

Short Communication

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

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 obese mice fed a high-fat diet.

Study Design: The lipid accumulation in 3T3-L1 adipocytes was carried out Oil Red O staining. And the oral administration of uRC(50 and 100mg/kg/day) 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), 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 100 mg/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.

Conclusion: Non-invasive independent predictors for screening esophageal varices may decrease medical as well as financial burden, hence improving the management of cirrhotic patients. These predictors, however, need further work to validate reliability.

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

(Note: 1. *Case Reports* should follow the structure of Abstract, Introduction, Presentation of Case, Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables. Abstract (not more than 250 words) of the Case reports should have the following sections: Aims, Presentation of Case, Discussion and Conclusion. Only Case Reports have word limits: Papers should not exceed 2000 words, 20 references or 5 figures. Other Type of papers have no word limits.

2. *Review papers* may have different headings of the sections and are exempted from following these suggestions.

3. *Research Papers and Short Notes* should follow the structure of Abstract, Introduction, Methodology, Results and Discussion, Conclusion, Acknowledgements, Competing Interests, Authors' Contributions, Consent (where applicable), Ethical approval (where applicable), and References plus figures and/or tables.)

1. INTRODUCTION

Recently, due to changes in the patterns of food consumption, the incidence rates of metabolic syndromes 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]. The unripe fruit, in particular, is used in traditional medicine as a remedy for erectile dysfunction [11]. Recently, studies have been conducted to evaluate ripe *R. coreanus* (RC) for its anti-obesity effects, but only *in vitro* results have been published [12]. We previously reported that RC alleviated fatigue by elevating the anti-oxidative potential [13]. 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 [14]. However, the anti-obesity effects of uRC have not yet been reported.

The present study aimed to evaluate the anti-obesity 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. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

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. Extracts were obtained from unripe and ripe fruits using 20 volumes of 5% ethanol or distilled water at 100°C for 4 h. 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

Fruits of unripe and ripe *R. coreanus* were purchased from Gochang country (Jeollabuk-do, Korea) in 2013. Extracts were obtained from unripe and ripe fruits using 20 volumes of 5% ethanol or distilled water at 100°C for 4 h. 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

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) (designated as day-4) for 2 days until confluent. 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 10 µg/mL insulin, 0.5 mM 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 10 µg/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 (20 µM), a known anti-obesity drug, was used as the positive control.

2.3. 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 30 min. 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 500 nm.

2.4. 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±1°C), humidity (45±5°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), high fat diet (HFD), HFD + uRC 50 mg/kg/day (uRC 50), HFD + uRC 100 mg/kg/day (uRC 100), and HFD + Green tea extract 50 mg/kg/day as positive control (GT). Oral administration of uRC (50 and 100 mg/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.

2.5. Determination of serum lipid parameters

At the end of the experimental period, the mice were kept fasting for 12 h 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.

2.6. Statistical Analysis

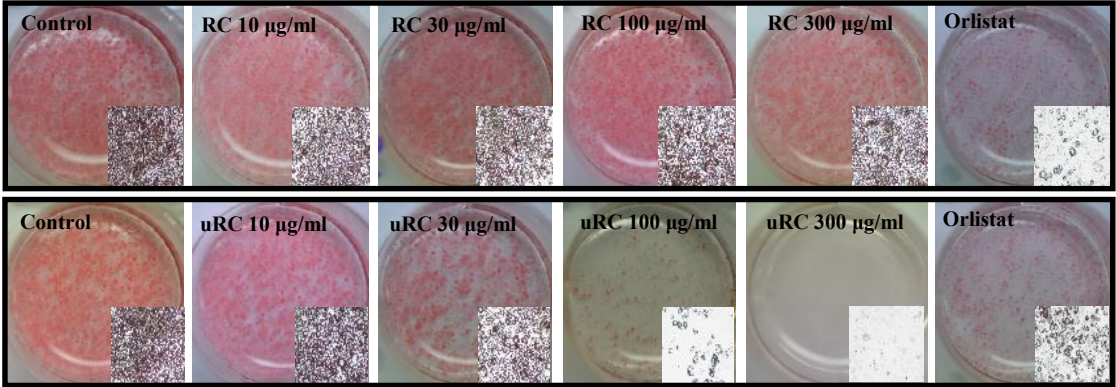
Data are presented as the mean ± S.D. The data were statistically evaluated using Student's t-test or one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test to compare significant differences between the groups at $p < 0.05$.

3. RESULTS AND DISCUSSION

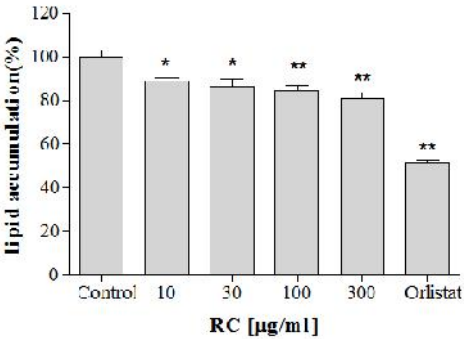
To examine the anti-adipogenic effects of uRC and RC, 3T3-L1 preadipocytes were treated with RC or uRC for 8 days. The differentiation of preadipocytes into adipocytes is associated

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, 1C). The inhibitory effects of RC and uRC on lipid accumulation during adipogenesis were dose dependent; treating differentiated cells with RC and uRC (300 µg/ml) decreased lipid levels by 18.8% and 79.7% on day 8, respectively. The mean percentage inhibition of uRC (300 µg/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 (Data not shown). These results indicate that uRC effectively blocks adipocyte differentiation in 3T3-L1 pre-adipocytes.

