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Original article

Short-term administration of *C. aronia* stimulates insulin signaling, suppresses fatty acids metabolism, and increases glucose uptake and utilization in the hearts of healthy rats

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ABSTRACT

This study evaluated the effect of *Crataegus aronia (C. aronia)* aqueous extract on cardiac substrate utilization and insulin signaling in adult male healthy Wistar rats. Rats (n = 18/group) were either administered normal saline (vehicle) or treated with *C. aronia* aqueous extract (200 mg/kg) for 7 days, daily. Fasting plasma glucose and insulin levels were not significantly changed in *C. aronia*-treated rats but were significantly reduced after both the intraperitoneal glucose or insulin tolerance tests. Besides, *C. aronia* significantly increased the left ventricular (LV) activities of phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH), two markers of glycolysis and glucose oxidation, respectively, and suppressed the levels of pyruvate dehydrogenase kinase 4 (PDK4), an inhibitor of PDH. Concomitantly, it significantly reduced the LV levels of carnitine palmitoyltransferase 1 (CPT1) and PPARα, two markers of fatty acid (FAs) oxidations. Under basal and insulin stimulation, *C. aronia* aqueous extract boosted insulin signaling in the LV of rats by increasing the protein levels of p-IRS (Tyr^{612}) and p-Akt (Ser^{473}) and suppressing protein levels of p-mTOR (Ser 2448) and p-IRS (Ser^{307}). In parallel, *C. aronia* also increased the protein levels of GLUT-4 in the membrane fraction of the treated LVs. All these effects were also associated with a significant increase in AMPK activity (phosphorylation at Thr^{172}), a major energy modulator that stimulates glucose utilization. In conclusion, short-term administration of C. *aronia* aqueous extract shifts the cardiac metabolism toward glucose utilization, thus making this plant a potential therapeutic medication in cardiac disorders with impaired metabolism.

1. Introduction

The heart is one of the highest energy-demanding organs that requires a continuous supply of oxygen and energy (i.e. ATP) to sustain contractility and ionic hemostasis (Bertrand et al., 2008; Lopaschuk and Jaswal, 2010). It is an omnivorous organ that has a virtue of metabolic flexibility (remodeling) to utilize different substrates including fatty acids (FAs), glucose, ketone bodies, amino acids, and lactate to meet its high energy demand in response to physiological alterations and pathological conditions (Lopaschuk, 2017). However, the preference of the heart toward its substrate utilization depends on several factors including the nutritional, hormonal status, substrate availability, workload, blood supply, metabolic status, and the existence of any cardiac disorders (Bertrand et al., 2008; Lopaschuk, 2017). Under normal conditions, the majority of the ATP produced in the heart (greater than95%) is generated from the mitochondria oxidative phosphorylation (MOP) whereas the remaining requirement comes from glycolysis (Karwi et al., 2018). In this view, it was shown that FAs oxidation is the major contributor in the MOP, providing about (40–60%) of the total energy whereas oxidation of the other substrates delineates the remaining amount of produced energy (Karwi et al., 2018; Ma and Li, 2015).

Currently, it is well accepted that cardiac energy remodeling is essential for normal cardiac function and the impairment of cardiac metabolism is associated with several forms of CVDs

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(Lopaschuk, 2017; Tripp, 1989). Although FAs are the preferred energy source for normal heart, glucose remains the highest energy-efficient substrate with a P/O (phosphate/oxygen) ratio of 2.58 as compared to that of 2.33 for FAs oxidation (FFAs paradox) (Karwi et al., 2018). However, a negative cross-talk between FFAs and glucose metabolisms has been established in the heart where FAs can suppress glucose utilization at multiple levels, and vice versa, in what is known as the Randle cycle (An and Rodrigues, 2006; Lopaschuk, 2016; Randle, 1963). However, increased FAs oxidation coincided with enhanced glycolysis and reduced glucose oxidation have been described in several conditions such as diabetic cardiomyopathy (DC), obesity, insulin resistance (IR), heart failure (HF), and reperfusion after cardiac ischemia (An and Rodrigues, 2006; Lopaschuk, 2017; Taylor et al., 2001; Tuunanen et al., 2008). Subsequently, this impaired cardiac metabolism results in a significant reduction in cardiac efficiency (O_2 utilization), mismatch between glycolysis and glucose oxidation, and accumulation of damaging end products such as protons and lactic acid which eventually lead to perturbations in cardiac contractility, function, and ionic homeostasis (Lopaschuk, 2017). Therefore, suppressing cardiac FAs oxidation or increasing glucose oxidation has been confirmed to be excellent strategies to preserve cardiac function during these pathological conditions (Fillmore and Lopaschuk, 2013).

However, the concept of metabolic modulation has been raised as a possible therapeutic approach to treat CVD. In this context, clinical and experimental trials to enhance cardiac function by shifting the metabolism toward the more energy-efficient substrate, glucose, or decreasing FAs oxidation have yielded promising results (Karwi et al., 2018; Lopaschuk, 2017; Tripp, 1989). Accordingly, several drugs targeting these approaches have been developed and showed efficient capabilities to reduce cardiac damage, improve cardiac output, and increase overall survival rates (Lopaschuk, 2017). These include glucose/insulin/potassium (GIK) solutions; carnitine palmitoyltransferase 1 (CPT1) inhibitors (e.g. oxfenicine, etomoxir, and perhexiline), β-adrenoceptor antagonists, 3-ketoacyl-coenzyme A thiolase (3KAT) inhibitors (e.g. trimetazidine), and pyruvate dehydrogenase activators (e.g. dichloroacetate) (Fillmore and Lopaschuk, 2013; Jaswal et al., 2011; Lopaschuk, 2017). However, some of these aforementioned drugs are associated with unfavorable adverse effects and showed poor pharmacokinetic and low potency profiles which compromise their clinical use and benefits (Fillmore and Lopaschuk, 2013; Jaswal et al., 2011). Therefore, searching for more long-acting safe drugs with similar efficiency on cardiac metabolism is encouraging.

Hawthorn (*Crataegus*), a commonly known plant worldwide with more than 170 species, has many health benefits including digestive, respiratory, and cardiovascular disorders (Wang et al., 2013). The currently available evidence has shown that the majority of hawthorn species have a positive impact on various cardiovascular disorders including myocardial infarction (MI), ischemia-reperfusion (I/R) injury, and HF by acting through different mechanisms including antioxidant, hypoglycaemic, hypolipidemic, anti-remodeling, anti-inflammatory, positive inotropic, vasodilation, and anti-arrhythmic effects (Tassell et al., 2010; Wang et al., 2013). In the Arabian region, the most dominant Hawthorn species is *Crataegus aronia* (Shatoor et al., 2019). During the last decade, we and others have extensively studied the systemic and cardioprotective effects of *C. aronia* in isolated heart preparations, as well as in animal models atherosclerosis and HF. The major findings obtained from these studies have shown a potent cardioprotective effect of *C. aronia* mediated mainly by positive inotropic, hypolipidemic, anti-thrombotic, antioxidant, and anti-inflammatory effects (Dallak, 2018; Keskin et al., 2012; Ljubuncic et al., 2005; Shatoor, 2013; Shatoor et al., 2019; Shatoor and Al Humayed, 2020).

However, *in vivo* evidence on the effect of *C. aronia* and other Hawthorn species on cardiac substrate utilization and metabolism in health and disease is completely lacking. Therefore, in this study, and using healthy rats, we aimed to investigate the effect of *C. aronia* on cardiac insulin glucose and FAs oxidation and investigate the possible underlying mechanism of regulation.

2. Materials and methods

2.1. Animals

All the male rats (7 weeks old, 130 ± 5 g) were supplied from the animal Department at King Khalid University, Saudi Arabia. All the rats were always housed under 12/12 h light/dark cycle, 22 ± 1 °C, and 59% humidity and had free access to chow and water. The experiments included in this study were approved by the ethical committee at the KKU which their instructions follow those regulations established by the National Institutes of Health Guide (1996, 7th edition).

