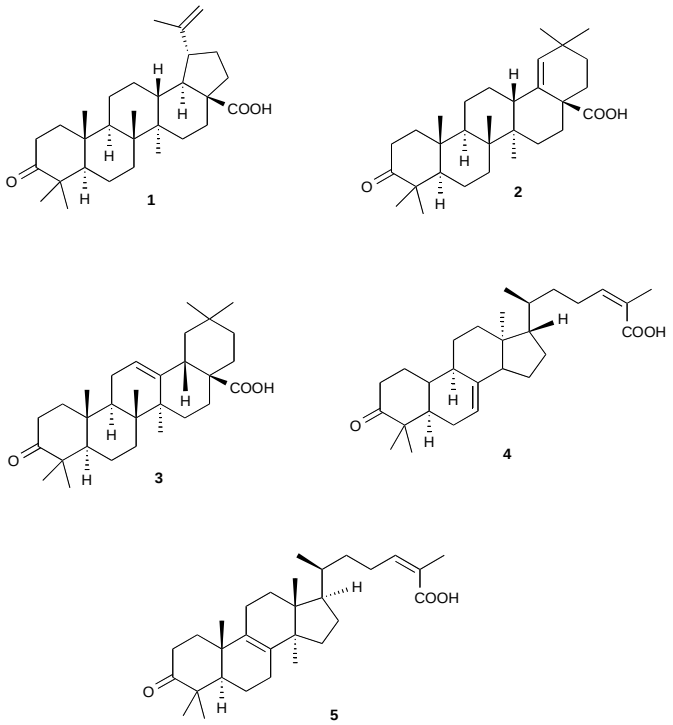

Figure 2: Structures of compounds **1**-**5**. **1**, betulonic acid; **2**, moronic acid; **3**, 3-oxo-oleanolic acid; **4**, masticadienoic acid; and **5**, anwuweizonic acid.

