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D-*chiro*-Inositol-Enriched Tartary Buckwheat Bran Extract Lowers the Blood Glucose Level in KK-A^y Mice

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D-*chiro*-inositol (DCI) is an active compound in tartary buckwheat [*Fagopyrum tataricum* (L.) Gaench] with an insulin-like bioactivity. The present study was performed to (i) prepare DCI-enriched tartary buckwheat bran extract (TBBE), (ii) evaluate its acute toxicity in mice, and (iii) examine its blood glucose lowering activity in diabetic mice. It was found that steaming buckwheat bran in an autoclave at 1.6 MPa and 120 °C for 60 min could significantly enrich the DCI level in TBBE from 0.03 to 0.22% and further to 22% after passage of the TBBE through activated carbon and ion exchange resins. An acute toxicity test demonstrated that the LD_{50} of TBBE was >20 g/kg of body weight in mice, suggesting that TBBE was in general nontoxic and safe in mice. Male KK-A^y mice (type 2 diabetic) and C57BL/6 mice (the control) were used to investigate the antidiabetic activity of TBBE. In KK-A^y mice, the blood glucose, plasma C-peptide, glucagon, total cholesterol, triglyceride, and blood urea nitrogen (BUN) levels were significantly higher than those in the C57BL/6 mice. In addition, KK-A^y mice showed an obvious decrease in insulin immunoreactivity in the pancreas. The present study clearly demonstrated that oral administration of DCI-enriched TBBE could lower plasma glucose, C-peptide, glucagon, triglyceride, and BUN, improve glucose tolerance, and enhance insulin immunoreactivity in KK-A^y mice.

KEYWORDS: Tartary buckwheat; DCI; steaming; blood glucose; antidiabetic activity

INTRODUCTION

Interest in tartary buckwheat [*Fagopyrum tataricum* (L.) Gaench] as a functional food ingredient is increasing (*1, 2*). D-*chiro*-Inositol (DCI), flavonoids, and sterols present in buckwheat have been claimed as the active ingredients responsible for benefits associated with the consumption of buckwheat (*3*). Acting as a component of a putative mediator of insulin action, DCI is a compound with an insulin-like bioactivity and is able to decrease plasma glucose in both streptozotocin-treated rats and obese rhesus monkeys with various degrees of spontaneous insulin resistance (*4*). In humans, non-insulin-dependent diabetes mellitus (NIDDM) has also been associated with decreased urinary DCI excretion (*5, 6*).

DCI exits naturally in two major forms, the free form and its galactosyl derivatives. Fagopyritols, chemically named as α-D-galactopyranosyl-D-*chiro*-inositols with one to three galactosyl moieties, are the major form of these derivatives present in not only buckwheat (*7*) but also other plants including soybean, lupin, lentil, chickpea (*8*), and jojoba (*9*). In addition, some fagopyritols have structural similarities with D-*chiro*-inositol phosphoglycan (IPGs), which has been proposed as an insulin mediator (*10*). It is believed that α-galactosidase is able to hydrolyze fagopyritols and release D-*chiro*-inositol (*11*). However, α-galactosidase does not exist in the human stomach (*12*); therefore, enrichment of DCI in foods by chemical methods seems to be necessary.

We have interest in enriching the DCI content in buckwheat extract. In view of a study by Wang et al. (*13*), who reported that a high-temperature steaming could change favorably the chemical constituents of ginseng, we optimized the extraction conditions and found that steaming buckwheat bran in an autoclave could significantly increase the yield of DCI in tartary buckwheat bran extract (TBBE). We reaffirmed that DCI-enriched TBBE could decrease the blood glucose level, modify favorably the lipid profile, and improve insulin immunoreactivity in KK-A^y mice.

