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AKADÉMIAI KIADÓ

Effect of *Aronia melanocarpa* fruit juice on glucose tolerance, lipid metabolism, and obesity in a rat model of metabolic syndrome

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ABSTRACT

Metabolic syndrome (MS) is a serious health condition. The purpose of this study was to investigate the effects of polyphenol-rich *Aronia melanocarpa* fruit juice (AMFJ) on glucose tolerance, triglyceride levels, and adipose tissue in rats with MS induced by high-fat high-fructose (HFHF) diet. Fifty rats were allocated in 5 groups: control, MS, MS+AMFJ$_{2.5}$, MS+AMFJ$_5$, and MS+AMFJ$_{10}$. In the course of 10 weeks, the control group was on a regular rat diet while the other groups received HFHF diet. During the experiment, control and MS groups were treated daily orally with distilled water (10.0 mL kg^{-1}) and the other three groups – with AMFJ at doses of 2.5, 5.0, and 10.0 mL kg^{-1}, respectively. In MS rats, glucose intolerance, hypertriglyceridemia, visceral obesity, and increased adipocyte size were observed. In AMFJ-treated groups, the serum glucose and triglycerides, as well as visceral fat and adipocyte size decreased significantly and did not differ from those of the control group. AMFJ at doses 2.5 and 5.0 mL kg^{-1} showed an anti-apoptotic activity in adipocytes, while at the dose of 10 mL kg^{-1} a pro-apoptotic effect was detected. In conclusion, AMFJ could antagonise most of the negative consequences of HFHF diet on carbohydrate and lipid metabolism in a rat MS model.

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KEYWORDS

Aronia melanocarpa fruit juice, glucose tolerance, metabolic syndrome, triglycerides, obesity

1. INTRODUCTION

Metabolic syndrome (MS) is a "modern" pathological condition, creating significant health and financial burden. Its effects on the overall health and potential therapeutic strategies are being investigated using animal models. Generally, the experimental models of MS are classified in two main groups – genetic and diet-induced. Genetic models require a shorter period of time for the development of MS. Knock-out mice with modified expression of the genes regulating food intake are commonly used (Lutz and Woods, 2012). These models require advanced technologies and are not achievable in some scientific laboratories. In addition, they do not reflect the etiology of MS in humans. In diet-induced models, carbohydrates (fructose, glucose, and sucrose) and/or saturated fats are used to induce MS and related biochemical abnormalities (Panchal and Brown, 2011). These models reflect closely the pathogenesis of the condition in humans and are able to replicate the clinical picture of MS.

Among the substances with therapeutic potential in MS are polyphenols (PPs). PPs are present in fruits, vegetables, beverages, and spices and they are traditionally classified into 2 groups: flavonoids (flavones, isoflavones, flavonols, flavanols, flavanones, and anthocyanins) and non-flavonoids (phenolic acids, stilbenes, and lignans) (Tsao, 2010). Phenolic compounds exert powerful antioxidant effects, and studies provide evidence about their potential to cause reduction of body weight and improvement of lipid profile and insulin resistance in individuals with MS (Williamson, 2017).

Aronia melanocarpa (black chokeberry) fruits are characterised by a very high polyphenolic content. Due to their astringent taste, they are usually used in the form of juice, jam, or wine. *A. melanocarpa* fruit juice (AMFJ) is rich in anthocyanins, proanthocyanidins (oligomeric flavonoids), and phenolic acids (Valcheva-Kuzmanova et al., 2014). Currently there are no studies investigating the effects of AMFJ on adipogenesis and biochemical abnormalities in rats with diet-induced MS.

The purpose of the current study was to investigate the effects of polyphenol-rich AMFJ on the glucose tolerance, triglyceride levels, and adipose tissue in rats with high-fat high-fructose (HFHF) diet-induced MS.

