

## ***Phaleria macrocarpa* reduces glomerular growth factor expression in alloxan-induced diabetic rats**

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### ABSTRACT

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#### **BACKGROUND**

Diabetic nephropathy (DN) is the most serious complication of diabetes, causing end-stage renal disease throughout the world. Recent studies have reported a direct role of vascular endothelial growth factor (VEGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) in DN pathogenesis. VEGF and TGF- $\beta$  are expressed early in glomeruli in response to hyperglycemia. Active substances of *Phaleria macrocarpa* (PM) pericarp are known to have nephroprotective effects. This study aimed to evaluate the effects of *Phaleria macrocarpa* (Scheff.) Boerl pericarp extract on VEGF and TGF- $\beta$  expression in alloxan-induced diabetic rats.

#### **METHODS**

An experimental study was conducted on twenty five male albino (Sprague Dawley) rats divided into five groups (of five each): normal control; diabetic; diabetic + metformin 100 mg/kgBW; diabetic + methanolic PM extract 250 mg/kgBW; and diabetic + aqueous PM extract 250 mg/kgBW. Diabetes was induced by alloxan monohydrate 150 mg/BW intraperitoneally. Treatment was given for 3 weeks. VEGF and TGF- $\beta$  expression analysis was performed by means of immunohistochemical technique. Differences between groups were assessed by one-way ANOVA.

#### **RESULTS**

VEGF expression in the PM extract group was significantly lower than that in the diabetic group and even metformin group ( $p < 0.01$ ). TGF- $\beta$  expression in methanolic PM extract group was significantly lower than in diabetic and metformin group ( $p < 0.01$ ), but aqueous PM extract group only showed significance when compared with diabetic group ( $p < 0.01$ ).

#### **CONCLUSIONS**

*Phaleria macrocarpa* pericarp extract reduces glomerular expression of TGF- $\beta$  and VEGF in alloxan-induced diabetic rats.

**Key words:** *Phaleria macrocarpa* (Scheff.) Boerl., growth factor expression, alloxan, diabetic nephropathy, rats

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## ***Mahkota dewa menurunkan ekspresi glomerular pada tikus diabetik diinduksi aloksan***

### **ABSTRAK**

#### **LATAR BELAKANG**

Nefropati diabetik (ND) merupakan komplikasi diabetes paling serius yang menyebabkan penyakit ginjal tahap terminal di seluruh dunia. Beberapa studi telah melaporkan peran vascular endothelial growth factor (VEGF) dan transforming growth factor- $\alpha$  (TGF- $\alpha$ ) dalam patogenesis ND. VEGF dan TGF- $\alpha$  diekspresikan di glomerulus pada tahap awal ND sebagai respons terhadap kondisi hiperglikemia. Susbtansi aktif pada daging buah mahkota dewa (*Phaleria macrocarpa* (Scheff.) Boerl =PM) diketahui memiliki efek nefroprotektif. Penelitian ini bertujuan untuk mengevaluasi efek ekstrak daging buah PM pada ekspresi VEGF dan TGF- $\alpha$  glomerular tikus diabetik yang diinduksi aloksan.

#### **METODE**

Rancangan penelitian eksperimental dilakukan pada 25 tikus putih *Rattus norvegicus* strain Sprague Dawley yang dibagi menjadi lima kelompok (masing-masing lima), yaitu: kontrol normal; diabetik; diabetik + metformin 100 mg/kgBB; diabetik + ekstrak metanol PM 250 mg/kgBB dan diabetik + ekstrak air PM 250 mg/kgBB. Kondisi diabetik diinduksi dengan injeksi aloksan monohidrat 150 mg/BB dosis tunggal secara intraperitoneal. Perlakuan diberikan selama 3 minggu. Ekspresi VEGF dan TGF- $\alpha$  dianalisis secara semikuantitatif dengan teknik imunohistokimia. Uji ANOVA digunakan untuk membandingkan perbedaan antara kedua kelompok.

#### **HASIL**

Ekspresi VEGF pada kelompok yang mendapatkan ekstrak PM lebih rendah dibandingkan kelompok diabetik dan bahkan kelompok diabetik yang mendapat metformin ( $p < 0,01$ ). Ekspresi TGF- $\alpha$  pada kelompok yang mendapat ekstrak metanol PM lebih rendah dibandingkan kelompok diabetik dan metformin ( $p < 0,01$ ), sedangkan kelompok yang mendapatkan ekstrak air PM lebih rendah dibandingkan kelompok diabetik saja ( $p < 0,01$ ).