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Table 1. Composition of the Experimental Diets

content	ICR	C57BL/6	KK-A^y
corn starch (%)	40.3	40.3	28.21
soybean meal (%)	16.6	16.6	11.62
soybean powder (%)	5.9	5.9	4.13
wheat flour (%)	20	20	14
wheat bran (%)	10.6	10.6	7.42
fish powder (%)	1.2	1.2	0.84
$CaCO_3$ (%)	1.9	1.9	1.33
$CaPO_4$ (%)	2.2	2.2	1.54
mineral (%)	0.8	0.8	0.56
vitamin (%)	0.1	0.1	0.07
soybean oil (%)	0.4	0.4	0.28
sucrose (%)	0	0	10
lard (%)	0	0	10
yolk powder (%)	0	0	10

MATERIALS AND METHODS

Materials. Tartary buckwheat [*F. tataricum* (L.) Gaench] seeds were obtained from the Institute of Comprehensive Utilization of Agricultural Products in Shanxi Province, China. Buckwheat bran (3 kg), accounting for 15% of the whole grain, was prepared in a grain polish machine. It was dried at 40 °C, ground in a laboratory mill, and passed through an 80 mesh screen sieve. DCI standard (99%) and phenyl α-D-glucoside were purchased from Sigma-Aldrich (Shanghai, China). *N*-Trimethylsilylimidazole was purchased from Shanghai Reagent No. 1 Factory (Shanghai, China). Pyridine was purchased from Beijing Reagent Factory (Beijing, China). Activated carbons were purchased from Tangshan Marine Chemical Co. Factory (Hebei, China). Strong acidic styrene cation ion-exchange resin [001×7(732)] and strong alkaline styrene anion ion-exchange resin [201×7(717)] were purchased from Taiyuan Resin Factory (Shanxi, China).

Preparation of TBBE. Buckwheat bran was extracted with 60% ethanol aqueous solution (30 L × 2) at 50 °C for 30 min. After vacuum filtration at 50 °C, the supernatants were combined and concentrated to one-third volume under a reduced pressure using a rotary evaporator and then stored at 4 °C until use.

Pressure Hydrolysis and Purification. Crude TBBE solution was steamed in a GSH-0.5A autoclave (Huixin, Weihai, China) at 1.6 MPa and 120 °C for 60 min. The solution was passed through the activated carbons to decolorize the sugar syrup. Desalination was followed by passing TBBE through the strong acidic styrene cation ion-exchange resin and strong alkaline styrene anion ion-exchange resin. The TBBE solution was then concentrated under a reduced pressure at 50 °C, followed by storage at 4 °C before use.

Determination of Moisture, Total Proteins, and Carbohydrates. AACC methods were used to determine moisture content. Protein was determined according to the Kjeldahl method, using a nitrogen to protein conversion factor of 5.75. Carbohydrate was determined by indirect iodometric titration (*14, 15*).

Determination of DCI. DCI was measured as previously described by Horbowicz et al. (*5*). The TBBE solution (40 μL) described above (filtered by a 0.45 μm filtration membrane) in a 1.5 mL tube, with the addition of 40 μL of 0.2% internal standard (phenyl α-D-glucoside), was dried under vacuum at 70 °C for 30 min. The dry residues were derivatized using 200 μL of a mixture of trimethylsilylimidazole/pyridine (1:1, v/v) in a tightly capped silylation vial (Supelco, Bellefonte, PA) at 70 °C for 30 min and then cooled at room temperature.

DCI was analyzed on a Shimadzu GC-9A gas chromatograph (Tokyo, Japan) equipped with a flame ionization detector equipped with an SE-30 capillary column (50 mm × 0.25 mm, 0.25 μm film thickness; J&W Scientific, Folsom, CA). The initial column temperature was set at 150 °C, increased to 200 °C at 3 °C/min, programmed to 325 at 7 °C/min, and then held for 20 min. The injection port and the detector temperature were set at 280 °C. The carrier gas (N_2) flow rate was operated at 2.0 mL/min.

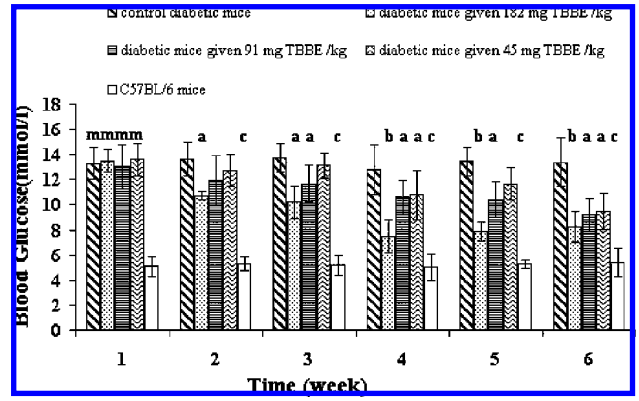

Figure 1. Changes in fasting blood glucose levels in diabetic KK-A^y mice given orally various doses of DCI-enriched tartary buckwheat bran extract (TBBE) compared with that in C57BL/6 mice. Data are mean ± SD of 10 animals. "m" indicates $P < 0.001$, compared with C57BL/6 mice. "a" indicates $P < 0.05$, "b" indicates $P < 0.01$, and "c" indicates $P < 0.001$, compared with C57BL/6 mice.

