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Inhibition of the hepatic glucose output is responsible for the hypoglycemic effect of *Crataegus aronia* against type 2 diabetes mellitus in rats

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Abstract: This study aimed to analyze the ameliorative effect of *Crataegus aronia* against type 2 diabetes mellitus (type 2-DM). Type 2-DM rats were treated with the extract and the changes in serum parameters (glucose, insulin, HbA_{1c} and lipids) and hepatic parameters (oxidative stress, inflammation and mRNA levels of GLUT-2 and gluconeogenesis enzymes) were compared to those of control and untreated type 2-DM rats. Also, levels of hepatic insulin receptors 1A (IR-1A) were measured immunohistochemically and compared between groups. In type 2-DM rats, *C. aronia* significantly improved the oral glucose tolerance test (OGTT), lowered plasma glucose, serum lipid levels and the hepatic glycogen content. Also, it significantly lowered the levels of hepatic lipid peroxidation, tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) and enhanced the level of reduced glutathione (GSH) and increased superoxide dismutase (SOD) activity. *C. aronia* enhanced hepatic mRNA expression of the insulin receptor A isoform (IR-A) and glucose 6-phosphatase (G6Pase), and lowered glucose transporter-2 (GLUT-2) and glycerol kinase (GK) mRNA expression. In conclusion, *C. aronia* ameliorates T2DM by inhibiting hepatic glucose output.

Key words: *C. aronia*; insulin receptor; liver; GLUT-2; type 2 diabetes mellitus

INTRODUCTION

Noninsulin-dependent diabetes mellitus (NIDDM or type 2-DM) is the most common form of diabetes mellitus and accounts for more than 90% of diabetes patients [1]. Current understanding of type 2-DM progression indicates that insulin resistance in peripheral tissues results in compensatory hyperinsulinemia, followed by β-cell failure, which initially leads to prandial and later to overt fasting hyperglycemia [1,2].

The liver is the main organ regulating glucose output through two main mechanisms, glycogenolysis and gluconeogenesis. These different processes are regulated via different mechanisms in response to plasma glucagon, nonesterified fatty acids (NEFAs), insulin [3] and neural input [4]. Circulatory NEFAs, glucagon, and catecholamines stimulate hepatic glucose synthesis and secretion, whereas circulatory insulin suppresses this process via stimulation of several intracellular protein substrates [5]. At present, there is still a debate as to whether muscle or hepatic insulin resistance is the key player in the progression of type 2-DM.

Muscle insulin resistance, as determined by the euglycemic hyperinsulinemic clamp, is a major risk factor for the development and pathogenesis of type 2-DM in both human and animal models [6]. However, the pathophysiology of hyperglycemia in established type 2-DM has been suggested to be related to hepatic not muscle insulin resistance. This is not surprising, as previously it was shown that calorie restriction in this animal model resulted in a fall in liver fat content, normalization of hepatic insulin sensitivity and fasting plasma glucose levels, with no change in

muscle insulin resistance [7]. Also, mice completely deficient in skeletal muscle insulin receptors (IR) do not develop diabetes [8], and individuals with inactive muscle glycogen synthase are not necessarily hyperglycemic [9].

Various triggers produce hepatic insulin resistance, and the associated hyperglycemia in patients or animals with type 2-DM is due to interference with insulin receptor expression or signaling. Accumulating evidence suggests that high levels of reactive oxygen species (ROS) [10-12], inflammation [13,14] and hepatic steatosis trigger type 2-DM-associated insulin resistance [15].

In this regard and based on the role of hepatic insulin resistance in the development of the hyperglycemic state, an ideal therapeutic agent should have hypoglycemic, hypolipidemic and antioxidant potentials. The currently available oral synthetic agents against type 2-DM-induced hyperglycemia have several serious side effects and are expensive, especially for patients in developing countries [16]. Therefore, the search for effective, low-cost hypoglycemic agents of plant origin with fewer side effects is promising and is recommended by many authors [17].

Crataegus aronia, which is a dominant species in the mountains of the Mediterranean basin, has been extensively used in traditional Arab medicine to treat various conditions, including cardiovascular disease, cancer, hyperlipidemia and diabetes [18]. Recently, *C. aronia* has been shown to have anti-obesity and hypoglycemic effects in animals via inhibition of pancreatic lipase activity [19]. However, in spite of the well-known effect of *C. aronia* in traditional medicine in decreasing blood glucose levels and reducing the associated macro- and microvascular complications, no strong scientific evidence is available in the literature. Hence, in this study, we investigated the ability of *C. aronia* to reverse some clinical complications linked with type 2-DM, such as hyperglycemia, hyperlipidemia and insulin resistance, and examined some of the mechanisms of its action by targeting the modulators of hepatic insulin signaling and gluconeogenesis.

