

## Betulinic acid reduces the complications of autoimmune diabetes on the body and kidney through effecting on inflammatory cytokines in C57BL/6 mice

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### Abstract

Autoimmune diabetes is one of the most common metabolic diseases with increasing prevalence in the past decades in which pancreatic Langerhans  $\beta$  cells are destroyed and lead to lack of insulin due to increased blood sugar. One of the consequences of diabetes is glomerular disease of the kidney, also called diabetes nephropathy. Different studies have been carried out on the effects of triterpenoids and their medicinal effects on diabetes mellitus. betulinic acid, a pentacyclic triterpenoid of Terpenes, is found in bushes and trees. Its medical effects are also approved by many studies. In this survey, we studied the effect of betulinic acid on diabetic inbred C57BL/6 male mice. They were randomly divided to three groups. Group A: Consisted of healthy mice which received citrate buffer. Group B: Diabetic mice without any treatment and group C: Treated diabetic mice with betulinic acid. The level of blood insulin level, fasting blood glucose, C-peptide, TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 cytokines were measured and pathologic studies of the kidney were performed. The results showed that betulinic acid could increase insulin and C-peptide, and decrease fasting blood sugar, kidney lesions and TNF- $\alpha$ , IFN- $\gamma$ , IL-1 in the treated groups. The differences were significant except for IL-1. Betulinic acid through reduction of inflammatory cytokines could have positive effects on inflammatory and autoimmune disease including autoimmune diabetes.

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### Introduction

Autoimmune diabetes (T1D) is an autoimmune disease, in which an inappropriate immune response is created against  $\beta$  cells in islets of Langerhans in the pancreas. This lack of insulin hormone leads to degradation of  $\beta$  cell in the pancreas and consequently reduces insulin level in the body. It is of note that insulin is produced by  $\beta$ -cells of the pancreas. Moreover, insulin reduces the glucose level of cells by affecting liver cells and depositing them as glycogen. Therefore, in T1D, the blood glucose level is increased. This ever-increasing disease can be seen in all ages and its clinical presentation is highly variable.<sup>1</sup>

The T1D is one of the most common metabolic diseases in childhood and adolescence that shows an increasing trend all around the world in recent decades. One of the chronic and permanent complications of diabetes is glomerular diseases called diabetic nephropathy (DN). Based on the reports, diabetes-induced kidney diseases

could include 10.00 to 50.00% of diabetic patients within 5 to 10 years of the onset of diabetes. The main characteristic of DN is kidney microangiopathy, causing gradual damaging on the kidney glomerular capillary wall that leads to proteinuria. The onset of DN is usually along with functional changes of kidney.<sup>2-6</sup>

The DN is one of the most important factors of deficiency in the physiological function of kidneys in mellitus diabetes, which significantly affects diabetic patients' life.<sup>7</sup> Among symptoms of DN, The thickness of basement membrane of glomerulus,<sup>8</sup> increasing and expansion of renal interstitial fibrosis, increasing the concentration of creatinine and clearance level of creatinine could be noted.<sup>9</sup> In addition, in developing countries, DN is one of the main progressive and inflammatory renal diseases which leads to dialysis, kidney transplantation and mortality.<sup>10</sup> In other words, diabetes causes these problems in the kidney by malfunctioning in the metabolism of glucose and proteins.

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Different factors are used to evaluate the kidney functions like the concentration of serum creatinine, creatinine clearance, serum urea, and Ur/Cr urinary ratio.<sup>11</sup> Reportedly, DN leads to renal problems by increasing blood glucose level and producing free radicals.<sup>12</sup>

C-peptide is a byproduct of insulin production pathway which is produced by  $\beta$ -cell of the pancreas and secretes into the blood. Measurement of C-peptide blood serum levels can be used to distinguish the function of  $\beta$ -cells of the pancreas. Its level associates with the type of diabetes and its duration. In particular, less than 0.20 nmol per L C-peptide level is diagnosed as type 1 diabetes (T1DM).<sup>13</sup>

