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Original article

Hematoprotective and Nephroprotective Effects of *Achillea millefolium* Aqueous Extract in Diabetic Mice

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Abstract

Background and objectives: *Achillea millefolium* has been used in medicine as an anti-fungal, antibacterial and antioxidant agent. The present study was carried out to assess the hematoprotective and nephroprotective activities of *Achillea millefolium* aqueous extract (AMAE) in diabetic mice. **Methods:** Seventy mice were used and diabetes was experimentally induced by intraperitoneal injection of streptozotocin (STZ) (60 mg/kg) in 60 mice. The mice with serum glucose level>250 mg/dL were considered diabetic. After three days, they were divided randomly into 7 groups. Group 1 and 2 were non-diabetic and untreated diabetic controls, respectively. Group 3 received 30 mg/kg glibenclamide orally. Groups 4, 5, 6 and 7 were given 10, 30, 90 and 270 mg/kg of AMAE, respectively for 20 days orally. At 20th day, the mice were dissected, and the blood and kidney samples were collected for hematological and pathological parameters analysis. **Results:** Daily treatment of diabetic mice with 10, 30, 90 and 270 mg/kg doses of AMAE at all doses especially 90 and 270 mg/kg significantly declined blood glucose, creatinine and urea levels and improved RBC (Red blood cell), platelet and WBC (White blood cell) parameters, compared to the untreated diabetic control. Also kidney of the treated diabetic mice with AMAE at all doses especially 270 mg/kg indicated significant improvement of the renal tissue compared to the untreated diabetic mice. **Conclusion:** The present research demonstrated the hypoglycemic properties of AMAE, offering to be suggested as an anti-diabetic supplement.

Keywords: *Achillea millefolium*; aqueous extract; hematoprotective effects; nephroprotective effects

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Introduction

Diabetes mellitus is one of the common metabolic disorders with micro-and macro-vascular complications that result in notable morbidity and mortality. It is considered as one of the five leading causes of death in the world [1,2]. Diabetes patients are five times more likely than non-diabetic patients to develop severe chronic leg ischemia, leading to foot ulceration and amputation most of the times [3]. Also in diabetic patients, hematological parameters such as hemoglobin, hematocrit and plasma proteins are often disturbed [4]. This resultant disturbance may be a risk factor for progression of retinal failure in diabetic retinopathy and renal failure in diabetic nephropathy [4]. Also some studies have revealed that in diabetes, the decrease of proximal and distal cell capacity, glomerular hyperfiltration and renal hypertrophy could be reversed by insulin treatment [5,6].

In modern medicine no satisfactory effective therapy is still available to cure diabetes mellitus [2]. There is increasing demand by patients to use natural products with anti-diabetic properties due to side effects associated with the use of insulin

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and oral hypoglycemic agents [1,2]. In previous studies hypoglycemic properties of medicinal plant (without any side effect) such as *Securigera Securidaca*, *Satureja khuzistanica*, *Plantago ovate*, *Opuntia streptacantha*, *Trigonella foenum graecum*, *Thea sinensis*, *Vaccinum arctostaphylos*, *Silybum morianum*, *Ipomoea betatas*, *Silybum marianum*, *Ocimum sanctum*, *Ginkgo biloba*, *Cuminum cyminum*, *Citrullus colocynthis*, *Allium sativum* and *Citrullus colocynthis* have been reported [7-10].

Iran has a rich flora that is widely distributed throughout the country [11-15]. In Iranian traditional medicine, herbal medicines have been the basis for prevention and cure of diabetic diseases [16,17]. One of the most important herbal medicines, which is widely consumed in Iranian traditional medicine for the treatment of diabetes is *Achillea millefolium* L. from *Asterales* Asteraceae family [18]. *Achillea millefolium* is native to temperate regions of the northern hemisphere in Asia, Europe, and North America [18]. It has been introduced as a feed for livestock in places like New Zealand and Australia. However, it is a weed in those places and sometimes also in its native regions. *A. millefolium* contains isovaleric acid, salicylic acid, asparagin, sterols, flavonoids, bitters, tannins, and coumarins [19]. It is one of the edible plants which has generated a lot of interest throughout human history as a medicinal plant. Several extracts of this plant have traditionally been used to treat gastric ulcer, parasitic, fungal, viral, and bacterial diseases [20]. Internally, it is used for loss of appetite and dyspepsia. Externally, it is used as a sitz bath for female disorders [21]. In rare cases, *A. millefolium* can cause severe allergic skin rashes; prolonged use can increase the skin's photosensitivity. This can be triggered initially when wet skin comes into contact with cut grass and yarrow together [22].

In comparison to many other pharmaceutical-industrial plants, there is very little data about hypoglycemic properties of AM; therefore, the present study was designed to investigate the hypoglycemic effects of AM aqueous extract (AMAE) in streptozotocin (STZ)-induced toxicity through hematological and histopathological assessment.