2. MATERIALS AND METHODS

2.1. AMFJ – preparation and composition

The juice was prepared by grinding, pressing, and squeezing the fresh fruit grown in the Balkan Mountains, Bulgaria. It was filtered, preserved with potassium sorbate (1.0 g L^{-1}), and stored at room temperature. The contents of PPs were as follows: total phenols (measured spectrophotometrically) – 5,461 gallic acid equivalents/L; total proanthocyanidins (measured gravimetrically) – 3,122.5 mg L^{-1}; anthocyanins (determined by high-performance liquid chromatography): cyanidin 3-galactoside – 143.7 mg L^{-1}, cyanidin 3-arabinoside – 61.7 mg L^{-1},

cyanidin 3-glucoside – 4.4 mg L^{-1}, cyanidin 3-xyloside – 11.6 mg L^{-1}, and phenolic acids (measured by high-performance liquid chromatography): chlorogenic acid – 585 mg L^{-1} and neochlorogenic acid – 830 mg L^{-1} (Valcheva-Kuzmanova et al., 2014).

2.2. Experimental animals, induction of MS and treatment protocol

Fifty male Wistar rats (at the age of 2 months) with an initial body weight of 180–280 g were included in the experiment. They were housed in plastic cages, at an ambient temperature of 20–25 °C, under 12-h light/dark cycle. They had access to food and drinking water *ad libitum*.

The rats were allocated into 5 groups (10 rats per group): control, MS, MS+$AMFJ_{2.5}$, MS+$AMFJ_5$, and MS+$AMFJ_{10}$. The control group was on regular rat chaw and tap water, while the other groups received HFHF-diet and 10% fructose in the drinking water to induce MS (according to the methodology described by Gancheva et al., 2015). HFHF diet was prepared by enriching the regular rat chow with 17% lard and 17% fructose. The caloric intake of the HFHF-diet was 405 kcal/100 g, where lard provided 38% of the energy intake and fructose – 17%. The fructose solution accounted for additional 40 kcal/100 mL.

In the course of 10 weeks, the experimental groups received a daily oral treatment through an orogastric probe. The control and MS group were treated with distilled water in a volume of 10 mL kg^{-1} body weight. Groups MS + $AMFJ_{2.5,}$ MS + $AMFJ_5$, and MS + $AMFJ_{10}$ were treated with increasing doses of AMFJ – 2.5, 5.0, and 10 mL kg^{-1} body weight, respectively. The doses of 2.5 and 5 mL kg^{-1} juice were diluted with distilled water to a total volume of 10 mL kg^{-1}.

All procedures concerning animal treatment and experimentation were conducted in conformity with the national and international laws and policies (EU Directive 2010/63/EU for animal experiments) and were approved by the Bulgarian Food Safety Agency (Document 177/07.07.2017).

2.3. Glucose tolerance test (GTT)

The test was performed at the end of the 10th week of the experiment. After 12 h of fasting, the rats were given an intraperitoneal injection of glucose at a dose of 2 g kg^{-1} body weight prepared as 40% solution. A blood sample from the distal end of the tail was collected. Blood glucose was measured with ACCU-CHEK Performa glucometer by using ACCU-CHEK Performa test strips immediately before injection (0 min), and 30, 60, and 90 min thereafter. The results of the blood glucose levels were presented both as absolute values (mmol L^{-1}) and percent (%) of the initial values.

2.4. Serum triglycerides (TGs)

At the end of the 10th week, the rats were anesthetised with diethyl ether. Blood was collected from the sublingual veins and then centrifuged at 2000 r.p.m. for 10 min. The obtained serum was stored at –20 °C until the time of analysis. Triglycerides were measured in the serum by using a colorimetric kit (Bio Maxima, Poland) at a spectrophotometer AURIUS 2021 (Cecil Instruments Ltd.).

2.5. Calculation of fat indices

After the sacrifice of the anesthetised animals, mesenteric, perigonadal, paranephral, and retroperitoneal fat pads were dissected and weighed. The total visceral adipose tissue was

calculated. The total and retroperitoneal fat indices were calculated according to the formula: fat pad weight/total body weight $\times 10^2$.

2.6. Histological examination of adipose tissue

Pieces from the retroperitoneal adipose tissue were fixed in 10% neutral buffered formalin and included in paraffin with a melting point of 52–54 °C in order to prepare paraffin blocks. Sections 5 μm thick were stained with hematoxylin-eosin (H and E) in the standard way to assess the histological changes in the adipose tissue.

