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ORIGINAL ARTICLE

Effect of strawberry (*Fragaria* × *ananassa*) leaf extract on diabetic nephropathy in rats

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[Correction added on 28 May 2015, after first online publication: this article has been further edited for language.]

SUMMARY

Diabetic nephropathy is a clinical syndrome characterized by albuminuria, hypertension and progressive renal insufficiency. The aim of this study was to investigate the effect of strawberry (*Fragaria* × *ananassa*) leaf extract on diabetic nephropathy in rats. Streptozotocin (STZ) diabetic rats were orally treated with three doses (50, 100 and 200 mg/kg) of strawberry leaf extract for 30 days. Nephropathy biomarkers in plasma and kidney were examined at the end of the experiment. The three doses of strawberry leaf extract significantly decreased the levels of blood glucose, urea nitrogen, plasma creatinine, kidney injury molecule (Kim)-1, renal malondialdehyde (MDA), tumour necrosis factor alpha (TNF-α), interleukin (IL)- 6 and caspase-3 in diabetic rats. Meanwhile, the levels of plasma insulin, albumin, uric acid, renal catalase (CAT), superoxide dismutase (SOD) and vascular endothelial growth factor A (VEGF-A) were significantly elevated in diabetic rats treated with strawberry leaf extract. These results indicate the role of strawberry leaf extract as anti-diabetic, antioxidant, anti-inflammatory and anti-apoptosis in diabetic nephropathy.

Keywords
diabetic nephropathy, kidney injury molecule-1, renal apoptosis, renal cytokines, renal function parameters, renal oxidative stress, strawberry leaf extract

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action or both leading to impaired carbohydrate, lipid and protein metabolism (Naik *et al.* 2014). DM is associated with microvascular, macrovascular and non-vascular complications (Abo-Salem *et al.* 2009). Diabetic nephropathy (DN) is considered one of the major microvascular complications of diabetes (Moresco *et al.* 2013).

Hyperglycaemia leads to overproduction of reactive oxygen species (ROS), which plays a central role in progression of DN. Antioxidant administration has a protective effect against diabetic nephropathy development (Wagener *et al.* 2009). Fruit and vegetables protect human from several chronic diseases, where they contain many antioxidants and bioactive compounds (Romandini *et al.* 2013). Strawberry (*Fragaria* × *ananassa*) is a widely grown hybrid species of the genus Fragaria. Its leaves contain many bioactive compounds including tannis, flavonoids, ascorbic acid and essential oil (Wang & Jiao 2000). Flavonoids and ascorbic acid have been reported as good antioxidant compounds that have been shown to neutralise the harmful effects which are associated with injury induced by ROS (Mandave *et al.* 2013). Strawberry leaves are used in traditional medicine as an appetizer, for treating hypercholesterolaemia, to lower blood pressure and to treat a host of other conditions such as gastrointestinal disorders and strictures. There is a long list of other effects reported in folk medicine: as a diuretic, to strengthen sight and dentition, to expel kidney stones and intestinal worms, to treat anaemia and hepatitis, to strengthen the nervous and immune system, to promote intestinal and liver activity, to suppress diarrhoea, to treat arthritis

and to speed up metabolism (Duru 2012). Strawberry fruits also have anti-carcinogenic activities (Wedge *et al.* 2001) and anti-thrombotic effects (Naemura *et al.* 2005).

This study was carried out to investigate the effects of three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract on hyperglycaemia, renal function markers, renal oxidative stress, renal inflammatory cytokines and renal apoptosis in diabetic nephropathy rats.

Materials and methods

Preparation of strawberry leaves extract

Strawberry (*Fragaria* × *ananassa*) leaves were collected in January 2014 from Benha, Egypt. Mature fresh and healthy leaves were collected in the morning and immediately taken to the laboratory.

Strawberry leaves were air-dried (20°C in the dark), then were milled. Dried leaf powder (100 g) was extracted with 1000 ml distilled water for 60 min and then filtered. Water extracted leaves were evaporated under vacuum (rotary evaporator Büchi R-110) to give a crude residue (yield: 50%).

Acute toxicity study (LD_{50})

The LD_{50} of the extract was estimated using the method described by Lorke 1983. The study was carried out using rats weighing 200 ± 10 g. Five groups (four rats each) were administered with graded doses (50, 100, 200, 400 and 800 mg/kg) of the extract intragastrically. Rats were observed for clinical signs of toxicity and death over a period of 14 days.