Material and Methods

Ethical considerations

All animal procedures were approved by standards of Payame Noor University of Kermanshah (No. 01/Z/G 1395/12/01) on Humane Care and Use of Laboratory Animals, in accordance with the Research Ethics Committee of the Ministry of Health and Medical Education in Iran (adopted on April 17, 2006), based on the Helsinki Protocol (Helsinki, Finland, 1975).

Plant collection

Achillea millefolium at maturity was collected Kerman, Iran during July 2017. The plant was identified by the herbarium of the Research Center of the Faculty of Agriculture, Kerman, Iran (Herbarium number KF1111).

Extraction

The leaves of the plant were shade-dried for one week. The dried aerial parts of the plant were ground and about 150 g of the obtained powder was extracted with 1500 mL distilled water for 2 h at 40 °C by continuous shaking. The extract was left for 24 h at room temperature; it was then filtered through Whatman paper No. 2. The extract was concentrated using a rotary evaporator and was lyophilized afterward.

Induction of diabetes

Seventy male Balb/c mice weighing between 38-40 g were procured from laboratory animal center of Kermanshah University of Medical Sciences, Kermanshah, Iran. The animals were housed in an air-conditioned room (22±2 °C) with 12 h light/dark cycle and had free access to standard pellet diet and water. Diabetes was induced by a single intraperitoneal (i.p.) administration of streptozotocin (STZ) (60 mg/kg.bw). Fasting blood glucose (FBG) level was assessed every day by Easy Gluco glucometer (Ames, Korea). The blood glucose level higher than 250 mg/dL was considered diabetic.

Experimental design

The mice were divided into seven following groups (n=10):

(I) Control group (C): which received 200 µL normal saline orally.

(II) Untreated-diabetic group (UD).

(III) Treated diabetic mice which received 30 mg/kg glibenclamide for 20 days (G).

(IV) Treated diabetic mice which received 10 mg/kg of the AMAE for 20 days (AMAE 10).

(V) Treated diabetic mice which received 30 mg/kg of the AMAE for 20 days (AMAE 30).

(VI) Treated diabetic mice which received 90 mg/kg of the AMAE for 20 days (AMAE 90).

(VII) Treated diabetic mice which received 270 mg/kg of the AMAE for 20 days (AMAE 270) [10,23].

Blood sampling and determination of biochemical parameters

Blood samples were obtained at days 0, 7, 13 and 20 from the tail vein in routine tubes to assess the blood glucose level by Easy Gluco glucometer (Ames, Korea). At the end of the day 20 of treatment, the animals of all groups were euthanized by xylazine (5 mg/kg) and ketamine HCl (40 mg/kg). Blood samples were drawn immediately from the animals' heart and inserted in plasma and serum tube. To separate the serum, the samples were centrifuged at 10,000 rpm for 15 min. Creatinine and urea levels were evaluated in the serum [7,9].

Determination of hematological parameters

Blood samples collected in EDTA bottles were analyzed for hematological parameters using a hematology analyzer (Mindray Auto Hematology Analyzer, BC-5200, USA) following the manufacturer's instructions. The parameters analyzed included red blood cell count (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets and white blood cell count (WBC) and the differentials and platelets [10].

Histopathological evaluation

Appropriate tissue samples were collected from the kidney at the end of the 20th day of the treatment and were then fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin-eosin staining for light microscopic examination. The sections were qualitatively (morphologically) evaluated.

Statistical analysis

All data were expressed as mean and standard deviation. Statistical comparison between group means was done by one-way ANOVA followed by Duncan's post-hoc test. $P \leq 0.05$ was considered significant.

Results and Discussion

Diabetes is now recognized as one of the major killing diseases and a leading cause of death, claiming many lives world over. Oral hypoglycemic chemical drugs have been commonly used in the management of the disease, although they have serious side effects [24]. Consequently, attention has been focused on the use of herbal remedies believed to be safer and devoid of serious side effects as alternatives in the treatment of diabetes [7-10].

In the present study, diabetes was induced in all mice by single intraperitoneal injection of 60 mg/kg of STZ. At this dose STZ partially destroys the beta cells of islets of Langerhans, nephron, hepatocytes and RBC resulting in inexpressive insulin secretion causing type 2 diabetes, nephrotoxicity, hepatotoxicity, hematotoxicity and hypercholesterolemia [9,10]. It destroys nephron through several mechanisms including production of reactive oxygen species (ROS) [25], activation of pancreatic NF-kβ [26] and induction of pronounced immune and inflammatory responses [27]. It has been suggested that overproduction of free radical NO under the influence of STZ may play a crucial role in destruction of the β-cells during the development of diabetes mellitus [28].

