

Anti-diabetic and anti-oxidative activity of fixed oil extracted from *Ocimum sanctum* L. leaves in diabetic rats

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Abstract. *Ocimum sanctum* L. (OS) leaves have been shown to exert diverse potential benefits in a variety of stress conditions. The present study was conducted to elucidate the effects of the fixed oil extracted from OS leaves on the blood glucose levels and serum lipid profile of streptozotocin-induced diabetic rats. In addition, the anti-oxidative activity of OS leaves to protect various organs including the liver, kidney and heart was investigated. The fixed oil of the OS leaves was extracted using hexane, and the various fatty acid contents of the oil were determined using gas chromatography-mass spectrometry. Male Wistar rats were allocated into three groups (n=7 per group): Normal control rats, diabetic rats and diabetic rats fed daily with the fixed oil for three weeks. The results showed that α -linolenic acid was the primary fatty acid contained in the fixed oil of OS. After 3 weeks of diabetic induction, the rats exhibited increased blood glucose levels and serum lipid profile, in addition to elevated serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase MB subunit (CK-MB), creatinine and blood urea nitrogen (BUN). The fixed oil significantly decreased the elevated levels of blood glucose, the serum lipid profile and the levels of serum creatinine and BUN ($P < 0.001$), without exerting significant effects on the elevated serum levels of AST, ALT, LDH and CK-MB. Furthermore, the fixed oil increased the diabetically-reduced levels of serum insulin and decreased the rat kidney weight. Fixed oil suppressed the elevated thiobarbituric acid reactive substances (TBARS) level and increased the activity of various antioxidative enzymes in the rat renal tissue. By contrast, the fixed oil had no effect on the elevated TBARS level and the inhibited activity of the antioxidative enzymes in the rat liver and cardiac tissues. Histopathological

results indicated that the fixed oil preserved the renal tissue against oxidative stress in diabetes. In summary, the results of the present study suggest that the fixed oil extracted from OS leaves exerted anti-hyperglycemic, anti-hyperlipidemic and free radical scavenging effects in diabetic rats, thus providing renal protection against diabetes. The α -linolenic acid contained in the fixed oil may be responsible for these effects.

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia over a prolonged period. The primary causes of DM are insulin deficiency or dysfunction, which impair the metabolism of carbohydrates, lipids and proteins, leading to oxidative stress and resulting in chronic health complications such as diabetic nephropathy, neuropathy, hypertension and cardiovascular disease (1,2). It has been estimated that the number of individuals with DM will increase to 360-380 million between 2025 and 2030 (3). The World Health Organization previously reported that DM was one of the ten leading causes of mortality globally between 2000 and 2012 (4). Although cardiovascular diseases, such as ischemic heart disease, have been the most significant causes of mortality over the past decade, the mortality rate associated with diabetic patients is continuously rising at an increased rate compared with cardiovascular diseases (4). Therefore, it has been clearly shown that DM is currently among the most challenging global health problems. Extensive interventions have been recommended for the treatment of DM, including diet control, exercise and hypoglycemic drugs, particularly insulin. However, diet control and exercise may not be successful interventions due to the modern lifestyle. Numerous factors may lead to patient noncompliance, including the undesirable side effects of the drug treatments coupled with the difficult treatment schedules and the expense and safety of long-term use (5). Therefore, the search for medicinal plants with anti-hyperglycemic and anti-oxidative activities and limited side effects remains a major challenge.

Numerous medicinal plants have been identified that exhibit anti-oxidative and anti-hyperglycemic activities (6-8). Among them, *Ocimum sanctum* L. (OS) is promising as it is routinely used in cooking vegetables, and also has been recommended for the treatment of a number of diseases by local people in

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various countries (9,10). Previous studies have shown that OS is safe for consumption as it contains no genotoxic or organotoxic effects (11,12). In addition, OS has been reported to possess therapeutic value for the treatment of a number of diseases, including bronchitis, diarrhea and dysentery (9,13). Our previous study showed that a diet containing 2% OS leaf powder resulted in a significant reduction in blood glucose levels in streptozotocin-induced DM rats (14). The administration of aqueous and alcoholic extracts of OS leaves also resulted in reduced blood glucose levels in streptozotocin-induced DM rats (15,16), and prevented insulin resistance in normal rats fed with a fructose diet (17). Prior studies have suggested that OS leaf extracts possess anti-hyperglycemic activity, in addition to protecting organs against various stress conditions such as hyperlipidemia (6), inflammation (18), cancer (19) and heavy metal toxicity (20,21). Furthermore, an aqueous extract of OS leaves has been shown to promote antioxidative activity, thus protecting various organs against DM (16,22). OS leaves are known to be a rich source of volatile and fixed oils. Our previous study showed that volatile and fixed oils extracted from OS leaves decreased the serum lipid profile and protected the hearts of rats fed with a high fat diet (23,24). However, the anti-hyperglycemic, anti-hyperlipidemic and organ protective effects of the fixed oil extracted from OS leaves against DM have not yet been investigated. It is known that the liver and kidney are the primary organs at risk in patients with DM. Therefore, the majority of previous studies have focused on the hepatic and renal protective activities of various medical plants in DM (8,16,20,25). However, DM may damage the heart in addition to the liver and kidneys (26). Furthermore, DM has a high correlation with cardiovascular diseases (27).

Therefore, the present study was conducted to elucidate the anti-hyperglycemic, anti-hyperlipidemic and anti-oxidative activities of the fixed oil extract of OS leaves, and to evaluate the ability of this extract to protect various vital organs including the liver, kidneys and heart in DM rats. In addition, the chemical composition of fixed oil was determined.

Materials and methods

Extraction of fixed oil from OS leaves. Fresh OS leaves were obtained from the National Institute of Thai Traditional Medicine, the Ministry of Public Health (Nonthaburi, Thailand), and were cut into small pieces (1 cm). The separated leaves were washed and air-dried at room temperature, and ground to powder using a blender (Phillips Avance HR2097/00; Phillips, Amsterdam, Netherlands). Fixed oil was extracted from the OS leaves using a Soxhlet extractor (Soxtherm Multistat/SX PC; C. Gerhardt GmbH & Co., Königswinter, Germany) with hexane as the solvent, as previously described by the Association of Official Analytical Chemists (28). The sample was filtered and evaporated. The extraction solvent was removed by rotary evaporation (Buchi R-124; Buchi Labortechnik, Flawil, Switzerland). In cases where the extraction solvent contained OS powder, the extraction solvent was filtered through a Whatman paper no. 1 (GE Healthcare Life Sciences, Little Chalfont, UK) prior to evaporation. From 100 g of dried OS leaf powder, the percentage yield of OS fixed oil was 1.046 g%. The OS fixed oil was collected and stored at 4°C.