Experimental animals

White male albino rats (*Rattus norvegicus*) weighing 200 ± 10 g were purchased from the Center of Laboratory Animals, Venoms & Crude Antisera Production, Helwan, Cairo, Egypt. Animals were maintained under laboratory conditions [Temperature (20 ± 2°C) and photoperiod (12 h light/12 h dark cycle)]. Rats were fed and watered *ad libitum*. They are fed a laboratory rodent chow (18–24% protein, 4–7% fat and 60–75% carbohydrate). Rats were acclimated to laboratory conditions for 1 week before the onset of the experiment. Body weight and food intake were not measured during the experimental period.

Induction of diabetes mellitus

Diabetes mellitus was experimentally induced in rats previously fasted for 12 h by a single intraperitoneal dose (45 mg/kg) of STZ (Sigma Co., USA) dissolved in citrate buffer (El-Gomhorya Co., Egypt) (pH 4.5) according to El Shafey *et al.* (2013). Animals were given 5% glucose solution instead of drinking water for 2 days to overcome the hypoglycaemic coma that occurs within the first 24 h following STZ injection. Rats were screened for blood glucose levels 48 h after STZ injection. Blood samples were withdrawn from the lateral tail vein, and glucose concentration was measured from overnight-fasted animals (10–12 h). Rats having glucose concentration exceeding 200 mg/dl were considered diabetic and included in the experiment according to El Shafey *et al.* (2013). The first dose of strawberry leaf extract was given intragastrically 72 h after STZ injection.

Experimental design

The rats under study were classified into five groups (Six rats each):

Group I: Normal rats.

Group II: Diabetic control rats intragastrically received vehicle alone (1 ml distilled water) once a day for 30 days.

Group III: Diabetic rats treated intragastrically with strawberry leaf extract (50 mg/kg) dissolved in 1 ml distilled water once a day for 30 days.

Group IV: Diabetic rats treated intragastrically with strawberry leaf extract (100 mg/kg) dissolved in 1 ml distilled water once a day for 30 days.

Group V: Diabetic rats treated intragastrically with strawberry leaf extract (200 mg/kg) dissolved in 1 ml distilled water once a day for 30 days.

Collection and preparation of samples

At the end of the experimental period, overnight-fasted rats were anaesthetized by inhalation of diethyl ether. Blood samples were collected from a postcaval vein and directly transported to tubes containing ethylenediamine tetra-acetic acid (EDTA) (El-Gomhorya Co., Egypt). All the tubes were centrifuged at 1500 ***g*** for 15 min by Hittech centrifuge, and plasma free of haemolysis was separated and frozen at −20°C.

Kidneys were removed from rats at the end of the experimental period and washed in ice-cold phosphate buffer saline (PBS) (pH 7.2) to remove excess blood. Samples were homogenized in ice-cold PBS and centrifuged at 5000 g for 5 min. The supernatants were stored at −20°C until used for analysis.

Biochemical assays in plasma

- The blood glucose level was determined by an enzymatic colorimetric method according to the method of Young and Friedman (2001) using a reagent kit purchased from Spinreact (Spain).
- The plasma insulin level was measured by a sandwich ELISA rat kit purchased from BioVendor – Research and Diagnostic Products (Japan).
- Plasma albumin was determined by the chemical colorimetric method according to the method of Doumas *et al.* (1971) using a reagent kit purchased from Diamond Diagnostics (Egypt).
- Blood urea nitrogen was determined by an enzymatic colorimetric method according to the method of Tietz (1995) using a reagent kit purchased from Diamond Diagnostics (Egypt).

- Plasma uric acid was determined by an enzymatic colorimetric method according to the method of Young (1995) using a reagent kit purchased from Spinreact (Spain).
- Plasma creatinine was determined by the kinetic colorimetric method according to the method of Burtis and Ashwood (1999) using a reagent kit purchased from Diamond Diagnostics (Egypt).
- Plasma kidney injury molecule-1 level was measured by a sandwich ELISA rat kit purchased from the Cloud Clone Corporation (USA).

Biochemical assays in the kidney

- Renal malondialdehyde content and catalase activity were determined by chemical colorimetric methods using kits purchased from BioVision (USA).
- Renal superoxide dismutase activity was determined by enzymatic colorimetric methods using kits purchased from BioVision (USA).
- Renal tumour necrosis factor alpha, interleukin-6, vascular endothelial growth factor A and caspase-3 were measured by ELISA rat kit purchased from the Cloud Clone Corporation (USA).

Statistical analysis

Data were expressed as mean ± SD for six readings. The data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan 1957). Statistical analysis was performed using the Statistical Package for Social Science (SPSS) computer program, Version 20.00 produced by IBM Software, Inc. Chicago, USA. Differences were considered significant at $P < 0.05$.

