

**Beneficial effects of the burdock ferment liquid on diabetic disorders in STZ-induced diabetic rats**

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**Abstract:** The present study was undertaken to characterize the effects of the burdock (*Arctium lappa* L.) ferment liquid (BFL) on diabetic disorders employing streptozotocin-induced diabetic rats (STZ-diabetic rats) as a type-1 diabetic model. There was a tendency towards a reduction in hypercholesterolemia after oral administration of BFL in diabetic rats for 2 consecutive weeks. BFL with abundant inulin was capable of alleviating significantly the hypertriglyceridemia and hyperglycemia. Diabetic-dependent alterations in serum creatinine concentrations, blood urea nitrogen and creatinine clearance were ameliorated after 2-week treatment with BFL in a dose-dependent manner. Additionally, BFL with polyphenolic components was able to scavenge 1,1-diphenyl-2-picrylhydrazyl radical as well as to attenuate the oxidative stress, evidenced by the reduction of hyperactivity in antioxidants including superoxide diamutase and glutathione peroxidase in plasma of diabetic rats. Thus, BFL has the ability to decrease the hyperlipidemia and hyperglycemia, and alleviate the hyperglycemia-associated oxidative stress in STZ-diabetic rats.

[Tsung-Hung Chang, I-Min Liu, Chi-Ting Horng, Feng-Chi Tsai, Daih-Huang Kuo, Po-Chuen Shieh, Shih-Chiang Lee, Jeng-Chuan Shiang, Fu-An Chen. **Beneficial effects of the burdock ferment liquid on diabetic disorders in STZ-induced diabetic rats.** Life Sci J 2012;9(2):718-726] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 107

**Keywords:** Burdock; *Arctium lappa* L.; STZ-diabetic rats; Antioxidant; Hyperlipidemia; hyperglycemia; Oxidative stress

**1. Introduction**

Diabetes, which ranks highly among the top ten causes of mortality around the world, often leads to disability from the vascular complications of coronary artery disease, cerebrovascular disease, renal failure, blindness, and limb amputation in addition to neurological complications and premature death [1]. It has been demonstrated that the use of pharmacological intervention in combination with lifestyle modifications that include diet and moderate exercise is particularly useful in the management of diabetes [2]. Accordingly, novel treatment with fewer side effects is feasible for the control of diabetic disorder, indicating the merit of additional medication for diabetic patients. In fact, diabetics and experimental diabetic animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation [3]. As a new strategy for alleviating the oxidative damage in diabetes, interest has grown in the usage of natural dietary antioxidants.

Burdock (*Arctium lappa* L.) has long been cultivated as a vegetable in Taiwan for dietary use. Burdock is also used as a folk medicine as a diuretic and antipyretic. It has become a popular health drink in Taiwan in the last decade. Several studies have reported that the root of burdock possesses various pharmaceutical activities including antibacterial activity [4-5], desmutagenic activity [6], antioxidant ability [7-10], hepatoprotective effect [11-12], gastroprotective activity [13-14] and anti-inflammatory activity [8], among which the gastroprotective activity, hepatoprotective efficacy, anti-inflammatory activity, and antioxidant activity are associated with the free radical scavenging activity. Additionally, burdock is claimed to be helpful for improve glycemic control in hyperglycemic subjects [15]; however, the related research on diabetes is not clear-cut.

Fermentation using yeast or lactic acid bacteria has long been applied in food industry due to its beneficial effects in flavor development, in inhibition of spoilage bacteria and pathogens, in intestinal health and other health benefits related to

cancer prevention, blood cholesterol levels and immune competence, which could be resulted from the modification and/or creation of nutrient, botanically-active components and microbial metabolites [16-19]. The streptozotocin induced-diabetic rats (STZ-diabetic rats) serve as an excellent model to study the molecular, cellular and morphological changes in tissue induced by stress during hyperglycemia [20]. The present study is thus to examine the efficacy of a burdock (*Arctium lappa* L.) ferment liquid (BFL) on diabetic disorders employing STZ-diabetic rats as a type-1 diabetic model.