Identification of fatty acids in OS fixed oil using gas chromatography-mass spectrometry (GC-MS). Fatty acids were transformed into methyl esters according to the ISO procedure (29). The fatty acid methyl esters (FAMES) were analyzed by GC-MS, using a 5840A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector for electron-impact mass spectrometry, and an integrator. The 1.5- μ l FAME sample was injected, and separation was conducted using a Agilent J&W DM-23 capillary column (30 m length, 0.25 mm i.d., and 0.25 mm film thickness of 5%; DEGS; Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium (pressure, 19 psi) with a split ratio of 50:1. The carrier gas and the column flow rate was 62.9 ml/min. The oven temperature was initially maintained at 80°C, then increased to 180°C at 10°C/min and finally maintained at 220°C for 7 min. The temperature of the injection port and the detector were set at 300°C. The fatty acids were identified by comparing their retention times with those of standards (Sigma-Aldrich, St. Louis, MO, USA). The content of each fatty acid was expressed as a percentage of the total fatty acid profile.

Animal preparation. In total, 21 male Wistar rats (age, 8 weeks; weight, 200-250 g) were purchased from the Animal Center of Salaya Campus, Mahidol University (Bangkok, Thailand). The rats were cared for in accordance with the principles and guidelines of the Institutional Animal Ethics Committee of Rangsit University (Pathumtani, Thailand), which is under the National Council of Thailand for Laboratory Animal Care. The rats were housed under a 12-h light-dark cycle at 25 \pm 2°C and fed with standard rat food and tap water *ad libitum*. DM was induced with an intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich) dissolved in citrate buffer (pH 4.5) at a dose of 65 mg/kg. At 5 days after STZ injection, fasting blood glucose was measured, and only rats with a blood glucose level of \geq 200 mg/dl were included in the study.

Experimental design. Three groups of 7 rats each were established as follows: i) Normal control rats; ii) normal untreated DM rats; and iii) DM + fixed oil rats.

In our previous study it was observed that supplementation with 2% dried OS leaf powder in the diet for three weeks induced anti-hyperglycemic and lipid-lowering effects in DM rats (14). The average dried OS leaf powder consumption was 4.45 g/kg/day. Therefore, the daily dose of OS fixed oil administered in the present study, calculated based on our previous data, was \sim 46.54 mg/kg/day.

Following diabetic induction, DM rats were administered OS fixed oil by intragastric intubation once a day for three weeks. Blood was collected weekly from rat tail veins to determine blood glucose levels. Body weight and food consumption were determined once a week.

Determination of serum lipid profile, serum insulin and biochemical evaluation of liver, kidney and cardiac injury. Following the OS fixed oil administration period, the rats were fasted overnight and anesthetized by an intraperitoneal injection of zoltilil (40 mg/kg; Virbac, Carros, France) and xylazine (3 mg/kg; L.B.S. Laboratory Ltd., Bangkok, Thailand). Blood was collected from the abdominal vein to determine serum insulin and lipid profile levels, including total cholesterol,

triglyceride, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Liver function was evaluated by determining the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In addition, cardiac injury was evaluated by measuring serum lactate dehydrogenase (LDH) and creatine kinase MB subunit (CK-MB) levels. Kidney function was evaluated by measuring the serum levels of creatinine and blood urea nitrogen (BUN).

Determination of lipid peroxide and the activity of anti-oxidative enzymes in the liver, kidney and heart. Following the experimental period, the rats were fasted overnight and anesthetized using zoltilil (40 mg/kg) and xylazine (3 mg/kg). Subsequently, the jugular vein was cannulated and perfused with ice-cold normal saline to remove the red blood cells. When all organs looked pale, the liver, kidney and heart were excised, cleaned and weighed. All organs were stored at -80°C until required for further analysis.

Determination of tissue lipid peroxide content. All organs were homogenized with 0.1 M phosphate buffer (pH 7.4). Lipid peroxides in the liver, kidney and heart were assessed using thiobarbituric acid reactive substances (TBARS), as previously described (30). TBARS was expressed in nmol malondialdehyde (MDA)/mg protein, using 1,1,3,3-tetraethoxy propane as a standard. Tissue protein levels were determined using Lowry's method, as previously described (31).

Determination of the activity of antioxidative enzymes in the various tissue samples. The levels of a number of antioxidative enzymes, including glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) were determined. The liver, kidney and cardiac tissue homogenates were prepared by homogenizing the tissues in 0.1 M phosphate buffer (pH 7.4). The homogenate was then centrifuged at 832 x g at 4°C for 10 min. The supernatant was collected and centrifuged again at 7,800 x g at 4°C for 30 min. The supernatant fraction was collected and further centrifuged at 136,000 x g at 4°C for 60 min. The final supernatant was analyzed to estimate the GPx, CAT and SOD activities, using the procedures described by Tapple (32), Luck (33), and Winterbourn *et al* (34), respectively.

Evaluation of tissue morphology. According to the experimental results, OS fixed oil only protected the kidney against DM. Therefore, only the histopathological appearance of renal tissue was evaluated. Following the study, kidneys were removed and washed with normal saline. All kidneys were longitudinally sectioned and fixed in 10% neutral-buffered formalin. Subsequently, the kidney samples were dehydrated and prepared under routine paraffin embedding protocol (35). The samples in paraffin blocks were serially sectioned at 5 µm, deparaffinized and stained with conventional hematoxylin and eosin (H&E) stain (Bio-Optica Milano SpA, Milan, Italy).