MATERIALS AND METHODS

Plant material and extraction

This procedure was done in accordance with our previous work [20]. In brief, fresh hawthorn *(C. aronia)* whole plants (stems, leaves and flowers) were purchased from a local market. The plant was identified, dried and extracted in the Department of Pharmacology, College of Pharmacy, King Khalid University. The dried plant material was ground to a powder and extracted by maceration using distilled water (1 kg/L, w/v) for 3 days at 37°C. The aqueous extract was then filtered and evaporated and the dried residue (32 g) produced was then stored at 4°C. At the time of use, the residue was reconstituted in distilled water and filtered through 0.2-μm filters to the desired concentration.

Animals

Adult healthy male Wistar rats (240±10 g, 9 weeks old) were included in the study and were supplied, maintained and treated in the animal house of the College of Medicine, King Khalid University, Abha, KSA. The rats were fed standard rat pellets and allowed free access to water before the experiment. Before and during the experimental procedures, the rats were housed in a controlled ambient temperature of 25±2°C and 50±10% relative humidity, with a 12-h light/dark cycle. All procedures used in this study were performed with the approval of the Research Ethics Committee (REC) at the College of Medicine, King Khalid University, Abha, Saudi Arabia, which are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

DEVELOPMENT OF TYPE 2-DM RATS

To produce type 2-DM experimental rats, we followed the protocol of Mansor et al. [21] with slight modifications. The rats were allocated two dietary regimens; either they were subjected to a normal standard chow diet (66% calories from carbohydrate, 22% from protein and 12% from fat) or to a high-fat diet (HFD) (58% fat, 25% protein and 17% carbohydrate) for 2 weeks. The HFD used in the current study was previ-

ously described by Srinivasan et al. [22] (Supplementary Table S1). To induce type 2-DM, rats were fasted overnight at the end of day 14 when they were injected a single low i.p. dose of streptozotocin (35 mg/kg, STZ in citrate buffer, pH 4). All rats continued feeding on their initial diets for another 7 days. On day 21, the animals fasted overnight and their blood glucose was measured. Rats with blood glucose higher than 300 mg/dL were considered diabetic and were included in the experimental design. All rats were returned to the standard diet once the experimental procedure was started.

Experimental design

Once type 2-DM was established in rats, the experimental animals were divided into three groups (n=6, each) and treated as follows: Group 1 was the control nondiabetic group that was administered 0.5 mL distilled water orally for 60 days. Group 2 was the type 2-DM group that was administered 0.5 ml distilled water orally for 60 days. Group 3 was the type 2-DM group of rats that were treated with an oral dose of *C. aronia* aqueous extract (500 mg/kg bw) in a final volume of 0.5 mL of distilled water for 60 days. The dosage was adjusted every week according to any change in body weight (bw) to maintain a similar dose/kg bw of the rat over the entire period of study. Food intake was also monitored weekly. At the end of the experiment, the animals were euthanized with sodium pentobarbital (75 mg/kg, i.p.) and blood samples and liver tissues were obtained.

Biochemical study

On the day before euthanization, all animals were weighed and an oral glucose tolerance test (OGTT) was performed. Blood samples were obtained from the lateral tail vein of rats deprived of food overnight (10-12 h). Successive blood samples were taken at 0, 30, 60, 90 and 120 min intervals following the administration of glucose solution (3 g/kg bw) through gastric intubation. Plasma glucose and insulin levels were measured using a special rat glucose colorimetric kit (Cat. No. 10009582, Caymen, Fl, USA) and an insulin ELISA kit (Cat No. 10-1250-01, Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Blood chemistry

From each rat, 1 mL of blood was collected in a heparinized (20 μL, 200 IU/ml) Eppendorf tube. The serum was prepared by centrifuging at 5000 rpm for 10 min and used to determine levels of serum total cholesterol (TC), triglycerides (TGs) and HDL cholesterol (HDL-c) using special commercially available kits according to the manufacturer's instructions. Serum LDL-cholesterol (LDL-c) level was calculated according to Friedewald's formula [23]: (LDL-c=TC-TGs/5-HDL-c), and the serum VLDL cholesterol (VLDL-c) concentration was calculated according to Nobert formula [24]: (VLDL-c=TGs/5). Blood glycosylated hemoglobin ($HbAC_1$) was determined in the heparinized blood samples by the Helena GLYCO-Tek affinity column method (Helena Laboratories, USA).

Tissue collection

Livers were rapidly excised, washed carefully with ice-cold phosphate buffer saline (PBS; pH 7.4) and then stored at -80°C for further biochemical and molecular analysis. Both left and right epididymal fat pads were excised, trimmed and weighed for assessment of adiposity.

Measurement of the liver glycogen content

Liver glycogen was determined using the method described by Lo et al. [25]. Briefly, samples were homogenized in potassium hydroxide (KOH) solution and incubated in boiling water for 30 min. After addition of 90% ethanol, the samples were incubated overnight at 4°C, followed by centrifugation at 1000 g for 30 min at 4°C. Absorbance was measured at 490 nm. Bovine liver glycogen (Sigma Chemical Co., St. Louis, MO, USA) was used as a standard.