The effect of AMAE on the fasting blood glucose in the diabetic mice has been shown in figure 1. There was no significant change in the blood glucose level of normal control mice throughout the study. The blood glucose levels of untreated diabetic mice increased to approximately 560% ($p \leq 0.05$) of control mice in a time-dependent manner. Also AMAE at all doses especially 90 and 270 apparently decreased serum glucose levels in diabetic compared to untreated diabetic mice which seems to offer that AMAE had the capacity to decrease the risk of other complications associated with people suffering with diabetes and hence could be used as a hypoglycemic agent. The difference between all doses of AMAE was significant ($p \leq 0.05$) at 7, 13 and 20 days. The AMAE showed the most effects on days 20th day of the experiment. In similar studies it has been indicated AM has antidiabetic potential [29]. It has been demonstrated that AM extract has caused a significant decrease in IL -1β and iNOS genes mRNA expression, which can combat the cytotoxic effect of STZ on pancreatic β-cells that was reflected in higher insulin level associated with lower glucose level and higher body weight of diabetic rats that received the extract compared to the control diabetic group [23]. The effect of the AMAE on the creatinine and urea in the diabetic mice has been shown in figure 2.

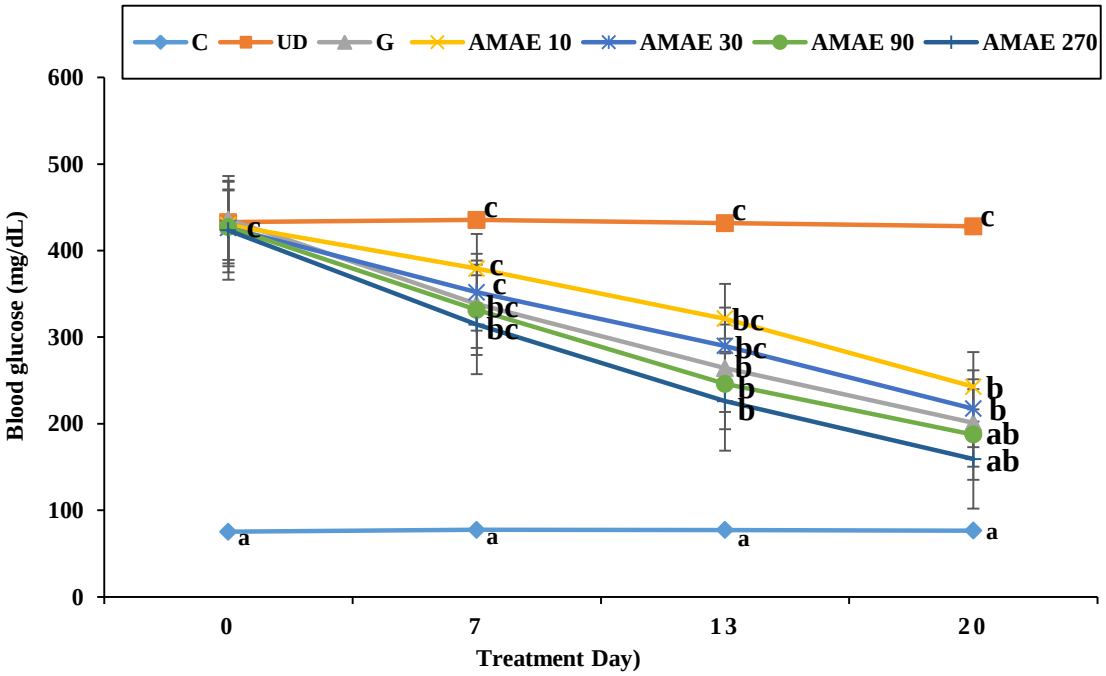

Figure 1. Blood glucose levels on different days in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

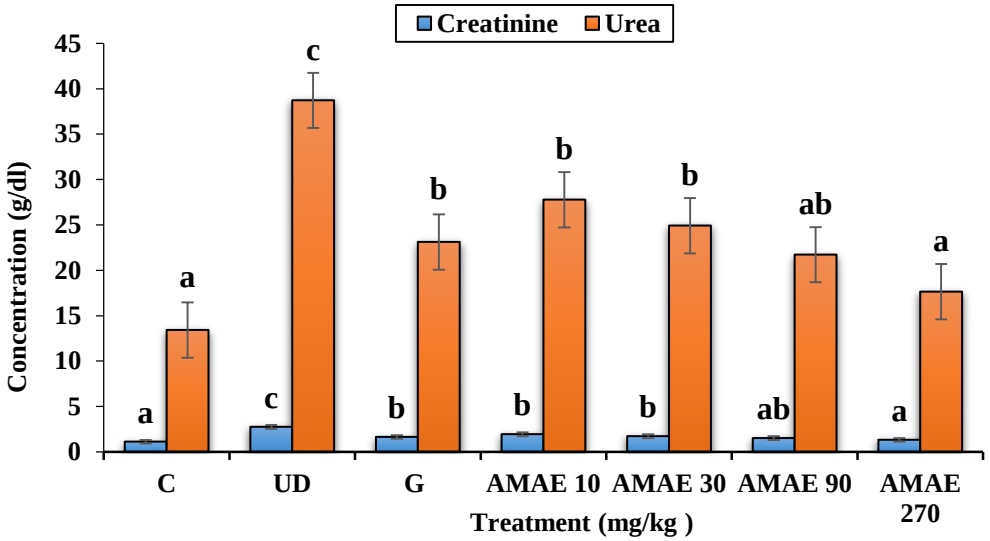

Figure 2. Creatinine and urea levels in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); Cr (Creatinine); U (Urea); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