It seems that Th1 cytokines execute a direct role in the autoimmune disease pathogenesis and progress. The evidence showed the direct role of Th1 cells and relevant cytokines in T1D pathogenesis and progress. Besides, the studies indicated an increase in expression of Th1 cytokines and a decrease in producing Th2 cytokines in newly diagnosed T1D. Also, Th1 cells indirectly affect the  $\beta$ -cell degradation by several mechanisms like suppressing the cytokines antagonists production, including IL-1 receptor antagonists. Th1 cytokines increase the production and effector actions of monokines (IL-1 and TNF- $\alpha$ ) and lead to improving the degradation cascade of  $\beta$ -cells.<sup>14</sup>

Many studies have been performed to show the possible role of cytokines in the pathogenesis of autoimmune diabetes. The studies indicated that swelling of pancreatic islets and degradation of  $\beta$ -cells are associated with an increase in pro-inflammatory cytokines expression such as IL-1, TNF- $\alpha$ , and IFN- $\alpha$  and Th1 cytokines such as IFN $\gamma$ , TNF- $\beta$ , IL-2, and IL-12. The performed studies showed the pathologic role of pro-inflammatory cytokines such as IL-1, TNF- $\alpha$ , and IFN- $\alpha$  and Th1 cytokines such as IFN- $\gamma$ , IL-2, and IL-12 in the progression of autoimmune diabetes.<sup>15</sup>

Betulinic acid (BA), 3 $\beta$ -Hydroxy-lup-20(29)-en-28-oic acid, is a pentacyclic triterpenoid of terpenes and found in bushes and trees.<sup>16</sup> Its medical effects are also approved by many studies.<sup>17</sup> BA can inhibit various enzymes associated with absorption and metabolism of carbohydrate/fat such as  $\alpha$ -amylase and several other enzymes.<sup>18-20</sup> Moreover, BA results in secretion of leptin and insulin.<sup>21</sup> In an in-vitro study on the effect to BA on human aortic smooth muscle cells (HASMC), reduce in intracellular reactive oxygen species (ROS), nuclear translocation suppression and IRB- $\alpha$  phosphorylation under an increasing glucose condition were reported. Accordingly, BA could inhibit diabetes-related cardiovascular disease.<sup>22</sup> BA has a prophylactic consequence on DN in conditions of diabetes.<sup>23</sup>

The DN is the main cause of renal failure, which is diagnosed by observing the excessive amount of extracellular matrix proteins such as fibronectin (FN) in glomerular mesangial and tubulointerstitial tissue.<sup>24</sup> Also, BA as a stimulant of insulin secretion might have strong anti-hyperglycemic effects.<sup>25</sup>

Therefore, considering the prevalence of diabetes and the therapeutic effects of BA, the aim of the present research was to evaluate the therapeutic effects of BA on autoimmune diabetes induced mice by evaluating the effect of BA on inflammatory cytokines, fasting blood glucose in serum, the blood insulin level and C-peptide as well as pathologic studies of kidney.

## Materials and Methods

In the present study, 30 C57BL/6 inbred male mice with 25.00 - 30.00 g weight and 6 - 8 week age were used and randomly divided into three groups. They were purchased from Pastor Institute. Group A (healthy control): Consisted of 10 healthy mice and only received citrate buffer (pH 4.5). Group B (diabetic control): Mice received streptozotocin (STZ; 50.00 mg kg<sup>-1</sup>; Sigma-Aldrich, Munich, Germany). Group C (treatment group): Consisted of mice that received 20.00 mg kg<sup>-1</sup> BA in 0.30 mL normal saline through gavage daily for two weeks after receiving the final dose of streptozotocin. Each group was kept in separated cages in a room with 25.00 °C temperature and 12 hr light-dark cycle with accessibility to enough food and water. The experimental method was confirmed by University of Tabriz Animal Ethical Committee (Ref No. ADBU-104-2016).

**Diabetes induction.** After 4 hr fasting, STZ was administrated by intraperitoneal injection for five consecutive days (10 min before injection; 50.00 mg kg<sup>-1</sup> of STZ was dissolved in 200 mL of citrate buffer). All tests were performed after the last dose of the BA.

**Cytokines measuring.** After blood sampling through the tail vein, IL-1, IFN- $\gamma$ , and TNF- $\alpha$  cytokines were measured in sera of mice using ELISA kit (Bendermed, Vienna, Austria) according to manufacturer's protocol.