2.2. Preparation of the extract

The dried whole plant (leaves, fruits, and stems) of *C. aronia* was purchased during the cultivation season from a certified local supplier in Amman Jordan (Kapatilo) and transferred directly to Saudi Arabia. Their records indicated that the plant was cultivated and dried. The preparation of the aqueous extract from the whole *C. aronia* plant was described in more detail in our previous studies (Shatoor and Al Humayed, 2020; Shatoor et al., 2019).

2.3. Experimental groups

Animals were segregated mainly into 2 major groups (n = 18) of either 1) control group that was administered normal saline (vehicle) or 2) *C. aronia*-treated group which was administered *C. aronia* aqueous extract (200 mg/kg). The vehicle or the drug was administered orally for 7 days daily (once/day). *C. aronia* was administered at this dose per our previous findings where such dose induced potent inotropic, hypotensive, and cardioprotective effects in the same strain of rats (Shatoor, 2013; Shatoor et al., 2019; Shatoor and Al Humayed, 2020).

2.4. Intraperitoneal glucose and insulin tolerance tests (IGTT & IPITT)

This has been done on the first 6 rats/group. IGTT and IPITT were performed as previously described by others (Wong and Sul, 2010). By the end of the last day of the experiment (day 7), all rats have fasted, for 12 h and then exposed to IPTT, in brief, glucose solution (1 g/kg) was injected (i.p) into each rat and then blood samples were withdrawn at 0.0, 15, 30, 60, 120 min. Then all rats were brought back to their special cages with free access to food and water. The next day, IPITT was conducted after 5 h fasting where each animal was injected with 0.75U/kg insulin and blood samples were withdrawn over the same time intervals for 1 h. For both tests, 200 μl of blood was collected each time into EDTA-supplied tubes and centrifuged (1200g/5 min/room temperature) to collect plasma which was further stored at −20 °C and used later to measure fasting glucose and insulin levels using the commercially available kits (Cat. No. 10009582, Cayman chemicals, USA and Cat No. ELR-Insulin-1, ga, USA, respectively).

2.5. Insulin stimulation and collection of left ventricles

The remaining 12 rats of each group were used in the next experimental procedures. Herein, and in the same line with many

other authors, we have used unfasted rats for the next experiments to minimize the effect of starvation on cardiac insulin signaling and energetic regulators including PGC-1α and AMPK levels (Di et al., 2018; Eid et al., 2019). Insulin stimulation was performed done as described by Wang et al. (Wang et al., 1999). In brief, rats of each group (n = 12/group) were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital. A surgical laparotomy was performed on the ventilated rats and a 10 U/kg insulin (6 rats/group) or vehicle (normal saline) (the remaining 6 rats/group) was injected through the superior vena cava. For this part of the experiment, each rat was also injected with 2.2 M glucose after insulin infusion to prevent sudden hypoglycemia. However, 60 min later, the hearts were rapidly removed on ice and their left ventricles (LVs) were isolated, washed into ice-cold phosphate buffer saline (BPS/pH = 7.4). Each isolated LV was sliced into smaller pieces, some of which were directly placed 10% buffered formalin (for 20 h) and sent to the pathology laboratories for routine histology. Other parts were collected and used later for the biochemical and molecular parts.

2.6. Preparation of total left ventricles homogenates

To prepare total LVs homogenates, 20 mg of each frozen LV were homogenized in 250 μl ice-cold PBS (pH = 7.4) and centrifuged (11,000*g*/10 min/4 °C). The supernatants were then separated and frozen at −80 °C until use. The preparation of total cell homogenates to be utilized in western blotting was performed by homogenizing another 30 mg of each LV in 500 μl 1x radioimmunoprecipitation (RIPA) buffer (Cat. No. R0278-50ML, Sigma-Aldrich, MO, USA) plus 10 μl protease inhibitor cocktail (Cat. No. Ab65621, Abcam, Cambridge, UK), followed by centrifugation at 12,000*g* to isolate the supernatant. This supernatant was stored at −80 °C and used later which was used later for the measurement of total proteins and western blotting.

2.7. Measurements in the LVs homogenates

All kits used for this part were rats' specific. Levels of glutathione (GSH) were determined by ELISA (Cat. No. MBS046356, ByBiosource, CA, USA). The quantification of MDA levels was performed by a colorimetric kit (Cat. No. KA3736, Abnova, Taipei City, Taiwan). The determination of the reactive oxygen species (ROS) was performed using a Fluorometric kit (Cat. No. STA-347, Cell Biolabs, CA, USA). The total amount of mitochondrial manganese superoxide dismutase (MnSOD) (SOD2) was determined by ELISA (Cat. No. MBS729914, ByBiosource, CA, USA). Levels of pyruvate dehydrogenase kinase 4 (PDK4) and activities and pyruvate dehydrogenase (PDH) and medium-chain acyl-CoA dehydrogenase (MCAD) were measured by ELISA kit (Cat. No. Ab215544, Cat. No. 109902, and Cat. No. 129734, Abcam, Cambridge, UK, respectively). The activity of phosphofructokinase1 (PFK1) was assayed calorimetrically (Cat. No. MAK093-1KT, Sigma Aldrich, UK). In all measurements, 6 samples/groups were used and all procedures were conducted per the kit instructions.

2.8. Preparation of the plasma membrane fraction

This has been done using a commercially available OrgFrontierTM Plasma Membrane Isolation Kit 3/18 (Cat. No. K414-10, Biovision, USA). In brief, 50 mg of each LV was homogenized in 0.5 ml working buffer solution provided with the kit and then sonicated on the ice at a rate of two 10 s pulses with 30 s between them, using a microsonicator. Each tube was then centrifuged (1200*g*/10 min/4°C) and the fatty residue on the top of each supernatant was removed. The remaining residues were collected, removed to a new pre-chilled tube, and maintained on ice. This step was repeated on the remaining pellets of the previous step to concentrate the supernatant which represents the post-nuclear fraction (PNS). This PNS was sonicated as above on ice. Then, 0.5 ml of the PNS was added to 2 ml of the pre-prepared Gradient Working Solution (GWS) and transferred into a new clean, ice-cold ultracentrifuge tube. After then, 5 ml of 25% gradient solution was transferred to the top of the PNS mixture carefully. Then, 1 ml of the 2.5% gradient solution was added to each tube. After this step, all tubes were ultracentrifuged (200,000 rpm/90 min/4°C) and the visible band at the interface of the 2.5%/25% gradients which contains the plasma membrane fraction was removed, collected, and frozen at −80 °C for further use.

2.9. Western blotting analysis

Frozen LVs samples collected from the rats with or without insulin treatment (30 mg) were homogenized in 1× 0.5 ml RIPA buffer as described above. The protein levels in each sample were determined using a Bradford protein assay kit (Cat. No. 23200, Thermo Fisher). Samples (40 μg/well) were separated on SDS-PAGE and then transferred onto nitrocellulose membrane, blocked, and incubated (2 h/room temperature/shaking) with the primary antibodies against GLUT-4 (Cat. No. Ab654, 45 kDa), CPT1A (Cat. No. ab53532, 80 kDa) (Abcam, Cambridge, UK), AMPKα (Cat. No. 5832, 62 kDa), p-AMPKα (Thr^{172}) (Cat. No. 2531, 62 kDa), IRS-1 (Cat. No. 2382, 180 kDa), p-IRS-1 (Ser^{307}) (Cat. No. 2381, 180 kDa), Akt (Cat. No. 9272, 60 kDa), p-Akt (Ser^{473}) (Cat. No. 4060), Nrf-2 (Cat. No. 12721, 100 kDa), mTOR (Cat. No 2983, 289 kDa), p-mTOR (Ser^{2488}) (Cat. No. 5366, 289 kDa), β-actin (Cat. No. 3700, 45 kDa) (Cell signaling Technology), p-IRS-1 (Tyr^{612}) (Cat. No. 44-816G, 180 kDa, ThermoFisher), and PPARα (Cat. No. sc-398394, 55 kDa) (Santa Cruze Biotechnology). Then, all membranes were plotted against the corresponding secondary antibody for another 2 h at room temperature. Skim milked and all antibodies were prepared/diluted in the washing buffer TBST buffer. Washing between steps was also done 3 times using the TBST buffer. Each membrane was stripped up to 5 times using Sep-pro stripping buffer (Cat. No. S4324, Sigma-Aldrich, MO, USA) and phosphorylated proteins were always detected first. Antigen/antibody interaction was developed using ECL substrate (Cat. No. Ab65623, Abcam, Cambridge, UK). Photographs were captured and analyzed using a gel scanner (LI-COR, USA).