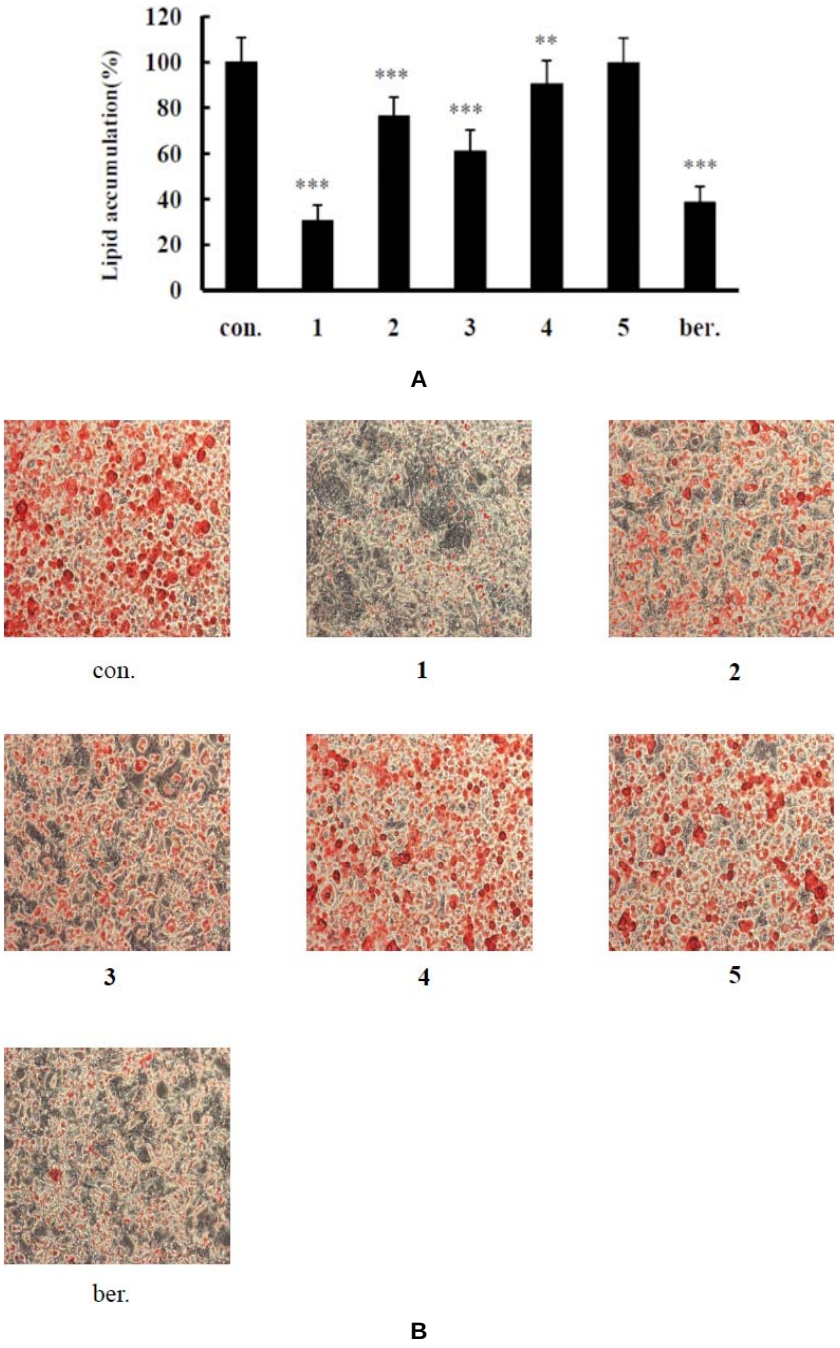

Figure 3: A: Effect of triterpenoids **1**-**5** on lipid accumulation in 3T3-L1 cells. Con, control; **1**, betulonic acid; **2**, moronic acid; **3**, 3-oxo oleanolic acid; **4**, masticadienonic acid; and **5**, anwnweizonic acid; ber., berberine chloride. Results are expressed as mean ± SD of three individual experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. control. The value of DMSO-treated cells was normalized to 100%.

B: Ablation of control, five triterpenoids, and berberine by Oil-red O staining. Con, control; **1**, betulonic acid; **2**, moronic acid; **3**, 3-oxo oleanolic acid; **4**, masticadienonic acid; **5**, anwnweizonic acid; and ber, berberine chloride.

and anwnweizonic acid (**5**) [18] was performed by a spectral comparison with literature data. Full details of the isolation and identification as well as the spectroscopic data are available on request from the corresponding author. The inhibitory effect of the five triterpenoids on intracellular lipid accumulation was examined. Berberine chloride was used as a positive control as it has been reported to have inhibitory effects on intracellular lipid accumulation [22]. The cytotoxicity of the five compounds was evaluated by the MTT assay in 3T3-L1 cells. The results indicated that the five compounds were nontoxic at concentrations up to 30.0 μM. Our results demonstrated that the five triterpenoids inhibited intracellular lipid accumulation at 30 μM (Figure **3A**). As presented in Figure **3B**, ablation of five triterpenoids, berberine chloride, and untreated

control resulted in inhibited lipid accumulation as shown by oil-red O staining. Anwnweizonic acid (**5**) exhibited less inhibitory effect on lipid accumulation. Moronic acid (**2**), 3-oxo oleanolic acid (**3**), and masticadienonic acid (**4**) exhibited a minimum inhibitory effect on lipid accumulation comparable with that of the positive control. Betulonic acid (**1**) was more effective than the other triterpenoids. As presented in Figure **4**, betulonic acid inhibited lipid accumulation in a concentration-dependent manner.

Recent studies have attempted to investigate the beneficial effects of lupeol and lupenone on intracellular lipid accumulation in 3T3-L1 cells [23, 24]. Our results indicate that triterpenoids may be important as antiobesity agents that inhibit the metabolic syndrome.

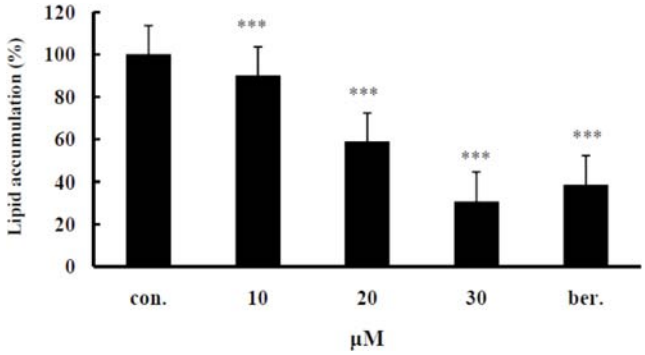

Figure 4: Betulonic acid (**1**) has a concentration-dependent effect on lipid accumulation in 3T3-L1 cells. Results are expressed as the means ± SD of three individual experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. control. The value of DMSO-treated cells was normalized to 100%.

CONCLUSION

We here investigated the inhibitory effect of various Brazilian propolis on intracellular lipid accumulation. In general, the chemical composition of Brazilian propolis depends on the vegetation of the area from where it is harvested. Lipid accumulation assay results demonstrated that the BP-AF-08 and AF-18 had inhibitory effects on intracellular lipid accumulation. In particular, the BP-AF-08 exhibited a potent inhibitory effect on intracellular lipid accumulation. Thus, our results suggest that the BP-AF-08 contributes to the prevention of metabolic syndrome. In addition, five known triterpenoids were isolated from the BP-AF-08. Four triterpenoids: betulonic acid (**1**), moronic acid (**2**), 3-oxo oleanolic acid (**3**), and masticadienonic acid (**4**) exhibited an inhibitory effect on the intracellular lipid accumulation. Furthermore, betulonic acid (**1**) exhibited the highest inhibitory effect on intracellular lipid accumulation as well as positive control. These results indicate that triterpenoids may be the source of antiobesity agents to improve the metabolic syndrome.

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