#### **KESIMPULAN**

Ekstrak daging buah *Phaleria macrocarpa* menurunkan ekspresi TGF- $\alpha$  dan VEGF glomerular pada tikus diabetik yang diinduksi aloksan.

**Kata kunci :** *Phaleria macrocarpa* (Scheff.) Boerl., ekspresi glomerular, aloksan, nefropati diabetik, tikus

## **INTRODUCTION**

According to the World Health Organization, the global prevalence of diabetes is estimated to increase from 2.8% in 2000 to 4.4% in 2030.<sup>(1)</sup> This number is predicted to rise due to longer life expectancy, urbanization, life style changes and higher prevalence of obesity. The increasing prevalence of diabetes is not only occurring in developed countries, but

also in developing countries including Asian countries.<sup>(2)</sup>

Diabetic nephropathy (DN) is one of the most serious complications affecting diabetes patients.<sup>(3)</sup> It is a major cause of morbidity and mortality in DM patients,<sup>(4,5)</sup> causing end-stage renal disease worldwide.<sup>(6)</sup> The diabetic milieu results in mesangial expansion and changes in the cellular and extracellular compartment of the glomerulus. Diabetes induced by aloksan in rats

results in development of severe hyperglycemia and reduced glomerular filtration rate similar to clinical DN.<sup>(7)</sup>

The hyperglycemic milieu increases the expression of many growth factors in the kidney. Recent evidence supports a direct role for vascular endothelial growth factor (VEGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) in the pathogenesis of DN. TGF- $\beta$  action in glomerular mesangial cells leads to changes that appear to be central to the development of DN. Enhanced renal expression of TGF- $\beta$  is reported to be responsible for initiation and progression of DN. Moreover, increased expression of TGF- $\beta$  induces accumulation of extracellular matrix proteins and is related to renal hypertrophy in DN.<sup>(8)</sup> VEGF expression is induced in glomerular cells by high glucose levels or advanced glycation end-products and reported to be increased in the early stages of DN. VEGF may contribute to renal matrix accumulation, since treatment with anti-VEGF antibodies attenuates glomerular basement membrane (GBM) thickening and mesangial expansion.<sup>(9)</sup>

Diabetes and its complications have been known to be amenable to control by natural products. Therefore, discovery and development of novel drugs for DM is very important. *Phaleria macrocarpa* (Scheff) Boerl (Thymelaeaceae) (PM), an endemic plant of Indonesia, has been used in traditional medicine for treating several diseases. PM pericarp has been reported to contain phenolic and flavonoid compounds with antioxidant activity.<sup>(10)</sup> In addition, PM reportedly possesses hypoglycemic activity, through inhibition of  $\alpha$ -glucosidase.<sup>(11)</sup> In a previous study, PM was found to reduce renal hypertrophy and blood urea nitrogen level in diabetic rats.<sup>(12)</sup> The present study examines the effect of PM pericarp extracts on the glomerular expression of VEGF and TGF- $\beta$  in alloxan-induced DN in rats. This study is part of a larger study on the nephroprotective effect of PM pericarp extract for diabetic nephropathy.

## METHODS

### Design of the study

A post-test only laboratory experimental study with control, conducted from July-December 2012 in the Pharmacology Laboratory, Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Purwokerto.

### Plant materials

Ripe fruits of *P. macrocarpa* were purchased from Merapi Herbal Farma, Yogyakarta, Indonesia. The plant species was identified by the Plant Taxonomy Laboratory at the Faculty of Biology, Jenderal Soedirman University, Purwokerto, Indonesia. The pericarps were sliced, dried at 70°C and ground into a powder using a milling machine. To obtain the methanolic extract, about 900 g of the powdered material was added to 5.5 liters of methanol and left to stand for 24 h. The filtrate was collected using Whatman No. 1 filter paper and the residue was re-extracted with 5.5 l methanol for 24 h and the re-extraction step was repeated for another 24 h. The filtrate from 3 days of methanol extraction was collected and evaporated by rotary evaporation. An aqueous extract as a mimic of traditional use was obtained from 900 g of the ground material by boiling in water for 30 minutes, filtering through Whatman No. 1 filter paper and evaporating the filtrate by rotary evaporation. The extracts were kept in the refrigerator (at 4°C) from where aliquots were withdrawn for the test procedures.<sup>(12)</sup>