2.10. Histology evaluation

Fresh formalin-fixed tissues (20 h) were dehydrated in various levels of ethanol. Then all samples were xylene cleared. After that, all tissues were embedded in paraffin and sliced using a microtome at sections 4–5 μm. All tissues were then routinely stained with hematoxylin and eosin (HE) and photographed under a light microscope (200X).

2.11. Statistical analysis

GraphPad Prism (version 8) was used for the statistical analysis. The comparison between the two groups was conducted by the student's *t*-test. Analysis of IPGT and IPITT, as well as the other comparison, included basal and insulin stimulation was done by the 2-way ANOVA followed by Tukey's *t*-test. The levels of significance were determined at $p < 0.05$ and all data were presented as mean ± SD.

3. Results

3.1. Changes in body weights, food intake, and plasma glucose and insulin

There was no change in food intake and final body weights between the rats that administered the vehicle or treated with the extract (Data not shown). Normal levels of fasting plasma glucose and insulin levels were observed between the control and *C. aronia*-treated rats extract (Fig. 1A and B). However, serum levels of FFAs have significantly decreased in *C. aronia*-treated rats as compared to control rats (Fig. 1E). Besides, *C. aronia* significantly lowered plasma glucose levels at all tested intervals (15–120 min) and significantly reduced their measured area under the curve (UAC) during the IGTT (Fig. 2A). Also, the treatment with the *C. aronia* extract significantly lowered insulin and glucose levels at 15 and 30 min and reduced their UAC during the IITT (Fig. 2B and C).

3.2. Changes in oxidative stress parameters

While *C. aronia* didn't affect the architectures and histological futures of the LVs tissues (Fig. 3A and B), it significantly suppressed the production of MDA and ROS and enhanced the endogenous levels of GSH and MnSOD in the LVs of treated rats (Fig. 3C–F). Besides, the hearts of *C. aronia*-treated rats had higher cardiac protein levels of Nrf2 relative to the control rats (Fig. 3G).

3.3. Changes in cardiac FAs and glucose metabolism

Concerning metabolism, LVs of rats administered *C. aronia* showed a reduction in the levels of PDK and activity MCAD but had higher activity of PDH and the levels of PFK (Fig. 4A–D). Aloes, administration of *C. aronia* significantly increased the membrane expression of GLUT-4 and decreased phosphorylation (activation) of AMPK (Thr^{172}) in the LVs of the rats under both basal and insulin stimulation, as compared to control rats (Fig. 5A–D).

3.4. Changes in insulin signaling

Insulin stimulation didn't significantly affect the LV expression of IRS-1 but significantly increased the phosphorylation of IRS (Tyr^{612}) and (Ser^{307}) as compared to basal conditions (Fig. 6A–D). However, total levels of IRS were not significantly different between control and *C. aronia* with or without insulin stimulation (Fig. 6A and B). However, protein levels of p-IRS (Tyr^{612}) were significantly increased but protein levels of p-IRS (Ser^{307}) were significantly decreased in the LVs *C. aronia*-treated rats under both the basal and insulin-stimulated conditions (Fig. 6C and D). On the other hand, total protein LV levels of Akt and mTOR levels were not significantly different between the control and *C. aronia*-treated under all treatments (Fig. 7A–D). However, insulin stimulation significantly increased the phosphorylation of Akt (Ser^{473}) and mTOR (Ser^{2448}) when compared to non-stimulated conditions (Fig. 7D). Nevertheless, the protein levels of pAkt (Ser^{473}) were significantly increased but protein levels of p-mTOR (Ser^{2448}) were significantly reduced in the LVs of *C. aronia*-treated rats under both the basal and insulin stimulation as compared to those administered the vehicle under both conditions (Fig. 7A and B).

Furthermore, insulin stimulation lowered the total protein levels of PPARα and CPT-1A in the LVs of rats as compared to those which were administered the vehicle under basal conditions (Fig. 8A and B). Similarly, *C. aronia* significantly reduced the total protein levels of these markers in the LVs of rats under both basal and insulin stimulation as compared to those administered the vehicle under both conditions (Fig. 8A and B). These data suggest that *C. aronia* inhibits fatty acid oxidation by downregulation of PPARα and CPT-1A.

4. Discussion

Previous studies have shown that suppressing FAs oxidation or stimulating the cardiac glucose utilization to couple glycolysis with glucose oxidation is an important strategy to prevent and alleviate clinical manifestations in several CVDs including DC, I/R, and HF (An and Rodrigues, 2006; Lopaschuk, 2017; Taylor et al., 2001; Tuunanen et al., 2008). Therefore, developing drugs with such efficiency will provide an excellent cardiac therapeutic option. In this area of research, the data obtained from the current study showed that subacute administration of *C. aronia* aqueous extract at a final dose of 200 mg/kg for 7 days not only stimulated cardiac insulin singling and glucose uptake but also suppressed FAs oxidation and enhanced glycolysis and glucose oxidation in healthy rats' LVs. These data validate the previously reported cardioprotective effect of this plant in several settings and suggest that modulating cardiac metabolism is one of its mechanisms of action. The graphical abstract demostrating these effects is shown in Fig. 9.

In general, the regulation of FAs metabolism in the heart is a complex process that involves multiple feedback regulation that is intertwined with glycolytic pathways (An and Rodrigues, 2006). The increase in circulatory levels of FFAs such as those observed in diabetic and IR patients is the major stimulator for cardiac FAs oxidation (An and Rodrigues, 2006; Lopaschuk, 2016). CPT1 is the rate-limiting step responsible for the mitochondria β-oxidation and is responsible for the transport of FAs to the mitochondria and is mainly stimulated by increasing levels of malonyl-CoA (Schreurs et al., 2010). Besides, any increase in the

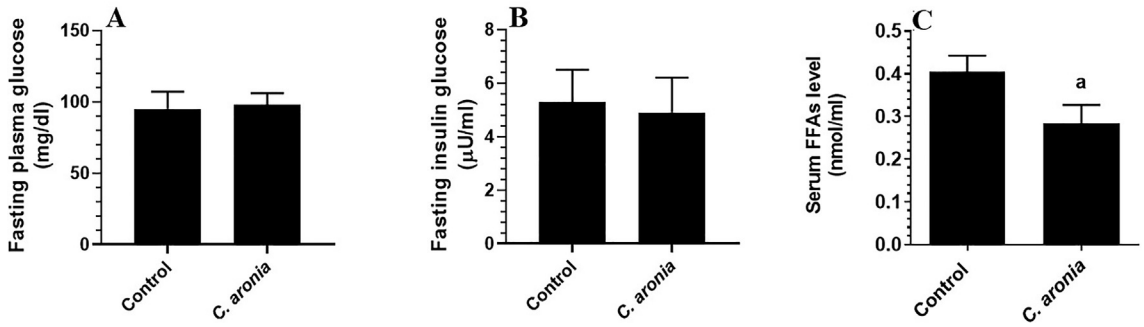

Fig. 1. Levels of Fasting plasma glucose (A) and insulin levels (B), as well as serum levels of free fatty acids (FFAs) (C) in the control and *C. aronia*-treated rats. Data are presented as mean ± SD of n = 6/group. [a]Significantly different as compared to control rats.

