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Phytochemical Analysis of the Phenolic Fat-Suppressing Substances in the Leaves of *Lactuca raddeana* in 3T3-L1 Adipocytes

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Abstract – *Lactuca raddeana* (Compositae) is used to treat obesity and complications due to diabetes. The five phenolic compounds including chlorogenic acid, chicoric acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, luteolin were qualitatively identified by LC-ESI-MS analysis. The contents were quantitatively determined by HPLC, under the condition of a Capcell Pak C18 column (5 μm, 250 mm × 4.6 mm i.d.) and a gradient elution of 0.05% trifluoroacetic acid (TFA) and 0.05% TFA in MeOH-H_2O (60 : 40). The contents of chicoric acid (100.99 mg/g extract) and luteolin 7-*O*-glucoside (101. 69 mg/g extract) were high, while those of other three phenolic substances were very low. The 3T3-L1 adipocyte cells treated with chicoric acid and luteolin 7-*O*-glucuronide significantly suppressed the accumulation of fat, suggesting they are effective against obesity. Since high level of peroxynitrite (ONOO) causes cardiovascular disease in obese patients, its scavenging activity was also studied.
Keywords – *Lactuca raddeana*, Compositae, Chicoric acid, Peroxynitrite, HPLC, Adipocyte

Introduction

Lactuca raddeana (Compositae) is used to treat complications due to diabetes and obesity in addition to fever, depression, insomnia and cardiovascular diseases. Recently, it is being revealed that several polyphenols present in fruits, vegetables or cereals exhibit anti-obesity effects.[1] Obesity increases the risk for insulin tolerance and cardiovascular disease.[2] Continued differentiation and fat accumulation of adipose cells are closely associated with obesity.[3]

It has been reported that many phenolic antioxidants attenuate obesity through apoptosis-inducing action in 3T3-L1 preadipocytes.[4-7] Studies have also shown a link between obesity and the the accumulation of reactive oxygen species (ROS) in adipose cells.[8] Pires *et al*.[9] reported that treatment of a peroxynitrite scavenger, Mn(III)tetrakis(4-benoic acid)porphyrin, reduced a weight gain and fat accumulation in adipose cells of mice on a high-fat diet. In addition, ONOO generation represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, and neurodegenerative disorders.[10]

Qualitative and quantitative analysis on *L. raddeana* were pursued based on results from our preliminary study that shown an inhibitory effect in 3T3-L1 adipocytes. Through a UPLC-ESI-MS/MS analysis, phytochemical phenolic constituents (chlorogenic acid, chicoric acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, and luteolin) were identified. Using the five compounds, quantitative analysis was also performed on the extracts and fractions by an HPLC method. In addition, the inhibitory effect on 3T3-L1 adipocyte cells and $ONOO^-$ scavenging effect was investigated.

Experimental

Instruments and reagents – Agilent 1200 Technologies (Agilent Technology, USA) was HPLC used for qualitative analysis by UPLC/Q-TOF-ESI-MS/MS and Synapt G2 (Waters, USA) was a MS detector used as a mode of Q-TOF MS. The HPLC system used for quantitative analysis consisted of two Prostar 210 pumps, Prostar 325 UV-Vis detector, and a Shiseido Capcell PAK C18 column (5 μm, 4.6 mm × 250 mm, Japan) equipped with

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its MetaTherm controller. Data were processed using Varian Star Workstation. HPLC solvents (HPLC grade of H_2O, MeOH, CH_3CN) were purchased from J.T.Baker (Phillisburg, NJ, USA). Three standard compounds used for both analysis and bioassay were chlorogenic acid (Lot# 104K0722, ≥ 95% by titration), chicoric acid (Lot# 060M1184V, ≥ 95% by HPLC), and luteolin (Lot# 11K4085, ≥ 95% by HPLC) purchased from Sigma-Aldrich (St. Louis, MO, USA), and other two standards, luteolin 7-*O*-glucuronide (> 98% purity) and luteolin 7-*O*-glucoside (> 98% purity), are the compounds preserved in our laboratory.

Plant material – *Lactuca raddeana* (Compositae) was collected during August in 2012, on a mountain area in Wonju, Korea. This plant was identified by Prof. Sang-Cheol Lim (Department of Horticulture and Landscape, Sangji University). A voucher specimen (natchem#-49) was deposited in the Laboratory of Natural Products Chemistry, Department of Pharmaceutical Engineering, Sangji University. The leaves of collected plants were dried in a shadow area, crushed, and prepared for extraction.