### Experimental animals

In accordance with the Federer formula, this study used a total of 25 healthy male Sprague Dawley rats (*Rattus norvegicus*) aged 6-8 weeks and weighing 160-200 grams. The rats were housed under standard laboratory conditions of 22-30°C temperature, 30% relative humidity, and 12 h light and dark cycle, with free access to standard pellet diet and water. Rats were adapted for 10 days and then divided into

five intervention groups (of five each): normal control group receiving only distilled water; diabetic group; diabetic + metformin 100 mg/kgBW, diabetic + PM pericarp methanolic extract (PMM) 250 mg/BW and diabetic + PM pericarp aqueous extract (PMW) 250 mg/kgBW. The treatments were conducted for 21 days, after which the animals were sacrificed.<sup>(12)</sup> The right kidney of each animal was taken for immunohistochemical staining.

### Induction of diabetes

After an overnight fast, rats were made diabetic with a single intraperitoneal injection of 150 mg/kgBW of alloxan monohydrate (Sigma, Germany) dissolved in distilled water.<sup>(12,13)</sup> After 72 h of alloxan administration, hyperglycemia was confirmed by measuring the fasting blood glucose level. Rats with a fasting blood glucose level above 130 mg/dl were considered diabetic and employed for further studies.<sup>(12,14)</sup>

### Immunohistochemical assay

For immunohistochemical staining, a renal tissue block was deparaffinized and rehydrated in graded alcohols to tap water. Kidney sections were incubated with H<sub>2</sub>O<sub>2</sub> 3% for 15-20 minutes and washed and rinsed in distilled water. Slides were transferred into solution for antigen retrieval at pH 6.0 and then microwaved for 10 min. After a water wash, slides were flooded with 1x PBS wash buffer and the protein blocker was applied for 5-10 minutes at room temperature. After the protein blocker was drained, the primary antibody, polyclonal rabbit anti-rat VEGF antibody (Thermo Scientific, USA) or TGF- $\alpha$  antibody (Novocastra, UK) was added for 30-60 minutes. Negative control sections were stained under identical conditions by omitting the antibody. Slides were washed in 2 changes of 1x PBS wash buffer for 2 minutes each. Slides were then incubated with biotinylated secondary antibody (Biocare, UK) for 15 minutes. After washing in 2 changes of 1x PBS wash buffer for 2 minutes each, slides were incubated with streptavidin peroxidase

(Biocare, UK) for 10 minutes. After washing in 2 changes of 1x PBS wash buffer for 2 minutes each, slides were incubated with chromogen: DAB for 3-5 minutes. After draining, the sections were counterstained with Mayer's hematoxylin for 1 minute. To evaluate VEGF and TGF- $\alpha$  staining, each glomerulus was graded semiquantitatively. Each score reflects changes in the extent rather than the intensity of staining. Five scores were awarded as follows; 0=very weak or absent staining and no localized increases in staining; 1=diffuse, weak staining with 1-25% of the glomerulus showing focally increased staining; 2=25-50% of the glomerulus demonstrating a focal, strong staining; 3=50-75% of the glomerulus stained strongly in a focal manner; 4=more than 75% of the glomerulus stained strongly. For each sample, 50-60 glomeruli were evaluated, and the average score was calculated.<sup>(15)</sup>

### Statistical analysis

Normally distributed data were expressed as mean  $\pm$  SD and analyzed using one way ANOVA whose results were further subjected to LSD post hoc test for multiple comparisons. All analyses were performed with SPSS 15.00 for Windows version 15 and differences between means were accepted to be significant at  $p < 0.05$ .

### Ethical clearance

Ethical approval for this study was obtained from the Research Ethics Committee, Faculty of Medicine and Health Sciences, Jenderal Soedirman University, Purwokerto.