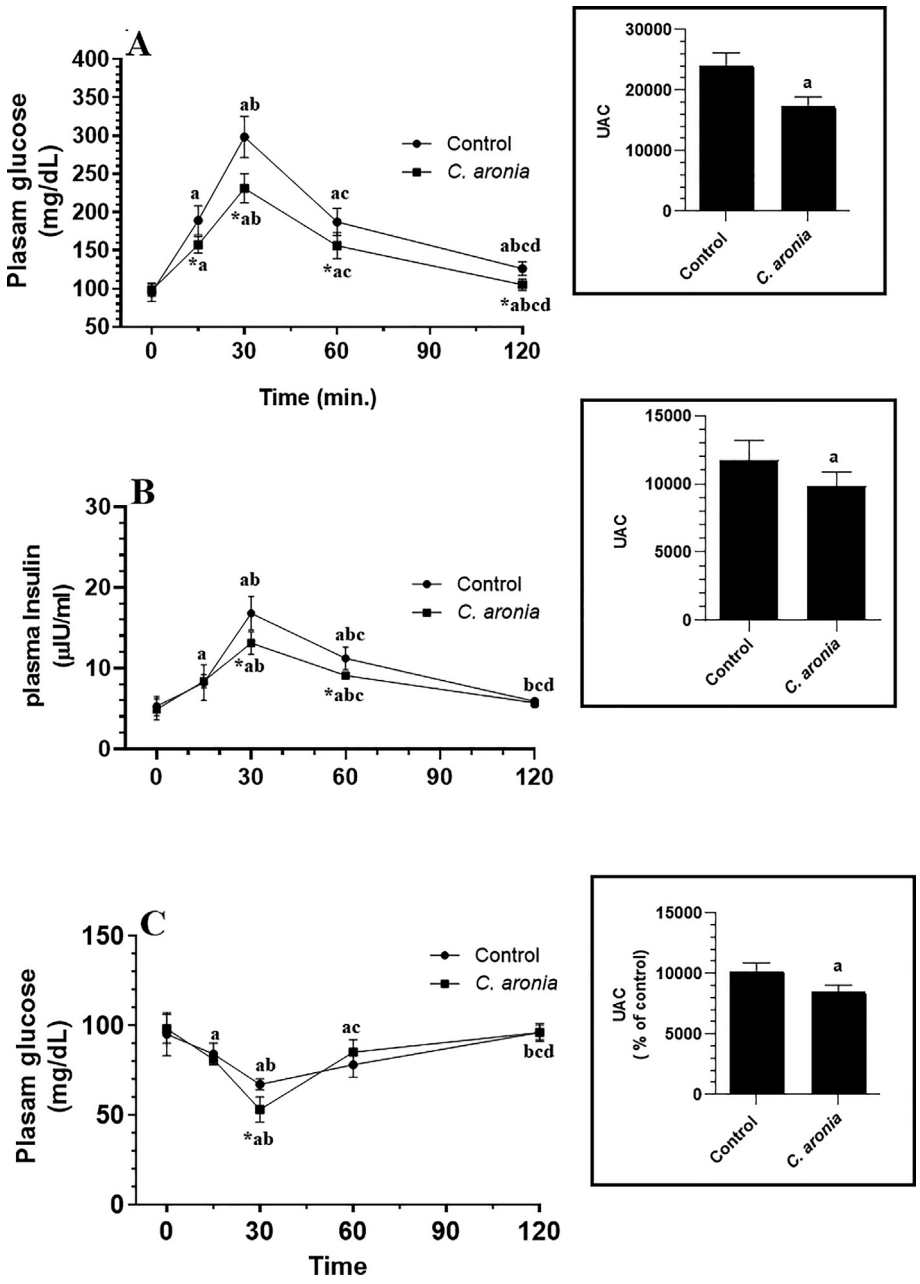

Fig. 2. Changes in glucose levels after the intraperitoneal glucose tolerant test (IGTT) (A) and changes in insulin (B) and glucose levels (C) after the intraperitoneal insulin levels in the control and *C. aronia*-treated rats. Data are presented as mean ± SD of n = 6/group. *Significantly different as compared to the corresponding time interval in the control group. [a]Significantly different compared with 0.0 min. [b]Significantly different with 30 min. [c]Significantly different with 60 min, and [d]Significantly different with 90 min. UAC: area under the curve.

activity of the transcription factor, PPARα, stimulates FAs oxidation by upregulating CPT1 and the medium-chain acyl-coenzyme A dehydrogenase (MCAD) (Fillmore et al., 2014; Foster, 2012; Minnich et al., 2001). Currently, it is largely accepted that targeting mitochondria FAs (β) oxidation is an effective strategy to improve the cardiac function in several CVDs including DC, ischemia, and HF by increasing cardiac glucose oxidation.

In this study, the administration of *C. aronia* significantly reduced cardiac of CPT1 and PPARα in the hearts of the treated healthy rats, thus suggesting suppressing of β-oxidation. These effects were also associated with a significant reduction in the levels of FFAs, possibly through increasing peripheral and cardiac insulin sensitivity. Indeed, insulin can decrease the circulatory levels of FFAs by increasing lipogenesis in the adipose tissue (Wong and Sul, 2010). Besides, insulin can suppress FAs uptake and oxidation by increasing the accumulation of malonyl CoA which allosterically inhibits CPT1 (Lopaschuk, 2016). This has been confirmed also in this study by the significant reduction in plasma glucose and insulin levels after both the glucose and insulin tolerance tests, even with no obvious effect on fasting serum and insulin levels. we have also previously shown that *C. aronia* enhances glucose tolerance and suppresses circulatory FFAs in high-fat diets-fed rats. These data could suggest that *C. aronia* stimulates insulin sensitivity independent of modulating glucose or insulin levels.

Given the concomitant increase in glucose metabolism in the hearts of *C. aronia*-treated rats (as discussed later), it seems reasonable that *C. aronia* aqueous extract enforces the myocardium

