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The synergistic effect of antiglycating agents (MB-92) on inhibition of protein glycation, misfolding and diabetic complications in diabetic-atherosclerotic rat

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Abstract

Protein glycation due to hyperglycemia resulting in misfolding and aggregation, which is known as one of the most important reasons of diabetes complications. We previously showed the beneficial effects of some antiglycating agents in diabetic rats. Here, the effect of MB-92, a combination of some amino acids and crocetin (Crt, a saffron carotenoid), was studied in the prevention of diabetic complications in diabetic-atherosclerotic rats. In addition, the inhibitory effect of these treatments on glycation intermediates, aggregation and misfolding of proteins was investigated both *in vivo* and *in vitro*. Thus, the streptozotocin-induced diabetic rats that underwent an atherogenic diet were treated with Crt, N-acetylcyctein and MB-92. Then, glycated products and markers of oxidation and inflammation, in addition to other markers of diabetes complications were studied. The results of the *in vivo* study indicated that the mentioned treatments prevented the atheromatos formation, reduced the increased blood glucose; inhibited the formation of various glycation products, induced glyoxalase system (I and II), diminished oxidation and inflammatory markers, and improved lipid profile and atherosclerotic index in the diabetic-atherosclerotic rats; but MB-92 was the most effective treatment. *In vitro* results also confirmed that MB-92 was the most effective treatment to inhibit protein glycation and misfolding in comparison with the other treatments. In conclusion, MB-92 showed the greatest potential for inhibition of glycation and oxidation products, atheromatose plaque formation and inflammation in diabetic-atherosclerotic rats, and to control protein glycation, misfolding and aggregation in high glucose concentration; thus, it can be suggested as a new drug to prevent diabetic complications**.**

Keywords: Combinatorial Therapy; Glycated Products; Misfolding; Aggregation; Diabetic Complications; Atheromatose Plaque; Crocetin; Amino Acids and Streptozotocin.

Introduction

The health organizations around the world have announced that diabetes mellitus is going to become an epidemic disease all over the world in near future. Diabetes mellitus has been categorized as a conformational disease in the last decade [1, 2]. In conformational diseases, misfolded (or non-conformationally-native) proteins aggregate and produce dysfunctional proteins. In Type 2 diabetes mellitus a constituent protein of beta cells, islet amyloid polypeptide, misfolds. The change in its tertiary structure results in self-aggregation and tissue deposition that cause some damage in the tissue [2, 3]. In addition, the persistence hyperglycemia induces conformational changes in the structures of various biomacromolecules like proteins, DNA, RNA and enhances oxidative stress, leading to the development of diabetic complications [4]. Serum albumin is one of the most important proteins that is affected by high levels of glucose (Glc) concentration that result in the formation of glycated albumin (gAlb). Fig.1 is the schematic representation of the direct or indirect roles of gAlb in various complications of diabetes. These complications are mediated by early, intermediates and advanced glycation end products (AGEs) [5].

L.C. Maillard, a French chemist, firstly explained the reaction between sugar and amino acids. In the course of the Maillard reaction, the firstly formed Schiff base adduct, which is produced in a reversible phase, then rearranges and converts to a more stable compound named Amadori product. The Amadori products then undergo further rearrangement to form irreversibly bound AGEs, in a cascade of chemical reactions. In the named process, numerous intermediates or secondary products are formed. Some of them are low molecular weight compounds such as methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG). These compounds are also named as α-oxoaldehydes that are very reactive carbonyls. They have the potential to react with

free amino groups of other molecules or biomolecules, leading to cross-linking and formation of AGEs in the late stage of Maillard reaction [6], the process has been called as carbonyl stress [7].

As mentioned above, gAlb is one of the most important Amadori products [8]. Elevated levels of gAlb, which can be determined by the fructosamine test (in human and animals), cause permanent damages connected with the metabolic disorders observed in diabetes mellitus, such as retinopathy, nephropathy, neuropathy and coronary artery diseases [9]. The deleterious effects on gAlb and production of AGEs have been considered in many *in vivo* and *in vitro* studies [10, 11]. In diabetes, glycation-oxidative stress-inflammation cycle may be the main cause of vascular complications. Therefore, inhibiting the glycated product formation at different stages is the most common target for prevention and/or treatment of not only diabetic complications, but also other related consequences like aging. Given the above mentioned mechanisms, various AGE inhibitors have been designed and introduced in the literature [12-15].

Although, various chemicals have been introduced as the inhibitor of non-enzymatic glycation, but some of them, are toxic and are not suitable for body health [16]. Therefore, using naturally occurring compounds that are more compatible with life and less harmful to health has been suggested [17].

Following to our previous *in vivo* and *in vitro* studies on application of some chemical chaperones of amino acid family [18, 19] and natural products [20] on preventing the AGEs formation and diabetic complications, in the present study we designed a new combinatorial treatment. So that, by using the synergism between the mentioned compounds we hope to achieve a more potentially powerful treatment to inhibit the glycation products and prevent the diabetic complications. Therefore, crocetin (Crt) as a natural carotenoid obtained from saffron was chosen. Some of the known pharmacological activities of Crt include: antioxidant activity

[21], prevention of AGEs-induced vascular endothelial cell apoptosis [22], inhibition of leukocyte adherence to vascular endothelial cells induced by AGEs [23] and inhibition of β-amyloid formation [24]. In addition, glycine (Gly) as an inhibitor of non-enzymatic glycation and AGEs formation [13, 18, 25], with a known antioxidant property was used. The third component was N-acetylcystein (NAC). It has been known as an antioxidant and a precursor of glutathione that scavenges GO and MGO [26], which represents the anti-AGEs property [27]. This combinatorial package was named as MB-92. Then, MB-92 and each of the components of this package were separately tested for their antidiabetic and antiaggregation effects in both *in vivo* and *in vitro* conditions and the results was compared.

Materials and Methods

Materials

Crocetin (Crt) was extracted and purified from Iranian saffron using the method described previously by us [28]. Standard of pentosidine was gifted by Professor Dr. Vincent M. Monnier. All other materials were in analytical grade and purchased from Sigma or Merck Chemical Companies.

In vivo studies

Animal Model of diabetes-atherosclerosis

Male Wistar rats, 8 weeks old and weighing 180±15 were purchased from the *Pasteur Institute* of Iran, Karaj. Animals housed under controlled temperature conditions with a 12-h light and 12-h dark cycle with free access to food and water. After 2 weeks, they were divided into 2 main groups. Group 1, after overnight fasting, was injected intraperitoneally (i.p.) with

streptozotocin (45 mg/kg body weight in Na-citrate buffer, pH 4.5) [29]. After three days, animals with fasting blood sugar (FBS) > 11 mmol/L, was accepted as diabetic. Group 2, normal or healthy animals were considered as control and were injected with vehicle only. Normal groups feed on a standard chow diet, while diabetic groups were on an atherogenic diet (Chow diet containing 1% Chol and 0.5 % cholic acid). Then, each of the two main (diabetic-atherosclerotic and normal) groups was divided into four subgroups, #10 rats in each. The rat groups were named as follows:

Subgroups 1 or control groups, with no more treatments that were named as D and N, for diabetic-atherosclerotic and normal rats, respectively.

Subgroups 2 received 100 mg/kg Crt by i.p. injection, once a month for three months and named as D-Crt (for diabetic group) and N- Crt (for the control group).

Subgroups 3 received NAC 60 mg/kg, daily in drinking water and were named as D-NAC and N-NAC.

Subgroups 4 were treated with a combination of NAC (60 mg/kg) and Gly (1%) in drinking water and injection of Crt as subgroup 2, and was named as D-MB-92 and N-MB-92.

The chemical structure of the mentioned treatments is shown in Fig.2. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guidelines for the care and use of laboratory animals prepared by Tarbiat Modares University.

At the end of the experiment, after 16 h fasting, rats were anesthetized with an i.p injection of ketamine – xylosine (90 + 10 mg/kg body mass), a blood sample was collected from their heart and transferred into the test tubes with and without EDTA. Serum samples were prepared by 15 min centrifugation of blood at 5000×g and were aliquoted and stored at −70 °C until the measurements.

Determination of Biochemical Parameters

FBS, TG, Chol and HDL were measured by enzymatic colorimetric methods. LDL was calculated from Friedwald equation [19]. Then, the atherogenic index (LDL/HDL ratio) was estimated from these data, as the cardiovascular risk factor.

