

Anti-Obesity Effects on Unripe *Rubus coreanus* Miquel Extract in High Fat Diet-Induced Obese Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author MAJ performed all the experiments and statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SHC managed the literature searches and participated in preparation of sample. Authors YSK and GSY participated in preparation of sample. Authors SYL, JHK and KNO participated in blood sampling and statistical analysis. Authors DWL and SOK designed the study and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: The objective of the present study was to evaluate the anti-obesity effects of unripe *Rubus coreanus* Miquel (uRC) in 3T3-L1 adipocytes and body weight, epididymal fat and perirenal fat weight, and lipid profiles in diet-induced obese (DIO) C57BL/6 mice.

Methodology: The lipid accumulation in 3T3-L1 adipocytes was carried out Oil Red O staining. And uRC (50 and 100 mg/kg/day) were orally administered for 90 days from the day of feeding with high fat diet (HFD). The serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL)-cholesterol and low density lipoprotein(LDL)-cholesterol and glucose levels were measured using Alere cholesterol LDX[®] system. And the serum glutamic oxaloacetic transaminase (GOT),

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glutamic pyruvic transaminase (GPT), blood urea nitrogen(BUN) and creatinine levels were measured using the respective kits.

Results: Our results indicated that treatment with uRC dose-dependently inhibited lipid accumulation in 3T3-L1 adipocytes. Moreover, after oral administration for 12 weeks, uRC (50 and 100mg/kg/day) extract produced a significant decrease in the serum total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, glucose and glutamic-oxaloacetic transaminase (GOT) levels of HFD-induced obese mice. Similarly, uRC extract elevated serum high density lipoprotein (HDL) cholesterol. These results suggest that uRC extract may be a useful resource for the management of obesity.

Conclusion: These results suggest that uRC extract may be a useful resource for the management of obesity.

Keywords: *Unripe Rubus coreanus*; anti-obesity; 3T3-L1 adipocyte; high-fat diet (HFD)-induced obese mice.

1. INTRODUCTION

Recently, due to changes in the patterns of food consumption, the incidence rates of metabolic disease such as obesity, stroke, atherosclerosis, hypertension, diabetes and cancer have been increasing considerably [1,2]. Obesity is defined as excess adipose tissue accumulation in the human body and can produce adverse health effects [3]. The mechanisms that control the number and size of adipocytes are not fully understood, despite major efforts [4,5]. Adipose tissue is also considered a major endocrine organ that secretes a number of cytokines and hormones [6,7]. Therefore, the modulation of adipose tissue mass is critical for anti-obesity efforts and for the control of energy balance and endocrine function.

The fruit of *Rubus coreanus* Miquel, commonly called Bokbunja in Korea, is native to far-eastern Asia countries (South Korea, China, and Japan). *R. coreanus* has been used for centuries as a traditional alternative medicine. *R. coreanus* fruits are usually harvested at the red stage (ripe). When ripe, *R. coreanus* are soft and differ from unripe fruits in color (red vs. green, respectively). The fruits of this plant can be used as a promising agent to reduce the risk of many diseases, including asthma and allergies, and they are effective in reducing inflammation and oxidation [8-10]. There are reports on water extract of unripe *R. coreanus* attenuates atherosclerosis and regulate lipid metabolism [11-12]. The unripe fruit, in particular, is used in traditional medicine as a remedy for erectile dysfunction [13]. We previously reported that RC alleviated fatigue by elevating the anti-oxidative potential [14]. Furthermore, unripe *R. coreanus* (uRC) fruits elicit a strong inhibitory effect on the production of inflammatory mediators, whereas

ripe fruit extracts have a weak inhibitory effect [15]. Recently, studies have been conducted to evaluate ripe *R. coreanus* (RC) for its anti-obesity effects, but only *in vitro* results have been published [16]. However, the effects of uRC have not yet been reported.