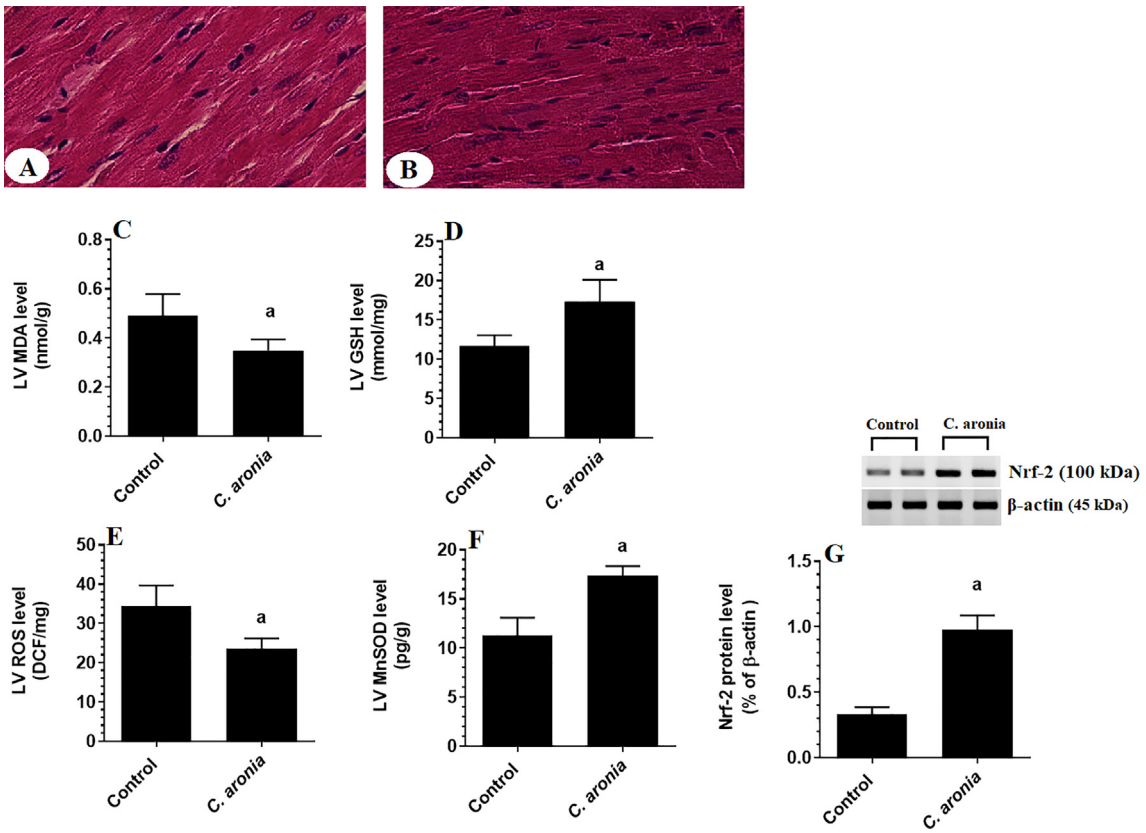

Fig. 3. Photomicrographs of the sections of the left ventricle (A and B) and left ventricular levels of malondialdehyde (MDA) (C), glutathione (GSH) (D), reactive oxygen species (ROS) (D), and manganese superoxide dismutase (MnSOD) (D) in the control and *C. aronia* treated rats. A: was taken from control rats. B: was taken from *C. aronia*-treated rats. Both A and B show normal myocardial tissue structure with intact nuclei and striated myofibrils. [a]Significantly different as compared to control rats.

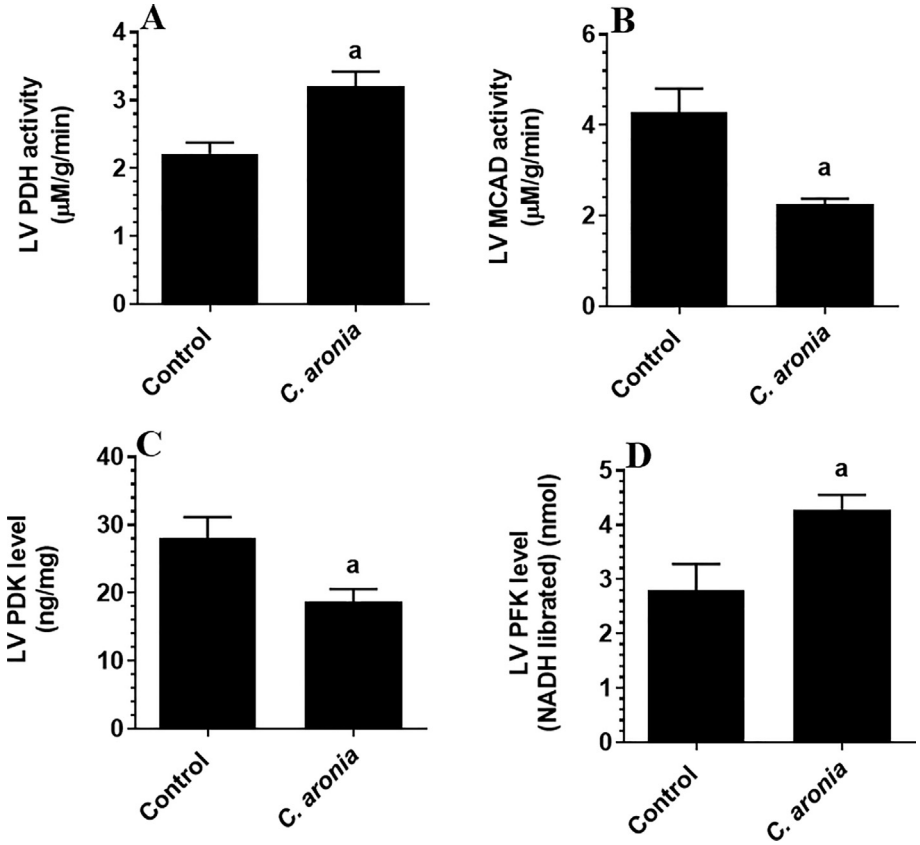

Fig. 4. Levels of pyruvate dehydrogenase (PDH) (A), medium-chain acyl-CoA dehydrogenase (MCAD) (B), pyruvate dehydrogenase kinase 4 (PDK) (C), and phosphofructokinase (PFK) (NADPH liberated) (D) in the left ventricles of the control and *C. aronia* treated rats. [a]Significantly different as compared to control rats.

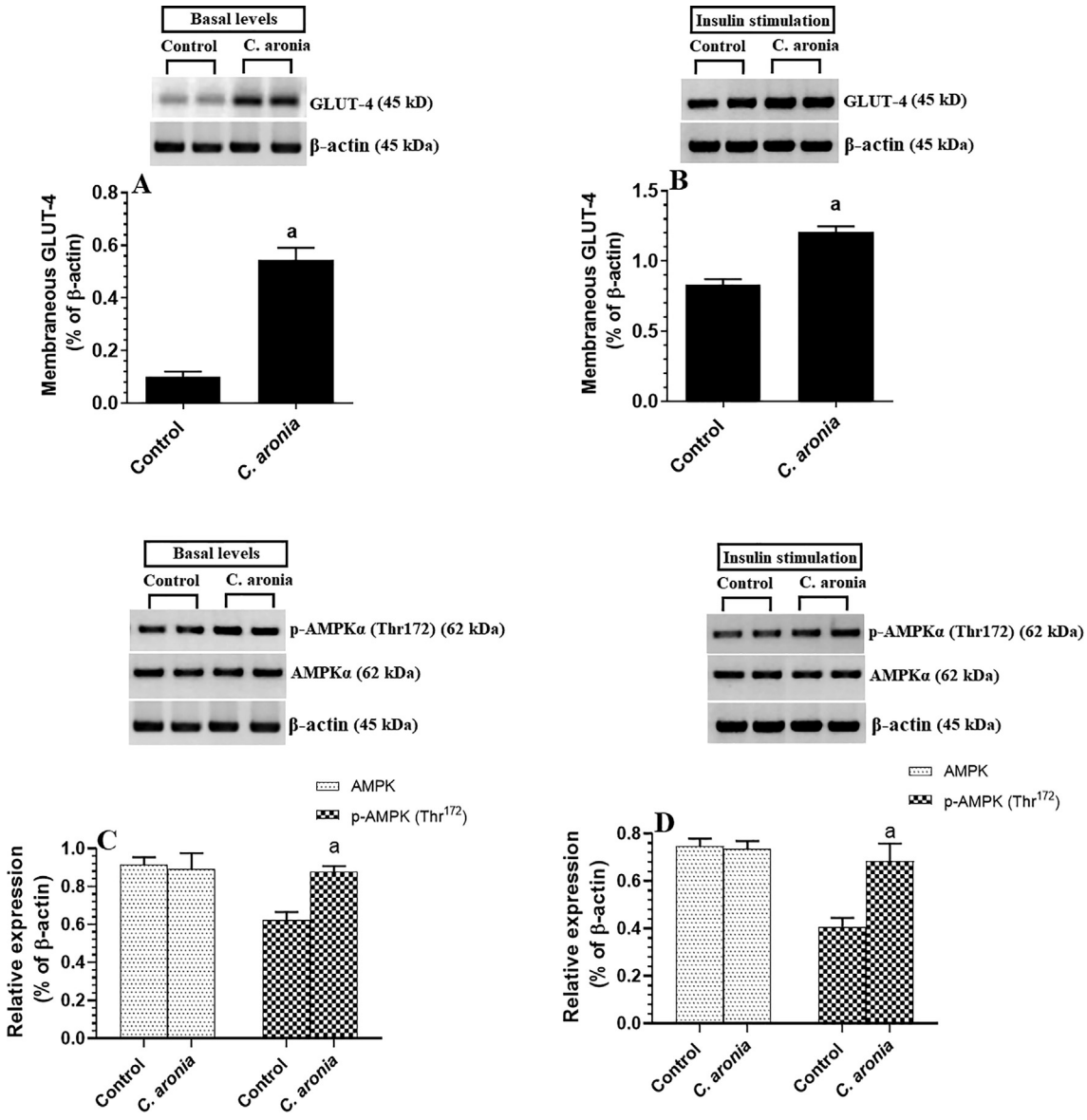

Fig. 5. Membranous protein levels of glucose transporter-4 (GLUT-4) (A and B) and total protein levels of 5′ AMP-activated protein kinase (AMPK) and p-AMPK (Thr^{172}) (C and D) in the left ventricles of the control and *C. aronia* treated rats under basal and insulin stimulation. [a]Significantly different as compared to control rats.

metabolism toward glucose utilization and oxidation by inhibiting FAs oxidation. Similar to this effect, oxfenicine, etomoxir, and perhexiline, are clinically prescribed drugs that are used to alleviate clinical symptoms in patients or animals with DC, aortic stenosis, angina pectoris, and ischemic and non-ischemic HF, mainly by suppressing FAs oxidation through inhibiting CPT1 and shifting the heart metabolism toward glucose metabolism (Cole et al., 1990; Jaswal et al., 2011; Jeffrey et al., 1995; Lopaschuk et al., 1988, 1990; Unger et al., 1997).