Oxidative stress assays

Lipid peroxidation in the liver homogenates was measured by the thiobarbituric acid (TBA) reaction using an assay commercially available kit (Cat No. NWK-MDA01, NWLSS, USA). Superoxide dismutase (SOD) activity in liver homogenates was measured using an assay commercially available kit (Cat. No. 706002, Cayman Chemical, Ann Arbor, MI, USA). All procedures

were carried out as per the manufacturer's instructions. Levels of total reduced glutathione (GSH) were determined manually according to the method of Jollow et al. [26] with slight modification. In brief, the liver homogenate was mixed with 4% sulfosalicylic acid (w/v) at a 1:1 ratio (v/v). The samples were incubated at 4°C for 1 h and centrifuged at 1200 × g for 15 min at 4°C. The assay mixture contained 0.1 mL of supernatant, 1.0 mM DTNB and 0.1M PB (pH 7.4) in a total volume of 1 mL. The yellow color that developed was measured immediately at 412 nm in a spectrophotometer. The GSH content was calculated as nmol GSH/mg of protein.

RNA extraction and RT-PCR

The procedure was optimized for semiquantitative detection using primer pairs and conditions described in Supplementary Table S2 and previously used by Farsi et al. [27]. Total RNA was extracted from frozen liver parts (30 mg) using an RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia) according to manufacturer's instructions. The purity of the extracted RNA was estimated by the 260/280nm absorbance ratio. Single-strand cDNA synthesis was performed as follows: 30 µL of reverse transcription mixture contained 1 µg of DNase I pretreated total RNA, 0.75 µg of oligo d(T) primer, 6 µL of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor and 240 U of reverse transcriptase (Invitrogen). The RT reaction was carried out at 40°C for 70 min, followed by heat inactivation at 95°C for 3 min. The tested genes and the internal control (β-actin) were amplified by PCR using 2 µL RT products from each sample in a 20-µL reaction containing Taq polymerase (0.01 U/mL), dNTPs (100 mM), $MgCl_2$ (1.5 mM) and buffer (50 mM Tris-HCl). PCR reactions comprised a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification, consisting of denaturation at 96°C for 30 s, annealing for 30 s and extension at 72°C for 1 min. A final extension cycle of 72°C for 15 min was included. The annealing temperature was adjusted for each target as shown in Table S2. A control reaction without reverse transcriptase was included for every sample of RNA isolated to verify the absence of contamination. PCR products (10 µL) were separated by electrophoresis on 2% agarose gels containing 100 ng/mL of ethidium bromide and photographed under ultraviolet illumination. Image J was used to analyze the percentages of expression of each mRNA against its corresponding β-actin mRNA.

Immunohistochemistry procedure

Histological sections of livers (5 µm) were cut from paraffin blocks with a rotary microtome. Immunohistochemistry was performed to localize GLUT-2 receptors by the avidin-biotin complex (ABC) technique. In brief, all sections were deparaffinized and rehydrated in a descending ethanol series, followed by immersion in a retrieval solution and washing (DAKO 51700, DAKO Diagnostics S.A., Barcelona. Spain). Inhibition of endogenous peroxidase activity was achieved with 3% H_2O_2 in absolute methanol for 30 min and then washing. Then sections were blocked by incubating with normal goat serum (DAKO X 909, DAKO Diagnostics S.A., Barcelona, Spain) with PBS diluted 1:4, followed by incubation with rabbit polyclonal antibody against GLUT-2 receptor protein, diluted 1:400 for 2 h at room temperature. Slides were washed with PBS and incubated with biotinylated goat anti-rabbit IgG (ab64256, Abcam, USA), washed and incubated with ABC complex (DAKO LSAB 2Kit, DAKO Diagnostics S.A). After successive washings, peroxidase was detected using 3',3'diaminobenzidine tetrahydrochloride as a chromogen (DAB). Hematoxylin was used as a counterstain and PBS was used instead of the monoclonal antibody as a negative control.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism statistical software package (ver. 6). The data are presented as means with standard deviation (mean±SD). All comparisons were analyzed by one-way ANOVA, followed by post hoc Tukey's t-test and accepted as significant at $p<0.05$.

RESULTS

Changes in food intake, final body weight and epididymal fat pad weight

The average daily food intake and changes between initial and final body and epididymal final weights are presented in Table 1. Type 2-DM group (Group 2) rats

Table 1. Changes in food intake, body weight and epididymal fat content in all groups of rats.

epididymal fat pad weight (g)	final body weight (g)	initial body weight (g)	daily food intake/rat (g)	Group
3.78±2.65	288.8±12.3	210.4±9.9	13.4±2.5	**Control**
7.67±2.35[a]	343.9±7.8[a]	206.6±7.6	21.3±3.2[a]	**DM2**
5.4±1.7[ab]	302.9±9.8 [ab]	209.8±5.6	16.7±3.4[ab]	**DM2+*C. aronia***

Values are expressed as the mean±SD for 6 rats in each group for results obtained in triplicate for each sample. Values were considered significantly different at p<0.05. DM-2 – type 2-DM; [a] indicates significant difference when compared to the control group; [b] indicates significant difference when compared to DM-2.