The present study aimed to evaluate effects of unripe and ripe *R. coreanus* in 3T3-L1 adipocytes. Moreover, we evaluated the influence of uRC on body weight, epididymal fat and perirenal fat weight, and lipid profiles in obese mice fed a high-fat diet.

2. MATERIALS AND METHODS

2.1 Preparation of Unripe and ripe *R. coreanus* Extract

Fruits of unripe and ripe *R. coreanus* were purchased from Gochang country (Jeollabuk-do, Korea) in 2013. uRC and RC were extracted with 20 volumes of 5% ethanol or water, respectively, at 100°C for 4h. The extracted solution was then filtered, concentrated and lyophilized. Finally, the dried extract was stored at 4°C until it was used in the assays.

2.2 Cell Culture and Differentiation

3T3-L1 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine serum (BS). 3T3-L1 cells were seeded at a density of 1×10^5 cells/mL in 6 well plate for 2 days until confluent (designated as day-4, n=3 replicates). Two days later (designated as day -2), the cells' media were changed to DMEM with 10% BS. Next, two days after confluence (designated as day 0), the cells

were cultured in DMEM 10% fetal bovine serum (FBS) and a differentiation cocktail (MDI) consisting of 10µg/mL insulin, 0.5mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexamethasone (DEX), with or without a sample. After 2 days, the cells were cultured in DMEM containing 10% FBS and 10µg/mL insulin and then changed DMEM containing 10% FBS (designated as day 4). Two days later (designated as day 6), the cells' media were changed to DMEM with 10% FBS. Orlistat (20µM), a known anti-obesity drug, was used as the positive control [17,18].

2.3 Cell Viability

Cell viability was carried out using MTT assay. 3T3-L1 cells were seeded at a density of 1×10^4 cells/well in 96 well plate (n=3 replicates). Then, various concentration RC and uRC were treated for 24h. Then 10 µg/mL of 5mg/mL MTT solution was added in each well. After 4h of addition of MTT, removed and the formazan crystals were solubilized in 100 µL of dimethyl sulphoxide (DMSO) and absorbance was taken at 540nm.

2.4 Oil Red O Staining

Oil Red O staining was performed on day 8. The differentiation cells were washed with phosphate buffered saline (PBS) and then fixed 10% formalin for 30min. The cells were washed with distilled water. The fixed cells were stained with Oil Red O working solution for 30 min and then washed with distilled water 3 times. The cells were photographed using a digital camera, and the lipid droplets were dissolved in 100% isopropanol and measured at 500nm.

2.5 Animals and Design

Male four-week-old C57BL/6 mice (16-21 grams body weight) were purchased from Orient Bio (Seongnam, Korea). The mice were kept in plastic cages under controlled temperature ($23 \pm 1^\circ\text{C}$), humidity ($45 \pm 5^\circ\text{C}$), 100% fresh HEPA-filtered air, and a 12-hour light-dark cycle (lights on from 06:00 to 18:00) in the Animal Research Center of Jeonnam Institute of Natural Resources Research. Animal care was in accordance with the guidelines of the Korean Council on Animal Care. The mice were divided into five groups of five mice each: normal diet (ND, n=5), high fat diet (HFD, n=5), HFD + uRC 50mg/kg/day (uRC 50, n=5), HFD + uRC 100 mg/kg/day (uRC 100, n=5), and HFD + Green

tea extract 50mg/kg/day as positive control (GT, n=5). Oral administration of uRC (50 and 100mg/kg/day) and GT was continued for 12 weeks from the day of feeding with HFD. The normal diets consisted of 10% kcal from fat (Research Diets, D12450B, New Brunswick, NJ, USA), whereas the high-fat diets consisted of 60% kcal from fat (Research Diets, D12492, New Brunswick, NJ, USA). Body weights and food intakes were measured twice per week.