However, insulin stimulates cardiomyocyte glucose uptake (through GLUT 4), suppresses FAs oxidation, and stimulates glycolysis and glucose oxidation (Becker et al., 2001; Bertrand et al., 2008; Brownsey et al., 1997). The increase in FAs uptake and oxidation impairs cardiac insulin signaling and glucose oxidation. This is well-reported in both humans and experimental animals with DC and HF (Karwi et al., 2018; Swan et al., 1997; Zhang et al., 2013). However, in clinical trials in patients with DC and MI, the polarizing treatment by infusion of GIK solution improved IR, cardiac efficiency, and function (Jaswal et al., 2011).

Under normal conditions, the serine phosphorylation of IRS suppresses insulin signaling by decreasing the tyrosine phosphorylation of the same receptor (Bertrand et al., 2008). This is achieved by a negative feedback mechanism mediated by the activation of Akt/mTOR/P70S6K axis (Bertrand et al., 2008). In this study, administration of *C. aronia* stimulated insulin signaling as noticed by the significant increase and decrease in the tyrosine and serine phosphorylation, respectively, as well as the increase in Akt phosphorylation. This could be explained by the concomitant reduction in FAs oxidation., Indeed, fatty acyl CoA, diacylglycerol, and ceramide, products of FAs metabolism suppress cardiac insulin signaling by increasing the serine phosphorylation of the IRS in a mechanism that requires activation of intracellular kinases including PKC IκB kinase (Kim et al., 2004). Also, inhibiting CPT1 by the malonyl CoA decarboxylase (MCD) suppressed FAs oxidation and improved cardiac insulin sensitivity and glucose oxidation (Dyck et al., 2004; Stanley et al., 2005).

Surprisingly, although *C. aronia* induced Akt activation, it suppressed its target, mTOR. Therefore, besides a possible action

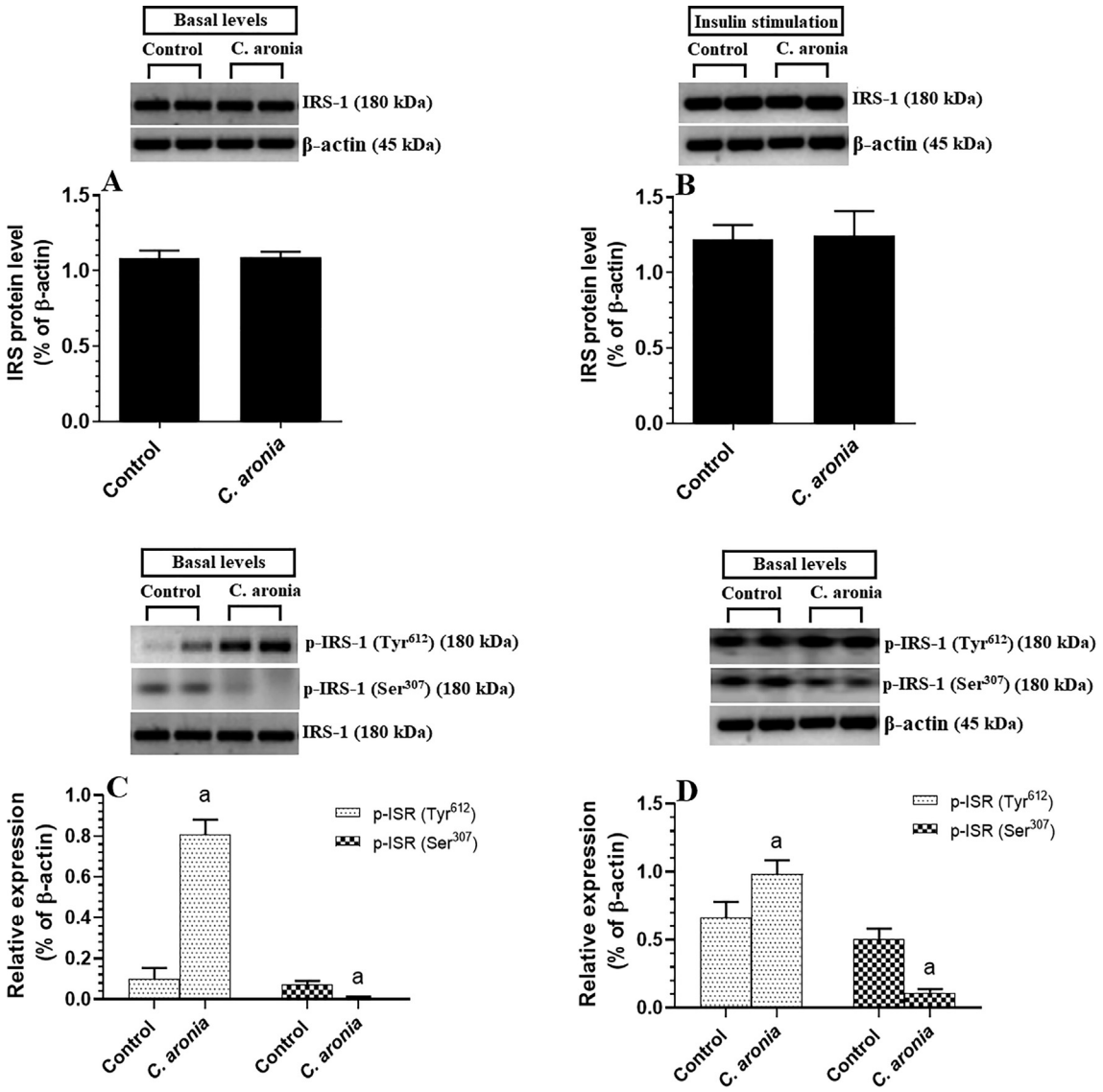

Fig. 6. Total protein levels insulin receptor substrate (IRS), p-IRS (Tyr^{612}), and p-IRS (Ser^{307}) in the left ventricles of the control and *C. aronia* treated rats under basal (A and C) and insulin stimulation (B and D). [a]Significantly different as compared to control rats.

through suppressing FAs oxidation, our data suggest that *C. aronai* also induces serine phosphorylation of IRS by inhibiting mTOR. However, and in parallel with the improved insulin signaling, *C. aronai* also stimulated glycolysis and glucose oxidation as observed by the significant increase in the activities of PFK1 and PDH, respectively. These effects were also associated with a significant reduction in the activity of PDK4, a natural inhibitor of PDH. Based on the Randle theory, FAs and glucose oxidation can inhibit each other at multiple levels in a bidirectional relationship. Accordingly, the increase in FAs derivatives (citrate, acetyl CoA, and NADH) decreases glucose oxidation by targeting the key enzymes PDH, thorough activating PDK4 (An and Rodrigues, 2006). On the contrary, the levels acetyl CoA produced induced by PDH or the increase in NADH levels produced by glucose oxidation inhibit β-oxidation by suppressing the enzyme, 3-KAT (An and Rodrigues, 2006). Besides, insulin represses FAs oxidation by increasing the activity of ACC and downregulating CPT1 (Fillmore et al., 2014). Therefore, based on these data, we can conclude that *C. aronai* stimulates the cardiac glycolysis and glucose oxidation by stimulating insulin signaling and repressing FAs oxidation.

