



## SERUM LEVELS OF C-REACTIVE PROTEIN, COMPLEMENT 3 AND COMPLEMENT 4 IN IRAQI DIABETIC PATIENTS ON METFORMIN THERAPY

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### ABSTRACT

Metformin is considered as an oral anti-diabetes agent. It is regularly used as a first-drug for the controlling of type-2 Diabetes Mellitus (T2DM). However, we aimed to evaluate whether the inflammatory biomarkers C - reactive protein (CRP), Complement 3 (C3) and Complement 4 (C4) levels are affected by metformin therapy in (T2DM) patients. Data from 150 male patients were classified into five groups (40 diabetics metformin users only, 40 diabetics without treatment, 25 diabetic insulin users only, 25 diabetic insulin plus metformin users and 20 nondiabetic healthy groups). The age ranged from 40-70 years old; samples were collected from patients who underwent treatment at National Center for Diabetes Research and Treatment/ Baghdad between the periods of October 2016 and June 2017. Blood sampling was collected separated and determined by using immunoassays. Our study revealed that serum levels of C - reactive protein, C3 and C4 significantly increased in patients with (T2DM) without metformin treatment (Uncontrolled). Serum levels of all indicated markers were markedly reduced in the metformin-treated group. Patients using insulin alone showed marked reduction in C4 level. While in patients using both insulin and metformin, C - reactive protein and C3 were highly reduced than C4 which was approximately 50 % of decrement. Study outcomes demonstrated an elevation of some inflammatory biomarkers in uncontrolled diabetic patients. Metformin has a potential role in alleviating these indicated biomarkers.

**KEYWORDS:** *Metformin, C - reactive protein, Complement 3, Complement 4 and Type-2 Diabetes Mellitus*



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## INTRODUCTION

Metformin remains the commonest oral antidiabetic drug used in the world. It is managed in all kinds of diabetes as a first choice treatment, but more important with type 2 diabetes mellitus.<sup>1</sup> The incidence of Type-2 Diabetes Mellitus (T2DM) is increasing rapidly worldwide. Clinically, metformin plays an essential role in the reduction of glucose level in (T2DM) patients. Many diabetic patients may develop cardiovascular complications such as myocardial infarction; metformin may contribute in preventing or delaying these complications.<sup>2-4</sup> Also, metformin has advantages for nondiabetic patients, for example decreasing the prevalence of diabetes, management of hyperandrogenism, dyslipidaemia and obesity.<sup>5,6</sup> Low-grade inflammation is highly associated with (T2DM), which confirmed by an elevation of C-reactive protein (CRP), an inflammatory biomarker in diabetic patients. Several studies suggested that inflammation may be involved in the pathogenesis of long-term complications of diabetes mellitus, especially cardiovascular diseases.<sup>7,8,9</sup> C-reactive protein is presented as a pentameric protein circulated in blood; this mediator increased in response to infection and inflammation.<sup>9</sup> Clinical evidence displayed higher CRP levels in (T2DM) patients compared with non-diabetic people. In addition, elevated levels of CRP levels were positively correlated with atherosclerosis in (T2DM). Since the previous studies investigated the role of the antidiabetic drug on inflammatory process, type-2 Diabetes Mellitus patients showed a decreased level of CRP after taking the metformin.<sup>10</sup> Overall, metformin plays a crucial role to reduce the CRP concentration which contributes to reduce the inflammation and prevent the development of cardiovascular complications.<sup>11,12</sup> Negligible inflammation can trigger classical pathway of the complement system, which is composed of subunits protein found in the blood. The proteins such as complement 3 (C3) and complement 4 (C4) play an essential role in inflammation through their functions in innate and adaptive immunity. However, the cellular and molecular mechanisms which induced inflammation and organ damage in diabetic complications are related to increased activation of complement system via the inhibition of CD59 molecules. High glucose levels in diabetic patients inactivated this Cluster Differentiation 95, prototype death receptor (CD95) molecules. Also, high glucose levels may

affect the complement proteins leading to activating more membrane attack complex (MAC) deposition on the cells which developing the inflammatory process in diabetic patients.<sup>13-15</sup> We supposed that metformin has a potential role to regulate the markers of inflammation like CRP, in addition, to other inflammatory marker, such as immunological marker C3 and C4. However, there is relatively little information regarding the effects of metformin. Therefore, this study focused on measuring inflammatory and immune biomarkers in Iraqi diabetic patients and on evaluating the effects of metformin to understand better how antidiabetic drugs could influence CRP, C3 and C4.