2.6 Determination of Serum Lipid Parameters

At the end of the experimental period, the mice were kept fasting for 12h prior to sacrifice. Blood samples were centrifuged at 4,000 rpm for 20 min at 4°C , and the serum was stored at -70°C until analysis. Serum concentrations of total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol and glucose were measured using an Alere cholesterol LDX[®] system (cholesteck LDX, Hayward, CA, USA). Serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN) and creatinine levels were measured using appropriate kits (DRI-CHEM 4000i, FUGI-FILM, Tokyo, Japan). After blood collection, the adipose tissue (epididymal fat, perirenal fat) and liver were removed from the mice and weighed immediately.

2.7 Statistical Analysis

Data are presented as the mean \pm S.D. The data were statistically evaluated using Student's t-test or one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test to compare significant differences between the groups at $p < 0.05$.

3. RESULTS AND DISCUSSION

To examine the anti-adipogenic effects of uRC and RC, 3T3-L1 preadipocytes were treated with RC or uRC for 8 days. The differentiation of preadipocytes into adipocytes is associated with an increased number of Oil red O-stained cells due to lipid accumulation. Microscopic observations of the Oil red O staining revealed a gradual reduction in the number of lipid droplets as the concentration of RC or uRC increased (Fig. 1A). The lipid accumulation rate was analyzed on day 8, and the cells treated with RC or uRC had significantly lower lipid content at this

time (Fig. 1B). The inhibitory effects of RC and uRC on lipid accumulation during adipogenesis were dose dependent; treating differentiated cells with RC and uRC (300µg/ml) decreased lipid levels by 18.8% and 79.7% on day 8, respectively. The mean percentage inhibition of uRC (300µg/ml) was significantly larger than that

of RC ($P<0.001$). This anti-adipogenic effect was achieved at a concentration that did not affect cell viability, according to the MTT assay (Fig. 1C). These results indicate that uRC effectively blocks adipocyte differentiation in 3T3-L1 pre-adipocytes.