Table 3. Effect of D-*chiro*-Inositol-Enriched Purified Tartary Buckwheat Bran Extract (TBBE) on Body Weight in Diabetic KK-A^y and C57BL/6 Mice

	initial body wt (g)	final body wt (g)
control diabetic mice	36.06 ± 1.20	37.53 ± 1.06
diabetic mice given 182 mg of TBBE/kg	35.57 ± 1.26	37.27 ± 0.73
diabetic mice given 91 mg of TBBE/kg	35.83 ± 1.06	36.37 ± 1.86
diabetic mice given 45 mg of TBBE/kg	37.03 ± 1.12	38.77 ± 0.73
C57BL/6 mice	16.70 ± 0.54	16.80 ± 0.42

Oral Acute Toxicity Test. Forty healthy ICR male and female mice (18−22 g) were obtained from the Department of Laboratory Animal Science Center (Beijng, China). Mice were randomly divided into two groups with 10 males and 10 females in each group and housed in a temperature-controlled animal room (21−25 °C) with a relative humidity of 40−70% for 3 days. TBBE was dissolved in redistilled water, and a dose of 20 g/kg was orally administered in the treated group, whereas the control group received the same volume of redistilled water. Possible toxic symptoms and mortality in mice were monitored for 14 days after administration. Finally, the median lethal dose (LD_{50}) and acute toxic classification were determined.

Diabetic Mice. Experiments were carried out according to the method of Yao et al. (*16*). Ten male C57BL/6 control mice and 40 male diabetic KK-A^y mice (*17*−*20*) were obtained from the Department of Laboratory Animal Science Center (Beijing, China). The diabetic mice were divided into four groups according to their weights and blood glucose levels to make the average weights and blood glucose levels similar among the groups: group I, control diabetic animals ($n = 10$); group II, diabetic animals given 182 mg of TBBE/kg (equivalent to DCI 40 mg/kg, $n = 10$); group III, diabetic animals given 91 mg of TBBE/kg (equivalent to DCI 20 mg/kg, $n = 10$); group IV, diabetic animals given 45 mg of TBBE /kg (equivalent to DCI 10 mg/kg, $n = 10$). C57BL/6 mice were regarded as group V. Body weights were measured during the treatment. All mice were housed individually in

Table 2. Effect of D-*chiro*-Inositol-Enriched Purified Tartary Buckwheat Bran Extract (TBBE) on Body Weight in ICR Mice

	male			female		
group	*n*	initial body wt (g)	final body wt (g)	*n*	initial body wt (g)	final body wt (g)
0.2 mL/10 g of redistilled water	10	22.21 ± 0.74	27.05 ± 1.96	10	22.94 ± 1.03	35.59 ± 1.96
20 g/kg of purified extract	10	22.22 ± 0.75	27.11 ± 1.85	10	22.96 ± 1.07	35.57 ± 3.14

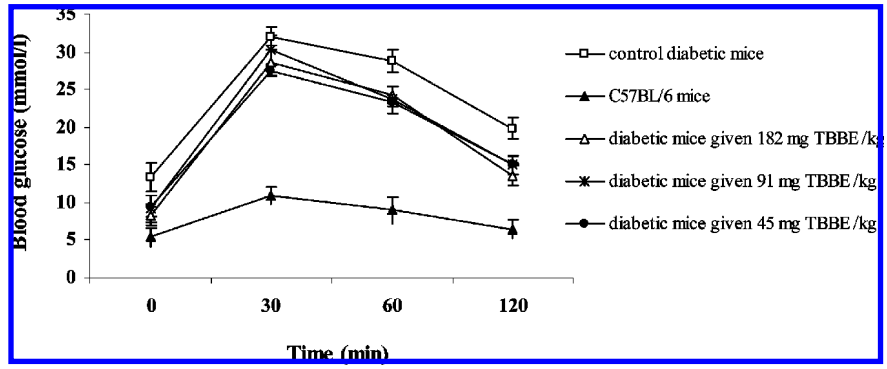

Figure 2. Oral glucose tolerance test (OGTT) in C57BL/6 mice and diabetic KK-A^y mice given orally various doses of DCI-enriched tartary buckwheat bran extract (TBBE) at week 5. Each time point represents the mean ± SD of 10 animals.