## RESULTS

Figure 1 shows significantly elevated glomerular expression of VEGF in diabetic rats compared with normal rats ( $p < 0.01$ ). Treatment with metformin and PM extracts (methanolic and aqueous) significantly reduced VEGF expression ( $p < 0.01$ ), but treatment with PM extracts showed better reduction in VEGF

expression as compared with metformin ( $p < 0.01$  for both extracts). No significant difference in VEGF expression was found between diabetic rats treated with methanolic PM pericarp extract and those treated with aqueous PM extract. Figure 2 shows a series of glomeruli with various degrees of glomerular VEGF expression. Glomeruli of diabetic rats showed the highest score of VEGF expression (Figure 2B), while lower scores of VEGF expression were shown by metformin and PM treated diabetic rats as well as normal rats (Figure 2A, 2C, 2D).

As shown in Figure 3, glomerular TGF- $\beta$  expression in the diabetic rats showed significant elevation compared with normal rats ( $p < 0.01$ ). Treatment with metformin and PM significantly reduced glomerular TGF- $\beta$  expression ( $p < 0.01$ ). Treatment with methanolic PM extract significantly reduced glomerular TGF- $\beta$  expression compared with metformin ( $p < 0.01$ ), whereas aqueous PM extract showed no significant difference with metformin in glomerular TGF- $\beta$  expression. The various degrees of glomerular TGF- $\beta$  expression are shown in Figure 4. The highest glomerular TGF- $\beta$  expression is shown in diabetic rats (Figure 4B), while lower scores of TGF- $\beta$  expression are shown by metformin and PM treated diabetic rats as well as normal rats (Figure 4A, 4C, 4D).

## DISCUSSION

Diabetes produces several changes in the composition of the basement membrane. This altered material undergoes accelerated glycosylation and further rearrangement to form advanced glycation end-products (AGEs), which stimulate protein synthesis, further decrease degradability of the basement membrane, increase its permeability and cause endothelial dysfunction. Consistent with previous studies,<sup>(9,16,17)</sup> this study showed that the untreated diabetic group showed significant elevation of glomerular VEGF and TGF- $\beta$

expression. Both TGF- $\beta$  and VEGF may contribute to the cellular hypertrophy and enhanced collagen synthesis observed in diabetic nephropathy as glomerulosclerosis.<sup>(18)</sup> The hyperglycemic condition in diabetes enhances glucose uptake by mesangial cells and increases production of AGEs and reactive oxygen species (ROS) that activate several responses, including inflammatory reactions through activation of protein kinase C (PKC) and ERK pathways. These pathways will further stimulate synthesis of TGF- $\beta$  and VEGF.<sup>(19)</sup>

In the present study, diabetic animals treated with PM showed reduction in glomerular TGF- $\beta$  and VEGF expression. These results indicate that PM attenuates the progression of glomerular damage, i.e., PM has a nephroprotective effect in alloxan-induced diabetic rats. This study also reports that attenuation of renal damage by PM extract is better than that by metformin. The attenuation of renal damage by PM pericarp extract is presumably due to active substances contained in the pericarp, such as phenolic constituents, quercetin, kaempferol, and terpenoids.<sup>(10)</sup> The active substances in PM have biological actions mediated by the inhibition of oxidative stress and inflammation,<sup>(20)</sup> and also have antihyperglycemic effects.<sup>(11)</sup> Triastuti et al.<sup>(13)</sup> reported that PM extract restored decreased renal antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, and glutathione (GSH) level in alloxan-induced diabetic rats, and also reduced renal hypertrophy and blood urea nitrogen (BUN) in diabetic rats. The role of oxidative stress in diabetes and diabetic tissue damage is reported elsewhere.<sup>(21,22)</sup> Hyperglycemia not only increases oxidative stress but also decreases antioxidant defenses in diabetic individuals.<sup>(22)</sup> Administration of antioxidants in experimental diabetes reduced apoptotic cell loss, which could be a major cause of glomerular sclerosis in diabetic nephropathy.<sup>(23)</sup> Our study also