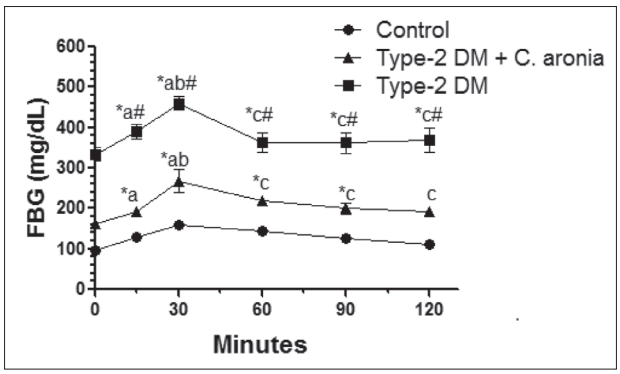

Fig. 1. OGTT of all groups of rats. Values are expressed as the mean±SD for 6 rats in each group in triplicates of 3 for each sample. Values were considered significantly different at P<0.05; [a] indicates significant difference when compared to a 0 min reading; [b] indicates significant difference when compared to a 15 min reading; [c] indicates significant difference when compared to a 30 min reading; [*] indicates significant difference when compared to the same time interval measured in the control group; [#] indicates significant difference when compared to the same time interval measured in the DM2+*C. aronia* group.

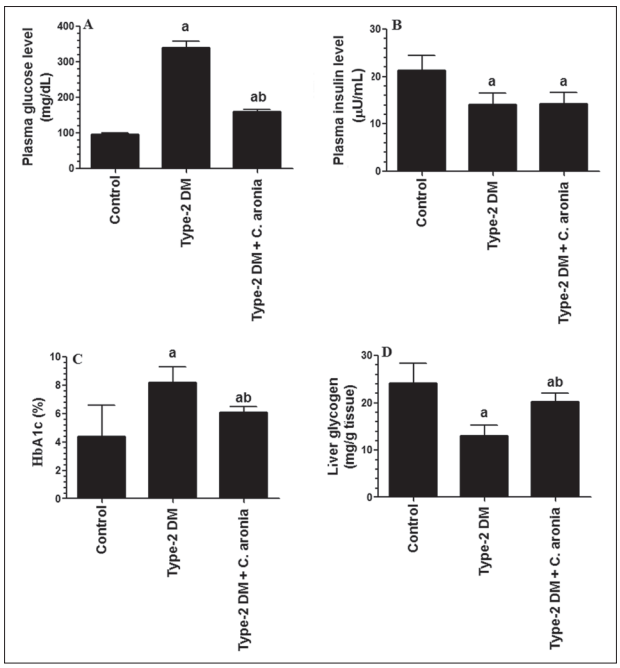

Fig. 2. Levels of plasma glucose (**A**), insulin (**B**) blood glycosylated hemoglobin (**C**) and hepatic glycogen (**D**) in all groups of rats. Values are expressed as the mean±SD for 6 rats in each group in triplicates of 3 for each sample. Values were considered significantly different at P<0.05; [a] indicates significant difference when compared to the control group; [b] indicates significant difference when compared to the DM-2 group. DM-2 – type 2-DM.

exhibited a significant increase in average daily food intake with concomitant increases in both final body and epididymal weights as compared to the control. Significant decreases in the levels of all these parameters were detected in type 2-DM rats that received the *C. aronia* aqueous extract for 60 days (Group 3) as compared to diabetic rats (Group 2). However, in spite of these reductions, analysis showed that the levels of these parameters remained significantly higher than their corresponding levels in control rats.

Changes in blood biochemistry and liver glycogen content

Fig. 1 shows the changes in OGTT in all groups of rats. Fig. 2 shows the changes in fasting plasma glucose (FPG) (Fig. 2A) and insulin (FPI) (Fig. 2B), blood percentages of glycosylated Hb (HBA1c%) (Fig. 2C) as well as liver glycogen content (Fig. 2D) in all experimental groups. As compared to the control rats, type 2-DM rats (Group 2) had significantly higher levels of FPG and HbA1c, which coincided with a decrease in both FPI levels and hepatic glycogen contents (Fig. 2). OGTT displayed disturbed glucose disposal and impaired glucose tolerance (Fig. 1). On the other hand, even if it did not affect FPI levels, administration of the *C. aronia* aqueous extract to diabetic rats (Group 3) significantly lowered FPG, HbA1c% and enhanced the glycogen content when compared to the type 2-DM group (Group 2). Except for HbA1c%, the levels of FPG and hepatic glycogen content remained

Table 2. Levels of triglycerides (TGs), total cholesterol (TC), very low-density low lipoproteins cholesterol (VLDL-c), low-density lipoproteins cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) in the sera of all groups of rats.