Biochemical assay for blood glucose, total cholesterol, triglyceride, HDL-C, LDL-C, AST, ALT, creatinine, BUN, LDH, CK-MB and serum insulin. Blood glucose levels were determined using a blood glucose strip (Abbott Laboratories Ltd., Maidenhead, UK). The concentrations of total cholesterol, triglyceride and HDL-C were assayed by using the cholesterol

(cat. no. 10028) and triglyceride (cat. no. 10724) enzymatic assay kits (Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany). In order to measure the serum cholesterol and triglyceride levels, 10 µl serum was added to 1 ml of assay reagent, the samples were incubated at 37°C for 5 min, and then the absorbance was measured at 500 nm (for serum lipid profile: Genesys 20 spectrophotometer, model 4001/4, Thermo Fisher Scientific, Inc., Haverhill, MA, USA; for other serum levels: UV spectrophotometer, model UV-2501PC, Shimadzu Co., Ltd., Kyoto, Japan). The serum levels of the various parameters were calculated according to the manufacturer's instructions in the assay kits.

For HDL-C determination, 200 µl serum was added in 500 µl of precipitating reagent, mixed and incubated at room temperature for 10 min. The samples were then centrifuged at 4,000 x g for 10 min and 200 µl supernatant was collected and used to determine the HDL-C using assay reagent. Subsequently, LDL-C was calculated using the following equation: $LDL-C = [TC - (HDL-C)] - (triglyceride/5)$.

The serum levels of AST, ALT, creatinine, BUN, LDH and CK-MB were measured using various enzymatic kits. In order to determine the AST and ALT levels (AST assay kit, cat. no. 12011; ALT assay kit, cat. no. 120120; Gesellschaft Für Biochemica und Diagnostica GmbH), 200 µl serum was added to 1 ml of working reagent, the samples were incubated at 37°C for 1 min, and then the absorbance was measured at 340 nm every 1 min for 3 min.

The assay kits used for creatinine (cat. no. CR 510), urea (cat. no. UR 446), LDH (cat. no. LD 401) and CK-MB (cat. no. CK 1296) were purchased from Randox Laboratories Ltd. (London, UK). For serum creatinine and urea determination, 10 µl serum was added to 1 ml of working reagent and the samples were incubated at 37°C for 30 sec. The absorbance of the creatine sample was read immediately and 2 min later at 492 nm, while that of urea was read immediately and 1 min later at 340 nm. The serum BUN level was then calculated by dividing the serum urea level by 2.14, which is the conversion factor derived based on the molecular weights of BUN and urea. For serum LDH and CK-MB determination, 20 and 40 µl serum, respectively, was added to 1 ml of working reagent, and the samples were incubated at 37°C for 30 sec and 10 min, respectively. The absorbance at 340 nm was measured immediately and every 1 min for 3 min for LDH, and immediately and after 5 min for CK-MB.

Serum insulin was assayed using a radioimmunoassay kit (cat. no. TKIN1; Diagnostic Product Co., Ltd., Los Angeles, CA, USA). Briefly, 200 µl serum was added to the Coat-A-count tube (included in the kit), followed by 1 ml ¹²⁵I-Insulin, and then the sample was mixed and incubated overnight at 15-28°C. The remaining insulin was decanted, the sample tubes were inverted and then counted in a γ counter for 1 min (Genesys Gamma 1, Laboratory Technologies Inc., Elburn IL, USA), according to the manufacturer's instructions.

Statistical analysis. Values are presented as the mean ± standard error of the mean. The results were analyzed by one-way analysis of variance. Duncan multiple rank test was performed to determine statistical significance among groups using SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

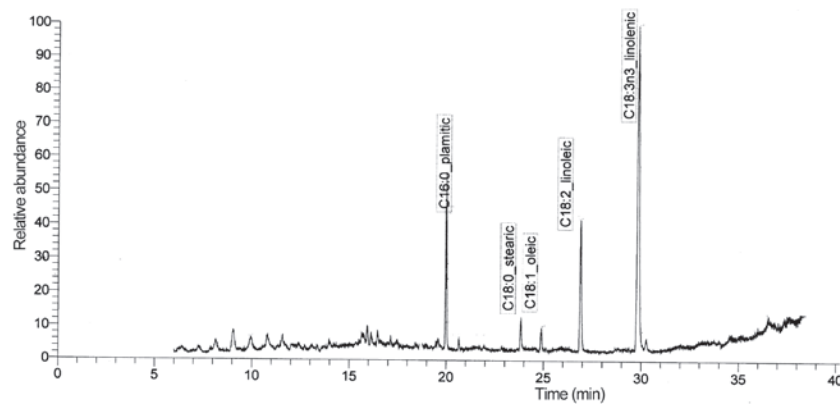


Figure 1. Gas chromatogram of fatty acids extracted from *Ocimum sanctum* L. leaf fixed oil.

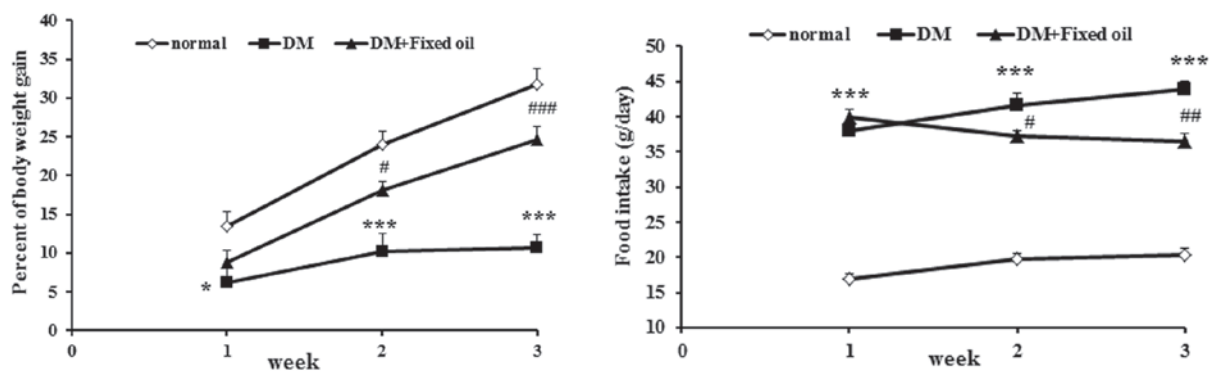


Figure 2. Differences in percentage body weight gain and food intake in normal rats and DM rats treated with or without fixed oil extracted from *Ocimum sanctum* L. for three weeks. * $P<0.05$ and *** $P<0.001$, vs. normal group; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$, vs. DM group. DM, diabetes mellitus.