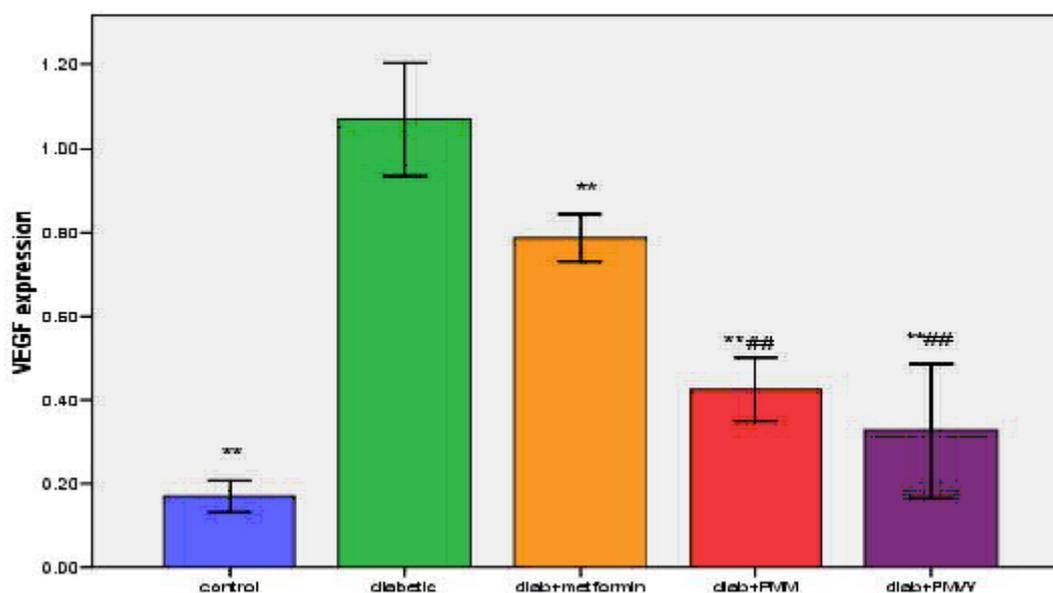


Figure 1. Semiquantitative analysis of glomerular expression of VEGF, values are mean  $\pm$  standard deviation, \*\* $p < 0.01$  compared with diabetic group, ## $p < 0.01$  compared with metformin group; ANOVA followed by LSD test; VEGF = vascular endothelial growth factor; PMM = methanolic extract of *Phaleria macrocarpa*; PMW = water extract of *Phaleria macrocarpa*