Extraction and fractionation – The leaves of *L. raddeana* (100 g) were extracted with 3 L of 70% MeOH three times under reflux. The extracted solution was filtered, evaporated under reduced pressure, and freeze-dried to give a 70% MeOH extract (aq. MeOH extract, 21.4 g). The extract was partitioned between aqueous phase and $CHCl_3$ phase, and then the latter phase was further concentrated *in vacuo* to give a $CHCl_3$ fraction (7.42 g). In the same way, the aqueous phase was successively fractionated with EtOAc and BuOH, respectively, to give an EtOAc fraction (1.68 g) and BuOH fraction (3.10 g). The aqueous extract was prepared as the same way with aq. MeOH extract: 30.6 g aqueous extract was obtained from 100 g of plant material.

Conditions of UPLC and MS – The conditions of UPLC and MS on qualitative identification are described. A column used in this experiment was Acquity@BEH C18 (1.7 μm, 2.1 × 50 mm). The two solvents, 0.05% trifluoroacetic acid (TFA) and 0.05% TFA MeOH-CH_3CN (60 : 40), were used as the A- and B solvents, respectively. Gradient elution was programmed at the flow rate of 0.3 ml/min ad follows: 0 min (100% A), 4 min (85% A), 20 min (60% A), 30 min (0% A), 35 min (100 A). LC system was coupled to a Q-TOF MS equipped with ESI source. In a TOF MS analysis, mass spectra were measured in a positive ion mode. The condition for ESI source was as follows: ESI capillary (3.0 kV), sampling cone (40 V), temp. source (120°), desolvation (300°), cone gas (100 L/h), desolvation gas (600 L/h). A UV wavelength was fixed at 254 nm for detection.

Preparation of sample solution – A freeze-dried aq. MeOH extract were used for preparation of sample solutions. Five concentrations (1,000, 1,500, 2,000, 2,500, and 3,000 μg/ml) were prepared according to ICH (International Conference on Harmonization) guidelines and used for the repeatability test. Of the five concentrations, the 1,500 μg/ml solution was used for intra-day- and inter-day variability tests. Sample solutions were filtered through a 0.50-μm syringe filter prior to injection to HPLC.

Condition for HPLC quantification – To prepare standard solutions, five standard compounds were completely dissolved in MeOH by vortexing. These solutions were filtered through a 0.50-μm syringe filter prior to injection onto an HPLC system. The two solvents, 0.05% TFA-H_2O solution and 0.05% TFA-MeOH/CH_3CN (60 : 40), were the A and B solvents, respectively, for the mobile phase. Gradient elution was programmed as follows: (A)/(B) = 85/15 (0 min) → (A)/(B) = 35/65 (35 min) → (A)/(B) = 0/100 (47 min; hold for 6 min to wash the column) → (A)/(B) = 85/15 (54 min; hold for 6 min to equilibrate the column condition). Chromatograms were recorded during a period of 0 - 37 min. The column was maintained at 40 °C, and a wavelength 254 nm was chosen because it was more sensitive than 280 and 360 nm for the simultaneous detection of caffeoylquinic acid, caffeoyltartric acid and luteolin glycosides.

Validation on the HPLC method – Validation experiments were performed in terms of linearity, sensitivity, precision and accuracy. Serial-dilution was conducted to prepare 3.13 - 100.0 μg/ml concentrations of chlorogenic acid, chicoric acid, and luteolin 7-glucuronide, and 1.56 - 50.0 μg/ml of luteolin 7-glucoside and luteolin. Peak areas (y) were plotted against the concentrations (x axis), and the linearity of regression equations was assessed by R^2 (correlation coefficient) values. Sensitivity was evaluated by LOD (limit-of-detection) and LOQ (limit-of-quantification) values which were determined by the signal-to-noise (S/N) method. Intra-day and inter-day variability tests were performed to evaluate precision of the method. Intra-day variability was completed within 24 h, while inter-day variability was conducted on four different days by injecting the same solutions five times a day. Relative standard deviations (RSDs) were obtained by injecting the same solution five times, and were considered a measure of precision. Accuracy was evaluated from the mean recovery rates (%) of standards from the spiked extract versus non-spiked solution extract sample.