The serum insulin level was determined by the enzyme-linked immunosorbent assay (ELISA) method using a rat insulin kit (Mercodia, Uppsala, Sweden). HOMA-IR (homeostasis model assessment of insulin resistance) and pancreas cell function were calculated using Eq. 1 [20]:

$$\text{HOMA-IR} = [(\text{fasting serum insulin } (\mu\text{U/ml}) \times \text{FBS [mmol/l]})/22.5] \quad \text{Eq.1}$$

Determination of the oxidation products

For measurement of the diene conjugates (DC) as the early LDL oxidation product, LDL was isolated by heparin precipitation method [38]. Lipids were extracted from LDL (100 µl) by adding 1 ml of chloroform: methanol (2:1, v:v), mixed and centrifuged at 1000×g, and then the lower organic layer were removed and dried under nitrogen. The dry residue was dissolved in cyclohexane, and analyzed spectrometrically at 234 nm. The molar extinction coefficient of DC is 2.95×10^4 M^{-1} and the results expressed as µmol/L. For measuring the end oxidation product of LDL, which was named oxidation fluorescent product (OFP), the dried residue was diluted 20 times with phosphate buffer 100 mmol/L, pH 7.4; and its emission was measured at 430 nm with excitation at 360 nm [30].

Determination of advanced oxidation protein products (AOPP) was based on the spectrophotometric detection, according to the method of Witko-Sarsat *et al.* [31]. Briefly, 200 µl

of the serum diluted 1:5 with PBS, 200 µl of chloramine T (0-100 µmol/L) for calibration and 200 µl of PBS as blank were applied. 10 µl of potassium iodide (1160 mmol/L) and 20 µl of acetic acid glacial were added and the absorbance of the solution was immediately measured at 340 nm. Concentration of AOPP was expressed in chloramine units (µmol/L).

Determination of High sensitivity C-reactive protein (Hs CRP)

Hs CRP was measured with the CUSABIO ELISA kit (Wuhan, Hubei, *China*).

Enzymatic assay

The activity of glyoxalase system, glyoxalase I and glyoxalase II, was measured in hemolysate. The activity of glyoxalases I was assayed in 37 ºC by measuring the initial rate of formation of S-D-lactoylglutathione. The assay mixture contained 100 mmol/L sodium phosphate buffer pH 7.2, 3.5 mmol/L MGO, 1.7 mmol/L glutathione (GSH) and 16 mmol/L $MgSO_4$, in a final volume of 1 ml. The mixture was incubated for 10 min followed by the addition of 20 µl of the hemolysate and its absorbency was read after 2 minutes at 240 nm.

The activity of glyoxalase II was assayed by measuring the initial rate of hydrolysis of S-D-lactoglutathione (0.3 mmol/L in 50 mmol/L Tris/HCl, pH 7.4) to reduce GSH and D-lactic acid. The rate of S-D-lactoglutathione hydrolysis, after 2 minutes of adding hemolysate, was followed by measuring the absorbance at 240 nm. The activities of both glyoxalase I and glyoxalase II were expressed as unit/ml [32].

Determination of the glycation products

The gAlb, MGO, GO, pentosidine and fluorescent AGEs in the serum of all rat groups was measured using the methods will be explained in the *in vitro* experiments and the percentage of inhibition of the glycated product was calculated according to Eq. 2 [3], by determination of different glycated products in the presence or absence of various treatments.

$$\text{Glycation inhibition (\%)} = 100 \times \left[1 - \frac{(\text{DT} - \text{N})}{(\text{D} - \text{N})}\right] \ldots\ldots\ldots\ldots\ldots\ldots Eq.2$$

Where N, D and DT were presented as, normal, diabetic-atherosclerotic and diabetic-atherosclerotic rats under treatment, respectively.

Pathological study

At the end of the experiment, animals were killed and the whole aortas were collected. After removal of any adhering tissues, all specimens were fixed at 10% buffered formalin, processed and paraffin embedded. Transverse and longitudinal sections of each segment were submitted on one cassette, H&E stains were used in sections for pathological examination by a pathologist.

***In vitro* studies**

Albumin extraction and glycation

Rat serum albumin (RSA) was extracted from the serum using trichloroacetic acid and ethanol, by the method of Ohkawara et al. [33]. RSA concentration was determined using A_{280}= 0.69 of 1 mg/ml of protein [34]**.**

Then 10 mg/ml of RSA was incubated in a series of the caped tubes with glucose (50 mmol/L) in 100 mmol/L sodium phosphate buffer containing sodium azide (0.1 mmol/L) to prevent degradation and inhibition of bacterial growth [35], EDTA (1 mmol/L) to prevent artificial oxidation [36], in the presence or absence of the mentioned inhibitors as defined in Table 1. The concentration of each of the mentioned treatments was chosen according to the literature [18, 37] and our personal experience. They were as follows: Crt (0.3 mmol/L), NAC (7.5 mmol/L) and Gly (1 %). The nomenclature of each series of tubes was done according to their contents and presented in Table 5. All series was prepared as duplicate and all

measurements were done at least three times. The results were presented as mean ± standard deviations (SD).

The reaction mixtures were then sterilized by filtration (0.45 µm pore filter) and allowed to incubate in the dark, at 37 ºC for up to 90 days. The aliquots of samples were prepared after 21 days for measuring gAlb, GO and MGO; and after 90 days for determination of AGEs and pentosidine; all aliquots were maintained at -70 °C until the experiment.

Determination of glycated protein products

The gAlb was quantified by a colorimetric method using nitroblue tetrazolium chloride (NBT) [38]. Briefly, 50 µl of the extracted albumin from serum was incubated with 100 µl of iodoacetamide (5 mmol/L) for 30 minutes at 37 ºC. Then 1000 µl of NBT reagent (200 mmol/L carbonate buffer, pH 10.3, containing 500 µmol/L NBT and 2 % Triton X-100) was added and incubated for 30 min at 37 °C. The absorbency of the sample was measured at 530 nm on a Shimadzu spectrophotometer model UV-3100 (Tokyo, Japan). The dihydroxyacetone, between 0 and 1000 µmol/L, was used as standard to draw the calibration curve.

Glyoxal (GO) and methylglyoxal (MGO) were measured by HPLC using the method of Deng *et al*. [39]. The HPLC system consisted of a KNAUER, Smartline Manager 5000 manual, Smartline pump 1000, a Smartline UV detector 2000 (KNAUER Corporation, Germany) and An analytical column, KENUER C18 (250 mm 4.6 mm; 5µ). The 2,4-dinitrophenylhydrazine (DNPH) derivatives of GO and MGO calibrators were used for chromatography assay. The elution was isocratic with 20 mmol/L phosphate buffer, pH 4.6, containing 32% acetonitrile and 8% of 2-methylpropanol at a flow rate of 1.0 ml/min. The DNPH derivatives were analyzed via a UV-detector at 330 nm.

Pentosidine was measured by the modified method of Slowik-Zylka *et al*. [40] to do the experiment at a shorter run time. The HPLC system consisted of a KNAUER HPLC system with a Rf-10AXL Shimadzu fluorescent detector and the same column as above. Chromatography assay was briefly done as follows: 100 µl of sample were lyophilized and then hydrolyzed by 50 µl of HCl (6 mol/L) at 110°C under nitrogen atmosphere for 16 h, subsequently neutralized with 100 µl of 5 N NaOH and 200 µl of 500 mmol/L of phosphate buffer (pH 7.4), and then filtered through a 0.45-µm Millipore filter. After that, it was diluted 20-fold with phosphate-buffered saline (PBS). Filtered samples (50 µl) were then injected into a C-18 reverse-phase analytical column using an online fluorescence detector at excitation/emission wavelengths of 335/372 nm. A linear gradient of three eluents (A, B and C) was used. Eluent A was 10 mmol/L of heptafluorobutyric acid (HFBA) in water and eluent B was 25% acetonitrile + 75% H_2O + 10 mmol/L of HFBA; and eluent C was acetonitrile. The elution profile was as follows: 0 to 7 min, 60% A and 40% B; 7 to 11 min, 100% C; 11.5 to 15 min, 60% A and 40% B. The flow rate was maintained at 1.2 ml/min through the chromatographic run.