**Fasting blood sugar measuring.** To evaluate fasting blood glucose level, blood was taken through the tail vein of mice after restraining and their blood glucose level was measured by an automatic glucometer (Accu-Chek®; Compact Plus, Dublin, Ireland). The mice were kept fasting for 4 hr prior to evaluation.

**Evaluation of C-peptide in plasma.** After blood sampling and separating plasma, C-peptide was measured using radioimmunoassay kit (Linco Research Inc., St. Charles, USA) according to manufacturer protocol.

**Insulin plasma level measuring.** After blood sampling through train vein of mice and separating plasma, insulin level of plasma was measured using ELISA kit (Crystal Chem Inc., Chicago, USA) according to manufacturer protocol. To evaluate the pathologic variations, kidneys of mice were removed a day after last BA injection and kept in cold 10.00% buffered formalin. After preparing paraffin embedded blocks, lamination was done and prepared sections were stained by Hematoxylin and Eosin (H & E) method.

Then they were studied in terms of hyperemia, infiltration of the inflammatory cells and degeneration in the kidney tissue by a pathologist under a light microscopy (YS100; Nikon, Tokyo, Japan). The severity of the lesion was determined by the following scores: 0= no lesion; 1= low; 2= medium; 3= severe.<sup>26</sup>

**Statistical analysis.** To analyze pathologic data, one-way ANOVA test was used. Besides, non-parametric data were converted to parametric ones by scoring the severity of the lesion. In addition, Tukey's test of SPSS (version 21.0; IBM Corp., Armonk, USA) was used to analyze other data. In all evaluations, the value was considered as significant level and the graph was plotted using Excel Software (version 16.0; Microsoft Corporation, Redmond, USA).

## Results

**Cytokines levels measuring in blood serum.** In group C, cytokines were reduced in contrast to group B and the difference was significant. However, in terms of IFN- $\gamma$ , no significant difference was observed (Fig. 1A). The variations of cytokines are as follow:

**IL-1 level measuring in mice serum.** The results showed that IL-1 level in group C was significantly reduced compared to group B ( $p < 0.05$ ), (Fig. 1A).

**TNF- $\alpha$  level measuring in mice serum.** The results showed that TNF-  $\alpha$  level in group C was significantly reduced compared to group B ( $p < 0.05$ ), (Fig. 1A).

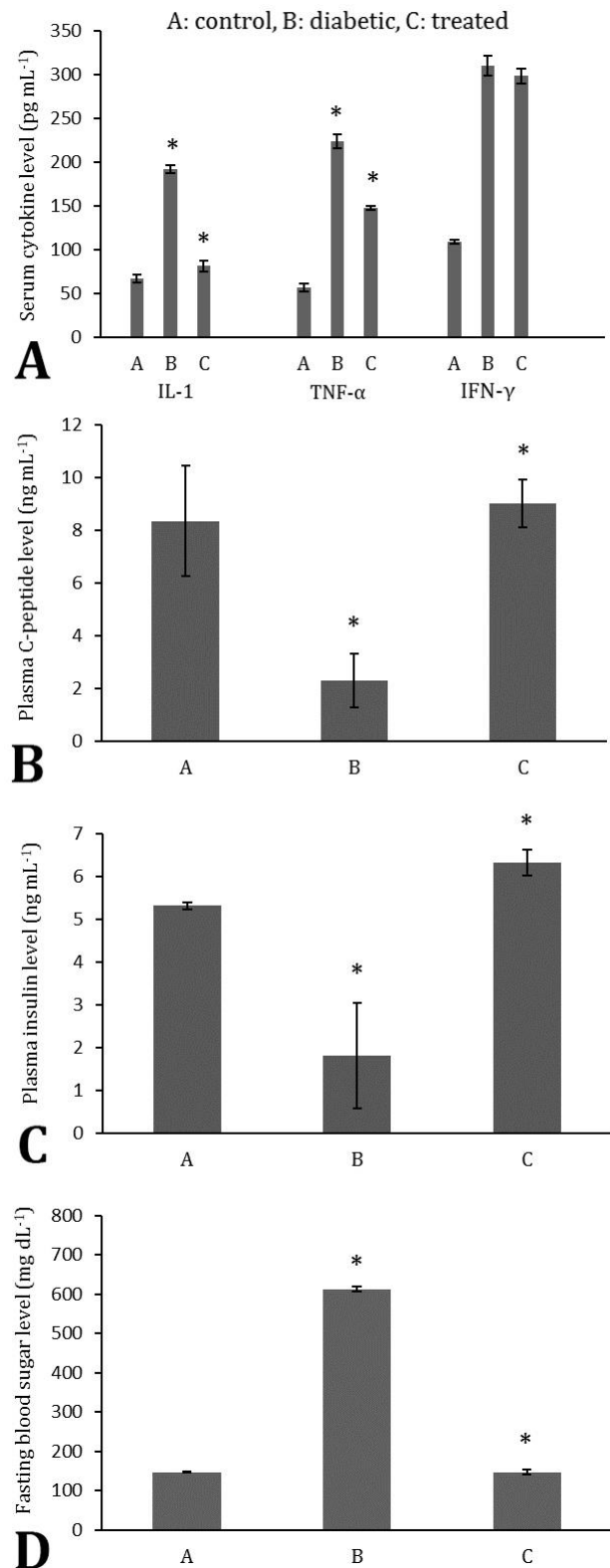
**IFN- $\gamma$  level measuring in mice serum.** The results showed that IFN- $\gamma$  level in group B significantly was increased compared to group A. However, in group C, it was reduced compared to group B that was not significant (Fig. 1A)