Cell Culture and adipocyte differentiation – 3T3-L1 mouse embryo fibroblasts were obtained from the American

Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FBS with 100 units/ml of penicillin-streptomycin solution at 37 °C, in 5% CO_2, at 95% humidity until confluence. Two days after confluence (Day 0), the cells were stimulated to differentiate with differentiation inducers (1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine, and 1 μg/mL insulin, MDI) that were added to DMEM containing 10% FBS for two days (Day 2). Preadipocytes were then cultured in DMEM, 10% FBS supplemented with 1 μg/mL insulin for another two days (Day 4), followed by culturing with 10% FBS/DMEM medium for additional two days (Day 6), at which time more than 90% of cells were mature adipocytes with accumulated fat droplets. On Day 2, the samples of the aq. MeOH extract, fractions ($CHCl_3$-, EtOAc- and BuOH fractions) and chicoric acid and luteolin 7-*O*-glucoside were prepared in a differentiation medium at three concentrations (50 μg/ml, 100 μg/ml, and 200 μg/ml).

Cell cytotoxicity assay – Cell viability was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, USA) according to the manufacture's instruction. Briefly, the cells (5 × 103 per 96 well) were incubated at 37 °C in 5% CO_2 and 95% air with samples. After 48 h for 3T3-L1 preadipocytes, 20 μL of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt] solution was added to each well and incubated for 4 h. Absorbance at 490 nm was measured using a VERSA maxmicroplate reader (Molecular Devices, Sunnyvale, CA, USA) to determine the formazan concentration, which is proportional to the number of live cells.

Oil red O staining – Intracellular fat accumulation was measured using Oil Red O. The Oil Red O working solution was prepared as described by Ramirez-Zacarias *et al*.[11] The 3T3-L1 cells were washed twice with phosphate-buffered saline (PBS) and were then fixed in 10% formaldehyde in PBS for 1 h. After washing with 60% isopropanol, the cells were stained with Oil Red O solution for 30 min at room temperature. The cells were washed with water four times to remove the unbound dye. The stained cells were observed with an Olympus IX71 Research Inverted Phase microscope (Olympus Co., Tokyo, Japan). Following the microscopic observation, 100% isopropanol was added to extract the excess staining dye from the cells.

Peroxynitrite-scavenging activity – An assay method described by Kooy *et al*.[12] was used to measure the peroxynitrite-scavenging activity of the aq. extract from the leaves of *L. raddeana.* The principle of this method is to monitor the intensity of highly fluorescent rhodamine formed from non-fluorescent DHR 123 under the presence of $ONOO^-$. Rhodamine buffer (pH 7.4) was consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final concentration of DHR 123 was 5 μM. The samples were dissolved in 10% DMSO (concentration: 5 μg/mL). The final fluorescent intensity was measured with or without the treatment of 10 μM $ONOO^-$ in 0.3N NaOH. The fluorescence intensity of oxidized DHR 123 was measured at the excitation and emission of 480 nm and 530 nm using microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc., Winooski, VT, USA). Through the detection of the oxidation of DHR-123, peroxynitrite-scavenging activity was calculated by subtracting the background fluorescence from the final fluorescent intensity. L-penicillamine with a great peroxynitrite-scavenging activity was used as a positive control.

Results and Discussion

Qualitative analysis by a UPLC-ESI-MS method – Five compounds of *L. raddeana* were identified on the