The fluorescent AGEs were determined according to the method of Kalousova *et al*. [41]. The sample was diluted 1:50 with 100 mmol/L phosphate buffer (pH 7.4) and fluorescence intensity was recorded at the emission maximum (440 nm) upon excitation at 370 nm using the Spectrofluorometer Shimadzu, Model RF-5000 (Shimadzu, Kyoto, Japan). Fluorescence intensity (FI) was expressed as arbitrary unit (AU).

Determination of the effectiveness of treatments on glycation inhibition

The percentage of inhibition of the glycated product was calculated according to the Eq.3 [3], by determination of different glycated products of albumin in the presence or absence of various treatments.

$$\text{Inhibition (\%) of glycation} = 100 \times [1 - \frac{(T-B)}{(G-B)}] \qquad \text{Eq.3}$$

Where the abbreviations B (Blank, as a negative control), G (glycated, as a positive control), and T (under treatment), represent the mentioned property of each solution containing RSA, RSA+Glc and RSA+Glc+the mentioned treatment, respectively.

Structural study of gAlb

Circular dichroism (CD) spectra were measured on a JASCO-810 Spectropolarimeter (Tokyo, Japan). The samples with a concentration of 0.1 mg/ml RSA were used for secondary structure estimation in the far-UV region. The spectra have been smoothed and are presented in units of mean residue molar ellipticity. All measurements were performed at room temperature. The amounts of the secondary structures of the protein were calculated by the J-800 for windows, Protein Secondary Structure Estimation Program (Model JWSSE-480).

Statistical analysis

All data were expressed as mean±S.D. Statistical comparisons were performed by Tukey HSD multiple analysis of variance (MANOVA) test using SPSS version 16.

Results

***In vivo* results**

Table 1 represents the levels of FBS, insulin and HOMA-IR in addition to the various glycation products in the serum of normal and diabetic-atherosclerotic rats with or without receiving the named treatments. FBS and HOMA-IR in diabetic-atherosclerotic groups were significantly higher than normal groups, but level of insulin was significantly decreased (P

<0.001). The most decrease in FBS was observed in D-MB-92. The FBS of the groups treated with NAC and Crt alone was in the second and third orders, respectively. The levels of insulin and HOMA-IR in the D-NAC were higher than the other treated groups, and there was no significant difference between diabetic groups with or without NAC treatment. HOMA-IR in the diabetic group treated with Crt and MB-92 were significantly lower than diabetic group without treatment ($p<0.001$).

The levels of various glycation products in diabetic-atherosclerotic rats were significantly higher than those in normal rats. The differences between these parameters in diabetic-atherosclerotic rats after treatment in comparison with the untreated group were also significant ($p<0.001$). As Table 2 shows, all of the mentioned treatments had no significant effect on the normal rats; thus, these data were not shown in Fig.3.

Table 2 also shows a significant difference on gAlb, MGO and fluorescent AGEs in the diabetic groups treated with MB-92 in comparison with the groups received Crt and NAC alone; furthermore, there were no significant differences in these parameters between two later groups. Effect of NAC on GO inhibition was significantly ($P<0.001$) more than the other treatments in diabetic-atherosclerotic groups, but there were no significant differences in this parameter between Crt and MB-92. All of the mentioned treatments significantly ($P<0.001$) reduced the pentosidine formation in diabetic-atherosclerotic rats. Fig.3 compares the contribution of each of the Millard reaction products to a total (100%) inhibition of glycation in the treated diabetic-atherosclerotic rats.

Table 3 represents the results of the effect of the mentioned treatments on lipid profile in all groups. Levels of TG, Chol, HDL and LDL, as well as the LDL/HDL ratio that was significantly

raised in the diabetic-atherosclerotic rats, were significantly decreased after the mentioned treatments. The best treatment was MB-92.

Comparison of the effect of the mentioned treatments on oxidation markers and Hs CRP in the named groups is shown in Table 4. Diabetic-atherosclerotic rats under the combinatorial therapy showed the lowest level of DC, OFP and AOPP. Between these groups, the highest levels of DC and OFP was observed in the D-NAC group and the highest level of AOPP was seen in the D-Crt group. The lowest and highest levels of Hs CRP were observed in the D-MB-92) and the D-NAC groups, respectively. MB-92 treatment induced the maximum activities of both glyoxalases I and II (Fig.4), which was significantly higher than other diabetic-atherosclerotic groups ($P<0.001$).

The effect of the mentioned treatments on atheromatous plaque formation in the normal and diabetic rats represented in Figs.5a to 5c. The atheromatous plaque was only formed in the diabetic rats with no treatment, but the neointima was formed in both diabetic-atherosclerotic rats and D-NAC. Like normal group, there were no aorta lesions in the diabetic groups received Crt and MB-92.

***In vitro* studies**

The effect of various treatments on the formation of various RSA glycation products is presented in Table 5. Fig.6 compares the contribution of each of the Milliard reaction products to a total (100%) inhibition of glycation by each treatment.

Glycation alters the folding and stability of RSA. Thus the secondary structures of RSA samples were also investigated by CD, and was shown in Fig.7. The percentages of the secondary structures of all samples are presented in Table 6. As it is seen, the secondary structure

of protein incubated with MB-92 was in closer similarity to the native RSA than other treatments.

Discussion

In this study, various antiglycating compounds, alone or in combination, were used and their efficiency in preventing the formation of glycation and oxidation products, inhibition of atherogenesis and diabetic complications was compared in the treated and untreated diabetic-atherosclerotic rat. The pathologic study indicated no alterations in the aorta tissue of the diabetic-atherosclerotic rats treated with Crt and MB-92. All of the mentioned treatments improved the glycemic control and lipid profile. They also reduced the concentrations of various glycation and oxidation products, and inflammatory marker; and induced the glyoxalase system. The inhibitory effect of these treatments on the *in vitro* glycation of RSA was also demonstrated. The obtained data indicated that MB-92 (as a combinatorial therapy) was more effective than each treatment alone.

Table 1 indicates a significant decrease in FBS due to the administration of all of these treatments in diabetic-atherosclerotic rats, but the best one was MB-92; NAC and Crt were in the following order. The hypoglycemic effects of the aqueous [42] and ethanolic [43] extracts of saffron, crocin [20], Gly [18, 25] and NAC [44] has been reported, previously. In addition, since the hypoglycemic effect of Crt was appeared at lower concentration than saffron aqueous extract [42] and crocin [20], it could be more effective than those. Diabetic-atherosclerotic rats under single and combined treatments of Crt had significantly lower levels of HOMA-IR, it means more insulin sensitivity than the diabetic rats and the diabetic group treated with NAC ($P<0.001$). In accordance with our results, it has also been reported previously that Crt decreased insulin

resistance in rat [45]; thus, one of the suggested mechanisms of the hypoglycemic effect of Crt and Crt containing treatment is the improvement of insulin sensitivity.

Chronic hyperglycemia results in the production of aggregated proteins and AGEs at different stages that subsequently causes the activation or inactivation of metabolic and/or signaling pathways [46]. Most studies on the antiglycating compounds have focused on the inhibition of protein aggregation and AGEs formation. While, all of the early, intermediates and end products of glycation have an important role in the initiation, propagation and development of diabetes complications [10, 11].

As indicated in our *in vivo* results (Table 2), all of the mentioned treatments showed the inhibitory effect on the early, intermediates and end products of glycation in diabetic rats. However, their effectiveness in various stages is slightly different. For example, gAlb concentrations in diabetic-atherosclerotic rats treated with Crt and NAC was significantly lower than MB-92. It means that the inhibitory effect of Crt and NAC alone was exerted in the first stage of glycation. While, the inhibitory role of MB-92 was mostly shown in the levels of pentosidine and fluorescent AGEs formation, which were significantly higher than those of Crt and NAC in the diabetic-atherosclerotic rats. Previous studies also showed the anti-AGEs properties of NAC [44] and Gly [18, 25] in diabetic rats. In addition, it has been shown that NAC has reduced MGO [47], but, combination of NAC with taurine has had no effect on the AGE inhibition [44]. The preventive effect of Crt on the pathologically AGEs-induced human vascular endothelial cell apoptosis have also been reported [22, 23].

The activity of glyoxalase system that was significantly decreased due to diabetes induction, was raised after administration of the mentioned treatments. Glyoxalase system has been introduced as a potent enzymatic defense system against glycation. Both glyoxalase I and

glyoxalase II was increased in all groups after treatment and had the highest level ($P<0.001$) in the group treated with MB-92.