## MATERIALS AND METHODS

### *Patient's sample collection*

140 male patients have been included in this study; patients were divided into five categories according to the specific criteria. Again, 150 male patients were classified into five groups (40 diabetic metformin users only, 40 diabetic non-metformin users, 25 diabetic insulin users only, 25 diabetic insulin plus metformin users and 20 nondiabetic healthy groups). Clinical samples (blood) were collected according to<sup>16</sup> from five groups of patients. The patient's sample was taken from the people referred for treatment at National Center for Diabetes Research and Treatment / Baghdad / Al-Yarmook and from private medical laboratory between the periods of October 2016 until June 2017. Written informed consent was obtained from every patient giving blood samples for the study. The study was approved by the Ethics National Center for Diabetes Research and Treatment/ Baghdad. Patient agreements to participate in scientific research have been taken.

### *Serum Preparation*

Blood samples have been collected aseptically by venipuncture into a dry clean and sterile tube without anticoagulant substances and allow it to clot. The name, gender, age, medication have been written on the tube from the provided patient's list history. Blood samples allowed to stand for 20-30 min for clot formation and centrifuged. The supernatant serums were stored in Eppendorf tube at (-20 C to - 80 C) for subsequent analysis or use.

### *Laboratory measurements of indicated proteins*

On the day of the laboratory analysis, patient's information, including the age, the personal medical, family history were documented by interviewer-administered questionnaire form.

Assays for serum CRP, C3 and C4 levels achieved as routine clinical tests by clinical laboratory staff. CRP levels were assessed by automated nephelometric immunoassay by Beckman Coulter SYNCHRON LX-20 (Beckman Coulter, Inc. America).<sup>17,18</sup> While the determination of the C3 and C4 protein was made by radial immunodiffusion plate (C3 & C4 RID) according to Mancini & coll.-Immunochemistry manufactured by the Meridian Healthcare Srl Company.<sup>19</sup>

## STATISTICAL ANALYSIS

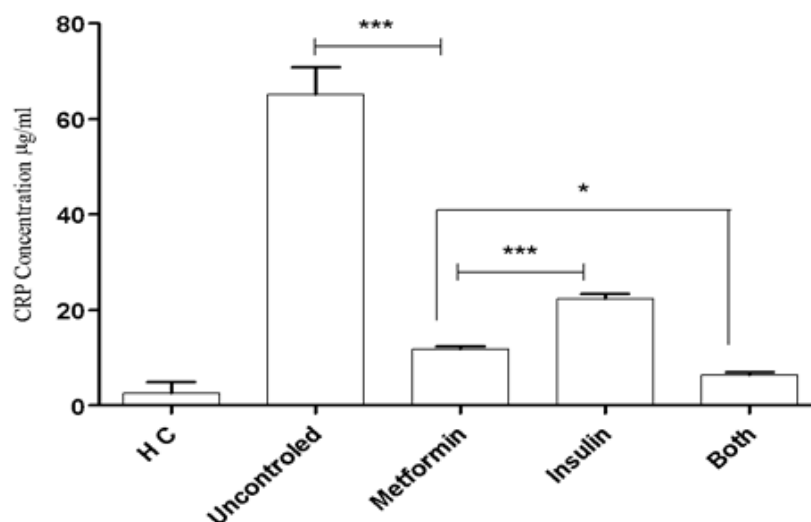
Statistical meaning of variances among means values from control and treated groups applied by specific (ANOVA) test via Graph Pad Prism® Version 5.0 software or Bonferroni Multiple Comparison test.  $p < 0.05$  established as significant.<sup>20</sup>