Results

Acute toxicity study

There was no mortality in animals at all doses of the extracts up to 800 mg/kg. The oral LD_{50} of the extracts is < 800 mg/kg

Effect of strawberry leaves extract on blood glucose and plasma insulin in diabetic rats

The blood glucose level was significantly increased ($P < 0.05$), and plasma insulin level was significantly decreased in diabetic rats compared with those in normal rats. However, blood glucose level was reduced, and plasma insulin level was elevated in treated diabetic rats compared with those in diabetic rats. The high dose (200 mg/kg) of strawberry leaf extract decreased blood glucose level and increased insulin level more than those with lower doses (50 and 100 mg/kg) of strawberry leaf extract (Table 1).

Effect of strawberry leaf extract on renal function parameters in diabetic rats

Plasma albumin and uric acid levels showed significantly decreased levels, ($P < 0.05$), and blood urea nitrogen, plasma creatinine and kim-1 levels showed significantly increased levels in diabetic rats compared with those in normal rats. Plasma albumin and uric acid levels were elevated and blood urea nitrogen, plasma creatinine and kim-1 levels were reduced in treated diabetic rats compared with those in diabetic rats. The high dose (200 mg/kg) of strawberry leaf extract elevated plasma albumin and uric acid levels and reduced blood urea nitrogen, plasma creatinine and kim-1 levels more than those in lower doses (50 and 100 mg/kg) of strawberry leaf extract (Table 2).

Effect of strawberry leaf extract on oxidative stress in diabetic rats

Renal MDA level showed significant increased levels ($P < 0.05$), and renal CAT and SOD activities showed significant decreased levels in diabetic rats compared with those in normal rats. Renal MDA level reduced, and renal CAT and SOD activities elevated in treated diabetic rats compared with those in diabetic rats. The high dose (200 mg/kg) of strawberry leaf extract reduced renal MDA level and elevated renal CAT and SOD activities more than those in lower doses (50 and 100 mg/kg) of strawberry leaf extract (Table 3).

Effect of strawberry leaf extract on inflammatory cytokines in diabetic rats

In diabetic rats, renal TNF-α and IL-6 levels were elevated significantly ($P < 0.05$) compared with those in normal rats. Renal TNF-α and IL-6 levels decreased significantly in treated diabetic rats compared with those in diabetic rats. The high dose (200 mg/kg) of strawberry leaf extract reduced renal

Table 1 Blood glucose and plasma insulin levels in normal rats (Group I), STZ-diabetic rats (Group II) and diabetic rats treated with three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract (Groups III, IV and V, respectively)

	Groups				
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
Glucose (mg/dl)	118.33 ± 3.51[¶]	264.67 ± 1.53*	162.67 ± 1.53[†]	144.00 ± 2.00[‡]	134.67 ± 3.51[§]
Insulin (ng/ml)	6.18 ± 0.01*	0.95 ± 0.12[§]	1.17 ± 0.20[‡]	1.26 ± 0.01[‡]	3.76 ± 0.09[†]

All data expressed as mean ± SD for six rats.
[*,†,‡,§,¶]Means superscripted with different letters are significantly different at ($P < 0.05$).

Table 2 Plasma albumin, urea nitrogen, uric acid, creatinine and kidney injury molecule-1 levels in normal rats (Group I), STZ-diabetic rats (Group II) and diabetic rats treated with three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract (Groups III, IV and V, respectively)

	Groups				
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
Albumin (g/dl)	3.73 ± 0.15*	3.03 ± 0.04¶	3.40 ± 0.03§	3.50 ± 0.03‡	3.63 ± 0.04†
Urea nitrogen (mg/dl)	28.72 ± 1.44§	49.03 ± 0.85*	43.33 ± 0.42†	41.43 ± 0.40†	32.33 ± 2.51‡
Uric acid (mg/dl)	3.85 ± 0.14*	1.16 ± 0.05§	1.61 ± 0.03‡	2.45 ± 0.06†	2.69 ± 0.27†
Creatinine (mg/dl)	0.19 ± 0.01¶	0.53 ± 0.06*	0.42 ± 0.02†	0.33 ± 0.02‡	0.26 ± 0.04§
Kim-1 (pg/ml)	64.11 ± 1.93§	263.41 ± 3.33*	259.75 ± 4.09*	247.61 ± 7.52†	233.29 ± 7.28‡

All data expressed as mean ± SD for six rats.
*,†,‡,§,¶Means superscripted with different letters are significantly different at ($P < 0.05$).

TNF-α and IL-6 levels more than those in lower doses (50 and 100 mg/kg) of strawberry leaf extract (Table 4).

