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Anti-hyperglycemic and Anti-hyperlipidemic Effects of the Triterpenoid-Rich Fractions from *Rubus coreanus* and *Rubus crataegifolius* and Their Main Component, Niga-ichigoside F_1, in Streptozotocin-induced Diabetic Rats

Jongwon Choi[1], Yeong-Min Yoo[2], Min-Young Kim[2], Jung-Hwan Nam[3], Agung Nugroho[2], and Hee-Juhn Park[2]*

[1]*College of Pharmacy, Kyungsung University, Busan 608-736, Korea*
[2]*Department of Pharmaceutical Engineering, Sangji University, Wonju 220-702, Korea*
[3]*Highland Agriculture Institute, Rural Development Administration, Pyongchang 232-950, Korea*

Abstract – To find the antidiabetic activity of the tripterpenoid-rich fractions of *Rubus coreanus* (TRF-cor) and *R. crataegifolius* (TRF-cra) leaves or its main component niga-ichigoside F_1 (Niga-F_1), anti-hyperglycemic and anti-hyperlipidemic effects were investigated in the diabetic rat model induced by streptozotocin (STZ). Treatments of rats with 200 mg/kg of the TRF-cor, TRF-cra (each, *p.o.*) or 20 mg/kg of Niga-F_1 significantly inhibited the increase of blood glucose concentration about 44.8%, 28.7% or 20.6%, respectively, in the diabetic rats. In addition, treatments with those fractions inhibited the increase of serum concentrations of triglyceride, total cholesterol or LDL-cholesterol caused by STZ. The inhibitory rate on atherogenic index (AI) values of the TRF-cor (200 mg/kg), TRF-cra (200 mg/kg) or Niga-F_1 (20 mg/kg)-treated groups were decreased about 55.7%, 36.3% or 22.6%, respectively, comparable to STZ-treated group. In the oral glucose tolerance test, treatment of TRF-cor or TRF-cra inhibited the increase of blood glucose concentration in the STZ-induced rats. Administration of 20 mg/kg of Niga-F_1 (*p.o.*) also exhibited similar effects with the effects of both TRFs at 200 mg/kg dose (*p.o.*). These results support that the triterpenoids, in particular Niga-F_1, are contributed to the antidiabetic effects of *R. coreanus* or *R. crataegifolius*.

Keywords – *Rubus coreanus*, *R. crataegifolius*, triterpenoid, niga-ichigoside F_1, anti-hyperglycemic, anti-hyperlipidemic

Introduction

Rubus coreanus (Rosaceae) is a woody plant that grows wild in Korea. Its ripe fruit is an edible food, whereas the unripe fruit (Rubi Fructus) is used as an oriental herb medicine to treat diabetes mellitus and sexual impotency (Moon, 1994). We previously reported that Rubi Fructus had anti-nociceptive and anti-inflammatory (Choi *et al*., 2003), also anti-rheumatic and gastroprotective effects (Nam *et al*., 2006).

R. coreanus fruit turns black when ripe, while those of *R. crataegifolius* become red. The *R. coreanus* and *R. crataegifolius* fruits growing wild in Korea are applicable to black and red raspberries, respectively, in the western countries. Unripe *R. coreanus* fruit contains 19a-hydroxyursane-type triterpenoids, as does ripe *R. crataegifolius*. The leaves of these plants also contain large amounts of these triterpenoids (Nam *et al*., 2007).

*Author for correspondence
Tel: +82-+33-730-0564; E-mail: hjpark@sangji.ac.kr

Diabetes mellitus, obesity, and atherosclerosis are increasing in developed countries, mainly due to changes in the diet. Diets rich in vegetables and functional foods as well as restriction of high calorie foods, can help treat or to prevent diabetes mellitus or hyperlipidemia. In this work, we elucidated the anti-diabetic effects of both the triterpenoid-rich fraction (TRF) of the leaves of *R. coreanus* and *R. crataegifolius* or its main component niga-ichigoside F_1 (Fig. 1) in streptozotocin (STZ)-induced rats by measuring body weight gain and food intake, blood glucose levels, levels of serum cholesterol and glucose tolerance.

Materials and Methods

Plant material – The leaves of *R. coreanus* and *R. crataegifolius* were collected in June near Wonju, Kangwon-Do, dried, and crushed for extraction.