**C-peptide level measuring in plasma.** The results showed that the level of C-peptide in group B significantly was reduced compared to group A. However, in group C, it was significantly increased compared to group B ( $p < 0.05$ ), (Fig. 1B).

**Insulin level measuring in plasma.** The results showed that insulin level in group B was significantly reduced compared to group A. However, in group C, it was significantly increased compared to group B ( $p < 0.05$ ), (Fig. 1C).

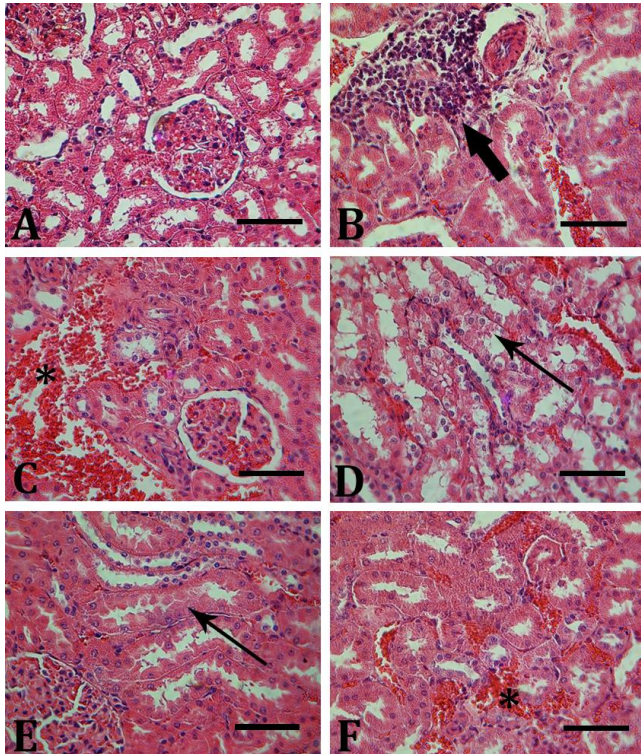
**Fasting blood glucose level measuring.** The results showed that fasting blood glucose level in group B was significantly increased compared to group A. However, in group C it was significantly decreased compared to group B ( $p < 0.05$ ), (Fig. 1D).

**Assessment and comparison of the renal lesions among experimental groups.** The architecture of kidney in control group was normal, with normal appearance of the glomeruli, tubules and interstitial tissue under the light microscopy. No inflammatory cell infiltration or hyperemia were observed (Fig. 2A).



**Fig. 1. A)** Serum cytokines levels, **B)** Plasma C-peptide level, **C)** Plasma insulin level and **D)** Fasting blood level. Asterisks show significant difference between group B and C ( $p < 0.05$ ).

