

AQUEOUS EXTRACT OF *Etlingera elatior* FLOWERS IMPROVED BLOOD GLUCOSE CONTROL, KIDNEY FUNCTION AND HISTOLOGY OF STREPTOZOTOCIN-INDUCED DIABETIC RAT

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Abstract: Diabetes mellitus (DM) is the most critical health problem of chronic renal disorder that has become a major reason in medical treatments of dialysis and kidney transplantation. In Malaysia, *Etlingera elatior* has been used as a native medicine to reduce blood sugar among diabetic patients. This study aims to evaluate the therapeutic effect of crude aqueous *E. elatior* flowers extract (AEEFE) on streptozotocin (STZ)-induced diabetic rats taken orally. The effective dose in lowering blood glucose level in acute test was found to be 300 mg/kg in the present study, and this was further evaluated in sub-acute test. The fasting blood glucose level and the body weight of the rats were weekly monitored. At the end of the study, biochemical parameters were subjected and the kidney histology was examined using hematoxylin and eosin (H&E) staining and scanning electron microscope (SEM). From the results, AEEFE appeared to significantly reduce the blood glucose level compared to the untreated diabetic rat with consistent body weight. In addition, blood urea nitrogen (BUN) has also shown a significant decrease in AEEFE-treated diabetic rats compared to the control group. The histological analysis of kidney rats treated with AEEFE illustrated no change in the size and structure of glomeruli with well-defined glomerular capillaries. Likewise, no mesangial hypercellularity was found. SEM analysis has supported present findings by demonstrating well branched podocytes with fenestrated foot processes arranged in parallel. Consequently, it is suggested that AEEFE has high potential to reduce blood glucose level without histological changes in renal function system of diabetic patients.

Keywords: Diabetes mellitus, *Etlingera elatior*, Kidney histology, Hematoxylin & eosin staining, Scanning electron microscopy

Introduction

DM is one of the worldwide chronic diseases associated with exceeding glucose level in the body due to insulin secretion and action defects. It is commonly characterised by hyperglycaemia or glycosuria and can lead to both micro and macro-vascular complications (Schalkwijk & Stehouwer, 2005). The hyperglycaemia will generate reactive oxygen species and contribute to massive damage to the body cells in many ways (Giacco, 2011; Nicolle *et al.*, 2011; Ullah Shirazi *et al.*, 2014). These damaged cells would ultimately result in secondary complications of DM. The National Health and Morbidity Survey (NHMS) has reported that, in 2011, 2.6 million Malaysian adults aged 18 years and above have been diagnosed with diabetes and this number has increased to 17.5% (3.5 million) in 2015 (Institute for Public Health, 2015). In 2020, the prevalence of diabetic patients in Malaysia is estimated to be greater than 4.5 million. This medical issue has evidently affected productivity and which would have negative impacts on the socio-economic development of our nation.

DM has become a world health challenge and therefore, it requires therapeutic strategies in managing this issue. Presently, the available drugs for diabetes treatment are costly and possess adverse

effects. These drugs like sulfonylureas, meglitinides, biguanides, thiazolidinediones and α -glucosidase inhibitors may cause bloating, diarrhea and even kidney complications to the treated patients (Luna & Feinglos, 2001). Nowadays, medicinal plants are gaining more attention as an alternative in diabetes medical management due to their lower side effects compared to synthetic pharmaceutical drugs (Gaikwad *et al.*, 2014). Natively, the prevention of DM has been associated with regular ingestion of raw vegetables, fresh fruits or plants that are rich in natural antioxidants. Thus, one of the most effective ways to avoid getting DM is by consuming various natural food sources that are high with antioxidant properties. Previously, phytochemicals in the plants, for example flavonoids have proven to give positive effects and can be used as an approachable option for diabetes treatment and its related complications (Hajiaghaalipour *et al.*, 2015).

Etlingera elatior or torch ginger (bunga kantan) originated from genus *Etlingera* in the ginger family known as Zingiberaceae, one of the largest plant kingdom families in the world. The genus *Etlingera* brings many beneficial products including foods, spices, medicines, fragrances, dyes, essential oils and male aesthetics (Jaafar *et al.*, 2007). In Malaysia, the flowers of *E. elatior* are traditionally used by the local folks for food flavouring and medicinal

purposes. In recent years, the phytochemical in *E. elatior* has received global attention due to its importance for biological properties for example, for antioxidant (Jackie *et al.*, 2011), anticancer (Ghasemzadeh *et al.*, 2015) and antimicrobial activities (Chan *et al.*, 2011).