Preparation of TRF from *R. coreanus* and *R. crataegifolius* – Plant material (500 g) was extracted with

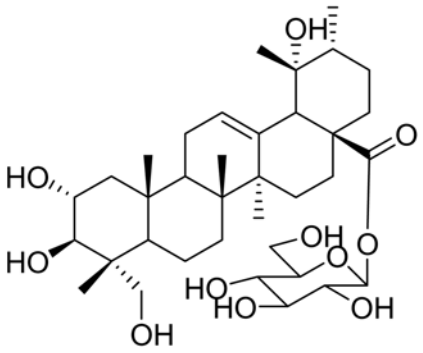

Fig. 1. Structure of niga-ichigoside F_1.

MeOH under reflux three times. After cooling, the methanolic extracts were filtered and evaporated *in vacuo* using a rotary evaporator. The extracts were suspended in 800 mL distilled water and partitioned with 800 mL hexane three times for fractionation. The water layer was fractionated with 800 mL ether three times, followed by 800 mL water-saturated BuOH three times. The BuOH-soluble portion was evaporated to dryness, suspended in 500 mL water, and then poured over a charcoal (100 g) column to remove any pigments or phenolic substances. The eluted solution was subjected to Diaion HP-20 (320 g, 5 × 70 cm) and then eluted with 2000 ml distilled water to remove any sugars. This column was then eluted with 2.0 L MeOH and the eluate was concentrated to dryness. The triterpenoid-rich fractions (TRFs) from *R. coreanus* and *R. crataegifolius* were labeled as TRF-cor (25.2 g) and TRF-cra (5.1 g), respectively. On TLC, no spots other than the triterpenoid were observed.

Isolation of Niga-F_1 – The TRF-cor (15 g) was chromatographed on a silica gel column (280 g, 5 × 55 cm) using $CHCl_3$-MeOH (4 : 1) as an eluent. The retention volumes of 0.90 - 1.20 L, 1.4 - 1.75 L, and 2.2 - 2.5 L were concentrated to dryness *in vacuo* to give Cor-Fr.1 (1.1 g), Cor-Fr.2 (2.9 g), and Cor-Fr.3 (4.2 g), respectively. The Cor-Fr.3 fraction was purified using silica gel column chromatography with $CHCl_3$-MeOH-H_2O (75 : 25 : 10, lower phase) to yield Niga-F_1. TRF-cra was also purified by same chromatography steps to yield Niga-F_1 (Nam *et al*., 2006).

Niga-F_1 – Amorphous powder from MeOH, mp, 233 - 6 °C, $[\alpha]_D^{26}$ +11.2° (c = 0.021, MeOH); ^{1}H- NMR and ^{13}C-NMR: Literature (Nam *et al*., 2006).

Experimental animals – Male Sprague-Dawley rats were purchased from Daehan Bio Link Co., allowed to adapt to laboratory conditions (temperature: 20 ± 2 °C, dampness: 40 - 60%, light/dark cycle: 12 h) for a week, and rats weighing 200 ± 10 g were used for animal experiments. 24 h before the experiment, only water was offered to the animals. Considering variations in enzyme activity during one day, the animals were sacrificed at a fixed time (10:00 A.M. - 12:00 P.M.). These experiments were approved by the University of Kyungsung Animal Care and Use Committee. All procedures were conducted in accordance with the "Guide for Care and Use of Laboratory Animals" published by the National Institutes of Health.

Induction of experimental diabetes – Diabetes mellitus was induced by intravenous injection of STZ in the tail vein (Sigma, St. Louis, MO) dissolved in 0.1 M cold sodium citrate buffer (pH 4.5) at 50 mg/kg body weight. After 48 h, only rats with fasting blood glucose levels 300 - 400 mg/dl were considered diabetic and used in the study.

Experimental design – Untreated (normal), diabetic (control), and TRF-treated rats were randomly assigned to 3 different groups (n = 9). One group received distilled water, a second group (control) had diabetes mellitus induced by STZ treatment, and the third STZ-treated groups received TRF-cor (100, 200 mg/ml), TRF-cra (100, 200 mg/ml), or Niga-F_1 (10, 20 mg/ml) for consecutive 4 weeks. The rats were treated orally. Rats were fasted 7 h after the final sample treatment, anesthetized with CO_2, and blood was collected from abdominal aortas. Serum was separated by centrifugation at 2,500 × g for 15 min.

Measurement of blood glucose concentrations, body weight, and food intake – Blood glucose concentration was measured by the method of Shirwaikar *et al*. (2006). Body weights were measured 24 h before STZ treatment and 24 h after sample treatment to calculate the change of body weights. Food intake was measured one day before sacrifice.

Oral glucose tolerance test – The STZ-induced diabetic rats were orally administered TRF-cor, TRF-cra and Niga-F_1, fasted for about 10 - 14 h, treated orally with 1.5 g/kg of glucose, and blood glucose concentration measured at 0, 30, 60 and 120 min.