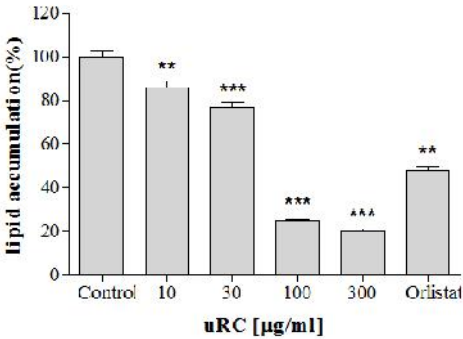
(A)



(B)



(C)



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Figure 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, c) 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±SE (n=3). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with the control.

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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 $88.5\pm5.5\%$, $82.0\pm5.6\%$, $29.5\pm1.9\%$, $12.0\pm0.7\%$ ($P<0.01$ and $P<0.001$), respectively. As the positive control, orlistat inhibited the lipid accumulation on rate to $56.2\pm2.5\%$ ($P<0.01$). 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

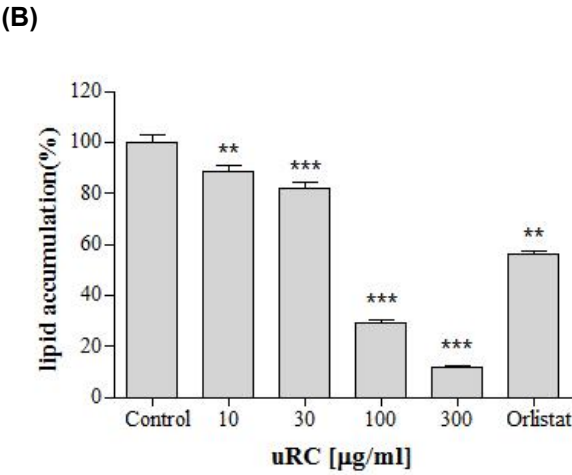
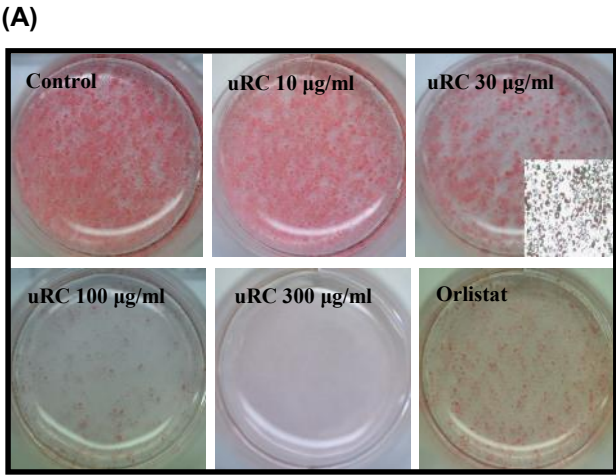


Figure 2. The effect of a 5% ethanol extract of unripe *R. coreanus* on lipid accumulation in 3T3-L1 adipocyte. (a) 3T3-L1 cells were differentiated with differentiation medium containing uRC for 8 days. Lipid accumulation was evaluated by Oil Red O staining (magnification,

186 100×). (b) The intracellular lipid accumulation was quantified by Oil Red O staining. Orlistat
187 (20 µM) was used as a positive control. Values are expressed as the mean±SE (n=3).
188 $P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control
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190 We also also investigated the anti-obesity effect of 5% ethanol extracts (50 and 100
191 mg/kg/day, oral ad) from uRC in high fat diet-induced obese (DIO) mouse model for 12
192 weeks. The body weight, food intake, adipose tissue weights, and serum lipid parameters of
193 each group are shown in Table 1. After 12 weeks, the final body weight gain of the uRC 50
194 (38.4±1.7 g) and uRC 100 (33.4±1.5 g) mice was significantly lower than that that of the HFD
195 mice (38.9±0.5 g) ($P<0.01$). The food intake was not different significantly between the HFD
196 group and the uRC group. The epididymal fat and perirenal adipose tissue weights in the
197 uRC 50 (2.44±0.16 g and 1.15±0.0 g) and uRC 100 (1.61±0.2 g and 0.67±0.1 g) mice were
198 significantly decreased compared with those in the HFD group (2.73±0.1 g and 1.21± 0.1 g)
199 ($P<0.01$ and $P<0.001$). The observed decrease in body weight by uRC treatment without any
200 significant difference in food intake suggests that uRC has a physiological effect on the
201 processing of food expenditure. uRC effectively also decreased the serum levels of total
202 cholesterol, LDL cholesterol, and glucose compared with the HFD group ($P<0.05$ and
203 $P<0.01$). On the other hand, uRC significantly increased the levels of HDL cholesterol in the
204 serum ($P<0.001$). Furthermore, uRC (5% ethanol extract) alleviated the liver damage by
205 lowering the levels of two common markers for liver damage, GOT (glutamic oxaloacetic
206 transaminase) and GPT (glutamic pyruvic transaminase), which were in turn increased by
207 HFD treatment (Table 1). GOT/GPT activities were not increased in the uRC extract (5%
208 ethanol extracts)-administered groups; instead, they were decreased. However, the relative
209 weights of the livers were not significantly different between the groups. These results
210 suggest that the organ and body weight reductions were not caused by liver toxicity, further
211 suggesting a beneficial role for *Rubus coreanus* extracts against obesity
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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 ^{**}

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. 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 obese mice.

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