The phytochemical screening of *E. elatior* inflorescence indicated the presence of phenolic, flavonoid, anthocyanin and tannins contents (Wijekoon *et al.*, 2011) while chlorogenic acid, quercetin and kaempferol in the leaves and diarylheptanoids, labdane, diterpenoids and steroids in the rhizomes' (Habsah *et al.*, 2005). In addition, its inflorescence has also been reported to contain a significant amount of crude protein, leusine and lysine, unsaturated fatty acid and fibre contents (Jeevani *et al.*, 2011). Conventionally, it is believed that the daily intake of raw inflorescence can reduce diabetes and hypertension diseases. A study by Srey *et al.*, (2014) has revealed that *E. elatior* rhizomes have significantly exhibited anti α -glucosidase and anti α -amylase, anti-oxidant, anti-inflammatory properties and have been recommended to be used for nutraceutical purpose in diabetic care. However, up to date, no study has been reported to scientifically evaluate the efficacy of *E. elatior* as a preparation for medical use. Hence, this study is aimed to evaluate the effects of *E. elatior* inflorescence (bunga kantan) in reducing blood sugar level and maintaining normal kidney structure in Type-2 diabetes rat model. Flowers of the plant were chosen for this study after taking into consideration that this part has been mostly consumed by many communities and used as a traditional herb locally. On the other hand, water was used as its solvent to emulate the herbal decoction by traditional practitioners.

Materials and Methods

Chemicals

Streptozotocin was purchased from Santa Cruz; Metformin from Tokyo Chemical Industry; Sodium pentobarbitone from Alfasan Woerden Holland; formalin, xylene, hematoxylin, eosin and ethanol were purchased from Merck, Germany; paraffin wax and DPX mounting medium from Leica Biosystems Richmond, Inc. Rats were procured by Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. This study was approved by Animal Ethics Committee USM (USM/Animal Ethics Approval/2016(760)).

Collection of Plant Materials

E. elatior flowers were purchased from Kota Bharu Central Market, Kelantan and were authenticated at International Islamic University Malaysia (IIUM), Kuantan, Pahang with plant voucher PIIUM 0275. Flowers selected for this study were fresh, unopened, of relatively equal maturity, uniform colour and no

apparent physical defect. The petals were detached from the stalks and washed under tap water to remove any dust and foreign particles, cut into small uniform pieces and were oven-dried at 50°C until a brownish colour was obtained. The dried sample was ground finely till it became powder and was stored at 4°C in amber bottles, covered with aluminium foil to prevent direct light exposure until further analysis.

Extraction of *E. Elatior* Flowers

Aqueous *E. elatior* flower extract (AEEFE) was prepared at the School of Health Sciences (PPSK), USM Kelantan. Ultrasound-assisted extraction method with minimum heat was applied in the study due to its benefits to facilitate more extractable compound release, lesser time taken and solvent consumption during the procedure (Annegowda *et al.*, 2012; Falleh *et al.*, 2012). Initially, 250 g sample powder was boiled three times with 1200 mL, 610 mL and 600 mL of distilled water consecutively with alternate sonication to prepare AEEFE. The boiling process was performed at 80°C for 30 minutes while the sonication period was 30 minutes each time at room temperature. The resultant combined filtered extract was frozen overnight at -20°C prior to freeze drying. The AEEFE was stored in the amber vial at -20°C until further use.

Phytochemicals Screening

The phytochemical analysis of AEEFE was done to screen the presence of phenolic, flavonoid, tannin, coumarin, alkaloid, glycosides, cardiac glycosides, saponin, steroid, terpenoid, quinone and reducing sugar based on previously described methods (Soni & Sosa, 2013; Banu & Cathrine, 2015; Gul *et al.*, 2017).

Induction of Diabetes

Sprague-Dawley (SD) rats were induced with streptozotocin (STZ) drug to exhibit diabetic rats. Prior to STZ induction, the rats fasted overnight and were given an intraperitoneal injection of STZ with a dosage of 55 mg/kg based on their fasting weights (Pandit *et al.*, 2010). The injected rats were returned to the cage and were allowed normal pallet diet *ad libitum*. The rats were housed individually. Blood glucose level of induced rats was measured at Day Seven to confirm the diabetic attribution in the rats. The level of blood glucose of the rats should be higher than 11.1 mmol/L before being assigned as diabetic rats.