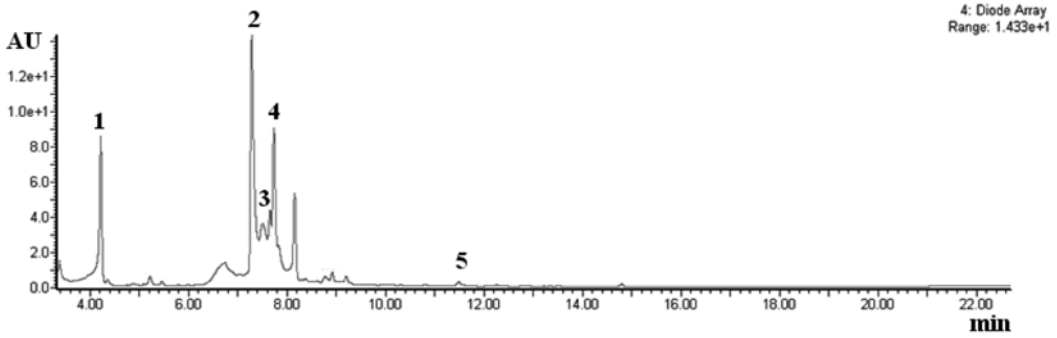

Fig. 1. UPLC chromatogram of the MeOH extract of *L. raddeana.*

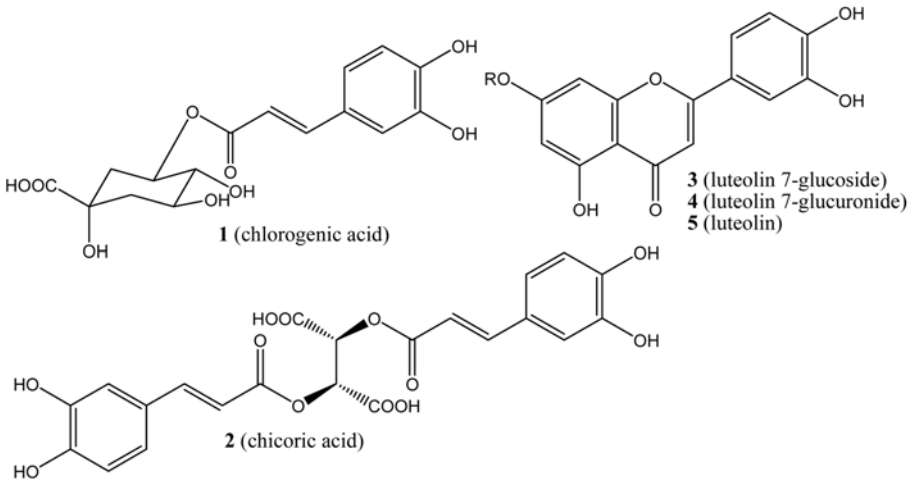

Fig. 2. Structure of compounds **1** - **5** identified from *L. raddeana*.

Table 1. UPLC-ESI-MS data of compounds **1** - **5** identified from *L. raddeana*

Compound	t_R (min)	Mw	$[M+H]^+$ (*m/z*)	Fragment (*m/z*)
1	4.21	354.31	355.1014	163.0389
2	7.28	474.37	475.3253	163.0385
3	7.57	448.37	449.1	287.0557
4	7.73	462.36	463.0867	287.0552
5	11.52	286.24	287.0558	287.0556

basis of pseudomolecular and fragment ions (*m/z*) on the mass spectra measured by a UPLC-ESI-MS experiment. The chromatogram and mass spectral data of pseudomolecular and fragment ions measured by this experiment were shown in Fig. 1 and Table 1, respectively. Mass spectra of compounds **1** and **2** exhibited a caffeoyl moiety at *m/z* 163.03, and those of **3**, **4** and **5** displayed a luteolin moiety at m/z 287.05. The five compounds were identified as chlorogenic acid (**1**), chicoric acid (**2**), luteolin 7-glucoside (**3**), luteolin 7-glucuronide (**4**), luteolin (**5**). The structures were shown in Fig. 2. The identification of compounds **1** - **5** were confirmed by comparisons of retention times (t_Rs) on the chromatogram and each mass spectrum with standard compounds.

Optimization and validation of HPLC method – Four parameters, mobile phase composition, gradient elution, UV wavelength and column temperature, were optimized to establish a more reliable method. The A and B solvents, 0.05%-trifluoroacetic acid (TFA)/H_2O and 0.05%-TFA in MeOH-CH_3CN (60 : 40), respectively, were chosen because they were more selective, environmentally-friendly and economic. The A and B solvents were adjusted with 0.05% TFA concentration so that the acidified solution could inhibit release of protons from phenolic substances. Solvents showed more selective peaks than acid-free solvent. Gradient elution was employed to cover a wide range of peaks within a shorter time. This method produced a more selective and a more repetitive chroma- togram at a fixed temperature of 40 °C than at room temperature.