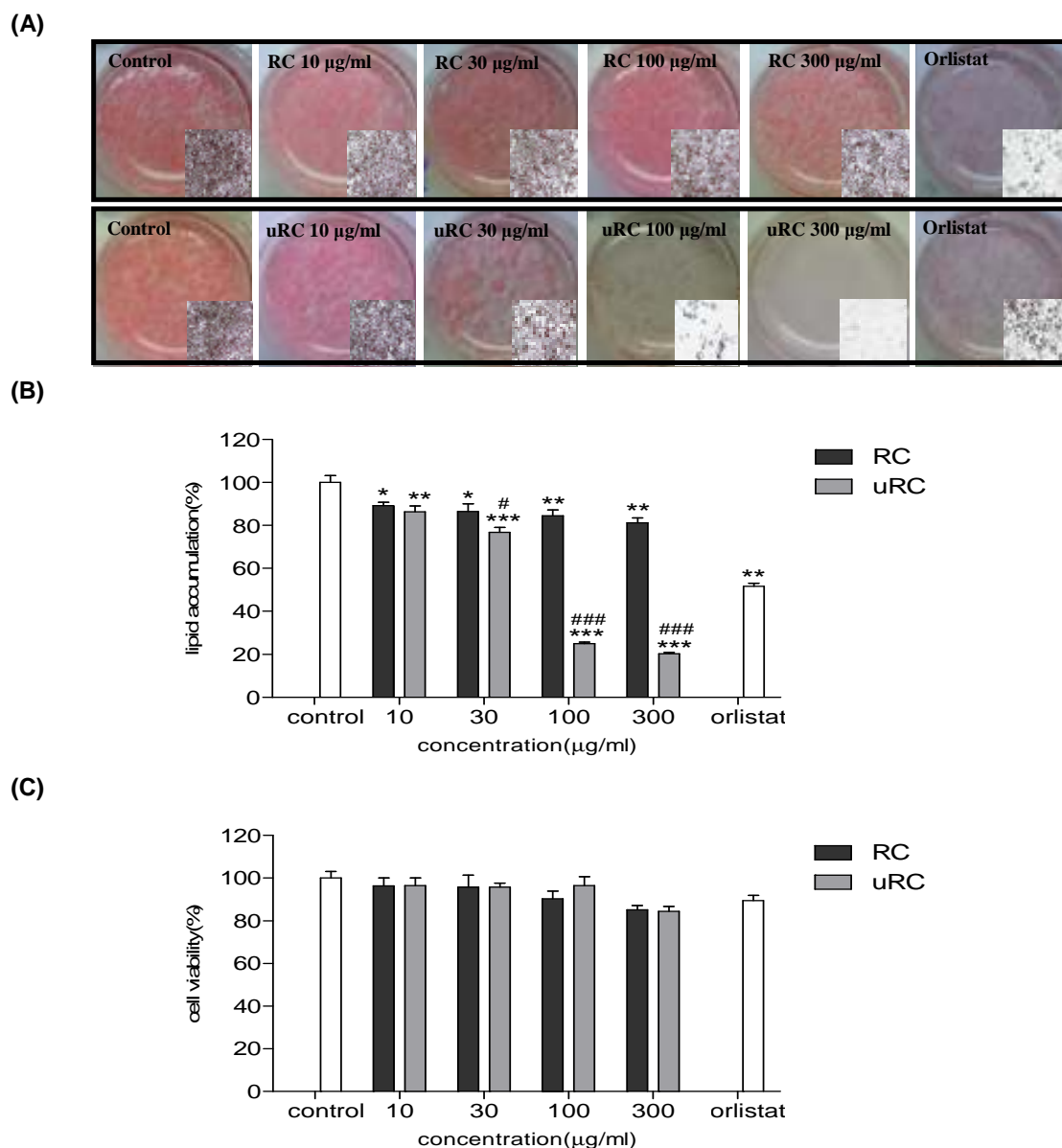


Fig. 1. The effect of a water extract of unripe and ripe *R. coreanus* on lipid accumulation in 3T3-L1 adipocytes. (a) 3T3-L1 cells were differentiated with differentiation medium containing uRC for 8 days. Lipid accumulation was evaluated by Oil Red O staining (magnification, 100×). (b) The intracellular lipid accumulation was quantified by Oil Red O staining. Orlistat (20 µM) was used as a positive control. Values are expressed as the mean ± S.E. (n=3). * $P<0.05$, ** $P<0.01$ and * $P<0.001$ compared with the control. # $P<0.05$ and ### $P<0.001$ compared with the RC group. (c) Cell viability was evaluated by MTT assay**

Therefore, we screened for the optimal inhibition of lipid accumulation in the uRC extracts using different solvent extraction conditions (water, 5% ethanol, 30% ethanol, 50% ethanol and 70% ethanol) in 3T3-L1 cells. The results indicated that the 5% ethanol extract of uRC had the highest activity (data not shown). The 5% ethanol extract of uRC dose-dependently inhibited the lipid accumulation in 3T3-L1 adipocytes, as indicated by Oil Red O staining (Fig. 2). uRC at concentrations of 10, 30, 100, and 300 $\mu\text{g/ml}$ significantly inhibited the lipid accumulation rates to $88.5\pm 5.5\%$ ($P<0.01$), $82.0\pm 5.6\%$ ($P<0.001$), $29.5\pm 1.9\%$ ($P<0.001$), $12.0\pm 0.7\%$ ($P<0.001$), respectively. As the positive control, orlistat inhibited the lipid accumulation on rate to $56.2\pm 2.5\%$ ($P<0.01$). These results might be relevant in determining whether the chemical composition changes that occur during ripening are associated with the anti-obesity effects of the unripe and ripe fruits.