The levels of these parameters significantly ($p \leq 0.05$) increased in untreated diabetic mice. Treatment with AMAE at all doses especially 90 and 270 doses significantly ($p \leq 0.05$) decreased the levels of creatinine and urea, compared to the untreated diabetic mice. Also, a study has shown that due to antioxidant components in most herbal extracts, the levels of creatinine and urea

had decreased in the serum [12]. *Achillea millefolium* is rich of antioxidant components such as eucalyptol, camphor, α-terpineol, β-pinene, and borneol [23] so it reduced the levels of creatinine and urea in diabetic mice. Alteration in various hematological parameters and the immune system during the course of diabetes have been reported [30]. Anemia has been noted to be a common pathophysiological feature and a complication of diabetes mellitus [31]. Diabetes-associated anemia is reported to be due to the increased non-enzymatic glycosylation of RBC membrane proteins which correlates with hyperglycemia [32].

Oxidation of these membrane proteins in the presence of chronic hyperglycemia in uncontrolled diabetes mellitus increases the production of lipid peroxides, leading to hemolysis of RBC [33]. The decrease in the RBC and its indices following treatment with diabetogenic agent in experimental diabetes is an indication of reduced and abnormal erythropoiesis. This observation is consistent with earlier reports [34] but differs from the report of some others [35,36]. Although the RBC membrane lipid peroxide level in diabetic mice was not measured in this study, other RBC parameters such as Hb, PCV, MCV, MCH, and MCHC were measured so as to investigate the effect of AMAE on the anemic status of STZ-induced diabetic mice. The levels of RBC, MCV, Hb, MCH and MCHC and percent of PCV significantly ($p \leq 0.05$) decreased in untreated diabetic mice. Treatment with AMAE at all doses especially 90 and 270 significantly ($p \leq 0.05$) increased the levels of these parameters, compared to the untreated diabetic mice (figure 3-7). There is a report that platelet count was significantly higher among diabetics compared to non-diabetics and a positive correlation between platelet count and poor glycemic control exists [37]. Raised platelet values are commonly observed in inflammatory and infectious diseases [38] and are considered as an acute phase reaction to infection or inflammation, as is the case with STZ-induced diabetogenesis caused by free radicals [39]. In the present study, the number of platelet significantly ($p \leq 0.05$) increased in untreated diabetic mice. Treatment with AMAE at all doses significantly ($p \leq 0.05$) decreased platelet number, compared to the untreated diabetic mice (figure 8).

WBC serves as a scavenger that removes foreign substances. The number of WBC is known to rise as a body defense mechanism in response to toxic environment [40]. Changes in WBC have been associated with insulin resistance and cardiovascular complications [41]. Leukocytosis is reported to be associated with insulin resistance, type 2 diabetes mellitus, stroke and diabetes induced macro and microangiopathy [42]. The result of this study demonstrated that WBC and eosinophils and basophils significantly ($p \leq 0.05$) increased in untreated diabetic mice. Also, lymphocytes and monocytes significantly ($p \leq 0.05$) decreased in untreated diabetic mice.

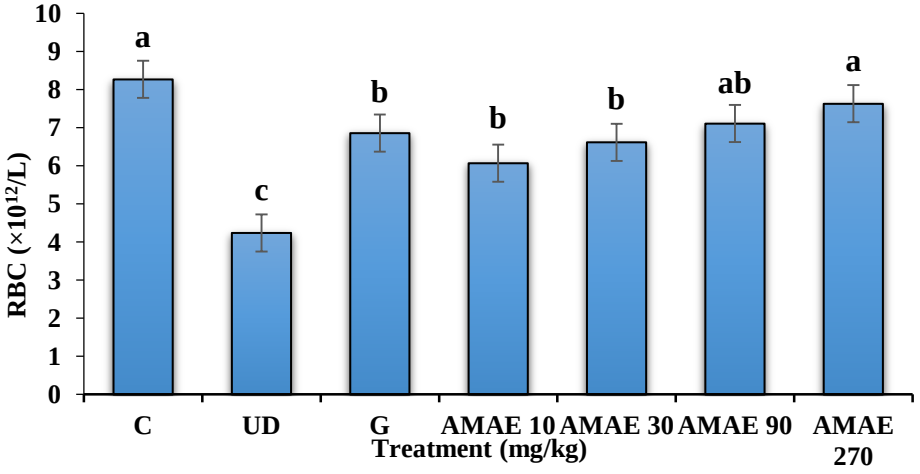

Figure 3. RBC count in of the experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); RBC (red blood cell); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