Measurement of total cholesterol levels – Total serum cholesterol levels were measured by following the manufacturer's protocol with an AM 202-K assay kit (Asan Pharm. Co., Korea) prepared by Richmond (1976). Three milliliter of solution (cholesterol esterase 20.5 U/l, cholesterol oxidase 10.7 U/l, sodium hydroxide 1.81/l, potassium phosphate monobasic 13.6 g/l, phenol 1.88 g/l) was mixed with 20 μL of serum and incubated at 37 °C for 5 min. For determination of total cholesterol levels, the absorbance of the reaction mixture was measured at

550 nm and levels were calculated using a standard calibration curve.

Measurement of triglyceride levels – Triglyceride levels were measured by following the manufacturer's protocol with an AM 157S-K assay kit (Asan Pharm. Co., Korea) prepared by McGowan *et al*. (1983). Three milliliter of solution [lipoprotein lipase 10,800 U, glycerol kinase 5.4 U, peroxidase 135,000 U, L-α-glycero phosphooxidase 160 U, N,N-bis(2-hydroxyethyl)-2-aminomethane sulfonic acid 0.427 g/dl] was mixed with 20 μL of serum and incubated at 37 °C for 10 min. For determination of serum triglyceride levels, the absorbance of the reaction mixture was measured at 550 nm and the levels were calculated using a standard calibration curve.

Measurement of high density lipoprotein-cholesterol (HDL) levels – Serum HDL-cholesterol levels were measured by following the manufacturer's protocol with an AM 203-K assay kit (Asan Pharm. Co., Korea) prepared by Noma *et al*. (1978). Twenty microliter of serum was mixed with two-hundred microliter of solution (dextran sulfate 0.1%, magnesium chloride 0.1 M), incubated at room temperature for 10 min, and centrifuged at 2,500 × g for 10 min. The 100 μL of supernatant was mixed with 3 mL of solution [lipoprotein lipase 10,800 U, glycerol kinase 5.4 U, peroxidase 135,000 U, L-α-glycero phosphooxidase 160 U, N,N-bis(2-hydroxyethyl)-2-aminomethane sulfonic acid 0.427 g/dl] and incubated at 37 °C for 5 min. For determination of serum HDL levels, the absorbance of the reaction mixture was measured at 500 nm and their levels were calculated as mg/dl using a standard calibration curve.

Calculation of low density lipoprotein-cholesterol (LDL) levels – LDL levels were calculated using the Friedewald's equation (Friedewald *et al*., 1972). LDL-cholesterol = total cholesterol HDL-cholesterol (triglyceride/5).

Statistical analysis – Results are expressed as means ± S.D. (n = 9). Statistical analysis was performed with Duncan's multiple range tests. Differences were considered significant at $p < 0.05$.

Results and Discussion

The STZ-treated group (control group) showed decreased body weight and a marked increase of food intake compared with the untreated group (Table 1). TRF-cor (200 mg/kg) and TRF-cra (200 mg/kg) both significantly inhibited these changes, but lower doses (100 mg/kg) did not. TRF treatment decreased food intake compared to the STZ group. Niga-F_1 (20 mg/kg), the main component of TRF, significantly inhibited changes in body weight and food intake by STZ treatment.

Table 1. Effect of TRF-cor (triterpenoid-rich fraction of *R. coreanus*), TRF-cra (triterpenoid-rich fraction of *R. crataegifolius*) or Niga-F_1 on body weight gain and food intake in STZ-treated hyperglycemic rats

Treatment	Dose (mg/kg)	Body weight (g)	Food intake (g/day/rat)
Utreated		68.9 ± 7.23^{a}	23.8 ± 5.47^{b}
STZ		–30.7 ± 6.47^{e}	35.9 ± 4.26^{a}
TRF-cor	100	–17.3 ± 5.43^{d}	30.7 ± 5.11^{a,b}
	200	20.6 ± 4.97^{b}	28.4 ± 3.56^{a,b}
TRF-cra	100	–22.4 ± 5.11^{d,e}	33.4 ± 4.16^{a}
	200	13.2 ± 3.97^{b,c}	30.5 ± 3.48^{a,b}
Niga-F_1	10	–25.6 ± 4.56^{d,e}	33.9 ± 5.10^{a}
	20	7.35 ± 3.42^{c}	31.3 ± 4.18^{a,b}

Streptozotocin (STZ, 50 mg/kg) in 0.2 ml of 10 mM citrate buffer (pH 4.5) was injected via the tail vein. Rats were orally administered TRF-cor, TRF-cra (triterpenoid-rich fraction of the leaves of *R. coreanus*) or Niga-F_1 (niga-ichigoside F_1) daily for 4 weeks after a STZ-induced hyperlipidemic state. The rats were sacrificed 7 h after the last treatment. Assay procedures are described in the materials and methods. Data represents means ± S.D. (n = 9). Values followed by the same superscript letter are not significantly different from each other ($p < 0.05$) by Ducan's multiple range test.