In group 2, the diabetic group, severe inflammatory cell infiltration, hyperemia and severe hemorrhage, notable degenerative processes including hydropic degeneration in proximal convoluted tubules epithelium were observed in kidney tissue sections (Figs. 2B- D). In group 3 in which animals received BA the degenerative process and all other lesions of diabetic group including hyaline cast formation and hemorrhage were diminished throughout the renal tissue and only mild hydropic degeneration and scant hyperemia in interstitial tissue were observed (Figs. 2E and 2F).

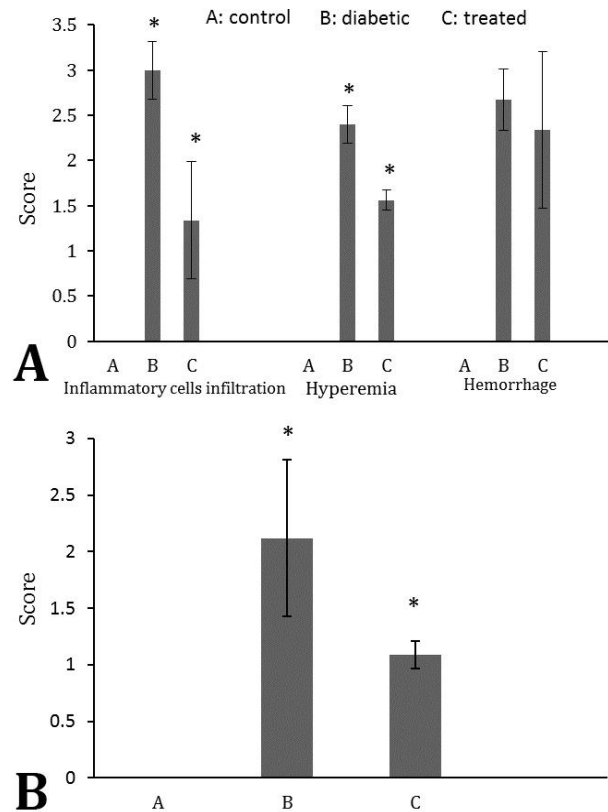


**Fig. 2.** Photomicrograph of kidney tissue. **A)** Control group shows normal architecture of kidney with normal glomeruli and normal proximal convoluted tubules. **B, C and D)** Diabetic group shows massive inflammatory cell infiltration (thick arrow), notable hemorrhage (asterisk) and severe hydropic degeneration of proximal convoluted tubules (thin arrow). **E and F)** Treatment group shows decreased hydropic degeneration (thin arrow) and little to moderate hyperemia in the interstitial tissue (asterisk), (H & E, Scale bar = 100  $\mu$ m).

**Comparison of the degeneration severity in renal interstitial tissue.** The results showed that in group B, the severity of degeneration in renal interstitial tissue was significantly increased compared to the control group ( $p < 0.05$ ). However, in group C, it was reduced significantly compared to group B ( $p < 0.05$ ), (Fig. 3A).

**Comparison of the severity of hemorrhage, hyperemia, and filtration of inflammatory cells in kidney.** The results showed that the severity of hyperemia and filtration of inflammatory cells in kidney

tissue were significantly increased in group B compared to group A. However, in group C they were significantly reduced compared to group B ( $p < 0.05$ ) (Fig. 3B). In terms of hemorrhage in kidney cells, its reduction in group C compared to B was not significant ( $p > 0.05$ ).



**Fig. 3. A)** The degradation severity in renal interstitial tissue. **B)** Severity of hemorrhage, hyperemia, and filtration of inflammatory cells in kidney tissue. \* shows significant difference between B and C group ( $p < 0.05$ ).

## Discussion

In the current study, the BA effect was evaluated on autoimmune diabetic C57BL/6 inbred male mice. Lack of insulin hormone leads to degradation of  $\beta$  cell in the pancreas and consequently reducing insulin level in the body. It is of note that insulin is produced by  $\beta$ -cells of the pancreas. Moreover, insulin reduces the glucose level of cells by affecting liver cells and depositing them as glycogen. Therefore, in T1D, the blood glucose level is increased. The result of our study showed that BA could increase insulin level and reduce fasting blood glucose level in the treatment group (C) compared to group B.