Acute and Sub-Acute Study

Thirty male SD rats were divided into 6 groups (n=5) for acute study (24-hour observation): Group 1 as the control non-diabetic rats, Group 2 as the control

diabetic rats without treatment, Group 3 as diabetic rats treated with 250 mg/kg AEE, Group 4 as diabetic rats treated with 300 mg/kg AEE, Group 5 as diabetic rats treated with 350 mg/kg AEE and Group 6 as diabetic rats on oral metformin (anti-hyperglycaemic agent) of 250 mg/kg (Chakrabarti *et al.*, 2005). The range of doses in the acute study was selected according to our pilot study. The blood glucose levels were monitored at 0, 2, 4, 6, 8 and 24-hour intervals. After the completion of the study, all rats were euthanized using 100mg/kg sodium pentobarbitone via intraperitoneal injection (IP).

In sub-acute study (4 weeks intervention), rats were divided into 4 groups (n=8); Group 1 as the control non-diabetic rats, Group 2 as control diabetic rats without treatment, Group 3 as diabetic rat treated with 300 mg/kg AEE (the effective dose from the present acute study) and Group 4 as diabetic rats on oral metformin 250 mg/kg. At the end of the experiment, the rats were euthanized with sodium pentobarbitone at 100 mg/kg (IP). The blood was collected through cardiac puncture for biochemical analysis. The kidneys were removed immediately, washed in ice-cold, isotonic saline and blotted individually on the filter paper for histological examination.

Assessment of Biochemical Parameters

Fasting blood glucose level and body weight were measured at Day 7, 14 and 21 days of the treatments. Blood from the tail's artery was used to measure glucose level using portable Accu-Check Advantage glucometer (One Touch Ultra). At the end of the study, all rats were euthanized and the blood was collected through cardiac puncture and was sent to a local laboratory service for the measurement of renal function test (RFT). The parameters for RFT were blood urea nitrogen (BUN) and serum creatinine.

Histological study

Kidneys were harvested from each rat, weighed and washed in saline. The organs were fixed in 10% neutral buffered formalin to preserve its structural integrity. The fixed specimens were processed (Leica tissue processor, ASP 300s) and were embedded into paraffin blocks (Leica EG1120). Then, the blocks were cut into 3µm paraffin sections by using a rotary microtome (Leica Biosystems GmbH, Germany). Every section was stained with hematoxylin and eosin (H&E) for histological assessment of the kidney structure.

Scanning Electron Microscope (SEM) Image Analysis

The dissected kidneys were fixed with McDowell Trump fixative for 24 hours at 4°C as a primary

fixative. The samples were then washed in triplicate with 0.1 M phosphate buffered saline (PBS) and were fixed in 1% osmium tetroxide for 2 hours as a secondary fixative. Later, samples were rewashed with PBS followed by dehydration process in different concentrations of acetone and were allowed to dry using critical point dryer (Quantum Technologies, E3000). Finally, the samples were gold coated using sputter coating (Leica SCD005) and their images were viewed under SEM (Fei, Quanta FEG540).

Statistical Analysis

The results of biochemical parameters, body weights and blood sugar levels were expressed in mean $\pm$ standard deviation (SD). Data were analysed using GraphPad Prism version 6.01 software (GraphPad, San Diego, CA). The biochemical parameters were analysed by means of one-way ANOVA followed by Bonferroni post-hoc test. Two-way ANOVA was used to evaluate the differences of blood sugar levels of rats in acute and sub-acute studies. All tests were two-tailed and the significant level was set at $p < 0.05$.