## RESULTS

### *Comparison of serum CRP following treatment with Metformin, Insulin and both*

To assess the potential action of metformin in

diabetic patients on inflammatory biomarkers, a CRP, C3 and C4 concentration's measurement was utilised. This was initially tested for effect upon the serum levels of mentioned biomarkers specifically for uncontrolled diabetic patients. However, a serum concentration in healthy control was suggestively undetectable. While, serum CRP concentrations in the uncontrolled group were significantly higher in patients with type 2 DM as compared to healthy controls (mean  $\pm$  SEM  $2.50 \pm 2.454$  versus  $65.113 \pm 5.639$ ,  $p = 0.001$ ). We also sought to determine the effect of metformin on endogenous inflammatory proteins production. As expected, patients with metformin group had significant lower serum concentrations for CRP ( $p = < 0.001$ ). Figure 1 illustrates the variability of CRP levels among different groups of patients in this study. CRP levels in patients receiving therapeutic agents (metformin, insulin, or both) have been declined. The high reduction was in patients treated with both agents (mean  $\pm$  SEM  $6.400 \pm 0.577$ ). Figure 1 also demonstrated that metformin decrease CRP levels more than insulin (mean  $\pm$  SEM  $11.733 \pm 0.577$  versus  $22.400 \pm 0.881$ , metformin; insulin respectively,  $p = < 0.5$ ).



**Figure 1**

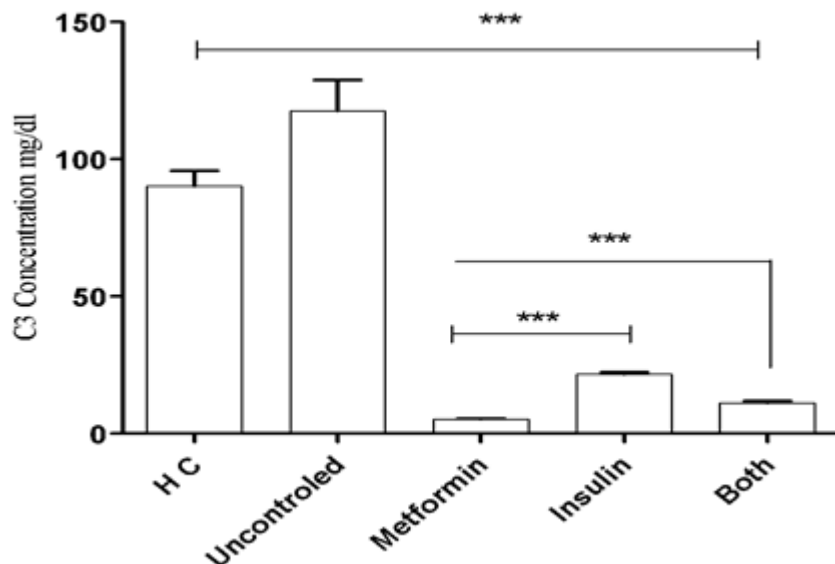
*The effect of metformin, insulin and both on serum CRP concentration in type 2 DM patients.*

Serum samples were analysed for CRP concentration for indicated group. Preparation of samples, latex agglutination methods used for proteins detection, are outlined in Section 2.3.

### *Characterization of serum C3 following treatment with Metformin, Insulin and both*

The serum C3 concentration has been considered in response to metformin, insulin and both as shown in (Figure 2) a strong reduction in C3 level

in patients on metformin therapy (mean  $\pm$  SEM  $4.993 \pm 0.340$ ). In contrast, healthy control (H C) represents a substantial normal value of C3 concentration as expected (mean  $\pm$  SEM  $90.0 \pm 5.773$ ). Further significant reduction in C3 levels was observed in patients using insulin alone or both insulin and metformin therapy (Insulin= mean  $\pm$  SEM  $21.553 \pm 0.709$ , both= mean  $\pm$  SEM  $11.130 \pm 0.554$ ).



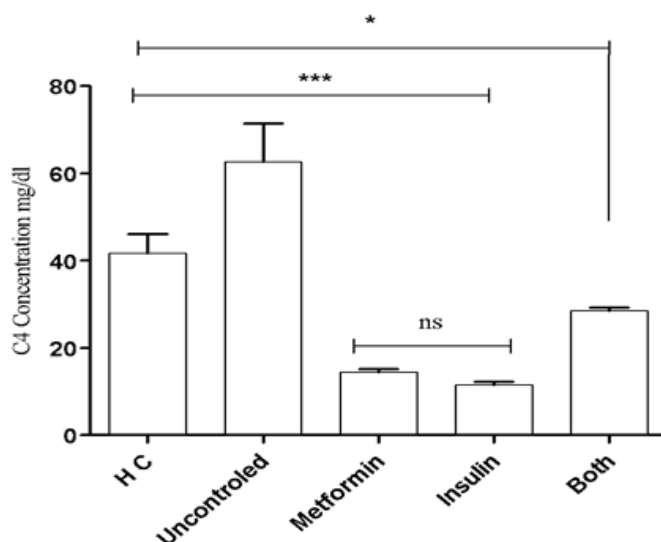
**Figure 2**  
*The effect of metformin, insulin and both on serum C3 concentration in type 2 DM patients.*