These results indicated that the mentioned treatments, especially MB-92 not only significantly reduced FBS, as the glycation substrate, but also inhibited the formation of glycated products and induced the glyoxalase system, in diabetic-atherosclerotic rats. All of these are the reasons for the *in vivo* decreased glycation products.

The glycation pathway is accompanied with the oxidation; the process that has been named the glyoxidative pathway [27]. Thus, the antiglycating compounds that have the antioxidant property are the most powerful inhibitors of glycoxidation. Previous studies indicated that all of the compounds used in this study (Crt [21], NAC [48], Gly [18, 49]) possess the antioxidant activity. NAC also has the role as scavengers of GO [2] and MGO [26]. The inhibitory effect of Crt on some markers of oxidative stress, such as malondialdehyde has also been shown previously in model animals [37]. We recently showed the inhibitory effect of Gly on HbA1c and AGE formation [18]. In addition, Gly therapy inhibited the production of glycated and aggregated protein products in the lens of diabetic rats, increased the serum FRAP activity and induced the antioxidant enzymes in the lenses of diabetic rat. All of these caused a significant delay in the cataract appearance in rats [18].

Here, we investigated the effect of the mentioned treatments on various oxidative markers in diabetic-atherosclerotic rats. The results show that AOPP, a marker of oxidative stress, was increased in diabetic-atherosclerotic rats. However, it was significantly ($P<0.001$) lower in all treated normal and diabetic-atherosclerotic rats than the untreated groups. This parameter showed the lowest and highest values in the groups receive MB-92 and NAC, respectively.

Another marker of oxidative stress is the diene conjugates or DC as the early LDL oxidation marker that was significantly increased in diabetic-atherosclerotic rats. The inhibitory activity of NAC on DC formation has been reported, previously [50], which is confirmed in this study; but the lowest level ($P<0.001$) of DC was observed in the rat treated with MB-92 in both normal and diabetic groups.

The OFP level, as the end LDL oxidation marker, which was significantly increased in diabetic-atherosclerotic rats, also decreased significantly in the diabetic group treated with Crt and MB-92; but its level was in higher order in NAC treated rats. It means that NAC alone has a slight preventive effect on the formation of oxidation fluorescent products of LDL, but it is more useful in combinatorial therapy.

Hs CRP, as an inflammatory marker in all treated diabetic-atherosclerotic groups were significantly lower than the untreated group. It has been shown in the previous study that the NAC decreased the Hs CRP in the silica-induced lung fibrosis in rat [51]. Our results also confirmed this effect, but Crt and MB-92 was more effective than NAC alone. The level of this parameter in N-Crt was even significantly lower than normal group without treatment. This indicates the high potential of Crt as an anti-inflammatory compound.

Statistical analysis showed the significant ($p<0.001$) and good correlations ($r^2>0.97$) between FBS and all of the mentioned parameters of insulin resistance (HOMA-IR), glycated products (gAlb, GO, MGO, pentosidine, AGEs), oxidation products (AOPP, DC, FOP), an inflammatory marker (HsCRP) and atherogenic index in the treated and untreated diabetic rats. While there was a negative correlation ($r^2> -0.98$) between FBS and both glyoxalases I and II.

These data confirmed the effectiveness of the treatments, especially MB-92 in diabetic-atherosclerotic rats.

In addition to the above mentioned explanations, all treatments in this study showed beneficial effect on the TG, Chol, HDL and LDL, as well as the atherogenic index in the diabetic-atherosclerotic rats in comparison with the untreated group. There were no significant differences between the TG levels in D-NAC with D-Crt and D-MB-92; but the level of TG was lower significantly ($P<0.001$) in D-MB-92 than in D-Crt. The potential of the mentioned treatments to reduce the Chol was in the following order: MB-92> NAC>Crt. As mentioned above, the HDL was increased due to all treatments in the diabetic-atherosclerotic rats and no significant differences were observed between them. The LDL levels were also decreased due to the treatments, but the combinatorial therapy the most effective on decreasing the LDL level and thus, the improvement of the atherogenic index. The beneficial effect of Crt on TG, Chol and LDL in non-diabetic quail under atherogenic diet has been shown previously, and the most effective dose has been reported as 100 mg/kg [37]. Therefore, based on the obtained results in the present study, the synergism between NAC, Gly and Crt caused the most beneficial effect on the lipid profile of the diabetic-atherosclerotic rats.

Pathologic data confirmed our experimental data on the prediction of atherogenesis. The untreated diabetic-atherosclerotic rats demonstrated both of the neointima and atheromatous plaque in the aorta, Fig.5. However, no lesions were observed in the aorta of the normal and diabetic groups received Crt and MB-92. It is in accordance with the previously reported data about the role of Crt in decreasing the accumulation of macrophage-derived foam cells in the aortic intima of quails and inhibited the thickening of intima [37]. Between the treated groups,

neointima formation was only observed in the diabetic-atherosclerotic rats treated with NAC. Since NAC showed the lower inhibitory effect in the formation of some glycation, oxidation and inflammatory markers (like GO, AGEs, DC, FOP and HsCRP) than two other treatments in these rats, the aorta predisposed to neointima or hyperplasia. It is important to note that this effect was not observed in the proposed combinatorial therapy. It means that the synergism removed the side effect of NAC.

To ensure about the inhibitory effect of the mentioned treatments on protein glycation, aggregation and misfolding, the RSA glycation and glycation-inhibition was studied as a model protein in the *in vitro* condition. For this purpose similar treatment as the *in vivo* experiments in addition to some other combinations were tested. As depicted in Table 5 and Fig.6, all of the applied treatments showed antiglycating activity and inhibited gAlb formation; however Crt and NAC were better than Gly. Then, the effect of various combinations was tested. Among them, Crt+NAC, and MB-92 showed the better inhibitory effect. According to our literature survey, the inhibitory effect of Crt on β-amyloid formation has been shown [24]; here we also observed the Crt antiglycating effect and inhibition of gAlb formation and aggregation in both *in vivo* and *in vitro* studies.

Our results showed that NAC is in the second order after Crt, in the antiglycating activity. These data indicated that 7.5 mmol/L of NAC acts as a potent inhibitor of the Amadori product, but the previously reported data indicated the ineffectiveness of its lower concentration (2 mmol/L) on BSA glycation [27]. Therefore, the inhibitory role of NAC on protein glycation is concentration dependent.

The role of NAC as the scavenger of both GO and MGO [52] and fluorescent AGEs have been reported previously [27, 44]. Here, we investigated the effect of the mentioned treatments

on the formation of the glycation intermediates and end products, including GO, MGO, pentosidine and fluorescent AGEs. The orders of the inhibitory effect of the mentioned treatments on *in vitro* formation of these compounds are as follows:

gAlb: Crt>NAC>Crt+NAC>MB-92>Crt+Gly>Gly

GO: Crt+NAC>MB-92> Crt>Gly>NAC> Crt+Gly

MGO: Crt+NAC> MB-92> Gly> Crt> NAC> Crt+Gly

Pentosidine: Crt+Gly> MB-92> Gly> NAC> Crt+NAC> Crt

Fluorescent AGEs: MB-92> Crt+Gly> Crt+NAC>NAC> Gly> Crt.

These data indicated that Crt and NAC are more effective in the inhibition of the first stages of glycation, while MB-92 is more effective in the inhibition of the last stage. Comparing the inhibitory effect of the chosen treatments on *in vitro* RSA glycation products with those obtained in the rat model of diabetes-atherosclerosis also revealed some differences. For example, Crt alone, mostly inhibited the first stages (gAlb and MGO formations) in the *in vitro* condition, but its *in vivo* effect was mostly on the last stages (AGEs, GO and MGO formations). These differences may be related to the direct interaction of each ligand with the protein that maintain it from the glucose attack, as previously shown the direct interaction of Crt with Alb [53]. However, in the *in vivo* condition other parameters are also involved. For example the glyoxalase system, antioxidant activity and so on, that we investigated some of them here. The presence of this system, potentiates the *in vivo* antiglycating activity of the mentioned treatments. Comparing Fig.3 with Fig.6 indicated that the observed synergistic effect of MB-92 in both *in vivo* and *in vitro* conditions couldn't simply be a concentration effect of depleting effective glucose concentration.