Results and Discussion

Phytochemical Screening

The preliminary phytochemical analysis of AEEFE in the study has revealed the presence of phenolic, flavonoid, coumarin, tannin and quinone contents in the plant (Table 1). These bioactive compounds have proved to support numerous therapeutic activities and consequently, have driven to various uses of the plant in traditional medical practice. A number of studies have demonstrated that phytoconstituents of medicinal plants, particularly its antioxidant substance, play a pertinent role in DM management (Dembinska *et al.*, 2008; Feshani *et al.*, 2011; Arya *et al.*, 2015). In some of the research carried out, certain polyphenols compounds such as flavonoids appear to have hypoglycemic potential (Lordan *et al.*, 2013; Ayodeji *et al.*, 2014) which possess an ability to inhibit kidney damage in the diabetic rats (Zou *et al.*, 2014, Mohan & Nandhakumar, 2014). Back then, phenolic was reported to be a major group compound in the plants which act primarily as an antioxidant agent (Yaqub *et al.*, 2016). In the case of DM clinical management, antioxidant will commonly act as a free radical scavenger s which helps to repair damaged cells and prevents the development of diabetic complications caused by hyperglycaemia-induced oxidative stress (Zatalia & Sanusi, 2013). In this study, the bioactive compounds from AEEFE have showcased accountability in lowering blood glucose level in rats and being responsible for the anti-diabetic activity. Therefore, extensive studies need to be done to further analyse the phytochemical compounds present in *E. elatior* to strengthen the present findings.

Table 1: Preliminary phytochemical analysis of AEEFE

Phytochemical	BKAE
Alkaloids	–
Coumarin	+
Flavonoids	+
Phenol	+
Quinones	+
Saponins	–
Steroids	–
Tannins	+

Note: + = presence; - = absence

Effects of AEEFE on blood glucose level

In the study, two parts were conducted to determine the blood glucose changes among rats; acutes and sub-acutes. The acute study is to assess any effects in blood sugar in three different dosages of AEEFE as well as to find out the effective dose to be further analysed in the sub-acute study. Three different dosages of the extract are 250 mg/kg, 300 mg/kg and 350 mg/kg. Figure 1 demonstrated that 300 mg/kg AEEFE significantly decreased the blood glucose level (8.60 ± 7.53 mmol/L) of diabetic rats compared to the untreated group (19.52 ± 3.01 mmol/L; $p < 0.05$) after 24 hours. No significant reduction of blood glucose level was observed in 250 mg/kg and 350 mg/kg of AEEFE. The result clearly showed that oral administration of 300 mg/kg was able to regulate positive effect in reducing the blood glucose levels of diabetic rats compared to 250 mg/kg and 350 mg/kg of AEEFE. Thus, this concentration was considered as an effective dose and was further investigated in sub-acute analysis.

The sub-acute test performed in this study was to evaluate long-term effects of AEEFE in blood sugar level and the kidney function and structure. Figure 2 summarizes the sub-acute outcomes of AEEFE on blood glucose level which significantly

decreased when compared to untreated diabetic rats ($p < 0.0001$). Overall, 300 mg/kg AEEFE-treated diabetic rats have a lower sugar level, 13.38 ± 4.89 , compared to the control DM group, 25.42 ± 2.97 on Day 14. Notably, it was also observed that blood glucose level appeared to be almost constant in all study groups within the first week which probably was the transition period for the rats after the introduction of anti-hyperglycaemic agent and potential anti-diabetic agent, AEEFE. Based on the weekly observation, a dropping trend in blood glucose level among AEEFE-treated rats was seen beginning from Week 2 to Week 4 of the treatment. Hence, the finding presumably suggested that blood sugar level will possibly be descending persistently with extensive AEEFE oral intake. On the other hand, no significant reduction of blood glucose level was discovered in metformin-treated group as reported by previous studies that single treatment of metformin in *in-vitro* or clinical trial showed no significant change in scaling down the level of plasma blood glucose (Diniz Vilela *et al.*, 2016; Pawlyk *et al.*, 2014). Meanwhile, comparing to the untreated group, the urine volume of rats in 300 mg/kg AEEFE-treated and metformin-treated groups reduced but their body weight remained unchanged during the intervention.