Table I. Fatty acid compositions in the fixed oil extracted from *Ocimum sanctum* L. leaves.

Fatty acid	Molecular formula	%
Palmitic	16:0	15.65
Stearic	18:0	3.08
Oleic	18:1 (n-9) cis	2.81
Linoleic	18:2 (n-6) cis	17.86
α -Linolenic	18:3 (n-3)	60.60

Results

Fixed oil composition, body weight and food intake. Saturated and unsaturated fatty acids were identified in the OS fixed oil, as shown in Fig. 1 and Table I. The predominant fatty acid identified in the fixed oil was α -linolenic acid (60.60%). Fig. 2 shows the percentage body weight gain and food intake of all groups. Body weight gain of the untreated DM rats was significantly reduced compared with the normal control rats throughout three weeks ($P<0.05$ at the first week and $P<0.001$ at the second and third weeks). The reduction in body weight gain was more prominent in the DM rats treated with the OS fixed oil. Food intake was significantly increased in DM rats ($P<0.001$), and was slightly lowered at the second and third week in the DM rats treated with the OS fixed oil.

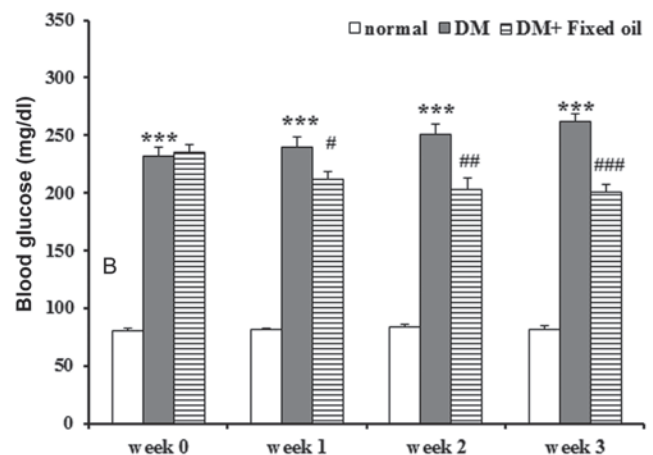


Figure 3. Alterations of blood glucose in normal rats and DM rats treated with or without fixed oil extracted from *Ocimum sanctum* L. for three weeks. *** $P<0.001$, vs. normal group; # $P<0.05$, ## $P<0.01$ and ### $P<0.001$, vs. DM group. DM, diabetes mellitus.

Blood glucose, serum insulin, lipid profile, and organ weight and function. Fixed oil significantly reduced high blood glucose of DM rats throughout three weeks of treatment (Fig. 3; $P<0.001$). Serum insulin levels were significantly decreased in the DM rats ($P<0.001$), an effect which was more marked in DM rats treated with the OS fixed oil (Table II). Liver, kidney and heart weight were significantly increased in the untreated DM rats (Table II; $P<0.001$). Only the high level

Table II. Alterations of serum insulin, and liver, kidney and heart weight in the three groups.

Group	Serum insulin (μ U/ml)	Liver weight (g/kg)	Kidney weight (g/kg)	Heart weight (g/kg)
Normal	5.98 \pm 0.26	34.9 \pm 1.3	6.43 \pm 0.11	3.5 \pm 0.07
DM	3.22 \pm 0.18 ^a	52.3 \pm 2.0 ^a	13.3 \pm 0.7 ^a	4.0 \pm 0.1 ^a
DM + fixed oil	4.50 \pm 0.24 ^{a,b}	51.0 \pm 1.4 ^a	11.8 \pm 0.2 ^{a,b}	4.1 \pm 0.1 ^a

Data are presented as the mean \pm standard error of the mean. ^aP<0.05 vs. normal group; ^bP<0.05 vs. DM group. DM, diabetes mellitus.

Table III. Differences in the serum lipid profile in the three groups.

Group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Normal	49 \pm 1	55 \pm 3	26 \pm 1	12 \pm 1
DM	93 \pm 7 ^a	92 \pm 7 ^a	20 \pm 1 ^a	55 \pm 6 ^a
DM + fixed oil	70 \pm 4 ^{a,b}	45 \pm 8 ^b	23 \pm 2	39 \pm 3 ^{a,b}

Data are presented as the mean \pm standard error of the mean. ^aP<0.05 vs. normal group; ^bP<0.05 vs. DM group. HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; DM, diabetes mellitus.

Table IV. Differences in ALT, AST, LDH, CK-MB, creatinine and BUN in serum of rats in the three groups.

Group	AST (U/l)	ALT (U/l)	LDH (U/l)	CK-MB (U/l)	Creatinine (mg/dl)	BUN (mg/dl)
Normal	82 \pm 7	36 \pm 3	279 \pm 46	420 \pm 49	0.92 \pm 0.07	14.4 \pm 0.5
DM	132 \pm 12 ^a	75 \pm 11 ^a	545 \pm 34 ^a	593 \pm 47 ^a	2.18 \pm 0.22 ^a	25.2 \pm 0.5 ^a
DM + fixed oil	138 \pm 6 ^a	79 \pm 5 ^a	452 \pm 63 ^a	603 \pm 40 ^a	1.27 \pm 0.18 ^b	20.2 \pm 0.7 ^{a,b}

Data are presented as the mean \pm standard error of the mean. ^aP<0.05 vs. normal group; ^bP<0.05 vs. DM group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK-MB, creatine kinase MB subunit; BUN, blood urea nitrogen; DM, diabetes mellitus.

of kidney weight was attenuated in the DM rats treated with the OS fixed oil. Total cholesterol, triglyceride and LDL-C were significantly increased (P<0.001), whereas HDL-C was reduced in the untreated DM rats (Table III; P<0.05). The high serum lipid profile was markedly reduced, whereas HDL-C was slightly increased in the DM + OS fixed oil rats. Serum levels of AST, ALT, LDH, CK-MB, creatinine and BUN in the untreated DM rats were significantly increased compared with the normal control rats (Table IV). Only high serum levels of creatinine and BUN were reversed in DM + fixed oil rats.