Nevertheless, it was later of our interest to reveal the precise mechanism by which *C. aronia* regulates cardiac metabolism. For this reason, we have targeted AMPK, being the major energy sensor that regulates energy utilization and substrate selection in response to energy deficits (Qi and Young, 2015; Srivastava et al., 2012). In this regard, AMPK acts on all levels of energy regulation by regulating gene transcription, substrate utilization, and mitochondria biogenesis (An and Rodrigues, 2006). Besides, the mechanism regulating cardiac energy of AMPK is board and includes regulation of glycolysis, glucose and FAs oxidation, and protein and glycogen synthesis (An and Rodrigues, 2006; Qi and Young, 2015; Srivastava et al., 2012). Using these mechanisms, the current investigation has shown that AMPK regulates cardiac metabolism under physiological and pathological conditions DC, MI, I/R, HF, and hypertrophy (Li et al., 2019; Lu et al., 2019; Qi and Young, 2015; Russell et al., 2004). Currently, a consensus in the literature is confirming that the primary effect driven by activating AMPK in the heart is mediated mainly by stimulating glucose uptake (Glut-4 expression), glycolysis, and glucose oxidation, thereby shifting the cardiac metabolism toward glucose utilization and inhibiting FAs

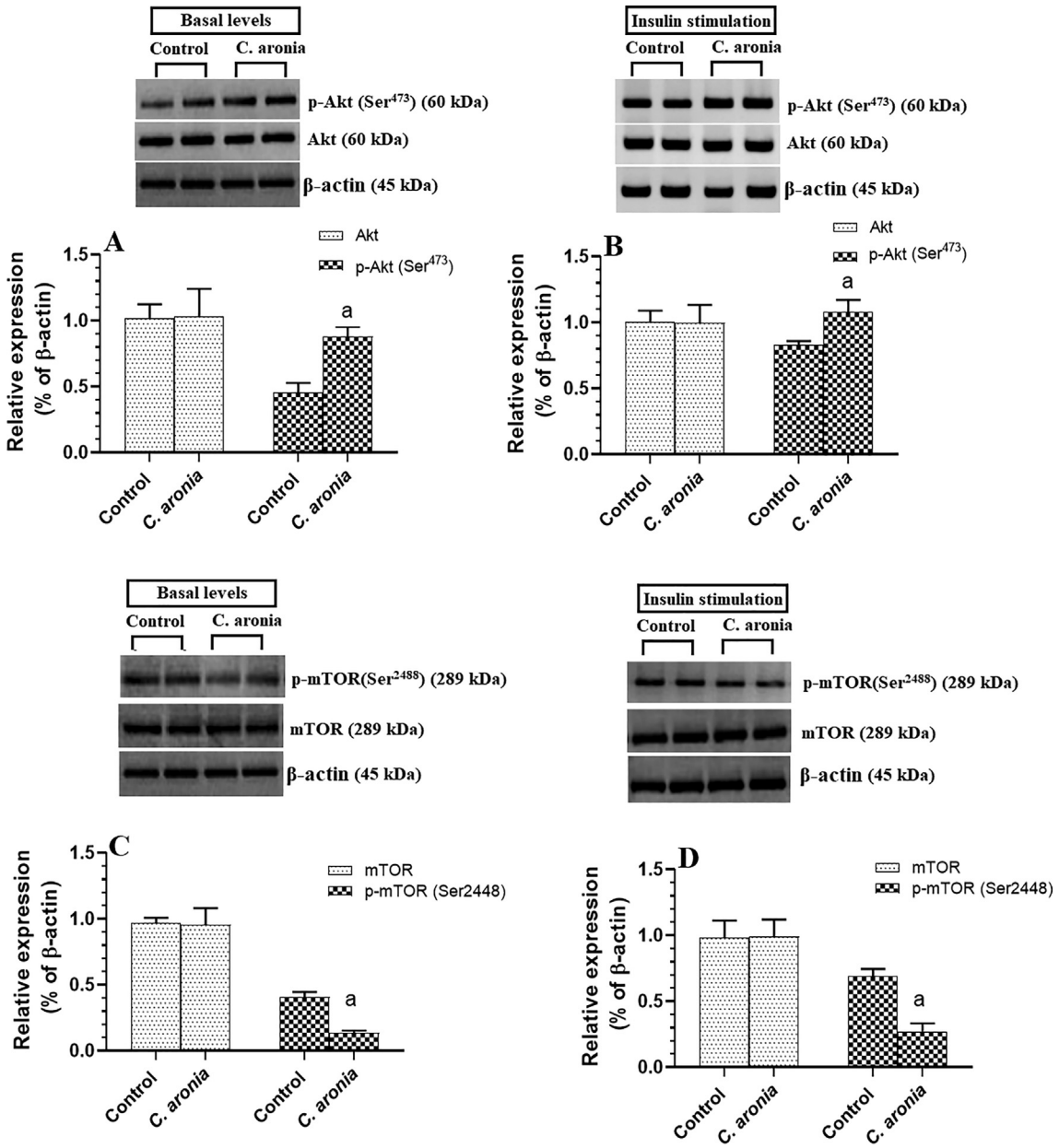

Fig. 7. Protein levels total protein kinase B (Akt), p-Akt (Ser^{473}), mTOR, and p-mTOR (Ser^{2488}) in the left ventricles of the control and *C. aronia* treated rats under basal (A and C) and insulin stimulation (B, D). [a]Significantly different as compared to control rats.

oxidation (Srivastava et al., 2012). Indeed, genetic mutation of AMPK resulted in cardiac metabolic storage disease, impaired conduction, and hypertrophy (Arad et al., 2002). Using kinase-dead (KD) transgenic mice, AMPK was demonstrated mainly to be involved in glucose uptake during I/R cardiac injury (Russell et al., 2004). Besides, infusion of AICAR, an AMPK activator stimulated cardiac AMPK levels and increased myocardial GLUT-4 translocation (Russell III et al., 1999).

The most interesting finding in this study is the observation that administration of *C. aronia* significantly activated the cardiac levels of AMPK which may explain all the observed effects including the increase in glucose uptake and oxidation. Besides, it may explain the previously observed significant reduction in the activity of mTOR. In support, several authors have shown that AMPK can inhibit protein synthesis and modify insulin signaling (decreasing (p-Ser^{307}) by inhibiting the TSC2/mTORC1/p70S6K and eIF2B pathways (Bertrand et al., 2008; Bolster et al., 2002; Krause et al., 2002). Based on these data, it becomes evident to our knowledge that the concomitant reduction in FAs oxidation afforded by *C. aronia* is secondary to its stimulatory role of glucose oxidation.