We investigated the anti-obesity effect of 5% ethanol extracts (50 and 100mg/kg/day, oral ad) from uRC in high fat diet-induced obese (DIO) mouse model for 12 weeks. The body weight, food intake, adipose tissue weights, and serum lipid parameters of each group are shown in Table 1. After 12 weeks, the final body weight gain of the uRC 50 ($38.4\pm 1.7\text{g}$) and uRC 100 ($33.4\pm 1.5\text{g}$) mice was significantly lower than that of the HFD mice ($38.9\pm 0.5\text{g}$) ($P<0.01$). The food intake was not different significantly

between the HFD group and the uRC group. The epididymal fat and perirenal adipose tissue weights in the uRC 50 ($2.44\pm 0.16\text{g}$ and $1.15\pm 0.0\text{g}$) and uRC 100 ($1.61\pm 0.2\text{g}$ and $0.67\pm 0.1\text{g}$) mice were significantly decreased compared with those in the HFD group ($2.73\pm 0.1\text{g}$ and $1.21\pm 0.1\text{g}$) ($P<0.01$ and $P<0.001$). The observed decrease in body weight by uRC treatment without any significant difference in food intake suggests that uRC has a physiological effect on the processing of food expenditure. uRC effectively also decreased the serum levels of total cholesterol, LDL cholesterol, and glucose compared with the HFD group ($P<0.05$ and $P<0.01$). On the other hand, uRC significantly increased the levels of HDL cholesterol in the serum ($P<0.001$). Furthermore, uRC (5% ethanol extract) alleviated the liver damage by lowering the levels of two common markers for liver damage, GOT (glutamic oxaloacetic transaminase) and GPT (glutamic pyruvic transaminase), which were in turn increased by HFD treatment (Table 1). GOT/GPT activities were not increased in the uRC extract (5% ethanol extracts)-administered groups; instead, they were decreased. However, the relative weights of the livers were not significantly different between the groups. These results suggest that the organ and body weight reductions were not caused by liver toxicity, further suggesting a beneficial role for *Rubus coreanus* extracts against obesity.

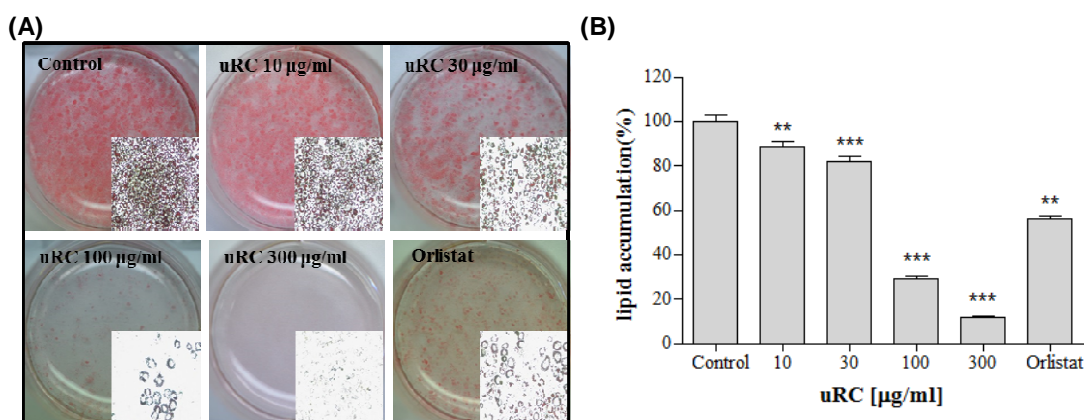


Fig. 2. The effect of a 5% ethanol extract of unripe *R. coreanus* on lipid accumulation in 3T3-L1 adipocyte. (a) 3T3-L1 cells were differentiated with differentiation medium containing uRC for 8 days. Lipid accumulation was evaluated by Oil Red O staining (magnification, 100 \times). (b) The intracellular lipid accumulation was quantified by Oil Red O staining

Orlistat (20 μM) was used as a positive control. Values are expressed as the mean \pm SE ($n=3$). $P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control

Table 1. The effect of 5% ethanol extract of unripe *R. coreanus* on body weight, food intake and serum lipid parameters