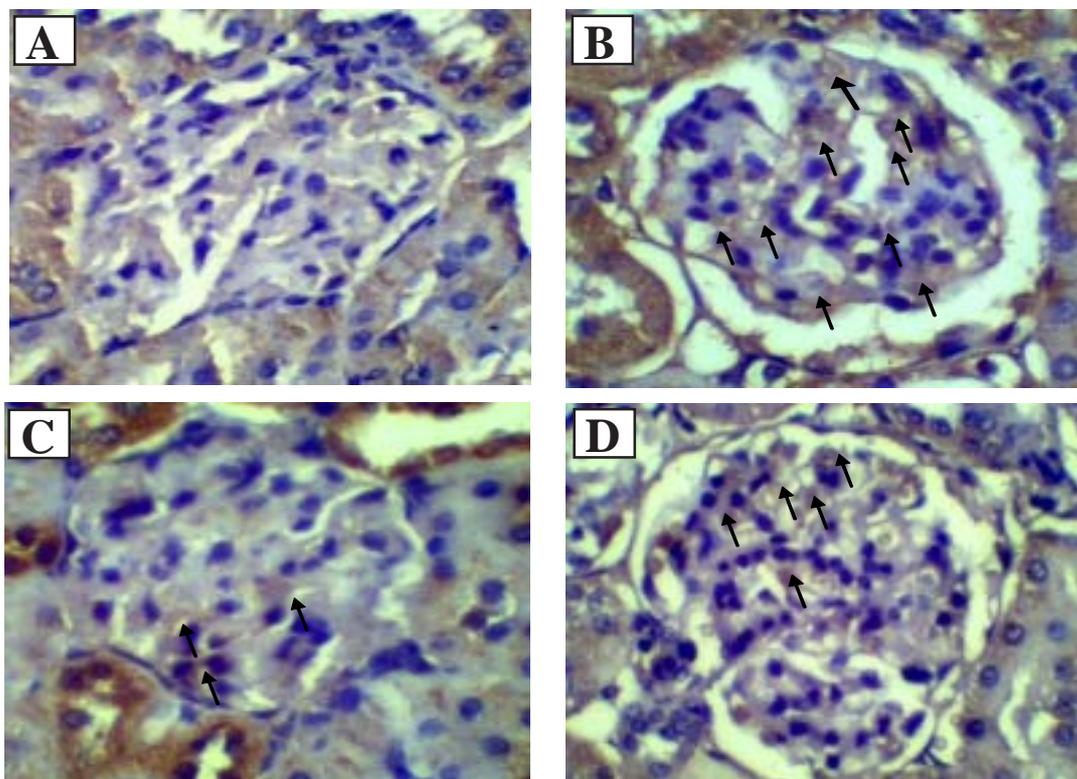


Figure 2. Glomerular expression of VEGF. A. Normal glomerulus of control group showed no expression of VEGF (score 0); B. Glomerulus of diabetic group showed expression of VEGF in 50-75% of the glomerulus (score 3); C. Glomerulus of diabetic group treated with *Phaleria macrocarpa* showed smaller area of expression of VEGF of up to 25% of the glomerulus (score 1); D. Glomerulus of diabetic group treated with metformin showed VEGF expression in 25-50% of the glomerulus (score 2); (arrows indicate VEGF)  
VEGF = vascular endothelial growth factor

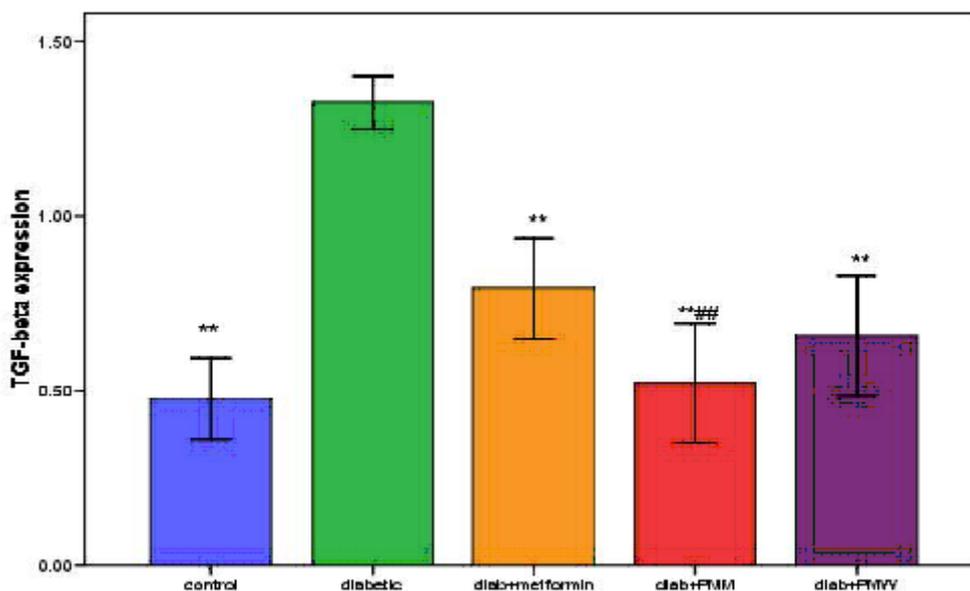


Figure 3. Semiquantitative analysis of glomerular expression of TGF- $\beta$ ; Values are mean  $\pm$  standard deviation \*\*p < 0.01 compared with diabetic group; ### p < 0.01 compared with metformin group; ANOVA followed by LSD test; TGF- $\beta$  = transforming growth factor- $\beta$ ; PMM = methanolic extract of *Phaleria macrocarpa*; PMW = water extract of *Phaleria macrocarpa*