Numerous researches have evaluated the effect of triterpenoid plants on mellitus diabetes. In agreement with the results of our study, Lee *et al.* demonstrated that ursolic acid (a triterpenoid) reduced the fasting blood sugar level in STZ-NA-induced diabetic mice.

They recommended that this effect could occur because of inhibition of glucose production in the liver.<sup>26</sup> Besides, Chia *et al.* demonstrated that glycyrrhetic acid reduced blood glucose level in diabetic rats.<sup>27</sup> In line with the current study, previous studies showed the BA effect on high fat diet-induced diabetic rats,<sup>28</sup> and oleanolic acid in STZ-induced diabetic rats.<sup>29</sup>

In comparison with our study, BA extracted from *Syzygium cumini* resulted in a reduction in plasma level of amylase- $\alpha$ .<sup>30</sup> Since enzymes are catalyzed the most major biochemical pathways, enzyme inhibitors, especially amylase inhibitor can be important to control treatment of disease.<sup>31</sup> In a study, the effect of BA on diabetes was compared to that of metformin. The results showed the effect of BA on blood glucose level was the same as metformin.<sup>31</sup> Also, the desirable effects applied by betulinic acid on insulin biomarkers were the same as treatment by metformin.<sup>32</sup>

Two different studies showed that insulin sensitivity was improved by betulin treatment.<sup>32,33</sup> In a study treating with BA a reduction in blood glucose and insulin was resulted, which showed the protective effect of BA against diabetes. Generally, the data showed that betulinic acid could be able to impede diabetes in STZ-induced mice.<sup>34</sup> In a study, the inhibitory effect of BA on producing liver glucose was evaluated in HepG2 cells and ICR mice. BA significantly inhibited the liver gluconeogenesis and the level of gene expression required for enzymes in this pathway, such as glucose 6 phosphate. Briefly, BA effectively reduced hyperglycemia by inhibiting liver gluconeogenesis.<sup>35</sup>

A study showed that betulinic acid acted as a stimulus for insulin static secretion in the islets of pancreas. Accordingly, BA stimulated insulin secretion by activating electro-physiologic mechanisms such as closing potassium channels and opening calcium and chloride channels inducing depolarization of cellular in company with metabolic-biochemical effects, followed by activating PKC and eventually insulin secretion.<sup>25</sup>

In one study, BA showed anti-diabetic activities via reducing insulin resistance and increasing the power and mass of beta cells. Several studies have shown that BA improved glucose metabolism and increased glucose uptake in the adipose and liver tissue of diabetic rats.<sup>36</sup> Diabetic nephropathy is one of the most important factors of deficiency in the physiological function of kidneys in mellitus diabetes, which significantly affects diabetic patients' life. Among symptoms of DN, one could name an increase in the thickness of basement membrane of the glomerulus, increasing and expansion of renal interstitial fibrosis, increasing the concentration of creatinine and clearance level of creatinine. In the present research, the diabetic mice kidneys were evaluated histopathologically and the results showed the reduction of kidney lesions by consuming BA.

A study indicated histopathologic observation of diabetic kidney sections, degeneration or severe renal tubule necrosis, moderate degrees of dilatation of glomerular, vascular wall thickness and inflammation of interstitial tissue, in contrast, in our study, BA weakened these changes.<sup>34</sup> Another study suggested that 40.00 mg kg<sup>-1</sup> BA may prevent DN, therefore, protective findings of the kidney might be produced by the anti-diabetic and hypoglycemic effects induced by BA injection.<sup>36</sup>

Histopathology in DN is characterized by changes such as inflammation of the tubulointestesia, reduction of brush border and extracellular matrix accumulation.<sup>37</sup> Studies of Jheng *et al.* showed a significant loss in the brush borders and also tubular distention.<sup>38</sup> Lingaraju *et al.* and chen *et al.* showed that BA was successful in improving the histopathological changes of the kidney resulting from an experimental model of poly-microbial sepsis in mice. Also, one of the recommended mechanisms of these changes was the antioxidant activity of BA.<sup>39,40</sup> Ahangarpour *et al.*,<sup>36</sup> confirmed the results of Lingaraju *et al.*<sup>39</sup> A study showed that BA could inhibit I $\kappa$ B $\alpha$  (NF- $\kappa$ B inhibitor protein) destruction and NF- $\kappa$ B activity in kidneys of diabetic rats and caused a decrease in fibronectin expression, thereby preventing kidney fibrosis in diabetic patients.<sup>24</sup>