2.7. Immunohistochemical examination of adipose tissue

An indirect immunoperoxidase method was used for immunohistochemical analysis of retroperitoneal adipose tissue samples by using the mini KIT high Ph DAKO K8024. Bax and Bcl-2 antibodies, at a dilution of 1:50, were used as markers for apoptosis and apoptosis suppression, respectively.

Evaluation of the immunohistochemical study was performed by examining 2 fields at maximum magnification for each individual case. Digital photos were taken with scanning device Leica A-perio Scan Scope AT2 (Aperio Technologies, Vista, CA), and subsequent analysis of the scanned images was performed with Image Scope V12.1.0.5029 (Aperio).

In order to represent the results quantitatively, a morphometric method was used: at least fifty cells per adipose tissue sample were examined in randomly selected fields. Bax and Bcl-2 were determined as present (score 1) or lacking (score 0) based on the presence/absence of intranuclear immune deposits, and their expressions were presented as mean number of positive cells per field. Finally, Bax/Bcl-2 ratio was calculated.

2.8. Statistical analysis

GraphPad Prism 5.00 statistical software (California, USA) was used to analyse the scientific data. The results are presented as mean ± SEM. The results from the glucose tolerance test were assessed by Student's *t*-test and two-way ANOVA. For the other results, one-way ANOVA was used. ANOVA was followed by Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Glucose tolerance test (GTT)

The results from the GTT are presented in Table 1 and Fig. 1.

Student's *t*-test found a borderline significance ($P = 0.059$) in the absolute glucose values at the 30th minute of the MS group compared to the control group. Presented as a percent of the initial value during the same time interval, there was a significant ($P < 0.05$) increase in the glucose in MS group compared to the control group (Table 1, Fig. 1).

Two-way ANOVA with Dunnet's multiple comparison test showed a significant increase in the blood glucose (%) of MS compared to the animals from the control group at the 30th ($P < 0.01$) and 60th ($P < 0.05$) minute. At the 30th and 60th minute the glucose levels of rats belonging to MS+AMFJ$_{2.5}$, MS+AMFJ$_5$, and MS+AMFJ$_{10}$ groups did not differ significantly from those of control animals (Table 1, Fig. 1).

Table 1. Glucose values (mmol L^{-1} and %) at the 30th, 60th, and 90th minute during the GTT

	30th min		60th min		90th min	
	(mmol L^{-1})	(%)	(mmol L^{-1})	(%)	(mmol L^{-1})	(%)
Control	15 ± 1.11	252.7 ± 21.76	11.35 ± 0.77	194.1 ± 17.22	8.51 ± 0.51	146.1 ± 9.33
MS	18.19 ± 1.05$^{(\&)}$	341.1 ± 25.28$^{\&**}$	13.13 ± 1.16	259.8 ± 28.08*	9.35 ± 0.63	184.9 ± 16.41
MS+AMFJ$_{2.5}$	15.48 ± 1.13	287.2 ± 21.00	12.36 ± 0.66	231.2 ± 17.18	9.34 ± 0.51	174.5 ± 12.70
MS+AMFJ$_{5}$	14.19 ± 0.73	291.6 ± 10.71	11.59 ± 1.12	235.6 ± 14.53	9.34 ± 0.61	193.1 ± 14.19
MS+AMFJ$_{10}$	17.03 ± 1.35	318.9 ± 26.28	14.11 ± 0.098	271.5 ± 24.27	10.21 ± 0.54	194.7 ± 15.79

$^{(\&)}$: $P = 0.059$ vs. Control group; $^{\&}$: $P < 0.05$ vs. Control group – evaluated by Student's *t*-test; *: $P < 0.05$ vs. Control group; **: $P < 0.01$ vs. Control group – evaluated by two-way ANOVA.