## Material and Methods

### Materials

The root of burdock (*Arctium lappa* L.) with Good Agriculture Practice (GAP) certification cultivated at Gueilai Area, Pingtung, Taiwan was applied as materials for preparation of burdock ferment liquid (BFL). BFL was kindly supplied by Dong Yuan Biotech Pharmaceutical Co., Ltd. (Kaohsiung, Taiwan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), inulin, gallic acid, hydroxymethylfuraldehyde (HMF) and streptozotocin (STZ) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Folin-Ciocalteu reagent was from Merck (Darmstadt, Germany). Cosmosil 5C18-AR-II column was purchased from Nacalai Tesque (Kyoto, Japan). Acetonitrile and methanol were LC grade from Tedia (Fairfield, USA). The diagnostic kits for determinations of glucose (Cat. No. COD12503), cholesterol (Cat. No. COD11539) and triglyceride (Cat. No. COD11529) in plasma were purchased from BioSystem (Barcelona, Spain). Nephrot II enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. NR002) was obtained from Exocell, INC. (PA, USA). The diagnostic kits for determinations of creatinine concentration in serum or urine (Cat. No. 221-30), and kinetic reagent for measurement of blood urea nitrogen (BUN) (Cat. No. 283-30) were purchased from Diagnostic Chemicals Limited (Connecticut, USA). The colorimetric assay kits for measurements for superoxide dismutase (SOD, Cat. No. 706002) and glutathione peroxidase (GSH-Px, Cat. No. 703102) activities in plasma were purchased from Cayman Chemical (Michigan, USA). All other reagents were from standard sources.

### Determination of total polyphenol and inulin in BFL

Total polyphenols in BFL were determined spectrophotometrically using the Folin-Ciocalteu reagent based on a colorimetric oxidation/reduction reaction. To 0.2 ml of diluted aqueous acetone sample, 1 mL of Folin-Ciocalteu reagent (diluted 10

times with distilled water) was added. After that, 0.8 mL of 7.5 %  $\text{Na}_2\text{CO}_3$  was added and mixed thoroughly. After 30 min of standing, the absorbance was measured at 765 nm. The amount of total polyphenols was calculated as a gallic acid equivalent based on a calibration curve of gallic acid standard, and expressed as mg gallic acid/mL BFL. All measurements were done in triplicate.

Inulin in BFL was measured using a HPLC method as described by Dall'Amico *et al.*, with minor modifications [21]. Sample preparation for HPLC measurement was performed by diluting BFL with distilled water and then mixed with 200  $\mu\text{l}$  of 70%  $\text{HClO}_4$ . After boiling for 10 min to hydrolyze inulin to fructose and to convert fructose to hydroxymethylfuraldehyde (HMF), the sample was cooled on ice for 5 min and aliquots of 20  $\mu\text{l}$  were subjected to HPLC analysis. A Hitachi (Tokyo, Japan) L-2130 HPLC pump system equipped with an L-2450 diode array detector, and an L-2200 autosampler was used to analyze HMF on a Cosmosil 5C18-AR-II column (5  $\mu\text{m}$ ; 4.6  $\times$  250 mm i.d.) with a flow-rate of 1 ml /min and monitored at 280 nm. Mobile phase system A was 3.2 mM HCl, pH 2.8, and B was acetonitrile/3.2 mM HCl (60:40, v/v). The HMF in sample was eluted chromatographically at ambient temperature with a gradient program: 0 % B (0-1 min), 0 %-35% B (1-9 min) and 0 % B (9.1-10 min). The amount of inulin in BFL was calculated based on a calibration curve constructed by plotting the HMF response area vs. inulin concentration. All measurements were done in triplicate.

### Evaluation of free radical-scavenging activity of BFL

The free radical-scavenging activity of BFL was evaluated using DPPH free radical-scavenging assay as described previously [22]. BFL was diluted with methanol and an aliquot of 50  $\mu\text{L}$  of each dilution was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). A working solution of DPPH (250  $\mu\text{M}$ ) in methanol was freshly prepared and then an aliquot of 150  $\mu\text{L}$  was added to each well. After incubation for 30 min, the remaining percentage of DPPH was measured at 490 nm on an ELISA reader (ThermoLabsystems, Cheshire, UK). Each dilution was performed at least in triplicate.