Effect of strawberry leaf extract on caspase-3 and VEGF-A in diabetic rats

In diabetic rats, renal caspase-3 activity were elevated significantly ($P < 0.05$) and renal VEGF-A level reduced significantly compared with those in normal rats. Renal caspase-3 activity reduced significantly and renal VEGF-A level elevated significantly in treated diabetic rats compared with those in diabetic rats. The high dose (200 mg/kg) of strawberry leaf extract reduced renal caspase-3 activity and elevated renal VEGF-A level more than those in lower doses (50 and 100 mg/kg) of strawberry leaf extract (Table 5).

Discussion

Hyperglycaemia is the most important cause of DN progression (Mima 2013). This study showed the hypoglycaemic effect of strawberry leaf extract on diabetic rats. In agreement with this result, Mandave *et al.* (2013) reported that *in vitro* aqueous extract of strawberry fruits delayed carbohydrate absorption by inhibiting α-amylase and α-glucosidase enzyme activities so they might control blood glucose. Strawberry leaves extract also elevated plasma insulin level in diabetic rats. Insulin has been suggested to be an endogenous protective factor that prevents the progression of DN (Mima 2013).

Albuminuria, creatinine and blood urea nitrogen are considered as renal function markers (Zou *et al.* 2014). Diabetes is characterized by elevated levels of albuminuria (Yassin & Mwafy 2007), urea nitrogen (Hamden *et al.* 2009) and creatinine concentration (Katyal *et al.* 2009) and low uric acid concentration (Waring *et al.* 2006). In this study, strawberry leaf extract elevated plasma albumin and uric acid levels in diabetic rats. Meanwhile, it lowered blood urea nitrogen and plasma creatinine levels in diabetic rats. These results indicate that strawberry leaf extract ameliorated renal dysfunctions biomarkers in DN rats.

Table 3 Renal oxidative stress parameters in normal rats (Group I), STZ-diabetic rats (Group II) and diabetic rats treated with three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract (Groups III, IV and V, respectively)

	Groups				
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
MDA (nmol/mg protein)	1.25 ± 0.03§	3.35 ± 0.04*	2.66 ± 0.10†	2.25 ± 0.90‡	2.11 ± 0.06‡
CAT (nmol/mg protein)	3.56 ± 0.11*	1.59 ± 0.03¶	1.75 ± 0.08§	2.05 ± 0.11‡	2.28 ± 0.06†
SOD (U/mg protein)	4.79 ± 0.17*	2.20 ± 0.06¶	2.45 ± 0.06§	2.96 ± 0.09‡	3.28 ± 0.04†

All data expressed as mean ± SD for six rats.
*,†,‡,§,¶Means superscripted with different letters are significantly different at ($P < 0.05$).

Table 4 Renal inflammatory cytokines parameters in normal rats (Group I), STZ-diabetic rats (Group II) and diabetic rats treated with three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract (Groups III, IV and V, respectively)

	Groups				
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
TNF-α (ng/ml homogenate)	35.37 ± 1.56§	64.69 ± 1.93*	60.82 ± 0.46*†	57.56 ± 1.66†	42.13 ± 1.77‡
IL-6 (pg/mg protein)	19.72 ± 0.99¶	49.46 ± 1.32*	43.28 ± 1.77†	39.16 ± 0.79‡	34.13 ± 0.88§

All data expressed as mean ± SD for six rats.
*,†,‡,§,¶Means superscripted with different letters are significantly different at ($P < 0.05$).

Table 5 Renal caspase-3 and vascular endothelial growth factor A levels in normal rats (Group I), STZ-diabetic rats (Group II) and diabetic rats treated with three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract (Groups III, IV and V, respectively)

	Groups				
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
Caspase-3 (pg/mg protein)	$29.42 \pm 1.09^{\S}$	$42.89 \pm 2.09^{*}$	$38.67 \pm 1.26^{\dagger}$	$34.81 \pm 0.74^{\ddagger}$	$32.42 \pm 1.31^{\ddagger}$
VEGF-A (pg/mg protein)	$52.36 \pm 2.71^{*}$	$40.73 \pm 0.81^{\ddagger}$	$44.82 \pm 0.77^{\dagger}$	$45.91 \pm 0.21^{\dagger}$	$47.14 \pm 0.46^{\dagger}$

All data expressed as mean ± SD for six rats.
*,†,‡,§Means superscripted with different letters are significantly different at ($P < 0.05$).

Kidney injury molecule-1 is a biomarker for proximal tubule injury in diabetic nephropathy (Alter *et al.* 2012; Moresco *et al.* 2013). The present data revealed significant decreases in plasma Kim-1 level in diabetic rats treated with strawberry leaf extract compared to diabetic rats.