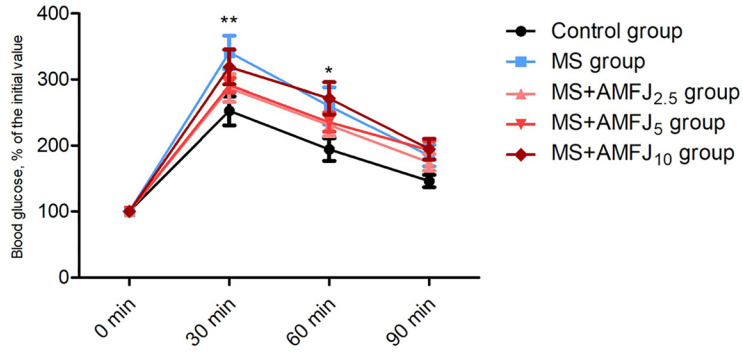

Fig. 1. Blood glucose levels in the GTT presented as a percent (%) from the initial values at 0, 30, 60, and 90 min; *: $P < 0.05$ and **: $P < 0.01$ of MS vs. Control group evaluated by two-way ANOVA

Insulin resistance is the main pathogenetic mechanism in the development of glucose intolerance in MS individuals. The most common method used to assess insulin resistance in experimental animals is the GTT. HFHF-diet used in this study has been shown to induce insulin resistance and hyperglycaemia (Gancheva et al., 2015). The results from the current study confirmed these findings as MS impaired the glucose control and presumably induced insulin resistance after 10-weeks of experimentation. AMFJ improved this impaired regulation by attenuating the effect of the HFHF-diet, thus maintaining the glucose at levels that were not different from the control values. The glucose-lowering effect of AMFJ has been demonstrated in an experimental model of diabetes (Valcheva-Kuzmanova et al., 2007b). On the one hand, the influence of *A. melanocarpa* on blood glucose could be explained with its possible insulin-sensitising effect after sub-chronic treatment period. As studies have reported, *A. melanocarpa* and its phenolic compounds could stimulate the hepatic effects of insulin (glycogen synthesis) and decrease the serum glucose level (Mu et al., 2020). On the other hand, mechanisms associated with carbohydrate absorption and transport could be involved. Alpha-glucosidase is an enzyme responsible for carbohydrate breakdown and absorption in the small intestine. Thus, its inhibition could reduce the serum glucose. The α-glucosidase-inhibiting activity was demonstrated for *Aronia* juice (Yamane et al., 2017) and *A. melanocarpa* fruit extract (Bräunlich et al., 2013). The authors found that these effects were most pronounced with the anthocyanin cyanidin 3-arabinoside and the least pronounced with cyanidin 3-xyloside (Bräunlich et al., 2013). The juice used in this study had a high cyanidin 3-arabinoside content. The potential hypoglycaemic effect of the juice could be due to stimulation of insulin secretion, as the fruit of *A. melanocarpa* is extremely rich in anthocyanins, which demonstrate such an effect *in vitro* (Jayaprakasam et al., 2005). Dipeptidyl peptidase-IV (DPP-IV) is an enzyme that degrades the endogenous insulin secretagogues glucagon-like peptide-1 and glucose-dependent insulinotropic peptide. DPP-IV inhibitory activity of Aronia juice was reported by Yamane et al. (2017).

3.2. Serum TGs

A significant increase in the TG levels was observed in the MS group compared to the control group ($P < 0.05$) (Fig. 2). AMFJ administration antagonised the TG elevation in a dose-

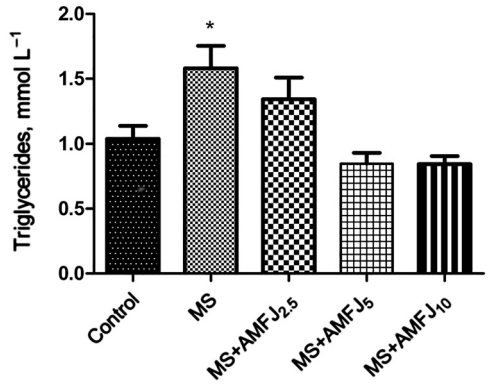

Fig. 2. Serum triglycerides in an experimental model of metabolic syndrome (MS) in rats treated with AMFJ at doses of 2.5, 5.0, and 10.0 mL kg^{-1}; *: $P < 0.05$ vs. Control

dependent manner. Thus, there was a non-significant difference between the triglyceride levels of the AMFJ-treated groups and the control group (Fig. 2).