Lipid peroxide and antioxidant levels. The high level of tissue lipid peroxide as indicated by TBARS was significantly increased (P<0.001), whereas GPx (P<0.05), CAT (P<0.001) and SOD (P<0.001) activities were significantly decreased, in the liver tissue of the untreated DM rats (Table V). TBARS and CAT were increased, whereas no significant changes in GPx and SOD activity were observed in the cardiac tissue of the untreated DM rats. The OS fixed oil treatment had no effect on TBARS or the activities of any antioxidative enzymes in the liver and cardiac tissues. However, renal TBARS was increased, while the activities of GPx and SOD were decreased, without significant changes of CAT activity, in the untreated DM rats.

The OS fixed oil treatment appeared to reduce the elevated level of TBARS to below normal levels, and normalized the low levels of GPx and SOD activities, while markedly increasing CAT activity in the renal tissue of the DM + fixed oil rats.

Histopathological examination. Fig. 4 shows the histopathological examination of renal tissues in all groups. The renal tissue of normal rats exhibited normal cortex and medullary features (Fig. 4A). The renal glomeruli and intact lining epithelium of the Bowman's capsule were normal. The glomerular capillary loops were lining with normal mesangial cells, which exhibited basic appearance of the loops. The quantities of vascular endothelial and mesangial cells were within normal limits. Furthermore, the proximal convoluted tubules were preserved and contained within the luminal spaces indicated by well-differentiated columnar epithelial cells. The renal tissue of the DM rats showed moderate to severe multifocal necrosis, with interstitial infiltration of mononuclear cells (Fig. 4C and D). The glomerular membranes were thickened, with interposition of mesangial cells between two layers. There was an increased mesangial cellularity and matrix. Furthermore, the proximal convoluted tubules exhibited numerous cellular vacuolizations within the cytoplasm of the epithelial cells lining the proximal convoluted tubules.

Table V. Effect of fixed oil of *Ocimum sanctum* L. leaves on lipid peroxide and antioxidative enzymes activity in the rat liver, heart and renal tissues.

Group	TBARS	GPx	CAT	SOD
Liver				
Normal	1.05±0.05	1.01±0.06	254±27	112±10
DM	1.34±0.03 ^a	0.61±0.06 ^a	138±9 ^a	51±7 ^a
DM + fixed oil	1.29±0.03 ^a	0.72±0.02 ^a	166±10 ^a	54±4 ^a
Heart				
Normal	0.72±0.03	0.27±0.01	9.2±0.8	41±5
DM	0.98±0.03 ^a	0.25±0.01	19.9±0.8 ^a	40±5
DM + fixed oil	0.93±0.02 ^a	0.28±0.02	21.0±0.9 ^a	45±4
Kidney				
Normal	1.02±0.03	1.02±0.02	4.99±0.38	52±4
DM	1.20±0.05 ^a	0.80±0.06 ^a	4.71±0.24	31±4 ^a
DM + fixed oil	0.89±0.02 ^{a,b}	1.13±0.11 ^b	76.4±9.5 ^{a,b}	45±5 ^b

Data are presented as the mean ± standard error of the mean. ^aP<0.05 vs. normal group; ^bP<0.05 vs. DM group. TBARS, thiobarbituric acid reactive substances (nmole MDA/mg protein); GPx, glutathione peroxidase (mmole/min/mg protein); CAT, catalase, (mmole/min/mg protein); SOD, superoxide dismutase (units/mg protein); DM, diabetes mellitus.

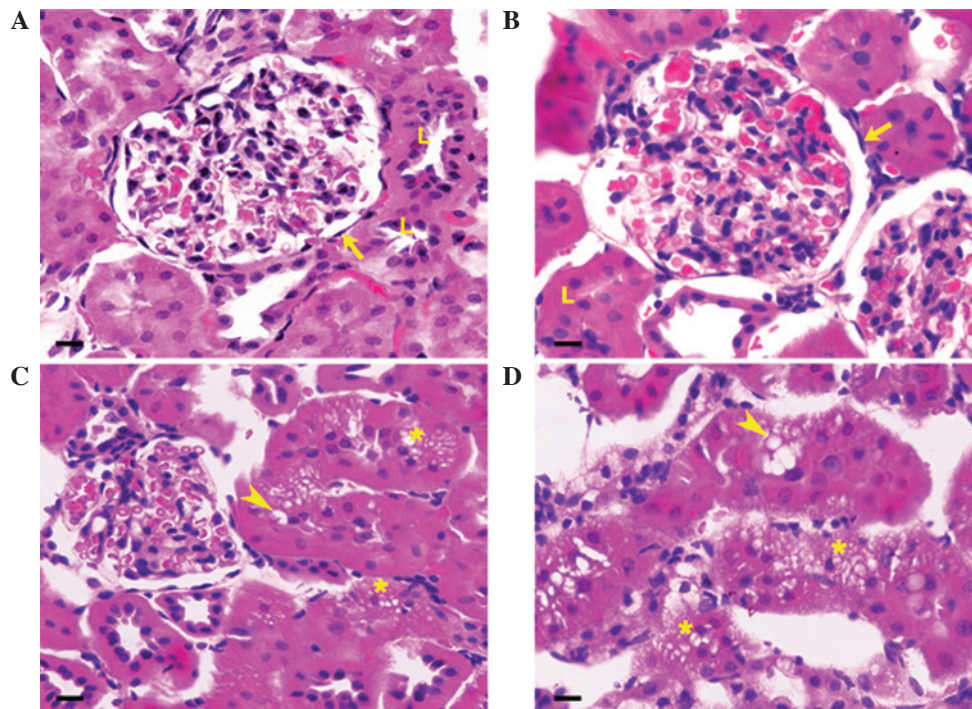


Figure 4. Histopathological appearance of renal tissues from the various groups (scale bar, 25 μ m). (A) Renal tissue of the control group rats exhibited normal kidney glomeruli and epithelium of the Bowman's capsule. Parietal podocytes were forming and intact in the Bowman's capsule (arrows). The proximal convoluted tubules were preserved and contained with their luminal spaces by well-differentiated columnar epithelial cells (L). (B) For diabetes mellitus (DM) rats treated with *Ocimum sanctum* L. fixed oil, the general renal appearance was improved, and the glomeruli remained defined. The mesangial cells and cellular matrix showed reduced proliferation and accumulation, respectively. The cytoplasmic vacuolizations were markedly reduced in the epithelium of the proximal convoluted tubules. (C) Renal glomeruli and (D) renal tubules. The glomerular membranes exhibited thickening, with interposition of mesangial cells between two layers, which resulted in increased mesangial cellularity and matrix. In (C), parietal podocytes were detached in dilated Bowman's capsule. Furthermore, the proximal convoluted tubules exhibited numerous cellular vacuolizations (*) and predominant clusters of large vacuoles were observed in certain tubular epithelial cells (arrows).