### Animal Models

Male Wistar rats, weighing 200-250 g were obtained from the Animal Center of National Cheng Kung University Medical College (Tainan, Taiwan). They were maintained in a temperature-controlled room ( $25 \pm 1^\circ\text{C}$ ) and kept on a 12:12 light-dark cycle (light on at 06:00 h). Standard rat chow and water were available *ad libitum*. STZ-diabetic rats were

prepared by intravenously (i.v.) injecting STZ (60 mg/kg) into male Wistar rats. Animals were considered to be diabetic if they had plasma glucose concentrations of 350 mg/dL or greater in addition to polyuria and other diabetic features. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) with IACUC approval number 9616.

### Treatment protocols

BFL solution were prepared by dilute with distilled water to have the tested solution at the indicated concentration as following: one part of original BFL diluted with one part of distilled water (1:1, v/v) to have solution with 50% BFL; one part of original BFL diluted with three part of distilled water (1:3, v/v) to get solution with concentration of 25% BFL; one part of original BFL diluted with five part of distilled water (1:5, v/v) to get solution with 17% BFL. A metabolism coefficient of 6.25 was employed to convert the recommended daily oral dosage of BFL (20 ml) for adult into rats, assuming that average body weight of an adult is 60 kg. Thus, diluted BFL solution was given by oral gavage at the indicated concentration for 2 mL/kg, twice a day, to the separate groups of the STZ-diabetic rats. Another group of STZ-diabetic rats was received the equivalent volume of distilled water used to dissolve the preparations of interest. All animals were administered twice a day via gastric tube. The standard rat diet and water were available *ad libitum* throughout the entire treatment period. Two weeks after the treatment, rats were weighed and blood samples were collected from a tail vein; meanwhile, individual rats were placed in metabolic cages (Shinete Instruments Co., Ltd, Taipei, Taiwan) to obtain 24-hour urine collections for measurements of urine creatinine (Cr). The systolic blood pressure (SBP) of the tail artery was also measured at weekly intervals.

### Blood sampling and analysis

Blood sample of rats were centrifuged at 2,000 g for 10 minutes at 4°C, plasma was removed and aliquot for the respective analytical determinations. Plasma glucose concentration was measured by glucose oxidase method. Levels of cholesterol and triglycerides in total plasma were analyzed enzymatically. The serum Cr concentration was determined by the modified Jaffe' method. BUN was determined according to the urease procedure.

Plasma SOD (E.C.: 1.15.1.1) activity was determined by commercial kit for measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in absorbance at 450 nm. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The activity assay for GSH-Px (E.C.: 1.11.1.9) determined by commercial kit was based on the oxidation of NADPH to NAD<sup>+</sup>, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm. All analyses were performed in accordance with the manuals provided by the manufacturers.

### Analysis of urine parameters

The 24-h urine collected from each diabetic rat and age-matched control was centrifuged at 2,000 g for 10 min. Urinary albumin concentrations were measured by ELISA assay using an anti-rat albumin antibody. The Cr concentration in pooled urine samples was determined by the modified Jaffe' method using the commercial assay kit. All analyses were performed in accordance with the manuals provided by the manufacturers. Creatinine clearance (Ccr) was calculated using the following equation:  $Ccr \text{ (mL/min/kg)} = [\text{urinary Cr (mg/dL)} \times \text{urinary volume (mL)} / \text{serum Cr (mg/dL)}] \times [1000 / \text{body weight (g)}] \times [1 / 1440 \text{ (min)}]$ .

### Blood pressure measurement

SBP of the tail artery was measured by non-invasive blood pressure system (MODEL BP-6, Diagnostic & Research Instruments Co., Ltd., Taoyuan, Taiwan). The measurements for SBP were recorded in quadruplicate for each rat and the average blood pressure was calculated.

### Statistical analysis

Data are presented as the mean  $\pm$  SD. The statistical significance between groups was analyzed by an analysis of variance (ANOVA) test, a *p* value of less than 0.05 was considered to be significant.