Swelling of pancreatic islets and destruction of  $\beta$ -cells take place in parallel with boosted pro-inflammatory cytokines expressions such as TNF- $\alpha$ , IL-1, INF- $\alpha$  and Th1 cytokines including INF- $\gamma$ , IL-12, TNF- $\beta$ , IL-2. Various studies have shown the pathological role of these cytokines in developing autoimmune diabetes.<sup>14</sup>

Many studies have been conducted on the possible role of cytokines in the pathogenesis of autoimmune diabetes. The results showed that swelling of pancreatic islets and destruction of  $\beta$ -cells took place in parallel with boosted pro-inflammatory cytokines expressions such as IFN- $\alpha$ , IL-1, TNF- $\alpha$ , and Th1 cytokine inclusive of IL-2, IFN- $\gamma$ , IL-12, and TNF- $\beta$ . Various studies performed on the evacuation of TNF- $\alpha$ , IL-1, IL-6 cytokines, and Th1 cytokines, such as IFN- $\gamma$ , IL-2, IL-12, showed a pathological role in the development of autoimmune diabetes.<sup>15</sup>

In our study, the effect of BA on cytokines in diabetic mice was observed as a decrease in IL-1, TNF- $\alpha$  and IFN- $\gamma$  cytokines, which was not significant for IFN- $\gamma$ . Inflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$  and IL-6 are involved in the diabetic nephropathy development. As well, IL-1 $\beta$  is involved in the irregularities progressions in intra-glomerular hemodynamics associated with synthesis of prostaglandin.<sup>41,42</sup> IL-6 enhances the level of fibronectin, exacerbates the proliferation of mesangial cells, disrupts the dynamics of the extracellular matrix and increases endothelial permeability.<sup>43</sup> TNF- $\alpha$  is cytotoxic for mesangial cells of glomeruli and epithelial cells. Also TNF- $\alpha$  could cause explicit renal damage as a result of the reactive free radicals production.<sup>44</sup>



The results showed that betulinic acid considerably decreased the inflammatory cytokines in serum and kidney tissues of diabetic rats. In another study that the effect of BA on endotoxin mice was evaluated, a decrease in TNF- $\alpha$  levels was observed in BA treated mice. Besides, the results showed that an increase in the serum level of IL-10, an anti-inflammatory cytokine, in BA-treated mice indicated the important role of this cytokine in BA-induced protection.<sup>45</sup>

Other findings indicated that betulin was not effective in the induction of cytokines such as IFN- $\gamma$  and IL-10. Compared to betulin, its oxidized form, BA, confirmed a slight decrease in IFN- $\gamma$  production and increase in IL-10 production.<sup>46</sup> The BA extracted from *Diospyros leucomelas* showed anti-inflammatory effect in different testes.<sup>47,48</sup>

The C-peptide is a polypeptide that its reduction is associated with an increase in glucose levels in type 1 diabetes. In the present study, the results showed an increase in C-peptide in group C compared to group B. Low levels of C-peptide and decreased function of  $\beta$ - cells are associated with higher glucose levels.<sup>47,48</sup> In experimental condition, C-peptide was indicated to prevent the formation of endothelial cell reactive oxygen species (ROS) in the presence of hyperglycemia.<sup>49</sup>

It is noteworthy that, according to studies conducted on anti-neoplastic activities, BA is a highly tolerated drug and its consumption in high dosage (> 500 mg kg<sup>-1</sup>) is safe.<sup>50</sup> Overall, it seems that treatment with the BA leads to several advantages in reducing diabetes so that these mice show a lower level of blood glucose than untreated diabetic ones and at the same time the consequences of diabetes such as hypertriglyceridemia. Also BA markedly decreased the production of pro-inflammatory cytokines. Moreover, BA is successful in improving the histopathological changes of the kidney. It seems that the BA can be considered as a useful agent in the treatment of type 1 diabetes

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### Conflict of interest

All of the authors declared that there was no conflict of interest.

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