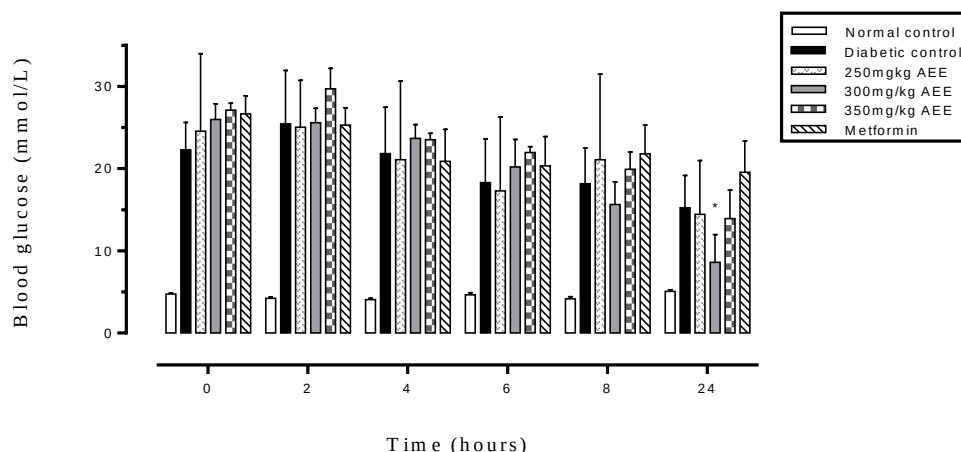


Figure 1: The effects of different treatments on blood sugar within 24 hours (acute study)

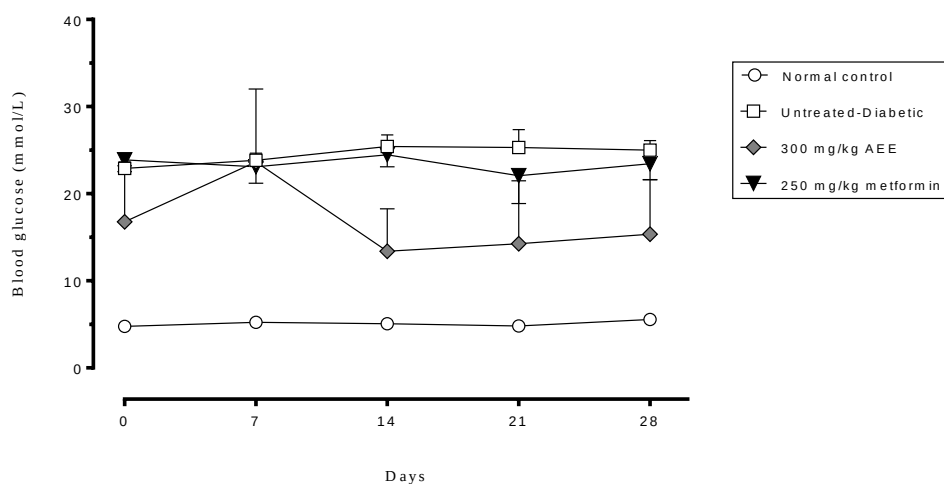


Figure 2: The effects of different treatments on blood sugar within 28 days (sub-acute study)

Effects of AEEFE on Body Weight

AEEFE treatment prevented weight loss among diabetic rats, thus their body weight was consistent throughout the study (Figure 3). In fact, the body weight of AEEFE-treated rats in Group 3 was comparable with metformin-treated diabetic rats, Group 4. There was a significant weight loss observed in the untreated diabetic rats at the end of the study. This severe body weight loss might be due to the

catabolic effects on insulin deficiency which led to high muscle wasting and the degradation of structural protein in diabetic patients (Ahmed *et al.*, 2014). Nevertheless, with 300 mg/kg AEEFE treatment, a remarkable body weight gain occurred in Group 3 starting from Days 21 to 28 ($p < 0.05$). Thus, it can be reported that AEEFE is able to decrease blood glucose level and enhance body weight in rats with diabetic complications.

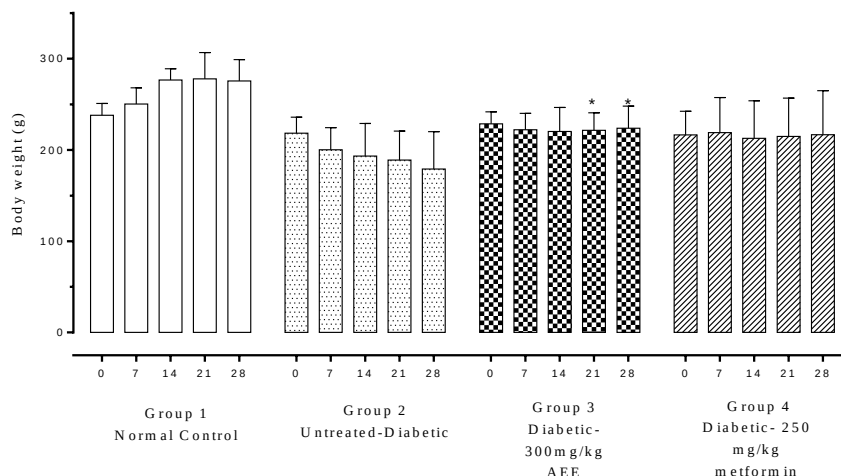


Figure 3: The effects of different treatments on the body weight (g) in sub-acute study. * $p < 0.05$ when compared to untreated-diabetic (Group 2)