Table 4. Plasma Parameters in Diabetic KK-A^y and C57BL/6 Mice[a]

	C-peptide (ng/mL)	glucagons (pg/mL)	triglycerides (mmol/L)	total cholesterol (mmol/L)	BUN[b] (mmol/L)
control diabetic mice	1.40 ± 0.04	108.87 ± 3.54	1.32 ± 0.03	5.50 ± 0.35	10.09 ± 0.28
diabetic mice given 182 mg of TBBE/kg	0.56 ± 0.06 b	50.41 ± 4.52 c	1.02 ± 0.07 a	5.15 ± 0.42	8.80 ± 0.71 a
diabetic mice given 91 mg of TBBE/kg	0.57 ± 0.03 b	55.08 ± 2.34 c	1.09 ± 0.08	5.05 ± 0.57	9.67 ± 0.64
diabetic mice given 45 mg of TBBE/kg	1.07 ± 0.07	116.46 ± 3.36	1.05 ± 0.05	5.14 ± 0.38	10.82 ± 0.49
C57BL/6 mice	0.48 ± 0.04 c	60.07 ± 5.15 c	0.88 ± 0.02 b	2.83 ± 0.08 c	5.48 ± 0.52 b

[a] "a", $P < 0.05$; "b", $P < 0.01$; "c", $P < 0.001$, compared with diabetic control mice. [b] BUN, blood urea nitrogen.

stainless steel wire-bottom cages in an air-conditioned room kept at controlled ambient temperature (22 ± 1 °C), humidity (50 ± 10%), and a 12 h light/dark cycle. The composition of the diets fed to the mice was in accordance with the general quality standard for formula feeds of laboratory animals in China (GB 14924.1, 2001) (**Table 1**). They were allowed free access to the diet and water. Blood glucose levels were determined for each mouse throughout the experiment. The experiment was carried out according to the European Community Guidelines for the Use of Experimental Animals and approved by the Peking University Committee on Animal Care and Use.

Fasting Blood Glucose Levels and Oral Glucose Tolerance Test (OGTT). Blood samples were taken from the tail vein weekly after overnight fasting. Glucose was determined using a glucose analyzer (ACCU-CHEK Active, Roche, Shanghai, China). On the morning of OGTT, fasting animals were given glucose orally (2 g/kg). Blood glucose levels were measured at 0 (before oral glucose), 30, 60, and 120 min after glucose administration.

Analysis of Plasma C-Peptide, Glucagon, Cholesterol, and Triglyceries. Plasma C-peptide was determined using an enzyme-linked immunosorbent assay kit (ADL, San Diego, CA). Glucagon was similarly determined by an enzyme-linked immunosorbent assay kit (RapidBio Laboratory, Calabasas, CA). Plasma total cholesterol, triglycerides, and blood urea nitrogen (BUN) were measured, using an autobiochemical analyzer (Hitachi 7600, Japan).

Immunohistochemical Evaluation on Pancreas. The pancreas was removed immediately after sacrifice and rinsed in ice-cold saline. The tissue samples were fixed in paraformaldehyde, dehydrated in a graded series of ethanol, and embedded in paraffin wax before sectioning. After being dewaxed, rehydrated, and washed in phosphate-buffered saline, sections were immersed in a solution of 3% H_2O_2 for 10 min. The sections were then preincubated with nonimmune serum for 15 min and subsequently replaced with the mouse anti-insulin antibody (1:200, SP-9000, Zymed, CA) for incubation at 4 °C for 16 h. Biotinylated goat anti-mouse immunoglobulin was used as a secondary antibody. They were labeled with streptavidin peroxidase followed by incubation with the secondary antibody at 37 °C for 30 min. The localization of the antigen was indicated by a brown color obtained with 3-amino-9-ethylcarbazole (AEC) as a chromogenic substrate for peroxidase activity. Slides were counterstained with hematoxylin for microscopic observation. The specificity of the immunohistochemical staining was checked by omission of the primary antibody or by using an inappropriate antibody (antigastrin).