## Results

### Contents of total polyphenol and inulin in BFL

The content of total polyphenol and inulin in various dilutions of BFL was shown in Table 1. The total polyphenol and inulin in original BFL was  $5.10 \pm 0.27$  mg/mL and  $304.0 \pm 26.7$  (mg/mL), respectively. Dilutions were in inverse proportion to its content of total polyphenol and inulin in BFL.

### DPPH radical-scavenging activity of BFL

BFL was able to scavenge significantly DPPH radical with concentration-dependant manner (Table 2). Additionally, the DPPH radical-scavenging activity of BFL was reached a plateau within 30 min

and maintained for 120 min (Table 2). In comparison of free radical scavenging activity of BFL with that of vitamin E (40 ppm), the dilution of BFL required to achieve similar effect was 1:10 (v/v) (data not shown).

**Table 1. The contents of total polyphenols and inulin in dilutions of BFL**

Dilutions (v/v)	Total polyphenol (mg gallic acid/mL)	Inulin (mg/mL)
100 % BFL (1:0, v/v)	5.10 ± 0.27	304.0 ± 26.7
50 % BFL (1:1, v/v)	2.92 ± 0.27	160.6 ± 2.0
25 % BFL (1:3, v/v)	1.49 ± 0.06	66.9 ± 7.9
17 % BFL (1:5, v/v)	0.76 ± 0.05	44.1 ± 1.0

Values (mean ± SD) were obtained for each group of 3 experiments.

#### General characteristics of STZ-diabetic rats repeatedly treated with BFL

Each diluted BFL solution made influences neither on body weight nor on SBP in STZ-diabetic rats at the end of the 2-week treatment period (Fig. 1A; Fig. 1B). The plasma glucose in STZ-diabetic rats receiving for 2-week treatment with higher concentration of BFL was lower than the corresponding values for vehicle-treated group (Fig. 2A). The higher plasma level of cholesterol was reduced in STZ-diabetic rats receiving for 2-week treatment with 50% BFL solution, but the values did not achieve statistical significance as compared to that of vehicle-treated counterparts (Fig. 2B). At the termination of 2-week treatment, the plasma level of triglyceride in STZ-diabetic rats tended to be reduced by 17% BFL solution. The action of BFL on the alleviation of hypertriglyceridemia was markedly in STZ-diabetic rats treated with 2-week 50% BFL solution (Fig. 2C).

#### Changes in renal function related parameters in STZ-diabetic rats repeatedly treated with BFL

Following 2-week treatment, STZ-diabetic rats received 17% BFL solution exhibited lower levels of serum Cr, whereas the difference did not achieve statistical significance as compared to those of vehicle-treated group at the corresponding time (Fig. 3A). The levels of BUN in STZ-diabetic rats received 17% BFL solution was also tended to be reduced at the end of 2 weeks treatment, but did not differ from their vehicle-treated counterparts (Fig. 3B). Both values for serum Cr and BUN of STZ-diabetic rats were significantly ( $p < 0.05$ ) lower by 50% BFL

solution as relative to their vehicle-treated counterparts at the end of 2 weeks treatment (Fig. 3A; Fig. 3B).

Furthermore, treatment with BFL from 2 weeks onward reduced the increase in urine volume of STZ-diabetic rats in a concentration manner (Fig. 3C). Also, treatment STZ-diabetic rat with 50% BFL solution significantly ( $p < 0.05$ ) reduced the increase in Cr as compared with their vehicle-treated counterparts at the end of 2 weeks treatment (Fig. 3D).

#### Changes on plasma antioxidant enzyme activity in STZ-diabetic rats repeatedly treated with BFL

The plasma SOD activity in STZ-diabetic rats was gradually reduced by 2 weeks treatment with diluted BFL solution in a concentration manner. Similarly, 2-week BFL administered diabetic rats displayed significantly lower activity of plasma GSH-Px as compared to their vehicle-treated counterparts (Fig. 1C; Fig. 1D).