Biochemical Effects of AEE

Renal function test (RFT) is essential for the evaluation of kidney function. In this study, two important parameters; creatinine and blood urea were measured. Accumulation of substances in the body indicated kidney disease and has been reported as biomarkers for kidney degeneration (Dabla, 2010). Administration of 300 mg/kg AEEFE demonstrated a significant reduction of blood urea level (9.77 ± 1.00) as compared to untreated group (15.10 ± 1.59) ($p < 0.05$) (Table 2). An earlier study reported by Mhammad *et al.*, (2015) also proved a significant

decrease of urea and blood urea nitrogen (BUN) in diabetic rat groups when treated with 300 mg/kg of cinnamon extract. However, no significant changes were assured for creatinine level either in treated and non-treated groups which might be due to the limited duration of observation in the study. This event was concordance to the finding made by Kaur *et al.*, (2018) where no significant changes of serum creatinine were discovered after 30 days treating with *Dillenia indica* extract but the level of serum creatinine decreased near to normal level after 70 days of treatment.

Table 2: The effects of different treatments on renal function test (RFT)

Groups	Creatinine ($\mu\text{mol/L}$)	Urea ($\mu\text{mol/L}$)
Normal (control)	51.60 ± 4.13	7.10 ± 0.30
Diabetic (control)	59.57 ± 3.56	15.10 ± 1.59
Diabetic (AEE 300 mg/kg)	64.23 ± 3.84	9.77 ± 1.00^a
Diabetic (Metformin 250 mg/kg)	64.83 ± 5.01	13.10 ± 2.91

^a $p < 0.05$ as compared to untreated-diabetic group.

Effects of AEEFE on Kidney Structure X Full Stop

The association of DM with renal function was further evaluated by H&E analysis. Gross examination and H&E staining clearly demonstrated an increase in the size of kidneys among untreated diabetic rats. The significant increase in kidney weight and size in diabetic *in-vivo* samples might be due to certain factors such as glucose over-utilization, glycogen accumulation, lipogenesis and protein synthesis in the kidney tissue (Sun *et al.*, 2002; Teoh

et al., 2010). The previous study by Pourghasem *et al.*, (2014) however concluded that glomerular hypertrophy and nephromegaly might contribute to the increase of kidney weight. Figure 4 demonstrates the histology of kidneys of rats of all groups. Figure 4a and 4b demonstrate a normal glomerulus with thin and delicate capillary loops. The size and number of endothelial and mesangial cells are within the normal range. H&E analysis of renal structure in untreated diabetic manifested large glomeruli with an expanded tuft, having a small capillary lumen and was difficult

to identify. As shown in Figure 4 (c and d), there are distinctive rise in the cells within the glomeruli. In addition, inflammatory cells infiltrations are detected in certain foci. Interestingly, AEEFE treated group showed a significant improvement in the glomerular structure with well-defined glomerular capillary loops and Bowman space that resemble the appearance of the normal glomerulus (Figure 4e and f). Besides, no mesangial hypercellularity and infiltration of inflammatory cells were identified. In the metformin-treated group, H&E stain attested normal appearance of glomerulus but some tubular dilatation and vacuolation were recognised. Cell vacuolization of tubules may correspond to the cell adaptation in new stressful situations resulting from hyperglycemia and later cell damage (Pourghasem *et al.*, 2015). However, no significant changes in glomerular basement membrane thickness were detected in all groups. To sum up, the histology results indicate that AEEFE treatment is able to restore the normal appearance of glomerulus, basement membrane and renal tubules.

Present findings were supported by SEM images which disclosed a normal arrangement of podocytes and foot processes in the glomeruli among AEEFE-treated (Figure 5e) and metformin-treated rats (Figure 5g). High magnification of SEM image analysis of the groups (Figure 5f and 5h) revealed high-branched podocytes and parallel arrangement of their foot processes which were consistent with the structure of normal kidneys shown in Figure 5a, b. In contrast, the number of podocytes dropped in the glomerulus of the untreated diabetic group (Figure 5c and d) and the foot process also became affected which indicated glomerular disease. Some other studies suggest that the decrease in podocytes could be detected within a short period of diabetes and may adversely affect podocyte reproduction and survival (Li *et al.*, 2007; Fioretto & Mauer, 2009). The results evidently indicated that kidneys of 300 mg/kg AEEFE-treated and metformin-treated rats improved significantly on the glomerulus structure compared to untreated diabetic rats.