On the other hand, AMPK can protect the cardiomyocytes and other cells by suppressing oxidative stress and inflammation. Recently, the inhibitory effect of AMPK on oxidative stress and inflammation was shown to be associated with upregulation and activation of the transcription factor, Nrf2 which is responsible for the synthesis of phase II enzymes including gamma-glutamyl Glutamate Cysteine Ligase (GCL), catalase, and SOD2 (Mo et al., 2014). In previous studies, the protective effect of *C. aronia* on the cardiomyocytes and other tissue was attributed to an antioxidant potential mediated by inhibiting the generation of the ROS and stimulating antioxidant levels (Dallak, 2018; Shatoor and Al Humayed, 2020). This has been also confirmed in this study where we have found a significant reduction in the levels of ROS and lipid peroxides (MDA) with a parallel increase in GSH and MnSOD2

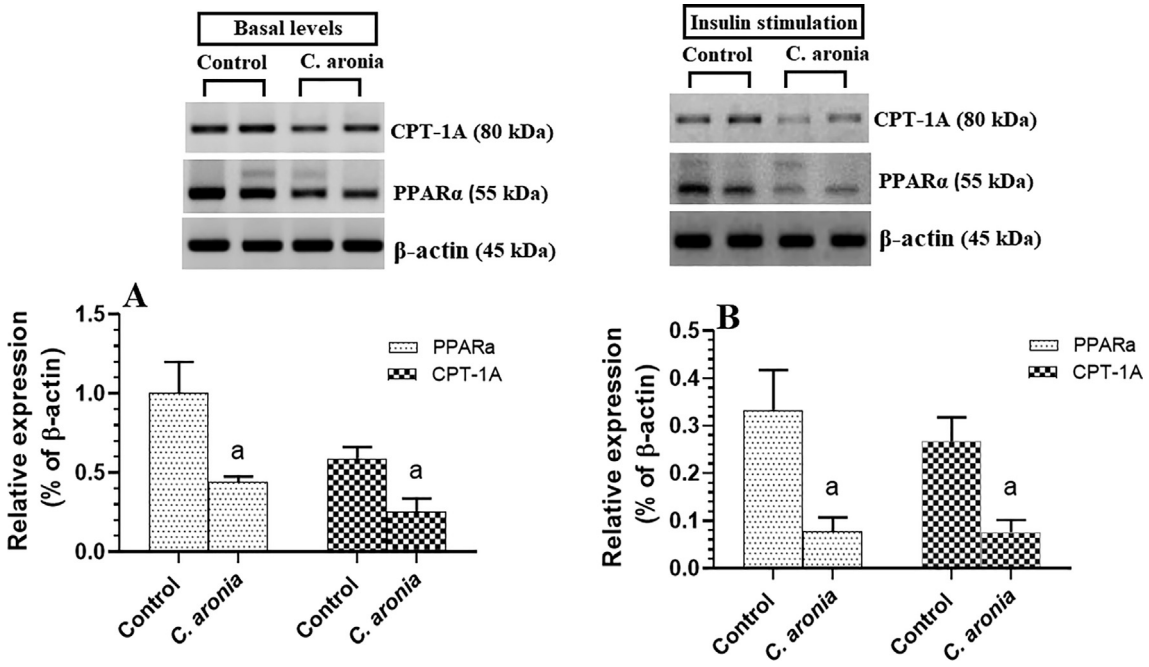

Fig. 8. Total protein levels of Carnitine palmitoyltransferase (CPT1A) and peroxisome proliferator-activated receptor-α (PPARα) in the left ventricles of the control and *C. aronia* treated rats under basal (A and C) and insulin stimulation (B, D). [a]Significantly different as compared to control rats.

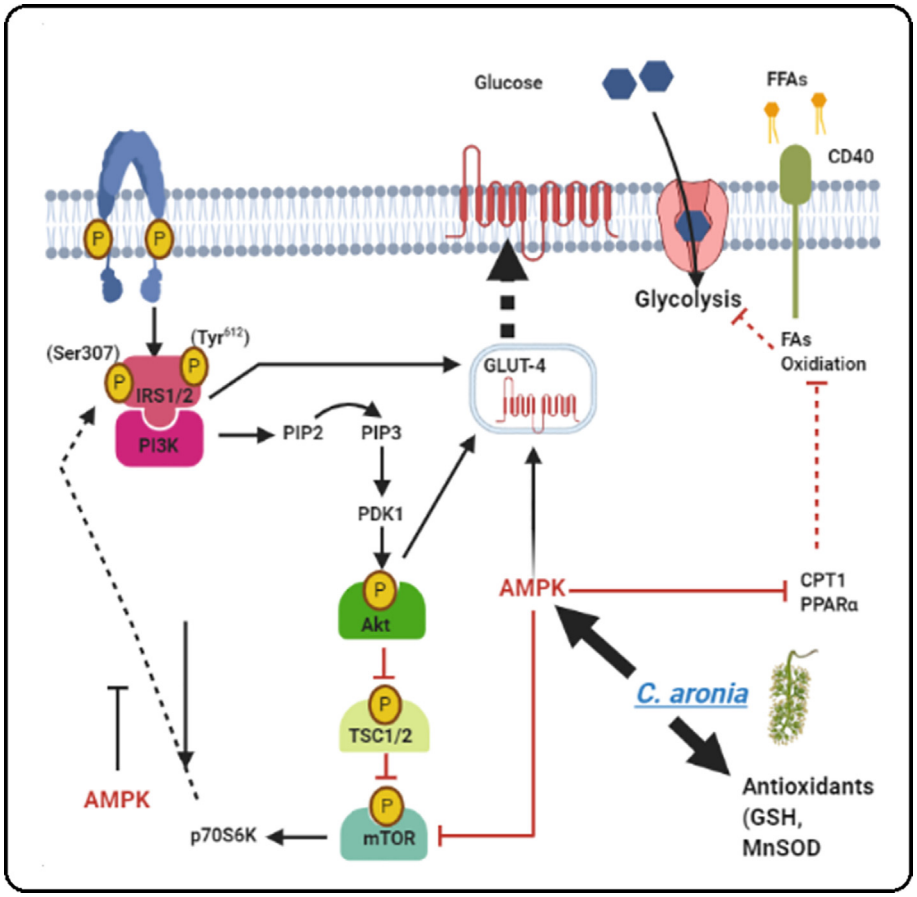

Fig. 9. A graphical abstract demonstrating the mechanistic role of *C. aronia* in the hearts of healthy rats. Under normal condition, insulin signalling is initiated by the phosphorylation of the insulin receptor. This auto-phosphorylates the insulin receptor substrate (IRS) at Tyr^{612} which then activates (phosphorylates) PI3K and subsequently Akt. Both PI3K and Akt stimulates GLUT-4 translocation. Also, Akt stimulates mTOR which in turn negatively supresses insulin signalling phosphorylating the IRS at Ser^{307} mediated by the activation of P70S6K. At the same time, the increased uptake of free fatty acids (FFAs) and higher levels of FAs oxidation supresses the cardiac glycolysis. AMPK also increases GLUT-4 translocation directly or through stimulating insulin signaling through supressing mTOR. Also AMPK supresses FAs metabolism by inhibiting the transcription factor PPARα and Carnitine palmitoyltransferase (CPT1) enzyme, thus stimulating glycolysis. The administration of *C. aronia* to rats stimulated cardiac insulin signalling, GLUT-4 membranous expression, glycolysis, and supressed FAs oxidation. These effects were associated with increased activation of AMPK. Besides, *C. aronia* is able to stimulate cardiomyocytes' antioxidant levels.

levels. However, the interesting observation is that we have also found a significant increase in the expression of Nrf2 in the LVs of C. *aronia*-treated hearts which may explain these observations. As discussed above, this was also associated with an increase in AMPK levels, thus highlighting the importance of the AMPK/Nrf2 axis in the mechanism of cardioprotection afforded by the extract of this plant.

Despite these findings, this study still has some limitations. Importantly, this study was done only in male rats. These findings should be also validated in future studies using female rats and other species. the effect of *C. aronia* on AMPK activities and its role in the observed metabolic effects remained observational. Besides, the effect of *C. aronia* is demonstrated in control hearts only. Therefore, further studies using animal models with cardiovascular disorders and AMPK inhibitor will widen our knowledge about the role of AMPK in the cardioprotective mechanism afforded by *C. aronia*. Another limitation of this study is that we are still unsure about the precise composition of the extract responsible for these cardiac metabolic effects. Hence, further chemical fractionation of the active compounds contained in this plant and their effect on cardiac insulin signaling, FAs metabolism, and AMPK activation are required.

In conclusion, the findings of this are novel and show for the first time the short-term administration of *C. aronia* is associated with increasing glycolysis and glucose oxidation −4 and suppression of FAs metabolism in the hearts of healthy rats. These effects are also associated with increased AMPK activation. These findings encourage further experimental and clinical trials to identify the active ingredients responsible for these effects in a trial to find more effective alternative therapeutic drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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