#### Discussion

Diabetics and experimental diabetic animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation (23,24). Although adequate control of blood glucose levels may prevent the development of complications, it is difficult to achieve strict blood glucose control, leading to a year-by-year increase in the number of patients with diabetes. Therefore, strategies to reduce oxidative stress in diabetes mellitus may exert favourable effects on the progression of diabetic complication [25]. Among diabetic complications, nephropathy is the most common cause of end-stage renal disease in developed countries and a major cause of morbidity and mortality in patients with diabetes [26]. In assays utilizing unnatural model radicals, the DPPH radical scavenging assay, BFL was shown to scavenge the DPPH radical significantly and concentration-dependently. Hence DPPH assay is one of the most common methods of assessing antioxidant activities [27], the in vitro antioxidant activity of BFL could be considered. STZ-Diabetes provides a relevant example of endogenous chronic oxidative stress and hyperglycemia [20]. Thus, we evaluated the oxidative stress and nephropathy induced by STZ in Wistar rats and examined the potential protective effects of BFL against the changes induced by STZ.

**Table 2. The free radical scavenging activity in dilutions of BFL**

Dilutions (v/v)	The remaining DPPH radicals (%)			
	30 min	60 min	90 min	120 min
Blank	95.25 ± 2.22	91.55 ± 2.60	91.06 ± 2.70	90.63 ± 2.65
1:45	73.23 ± 1.35*	68.13 ± 2.07*	64.85 ± 2.10*	62.18 ± 2.07*
1:40	70.06 ± 1.28*	64.98 ± 1.77*	61.44 ± 1.71*	58.27 ± 2.07*
1:35	65.19 ± 1.35*	59.64 ± 1.26*	55.67 ± 1.24*	52.15 ± 1.25**
1:30	64.64 ± 2.23*	58.04 ± 2.62*	53.02 ± 2.51*	48.74 ± 2.49**
1:25	63.19 ± 2.09*	56.79 ± 2.21*	51.73 ± 2.15**	47.34 ± 2.18**
1:20	52.92 ± 2.31**	44.15 ± 2.90**	37.65 ± 2.62**	32.07 ± 2.73**
1:15	30.93 ± 1.93**	19.94 ± 1.96**	14.65 ± 1.23**	13.22 ± 0.70**
1:10	15.37 ± 0.54**	13.83 ± 0.58**	13.53 ± 0.58**	12.87 ± 0.68**

Values (mean ± SD) were obtained for each group of 3 experiments. \*  $p < 0.05$  and \*\*  $p < 0.01$  compared to the values of blank at the indicated times, respectively.