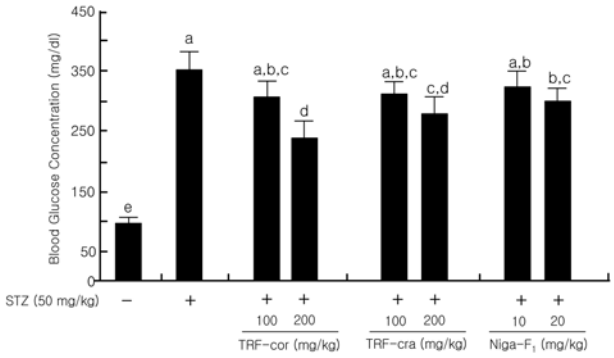

Fig. 2. Effect of TRF-cor, TRF-cra, or Niga-F_1 on the blood glucose concentration in STZ-treated hyperglycemic rats. Data represents means ± S.D. (n = 9). Values followed by the same superscript letter are not significantly different from each other ($p < 0.05$) by Ducan's multiple range test.

The STZ-treated group showed increased blood glucose concentrations, with TRF-cor and TRF-cra (both at 200 mg/kg) or Niga-F_1 (20 mg/kg) suppressing these increases by 44.8%, 28.7% and 20.6%, respectively (Fig. 2). These results suggest that the triterpenoids of *R. coreanus* and *R. crataegifolius* or their main component, Niga-F_1, may be effective in treating diabetes mellitus.

The atherogenic index (AI) is a measure of hyperlipidemia that incorporates: 1) increased chylomicron biosynthesis in the small intestine; 2) increased triglyceride,

Table 2. Effect of TRF-cor, TRF-cra, or Niga-F_1 on the concentration of triglyceride, total cholesterol (total), HDL-cholesterol, LDL-cholesterol (LDL), or atherogenic index (AI) in STZ-treated hyperglycemic rats

Treatment	Dose (mg/kg)	Triglyceride (mg/dl)	Total (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Untreated		74.3 ± 6.25^{g}	64.8 ± 5.49^{e}	38.9 ± 4.17^{c}	12.1 ± 2.42^{f}
STZ		140.8 ± 9.27^{a}	151.3 ± 4.57^{a}	$42.9 \pm 2.49^{b,c}$	80.3 ± 0.84^{a}
TRF-cor	100	$120.4 \pm 5.69^{c,d}$	136.4 ± 4.53^{b}	45.3 ± 3.17^{b}	65.1 ± 4.25^{c}
	200	96.8 ± 3.11^{f}	108.7 ± 5.17^{d}	51.4 ± 2.13^{a}	39.7 ± 3.97^{e}
TRF-cra	100	$128.8 \pm 7.28^{b,c}$	$144.6 \pm 4.25^{a,b}$	$43.8 \pm 3.09^{b,c}$	$74.3 \pm 5.11^{a,b}$
	200	$103.8 \pm 6.25^{e,f}$	123.9 ± 4.86^{c}	$47.6 \pm 3.11^{a,b}$	53.5 ± 4.18^{d}
Niga-F_1	10	$135.2 \pm 6.43^{a,b}$	150.9 ± 3.45^{a}	$43.1 \pm 2.25^{b,c}$	79.8 ± 4.25^{a}
	20	$112.4 \pm 8.20^{d,e}$	137.9 ± 4.21^{b}	$46.5 \pm 2.10^{a,b}$	$68.9 \pm 3.27^{b,c}$

The assay procedures are described in the materials and methods. Data represents means ± S.D. (n = 9). Values followed by the same superscript letter are not significantly different from each other ($p < 0.05$) by Ducan's multiple range test. LDL-Cholesterol: Total Cholesterol – (HDL-Cholesterol + Triglyceride/5); Atherogenic Index (AI): (Total Cholesterol – HDL-Cholesterol)/HDL-Cholesterol.