In the current study, HFHF-diet induced a notable increase in the serum TGs (one of the MS components) in MS group. AMFJ treatment prevented the elevation of the TG levels. The TG-lowering effect of AMFJ was described by Valcheva-Kuzmanova et al. in experimental models of hypercholesterolemia (Valcheva-Kuzmanova et al., 2007a, 2007c) and diabetes (Valcheva-Kuzmanova et al., 2007b). The mechanisms underlying this effect could be sought in the intestinal lipid absorption and the hepatic lipid metabolism. Dietary fats are degraded in the small intestine by the pancreatic enzyme lipase to FFAs, which are absorbed after forming bile acid micelles. Takahashi et al. (2015) reported that anthocyanins-rich *Aronia* fruit inhibited pancreatic lipase activity, the absorption of dietary fat and suppressed the postprandial hyperlipidaemia. Fatty acid synthase (FAS) is an enzyme responsible for hepatic *de novo* lipogenesis. Triglycerides are produced in the liver from accumulated FFAs. Peroxisome proliferator-activated receptor-gamma (PPAR-γ), a hepatic transcription factor, regulates the preadipocyte differentiation, but also the hepatic lipoprotein metabolism and lipolysis. PPAR-γ induces the activity of lipoprotein lipase, and there is a positive correlation between PPAR expression and formation of lipid droplets in the hepatocytes. It has been found that *A. melanocarpa* decreased the expression of PPAR-γ and FAS (Park et al., 2017). These events could decrease FFA influx into the liver and decrease the hepatic TG production.

3.3. Fat indices

The retroperitoneal fat index in MS group was significantly higher than that of the control group rats ($P < 0.05$). The retroperitoneal fat indices of AMFJ-treated groups were not significantly different from the control group (Fig. 3).

Although non-significant, the total fat index tended to be higher in MS rats compared to the control animals. In all AMFJ-treated groups, the total fat index had values similar to those of the control group (Fig. 3).

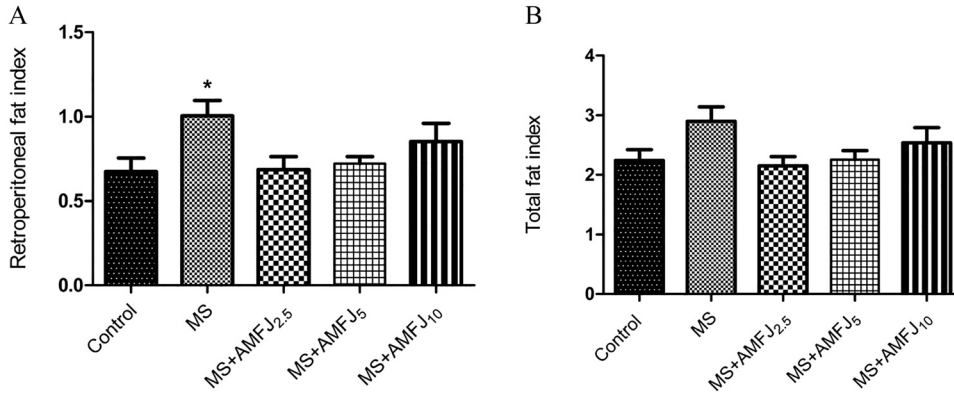

Fig. 3. Retroperitoneal (panel A) and total (panel B) fat indices in an experimental model of metabolic syndrome (MS) in rats treated with AMFJ at doses of 2.5, 5.0, and 10.0 mL kg^{-1}; *: $P < 0.05$ vs. Control

3.4. Histological examination of adipose tissue

The histological pictures of the retroperitoneal adipose tissue of the experimental groups are presented in Fig. 4. The HFHF diet induced changes in the adipose tissue of the rats from MS group, expressed as an increase in the size of adipocytes (curved arrows) compared to the control group (arrows). Restoration of the size (arrowheads) was observed in all three groups treated with AMFJ, with changes being more pronounced at higher doses.