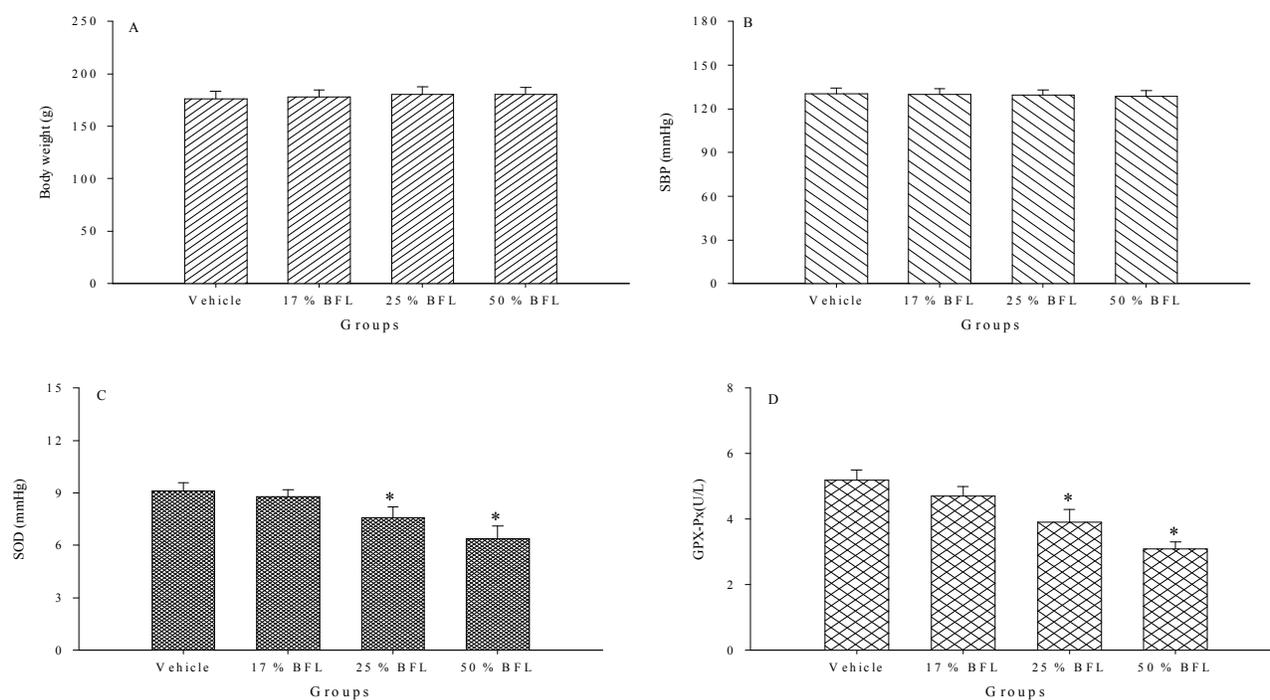


Fig. 1. Changes of the body weight, systolic blood pressure (SBP) and the antioxidant enzyme activity in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. \*  $p < 0.05$  compared to the values of vehicle-treated STZ-diabetic rats.

We observed that 2 week-administration of diluted BFL solution reduced the higher level of serum Ccr and BUN as well as creatinine clearance in STZ-diabetic rats significantly and dose-dependently. Otherwise, the typical characteristics of diabetes with large increase of urine output and the less body weight gain in STZ-diabetic rats were ameliorated by 2-week of BFL administration, implying the product be with the capacity to modify the renal hyperfiltration. After 2 week-treatment with diluted BFL solution, the results revealed significant reduction in hyperglycemia of diabetic animals. The beneficial effect of BFL on diabetic nephropathy may be associated with reduce severity of hyperglycemia.

It has long been known that hypertension is an aggravating factor in increased vascular pressure and may be the key hemodynamic determinant of diabetic renal injury as well; it is therefore well recognized that blood pressure control is important in diabetic patients [28]. Actually, BFL at any concentration was observed to make no influence on the blood pressure in STZ-diabetic rats, indicating that the beneficial effect of BFL on diabetic nephropathy was not linked with the amelioration of hypertension. Besides hyperglycemia and high blood pressure, other risk factors have been identified in the development or progression of diabetic kidney disease; hyperlipidemia has been associated with occurrence of severe renal failure secondary to development of glomerulosclerosis and tubulointerstitial disease [29-30]. The increased lipid peroxidation in the kidney implies the level of susceptibility to diabetic oxidative stress, leading to diabetic complications. From this view point, prevention of hyperlipidemia and/or lipid peroxidation resulting from oxidative stress is considered to play a crucial role in protection from disorders associated with diabetes [29-30]. We observed that BFL, especially at the higher concentration, be effective in the modification of hypertriglyceridemia as well as alleviation of hypercholesterolemia in STZ-diabetic rats. The beneficial effects of BFL on the amelioration of diabetic renal function are thought to be linked to the observed control of lipid metabolism.

Some dietary components that completely escape glucide digestion, such as resistant starch and oligofructose, have been demonstrated to exert systemic effects by modifying lipid metabolism [31-32]. In contrast to starch, inulin is fermentable dietary fiber, resistant to hydrolysis by pancreatic amylase and saccharidases in the upper gastrointestinal tract. Previous study have been demonstrated that inulin

was produced enzymatically from sucrose, and that supplementing inulin be helpful for reduced elevated hepatic levels of triacylglycerols in rats fed with a high-fat and high sucrose diet [33]. Actually, inulin is rich in BFL. It is possible to anticipate that the reduction in elevated levels of hepatic lipids in diabetes was associated with the inulin of BFL.