The optimized HPLC method was validated in terms of linearity, sensitivity, precision and accuracy. The R^2, LOD and LOQ values are shown in Table 2. Linearity was verified from the R^2 values > 0.9996. Sensitivity was validated from the LOD- (< 0.89 μg/ml) and LOQ values

Table 2. Linearities and limits of detection and quantification (LOD and LOQ) of the analytes

Analyte	Equation of the linear regression[a]	Linear range (μg/mL)	R^2 [b]	LOD[c] (μg/mL)	LOQ[d] (μg/mL)
1	$y = 166.80x + 58.46$	3.13-100.0	0.9998	0.19	0.63
2	$y = 84.43x + 35.13$	3.13-100.0	0.9997	0.65	2.17
3	$y = 200.82x + 40.73$	1.56-50.0	0.9998	0.25	0.82
4	$y = 48.71x + 46.73$	3.13-100.0	0.9999	0.89	2.96
5	$y = 399.67x + 64.06$	1.56-50.0	0.9996	0.07	0.22

[a]*y*, peak area at 254 nm; *x*, concentration of the standard (μg/mL); [b]R^2, correlation coefficient for 6 data points in the calibration curves (n = 4); [c]LOD, limit of detection (S/N = 3); [d]LOQ, limit of quantification (S/N = 10).

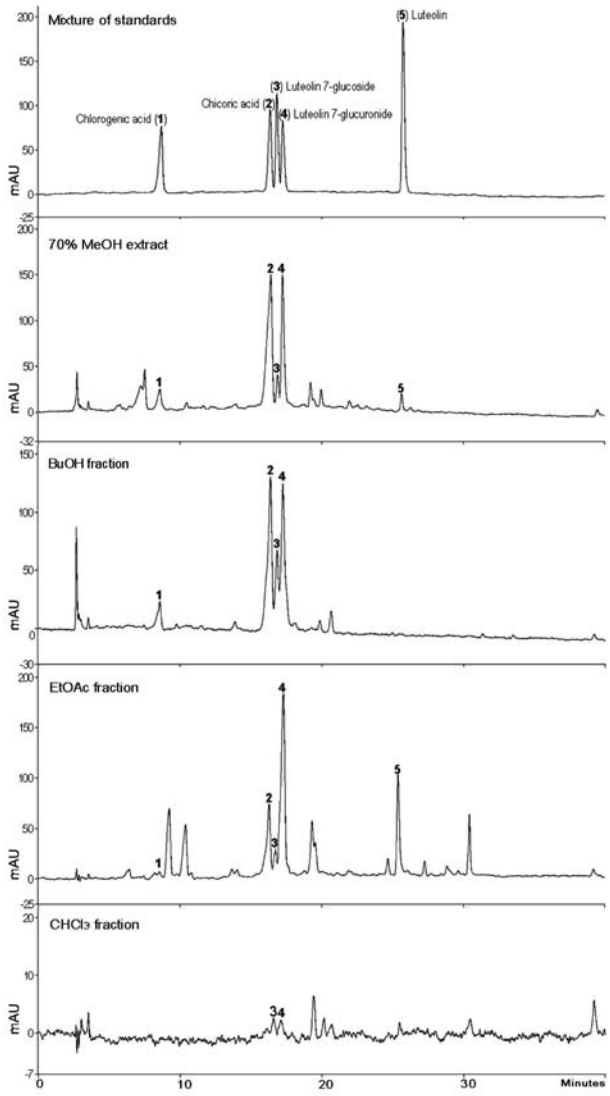

Fig. 3. HPLC chromatograms of extract and fractions of *L. raddeana*.

(< 2.17 μg/ml). Experimental results obtained from the intra-day- and inter-day variability and from the recovery test were shown in Table 3. The RSD values in the intra-day variability were shown from 0.48 - 2.42%, suggesting that it is sufficiently precise. And the RSD values of the inter-day variability were between 0.69 - 4.16%, suggesting that this method has sufficient stability. Accuracy was also established with recovery rates of 96.01 - 102.75%.

Contents of phenolic substances – Contents of five phenolic substances in the extracts and fractions were shown in Table 4. In plants, caffeic acid esters mainly occurred in the form of caffeoylquinic acids or caffeoytartartic acid, where their typical compounds are chlorogenic acid and caffeoyltartaric acid, respectively. The content of chicoric acid (100.99 mg/g) was considerably higher than that of chlorogenic acid (6.69 mg/g), suggesting that the former compound is the main caffeoylquinic acid in *L. raddeana*. Luteolin 7-*O*-glucuronide was a main substance among the two luteolin glycosides and their aglycone (luteolin). These five phenolic substances were quantitatively higher in the EtOAc- and BuOH fractions than $CHCl_3$ fraction. Further, the analysis was also performed to compare aq. MeOH and H_2O which solvent yielded the highest extracts. The aq. MeOH extract yielded higher contents than the aq. extract.