Previous studies have shown that glycation of bovine or human serum albumin leads to some changes in their secondary structure determined by CD spectroscopy [19, 54, 55]. Fig.7 and Table 6 indicated the reduction of α–helix from 67% in RSA to 3% in the gAlb, which was accompanied by increasing of ß-sheet from 3% to 30.7%, random coil from 21% to 48% and ß-turn from 9 to 19% in the protein of rat origin. Similar changes have been reported previously on human and bovine albumins [19, 54]. The effect of some antiglycating compounds on maintaining the secondary structure of serum albumin has also been shown previously [19]. All of these reports are consistent with the present study and indicated the decrease in the α-helix and increase in the ß-sheet contents of protein due to glycation that compensated in various degrees by different antiglycating compounds. Misfolding of a protein due to the increase in β-sheet content, result in the aggregate formation; and has been known as the origin of diabetic complications [1, 2]. Among the treatments used in this and previous studies, the combination of Crt with NAC and Gly (MB-92) was the most powerful treatment in maintaining the serum albumin secondary structure. Our previous study indicated the glycation induced conformational change of histone H1 is exactly the same in both *in vitro* and *in vivo* conditions [56].

Although each of the NAC and Crt here, and Gly in our previous study [18], was separately used to treat diabetic groups and showed various degrees of beneficial effects, but combinatorial therapy using MB-92 (Crt, NAC and Gly) with higher potency of antiglycating effect, inhibition of misfolded proteins and aggregate formation, increasing glyoxalase system activity, higher level of antioxidant and anti-inflammatory activities in addition to its beneficial effect on the lipid profile, and preventing the atherosclerotic plaque formation was batter than the other treatments in this study.

The result of the present study is applicable for diabetic patients, except the administration type. NAC and Gly were administered here in drinking water whereas Crt was injected. Since the injection is not a preferred method for human and Crt is sensitive to light, and is not water soluble, preparation of the tablet or capsule containing all three components is suggested for diabetic patients.

Conclusion. The synergism between Gly, Crt and NAC at the applied concentrations in MB-92 showed the most beneficial effect in diabetic-atherosclerotic rat. The hypoglycemic, antiglycating, antiaggregation, antioxidant and anti-inflammatory effects accompanying with the improvement in the lipid profile and insulin sensitivity, are factors that potentially inhibited the atheromatous plaque formation in diabetic-atherosclerotic rat.

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This manuscript is completely repeatable by any other research group all around the world and is recommended for application in human.

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Figures Legends

Fig.1. Formation of Amadori-glycated proteins and advanced glycation end products (AGEs) and their putative role in diabetes complications. This figure represents the mechanisms of glycated albumin and AGEs in the development of diabetes complications.

Fig.2. Chemical structure of crocetin, N-acetyl cysteine and glycine.

Fig.3. Comparison of the contribution of each of the Milliard reaction products to a total (100%) inhibition of glycation by each of the mentioned treatments in the serum of diabetic-atherosclerotic rats under different treatments.

Fig.4. Activity of the glyoxalase I (up) and glyoxalase II (down) in the serum of all rat groups.

[a] Indicates significance of data comparing group N with other groups ($P < 0.001$).

[b] Indicates significance of data comparing group D with other groups ($P < 0.001$).

[c] Indicates significance of data comparing group D-Crt vs. other groups ($P < 0.001$)

[d] Indicates significance of data comparing group D-NAC vs. other groups ($P < 0.001$)

Fig.5. Effect of the desired treatments on atheromatous plaque formation in normal and diabetic-atherosclerotic rats (stained by H&E & original magnification ×200).

(a) Normal aorta of the normal and diabetic rats treated with Crt and MB-92.

(b) Neointima formation in diabetic groups untreated and treated with NAC.

(c) Atheromatous plaque formation in untreated diabetic group.

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Fig.6. Comparison of the contribution of each of the Milliard reaction products to a total (100%) inhibition of glycation by each of the mentioned treatments on the RSA in the *in vitro* condition.

Fig.7. The CD spectra of RSA in far-UV region after 3 months incubation at 37 ºC in the absence and presence of glucose 50 mM with or without the mentioned treatments. Analysis of the percentage of secondary structure of RSA in each situation is presented in Table 6.

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Table 1. Levels of FBS, insulin and HOMA-IR in the treated and untreated normal and diabetic rats.

Group Name	Treatment	FBS (mmol/L)	Insulin (U/ml)	HOMA-IR
Normal	-------	4.48 ± 0.25^{b}	19.00 ± 0.90^{b}	3.78 ± 0.30^{b}
	Crt	4.76 ± 0.31^{b}	18.10 ± 1^{b}	3.83 ± 0.20^{b}
	NAC	4.44 ± 0.32^{b}	18.20 ± 0.80^{b}	3.63 ± 0.20^{b}
	MB-92	5.00 ± 0.29^{b}	$17.10 \pm 0.70^{a,b}$	3.80 ± 0.40^{b}
Diabetic	-------	15.55 ± 0.46^{a}	8.80 ± 0.60^{a}	6.20 ± 0.50^{a}
	Crt	$11.27 \pm 0.29^{a,b}$	$11.50 \pm 0.30^{a,b,d}$	$5.39 \pm 0.20^{a,b,d}$
	NAC	$10.22 \pm 0.29^{a,b}$	$12.80 \pm 0.70^{a,b}$	5.80 ± 0.40^{a}
	MB-92	$9.55 \pm 0.40^{a,b,c}$	$11.40 \pm 0.50^{a,b,d}$	$4.84 \pm 0.20^{a,b,d}$

[a] Indicates significance of data comparing group N vs. other groups ($P < 0.001$).

[b] Indicates significance of data comparing group D vs other groups ($P < 0.001$).

[c] Indicates significance of data comparing group D-Crt vs. other groups ($P < 0.001$)

[d] Indicates significance of data comparing group D-NAC vs. other groups ($P < 0.001$)

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Table 2. Levels of the glycation products in the absence and presence of the mentioned treatments in the normal (N) and diabetic-atherosclerotic (D) rats.

Group	Treatment	Glycation Product				
		gAlb (µmol/L)	Methylglyoxal (µmol/L)	Glyoxal (µmol/L)	Pentosidine (µmol/L)	Fluorescent AGEs (AU)
N	------	80.80±4.60[b]	16.80±3.55[b]	15.60±1.60[b]	42.00± 4.73[b]	47.66± 5.9[b]
	Crt	85.70±5.60[b]	13.80±3.40[b]	9.60±2.90[b]	34.80±5.40[b]	41.30±4.20[b]
	NAC	79.80±5.75[b]	8.90±1.45[b]	10.91±0.81[b]	40.33±4.80[b]	39.90±4.80[b]
	MB-92	91.00±7.00[b]	11.20±2.60[b]	12.30±1.70[b]	38.50±4.60[b]	45.50±3.90[b]
D	-------	402.00±10.70[a]	93.00±5.10[a]	103.80±6.00[a]	446.00±10.7[a]	363.70±9.00[a]
	Crt	302.50±8.10[a,b]	57.20±4.10[a,b]	43.70±4.10[a,b]	314.50±6.10[a,b]	176.80±6.30[a,b]
	NAC	291.55±5.90[a,b]	54.00±5.00[a,b]	66.00±8.30[a,b,c]	294.00±6.70[a,b,c]	184.80±6.70[a,b]
	MB-92	318.15±7.20[a,b,c,d]	29.30±6.60[a,b,c,d]	50.00±5.70[a,b,c,d]	252.80±6.30[a,b,c,d]	157.33±6.40[a,b,c,d]

[a] Indicates significance of data comparing group N vs. other groups ($P < 0.001$).

[b] Indicates significance of data comparing group D vs other groups ($P < 0.001$).

[c] Indicates significance of data comparing group D-Crt vs. other groups ($P < 0.001$).

[d] Indicates significance of data comparing group D-NAC vs. other groups ($P < 0.001$).

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Table 3. Effect of the mentioned treatments on the lipid profile of the normal (N) and diabetic-atherosclerotic (D) rats.