As established clearly, reactive oxygen species (ROS) are generated in augmented amounts in diabetes, the main mechanisms being increased glucose autooxidation and advanced protein glycation, activation of polyol pathway and attenuated antioxidant defence system. It is known that oxygen radical scavenging enzymes can respond to conditions of increased oxidative stress with compensatory increases in activity [34], our findings are consistent with this fact. SOD is reported to be the first induced enzyme; its higher activity could be due to its induction by increased superoxide anion production. The induction of SOD in turn leads to protection of GSH-Px against inactivation by superoxide anion the net effect being a higher GSH-Px activity [35]. However in BFL administered STZ-diabetic rats, the activities of both SOD and GSH-Px in plasma were lower in comparison to diabetic controls, indicating the lower levels of oxidative stress in these rats by BFL treatment. The above observation shows that BFL possesses antioxidant activity, which could exert a beneficial action against pathological alterations caused by the presence of free radicals in STZ diabetes. Taking the above results into consideration, the renoprotective effects of BFL in diabetes were not only attributable to improved dyslipidemia alone, but also likely reflected its antioxidant activity. The combined antioxidant and reducing hyperlipidemia and hyperglycemia actions of BFL should be particularly advantageous and perhaps even synergistic in preventing renal injury and other diabetic complications.

Phenolic compounds from fruits and vegetables have been receiving increasing interest from consumers and manufacturers because numerous epidemiological studies have suggested associations between consumption of polyphenol-rich foods or beverages and the prevention of certain chronic diseases dependent of their antioxidant properties[36]. Indeed, polyphenolic compounds were abundant in this burdock product. Application of the burdock product proved the positive influence of polyphenols on oxidative stress and hyperlipidemia through the antioxidants could thus be considerable.

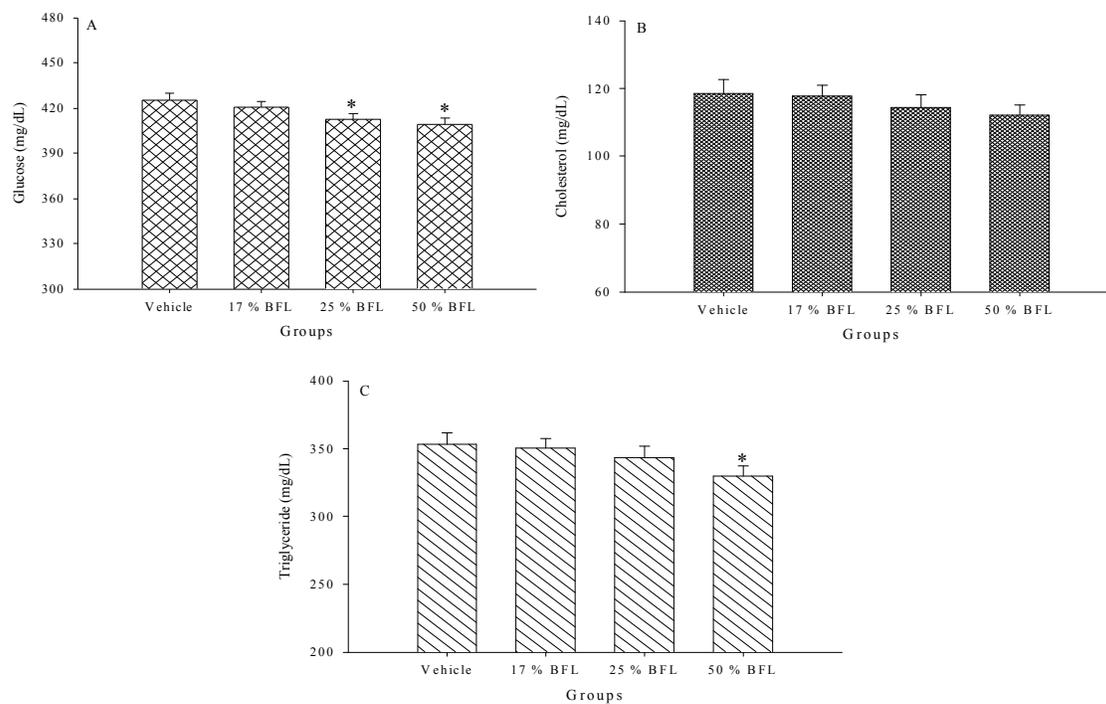


Fig. 2. Changes of the plasma parameters in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. \*  $p < 0.05$  compared to the values of vehicle-treated STZ-diabetic rats.