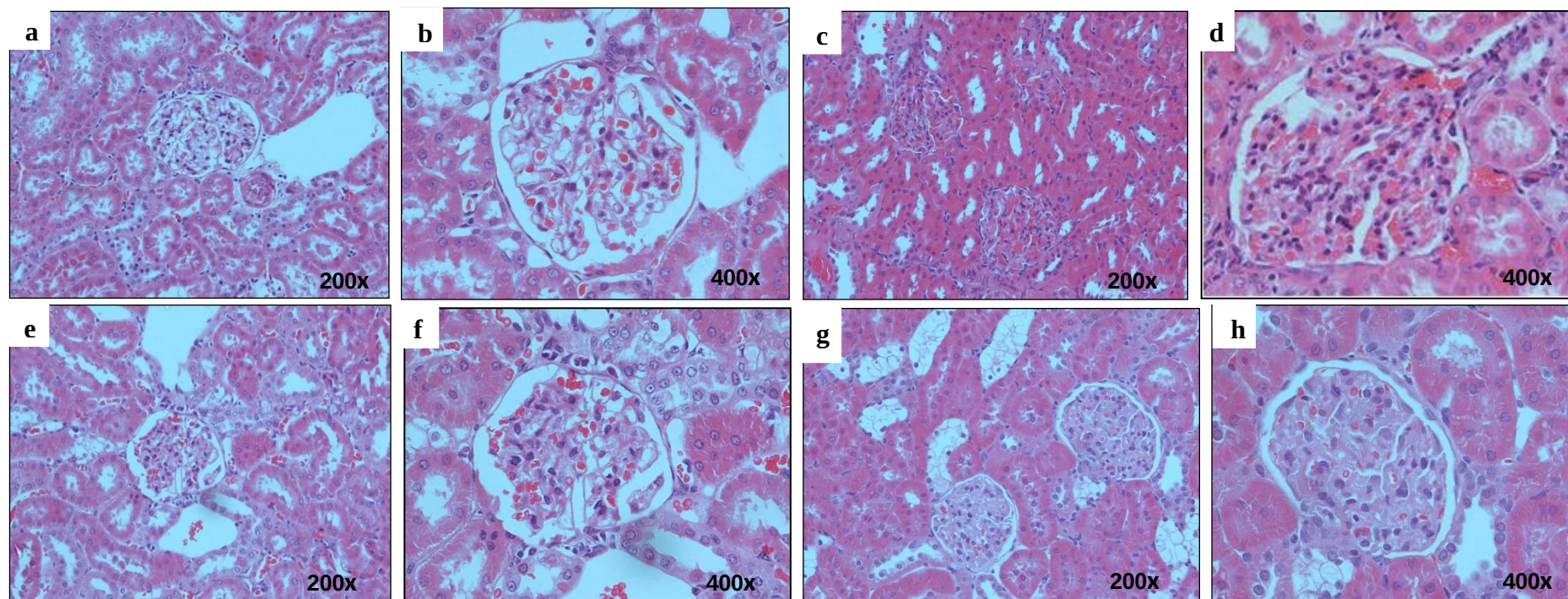


Figure 4: H&E kidney. In normal rat (a,b) the well-defined glomerular capillaries; in the untreated diabetic rat (c,d) a mesangial hypercellularity and reduced glomerular capillary lumen in the diabetic rat; improvements seen on 300 mg/kg AEEFE-treated (e,f) and 250 mg/kg metformin-treated groups (g,h)