Inhibition on fat accumulation in 3T3-L1 adipocytes and peroxynitrite formation – To determine the cytotoxicity of test samples, 3T3-L1 cells were treated with various concentrations (1.56 - 200 μg/mL), and the cell viability was measured by the MTS assay. As shown in Fig. 4, treatment with 1.56 - 200 μg/mL of samples did not have significant cytotoxic effects on 3T3-L1 cells. Further, we measured the effect on adipocyte differentiation. We used a differentiation mixture (MDI) to induce the

Table 3. Precision and recovery data of the analytes

Analyte	t_R (min)	Precision test				Recovery test					
		Intra-day variability RSD (%)		Inter-day variability RSD (%)		Initial conc. (μg/mL)	Amount added (μg)	Concentration after addition (μg/mL)		Recovery (%)	RSD (%)
		t_R	Area	t_R	Area			Expected	Measured		
1	8.65	0.21	2.42	0.29	4.16	14.32	12.50	26.82	25.75	96.01	1.03
2	16.40	0.14	0.48	0.20	0.69	54.03	50.00	104.03	101.83	97.88	0.66
3	16.73	0.12	0.95	0.17	1.28	11.26	12.50	23.76	22.97	96.67	1.25
4	17.05	0.11	1.21	0.16	1.42	54.40	50.00	104.40	107.27	102.75	0.83
5	25.66	0.11	2.32	0.18	3.53	2.14	3.12	5.26	5.17	98.27	1.23

Relative standard deviation (RSD) values were calculated for both retention time (t_R) and peak area of three experiments. Recovery tests were performed in the 70% MeOH extract spiked with each standard compound.

Table 4. Content of analytes in the lyophilized extract and fractions of *L. raddeana* (mg/g)

Analyte	Extracts		Fractions		
	70% MeOH	Aq. (H_2O)	$CHCl_3$	EtOAc	BuOH
1	6.69 (1.43)	2.05 (0.63)	< LOQ	1.30 (0.01)	6.95 (0.11)
2	100.99 (21.61)	66.65 (19.73)	< LOQ	42.77 (0.37)	78.54 (1.24)
3	5.26 (1.13)	0.85 (0.26)	0.59 (0.02)	5.21 (0.05)	12.66 (0.20)
4	101.69 (21.76)	30.23 (9.25)	2.19 (0.09)	190.62 (1.65)	116.18 (1.83)
5	0.50 (0.11)	<LOQ	< LOQ	8.48 (0.07)	< LOQ

Values in the parentheses are content of analytes in the dried plant materials (mg/g).

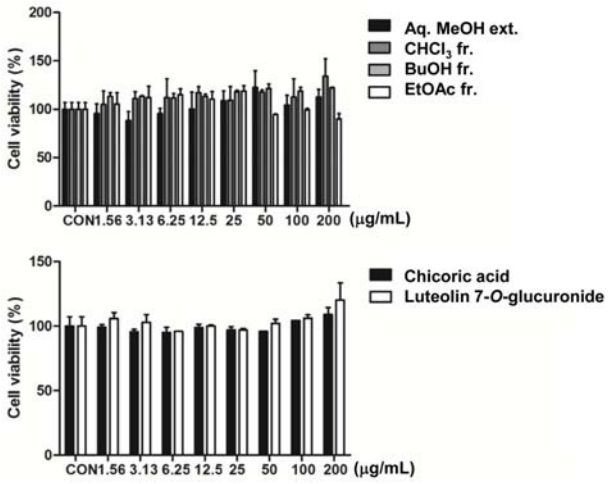

Fig. 4. Effect of aq. MeOH extract of *L. raddeana*, its fractions and its substances (luteolin 7-*O*-glucuronide and chicoric acid) on cell viability in 3T3-L1 cells. 3T3-L1 cells were treated with samples at various concentrations (1.56 - 200 μg/mL) for 48 h. Cell viability was determined by the MTS assay. Postconfluent 3T3-L1 cells were differentiated in the absence or in the presence of the aq. MeOH extract of *L. raddeana* and its fractions (1.56 - 200 μg/mL) and chicoric acid, luteolin 7-*O*-glucuronide (1.56 - 200 μg/mL) for 6 days.