Serum samples were analysed for C3 concentration for indicated group. Preparation of samples, Radial immunodiffusion plate used for proteins detection, are outlined in Section 2.3.

**Characterization of serum C4 following treatment with Metformin, Insulin and both**

Having established that from the previous figure the metformin reduced the C3 level, the effect of metformin on the C4 level was examined in the same manner. Figure 3 shows the reducing effect

of metformin upon C4, and there was a little more reduction in response to insulin alone (Metformin= mean  $\pm$  SEM 14.423  $\pm$  0.674, insulin= mean  $\pm$  SEM 11.466  $\pm$  0.726). In contrast, treatment with both agents doesn't induce the same effect of decreasing in C4 which was approximately 50 % (Both= mean  $\pm$  SEM 28.450  $\pm$  0.695). Under these conditions, metformin alone or insulin alone caused a significant decrease in C4 level at all established samples, which was not notable decreased by patients used both agents.



**Figure 3**  
*The effect of metformin, insulin and both on serum C4 concentration in type 2 DM patients.*

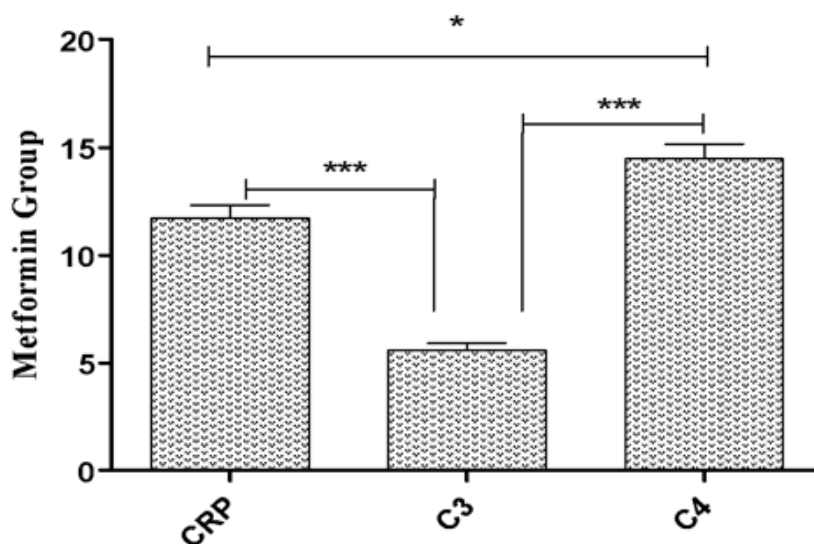
Serum samples were analysed for C3 concentration for indicated group. Preparation of

samples, radial immunodiffusion plate used for proteins detection, are outlined in Section 2.3.

**The assessment of metformin effect on serum CRP, C3 and C4 in type-2 DM**

Having established that metformin could reduce the activity of inflammatory biomarkers, the effects of metformin on CRP, C3 and C4 in (T2DM) patients were evaluated. Figure 4 shows the effect of metformin upon all mentioned

markers above in response to treatment. C3 levels reduction by the same group was much more and highly significant compared to CRP and C4. Again, metformin reduced CRP much more than C4 level (CRP= mean  $\pm$  SEM 11.733  $\pm$  0.577, C4= mean  $\pm$  SEM 14.493  $\pm$  0.674 and C3= mean  $\pm$  SEM 5.580  $\pm$  0.340).