HDL-C (mg/dL)	LDL-c (mg/dL)	VLDL-c (mg/dL)	TC (mg/dL)	TGs (mg/dL)	Group
19.3±2.1	34.17±3.5	12.3±1.7	65.0±10.6	79.2±7.5	**Control**
13.0±1.96[a]	104.7±8.2 [a]	32.8±4.5 [a]	147.8±9.6[a]	182.2±16.6[a]	**DM-2**
16.17±2.4 [ab]	55.6±5.5[ab]	18.4±1.7[ab]	96.3±9.9[ab]	112.5±13.2[ab]	**DM2+*C. aronia***

Values are expressed as mean±SD for 6 rats in each group. Values are expressed as mean±SD for 6 rats in each group in triplicates of 3 for each sample. Values were considered significantly different at $p<0.05$. DM-2 – type 2-DM; [a] indicates significant difference when compared to the control group; [b] indicates significant difference when compared to DM-2.

slightly but significantly higher than their corresponding levels in control rats.

Changes in the serum lipid profile

Changes in serum levels of TGs, TC and LDL-c, VLDL-c and HDL-c, in the serum of all groups are shown in Table 2. Significant increases in the levels of TGs, TC, LDL-c and VLDL-c with a parallel decrease in the level of HDL-c were observed in the serum of type 2-DM rats (Group 2) when compared to control rats. As compared to type 2-DM group (Group 2), rats that were administered *C. aronia* (Group 3) showed significant decreases (but not to normalization), in the levels of TGs, TC, LDL-c and VLDL-c, and a significant increase in HDL.

RT-PCR and immunohistochemistry

The levels of GK and IR-1A mRNA were significantly decreased, and the mRNA levels of G6Pase and GLUT-2 were significantly increased in the livers of type 2-DM rats (Group 2) (Fig. 3). Along with these changes, the immunohistochemical study revealed a significant increase in GLUT-2 expressions in type 2-DM rats (Figure 4 C and D). In contrast, significant decreases in the levels of G6Pase and GLUT-2 mRNAs and significant increases in GK and IR-1A mRNAs (Fig. 3), with a concomitant decrease in GLUT-2 immunoreactivity (Fig. 4E), were detected in the livers of type 2-DM rats that were treated with the *C. aronia* aqueous extract (Group 3) as compared to diabetic rats (Group 2).

Oxidative stress and inflammation

The concentration of GSH and the specific activity of total SOD were significantly decreased, while levels

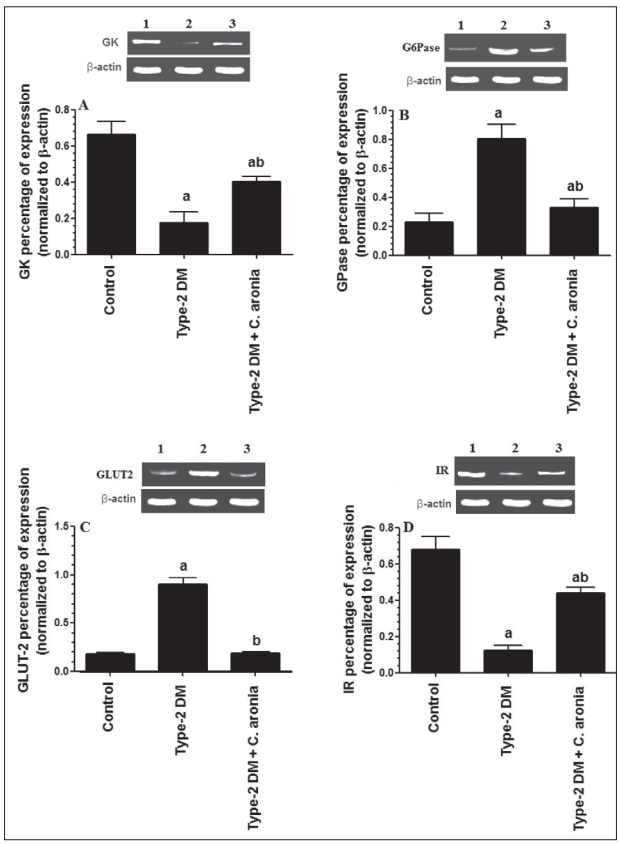

Fig. 3. Levels of mRNA expression for GK (**A**), G6Pase (**B**), GLUT-2 (**C**) and IR-1A (**D**) in the livers of all groups of rats; 1 – control rats, 2 – type2-DM, 3 –type 2-DM+*C. aronia*. Values are expressed as the mean±SD for 6 rats in each group in triplicates of 3 for each sample. Values were considered significantly different at $p<0.05$; [a] indicates significant difference when compared to the control group; [b] indicates significant difference when compared to the DM-2 group. DM-2 – type 2-DM.