Group	Treatment	TG (mmol/L)	Chol (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	Atherogenic Index (LDL/HDL)
N	------	0.93 ± 0.02^{b}	2.22 ± 0.07^{b}	1.4 ± 0.05^{b}	0.33 ± 0.01^{b}	0.36 ± 0.02^{b}
	Crt	0.95 ± 0.01^{b}	2.25 ± 0.08^{b}	1.34 ± 0.05^{b}	0.47 ± 0.02^{b}	0.41 ± 0.02^{b}
	NAC	0.85 ± 0.04^{b}	2.35 ± 0.04^{b}	1.3 ± 0.04^{b}	$0.68 \pm 0.01^{a,b}$	0.44 ± 0.01^{b}
	MB-92	0.84 ± 0.02^{b}	2.36 ± 7.00^{b}	0.97 ± 0.06^{b}	0.69 ± 0.02^{b}	0.32 ± 0.02^{b}
D	------	2.83 ± 0.12^{a}	6.46 ± 0.24^{a}	0.43 ± 0.01^{a}	4.73 ± 0.24^{a}	11.02 ± 0.44^{a}
	Crt	$1.42 \pm 0.06^{a,b}$	$5.11 \pm 0.18^{a,b}$	$0.82 \pm 0.03^{a,b}$	$3.62 \pm 0.16^{a,b}$	$4.51 \pm 0.31^{a,b}$
	NAC	$1.30 \pm 0.29^{a,b}$	$4.66 \pm 0.16^{a,b}$	$0.90 \pm 5.00^{a,b}$	$3.61 \pm 0.15^{a,b,c}$	$3.57 \pm 0.27^{a,b}$
	MB-92	$1.27 \pm 0.04^{a,b,c}$	$4.07 \pm 0.16^{a,b,c,d}$	$0.89 \pm 0.04^{a,b,c}$	$2.59 \pm 0.06^{a,b,c,d}$	$2.54 \pm 0.20^{a,b,c,d}$

[a] Indicates significance of data comparing group N vs other groups ($P < 0.001$).

[b] Indicates significance of data comparing group D vs other groups ($P < 0.001$).

[c] Indicates significance of data comparing group D-Crt vs. other groups ($P < 0.001$).

[d] Indicates significance of data comparing group D-NAC vs. other groups ($P < 0.001$).

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Table 4. Effect of the mentioned treatments on the oxidative and inflammarory markers of the normal (N) and diabetic-atherosclerotic (D) rats. DC: Diene conjugates, FOP: Fluorescent oxidation products of LDL, AOPP: Advanced oxidation protein products, and Hs CRP: High sensitivity C-reactive protein.

Group	Treatment	DC (µmol/L)	FOP (AU)	AOPP (µmol/L)	Hs CRP (ng/mL)
N	-------	15.98±0.90[b]	248.80±12.11[b]	28.85±0.99[b]	235.02± 13.07[b]
	Crt	15.08±0.63[b]	240.16±10.22[b]	16.90±0.87[a,b]	202.13±12.97[a,b]
	NAC	14.06±0.77[b]	244.38±11.07[b]	19.27±0.85[a,b]	234.09±15.43[b]
	MB-92	14.00±0.59[b]	195.71±9.11[a,b]	18.41±0.84[a,b]	225.66±13.74[b]
D	-------	108.33±3.40[a]	515.66±22.37[a]	89.31±3.25[a]	596.13±32.66[a]
	Crt	69.20±0.33[a,b]	471.45±18.12[a,b]	75.01±3.01[,b]	441.54±23.77[a,b]
	NAC	83.00±3.20[a,b,c]	500.22±23.29[a]	69.81±3.47[a,b,c]	538.76±29.54[a,b,c]
	MB-92	58.20±2.80[a,b,c,d]	457.11±19.05[a,b,c,d]	57.03±2.42[a,b,c,d]	403.81±24.12[a,b,c,d]

[a] Indicates significance of data comparing group N vs other groups ($P < 0.001$).

[b] Indicates significance of data comparing group D vs other groups ($P < 0.001$).

[c] Indicates significance of data comparing group D-Crt vs. other groups ($P < 0.001$).

[d] Indicates significance of data comparing group D-NAC vs. other groups ($P < 0.001$).

Table 5. Effect of different treatments (single or combination) on formation of various glycation products of rat serum albumin (RSA) in the *in vitro* experiment. The data in rows 1 and 2 show the values related to the native and glycated protein, respectively.

Row	Tube Content	Glycation Products				
		glycated albumin (µmol/L)	Methyglyoxal (µmol/L)	Glyoxal (µmol/L)	Pentosidine (µmol/L)	Florescent AGEs (AU)
1	RSA	120.00±3.40	3.85±0.10	9.45±0.10	4.60±0.15	55.00±1.00
2	RSA+Glc	1150.00±10.10	9.85±0.20	30.45±0.40	21.80±0.11	499.00±5.10
3	(RSA+Glc)+(Crt)	345.00±6.10	5.72±0.10	22.86±0.42	15.30±0.23	474.00±4.20
4	(RSA+Glc)+(NAC)	380.00±5.10	8.15±0.22	26.50±0.37	11.90±0.07	377.00±2.20
5	(RSA+Glc)+(Gly)	1047.00±7.50	4.85±0.11	25.02±0.39	11.00±0.22	443.00±5.00
6	(RSA+Glc)+(Crt+Gly)	663.00±6.20	7.98±0.11	27.03±0.22	7.00±0.16	234.00±2.50
7	(RSA+Glc)+(Crt+NAC)	388.00±3.50	4.51±0.18	19.64±0.28	13.60±0.19	322.00±3.10
8	(RSA+Glc)+(MB-92)	403.00±4.60	4.55±0.07	20.70±0.17	10.80±0.16	200.00±2.10

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Table 6. Comparison between the percentage of secondary structures of rat serum albumin (RSA) in the presence and absence of glucose (Glc) and the mentioned treatments in the *in vitro* study. The data in the rows 1 and 2 show the values for native and glycated RSA, repectively.

Row	Tube Content	Percentage of Secondary Structure			
		α%	β%	Turn%	Random Coil %
1	RSA	67.0	3.0	9.0	21.0
2	RSA+Glc	2.3	30.7	19.0	48.0
3	(RSA+Glc)+(Crt)	8.0	28.0	18.0	46.0
4	(RSA+Glc)+(NAC)	13.0	25.5	16.5	45.0
5	(RSA+Glc)+(Gly)	5.0	30.3	17.7	47.0
6	(RSA+Glc)+(Crt+Gly)	18.0	23.0	15.3	43.7
7	(RSA+Glc)+(Crt+NAC)	35.0	15.0	9.9	40.1
8	(RSA+Glc)+(MB-92)	53.0	8.0	5.7	33.3

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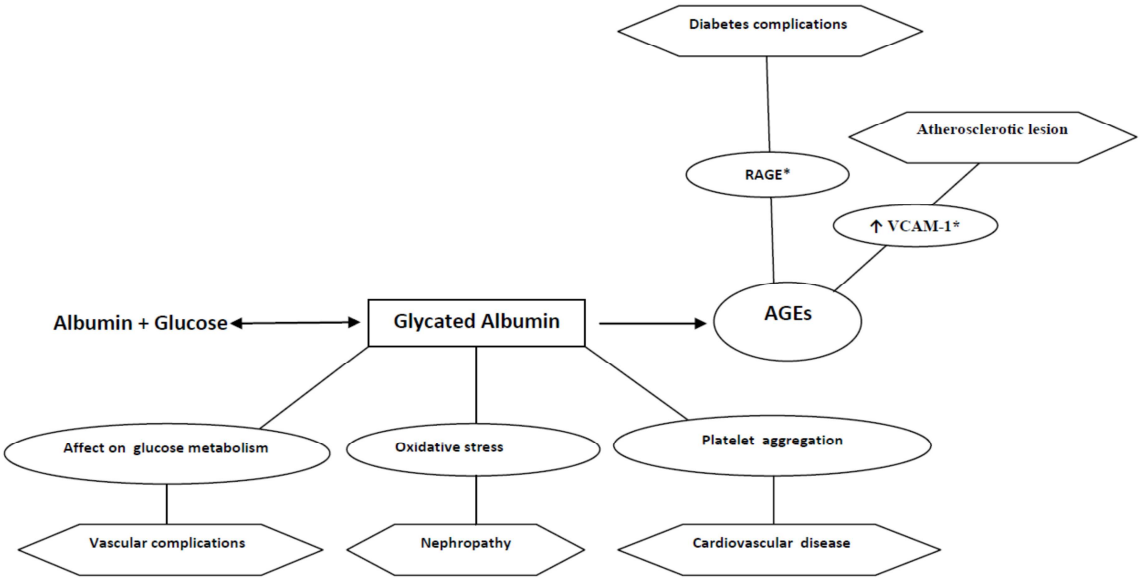
Fig. 1



* vascular cell adhesion molecule (VCAM-1)

* Receptor for AGEs(RAGE)