Statistical Analysis. All values were expressed as mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's t test. Differences with $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Buckwheat seed bran is rich in DCI and its derivatives. In general, DCI has been prepared from either natural sources or chemical synthesis (*21, 22*). Buckwheat bran, which contains seed coat and embryo tissues, is commonly used as an animal feed in China. In this study, TBBE prepared from buckwheat bran contained 72.7 g of protein, 278 g of lipids, and 5.7 g of carbohydrate/kg, with most DCI being in free form or galactosides, predominantly fagopyritol A_1 and fagopyritol B_1. The latter have to be converted to DCI in order to increase its absorption (*5*). In this regard, the bioavailability of fagopyritols is very low, as humans and other monogastric animals do not have α-D-galactosidase (*13*). In this study, 3 kg of tartary buckwheat bran was used to prepare 22.2 g of TBBE. Without a steaming procedure, DCI reached only 0.032%, whereas with an autoclave steaming treatment, it was increased significantly to 0.22%. Further enrichment through column chromatography of activated carbon and ion exchange resins could produce TBBE with DCI reaching 22%. The present results suggest that pressure and heat can disrupt galactosidic bonds and release the free form of DCI.

In the oral acute toxicity test, there was no difference in body weights between the control group and TBBE-treated mice (**Table 2**). The LD_{50} of the TBBE-treated group was >20 g/kg in mice. During the 14 day observation period, mice treated with and without TBBE all survived and were healthy in general. On the basis of these observations, we concluded that TBBE was not toxic but instead safe for use as an animal feed according to the criteria of acute toxic classifications (*23*).

Hyperinsulinemic KK-A^y mice have a high fasting blood glucose level similar to that of diabetic patients. KK-A^y mice with homozygous mutation exhibit metabolic abnormalities such as hyperglycemia and glucose intolerance that phenotypically resemble human type 2 diabetes (*24, 25*). With regard to the body weight, KK-A^y mice were heavier than the age-matched C57BL/6 control mice (**Table 3**). The present study found that

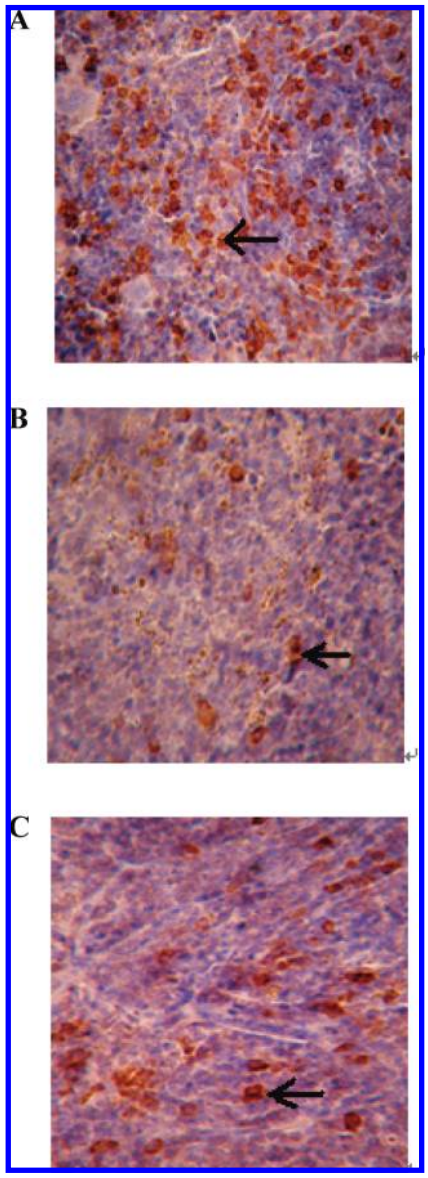

Figure 3. Immunohistochemical evaluation on pancreas (400×): (**A**) C57BL/6 mice showing normal insulin immunoreactivity; (**B**) diabetic KK-A^y mice showing decreased insulin immunoreactivity; (**C**) diabetic KK-A^y mice orally given DCI-enriched tartary buckwheat bran extract (TBBE) (equivalent to 40 mg/kg DCI), showing an improvement in insulin immunoreactivity. Insulin immunoreactivity is shown by arrows.