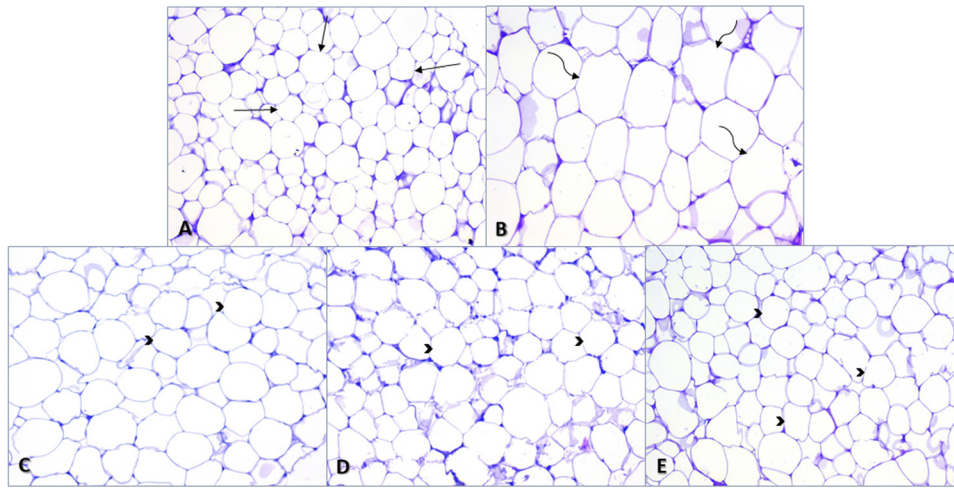

Fig. 4. Histological pictures of the retroperitoneal adipose tissue of animals from groups Control (panel A), MS (panel B), MS+$AMFJ_{2.5}$ (panel C), MS+$AMFJ_5$ (panel D), and MS+$AMFJ_{10}$ (panel E); arrows – normal adipocyte size, curved arrows – enlarged adipocytes, arrowheads – restored adipocyte size; H and E staining, magnification × 200

Visceral obesity is considered as a hallmark of MS. The HFHF-diet induced an increase in the visceral adipose tissue and adipocytes size. AMFJ treatment antagonised these effects of the HFHF-diet. These findings are similar to the reported in other experimental studies focused on the effect of *Aronia* extracts on weight (Jakovljevic et al., 2018; Kim et al., 2018; Lim et al., 2019). The observed anti-obesity effect in this experiment could be attributed to the high polyphenolic content of AMFJ. *A. melanocarpa* polyphenols have been shown to inhibit lipid accumulation in adipocytes and adipocyte differentiation – two key moments contributing to obesity (Lee et al., 2021). The expression of cytosine-cytosine-adenosine-adenosine-thymidine enhancer-binding protein alpha, and fatty acid-binding protein, which are involved in these two pathogenetic moments, decreased after *A. melanocarpa* administration – a finding described by Park et al. (2017).

3.5. Immunohistochemical examination of adipose tissue

As shown in Fig. 5, the expression of the apoptotic Bax protein (brown deposits, indicated by arrows) increased in the MS group compared to the control group. AMFJ down-regulated the pro-apoptotic marker in all treated groups.

The expression of Bcl-2 is presented in Fig. 6. Adipocytes of the animals from MS group had higher expression of Bcl-2 (brown deposits, indicated by arrows) compared to the control group. The doses of 2.5 and 5 mL kg^{-1} AMFJ upregulated Bcl-2. The dose of 10 mL kg^{-1}, however, reduced the expression of the anti-apoptotic protein.