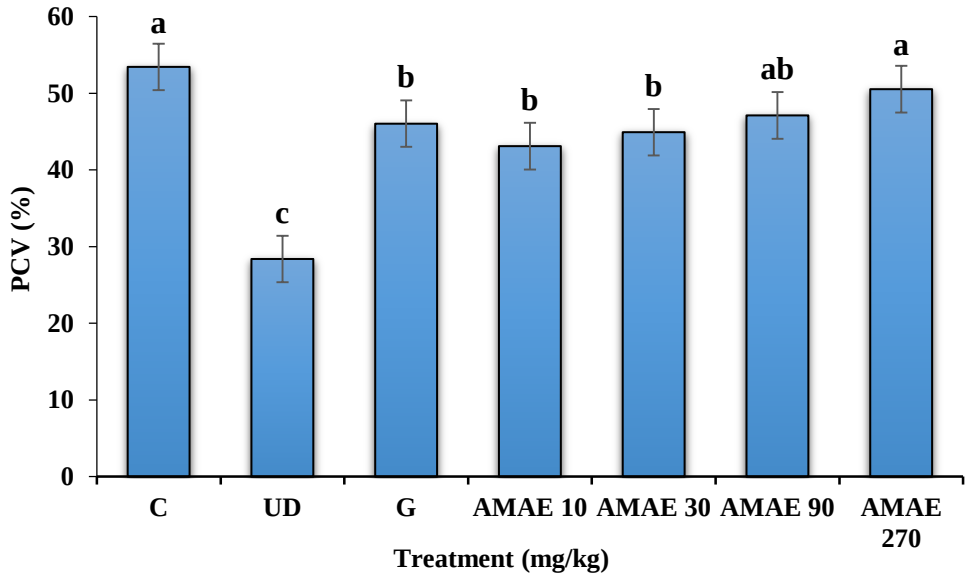

Figure 4. PCV percent in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); PCV (packed cell volume); Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

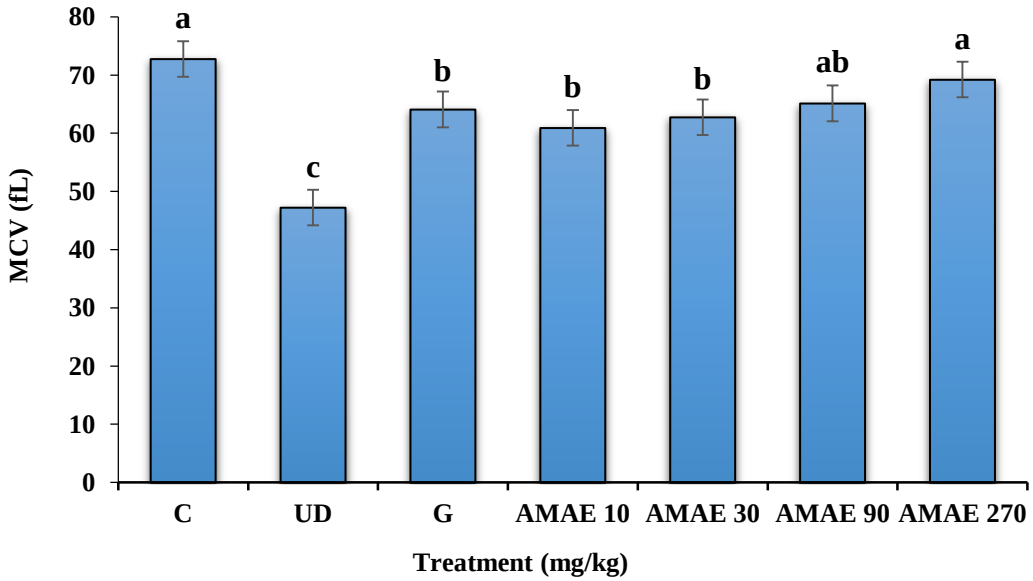

Figure 5. MCV in of the experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); MCV (mean corpuscular volume); Non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

Treatment with AMAE at all doses significantly ($p \leq 0.05$) decreased WBC and eosinophils and basophils and increased lymphocytes and monocytes compared to the untreated diabetic mice. This may have been as a result of the ability of the extracts to restore insulin sensitivity and reduce oxidative stress within the blood cells [35,42]. No significant changes occurred in the

neutrophils of all of the experimental groups (figures 9 and 10).

The kidneys of the normal control mice had normal structure and the proximal and the distal convoluted tubules, renal corpuscles, glomerulus and glomerular capsule had normal architecture.