TBBE had no effect on the final body weight of KK-A^y mice during the 5 week period of treatment.

TBBE was hypoglycemic in KK-A^y mice. Initially, the fasting blood glucose level in the four KK-A^y groups (I−IV) was significantly higher (by 163%, $P < 0.001$) than that in C57BL/6 mice (group V). The present study clearly demonstrated that oral administration of TBBE for 5 weeks caused a dose-dependent drop in blood glucose level compared with the control diabetic group (**Figure 1**). TBBE appeared to improve the glucose tolerance in KK-A^y (**Figure 2**). The blood glucose level in the C57BL/6 mice (group V) rose to its peak value 30 min after glucose loading and decreased to a normal level at 120 min. However, KK-A^y control mice (group I) showed sharply increased blood glucose concentration at 60 min after glucose loading and maintained this high level for over an additional 60 min. All TBBE-treated KK-A^y mice showed decreases in blood glucose levels at 60 and 120 min compared with the control KK-A^y mice. These results are in agreement with those observed by Kawa et al. (*26*), who reported that DCI in the buckwheat concentrate was primarily responsible for the serum glucose lowering activity in STZ rats.

Plasma C-peptide and glucagon levels were higher in the control KK-A^y mice than in the C57BL/6 mice ($P < 0.001$) (**Table 4**). Furthermore, TBBE (40 and 20 mg/kg DCI) significantly lowered the levels of plasma C-peptide ($P < 0.01$), which is a byproduct of insulin production. In this study, the KK-A^y mice displayed hyperglucagonemia ($P < 0.001$) and TBBE lowered the plasma glucagon level compared to the control diabetic mice. Glucagon is one of several hormones that possess antagonistic action against insulin that can exacerbate the metabolic consequences of insulin deficiency. The suppression of endogenous glucose production has been reported not just as a simple response to insulin but rather as a complex interplay between the action of glucagons and insulin (*27*). Thus, the improvement in hyperglycemia by TBBE could be partly attributable to the amelioration of hyperglucagonemia.

The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia (*28*). In the present study, serum lipids including cholesterol and triglycerides in diabetic mice were elevated. Compared with those in the C57BL/6 mice, serum triglyceride and total cholesterol concentrations were significantly higher in the control KK-A^y mice ($P < 0.001$, $P < 0.01$) (**Table 4**). In KK-A^y mice orally given TBBE (20 mg/kg DCI), the increase in triglyceride and total cholesterol levels appeared to be suppressed; however, no statistically significant differences were observed compared with the control KK-A^y mice. Diabetic hyperglycemia induces the elevation of plasma urea, which is considered to be a marker of renal dysfunction (*29*). In this study, plasma urea in the diabetic group was 45.7% higher than the C57BL/6 mice group and TBBE treatment (DCI 40 mg/kg) reduced significantly plasma urea by 12.8% ($p < 0.05$) compared with the value of the diabetic control group, indicating that TBBE is capable of ameliorating the impaired diabetic kidney function in addition to its hypoglycemic control.

The present study confirms that pancreatic β-cells are destroyed in KK-A^y mice (*25*). In the control diabetic mice, a significant decrease in insulin immunoreactivity was observed compared with the control C57BL/6 (**Figure 3A**,**B**). However, the TBBE treatment group (40 mg/kg DCI) had significantly improved insulin immunoreactivity compared with the diabetic group (**Figure 3C**), suggesting that TBBE had the ability to enhance insulin sensitivity and to modulate favorably the endocrine pancreatic function (*30*).

In conclusion, we developed a simple method for DCI enrichment in buckwheat bran extract. The method included two major steps with the first step being the steaming of tartary buckwheat bran in an autoclave at 1.6 MPa and 120 °C for 60 min and the second step being passage of the crude extract through activated carbon and ion exchange resins. The resulting TBBE could reach 22% DCI, which is 73-fold higher than that in buckwheat bran. TBBE used in the present study was considered to be nontoxic and safe at least in mice. Most importantly, TBBE exerted an antidiabetic activity in type 2 diabetic KK-A^y mice, at least in part, by improving glucose tolerance and insulin response to glucose metabolism without affecting body weight.

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