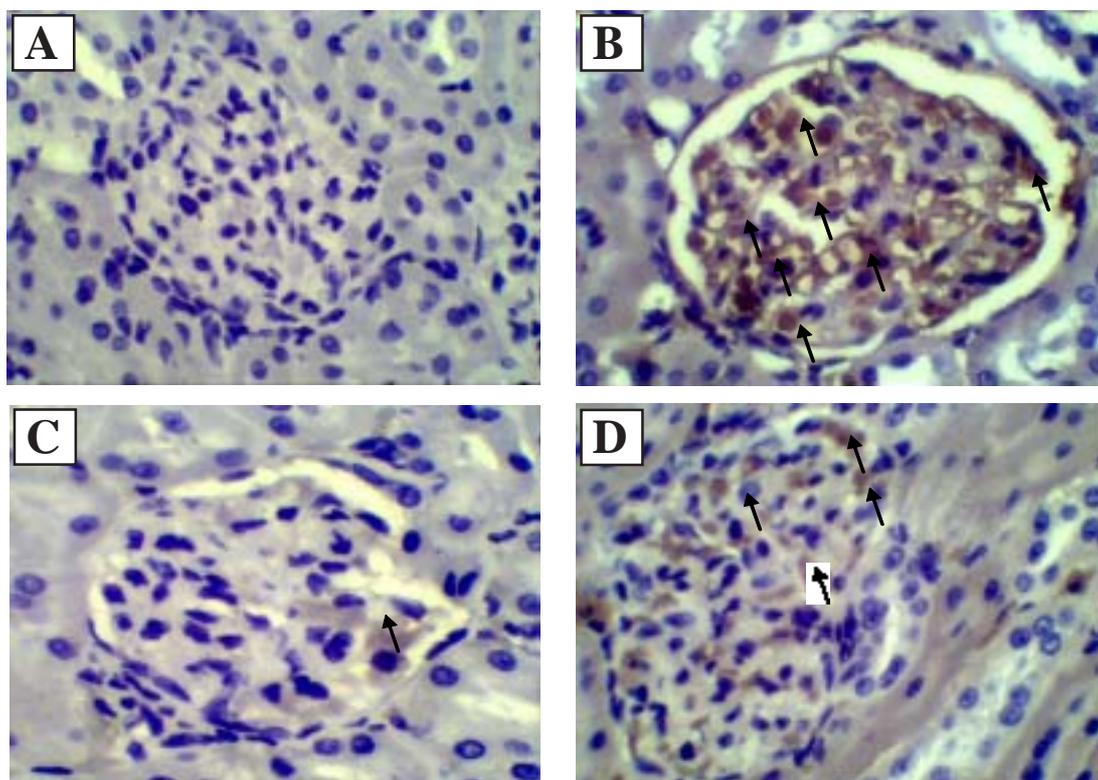


Figure 4. Various degrees of TGF- $\beta$  expression. A. Normal glomerulus of control group showed no expression of TGF- $\beta$  (score 0); B. Glomerulus of diabetic group showed expression of TGF- $\beta$  of more than 75% of the glomerulus (score 4); C. Glomerulus of diabetic group treated with *Phaleria macrocarpa* showed smaller area of expression of TGF- $\beta$  up to 25% of the glomerulus (score 1); D. Glomerulus of diabetic group treated with metformin showed TGF- $\beta$  expression in 50–75% of the glomerulus (score 3); (arrows indicate TGF- $\beta$ )  
TGF- $\beta$  = transforming growth factor- $\beta$

showed that PM extract reduced renal hypertrophy and glomerulosclerosis in diabetic rats.<sup>(12)</sup>

In addition to the antioxidant properties of PM extract, recent studies also reported antihyperglycemic and anti-inflammatory properties of PM. PM extract has an antihyperglycemic effect via intra- and extra-pancreatic mechanisms. Alkaloid substances of PM regenerate pancreatic  $\beta$  cells and increase glucose utilization and sensitivity of insulin receptors in peripheral tissues.<sup>(24)</sup> PM pericarp extract contains high amounts of flavonoids that inhibit NF- $\kappa$ B activation, which further inhibits proinflammatory cytokine synthesis.<sup>(25)</sup> There are differences in the nephroprotective effect of methanolic and aqueous PM extracts. The present study reports that methanolic PM extract reduces TGF- $\beta$  expression better than the aqueous extract. An earlier anti-hyperglycemic study with serial crude extracts of *Phaleria macrocarpa* (PM) fruits indicated the methanol extract as the most effective,<sup>(10)</sup> which might be due to the presence of flavonoid compounds.<sup>(26)</sup> However, the aqueous extract as a mimic of its traditional use, which is more applicable to rural populations in developing countries, showed the same effect in reducing VEGF expression. The present study shows that PM extract confers benefits in the early phases of DN development, and thus can be used as an alternative preventive agent for DN. However, our study was limited to two methods of extraction and two parameters of renal damage, therefore further investigations on PM pericarp extraction are still needed, as well as other parameters of nephroprotective effect to prevent diabetic complications.

## CONCLUSIONS

Alloxan-induced diabetes increases glomerular expression of VEGF and TGF- $\beta$ , while treatment with *Phaleria macrocarpa* (Scheff.) Boerl. is capable of decreasing glomerular VEGF and TGF- $\beta$  expression.

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