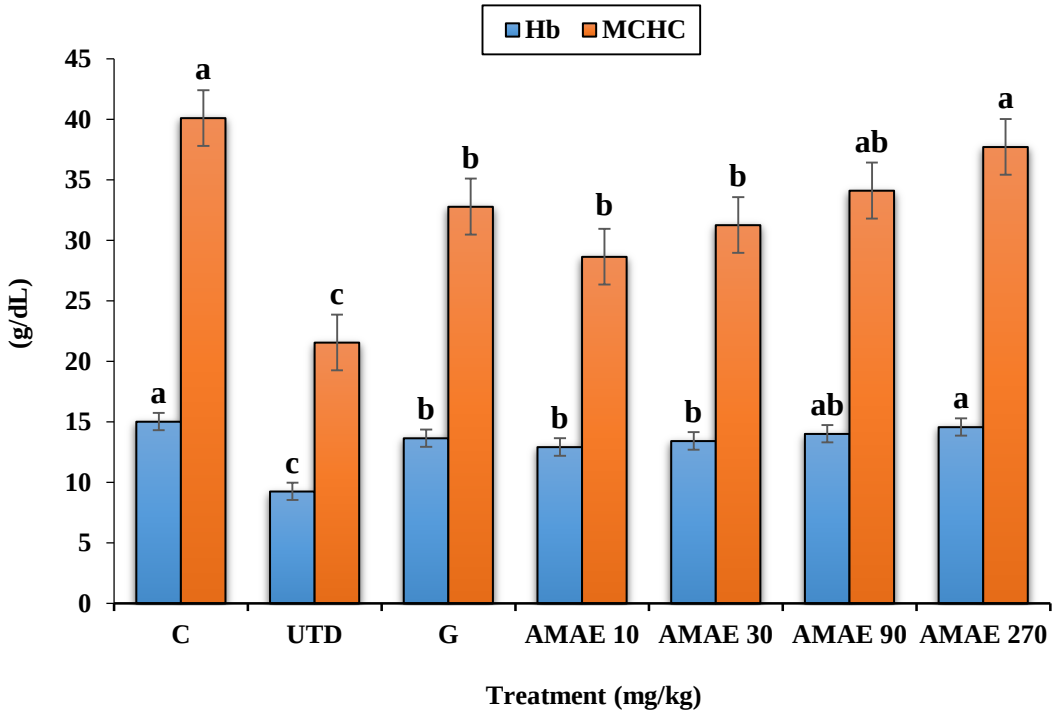

Figure 6. The levels of Hb and MCHC (g/dL) in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); Hb (Hemoglobin); MCHC (mean corpuscular hemoglobin concentration); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

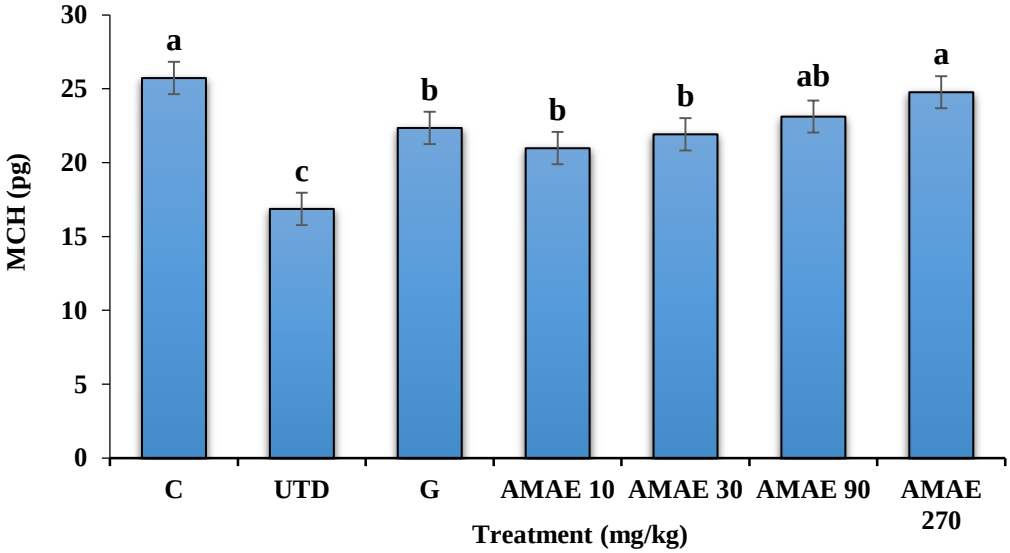

Figure 7. The levels of MCH (pg) experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); MCH (mean corpuscular hemoglobin); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