The results from the calculation of Bax/Bcl-2 ratio are presented in Fig. 7. The MS rats had higher Bax/Bcl-2 ratio compared to the control group, and this result showed a prevailing apoptotic potential of the adipocytes. At doses of 2.5 and 5.0 mL kg^{-1}, AMFJ upregulated Bcl-2, as a result of which it decreased the ratio – this indicated an anti-apoptotic phenotype. This

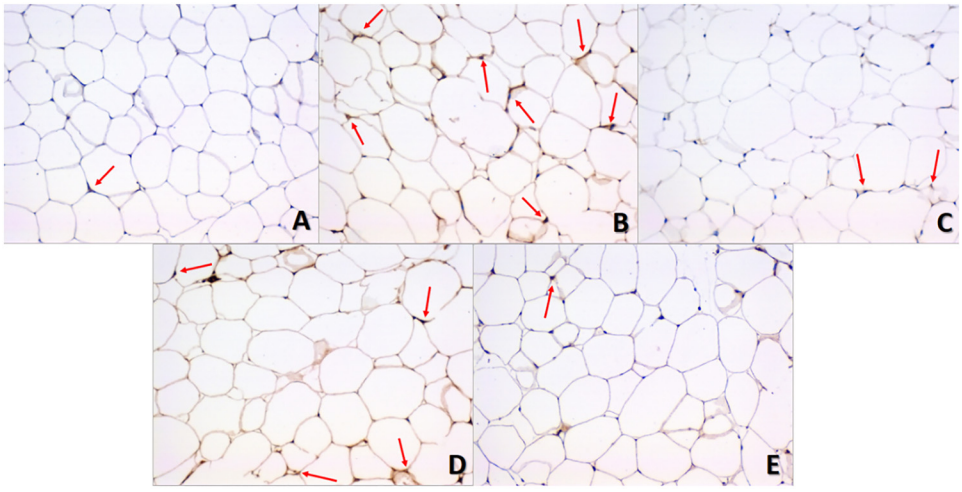

Fig. 5. Bax expression (arrows) in tissue samples from the retroperitoneal adipose tissue of animals from groups Control (panel A), MS (panel B), MS+$AMFJ_{2.5}$ (panel C), MS+$AMFJ_5$ (panel D), and MS+$AMFJ_{10}$ (panel E); H and E staining, magnification × 200

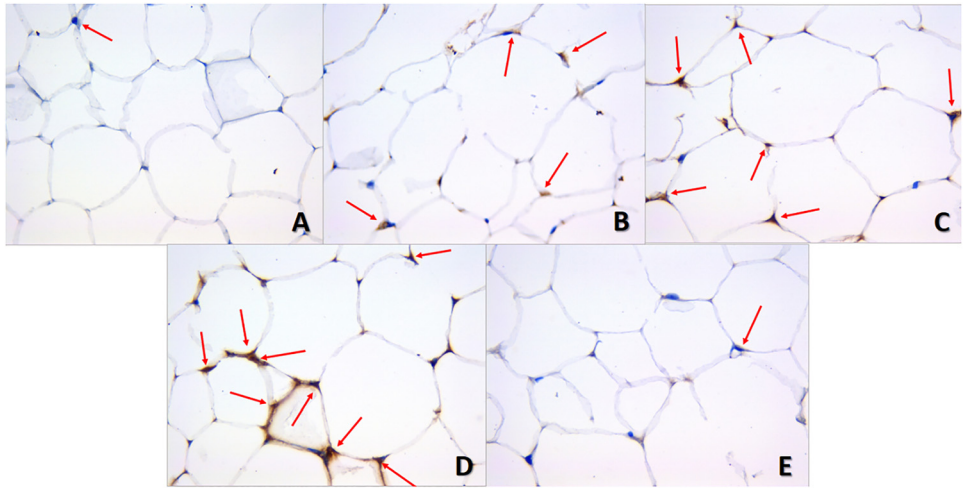

Fig. 6. Bcl-2 expression (arrows) in tissue samples from the retroperitoneal adipose tissue of animals from groups Control (panel A), MS (panel B), MS+AMFJ$_{2.5}$ (panel C), MS+AMFJ$_5$ (panel D), and MS+AMFJ$_{10}$ (panel E); magnification × 400