of TNFα, interleukin-6 (IL-6) and TBARS were significantly increased in the liver homogenates of type 2-DM rats (Group 2) when compared to control rats (Fig. 5). Even though not fully normalized, the administration of *C. aronia* to type 2-DM rats for 60 days (Group 3) significantly improved the concentration

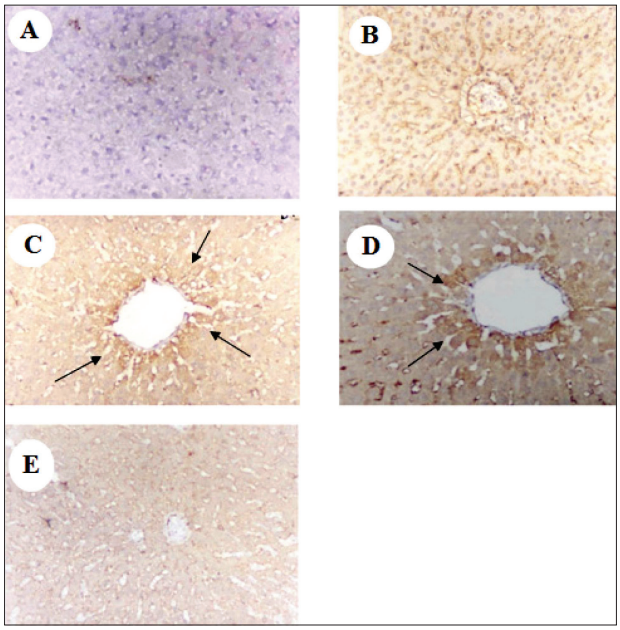

Fig. 4. Immunoreactivity of GLUT-2 receptors in cells of the liver of all groups of rats. **A** – negative control; B – control non-diabetic rats; **C**, **D** – type 2-DM. E – type 2-DM+*C. aronia*. Counterstaining was with hematoxylin; magnification: 200X. Brown coloration (arrows) indicate a positive reaction.

of GSH and SOD activity, and significantly inhibited the increases in the levels of TBARS, TNFα and IL-6 in the livers of these rats (Fig. 5).

DISCUSSION

The exact site of the insulin resistance that leads to decreased glucose disposal remains a matter of debate. Although insulin resistance appears to be the greatest in skeletal muscle in both animals and humans during very early phases of type 2-DM, [6,28], several studies have indicated the role of hepatic insulin resistance as a determining factor for fasting blood glucose (FBG) concentrations in all phases of type 2-DM. This is supported by normal whole-body glucose homeostasis in the muscle of rats deficient in IR [8]. Further studies have reported similar results, and the overall conclusion was that increased hepatic glucose production (HGP) is strongly correlated with fasting hyperglycemia in type 2-DM patients [28]. Hence, targeting hepatic insulin resistance rather than insulin resistance in skeletal muscles may be much more important in lowering FBG levels.

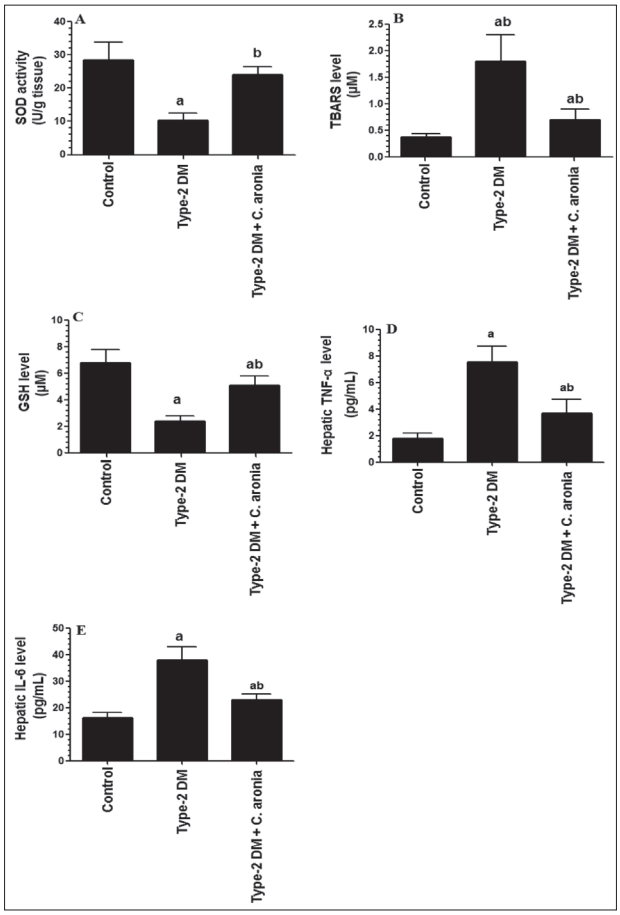

Fig. 5. Activity of SOD (**A**), the concentrations of TBARS (**B**), GSH (**C**), TNFα (**D**) and IL-6 (**E**) in liver homogenates of all groups of rats. Values are expressed as the mean±SD for 6 rats in each group, measured in triplicates of 3 for each sample. Values were considered significantly different at $p<0.05$; [a] – indicates significant difference when compared to the control group; [b] – indicates significant difference when compared to the DM-2 group. DM-2 – type 2-DM.