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Fig. 2

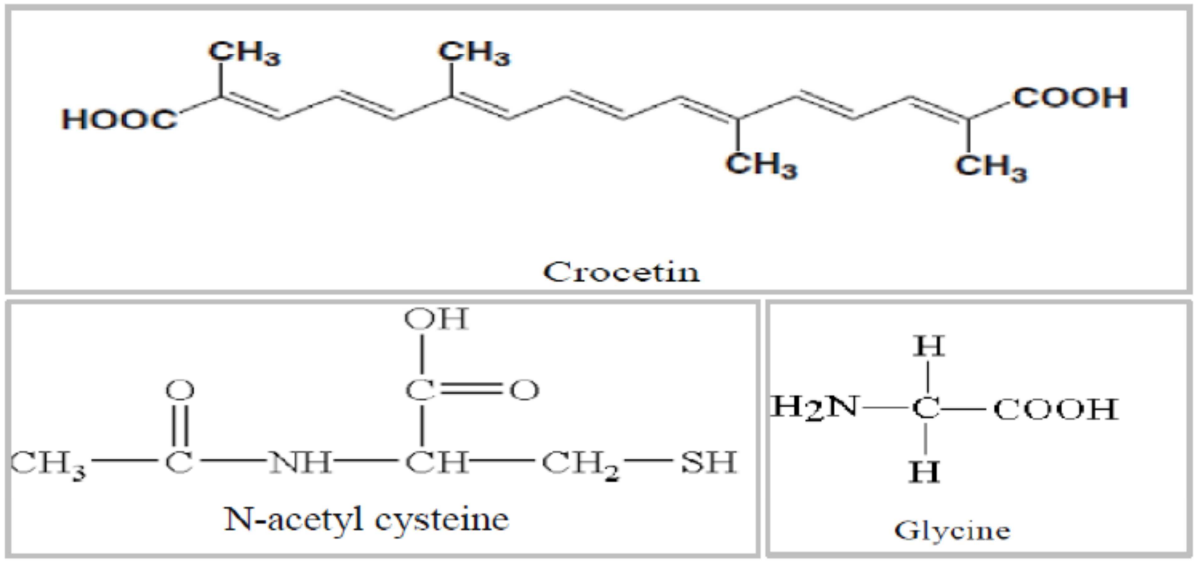

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Fig.3

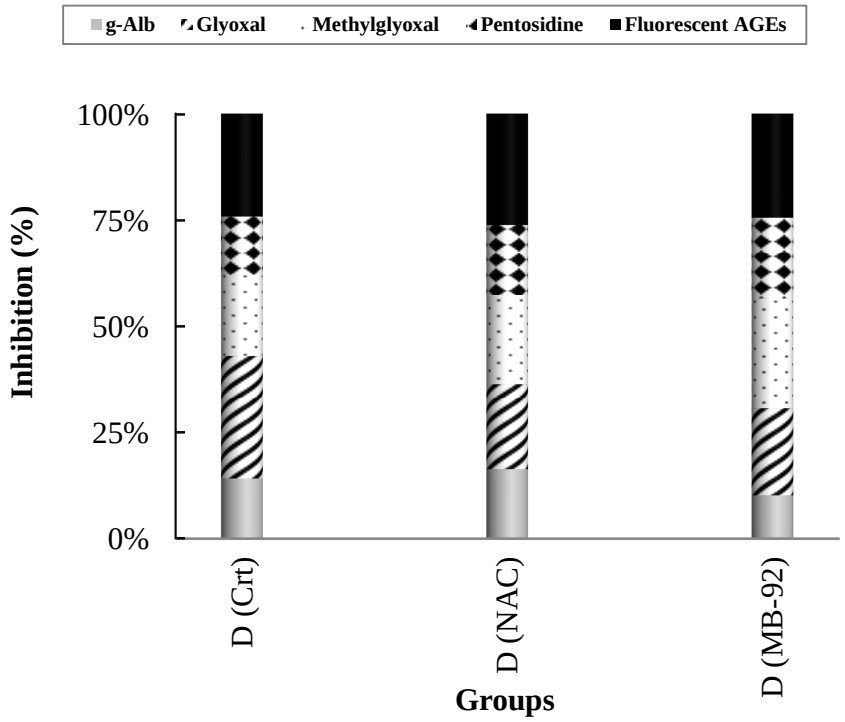

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Fig. 4

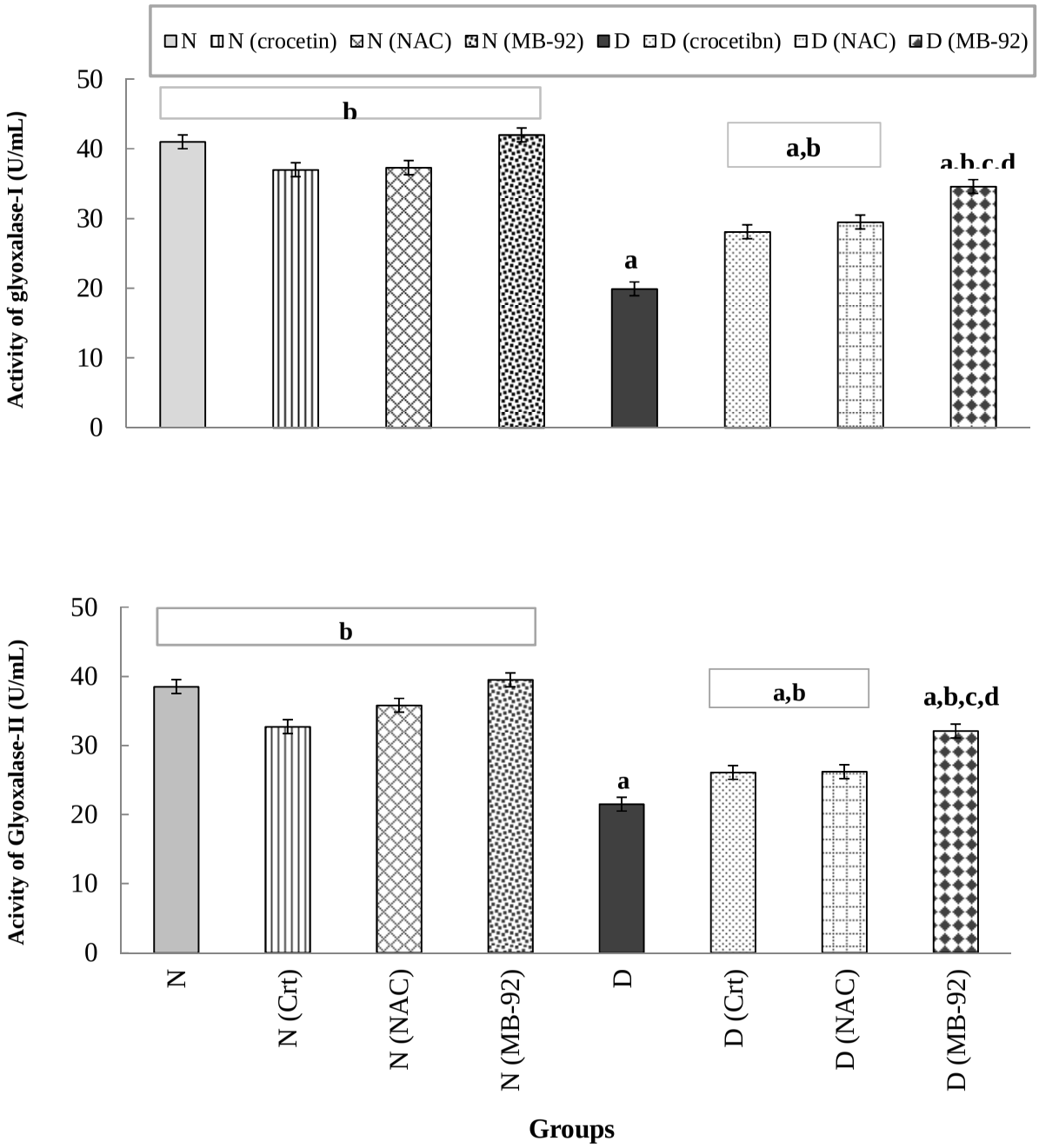

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Fig.5

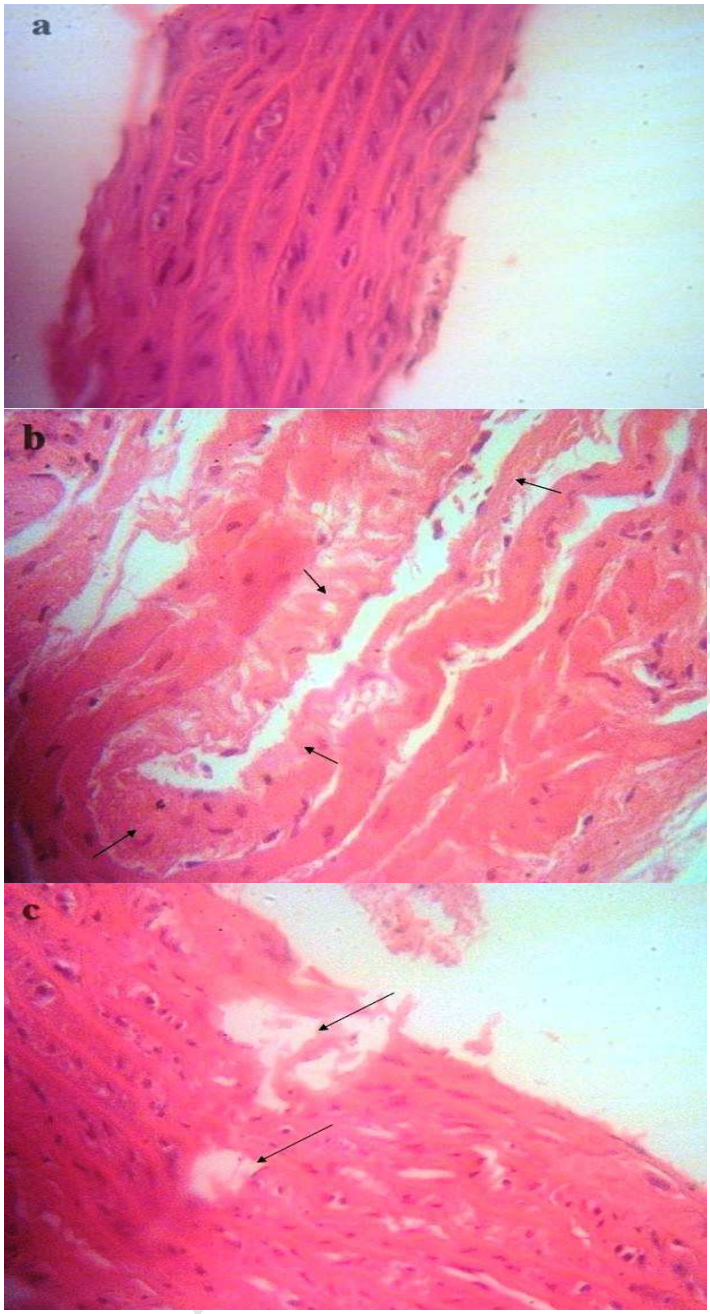

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Fig. 6

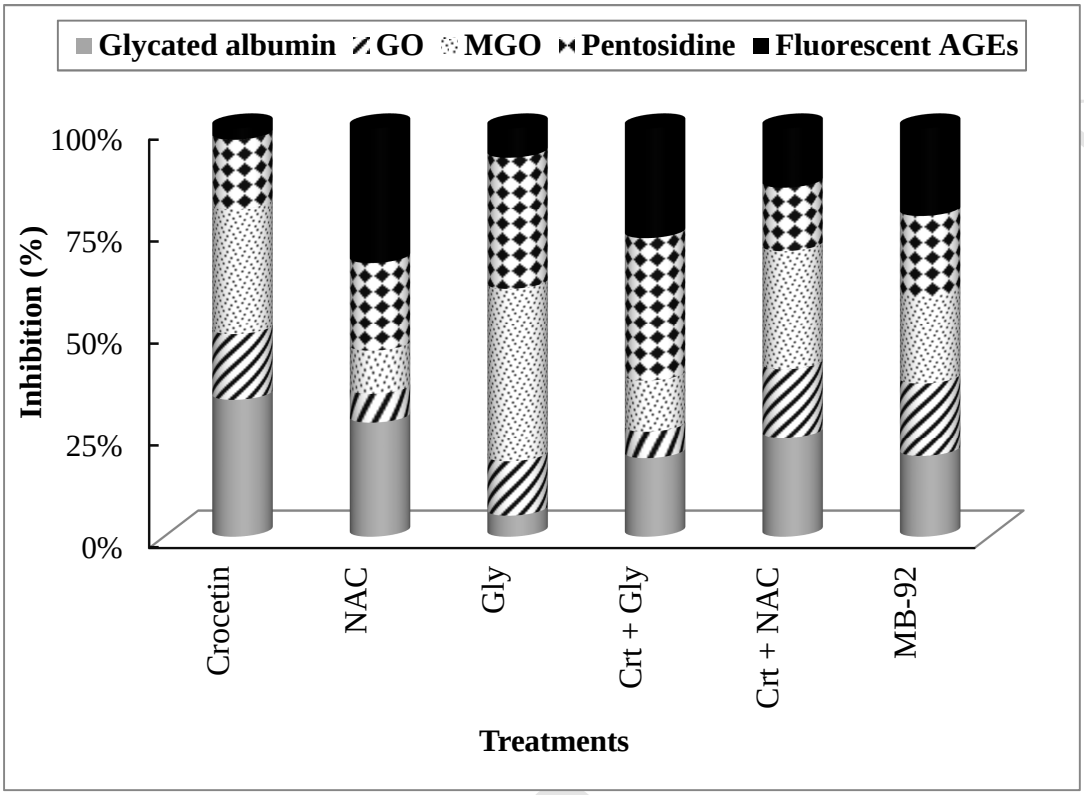

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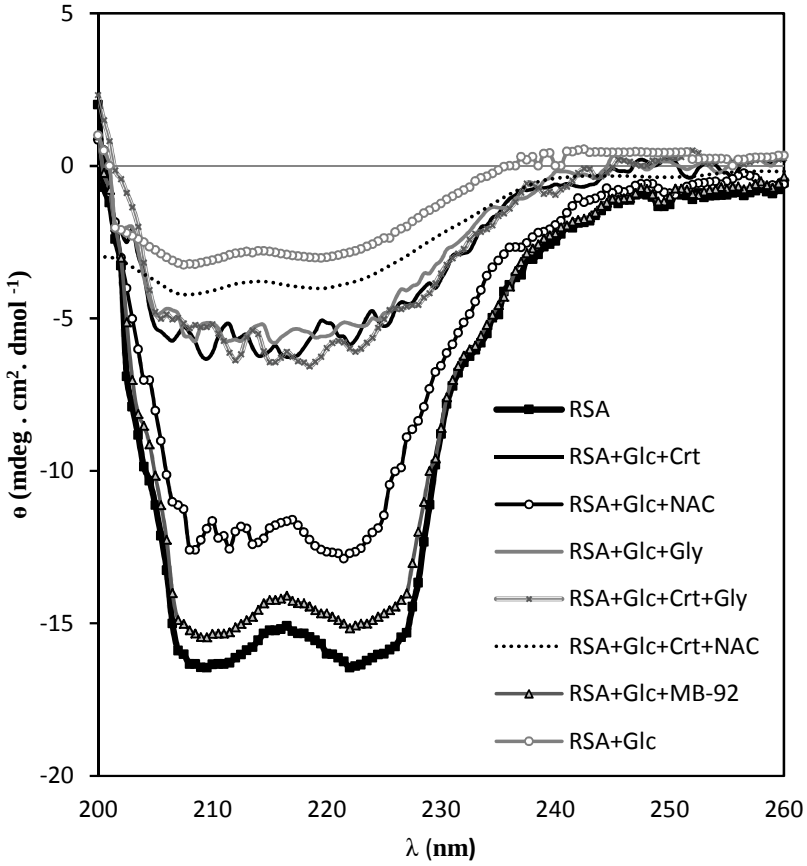
Fig.7



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- A combination of crocetin, glycine and N-acetyl cysteine (MB-92) was designed and tested here.

- MB-92 reduced FBS and insulin resistance, and improved lipid profile in diabetic rats.

- MB-92 reduced glycation, oxidation and inflammatory markers in these rats.

-MB-92 increases glyoxalases I and II activities in the mentioned rats.

- MB-92 prevents atheromatose plaque formation in the mentioned rats.

-MB-92 inhibited the glycation intermediates, aggregation and misfolding of proteins.

-MB-92 was more effective than each treatment alone.

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