Predominant clusters of large vacuoles were observed in certain tubular epithelial cells. Cellular swelling of the tubular epithelium is noticeable without its luminal space. In the DM + fixed oil rat tissues, general renal appearance was improved (Fig. 4B).

Glomeruli remained defined, mesangial cells showed reduced proliferation and the cellular matrix showed decreased accumulation. The cytoplasmic vacuolization was markedly reduced in the epithelium of the proximal convoluted tubules.

Discussion

In addition to cancer and cardiovascular diseases, diabetes mellitus (DM) has become a major global health problems in the 21st century, due to the gradually and continuous increased in the number of diabetic patients (3,4). Due to the wide range of affected organs and its influence on multiple organ systems, DM may lead to serious health complications and potentially mortality. Although a number of interventions are recommended for the management of DM, patients are often unsuccessful at following the regimes due to lifestyle and undesirable side effects, cost and the safety of the long-term use of synthetic drugs (5,36). Therefore, novel treatments that fulfil the required safety and efficiency of life-long treatments are needed. It has been widely accepted that medicines of herbal origin may be useful in treating various diseases, as many contain bioactive photochemical ingredients that may function as effective, safe and cheap therapeutic compounds (7,16). *Ocimum sanctum* L. (OS), commonly used as a cooking vegetable, has shown its potency as a therapeutic herb and has already been proven to be safe for long term consumption, as mentioned earlier. A number of experimental studies have evaluated the anti-diabetic activity of OS using alcoholic and aqueous extracts of its leaves (15-17). However, OS leaves are enriched in volatile and fixed oils. To the best of our knowledge, no previous studies have investigated the anti-diabetic and anti-oxidative effects of fixed oil extracted from OS leaves. The present study showed that the fixed oil extract of OS decreased the elevated blood glucose levels and serum lipid profile in DM rats (Fig. 3 and Table III). These results are consistent with those of our previous study, which demonstrated the anti-hyperlipidemic activity of OS fixed oil in rats fed with a high fat diet (24). The lipid-lowering effect of OS may be a beneficial property for the prevention of cardiovascular disease in DM.

β -cells are known to be highly susceptible to cytotoxic agents such as streptozotocin (STZ) (37). STZ induces DM by the rapid depletion of β -cells, leading to a reduction in insulin secretion. Insulin is a vital hormone for the maintenance of normal levels of blood glucose and lipids. The present results indicated that the low level of serum insulin in DM rats could be raised by treatment with OS fixed oil. Therefore, the anti-hyperglycemic and lipid-lowering abilities of OS fixed oil may be associated with its ability to improve pancreatic β -cell function. α -Linolenic acid, the primary fatty acid contained in OS fixed oil, is speculated to be the active ingredient underlying these activities, which is supported by several studies (38,39). In a previous study, α -linolenic acid agonist administration significantly decreased plasma glucose and augmented insulin release in mice (38). Furthermore, a diet rich in α -linolenic acid has been shown to improve insulin sensitivity by increasing GLUT4 protein content in the gastrocnemius muscle membranes of STZ-induced diabetic rats (38).

Lipid peroxidation is a free radical-mediated process that occurs following oxidative stress. If this process is localized in biological membranes it may cause a variety of types of cellular membrane damage, including change in membrane fluidity, increased membrane permeability and finally membrane rupture (40). Therefore, free radicals serve a crucial function in the induction of a variety of stress-related diseases. In order to prevent the cellular deterioration induced by free radicals, cells

possess an antioxidant defense system. An elevation of blood glucose induces oxidative stress, resulting in an increased production of oxygenated free radicals and decreased antioxidant enzyme activities (6,41). This may result in intracellular structure modification, and ultimately affect normal cellular function, leading to pathogenesis and the development of diabetic complications (41,42). The present results show that DM impaired the liver, kidney and cardiac functions of the DM rats, as indicated by the augmentation of serum levels of AST, ALT, creatinine, BUN, LDH and CK-MB (Table IV). In addition, TBARS levels were significantly increased, whereas the activities of various antioxidative enzymes were significantly suppressed in the liver, heart and renal tissues of untreated DM rats (Table V). These results demonstrate that three weeks of diabetic induction is sufficient to cause oxidative stress and damage to the liver, heart and renal tissues of rats.

Notably, the TBARS level was increased without significant differences in GPx and SOD, whereas CAT was markedly increased in the cardiac tissue of untreated DM rats. It has been reported that during periods of elevated oxidative stress, cells protect themselves by increasing activity of various antioxidative enzymes (6,43). Therefore, the augmentation of cardiac CAT activity without changes in GPx and SOD activity is potentially a compensatory response against the oxidative stress induced by DM (43,44). However, the enhancement of cardiac CAT activity may not markedly protect the heart against oxidative stress, as levels of cardiac TBARS remained elevated. This suggests that numerous free radicals were generated in the rat cardiac tissue, which could not be eliminated through increasing CAT activity alone.

The OS fixed oil exerted no hepatoprotective or cardioprotective effects against DM, as indicated by the unchanged elevations in the serum levels of AST, ALT, LDH and CK-MB, in addition to TBARS levels and the activities of the various anti-oxidative enzymes (Tables IV and V). The results of our previous study suggested that OS fixed oil exerted a cardioprotective effect in rats fed with a high fat diet (24); however, fixed OS oil appeared to have no effect in diabetic rats in the present study. This may be due to the difference in the severity of stress exposure. The hearts of the DM rats in the present study were exposed to hyperglycemic and hyperlipidemic conditions, whereas the hearts of the high fat-fed rats were exposed to hyperlipidemia only (24,45). In contrast to the liver and heart tissues, OS fixed oil normalized GPx and SOD activities and markedly promoted CAT activity, which subsequently decreased the high level of TBARS, in renal tissue. These results indicate that the OS fixed oil exerted a free radical scavenging activity, providing renal protection against DM. This hypothesis is supported by the normal general appearance of the rat renal tissue, as shown by histopathological analysis (Fig. 4).