Hence, in this study we sought to investigate the effect of the aqueous extract of *C. aronia* on the rat model of type 2-DM induced by a high fat diet combined with the administration of a low dose of STZ, with emphasis on HGP and uptake. We also examined the major mechanisms responsible for hepatic insulin resistance, including lipid accumulation, aspects of the oxidative stress and some inflammatory markers. One finding of this study is that type 2-DM rats (Group 2) exhibited hepatic IR-1A deficiency associated with an enhanced rate of gluconeogenesis, inhibition of glycogen synthesis, enhanced levels of $HbA1_C$ and overexpression of GLUT-2 mRNA and protein levels.

In addition, the livers of these rats displayed a lower endogenous antioxidant status and enhanced levels of inflammatory cytokines, paralleled by increases in serum levels of TGs, TC and LDL-c. Also, the administration of the *C. aronia* extract to type 2-DM rats for 60 days (Group 3) significantly reversed all of the abovementioned changes via a mechanism that is apparently mediated by the antioxidant and antiinflammatory potentials of this plant.

Type 2-DM rats displayed enhanced polyphagia and increased body weight. Coexistent with these signs were the high levels of plasma fasting blood glucose and $HbA1_C$. The plasma levels of insulin significantly decreased with impaired OGTT. Interestingly, liver glycogen levels were significantly decreased. Hence, we investigated the role of the hepatic insulin resistance by investigating aspects of hepatic insulin metabolism.

Once insulin binds to its receptor, it initiates a cascade of events that eventually suppresses hepatic glucose output [5]. In this process, insulin mediates the phosphorylation activation of PI3K/Akt, which in turns mediates the phosphorylation inhibition of glycogen synthase (GS) [29], the key enzymes in glycogenolysis. In addition, the phosphorylated PI3K/Akt phosphorylates forkhead box protein O1 (FOXO1) in turns mediates the inhibition of phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase, the key regulatory enzymes of gluconeogenesis [29]. On the other hand, hepatic GLUT-2 is expressed in response to high circulatory or hepatic glucose levels [30]. Hence, insulin has a negative impact on hepatic GLUT-2 expression in mice [31].

The work presented here revisits the role of the IR expression in the pathology of type 2-DM (Group 2) and suggests that abundant expression of liver IR-1A has an important role in insulin-mediated metabolic signaling and hepatic insulin resistance. In the current study, type 2-DM rats had decreased IR-1A mRNA, increased G6Pase mRNA and decreased GK mRNA levels and concentrations of hepatic glycogen. These data, in addition to the enhanced levels of plasma fasting glucose, could explain the increases in hepatic mRNA and protein levels of GLUT-2 in type 2-DM rats. Taken together, these data suggest that impaired insulin hepatic signaling due to downregulation of IR-1A expression or decreased plasma insulin levels plays an important role in the pathology of type 2-DM.

In this study, we are the first to confirm that the antidiabetic potential of *C. aronia* against type 2-DM in rats (Group 3) is mediated by decreased fat accumulation, enhanced hepatic insulin signaling and decreased hepatic glucose output. This is evident from the lower levels of FPG and $HbA1_C$, the higher glycogen content, enhanced GK mRNA and lower G6Pase mRNA levels in the livers of type 2-DM rats treated with *C. aronia*. Interestingly, *C. aronia* did not affect the circulatory insulin levels but significantly enhanced hepatic the level of IR-1A mRNA and downregulated the mRNA and protein levels of GLUT-2. Such increases in the expression of IR-1A could be the key mechanism by which *C. aronia* enhances insulin signaling in the liver of diabetic rats, instead of affecting the plasma insulin levels. The decrease in the concentration of plasma glucose and the inhibition of hepatic gluconeogenesis in the liver of diabetic rats explains the mechanisms by which *C. aronia* mediates the decreases in mRNA levels and expression of GLUT-2 in the livers of these diabetic rats. However, in spite of these observations, the definite effect of *C. aronia* on protein abundance and phosphorylation of insulin signaling mediators remains to be investigated.

It is accepted that ROS, inflammatory cytokines and hepatic steatosis are the major risk factors in the genesis of type-2 DM. Different mechanisms have been suggested to explain the roles of these alterations in inducing hepatic insulin resistance [11-15]. Indeed, most type 2-DM patients have fatty liver and hepatic insulin resistance [32-34]. Also, the hepatic levels of NF-κB and IL-6 were reported to be increased in the livers of diet- and genetically-induced type 2-DM rats with or without steatosis [13,14]. In addition, animals with very high levels of hepatic ROS had impaired insulin signaling and exhibited both hepatic and systemic insulin resistance [10,11]. Furthermore, disturbed mitochondrial function with increased ROS levels were reported in type 2-DM patients with fatty liver [35-39]. Moreover, palmitate-induced hepatic insulin resistance was dependent on the generation of mitochondrial ROS [12].