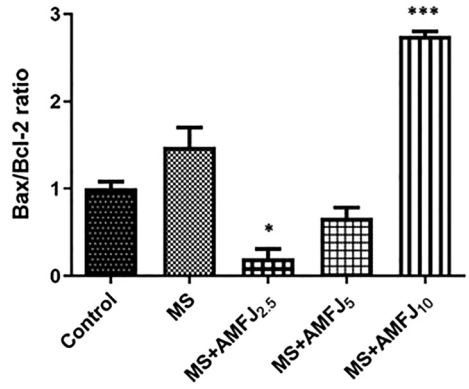

Fig. 7. Bax/Bcl-2 ratio in tissue samples from the retroperitoneal adipose tissue in an experimental model of metabolic syndrome (MS) in rats treated with AMFJ at doses of 2.5, 5.0, and 10.0 mL kg^{-1}; *: $P < 0.05$ vs. Control, ***: $P < 0.001$ vs. Control

decrease was significant compared to the control value in MS+AMFJ$_{2.5}$ group ($P < 0.05$), while the ratio of the MS+AMFJ$_5$ group did not differ significantly from the control group. Unexpectedly, the highest dose of AMFJ downregulated Bcl-2 expression, and the Bax/Bcl-2 ratio of MS+AMFJ$_{10}$ group was significantly higher ($^{***}P < 0.001$) in comparison with the control value showing a prevailing apoptotic activity of the adipocytes in this group (Fig. 7).

Studies have found a link between apoptosis and the metabolic consequences of MS. The expansion of adipocytes (which occurs in obesity) activates the two apoptosis pathways, which,

in turn, stimulates adipose tissue macrophages (ATM). ATM release proinflammatory cytokines, induce meta-inflammation, and subsequently lead to insulin resistance, dyslipidemia, and hepatic steatosis (Alkhouri et al., 2010). Since oxidative stress can activate apoptosis (Dingeldein et al., 2019), it is reasonable to expect that antioxidant compounds could suppress this cellular process. *A. melanocarpa* fruit juice contains strong antioxidants such as proanthocyanidins, anthocyanins, and phenolic acids. Therefore, in this study, the effect of AMFJ on the programmed cell death was also investigated. To clarify this effect, Bax/Bcl-2 ratio, which serves as a predictive tool for cell susceptibility to apoptosis (Raisova et al., 2001), was calculated. This study demonstrated that adipocytes of the animals from MS group exhibited a proapoptotic phenotype, while adipocytes from MS+AMFJ$_{2.5}$ and MS+AMFJ$_5$ groups exhibited an anti-apoptotic phenotype. These findings are in compliance with other studies describing the effect of *A. melanocarpa* on apoptosis. For instance, *A. melanocarpa* aqueous extract reduced the Bax/Bcl-2 ratio in the adrenal glands in a model of adrenal hexavalent chromium-induced injury (Savici et al., 2021). Meng et al. (2018) confirmed the anti-apoptotic activity of *Aronia*-derived anthocyanins, which increase the expression of Bcl-2 and decrease the same of cytochrome c, caspase-3, caspase-9, and Bax.

Contrary to the expectations, the group treated with the highest dose of AMFJ (10 mL kg^{-1}) had the highest Bax/Bcl-2 ratio, even higher than that of the MS group. According to a study on human leukaemia cell line, some polyphenols (chlorogenic acid, cyanidin, and quercetin derivatives) could trigger ROS production, and this could disrupt the mitochondrial membrane potential, stimulate cytochrome c release, and activate caspase-3-mediated apoptosis (Sharif et al., 2012). We could suppose that AMFJ at the highest dose used in this study might lead to activation of the programmed cell death of the adipocytes through the mechanism described above. Unfortunately, however, the effect of the juice on the oxidative stress markers was not investigated in the present experiment, which could be a limitation of this study.

4. CONCLUSIONS

In summary, this study demonstrated the beneficial effects of the polyphenol-rich *A. melanocarpa* fruit juice in reducing serum glucose and triglyceride levels, exerting a certain antiobesity effect and preventing adipocyte expansion in the high-fat high-fructose diet-induced metabolic syndrome in rats. In addition, *A. melanocarpa* fruit juice was able to suppress adipocyte apoptosis at doses of 2.5 and 5.0 mL kg^{-1}. Further experimental studies are needed to clarify the reason for the proapoptotic effect in the adipose tissue of the highest dose (10 mL kg^{-1}) of the juice.

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