Notably, the OS fixed oil exerted a marked anti-oxidative capacity in the renal tissue of the DM rats; by normalizing the reduced levels of GPx and SOD, in addition to enhancing the CAT activity to a higher than normal level. This may be the reason why the OS fixed oil suppressed the renal TBARS levels to below normal levels (Table V). The α -linolenic acid contained in the OS fixed oil may be responsible for the renal protective activity against DM. It has been shown that a high α -linolenic acid diet ameliorated diabetic nephropathy in

rats (46) and prevented high glucose-induced renal tubular cell damage by reducing the production of reactive oxygen species (47). The present results suggest that fixed oil extracted from OS leaves may be of therapeutic use as an anti-hyperglycemic and lipid-lowering agent, in addition to being a cytoprotective agent for the prevention of renal injury as a result of DM. As the present study was performed in rats with STZ-induced DM, which is type 1 DM, the effect of OS fixed oil in type 2 DM requires further investigation.

In conclusion, treatment with the fixed oil extracted from OS leaves for three weeks significantly lowered the diabetically-elevated blood glucose levels and serum lipid profile, while increasing serum insulin levels in STZ-induced DM rats. The OS fixed oil exhibited a free radical scavenging activity, which provided renal protection against DM via the suppression of high TBARS levels, and by enhancing the activity of various antioxidative enzymes in the rat renal tissue. Histopathological studies showed that the OS fixed oil protected rat renal tissues against DM. The α -linolenic acid contained in OS fixed oil may be mechanistically involved in its anti-hyperglycemic, anti-hyperlipidemic and renal protective activities against DM.

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References

- American Diabetes Association: Diagnosis and classification of diabetes mellitus. *Diabetes Care* 33 (Suppl 1): 62-69, 2010.
- Rochette L, Zeller M, Cottin Y and Vergely C: Diabetes, oxidative stress and therapeutic strategies. *Biochim Biophys Acta* 1840: 2709-2729, 2014.
- Wild S, Roglic G, Green A, Sicree R and King H: Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27: 1047-1053, 2004.
- World Health Organization: The top 10 causes of death. Fact sheet No. 310. <http://www.who.int/mediacentre/factsheets/fs310/en/>. Accessed May, 2014.
- Emilien G, Maloteaux JM and Ponchon M: Pharmacological management of diabetes: Recent progress and future perspective in daily drug treatment. *Pharmacol Ther* 81: 37-51, 1999.
- Suanarunsawat T, Ayuthaya WD, Songsak T, Thirawarapan S and Pongshompoo S: Lipid-lowering and antioxidative activities of aqueous extracts of *Ocimum sanctum* L. leaves in rats fed with a high-cholesterol diet. *Oxid Med Cell Longev* 2011: 962025, 2011.
- Taleb-Senouci D, Ghomari H, Krouf D, Bouderbala S, Prost J, Lacaille-Dubois MA and Bouchenak M: Antioxidant effect of *Ajuga iva* aqueous extract in streptozotocin-induced diabetic rats. *Phytomedicine* 16: 623-631, 2009.
- Sarkhail P, Rahmanipour S, Fadyevatan S, Mohammadirad A, Dehghan G, Amin G, Shafiee A and Abdollahi M: Antidiabetic effect of *Phlomis anisodonta*: Effects on hepatic cells lipid peroxidation and antioxidant enzymes in experimental diabetes. *Pharmacol Res* 56: 261-266, 2007.
- Pattanayak P, Behera P, Das D and Panda SK: *Ocimum sanctum* Linn. A reservoir plant for therapeutic applications: An overview. *Pharmacogn Rev* 4: 95-105, 2010.
- Cohen MM : Tulsi - *Ocimum sanctum*: A herb for all reasons. *J Ayurveda Integr Med* 5: 251-259, 2014.
- Chandrasekaran CV, Srikanth HS, Anand MS, Allan JJ, Viji MM and Amit A: Evaluation of the mutagenic potential and acute oral toxicity of standardized extract of *Ocimum sanctum* (OciBest™). *Hum Exp Toxicol* 32: 992-1004, 2013.
- Gautam MK and Goel RK: Toxicological study of *Ocimum sanctum* linn leaves: Hematological, biochemical and histopathological studies. *J Toxicol* 2014: 135654, 2014.
- Mahajan N, Rawal S, Verma M, Poddar M and Alok S: A phytopharmacological overview on *Ocimum* species with special emphasis on *Ocimum sanctum*. *Biomed Prevent Nutr* 3: 185-192, 2013.
- Suanarunsawat T and Songsak T: Anti-hyperglycemic and anti-hyperlipidemic effect of dietary supplement of white *Ocimum sanctum* L. before and after STZ-induced diabetes mellitus. *Inter J Diabetes Metab* 13: 18-23, 2005.
- Narendhirakannan RT, Subramanian S and Kandaswamy M: Biochemical evaluation of antidiabetogenic properties of some commonly used Indian plants on streptozotocin-induced diabetes in experimental rats. *Clin Exp Pharmacol Physiol* 33: 1150-1157, 2006.
- Suanarunsawat T, Ayuthaya WD, Thirawarapan S and Pongshompoo S: Anti-oxidative, anti-hyperglycemic and lipid-lowering effects of aqueous extracts of *Ocimum sanctum* L. leaves in diabetic rats. *Food Nutr Sci* 5: 801-811, 2014.
- Reddy SS, Karuna R, Baskar R and Saralakumari D: Prevention of insulin resistance by ingesting aqueous extract of *Ocimum sanctum* to fructose-fed rats. *Horm Metab Res* 40: 44-49, 2008.
- Shetty S, Udupa S and Udupa L: Evaluation of antioxidant and wound healing effects of alcoholic and aqueous extract of *Ocimum sanctum* linn in rats. *Evid Based Complement Alternat Med* 5: 95-101, 2008.
- Bhattacharyya P and Bishayee A: *Ocimum sanctum* Linn. (Tulsi): An ethnomedicinal plant for the prevention and treatment of cancer. *Anticancer Drugs* 24: 659-666, 2013.
- Sharmila Banu G, Kumar G and Murugesan AG: Effects of leaves extract of *Ocimum sanctum* L. on arsenic-induced toxicity in Wistar albino rats. *Food Chem Toxicol* 47: 490-495, 2009.
- Ramesh B and Satakopan VN: Antioxidant activities of hydroalcoholic extract of *Ocimum sanctum* against cadmium induced toxicity in rats. *Indian J Clin Biochem* 25: 307-310, 2010.
- Hussain EH, Jamil K and Rao M: Hypoglycaemic, hypolipidemic and antioxidant properties of tulsi (*Ocimum sanctum* linn) on streptozotocin induced diabetes in rats. *Indian J Clin Biochem* 16: 190-194, 2001.
- Suanarunsawat T, Devakul Na Ayuthaya W, Songsak T, Thirawarapan S and Pongshompoo S: Antioxidant activity and lipid-lowering effect of essential oils extracted from *Ocimum sanctum* L. leaves in rats fed with a high cholesterol diet. *J Clin Biochem Nutr* 46: 52-59, 2010.
- Suanarunsawat T, Boonnak T, Na Ayuthaya WD and Thirawarapan S: Anti-hyperlipidemic and cardioprotective effects of *Ocimum sanctum* L. fixed oil in rats fed a high fat diet. *J Basic Clin Physiol Pharmacol* 21: 387-400, 2010.
- Su J, Zhang P, Zhang JJ, Qi XM, Wu YG and Shen JJ: Effects of total glucosides of paeony on oxidative stress in the kidney from diabetic rats. *Phytomedicine* 17: 254-260, 2010.
- Li CJ, Lv L, Li H and Yu DM: Cardiac fibrosis and dysfunction in experimental diabetic cardiomyopathy are ameliorated by alpha-lipoic acid. *Cardiovasc Diabetol* 11: 73, 2012.
- Mercer BN, Morais S, Cubbon RM and Kearney MT: Diabetes mellitus and the heart. *Int J Clin Prac* 66: 640-647, 2012.
- AOAC International: Method 948.22. In: Official Methods of Analysis of AOAC International. 16th edition. Association of Analytical Communities, Washington, DC, 1995.
- International Organization for Standardization (ISO): Animal and vegetable fats and oils: Preparation of methyl esters of fatty acids (Method ISO 5509). ISO, Geneva, pp 1-6, 1978.
- Ohkawa H, Ohishi N and Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351-358, 1979.
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
- Tapple AL: Glutathione peroxidase and hydroperoxidase methods. In: *Methods in Enzymology*. Vol 2. Sidney F and Lester P (eds). Academic Press, New York, NY, pp506-513, 1978.
- Luck H: Catalase. In: *Method for Enzymatic Analysis*. Vol 3. Bergmeyer HU (ed). Academic Press, New York and London, pp885-894, 1965.
- Winterbourn CC, Hawkins RE, Brian M and Carrell RW: The estimation of red cell superoxide dismutase activity. *J Lab Clin Med* 85: 337-341, 1975.
- Hewitson TD, Wigg B and Becker GJ: Tissue preparation for histology: Fixation, embedding, and antigen retrieval for light microscopy. *Methods Mol Biol* 611: 3-18, 2010.