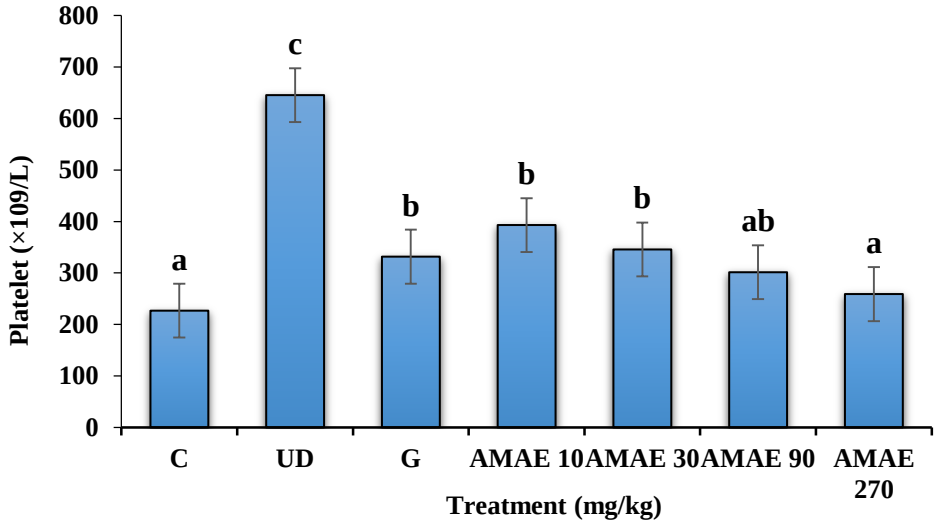

Figure 8. Platelet count in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

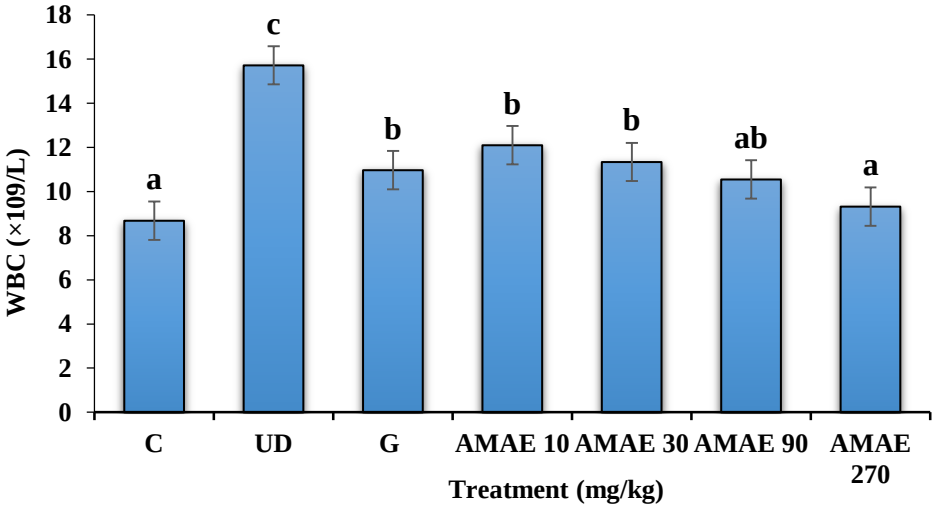

Figure 9. WBC count in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); WBC (white blood cell); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

Untreated mice revealed some degree of renal hypertrophy which was mainly due to the enlargement of the cortex, medullary and its subcomponents. These changes were ameliorated significantly with AMAE. The pathogenesis of renal hypertrophy can be attributed to the overproduction of oxygen-free radicals following toxic chemical administration which is expressed in response to cytokines [43-46]. Also microscopic examination of the kidneys of the treated diabetic mice with AMAE at all doses especially 270 did not show tubular necrosis with necrotic changes in the glomerular epithelium and diffused interstitial and glomerular hemorrhages. In fact, AMAE at all doses especially 270 not only did not have adverse toxic effects on the kidneys but also reduced the effects of diabetes mellitus on the kidney by reducing blood glucose levels (figure 11).