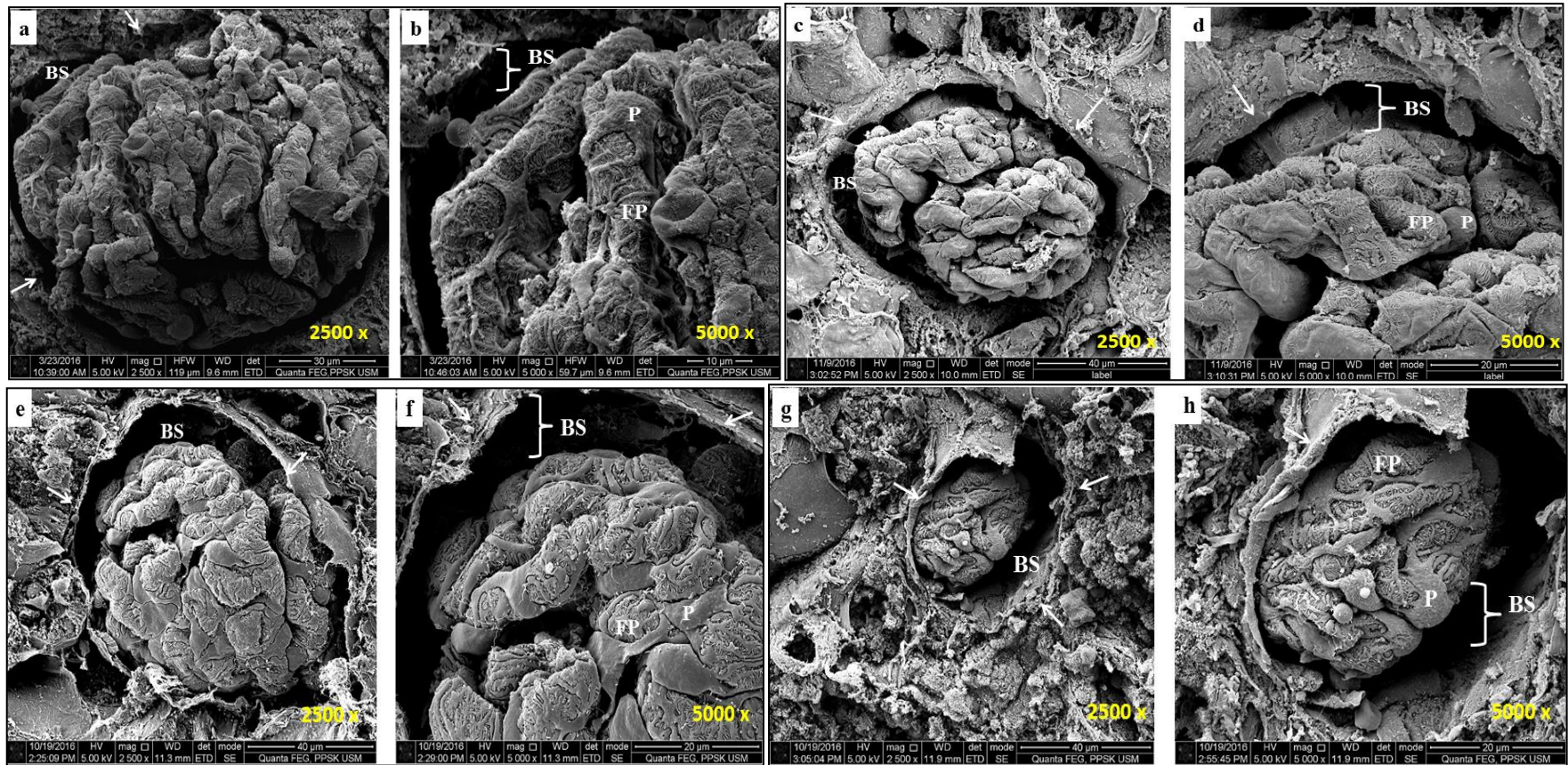


Figure 5: SEM of glomerulus. (a,b) normal rat; (c,d) untreated diabetic rat; (e,f) 300 mg/kg AEEFE-treated diabetic rat; (g,h) 250 mg/kg metformin-treated diabetic rat. (BS: Bowman's space; P: podocytes; FP: foot process)

Conclusion

As a conclusion, the findings from the present study indicate that aqueous extract of *E. elatior* inflorescences at dose 300 mg/kg, is able to improve the regulation of blood glucose level while managing the reduction of body weight in the diabetic rats, due to diabetic complications, as well. Through the observation of the kidney's histological and ultrastructure, AEEFE distinctly recovered the oxidative diabetic damaged organ and restored its normal functional structure. Thus, it can be reiterated that *E. elatior* is a good natural source of antioxidant which is potentially able to ameliorate the diabetic conditions of patients. However, a future comprehensive study on the phytochemical analysis of the plant should be performed to determine its bioactive compounds responsible for the remedy effects of *E. elatior* on the diabetic rats.

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