36. Reichard P and Pihl M: Mortality and treatment side-effects during long-term intensified conventional insulin treatment in the Stockholm Diabetes Intervention Study. *Diabetes* 43: 313-317, 1994.
37. Elsner M, Guldbakke B, Tiedge M, Munday R and Lenzen S: Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. *Diabetologia* 43: 1528-1533, 2000.
38. Moran BM, Abdel-Wahab YH, Flatt PR and McKillop AM: Evaluation of the insulin-releasing and glucose-lowering effects of GPR120 activation in pancreatic β -cells. *Diabetes Obes Metab* 16: 1128-1139, 2014.
39. Khamaisi M, Rudich A, Beeri I, Pessler D, Friger M, Gavrilov V, Tritschler H and Bashan N: Metabolic effects of gamma-linolenic acid-alpha lipoic acid conjugate in streptozotocin diabetic rats. *Antioxid Redox Signal* 1: 523-535, 1999.
40. Halliwell B and Chirico S: Lipid peroxidation: Its mechanism, measurement and significance. *Am J Clin Nutr* 57 (5 Suppl): 715S-725S, 1993.
41. Rochette L, Zeller M, Cottin Y and Vergely C: Diabetes, oxidative stress and therapeutic strategies. *Biochim Biophys Acta* 1840: 2709-2729, 2014.
42. King GL and Loeken MR: Hyperglycemia-induced oxidative stress in diabetic complications. *Histochem Cell Biol* 122: 333-338, 2004.
43. Kumar S, Prasad S and Sitasawad SL: Multiple antioxidants improve cardiac complications and inhibit cardiac cell death in streptozotocin-induced diabetic rats. *PLoS One* 8: e67009, 2013.
44. Zobali F, Avci A, Canbolat O and Karasu C: Effects of vitamin A and insulin on the anti-oxidative state of diabetic rat heart: A comparison study with combination treatment. *Cell Biochem Funct* 20: 75-80, 2002.
45. Suanarunsawat T, Ayutthaya WD, Songsak T and Rattanamahaphoom J: Anti-lipidemic actions of essential oil extracted from *Ocimum sanctum* L. leaves in rats fed with high cholesterol diet. *J Appl Biomed* 7: 45-53, 2009.
46. Barcelli UO, Weiss M, Beach D, Motz A and Thompson B: High linoleic acid diets ameliorate diabetic nephropathy in rats. *Am J Kidney Dis* 16: 244-251, 1990.
47. Jiang M, Zheng J, Yu W, Lv G, Xu Q, Zhou Y and Zhai C: Protective effect of ALA on high glucose induced cellular injury of LLC-PK1 cell. *Wei Sheng Yan Jiu* 41: 191-194, 2012 (In Chinese).