Similar to these studies, livers of type 2-DM rats (Group 2) showed severe reduction in endogenous

antioxidant potential (decreased SOD activity and GSH concentration), and enhanced lipid peroxidation as a marker of high levels of oxidative stress. In addition, the increased levels of IL-6 and TNFα detected in the liver homogenates of type 2-DM rats and their serum displayed prominent increases in the levels of TGs, CHOL and LDL-c. Administration of *C. aronia* to type 2-DM rats (Group 3) significantly reduced the levels of IL-6 and TNFα and significantly enhanced the activity of SOD and the concentration of GSH, leading to significant inhibition of hepatic lipid peroxidation. Also, *C. aronia* application significantly normalized serum lipids. Thus, it would appear that the antioxidant, antiinflammatory and hypolipidemic effects of *C. aronia* improve hepatic insulin signaling.

The chemical composition of the leaves and flowers of *C. aronia* have been identified previously by other authors [40,41]. The major ingredients include phenolics such as oligomeric proanthocyanidin, flavonoids (vitexine-2-O-rhamnoside, VOR) and polyphenols, such as chlorogenic acid, hyperoside and quercetin. Interestingly, proanthocyanidin and VOR have been shown to have a potent antioxidant potential against superoxide- and hydrogen peroxide-induced oxidative damage and can be safely used at a wide range of concentrations [42-46]. In addition, some authors have also shown that the flavonoid VOR has a potent hypolipidemic effect and is capable of treating fatty liver diseases [47]. Furthermore, isovitexin is a well-known antiinflammatory agent [48] with potent antidiabetic activity in a murine model of type 2 diabetes [49].

Recent evidence has highlighted the important role of chlorogenic acid as an antioxidant and of polyphenols in lowering glucose and lipid levels in different animal disease models [50]. Importantly, quercetin and its derivatives exert antidiabetic effects by acting through various mechanisms, including inhibition of intestinal starch digestion and hepatic glucose production by enhancing hepatic glucose kinase activity, improving skeletal muscle uptake of glucose and by protecting against pancreatic islet damage [51-56]. It also has a potent hypolipidemic effect and is capable of ameliorating the resultant insulin resistance in STZ-induced diabetic animal models [51,53]. Furthermore, quercetin has potent antioxidant and antiinflammatory actions in various tissues of diabetic animals [53,55].

Given all this information, we could speculate that the amelioration of oxidative stress and the antidiabetic mechanisms reported in this study are essentially related to the concomitant and synergistic effects of all these ingredients. However, there are some limitations in this study. Even though *C. aronia* enhanced the expression levels of IR-1, inhibited gluconeogenesis and activated glycogen synthesis, the effect of the extract on cellular insulin signaling cannot be confirmed by the available data. Further studies measuring the phosphorylation status and abundance of proteins involved in insulin signaling should be carried out to confirm the exact mechanisms by which HFD and *C. aronia* act. In addition, such studies should be done in other animal models and sexes in order to confirm the endpoints.

The novel findings of the current study are unique in confirming the role of hepatic insulin resistance associated with decreased IR expression as the major factor in the etiology of type 2-DM. Our results are the first to show a possible mechanistic effect of *C. aronia* in lowering blood glucose in rats with type 2-DM.

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Author contributions: DGM, EFK and GAA contributed equally to the current study. Both authors designed the experimental procedure and performed all statistical analyses as well as participated in writing the manuscript. EFK and GAA produced the animal model and performed all the treatments of rats, measured the biochemical parameters and performed the RT-PCR procedure and analysis. DGM helped in tissue and blood sampling, measurements and analysis of the biochemical parameters and performed the immunohistochemistry study.

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Supplementary Data

Supplementary Table S1. Composition of HFD used in the experimental procedure.

Ingredients	g/Kg
Powder normal chow	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dL-Methionine	03
Yeast powder	01
Sodium chloride	01

Supplementary Table S2. RT-PCR primers and annealing conditions.

Annealing temperature	sequence	Primer	Gene
61.7	5′-CATATGTGCTCCGCAGGACTAG-3′ 5′-CTTGTACACGGAGCCATCCA-3′	Forward Reverse	GK
62.7	5′-GGATCTACCTTGCGGCTCACT-3′ 5′-TGTAGATGCCCCGGATGTG-3′	Forward Reverse	G6Pase
63.4	5′-CATCAAAACGTAGAGCACGGTAA-3′ 5′-TATGGGCATTTAGTCTGCACGTA-3′	Forward Reverse	GLUT-2
62	5′- TGAAAAGTCACCTCCGTTCTCT-3′ 5′-CTCTCGTCATTCCAAAGTCTCC-3′	Forward Reverse	IR-1A
55	5′-GCTCTGGCTCCTAGCACCAT-3′ 5′-GCTGATCCACATCTGCTGGAA-3′	Forward Reverse	β-actin