Oxidative stress reflects an imbalance between ROS production and antioxidant defence system (Dwivedi & Sarkar 2010). It was apparent from the results of this study that renal MDA level (ROS indicator) increased, but renal SOD and CAT activities (antioxidant enzymes) decreased in diabetic rats compared to normal rats. This increase in renal oxidative stress might be due to hyperglycaemia. In support of this view, Abo-Salem *et al.* (2009) and Naik *et al.* (2014) reported that renal oxidative stress increased in diabetic nephropathy rats. Hyperglycaemia in diabetic nephropathy increased ROS production in mesangial and tubular epithelial cells (Arora & Singh 2013).

The present study showed a significant decrease in the renal MDA level and significant increase in renal SOD and CAT activities in treated diabetic rats compared to diabetic rats. These results might be explained as a result of high antioxidant capacity of strawberry leaves (Katalinic *et al.* 2006 and Buřičová & Réblová 2008) due to the presence of polyphenolic compounds (Wang & Jiao 2000) such as tannins, flavonoids and ascorbic acid (Duru 2012). In support of this view, Mandave *et al.* (2013) reported that strawberry fruits possess high antioxidant capacity due to the presence of polyphenolic compounds (Ninomiya *et al.* 2010) such as anthocyanins (Van de Velde *et al.* 2013), flavonols, ellagitannins (Aaby *et al.* 2005) and fistein (Mahe *et al.* 2011). Not only that, but they also contain vitamin C and glutathione (Guo *et al.* 1997) and inhibit free radicals (Wang & Jiao 2000).

Interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-a) are inflammatory cytokines, used as inflammatory biomarkers for early diabetic nephropathy diagnosis (Moresco *et al.* 2013). The present data revealed a significant increase in renal IL-6 and TNF-α levels in diabetic rats compared to normal rats. Increasing ROS might be the reason that increases the inflammatory cytokines. In support of this view, Wagener *et al.* (2009) reported that ROS can activate inflammation, resulting in production of cytokines. Tumour necrosis factor-α induces renal injury and disruption of the glomerular permeability barrier (Moresco *et al.* 2013).

This study showed significant decrease in renal IL-6 and TNF-α levels in treated diabetic rats compared to diabetic rats. This decreasing might be due to decreased renal ROS after strawberry leaf extract administration to diabetic rats. These results were in agreement with the result of Hannum (2004), who reported that strawberries have antioxidant and anti-inflammatory functions.

Apoptosis is the process of programmed cell death that occurs during normal development (Chandra *et al.* 2001). The apoptotic process is regulated by cysteine-dependent specific aspartate proteases or caspases (Wagener *et al.* 2009). Vascular endothelial growth factor is a protein secreted by podocytes that are necessary for survival of endothelial cells, podocytes and mesangial cells (Tufro & Veron 2012). It also increases the expression of the anti-apoptotic proteins Bcl-2 and prevents endothelial cell apoptosis (Mima 2013). In the present study, renal caspase-3 activity increased and VEGF-A level decreased significantly in diabetic rats compared to normal rats that indicated increase renal apoptosis in diabetic rats due to increased renal ROS levels. Wagener *et al.* (2009) reported that high ROS formation induced mesangial and proximal tubular epithelial cell apoptosis and development of diabetic nephropathy. Reduction of VEGF-A expression in diabetic nephropathy is associated with podocyte loss (Baelde *et al.* 2007).

It was apparent from the present study that renal caspase-3 activity decreased and VEGF-A level increased significantly in treated diabetic rats compared to diabetic rats and that might be due to decreasing renal ROS after strawberry leaves extract administration to diabetic rats. In support of this view, Wagener *et al.* (2009) reported that in diabetic nephropathy apoptosis was inhibited after antioxidant treatment with vitamin C in type 2 diabetic rats. Superoxide dismutase overexpression abrogated caspase-3 cleavage, DNA damage and mesangial apoptosis in type 2 diabetic mice (Kitada *et al.* 2011).

In conclusion, this study showed for the first time, the renal functional improvement which occurs after three doses (50, 100 and 200 mg/kg) of strawberry (*Fragaria* × *ananassa*) leaf extract on diabetic nephropathy rats. The renal improving properties of high dose (200 mg/kg) strawberry leaf extract was more pronounced than that of lower doses (50 and 100 mg/kg) of strawberry leaf extract. It is therefore

suggested that the use of strawberry leaf extract might be useful as an herbal medicine for diabetic nephropathy patients.

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