Groups ¹⁾	ND	HFD	uRC 50	uRC 100	GT
Initial body weight(g)	19.9±0.5	19.9±0.5	19.9±0.5	19.9±0.5	19.9±0.6
Final body weight(g)	27.8±0.7	38.9±0.5 ^{###}	38.4±1.7	33.4±1.5 ^{**}	33.5±0.9 ^{***}
Body weight gain(g)	7.9±1.0	18.7±0.9 ^{###}	20.4±1.1	13.6±.9	13.4±0.5 ^{**}
Food intake(g/day)	2.7±0.0	2.0±0.0	2.2±0.0	2.1±0.0	2.1±0.0
Epididymal fat(g)	0.71±0.1	2.73±0.1 ^{###}	2.44±0.2	1.61±0.2 ^{***}	1.47±0.3 ^{**}
Perirenal fat(g)	0.40±0.0	1.21±0.1 ^{###}	1.15±0.0	0.67±0.1 ^{***}	0.67±.1 ^{**}
Liver(g)	0.99±0.0	0.98±0.0	1.02±0.1	0.94±0.0	0.98±0.0
GOT (U/L)	99.6±6.0	128.2±14.1 [#]	113.4±8.9	88.6±5.6	128.0±7.8
GPT (U/L)	28.0±3.9	31.6±1.9	42.4±5.7	36.2±2.1	26.0±2.1
Total cholesterol (mg/dL)	130.7±5.9	156.3±2.3 ^{##}	152.3±3.6	131.8±4.8 ^{**}	138.1±9.7
Triglyceride (mg/dL)	107.0±7.0	108.1±5.3	102.6±5.0	101.9±16.1	82.8±8.2 ^{**}
LDL-cholesterol (mg/dL)	12.3 ±0.7	26.6±2.1 ^{###}	18.7±2.3	16.3±5.0	13.4±2.8 ^{**}
HDL-cholesterol (mg/dL)	121.9±3.8	103.4±1.4 ^{##}	123.4±1.4 ^{***}	126.2±3.3 ^{***}	124.4±3.5 ^{**}
Glucose (mg/dL)	93.7±11.6	159.3±7.3 ^{##}	113.4±16.9	98.4±1.9 ^{**}	109.0±9.9 ^{**}

Values are expressed as the mean±SE (n=5).[#] P<0.05, ^{##} P<0.01 and ^{###} P<0.001 compared with ND group. ^{*} P<0.05, ^{**} P<0.01 and ^{***} P<0.001 compared with the HFD group

These studies indicate that the uRC can be potential natural resource for the management of obesity. Our previous another study reported that the contents of malic acid from ripe *Prunus mume* were decreased more than unripe one [19]. Citric acid, succinic acid, fumaric acid, and ellagic acid were found in unripe and ripe *R. coreanus*, the citric acid and ellagic acid showed the highest concentration in the unripe *R. coreanus* [15]. Moreover, it has been shown that the citric acid and ellagic acid possesses anti-obesity and anti-oxidant properties both *in vitro* and *in vivo* [20]. Although ellagic acid is component that has anti-obesity properties in *R. coreanus*, further studies will be required in order to define the candidate chemicals that regulate the anti-obesity effects of uRC.

4. CONCLUSION

In conclusion, uRC administration significantly suppressed lipid accumulation, body weight gain, adipose tissue weight, GOT/GPT levels, and serum levels of TC, TG, LDL-cholesterol, and glucose in the 3T3-L1 adipocytes of high fat diet-induced obese mice. To the best of our knowledge, this study is the first to investigate the effect of the anti-obesity activity of unripe *R. coreanus* on high fat diet-induced obese mice. Hence, uRC can be considered a potential natural resource for controlling obesity. The *ob/ob* mice have a genetically recessive obesity because they do not produce leptin, an important regulator of energy homeostasis, which results in sterile adult mice with over 50% fat. Thus, this mouse model is one of the best animal models for obesity studies. Therefore, it is necessary to determine the chemical identity of the bioactive

constituents and to verify the roles of these bioactive compounds through further studies aimed at improving lipid metabolism in *ob/ob* mice.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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