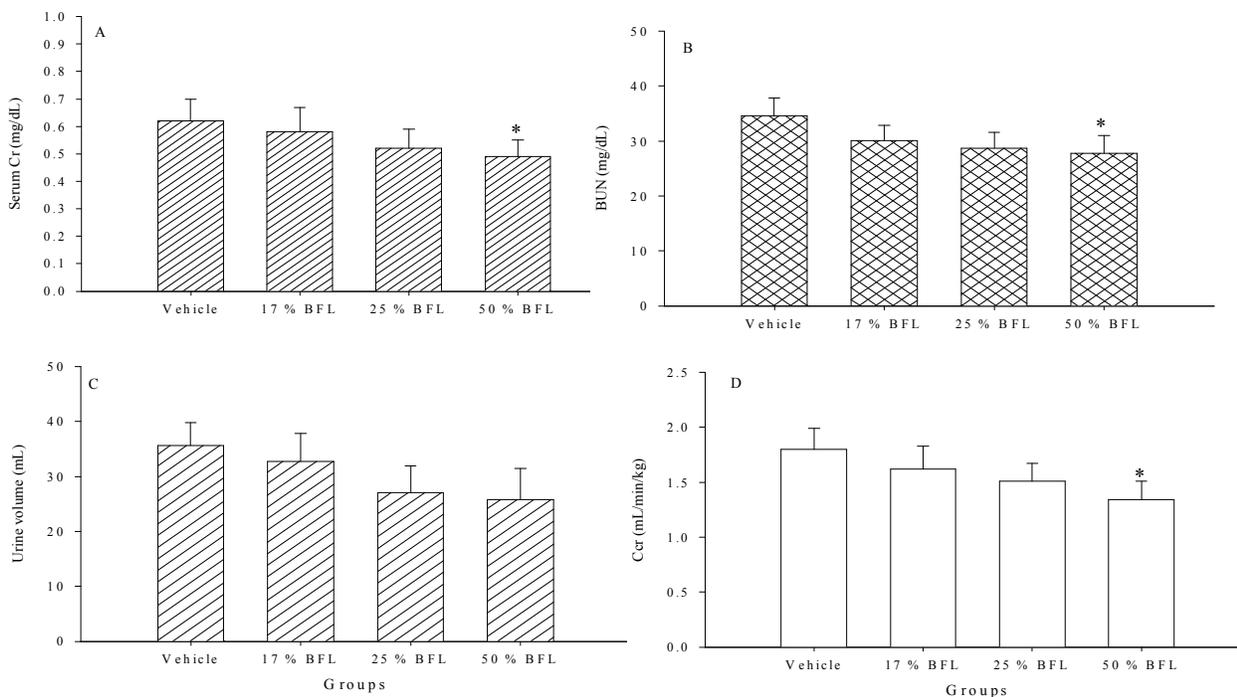


Fig. 3. Changes of the renal function related parameters in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. \*  $p < 0.05$  compared to the values of vehicle-treated STZ-diabetic rats.

The antioxidant responsiveness mediated by burdock may also be anticipated to have biological significance in eliminating reactive free radicals that may otherwise affect the normal cell functioning.

In conclusion, burdock showed an antidiabetic effect *via* reducing hyperlipidemia and hyperglycemia in STZ-diabetic rats. Besides, BFL possesses antioxidant activity which could exert a beneficial action against pathological alterations and diabetic renal complications caused by the presence of free radicals in hyperglycemia. The obtained data strengthen the basis for recommending BFL as an adjuvant for diabetic individuals.

#### Acknowledgments

The author would like to thank Gueilai Community Developmental Institute in Pingtung, Taiwan for providing burdock as materials for preparation of burdock ferment liquid. We appreciate Dong Yuan Biotech Pharmaceutical Co., Ltd. (Kaohsiung, Taiwan) for the kind supply of burdock ferment liquid.

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5/3/2012