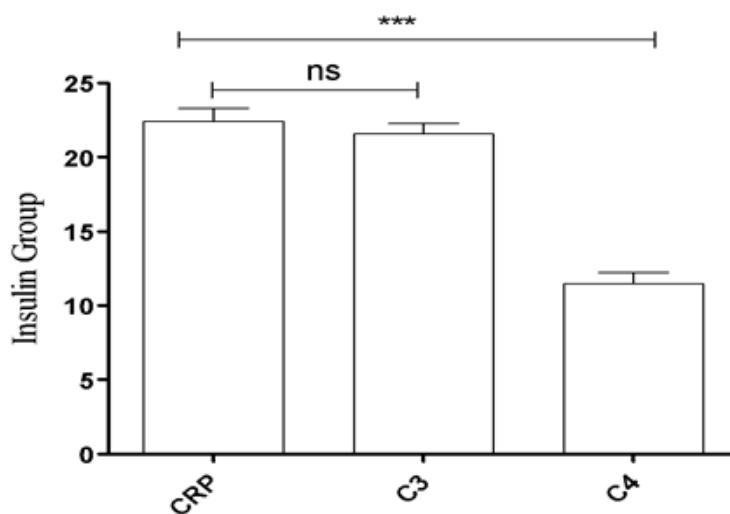
**Figure 4**  
*The effect of metformin on serum CRP, C3 and C4 concentration in type 2 DM patients.*

Serum samples were analysed for proteins above concentration as indicated group. Preparation of samples, radial immunodiffusion plate used for proteins detection, are outlined in Section 2.3.

**The estimation of insulin effect on serum CRP, C3 and C4 in type-2 DM**

The effect of insulin on inflammatory biomarkers in different groups was demonstrated. Figure 5

shows amazing outcomes in biomarkers concentration following insulin therapy. C4 levels were extremely decreased. While no significant reduction showed in CRP and C3 respectively, (CRP= mean  $\pm$  SEM 22.400  $\pm$  0.881, C3= mean  $\pm$  SEM 21.553  $\pm$  0.709, (C4= mean  $\pm$  SEM 11.466  $\pm$  0.726). \*\*\*p < 0.001.



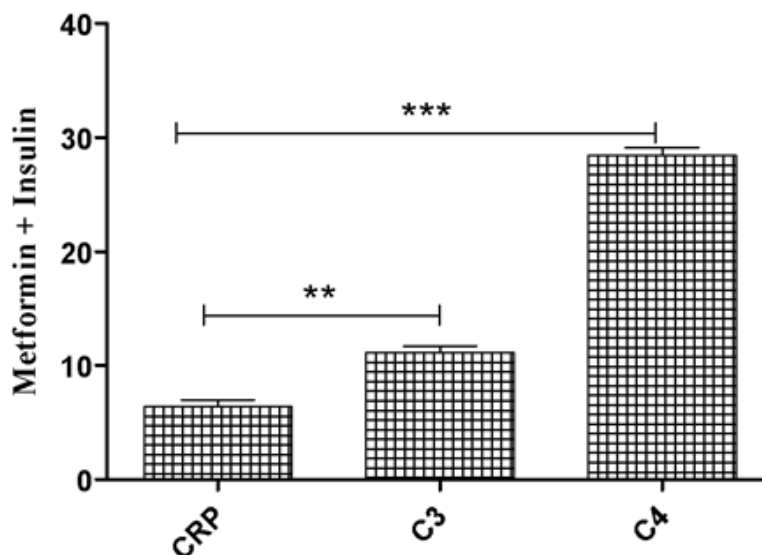
**Figure 5**  
*The effect of insulin on serum CRP, C3 and C4 concentration in type 2 DM patients.*

Serum samples were analysed for proteins above concentration as indicated group. Preparation of samples, radial immunodiffusion plate used for proteins detection, are outlined in Section 2.3.

### ***The estimation of metformin and insulin effect on serum CRP, C3 and C4 in type-2 DM***

The evidence of variation among CRP, C3 and C4 levels in patients using metformin or insulin were also considered. Metformin plus insulin caused a

marked effect on CRP and C3 levels. While surprisingly, analysis of the C4 level, in the same manner, was less impact. However, both therapeutic agents have significantly different alleviation regarding C4 concentration (Figure 6). This evidence is confirmed by high reduction of CRP and C3 compared to the reduction of the C4 level (CRP= mean  $\pm$  SEM  $6.40 \pm 0.577$ , C3= mean  $\pm$  SEM  $11.13 \pm 0.554$  and C4= mean  $\pm$  SEM  $28.45 \pm 0.695$ ).