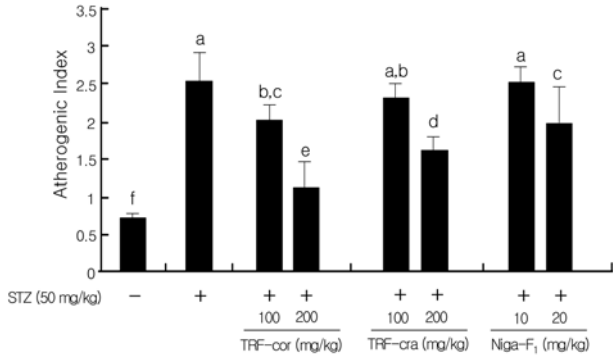

Fig. 3. Atherogenic index of TRF-cor, TRF-cra, or Niga-F_1 in STZ-treated hyperglycemic rats. Data represents means ± S.D. (n = 9). Values followed by the same superscript letter are not significantly different from each other ($p < 0.05$) by Ducan's multiple range test.

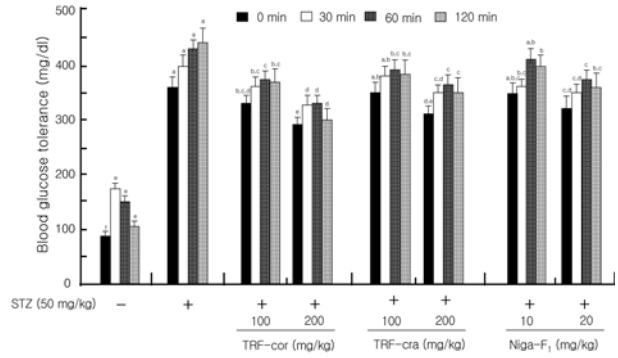

Fig. 4. Effect of TRF-cor, TRF-cra, or Niga-F_1 on the blood glucose tolerance test in streptozotocin-induced hyperglycemic rats. Data represents means ± S.D. (n = 9). Values followed by the same superscript letter are not significantly different from each other ($p < 0.05$) by Ducan's multiple range test.

LDL- and VLDL-cholesterol biosynthesis in the liver; 3) decreased HDL-cholesterol biosynthesis in the liver; 4) decreased lipase activity and removal of triglycerides in the peripheral tissue (Miller, 1978). Hyperlipidemia often leads to atherosclerosis. In addition, lipid peroxides and oxygen free radicals produced from LDL peroxidation are toxic to endothelial cells. Oxidized LDLs are adhesive to blood vessel walls, leading to further accumulation of other oxidized LDLs, platelets, and macrophage cells to the vessel walls (Steinberg, 1997). STZ treatment increased serum triglyceride, total- and LDL-cholesterol, but did not markedly change HDL-cholesterol, leading to an increased AI value compared to the untreated group. TRF treatment decreased the hyperlipidemia and high AI caused by STZ treatment. AI values of the STZ-treated group, TRF-cor (200 mg/kg dose) or TRF-cra (200 mg/kg dose)-treated groups were 2.53 ± 0.48, 1.12 ± 0.03, or 1.61 ± 0.17, respectively, while that of the Niga-F_1 (20 mg/kg dose) group was 1.96 ± 0.15. AI inhibition by TRF-cor, TRF-cra and Niga-F_1 were 55.7%, 36.3%, and 22.6%, respectively (Fig. 3). These results indicate that the triterpenoid in *R. coreanus* or *R. crataegifoius* and its main component, Niga-F_1, inhibits hyperlipidemia and atherosclerosis due to diabetes mellitus. This anti-hyperlipidemic effect, particularly decreasing the AI value, was stronger than the anti-hyperglycemic effect. The activity of TRF-cor was more potent than that of TRF-cra.

After oral administration of glucose (1.5 g/kg) to STZ-treated rats, blood glucose concentrations were measured from 0 to 120 min. Blood glucose concentrations in the STZ-treated group increased up to 120 min, indicating reduced glucose tolerance (Fig. 4). Treatment with TRF-cor (200 mg/kg), TRF-cra (200 mg/kg), or Niga-F_1 (20 mg/kg) inhibited the reduced glucose tolerance by STZ treatment, indicating that they contributed to blood glucose transport into cells. TRF-cor had more potent anti-hyperglycemic and anti-hyperlipidemic activitys than TRF-cra.

In conclusion, the triterpenoids of *R. coreanus* or *R. crataegifolius* contribute to the traditional therapeutic use in treating diabetes mellitus, with Niga-F_1 as a key component. Also, TRF-cor has greater potency than TRF-cra in animal models. The two TRFs exhibited more potent inhibitory effects on hyperlipidemia than on hyperglycemia.

Acknowledgements

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