differentiation of 3T3-L1 cells. The 3T3-L1 cells were treated with samples (50, 100, and 200 μg/mL; aq. MeOH extract, fractions, chicoric acid and luteolin 7-*O*-glucuronide) during differentiation. Six days later, cells were stained with Oil Red O. As shown in Fig. 5, the aq. MeOH extract and EtOAc and BuOH fractions (200 μg/ml concentrations) effectively suppressed fat accumulation in 3T3-L1 cells compared to the control. The two substances (chicoric acid and luteolin 7-*O*-glucuronide) significantly reduced fat accumulation in cells at 200 μg/ml, which suggests that the effect of the aq. MeOH extract is mainly attributed to the two substances. These effects were observed in dose-dependent manners (data not shown). The pharmacological actions of chicoric acid such as inducing apoptosis,[13] protecting against stress,[14] and antiviral[15] have been reported. Luteolin 7-*O*-glucuronide has anti-gastritis,[16] antidepressant,[17] and antimutagenic effects.[18] As shown in Table 5 and 6, $ONOO^-$-scavenging activities of the extract of *L. raddeana*, chicoric acid, and luteolin 7-*O*-glucuronide were potent. The IC_{50} values of *L. raddeana*, chicoric acid, and luteolin 7-*O*-glucuronide were 1.15 μg/ml, 0.76 μM, and 3.13 μM, respectively.

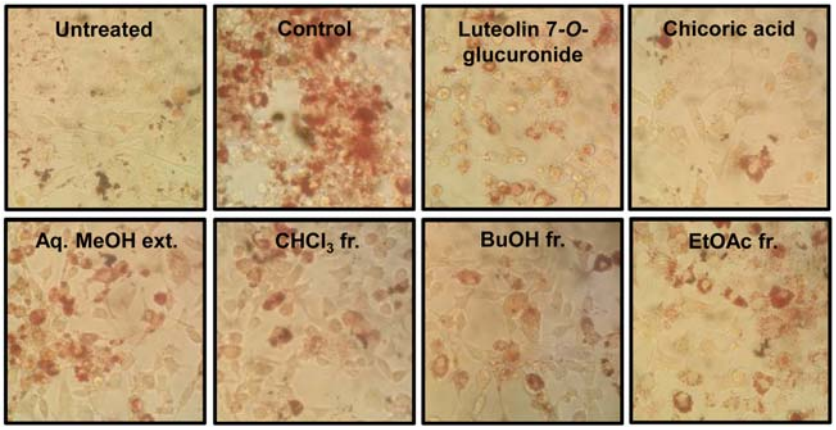

Fig. 5. Effect of Aq. MeOH extract of *L. raddeana*, its fractions and its substances (luteolin 7-*O*-glucuronide and chicoric acid) on lipid accumulation of 3T3-L1 adipocyte differentiation. Postconfluent 3T3-L1 cells were differentiated in the absence or presence of the extracts and fractions of *L. raddeana* (1.56 - 200 μg/mL), chicoric acid, and luteolin 7-*O*-glucuronide (1.56 - 200 μg/mL) for 6 days. (a) Fat droplets were observed by Oil Red O staining.

Table 5. Peroxynitrite-scavenging activity of the aq. MeOH extract

Sample	Conc. (μg/ml)	Inhibition (%)	IC_{50} (μg/ml)
Aq. MeOH ext.	0.4	25.87 ± 9.07*	1.15
	2	77.14 ± 0.98	
	10	95.15 ± 0.49	

*Value represents mean ± S.D. (n = 2).

Table 6. Peroxynitrite-scavenging activity of chicoric acid (**1**), luteolin 7-*O*-glucuronide (**4**) and penicillamine (positive control)

Sample	Conc. (μg/ml)	Inhibition (%)	IC_{50} (μM)
2	0.08	21.15 ± 1.37	0.76
	0.4	53.62 ± 3.47	
	2	85.34 ± 0.69	
4	0.4	22.53 ± 5.13	3.13
	2	64.29 ± 2.08	
	10	86.87 ± 2.05	
Penicillamine	0.4	34.18 ± 4.98	8.62
	2	62.74 ± 0.62	
	10	86.73 ± 0.35	

*Value represents mean ± S.D. (n = 2).

In conclusion, it is suggested that the leaves of *L. raddeana*, which are used to treat diabetic complications including obesity and cardiovascular disorders, contained high amounts of chicoric acid and luteolin 7-*O*-glucuronide. These two substances are regarded as the active substances in *L. raddeana* which are responsible for suppressing fat accumulation in 3T3-L1 adipocytes. Therefore, the leaves of *L. raddeana* could be used to prevent or treat obesity.

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