**Figure 6**  
***The effect of insulin on serum CRP, C3 and C4 concentration in type 2 DM patients.***

Serum samples were analysed for proteins above concentration as indicated group. Preparation of samples, radial immunodiffusion plate used for proteins detection, are outlined in Section 2.3.

## **DISCUSSION**

The role of metformin in diabetic patients has been poorly studied relative to other antidiabetic agents in particular insulin. However emerging studies indicate a possible role in some cardiovascular diseases including atherosclerosis. In this study, diabetic patients controlled on metformin therapy were used as an approach to determine the role of metformin in inflammatory biomarkers in diabetic patients. This is the first study in our country in which the effects of metformin on serum CRP, C3 and C4 concentration with type-2 DM patients have been investigated. The main findings were a significant reduction of CRP concentrations during metformin therapy and, conversely a significant elevation in uncontrolled patients or without metformin therapy. CRP is an identified as an

inflammatory biomarker; its elevation in the blood is considered risk factors for several clinical disorders such as cardiovascular diseases and other acute systemic inflammation.<sup>21</sup> The nonpharmacological management like lifestyle changes including increased exercise, weight reduction, smoking cessation and improved nutrition produced a minor reduction in CRP concentrations.<sup>22</sup> Some medications like antidiabetic and antihyperlipidemic may also decrease CRP levels.<sup>23</sup> This research aimed to investigate the effect of antihyperglycemic agents on CRP, C3 and C4 concentrations besides to their central indication for either glucose regulator. In vitro studies confirmed that CRP has a pro-inflammatory effect on endothelial cells. By its increasing the endothelial expression of some chemokines, CRP may be considered as an inflammatory mediator rather than marker.<sup>24</sup> Our data presented that the baseline CRP levels remained pointedly higher in uncontrolled than metformin therapy subjects with type-2 DM. This suggests that the increased CRP levels found in type-2 DM due to tissues damage. These findings were in agreement with the previous study on

women with PCOS.<sup>25</sup> Again, our consequences are in agreement with other studies outcomes,<sup>23</sup> which also demonstrates an association between metformin control and CRP concentrations. Numerous studies on diabetic patients illustrated that the metformin therapy together with glycemic control has promising effect in preventing or delaying cardiovascular complications mainly via alleviation of inflammatory process reflected by a reduction of CRP concentrations.<sup>6,12,26</sup> The underlying mechanism may be the interaction of metformin with the synthesis and secretion of CRP. On the other hand, the concentration of another inflammatory marker, complement factor C3 not affected by metformin therapy<sup>12</sup>, which was in contrast with our results that showed significant reduction of C3 levels. Central question may be involved in this research, as to why we have examined the effect of metformin on the other marker such as C3 and C4. Many studies demonstrated the role of metformin to regulate the immunological mediators like macrophage migration inhibitory factor (MIF), a cytokine contributed to innate and adaptive immunity. Also, the antiatherogenic effect of metformin may be associated with the reduction of MIF.<sup>27</sup> Regarding the group of patients using insulin and metformin; our data demonstrated a significant decline in CRP and C3 levels, these findings were in agreement with other previous findings. In addition to the beneficial effect of metformin, insulin also has an anti-inflammatory effect. Good outcome concerning reduction of CRP concentration has been recognized after intravenous infusion of insulin.<sup>27-29</sup> The results of our research showed that the significant effect of metformin alone or combined with insulin in the C3 and C4 concentrations. Patients with metformin alone showed an excellent response to the primary component of complement system C3,

while showed less effect on C4 protein. The complement system has a pivotal role in the pathogenesis of diabetic complications.<sup>30-32</sup> So, controlling activated complement parameters by metformin, insulin or both may alleviate these complications.

## CONCLUSION

Patients with type 2 DM among Iraqi population without metformin therapy displayed higher CRP, C3, and C4 levels comparing to the normal levels in healthy peoples. Also, this research confirmed a potential role for metformin in alleviation of some of the inflammatory and immunological markers in Iraqi diabetic patients. Variable reduction in concentrations of these biomarkers in response to metformin, insulin or both was established. Further studies are needed to investigate more specific inflammatory biomarkers its correlation with diabetic complications. Finally, metformin is considered the best anti-inflammatory treatment with type 2 diabetic patients besides its glucose regulator function and has a potential role in alleviating these indicated biomarkers.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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