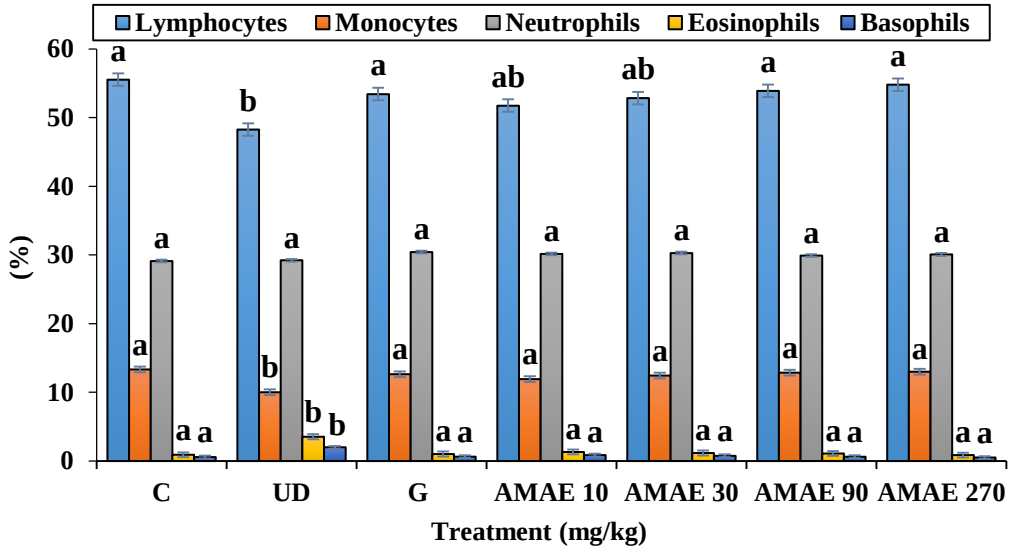

Figure 10. Lymphocyte, monocyte, neutrophil, eosinophil and basophil in experimental groups. C (control); UD (untreated diabetic); G (glibenclamide treated); AMAE 10 (treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract); AMAE 30 (treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract); AMAE 90 (treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract); AMAE 270 (treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract); non-identical letters indicate a significant difference between the groups ($p \leq 0.05$).

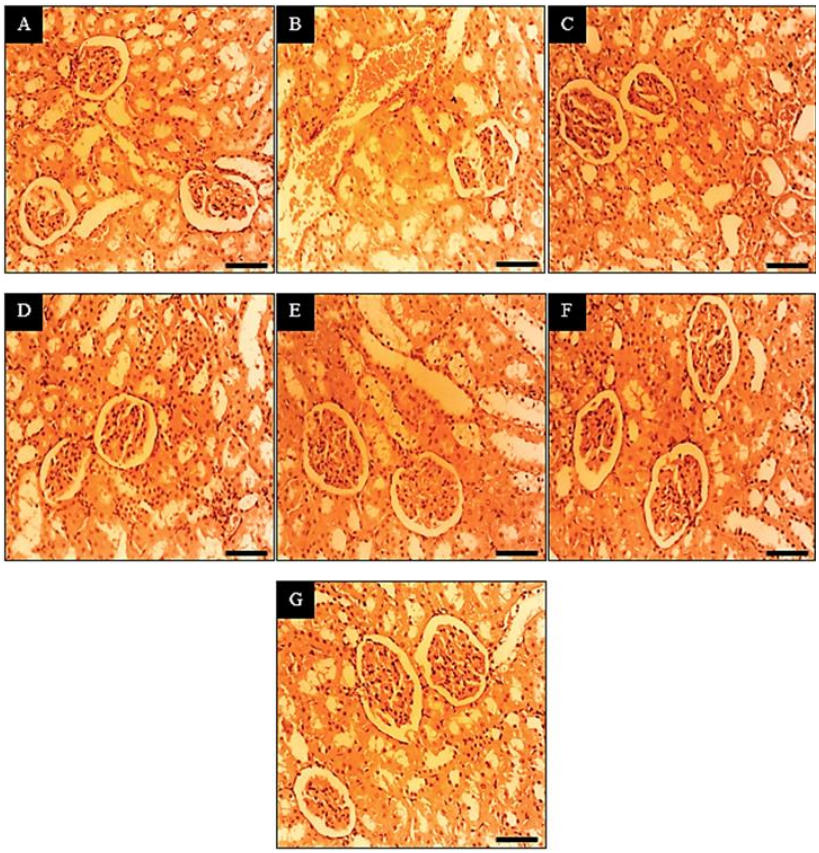

Figure 11. Longitudinal sections of kidney (scale bar for 150 µm) with Hematoxylin-Eosin staining. A: Control; B: Untreated diabetic; C: Glibenclamide treated; D: Treated diabetics with 10 mg/kg of *Achillea millefolium* aqueous extract; E: Treated diabetics with 30 mg/kg of *Achillea millefolium* aqueous extract; F: Treated diabetics with 90 mg/kg of *Achillea millefolium* aqueous extract; G: Treated diabetics with 270 mg/kg of *Achillea millefolium* aqueous extract

From the above results it could be concluded that AMAE at all doses (especially AMAE 90 and AMAE 270) exhibited significant anti-hyperglycemic effects similar to glibenclamide treated diabetic mice. This extract also indicated improvement in kidney histopathological and hematological parameters and might be of value in diabetes treatment. Further investigation is necessary to determine the exact phytoconstituent (s) responsible for anti-diabetic activity.

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Author contributions

The experimental procedures and manuscript preparation were carried out by Mohammad Mehdi Zangeneh.

Declaration of interest

The author declares that there is no conflict of interest. The author alone is responsible for the content of the paper.

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Abbreviations

STZ: Streptozotocin; AMAE: *Achillea millefolium* aqueous extract; RBC: Red blood cell. Hb: Hemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell