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PHARMACOLOGICAL INVESTIGATION OF THE EFFECTS OF ARONIA MELANOCARPA FRUIT JUICE IN AN EXPERIMENTAL MODEL OF METABOLIC SYNDROME

DOCTORAL THESIS

ABSTRACT

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USED ABBREVIATIONS:

CCL4: carbon tetrachloride

FAS: fatty acid synthase

GAE: gallic acid equivalent

GPx: glutathione peroxidase

GSH: glutathione

HDL: high-density lipoprotein

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

ICAM-1: intercellular adhesion molecule-1

IL: interleukin

LDL: low-density lipoprotein

MCP-1: monocyte chemoattractant protein-1

MDA: malondialdehyde

NADP: nicotinamide adenine dinucleotide phosphate

NAFLD: non-alcoholic fatty liver disease

NF-κB: nuclear factor-kappa beta

Nrf2: nuclear factor-erythroid factor 2-related factor 2

OFT: open field test

PPAR-γ: peroxisome proliferator-activated receptor-γ

PRT: place recognition test

SEM: standard error of the mean

SIT: social interaction test

SOD: superoxide dismutase

TBARS: thiobarbituric acid reactive substances

TNF-α: tumor necrosis factor-alpha

VCAM-1: vascular cell adhesion molecule-1

VLDL: very-low-density lipoprotein

ASCVD: atherosclerotic cardiovascular disease

HFHF: high-fat, high-fructose

GTT: glucose-tolerance test

DM: diabetes mellitus

IR: insulin resistance

MS: metabolic syndrome

AMFJ: *Aronia melanocarpa* fruit juice

PP: polyphenols

RAAS: renin-angiotensin-aldosterone system

ROS: reactive oxygen species

FFA: free fatty acid

CVD: cardiovascular disease

T2DM: type 2 diabetes mellitus

TG: triglycerides

Content

I. INTRODUCTION

Metabolic syndrome (MS) is a constellation of clinical and biochemical abnormalities that increase the risk of cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM). These abnormalities include: elevated fasting glucose level and serum triglycerides (TGs), low levels of HDL-cholesterol, central (visceral) obesity, and elevated blood pressure. A clear definition of the syndrome is necessary, as it helps identify the patients who are at risk of developing atherosclerotic cardiovascular disease (ASCVD) or T2DM.

The medical and social significance of MS is huge, as a steady trend of increasing incidence among adults and children is observed worldwide. The "western" lifestyle, characterized by consumption of high-calorie foods and beverages and reduced physical activity contributes to this high prevalence. Such an unhealthy lifestyle leads to the accumulation of fat around the abdomen and development of the visceral obesity.

Despite the advances in the study of MS and the identified risk factors, its pathogenesis has not been fully elucidated. The main accepted hypothesis is that visceral adipose tissue, considered the largest endocrine organ, produces so-called adipocytokines, which induce insulin resistance and low-grade inflammation. These two are considered key moments in the development of MS and its associated complications.

The main focus in the treatment of MS is to improve insulin sensitivity. Increased physical activity, dietary interventions aimed at reducing calorie intake and taking medications that stimulate the insulin response is recommended. However, treatment is not always effective. Therefore, the efforts are focused on finding new therapeutic alternatives.

Plants are being studied intensively in attempts to isolate biologically active substances that could have therapeutic potential in MS. These include polyphenols (PPs). They consist of two or more benzene rings with additional functional groups attached to them. PPs are of considerable interest to the scientific community as in recent years their effects have been widely studied both *in vitro* and *in vivo* and continue to be investigated. Their easily accessible nature and relatively low cost make them very suitable for studies in various experimental models of diseases (including models of MS) to test their potential effects.

Aronia melanocarpa (black chokeberry) stands out among the plants with very high polyphenolic content. There is strong evidence that chokeberry PPs possess insulin-sensitizing and anti-inflammatory activity. The effects of chokeberry PPs on the various components of MS (hypertriglyceridemia, hyperglycemia, obesity, hypertension) have been largely studied and beneficial effects have been reported in healthy subjects and in animals with experimental models of certain diseases (hypertension, diabetes, dyslipidemia).

If so, the use of PP-rich Aronia melanocarpa fruit juice (AMFJ) would have a beneficial effect on clinical and biochemical disorders in subjects (experimental animals) with diet-induced MS,

There are currently no data available about the effects of AMFJ in experimental animals with diet-induced MS. Further studies are needed to confirm the aforementioned effects in MS.

II. OBJECTIVE AND TASKS

The **objective** of this dissertation is:

To investigate and summarize the pharmacological effects of *Aronia melanocarpa* fruit juice (AMFJ) administered orally in three different doses to rats with an experimental model of metabolic syndrome (MS) induced by high-fat, high-fructose (HFHF) diet.

Tasks

1. To induce MS in the studied experimental animals through 10-week dieting with a HFHF diet.

2. In such obtained model of MS to study the effects of AMFJ on:

2.1. Behavioral indices providing information about:

2.1.1. The general locomotor activity;

2.1.2. The spatial memory;

2.1.3. Anxiety;

2.1.4. Depressive-like behavior;

2.2. Biochemical markers of energy metabolism:

2.2.1. Glucose regulation;

2.2.2. Serum triglycerides;

2.3. Serum activity of antioxidant enzymes;

2.4. Adipose tissue (retroperitoneal, mesenteric, perigonadal, paranephral, total) indices;

2.5. The structure of visceral adipose tissue, myocardium, coronary vessels and liver;

2.6. Immunohistochemical expression of markers of apoptosis (in the adipose tissue and liver) and inflammation (in the liver);

2.7. Carrageenan-induced acute inflammation of the left hind paw.

III. MATERIALS AND METHODS

1. Materıals

1.1. Experımental anımals

The experimental design includes 50 male Wistar rats (160-280 g) grown in plastic cages at an average room temperature of 20-25^{o}C, exposed to 12-hour light / dark cycle. They had free access to food and water. All procedures for the treatment of the animals and experiments were conducted in accordance with EU Directive 2010/63 / EU and approved by the Bulgarian Food Safety Agency (Document №177/07.07.2017).

1.2. Substances used

1.2.1 *Aronia melanocarpa* fruit juice (AMFJ)

AMFJ used in the present study was prepared by grinding, pressing and squeezing the fresh fruits *(*Valcheva-Kuzmanova et al., 2014). The obtained juice was filtered, preserved with potassium sorbate (1.0 g / l) and stored in a refrigerator until the experiment. **Table 1** represents the polyphenolic content of the juice.

Table 1. Polyphenolic content of *Aronia melanocarpa* fruit juice used in the experiment; GAE: gallic acid equivalents

Polyphenols	**Content**
Total phenols	5461 mg GAE/l
Total proanthocyanidins	3122.5 mg/l
Cyanidin 3-galactoside	143.7 mg/l
Cyanidin 3-arabinoside	61.7 mg/l
Cyanidin 3-glucoside	4.4 mg/l
Cyanidin 3-xyloside	11.6 mg/l
Chlorogenic acid	585 mg/l
Neochlorogenic acid	830 mg/l

1.3. Reagents and kits used

The antibodies, staining reagents, and operating concentrations used in the immunohistochemical assays are presented in **Table 2** and **Table 3** - Bax antibody (B-9), cat. № 7480, Bcl-2 cat. № 7382, Macrophage Marker Mac387 cat. № 66204. The antibodies are manufactured by Santa Cruz Biotechnology.

Table 2. Reagents used in the immunohistochemical analyses

Antibody	**Dilution**	**Positive control**	**Marker for**	**Manufacturer**
Anti-Bax	1:50	Lymph node	Apoptosis	Santa Cruz Biotechnology
Anti- Bcl-2	1:50	Lymph node	Apoptosis	Santa Cruz Biotechnology
Anti-Mac387	1:50	Lymph node	Macrophages	Santa Cruz Biotechnology

Table 3. Staining systems

HRP-DAB System	Original staining system	Dako (manufacturer)
Mayer's hematoxylin	Counterstaining	Dako(manufacturer)

Individual kits were used to determine the serum TG levels (Bio Maxima, Poland), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity (Calbiochem, Germany).

2. Methods

2.1. Induction of metabolic syndrome and treatment

The experimental animals were divided into 5 groups (10 rats per group): control, MS, MS + $AMFJ_{2.5}$, MS+$AMFJ_{5}$ and MS+$AMFJ_{10}$. Control animals received regular laboratory diet and drinking water, other groups – a high-fat, high-fructose (HFHF) diet and 10% fructose solution instead of drinking water for induction of MS (Gancheva et al., 2015).

The HFHF diet consisted of granules enriched with 17% lard and 17% fructose. The calorie intake of this diet corresponded to 405 kcal/100 g, as lard provided 38% of energy intake, fructose - 17%. The fructose solution provided an additional 40 kcal/100 g calorie intake.

The experimental groups were orally treated in a ten-week period with an orogastric tube once in a day. The control and MS groups were treated with distilled water in a total volume of 10 ml/kg body weight. The groups $AMFJ_{2.5}$, MS+$AMFJ_{5}$ and MS+$AMFJ_{10}$ were treated with increasing doses of fruit juice: 2.5 ml/kg, 5.0 ml/kg and 10 ml/kg, respectively. AMFJ in doses of 2.5 and 5.0 ml/kg was diluted with distilled water to a total volume of 10 ml/kg. The diet and treatment of the experimental groups are summarized in **Table 4**.

Table 4. Experimental protocol; HFHF*: high-fat, high-fructose*

Group	Diet	Treatment
Control	Regular	10 ml/kg water
MS	HFHF	10 ml/kg water
MS+AMFJ$_{2.5}$	HFHF	2.5 ml AMFJ, diluted to a total volume of 10 ml/kg
MS+AMFJ$_{5}$	HFHF	5.0 ml AMFJ, diluted to a total volume of 10 ml/kg
MS+AMFJ$_{10}$	HFHF	10 ml AMFJ

2.2. Induction of acute inflammation of the hind paw

At the end of the treatment period, carrageenan at a dose of 1 mg as 0.1 ml of freshly prepared solution in 0.9% saline was injected into the plantar surface of the left hind paw of the rats to induce acute inflammatory response. The paw volumes (ml) were evaluated initially (before injection), on the 30th minute and the 1st, 2nd 3rd, 4th and 5th hour after the injection by a digital plethysmometer LE7500 Panlab, Barcelona. The difference between the paw volumes before and after the injection correlated with the intensity of the inflammation induced.
The paw edema (ml) was calculated using the following formula:

$$\text{Paw edema (ml)} = (V_s - V_0),$$

V_s – the paw volume measured at the six-time intervals after the carrageenan injection;

V_0 – the initial paw volume.

Paw edema inhibition (%) was calculated using the formula:

$$\text{Paw edema inhibition (\%)} = (V_C - V_T)/V_C \times 100,$$

V_C – the mean paw edema volume of the control group (MS) for this experiment;

V_T – average volume of the paw edema for each treated group.

2.3. Behavioral research methods

After week 8, behavioral tests were performed to assess the locomotor activity, anxiety, spatial memory and depressive-like behavior in animals with already induced MS. The following methods were used for the purpose: open field test, social interaction test, place recognition test and forced swimming test.

2.3.1. Open field test (OFT)

Developed by Calvin Hall (), the open field test gives a general idea about the overall locomotor activity. To carry out the test, a wooden field (100 cm x 100 cm) surrounded by walls 40 cm high was used. It was divided by blue lines into 25 squares measuring 20 cm x 20 cm. The test rat was placed in the center of the field and its behavior was studied by direct observation for 5 minutes. The total number of squares crossed by the four paws was counted as an indicator of horizontal activity. The vertical activity was evaluated by the number of rearings.

2.3.2. Social interaction test (SIT

The test, introduced by File and Hyde (1978), serves to determine the level of anxiety of experimental animals, as judged by the time of social interaction of a rat with an unfamiliar test partner. Two rats from different cages were simultaneously placed in the opposite corners of the open field arena. The animals had the same treatment and similar weights (with a difference of no more than 10%). The time spent in active social interaction (grooming, sniffing, following or crawling under/over the partner) was measured as an index inversely related to anxiety. Passive contact (e.g. lying or sitting with bodies in contact) was not considered as a social interaction.

2.3.3. Place recognition test (PRT)

The test is a modification of the novel object recognition test and is adapted to study the spatial memory of the tested animals. A walled field, measuring 60 cm x 40 cm and evenly lit by a source of artificial light, was used. The test was conducted in two sessions, the first being a training one. Two identical objects were located symmetrically and firmly attached to the floor, so that they could not be moved by the animal. The rat was placed in the center of the field and allowed to explore the objects for 3 min; 30 minutes later, a test session was performed, in which the location of one of the objects was changed. Again, within 3 minutes, the animal behavior was observed and the exploration time of both objects was recorded as

approaching (to less than 1 cm), sniffing, climbing the objects. As an indicator of spatial memory, the discrimination index was calculated:

$$\text{Discrimination index} = B/(A + B),$$

A - the time of exploring the object with the original location;
B – the time of exploring the object with the new location.

The higher the discrimination index, the better the memory.

2.3.4. Forced swim test (FST)

The method was described by Porsolt *et al.* (1977). It is used to study the presence of depression-like behavior in rodents. When a rat is forced to swim with no possibility to escape, after an initial period of active struggling (swimming, climbing), the animal assumes an immobility posture with only minimal movements necessary to keep his head above the water. This condition of immobility is considered to reflect the animal's "despair" as a result of a depression-like state. A glass cylinder (17 cm in diameter and 50 cm high) was used, filled with water (~30 ºC) up to 30 cm to ensure that the animal could not touch the bottom of the pool with his hind paws or tail. The rat was placed in the cylinder for 5 minutes and its behavior was observed. The test was conducted in two identical sessions – a training and an experimental one, separated by 24 hours, with the immobility time during the second session only being recorded and analyzed.

2.4. Biochemical methods

2.4.1. Obtaining the blood serum

Experimental animals were anesthetized with diethyl ether, blood was collected from the sublingual veins and blood tubes were centrifuged at 2000 rpm for 10 minutes. The separated serum was stored at -20 ° C until the biochemical analyses.

2.4.2. Glucose tolerance test (GTT)

The test was performed in the 10th week of the experimentation. After 12 hours of fasting the rats were injected intraperitoneally with 40% glucose solution at a dose of 2 g/kg body weight. A blood sample from the distal end of the tail was used to test blood glucose level (obtained by incision according to the method of Fluttert et al., 2000). Blood glucose was

measured with an ACCU-CHEK Performa glucometer and ACCU-CHEK Performa test strips before injection (0.min) and 30, 60 and 90 minutes thereafter.

2.4.3. Determination of serum triglyceride (TG) level

TG were determined spectrophotometrically using a standard kit.

TGs are hydrolyzed by the enzyme lipoprotein lipase to glycerol and fatty acids. Glycerol is phosphorylated by ATP from the enzyme glycerol kinase, which synthesizes dihydroxyacetone phosphate and H_2O_2. H_2O_2 binds to 4-chlorophenol and 4-aminoantipyrine to form a colored complex:

TG + H_2O → glycerol + fatty acids

Glycerol + ATP → 3-P-glycerol + ADP

3-P-glycerol + O_2 → dihydroxyacetone-+ + H_2O_2

$2H_2O_2$ + 4-aminoantipyrine + 4-chlorophenol → quinonothine + $4H_2O$

The intensity of the color of the obtained compound, measured spectrophotometrically, corresponds to the TG concentration.

The concentration of triglycerides in the samples was calculated according to the formula:

Triglycerides [mmol/l] = A (Sample)/A (Standard) x concentration of the standard,

A (Sample) = Absorption of the sample,

A (Standard) = Absorption of the standard.

2.4.4. Determination of the serum activity of antioxidant enzymes

The serum SOD activity was determined by spectrophotometry using a standard kit.

Principle: The kit uses tetrazolium salt to detect superoxide radical generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the concentration of enzyme required for 50% dismutation of the superoxide radical.

Serum GPx activity was determined spectrophotometrically using a standard kit. GPx catalyzes the reduction of hydroperoxides with the help of reduced glutathione and protects cells from oxidative damage.

Principle: The method is based on an enzyme-linked immunosorbent assay of glutathione peroxidase activity indirectly by reaction with glutathione peroxidase. Oxidized glutathione produced after the reduction of hydroperoxide by the enzyme GPx is recycled to a reduced form of glutathione reductase and NADPH:

$R\text{-}O\text{-}O\text{-}H + 2GSH \rightarrow R\text{-}O\text{-}H + GSSG + H_2O$

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

Oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at a wavelength of 340 nm. The rate of decrease is directly proportional to the GPx activity in the respective sample.

2.5. Determination of mesenteric, paranephral, perigonadal, retroperitoneal and total fat indicies

Mesenteric, perigonadal, paranephral and retroperitoneal fat depots were dissected and weighed. Total visceral adipose tissue was calculated. The corresponding fat indices were calculated according to the formula: adipose tissue weight/total body weight * 10^2.

2.6. Histological and immunohistochemical methods

2.6.1 Histological methods

Tissue samples (1 cm wide, 1 cm long and 0.5 cm thick) of retroperitoneal adipose tissue, liver, myocardium and coronary artery were fixed in 10% neutral buffered formalin and after appropriate treatment were embedded in paraffin with a melting point 52-54 ^oC. Sections 5 μm thick were standardly stained with hematoxylin-eosin to assess the histological changes.

2.6.2. Immunohistochemical methods

An indirect immunoperoxidase method was used for immunohistochemical analysis using the mini KIT high Ph DAKO K8024. Negative controls were designed by incubating sections of the paraffin blocks used with normal non-immune serum instead of the primary antibody. For positive controls, lymph node tissues stained with Anti-Bax, Anti-Bcl-2, and Anti-Mac387 were used.

Expression of the test antibodies and interpretation of the results

Immunohistochemical expression of Bax and Bcl-2 and MAC387 was assessed semiquantitatively by determining the expression of at least fifty cells from each sample in randomly selected fields. For each cell, the intensity of the cytoplasmic expression was assessed as: 0 - no response, 1+ - weak positivity, 2+ - moderate positivity or 3+ - strong positivity. For adipose tissue, due to the presence of large cytoplasmic lipid inclusions and the peripheral location of the nuclei, expression was defined as: 0 - no reaction or positive 1+ depending on the absence or presence of intranuclear immune deposits.

Bax/Bcl-2 ratio was determined for each experimental group and the values between the groups were compared.

2.7. Statistical methods

The processing and analysis of the data were performed with the statistical software GraphPad Prism 7.00 (San Diego, California), using one-way and two-way variation analysis (one-way and two-way ANOVA) and unpaired two-tailed Student's t-test. ANOVA analyzes were followed by Dunnett's multiple comparisons post-test. Results are presented as mean ± standard error (mean ± SEM). Statistical reliability was assumed at a value of $p < 0.05$.

IV. RESULTS AND DISCUSSION

1. Effects of *Aronia melanocarpa* fruit juice on the behavior in an experimental model of metabolic syndrome

1.1. Effects of Aronia melanocarpa fruit juice in open field test

The results are presented in **Table 5** and **Figure 1**. After statistical analysis of the results no significant difference was found in the number of horizontal and vertical movements among the studied experimental groups.

Table 5. Number of horizontal and vertical movements in the open field of rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg

Parameter	Control	MS	MS+AMFJ$_{2.5}$	MS+AMFJ$_{5}$	MS+AMFJ$_{10}$	One-Way ANOVA
Number of squares crossed	60.7 ± 12.18	81.6 ± 13.15	64.7 ± 10.13	54.2 ± 10.37	43.8 ± 10.07	0.1963
Number of rearings	20.7 ± 3.01	35.35 ± 3.17	22.9 ± 4.09	18.6 ± 2.09	17.5 ± 2.26	0.3343

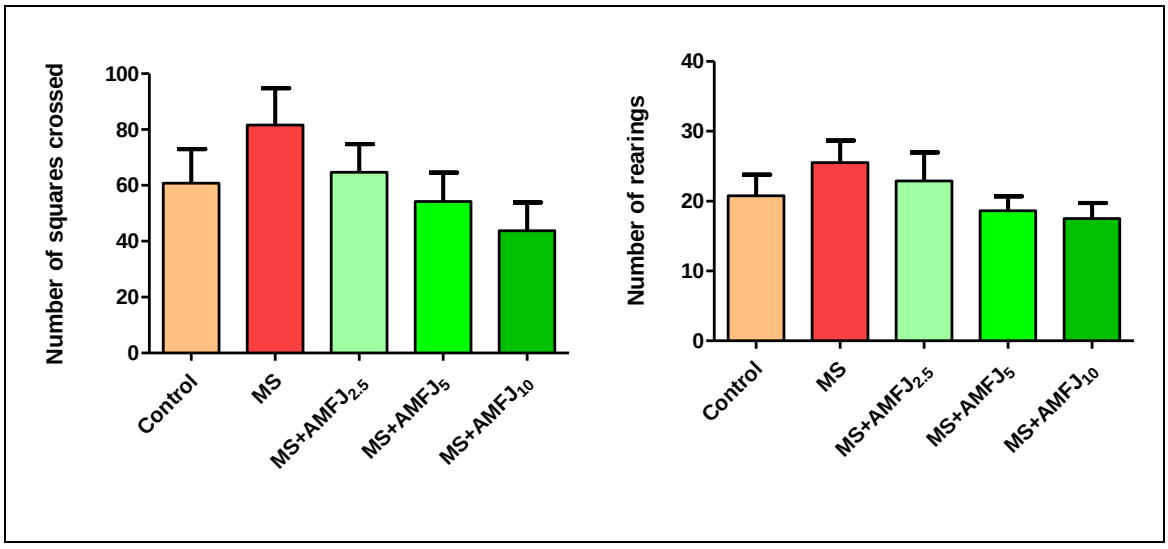

Figure 1. Number of squares crossed and rearings in the open field of rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg.

1.1. Effects of Aronia melanocarpa fruit juice in social interaction test

The results are presented in **Table 6** and **Figure 2**. One-way ANOVA reported a significant difference among the groups in the time spent in social interaction. Subsequent Dunnett's analysis showed a significant reduction (by 28.4%) in the social interaction with a test partner of rats in the MS group ($p < 0.05$ vs. Control group). The AMFJ-treated groups had a longer time of social interaction and the effect was significant in the MS + $AMFJ_{2.5}$ group ($p < 0.05$ vs. MS group) (improvement by 43%). In addition, there was no significant difference in the time spent in social interaction between the three AMFJ-treated groups and the control group (Figure 1).

Table 6. Time, spent in social interaction (seconds) with a test partner in rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg; *$p < 0.05$ compared to the control group, $^{\#}p < 0.05$ compared to the MS group

Parameter	**Control**	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Time spent in social interaction with a test partner (seconds) **(Mean ± SEM)**	28.96 ± 1.89	20.73 ± 1.86*	29.63 ± 2.21#	24.09 ± 3.49	24.93 ± 3.13

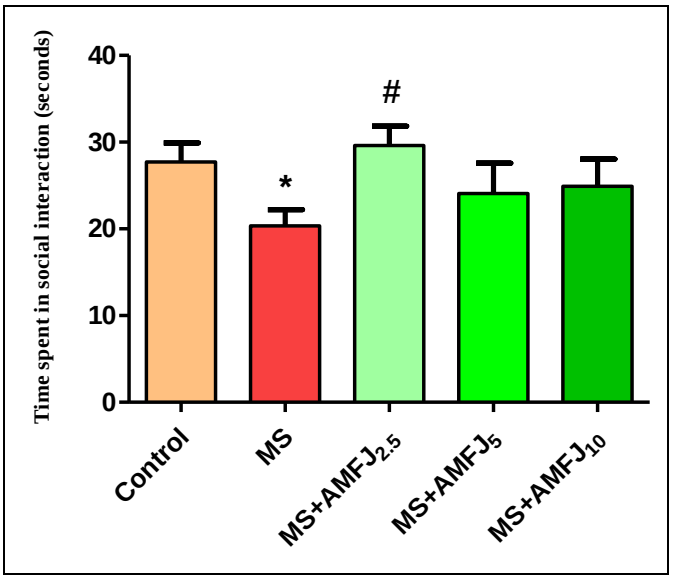

Figure 2. Time spent in social interaction with a test partner in rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; *$p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. MS group

1.2. Effects of Aronia melanocarpa fruit juice in place recognition test

The results are shown in **Table 7** and **Figure 3**. One-way ANOVA analysis highlighted a significant difference between groups (p=0.0076). Dunnett's post-hoc test showed a significant disturbance in the spatial memory of the rats from the MS group - the discrimination index in MS rats decreased by 32.3% ($p < 0.05$ vs. Control group). Significant improvement in the spatial memory was documented in rats from all groups treated with AMFJ - the index increased by 40.9% in group MS+AMFJ$_{2.5}$ ($p < 0.05$ vs. MS group), in group MS+AMFJ$_5$ - by 54.5% ($p < 0.01$ vs. MS group) and in the MS+AMFJ$_{10}$ group - by 38.6% ($p < 0.05$ vs. MS group). In addition, animals treated with the juice did not have a statistical difference in the index compared to the control animals.

Table 7. Discrimination index (B/(A+B) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; $^{*}p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. MS group, $^{\#}p < 0.01$ vs. MS group

Parameter	**Control**	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
B/ (A+B) (Mean±SEM)	0.65±0.07	0.44±0.04*	0.62±0.05$^{\#}$	0.68±0.04$^{\#\#}$	0.61±0.03$^{\#}$

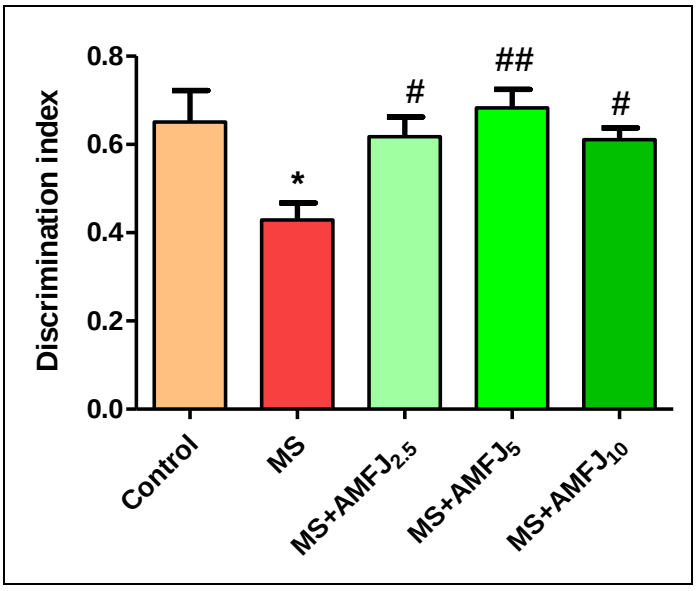

Figure 3. Discrimination index (B/(A+B) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; $^{*}p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. MS group, $^{\#}p < 0.01$ vs. MS group

1.3. Effects of Aronia melanocarpa fruit juice in forced swimming test

The results of the forced swimming test are shown in **Table 8** and **Figure 4.** After the statistical analysis, no significant difference was found in the time spent in immobility among the tested experimental groups.

Table 8. Time spent in immobility (seconds) in forced swimming test in rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg

Parameter	**Control**	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_{5}$**	**MS+AMFJ$_{10}$**	**One-way ANOVA (p)**
Time spent in immobility (seconds) **(Mean±SEM)**	122.1±42.22	152.4±48.30	128.3±27.03	153.2±34.54	123.1±36.83	0.2276

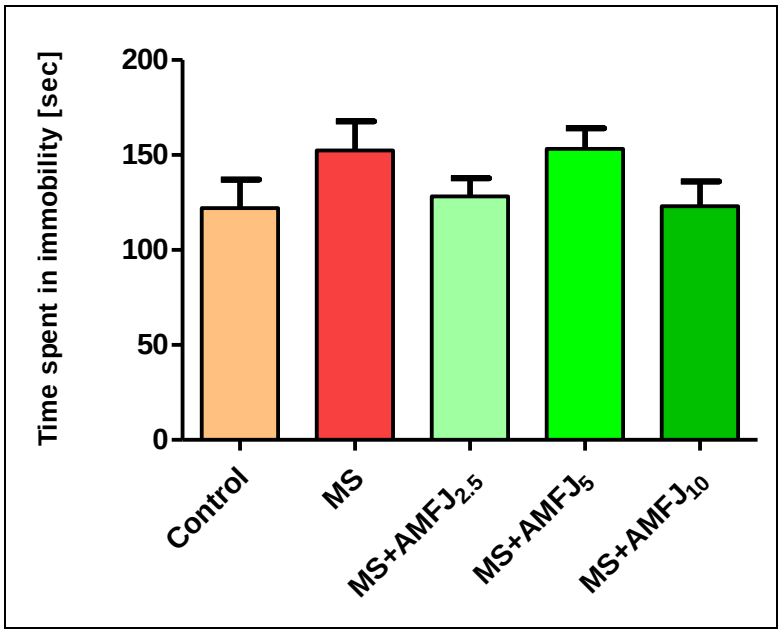

Figure 4. Time spent in immobility (seconds) in forced swimming test in rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg

1.4. Discussion

An increasing body of evidence has demonstrated a connection between MS, obesity and/or diabetes and neurocognitive performance. Common features of these conditions include insulin resistance, mitochondrial dysfunction and oxidative stress (Cardoso and Moreira, 2019).

It has been long believed that the liver, adipose tissue and skeletal muscle are the “classic targets” of insulin and the brain has been considered “insulin-insensitive”. Emerging data shows that insulin plays a significant role in regulating brain functions (Gray et al., 2014) as it affects food intake and energy homeostasis, odor sensitivity, neuronal survival and longevitiy, dendritic spine formation (Cardoso et al., 2009; Kellar and Craft, 2020; Taouis and Torres-Aleman, 2019). “Brain insulin resistance” is considered as a modern concept of the pathogenesis of MS and related disorders (Kellar and Craft, 2020). In such a resistant state, dysregulation of insulin signalling impaires the brain energy metabolism and the expression of insulin-responsive genes required for cognitive functions, and increases oxidative stress. Interestingly, peripheral insulin resistance influences or indicates a state of cerebral insulin resistance. In support of this, there are data indicating that synaptic plasticity and cognitive performance negatively correlate with hyperglycemia and peripheral insulin resistance in animal models of obesity or diabetes (Cardoso and Moreira, 2019). Especially, such a correlation was documented in high fat diet (HFD)-induced MS models in rats (Carvalho et al., 2015; Sa-Nguanmoo et al., 2016; Sa-Nguanmoo et al., 2017). Clinically these relations have been demonstrated by a longitudinal study in patients with prediabetes, revealing that insulin resistance is the major predictor of memory decline (Willmann et al., 2020).

Mitochondria are the main source of cell energy. Additionaly, they regulate thermogenesis, cell apoptosis, calcium homeostasis and reactive oxygen species (ROS) production and scavenging (Mahjoub and Masrour-Roudsari, 2012). Mitochondrial dysfunction is considered a common pathophysiological feature of MS (Dorszewska and Kozubski, 2018) and neurocognitive disorders (Guo et al., 2013). As a consequence of impaired mitochondrial function, oxidative damage may amplifiy neuronal damage and induce neurodegeneration. It has been demonstrated in experimental models of obesity, diabetes or MS (conditions, characterized by oxidative stress) (Asmat et al., 2016; Mahjoub and Masrour-Roudsari, 2012; Marseglia et al., 2014) that cognitive performance correlates negatively with the oxidative stress (Kim et al., 2016; Pintana et al., 2014).

Anxiety is a normal emotional response to real or potential threat, but when it is extreme and persistent, is considered pathological. Anxiety disorders are the most common type of mental disorders. There is also a high risk of developing other psychiatric co-morbidities such as depression (Ströhle et al., 2018). The association of MS with depression and anxiety has been a focus of considerable interest. While epidemiological observations and cross-sectional studies generally confirm the connection of MS with depression (Pan et al., 2012; Skilton et

al., 2007), the link with anxiety has been more illusive. Despite the prevailing negative results, a recent meta-analysis reports a significant association between anxiety and MS (Tang et al., 2017). Interestingly, age-related differences have been found in one study, with anxiety being more common in adults with MS and depressive behaviour prevailing in children (Kahl et al., 2015). The connections between MS and the anxious state appear to be bidirectional: anxiety increases the level of stress hormone cortisol and cortisol stimulates visceral adipogenesis . Additionally, it is supposed that interleukins, secreted by the abdominal adipose tissue, are able to stimulate cortisol release. Thus, interleukin antagonism in obese individuals has been associated with a decrease in cortisol, systolic blood pressure and heart rate (Urwyler et al., 2017). Insulin resistance may be another link between mood dissorders and MS, as Grillo et al. found that down-regulation of hypothalamic insulin receptors is associated with a higher risk of depression and anxiety (Grillo et al., 2011a; Grillo et al., 2011b; Grillo et al., 2007). In addition, a correlation has been established between lipid peroxidation and features of anxiety and depression in rats with diet-induced MS .
In the present study we aimed to test the effects of AMFJ on the behavior of rats subjected to diet-induced MS (Gancheva et al., 2015).

It has been previously established by our group that the HFHF-diet described here is able to induce symptoms of the MS such as visceral adiposity, glucose intolerance, insulin resistance, elevated triglycerides, oxidative stress in the experimental animals (Gancheva et al., 2015). These manifestations of MS have also been reproduced in the rats used in the present experiments. On the other hand, the effects of AMFJ on memory and mood disturbances have been tested previously in different experimental settings. The ability of AMFJ to improve learning and memory has been shown in young male rats subjected to subchronic treatment, using the two-way active avoidance task (Valcheva-Kuzmanova et al, 2013) or the passive avoidance test (Valcheva-Kuzmanova et al., 2014b). Anxiolytic effects have also been demonstrated by using the SIT and the elevated plus maze test in young, healthy male rats, as well as an antidepressant activity in the FST after a single or prolonged administration of the AMFJ (Eftimov and Valcheva-Kuzmanova, 2013; Valcheva-Kuzmanova et al., 2013; Valcheva-Kuzmanova and Zhelyazkova-Savova, 2009). In addition, AMFJ has been found to exert anti-inflammatory and antioxidant effects, both in *in vivo* and *in vitro* conditions (Olas et al., 2008; Valcheva-Kuzmanova et al., 2018; Valcheva-Kuzmanova et al., 2014a). Similar results have been reported by other authors in different experimental or clinical studies (Banach et al., 2020; Ryszawa et al., 2006; Zielinska-Przyjemska et al., 2007).

In the present study, the most prominent behavioral alterations in the MS group, were the increased anxiety-like state demonstrated in the SIT and the memory impairment observed in the PRT. The Porsolt test failed to demonstrate significant diet-induced depression-like state despite the increase in the immobility time in the MS group.

At the background of increased anxiety caused by the consumption of HFHF diet, the administration of AMFJ effectively normalized the rat behavior in the lowest concentration used, as evaluated by the SIT. Given the role of oxidative stress (Bouayed et al., 2009) and possibly also inflammation (Vogelzangs et al., 2013) in the genesis of anxiety, the protective effect of AMFJ can be potentially related to attenuating of these pathogenic mechanisms. Moreover, we have previously shown that the HFHF diet causes elevation of lipid peroxidation, which correlates with the anxiety measures in the rats (Gancheva et al., 2015). The antioxidant and anti-inflammatory actions of AMFJ are even more plausible explanation of the results from the spatial memory test, where a significant improvement was seen by all the doses used, antagonizing the deleterious effect of the diet. The spatial memory is known to be sensitive to manipulations with calorie-dense diets (McNay et al., 2010). Therefore, the antiamnesic effect in our study can be related to the antioxidant activity of AMFJ. In this line, *Aronia* juice has been demonstrated to counteract the reduction in the paraoxonase activity in brain tissue of rats fed HFHF diets (Francik et al., 2014). Neuroprotective effects of *Aronia* extract have been demonstrated also on mouse hippocampal cells *in vitro* (Lee et al., 2017), where the glutamate-induced cell death, ROS production and intracellular calcium levels were suppressed, together with increased activity of glutathione peroxidase and glutathione reductase. The role of anthocyanins for the cognitive-enhancing effect of *Aronia* fruits has been evaluated by Wen *et al*. in an amyloid-beta (Aβ)-induced memory damage model in rats. It was observed that purified anthocyanin treatment was associated with improved spatial memory in Morris water maze test as well as hippocampal cell protection against Aβ-toxicity (Wen et al., 2020). Another study aimed to test the effect of *Aronia*-derived anthocyanins on age-related degenerative changes in rat brain induced by D-galactose injection. Anthocyanin supplementation prevented the age-related cognitive decline and improved the antioxidant protection of the neurons as shown by the decreased level of malondialdehyde (MDA) and increased activity of SOD and GPx (Wei J et al., 2017).

Given the role that insulin resistance plays in the development of cognitive impairment, it can be regarded as a target through which AMFJ could improve the neurobehavioral alterations induced by the HFHF diet. The effects of *Aronia melanocarpa* on insulin resistance have been

studied in preclinical studies. In a HFD- and streptozotocin-induced type 2 diabetes in rats, 8-week *Aronia melanocarpa* berry extract treatment significantly decreased insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) score, improved glucose tolerance and increased glycogen content in the liver (Mu J et al., 2020). Kim *et al.* demonstrated *Aronia melanocarpa* methanol extract modulated adipogenesis and improved insulin resistance in HFD-induced obese mice. These effects were accompanied by an improvement in the lipid profile with a decrease in serum triglycerides and low-density lipoproteins (Kim et al., 2018).

The results from this experiment showing an improvement of the behavioral indices compromised by the intake of HFHF diet in rats, are well in accordance with the known antioxidant, anti-inflammatory and beneficial metabolic effects of AMFJ.

2. Effects of *Aronia melanocarpa* fruit juice on biochemical markers of energy metabolism

2.1. Effects of Aronia melanocarpa fruit juice on serum glucose level during glucose tolerance test

2.1.1. Results

The results from the GTT are presented in **Table 9** and **Figure 5.**

Student's t-test found a borderline significance ($p=0.059$) in the absolute glucose values at the 30^{th} minute of the MS group compared to the control group. Represented as a percent from 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 30^{th} ($p < 0.01$) and 60^{th} ($p < 0.05$) minute. At the 30^{th} and 60^{th} 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 9. Glucose values (mmol/l and % from the initial value) at the 30th, 60th and 90th minute during the GTT in rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg; (&)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

	30th minute		60th minute		90th minute	
	mmol/l	**% from the initial value**	**mmol/l**	**% from the initial value**	**mmol/l**	**% from the initial value**
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+AMFJ2.5	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+AMFJ5	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+AMFJ10	17.03±1.35	318.9±26.28	14.11±.098	271.5±24.27	10.21±0.54	194.7±15.79

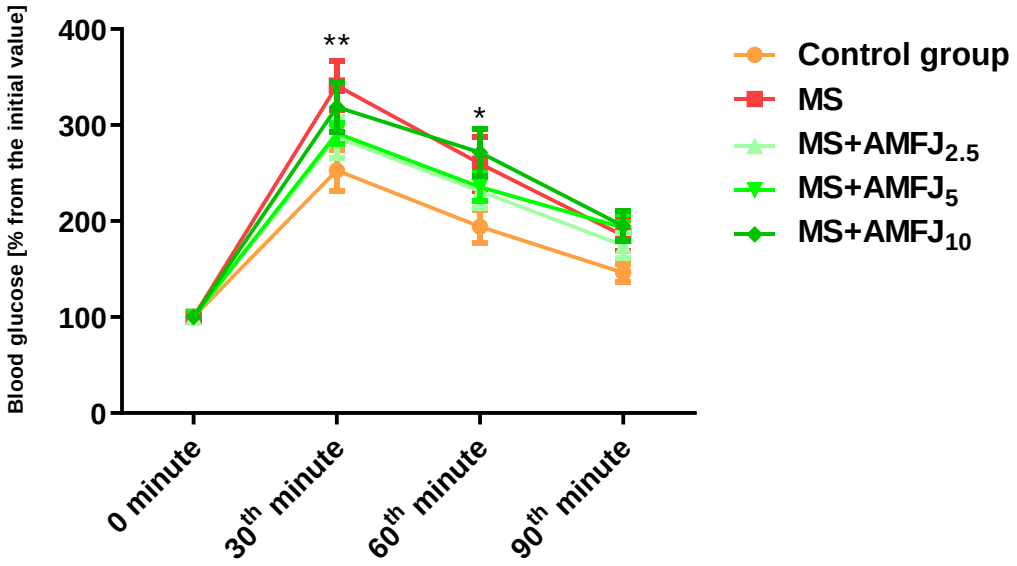

Figure 5. Blood glucose levels in the GTT presented as a percentage (%) from the initial values at 0, 30, 60 and 90 min in rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg; *p < 0.05 and **p < 0.01 of MS vs. Control group evaluated by two-way ANOVA

2.1.2. Discussion

Glycemic disorders are a leading cause of accelerating the atherosclerotic process (Aronson and Rayfield, 2002) and increasing the risk of cardiovascular and cerebrovascular diseases

and accidents in people with MS and / or diabetes mellitus. Interventions aimed at normalizing glucose tolerance would reduce these risks.

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 hyperglycemia (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 *Aronia melanocarpa* on blood glucose could be explained with its possible insulin-sensitizing effect after sub-chronic treatment period. As studies have reported, *Aronia 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 *Aronia melanocarpa* fruit extract (Bräunlich M 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 M et al., 2013). The juice used in this study had a high cyanidin 3-arabinoside content. The potential hypoglycemic effect of the juice could be due to stimulation of insulin secretion, as the fruits of *Aronia melanocarpa* are extremely rich in anthocyanins, which demonstrate such an effect *in vitro* (Jayaprakasam et al., 2005). Dipeptidyl peptidase-IV (DPP-IV) is an enzyme which 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).

2.2. Effect of Aronia melanocarpa fruit juice on the serum triglycerides

2.2.1. Results

The results are presented in **Table 10** and **Figure 6**. The HFHF diet in MS rats led to a significant increase (by 51.9%) in the level of serum TGs ($p < 0.05$ vs. Control group).

Treatment with AMFJ had a beneficial effect on TGs and dose-dependently reduced their levels - animals from groups MS+AMFJ$_5$ and MS+AMFJ$_{10}$ had significantly lower values ($p < 0.001$ and $p < 0.01$, respectively vs. MS group), as in these two groups 46.2% reduction in the levels was registered. AMFJ antagonized the hypertriglyceridemic effect of the diet and in the three AMFJ-treated groups the TG values did not differ significantly from those in the control group.

Table 10. Serum triglycerides (TGs, mmol/l) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; * $p < 0.05$ vs. Control group, $^{\#\#}p < 0.01$ vs. MS group, $^{\#\#\#}p < 0.001$ vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
TGs (mmol/l) **Mean±SEM**	1.04±0.10	1.58±0.17*	1.34±0.17	0.85±0.08$^{\#\#\#}$	0.85±0.06$^{\#\#}$

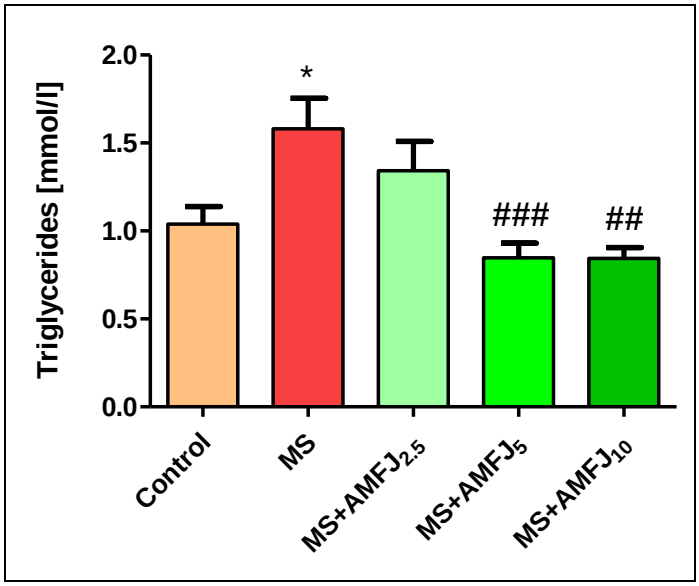

Figure 6. Serum TGs (mmol/l) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; * $p < 0.05$ vs. Control group, $^{\#\#}p < 0.01$ vs. MS group, $^{\#\#\#}p < 0.001$ vs. MS group

2.2.2. Discussion

MS-related dyslipidemia is characterized by large VLDL particles that induce the production of atherogenic LDL and lower the levels of the protective HDL particles. These factors directly affect the atherogenic risk and the development of atherosclerotic cardiovascular

disease. In addition, a recent study demonstrates that elevated levels in MS are an independent risk factor for activating platelet aggregation (Wang et al., 2020). Hypertriglyceridemia is both a component and a risk factor for MS (Nilsson et al., 2019). In addition, it has long been known that high TG values have been identified as a risk factor for developing acute pancreatitis (Yang and McNabb-Baltar, 2020).

The main goal of pharmacological treatment is to reduce the risk of developing acute pancreatitis in patients with severe hypertriglyceridemia and cardiovascular disease in patients with moderate hypertriglyceridemia (Simha, 2020). Fibrates, statins, omega-3 fatty acids, ezetimibe and PCSK9 inhibitors are currently used to control serum TG levels. In addition, hypertriglyceridemia could be controlled by optimizing the lifestyle, namely by increasing physical activity and avoiding prolonged periods of immobilization, avoiding alcohol consumption, controlling the blood glucose (in people with diabetes) and optimal diet (Parhofer and Laufs, 2019).

The available scientific data show that the PPs contain-ed in *Aronia melanocarpa* fruits had an antihypertriglyceridemic effect (Jurendić and Ščetar, 2021). Based on this fact, we aimed to study the effect of AMFJ on serum TG levels in rats with diet-induced MS.

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, c) 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 free fatty acids (FFAs), which are absorbed after forming bile acid micelles. Takahashi et al. (2015) reported that anthocyanins-rich Aronia fruits inhibited pancreatic lipase activity, the absorption of dietary fat and suppressed the postprandial hyperlipidemia (Takahashi et al., 2015). 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 Aronia 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. Effect of *Aronia melanocarpa* fruit juice on antioxidant enzymes

3.1. Effect of Aronia melanocarpa on the serum SOD activity

The results are shown in **Table 11** and **Figure 7**. One-way ANOVA showed statistically significant differences between groups ($p = 0.0174$), with subsequent analysis by Dunnett's multiple comparison's test reporting significantly higher enzyme activity in MS group compared to the control group (35.3% increase, $p < 0.01$ vs. Control group). AMFJ treatment reduced the activity of the antioxidant enzyme compared to the MS group, with the effect being most significant in MS+AMFJ$_{2.5}$ and MS+AMFJ$_5$ groups ($p < 0.05$ compared to the MS group) - in both groups the reduction in enzyme activity was by 23.2 %. The serum SOD activity in all AMFJ-treated groups was similar to that observed in the control group.

Table 11. Serum SOD activity (E/ml) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; **p < 0.01 vs. Control group, #p < 0.05 vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Serum SOD activity (E/ml) Mean±SEM	0.0051±0.0003	0.0069±0.0007**	0.0053±0.0003#	0.0053±0.0003#	0.0054±0.0003

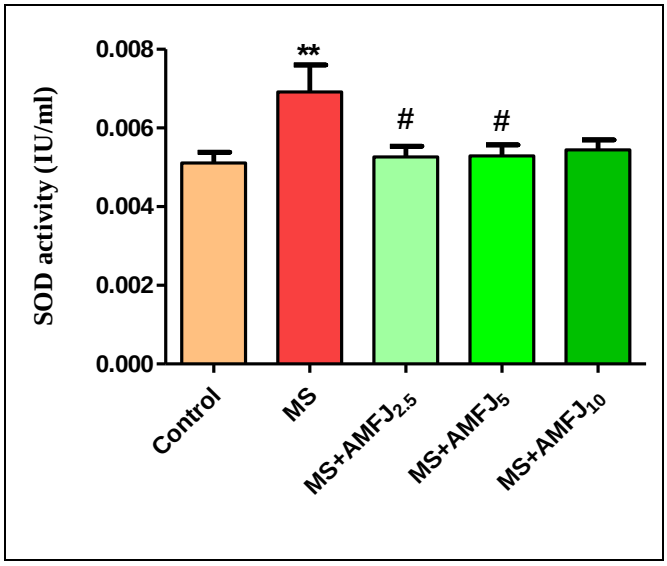

Figure 7. Serum SOD activity (E/ml) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; **p < 0.01 vs. Control group, #p < 0.05 vs. MS group

3.2. Effect of Aronia melanocarpa fruit juice on the serum GPx activity

The data are shown in **Table 12** and **Figure 8**. Despite the tendency to increase the activity of the enzyme in the AMFJ-treated groups, the statistical analysis did not show a significant difference among the tested experimental groups.

Table 12. Serum GPx activity (nmol/min/ml) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_{5}$**	**MS+AMFJ$_{10}$**	**One-way ANOVA**
GPx (nmol/min/ml) **Mean±SEM**	13.50±1.64	11.11±1.71	15.92±2.27	14.50±4.05	15.20±2.06	p=0.7067

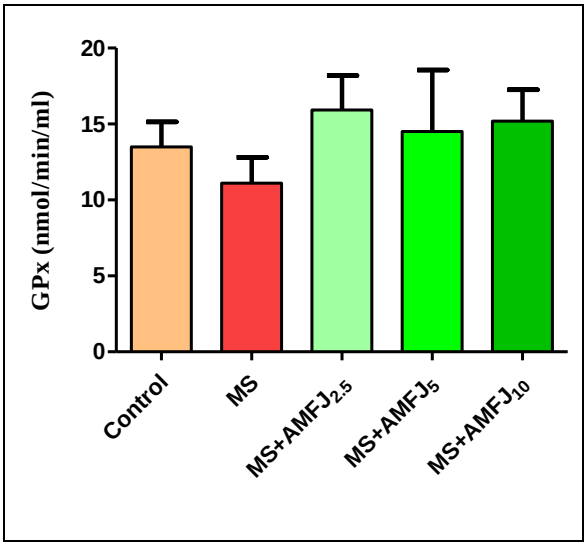

Figure 8. Serum GPx activity (nmol/min/ml) of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg

3.3. Discussion

Metabolic syndrome has been recognized as an important cardiometabolic risk factor (Tsai et al., 2018). The pathophysiology of MS is complex and involves multiple related factors. Many experimental and clinical studies reveal that insulin resistance, inflammation and oxidative stress play a significant role in the pathogenesis of MS. The visceral adipose tissue is an important source of ROS and contributes to the development of low-grade inflammation in MS. Oxidative stress (OxS) is associated with inflammation activated by the up-regulation

of nuclear factor-kappa B (NF-κB) which is implicated in the transcription of genes regulating the production of pro-inflammatory molecules – cytokines and adhesion molecules (Marseglia et al., 2014; Hussain et al., 2016). On the other hand, the inflammatory process induces oxidative stress. Pro-inflammatory cytokines, including tumor necrosis factor-alpha, interleukin (IL)-1β, and IL-6, that are secreted by the excessive adipose tissue, aggravate OxS through binding of specific receptors and promoting NF-κB signaling, thus inducing ROS generation (Marseglia et al., 2014). In addition, studies have also shown that the concentration of endogenous antioxidants and activity of antioxidant enzymes are generally reduced in MS (Ford et al., 2003; Hansel et al., 2004; Liu Y et al., 2020).

Our previous study with HFHF diet-induced MS demonstrated that it produced OxS and increased lipid peroxidation (measured by thiobarbituric acid-reactive substances, TBARS levels) (Gancheva et al., 2017). Therefore, in the present study we aimed to determine the activity of the endogenous antioxidant enzymes SOD and GPx in MS rats. We found that SOD activity was higher in MS group compared to the other groups while no difference was observed in the activity of GPx. These results are in accordance with the study of Vavrova *et al*., who have found, similarly, increased SOD activity and unaltered GPx activity in patients with MS (Vávrová et al., 2013). Interestingly, Yubero-Serrano *et al*. have reported an association between the number of MS components and SOD activity and suggested that it could be used as a predictive tool to determine the degree of the underlying OxS in MS (Yubero-Serrano et al., 2013). Other experimental and clinical studies also have revealed an association between obesity and increased activity of SOD (Stefanović et al., 2008; Fujita et al., 2005). Under physiological conditions, small amounts of superoxide radicals are produced during glucose autoxidation and cellular respiration (mitochondria). Enzymes such as NADPH oxidase, xanthine oxidase, cyclooxygenase and lipoxigenase can additionally contribute to superoxide production. The radicals are inactivated by SOD to hydrogen peroxide which is further converted to water by catalase, GPx or thioredoxin (Mahjoub and Masrour-Roudsari, 2012). It is known that nutrient excess (especially high-fat diet) induces up-regulation of NADPH oxidase (32). We could build up a hypothesis that the HFHF-diet, administered to the MS animals in our experiment, induced superoxide production and increased compensatory the activity of SOD, aiming to inactivate the radical.

Polyphenols are a large group of naturally occurring chemical compounds, bearing one or more phenolic ring. The powerful antioxidant action of these compounds is due to their ability to neutralize free radicals by donating an electron or hydrogen atom (Tsao R, 2010). *Aronia*

melanocarpa fruits contain a large amount of polyphenols and, therefore, exhibit strong anti-oxidant properties. Different *in vitro* assays demonstrate the ability of *Aronia* to scavenge a variety of active radicals, including superoxide anion, hydroxyl radical, and nitrogen radicals. In addition, *Aronia* polyphenols are potent inhibitors of lipid peroxidation and suppress the formation of reactive oxygen and nitrogen species (Banach et al., 2020; Valcheva-Kuzmanova et al., 2007; Valcheva-Kuzmanova S et al., 2014a). A number of experimental studies have demonstrated the ability of AMFJ to reduce OxS and ameliorate pathological conditions associated with it (Valcheva-Kuzmanova, 2015; Kondeva-Burdina et al., 2015; Valcheva-Kuzmanova et al., 2014; Valcheva-Kuzmanova et al., 2018).

In the current study, we aimed to assess the effect of AMFJ on the activity of antioxidant defense enzymes in animals with diet-induced MS. The animals receiving HFHF diet, which were treated with AMFJ, demonstrated lower activity of SOD compared to the untreated rats with MS, the effect being significant in MS + $AMFJ_{2.5}$ and MS + $AMFJ_5$ groups. In general, the SOD activity in all AMFJ-treated animals was similar to that of the control rats, receiving regular laboratory diet. We can explain our results with the antioxidant activity of the polyphenols present in the fruits. We suggest that AMFJ counteracted the OxS caused by the HFHF diet and prevented the superoxide production and the consequent compensatory activation of SOD in AMFJ-treated rats.

GPx activity remained unaffected in our study. Neither the HFHF diet, nor the AMFJ treatment affected the activity of this enzyme. Possible explanation of the observed result could be found in the relation between GPx function and oxidative stress, associated with MS. GPx acts as an antioxidant enzyme, scavenging lipid peroxidation products. Masurement and quantification of lipid peroxidation could be achieved by malondialdehyde (MDA) or thiobarbituric acid-reactive substrates (TBARS) assays (Gaschler and Stockwell, 2017; Montuschi et al., 2004). Although these markers are not tested in this experiment, there are studies revealing that elevation of lipid peroxidation is not mandatory in high-fat-induced obesity and diabetes (Boyne et al., 2007; Sohet et al., 2009) (and presumably MS) and the levels of MDA, TBARS or isoprostanes are low. This fact might be explained with the presence of vitamin E in the fats used as a component of high-fat diets for induction of these pathological conditions (Traber and Atkinson, 2007) as vitamin E can interact with the serum GPx activity.

The current study has some limitations. Only several flavonoids were measured in AMFJ. The content of other antioxidants presented in *Aronia* fruits and juice such as quercetin and other hydroxycinnamic acids were not investigated. The effect of the food preservative potassium sorbate was not taken into account. There is a study showing that the compound could induce oxidative stress and decrease the activity of the antioxidant enzyme catalase in female rats (Hasson, 2020). However, the content of potassium sorbate in AMFJ was considerably lower (0.1%) compared to the content of potassium sorbate (5%/10% in the diet) in the cited study (Hasson, 2020). We could suppose that at this low concentration potassium sorbate would not affect the activity of the antioxidant enzyme. The markers for lipid peroxidation were not investigated. Measuring MDA, TBARS or isoprostanes could clarify the effect of AMFJ on lipid peroxidation and the activity of GPx in MS.

4. Effects of *Aronia melanocarpa* fruit juice on the adipose tissue

4.1. Adipose tissue indices

The results are shown in **Table 13 and Figure 9**.

Table 13. Mesenterial, paranephral, perigonadal, retroperitoneal and total fat indices of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; *$p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. MS group

Group	Mesenterial fat index	Paranephral fat index	Perigonadal fat index	Retroperitoneal fat index	Total fat index
Control	0.64±0.05	0.17±0.03	0.89±0.1	0.67±0.08	2.24±0.18
MS	0.69±0.06	0.21±0.04	1.00±0.08	1.00±0.09*	2.90±0.24
MS+AMFJ$_{2.5}$	0.52±0.04	0.14±0.02	0.85±0.07	0.69±0.08$^{\#}$	2.15±0.15$^{\#}$
MS+AMFJ$_{5}$	0.57±0.04	0.14±0.02	0.78±0.06	0.72±0.04$^{\#}$	2.26±0.15
MS+AMFJ$_{10}$	0.63±0.05	0.15±0.02	0.90±0.10	0.85±0.11	2.54±0.26

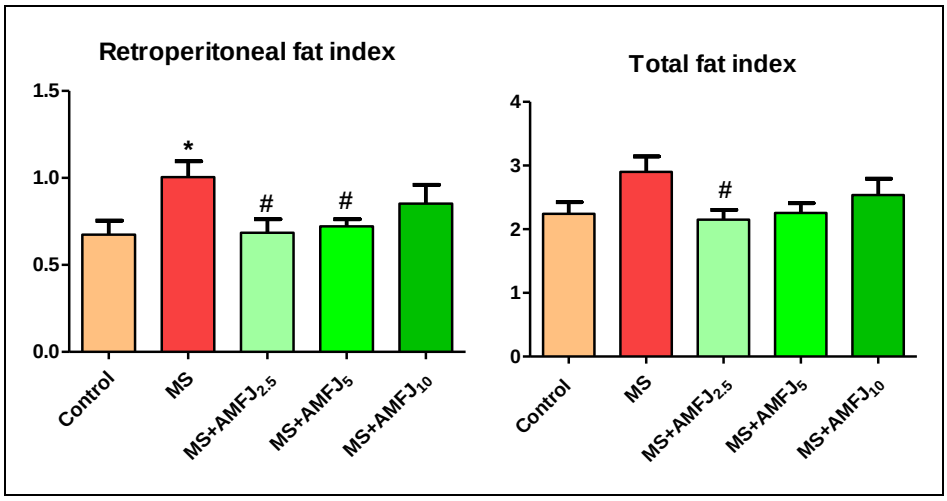

Figure 9. Retroperitoneal and total fat indices of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; *$p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. MS group

Retroperitoneal adipose tissue index was significantly higher in MS group than the index of Control group ($p < 0.05$) - an increase of 49.3% was registered. Compared to the MS group, AMFJ treatment reduced the retroperitoneal adipose tissue, with a significant effect observed in the MS+AMFJ$_{2.5}$ ($p < 0.05$) and MS+AMFJ$_5$ ($p < 0.05$) groups – the decreases were 44.9% and 38.9%, respectively. It is noteworthy that the index in all groups treated with fruit juice did not differ significantly from that of the control group (Figure 11).

There was no significance in the total adipose tissue index between Control group and metabolic groups. There was a tendency of decreasing the index after treatment with AMFJ, as Dunnet post-hoc analysis reported a significantly lower index in group MS+AMFJ$_{2.5}$ (reduction of 34.9%) compared to MS group ($p < 0.05$). In all treated groups, the total adipose tissue index had values close to those of the control group (Figure 11).

4.2. Adipose tissue histology

Histological findings are presented in **Figure 10**.

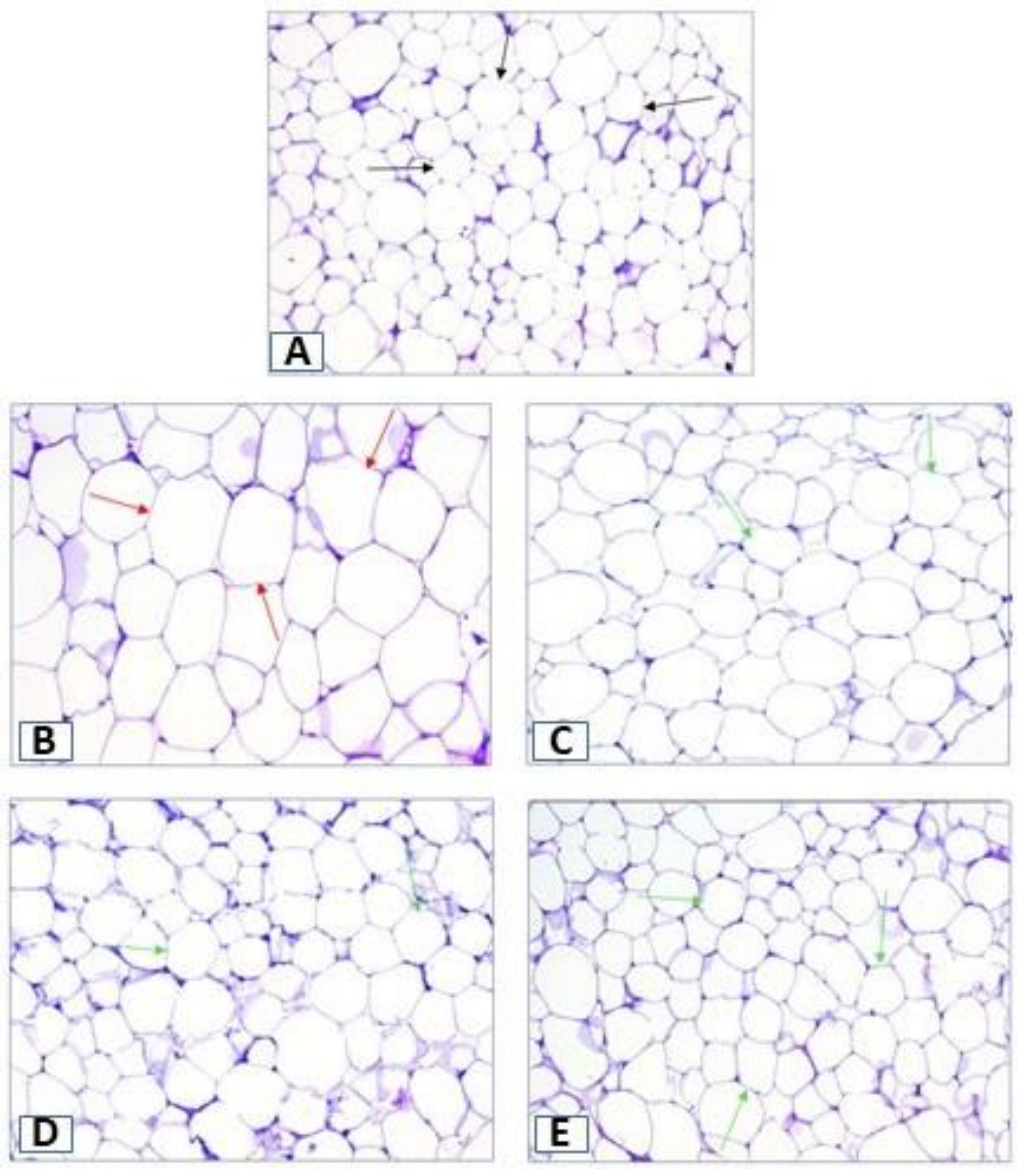

Figure 10. Adipose tissue of rats from the Control group (A), MS group (B), MS+AMFJ$_{2.5}$ group (C), MS+AMFJ$_5$ group (D) and MS+AMFJ$_{10}$ group (E); black arrows – normal adipocytes, red arrows – enlarged adipocytes; green arrows – restoration of the adipocyte size; hematoxylin-eosin staining; magnification x200

Retroperitoneal adipocytes of MS rats were increased in size compared to control animals. Unlike the control group, where small and medium-sized adipocytes predominate (Figure 12,A - black arrows), MS is characterized by presence of large adipocytes (Figure 12, B - red arrows). In animals treated with AMFJ, adipocyte sizes were reduced (Figure 12 B, D and E - green arrows), with changes being most pronounced at the highest dose of the juice. When treated with AMFJ at doses of 2.5 and 5.0 ml/kg, adipocytes decreased in size, with medium-

sized ones predominating, while in the MS + $AMFJ_{10}$ group, medium and small sizes predominated.

4.3. Apoptosis in the adipose tissue

4.3.1. Bax expression

The results are shown in **Table 14** and **Figures 11** and **12**. The expression of the apoptotic Bax protein was significantly increased (over 11-fold) in the MS group compared to the control group ($p < 0.01$) (Figures 13 and 14). AMFJ treatment resulted in a decrease in the pro-apoptotic marker expression in all three groups, and this effect was statistically significant in the MS+$AMFJ_{2.5}$ group, where a threefold decrease in expression was recorded ($p < 0.01$ vs. MS group). In addition, AMFJ antagonized the effect of the HFHF diet on the expression of Bax in the adipose tissue in all three doses and no statistically significant difference was found in its expression in the groups MS+$AMFJ_{2.5}$ and MS + $AMFJ_{10}$ compared to control values.

Table 14. Bax expression in retroperitoneal adipose tissue of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; **$p < 0.01$ vs. Control group, $^{\#\#}p < 0.01$ vs. MS group

	Control	**MS**	**MS+$AMFJ_{2.5}$**	**MS+$AMFJ_{5}$**	**MS+$AMFJ_{10}$**
Number of Bax (+) cells in a field (retroperitoneal adipose tissue) **Mean±SEM**	2.00±1.00	23.75±3..84**	8.00±2.52$^{\#\#}$	21.33±2.20	10.50±0.50

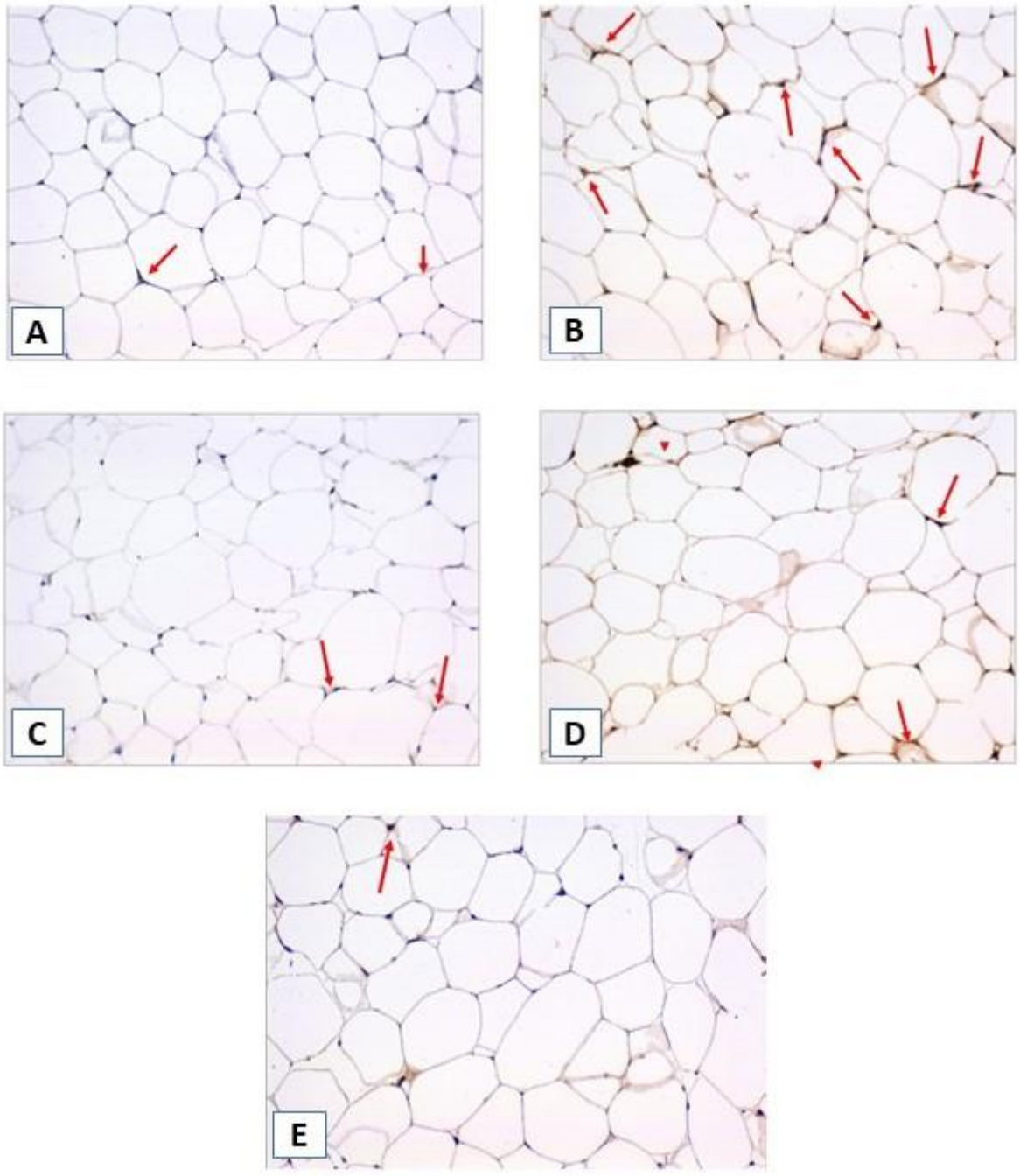

Figure 11. Bax expression (red arrows) in retroperitoneal adipose tissue of rats from the Control group (A), MS group (B), MS+AMFJ$_{2.5}$ group (C), MS+AMFJ$_{5}$ group (D) and MS+AMFJ$_{10}$ group (E)

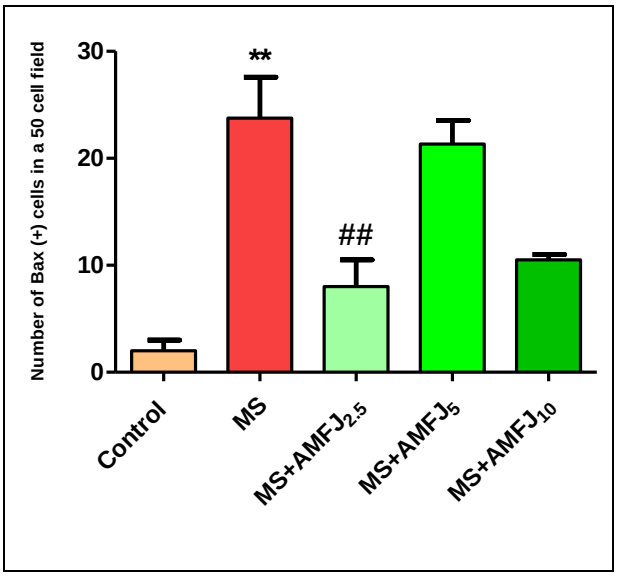

Figure 12. Number of Bax (+) cells in the retroperitoneal adipose tissue of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; **p < 0.01 vs. Control group, ##p < 0.01 vs. MS group

4.3.2. Bcl-2 expression

The results are shown in **Table 15** and **Figures 13** and **14**. Adipocytes of the rats from the MS group had higher Bcl-2 expression (over 7-fold increase) compared to the control group ($p < 0.001$). A statistically significant increase in the level of the marker was observed in MS+AMF|$J_{2.5}$ group ($p < 0.001$ vs. MS group) and MS+$AMFJ_5$ group ($p < 0.001$ vs. MS group) - by 71% and 100%, respectively. In MS+$AMFJ_{10}$ group, the expression of the anti-apoptotic protein decreased to a level close to that of the control group, and no statistical difference in marker expression was observed between these two groups.

Table 15. Expression of the anti-apoptotic marker Bcl-2 in retroperitoneal adipose tissue of rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg; ***p < 0.001 vs. Control group, ###**p** < 0.001 vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Number of Bcl-2 (+) cells in a 50-cell field (retroperitoneal adipose tissue) **Mean±SEM**	2.50±0.34	19.00±1.00***	32.50±0.50###	38.00±1.96###	4.67±0.92###

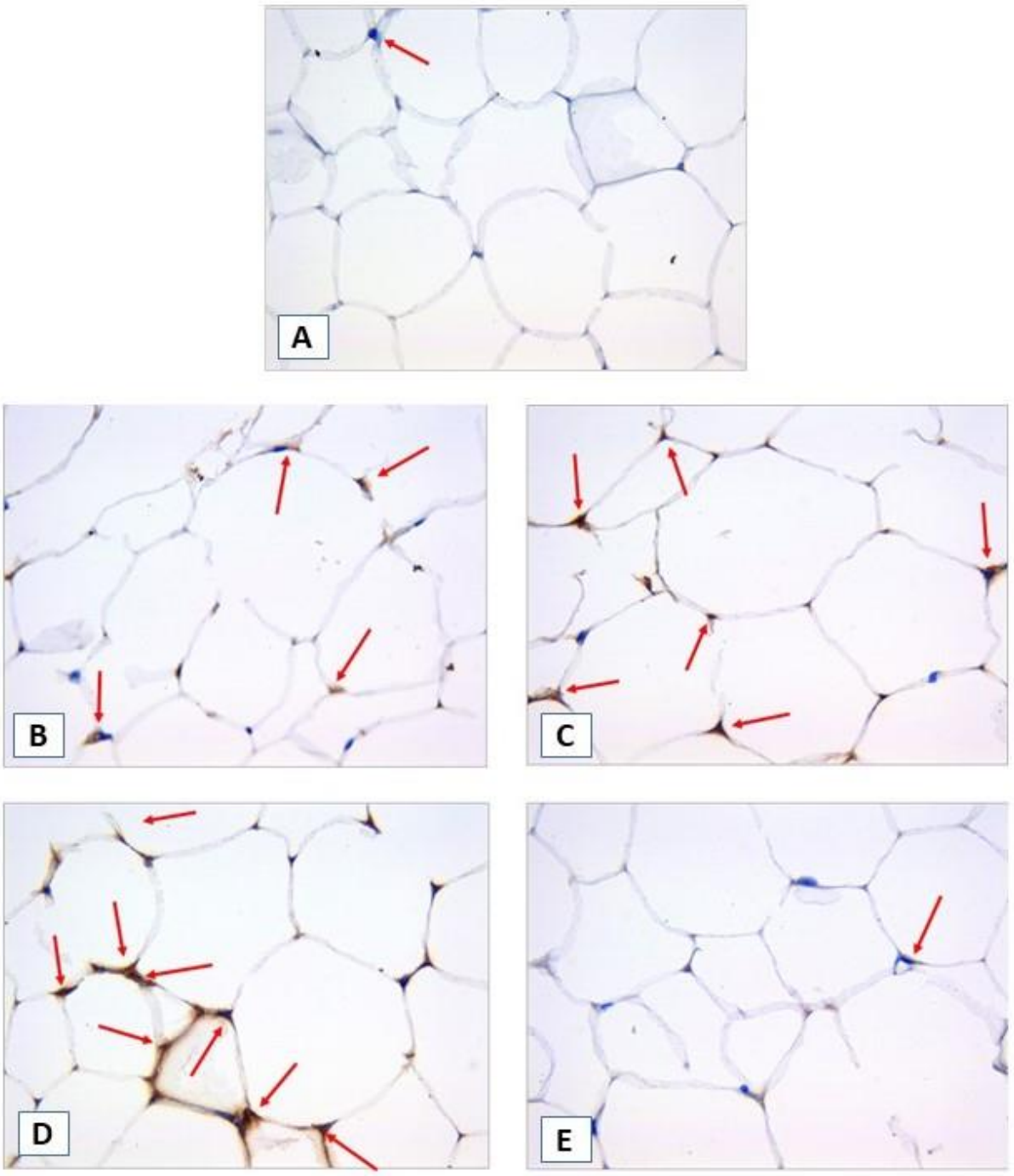

Figure 13. Bcl-2 **e**xpression (red arrows) in retroperitoneal adipose tissue of rats from Control group (A), MS group (B), MS+AMFJ$_{2.5}$ group (C), group MS+AMFJ$_{5}$ group (D) and MS+AMFJ$_{10}$ group (E)

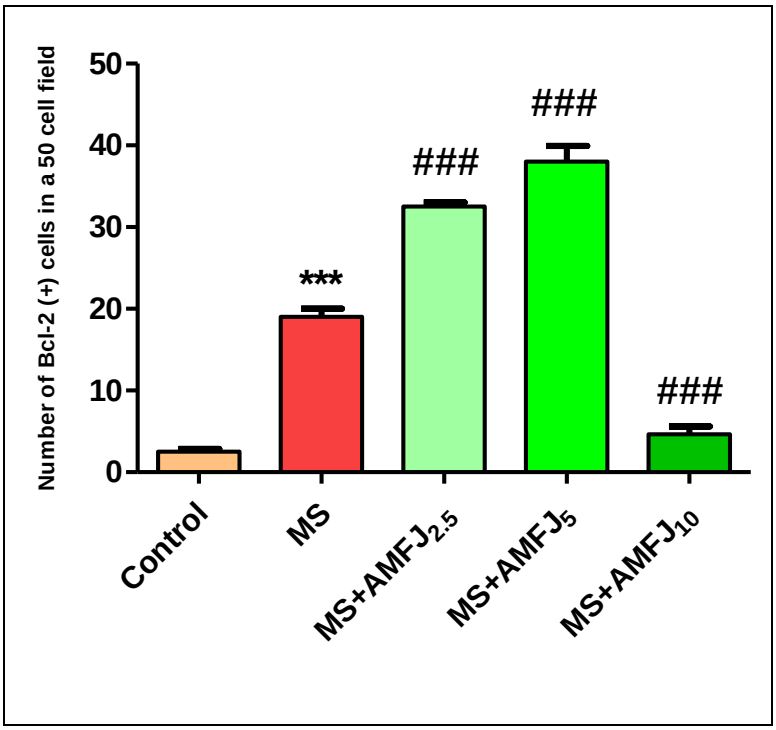

Figure 14. Number of Bcl-2 positive cells in the retroperitoneal adipose tissue of rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10 ml/kg; ***$p < 0.001$ vs. Control group, $^{###}p < 0.001$ vs. MS group

4.3.3. Bax/Bcl-2 ratio

The results are presented in **Table 16** and **Figure 15**. The Bax/Bcl-2 ratio in the MS group was higher (48% increase) compared to the control group, which meant that the adipocyte phenotype was apoptotic. At doses of 2.5 and 5.0 ml/kg, AMFJ increased Bcl-2 expression and decreased the ratio, resulting in cells acquiring an anti-apoptotic phenotype. This decrease was statistically significant compared to the MS group in the MS+$AMFJ_{2.5}$ group ($p < 0.01$vs. MS group), where a sevenfold reduction in the ratio was registered, and in the MS+AMFJ5 ($p < 0.05$ vs. MS group). The highest dose of AMFJ decreased the expression of Bcl-2 and the Bax/Bcl-2 ratio in the MS+$AMFJ_{10}$ group was significantly higher ($p < 0.01$) compared to the MS group (85.8% inccrease), indicating that the proapoptotic phenotype predominated in this group.

Table 16. Bax/Bcl-2 ratio in retroperitoneal adipose tissue samples from rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10.0 ml/kg; $^{\#}p < 0.05$ vs. MS group, $^{\#\#}p < 0.01$ vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_{5}$**	**MS+AMFJ$_{10}$**
Bax/Bcl-2 ratio (retroperitoneal adipose tissue) **Mean±SEM**	1.00±0.08	1.48±0.22	0.20±0.11$^{\#\#}$	0.67±0.12$^{\#}$	2.75±0.05$^{\#\#}$

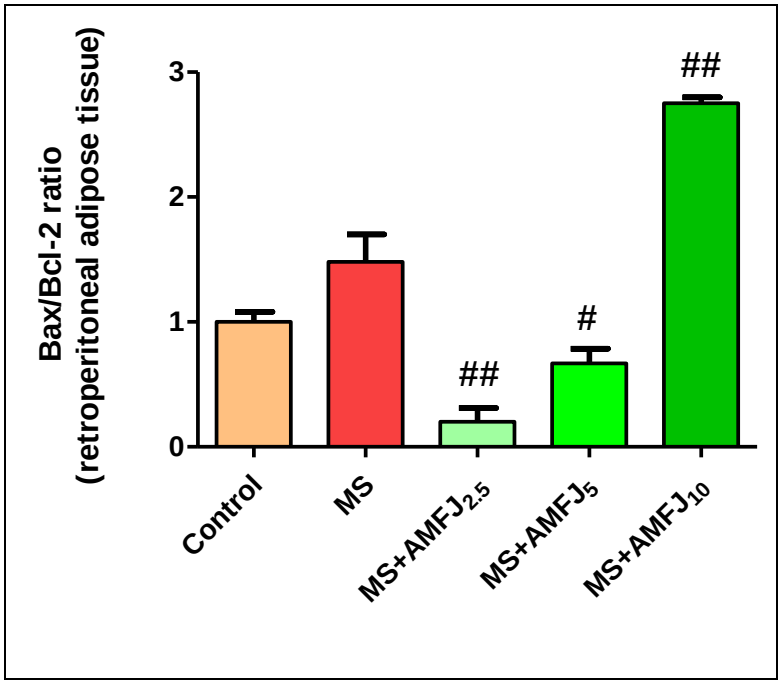

Figure 15. Bax/Bcl-2 ratio in retroperitoneal adipose tissue samples from rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10.0 ml/kg; $^{\#}p < 0.05$ vs. MS group, $^{\#\#}p < 0.01$ vs. MS group

4.4. Discussion

Visceral obesity is a hallmark of MS. It is estimated that by 2030, one in two adults will be obese and one in four will be severely obese (Ward et al., 2019). Visceral obesity is associated with the development of diabetes, atherosclerosis, hypertension, dyslipidemia, coronary heart disease, non-alcoholic fatty liver disease (NAFLD), osteoarthritis, malignancy (breast, uterine and esophageal cancer) (Pluta et al., 2022). In addition, overweight and obesity are associated with a more severe course of COVID-19, an infection that has endangered the lives of millions of people over the past 3 years (Foulkes et al., 2022).

Polyphenolic compounds are powerful antioxidants, characterized by a number of pleiotropic effects. A growing number of publications demonstrate their potential for beneficial effects on visceral obesity and body weight (Boccellino and D'Angelo, 2020). *Aronia melanocarpa* ranks among the plants whose fruits have a very high PF content (Sidor and Gramza-Michałowska, 2019). We could expect that AMFJ will have a beneficial effect on visceral obesity in rats with diet-induced MS.

The HFHF diet used in this study caused an increase in visceral adipose tissue and adipocyte size. MAFJ treatment antagonized these effects of diet. These results are similar to those reported in other experimental studies focusing on the effect of *Aronia* extracts on weight (Jakovljevic et al., 2018; Kim et al., 2018; Lim et al., 2019). The observed effect on visceral obesity in this experiment may be due to the high polyphenolic content of AMFJ. *Aronia melanocarpa* PPs have been shown to inhibit lipid accumulation in adipocytes and adipocyte differentiation - two key factors in the development of obesity. Expression of cytosine-cytosine-adenosine-adenosine-thymidine-enhancing protein-alpha (CCAAT)/enhancer-binding protein and fatty acid-binding protein which are involved in these two pathogenetic moments decreased after administration of *Aronia melanocarpa* - an effect described by Park et al. (Park et al., 2017).

Apoptosis is a programmed cell death that is controlled by pro-apoptotic and anti-apoptotic molecules. Proteins from the Bcl-2(B-cell lymphoma-2) family, expressed in the mitochondria and the smooth endoplasmic reticulum, are the main controllers of this process. Pro-apoptotic members of the family include multidomain proteins such as Bax and BH3-only proteins, while antiapoptotic members include Bcl-2, Bcl-xl, Bcl-w, Bcl-b, Mcl-1 and Bfl-1. Apoptosis can be initiated by activating the internal or external apoptotic pathways. The external (death-receptor) pathway is activated by tumor necrosis factor (TNF) and FAS-ligand after binding to their receptors (TNF-R / FAS-R). The internal pathway is activated by cellular stress, chemotherapy and radiation therapy. This leads to increased permeability of the outer mitochondrial membrane, release of cytochrome c and enzymes (caspases) in the cytosol. Activation of caspases (especially caspase-3 and caspase-7) leads to apoptosis (Suvarna et al., 2019).

Studies have linked apoptosis to the metabolic effects of MS. Adipocyte enlargement (observed in obesity) activates both pathways of apoptosis, which in turn stimulate adipose tissue macrophages (ATM). ATMs release proinflammatory cytokines that cause meta-

inflammation and subsequently IR, dyslipidemia, and hepatic steatosis (Alkhouri et al., 2021). Since oxidative stress can activate apoptosis (Dingeldein et al., 2019), it is reasonable to expect that antioxidant compounds may inhibit this cellular process. *Aronia melanocarpa* fruit juice contains strong antioxidants such as proanthocyanidins, anthocyanins and phenolic acids. In this study, the effect of AMFJ on programmed adipose tissue death was investigated. To elucidate this effect, the expression of Bax and Bcl-2 in adipocytes from retroperitoneal adipose tissue were studied and the Bax/Bcl-2 ratio was calculated, which serves as a tool to determine the susceptibility of cells to apoptosis (Raisova et al., 2001). This study showed that adipocytes of animals in the MS group showed a pro-apoptotic phenotype, while adipocytes in the MS+AMFJ$_{2.5}$ and MS+AMFJ$_5$ groups showed an anti-apoptotic phenotype. These results are consistent with other studies describing the effect of *Aronia melanocarpa* on apoptosis. For example, the aqueous extract of *Aronia melanocarpa* reduces the Bax/Bcl-2 ratio in the adrenal glands in a model of adrenal damage caused by hexavalent chromium (Savici et al., 2021). Meng et al. confirm the anti-apoptotic activity of *Aronia* anthocyanins, which increase the expression of Bcl-2 and decrease that of cytochrome c, caspase-3, caspase-9 and Bax (Meng et al., 2018).

Contrary to expectations, the group treated with the highest dose of AMFJ (10 ml/kg) had the highest Bax/Bcl-2 ratio among all experimental groups studied, even higher than that of the MS group. According to a study on cell cultures of acute lymphoblastic leukemia, AMFJ may have a pro-apoptotic effect at high concentrations. It has been found that some PPs in the juice (mainly derivatives of chlorogenic acid, cyanidin and quercetin) can induce ROS production, which can disrupt the mitochondrial membrane potential, stimulate cytochrome c release and activate caspase-3. These events lead to apoptosis (Sharif et al., 2012). We can assume that the highest dose of fruit juice used in this study has the potential to induce dose-dependent activation of programmed adipocyte cell death by the mechanism described above.

5. Effects of *Aronia melanocarpa fruit juice* on the myocardium and coronary artery

5.1. Histology of the myocardium

Histological findings are shown in **Figure 16**.

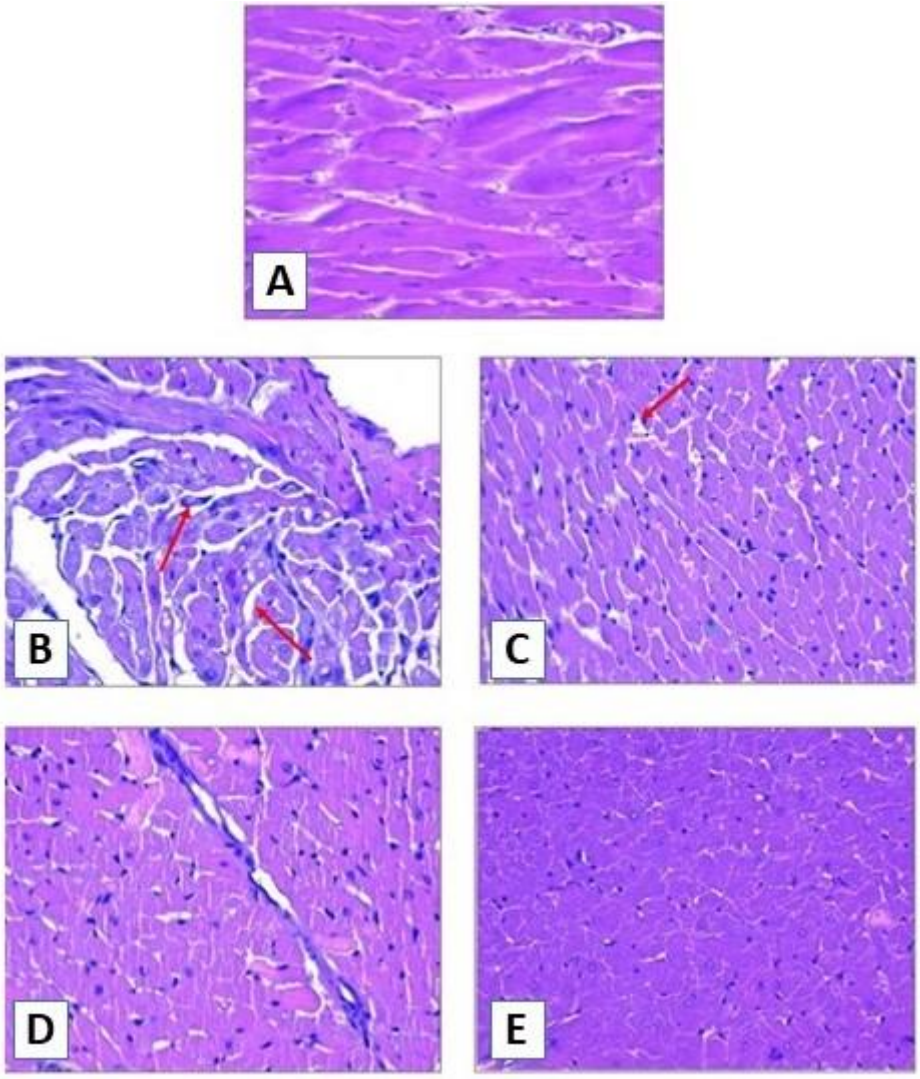

Figure 16. Myocardium of rats from the Control group (A), MS group (B), MS+AMFJ$_{2.5}$ group (C), MS+AMFJ$_5$ group (D) and MS+AMFJ$_{10}$ group (E); red arrows - distant cardiomyocytes; hematoxylin-eosin staining; magnification x 100

No morphological changes were detected in the myocardium of the control rats. In the MS group, degenerative changes were observed in the cardiomyocytes, which are distant from each other. Low dose AMFJ reduced the damage of the cardiac cells, but those with degenerative changes were also found, albeit in single cells. At higher doses (MS+AMFJ$_5$ and MS+AMFJ$_{10}$ groups) the myocardium showed no histological abnormalities.

5.2. Coronary artery histology

Histological findings are shown in **Figure 17**.

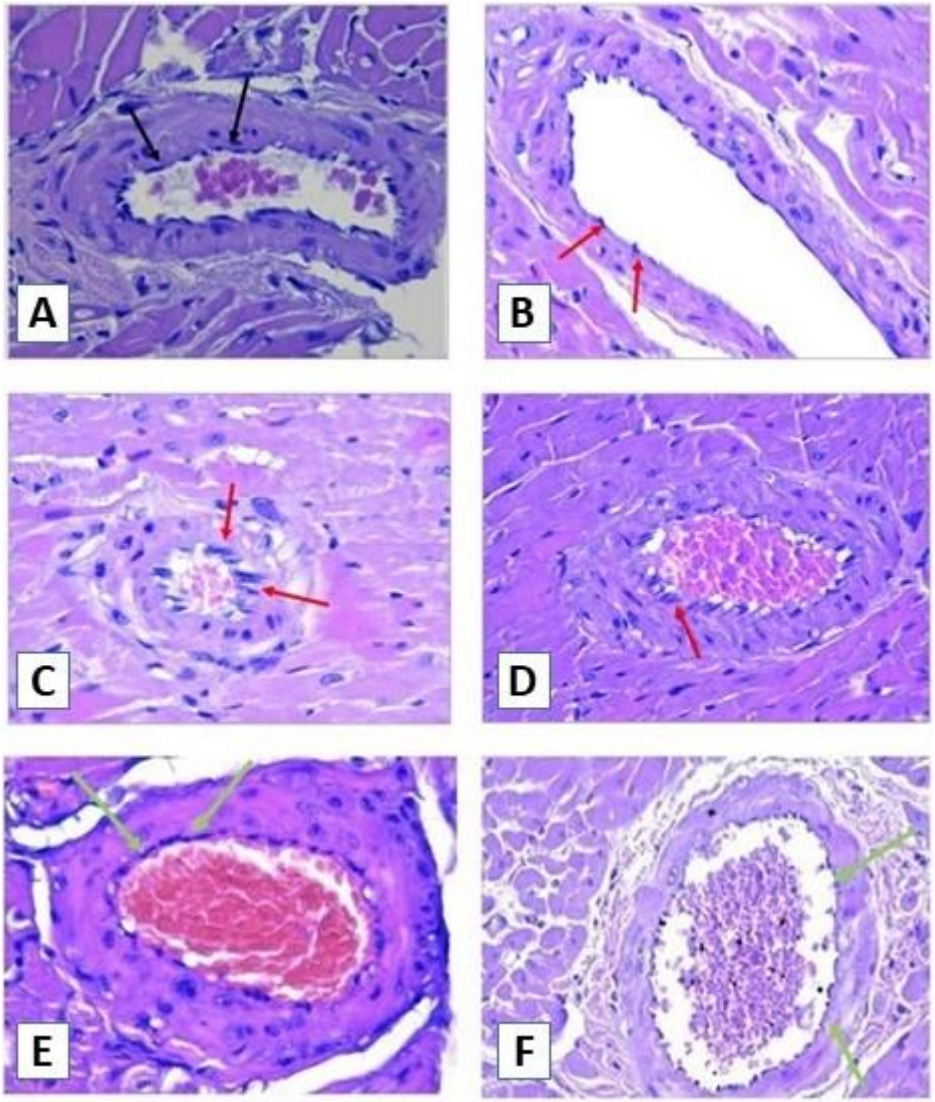

Figure 17. Coronary artery of rats from the control group (A), MS group (B, C), MS+AMFJ$_{2.5}$ group (D), MS+AMFJ$_{5}$ group (E) and MS+AMFJ$_{10}$ group (F); black arrows – normal endothelium, red arrows – activated endothelial cells, green arrows – intact endothelium

Coronary arteries in control rats showed a continuous endothelial layer and no structural changes were found in their wall. In MS group, the endothelium was damaged, there was necrosis of endothelial cells and the basement membrane was exposed. In MS+AMFJ$_{2.5}$ group, the endothelium was also damaged and the preserved cells showed signs of activity - the nuclei of endothelial cells were located perpendicular to the basement membrane. At higher doses (MS+AMFJ$_{5}$ and MS+AMFJ$_{10}$ groups) endothelial layer of the coronary arteries was intact.

5.3. Discussion

The link between MS and the increased risk of CVD is well known. Each component of MS represents an individual independent risk factor for the development of coronary calcifications, coronary dysfunction, coronary artery disease, myocardial dysfunction, acute myocardial infarction and/or heart failure (Tune et al., 2017). The pathogenetic substrates of these complications in MS have been identified by imaging and pathoanatomical studies. Magnetic resonance spectroscopy has shown that in MS and type 2 diabetes patients, myocardial TG accumulation was increased and correlated positively with myocardial deformity and myocardial perfusion disorders (Gao Y et al., 2020). Histologically, inflammation and fibrosis were found in myocardial infarction in advanced MS (Li et al., 2012). Such changes have also been described in the ascending aorta (Saraf et al., 2016). Using virtual histological methodology, it was further found that fibroatheromatous plaques were more common in the coronary arteries of patients with diabetes and/or MS, and their size was larger than those without MS (Zheng M et al., 2011).

There are several pathophysiological factors involved in myocardial and vascular damage in MS. Oxidative stress potentiates the activation of the sympathetic nervous system and the renin-angiotensin-aldosterone (RAAS system). In turn, these systems induce oxidative stress - that is, the relationship is two-way. (Koba, 2018; Fanelli and Zatz, 2011). The sympathetic nervous system and RAAS stimulate the cardiovascular system - they induce positive tropic effects in the myocardium, as well as vasoconstriction and endothelial damage in the blood vessels (Bruno et al., 2012; Aroor et al., 2013). Increased production of adipocytokines by abdominal adipose tissue, together with the aforementioned regulatory systems, contributes to an increase in the heart rate, circulating blood volume, end-diastolic volume in the ventricles, cardiac output and vascular resistance. These changes lead to impaired energy metabolism, abnormal oxygen demand, diastolic dysfunction, and concentric myocardial hypertrophy (Tune et al., 2017). Endothelial activation, described histologically, is a pro-inflammatory response (Blann, 2000) and is an expression of endothelial dysfunction in MS.

In MS, pre-diabetic conditions and diabetes the arterial vascular structure (endothelial layer and basement membrane) is damaged, which triggers significant micro- and macrovascular complications. Endothelial dysfunction has been shown to be a leading mechanism associated with vascular complications in MS. Decreased NO production and increased vasoconstrictor and ROS production damage the blood vessels, with the end effect being accelerated

atherosclerotic process and increased BP - changes observed in MS (Tran et al., 2020). In addition, it has long been known that adverse metabolic effects (hyperglycemia) can activate matrix metalloproteinases that degrade type IV collagen on the basement membrane of the endothelium and damage it (Death et al., 2003).

The present study examined the effect of MS on myocardial and coronary vessel histology in rats with diet-induced MS and the effect of AMFJ on the structure of these organs. The high-calorie diet induced degeneration of the cardiomyocytes and disrupted their microarchitectonics in MS rats. In a coronary vessel, MS lead to endothelial cell activation, endothelial necrosis, and damage to the basement membrane. AMFJ treatment improved the histological picture and eliminated these pathological changes both in the myocardium and coronary blood vessels, especially at higher doses.

It has long been known that a diet rich in fresh vegetables and fruits helps reduce cardiovascular risk and is associated with reduced cardiovascular mortality (Hemler and Hu, 2019). The French diet, which is characterized by a moderate intake of red wine, the Mediterranean diet, which is characterized by the intake of whole grains, fruits, vegetables, fish and olive oil, and the diet of regular consumption of green tea in the Middle East are all examples of diets rich in PPs and with proven CVD-preventive effects (Renaud and de Lorgeril, 1992; Huang and Sumpio, 2008; Arts and Hollman, 2005). This gives grounds to study the effects of AMFJ on the structure of the myocardium and coronary vessels in diet-induced MS.

Although studies on the effect of *Aronia melanocarpa* on cardiovascular structure are scarce, available publications show beneficial effects. A study conducted by Daskalova et al. showed that treating aging rats with *Aronia* juice slowed the age-related accumulation of collagen fibers in the aortic wall. In addition, the level of atherogenic LDL-cholesterol was reduced (Daskalova et al., 2015). The results of another studies by the same team regarding the effects of AMFJ on the vessel wall in aging rats (Kitova et al., 2021) were similar. A recent study showed that AMFJ supplementation in aging rats reduced collagen fiber accumulation and mast cells infiltration in the perivascular space in the left ventricle and slowed the age-related myocardial remodeling (Delchev et al., 2021).

The cytoprotective effects of AMFJ in the present study could be related primarily to the antioxidant effects of the PPs. Nrf2 (erythroid-derived 2)-like 2 is a transcription factor that

regulates the cellular response to oxidative stress (Jadeja et al., 2016) and increases the expression of the antioxidant enzymes SOD, GPx and NADP (H) quinone oxidoreductase. Under normal conditions (without free radical production) Nrf2 is coupled with ECH -associated protein 1 (Keap1). Keap1 induces proteasome lysis and termination of the Nrf2 effects (Nguyen et al., 2009). In oxidative stress these proteins dissociate from each other, Nrf2 enters the cell and induces the expression of genes encoding said antioxidant enzymes. This is thought to be the main mechanism underlying the antioxidant action of PPs. Thus, antioxidant protection is likely to prevent overactivation of the sympathetic nervous system and RAAS and protect the myocardium. Antioxidant-mediated cardioprotective effect has been described for the flavonol catechin as well as for the anthocyanidins cyanidin and delphinidin in a study of in H9c2 cardiomyoblasts (Akhlaghi and Bandy, 2012). In another study, cranberry extract rich in cyanidin-3 galactoside, cyanidin-3 arabinoside and cyanidin-3 glucoside, by preventing oxidative stress inhibited the activation of the apoptotic pathways in cardiomyocytes (Isaak et al., 2017). AMFJ used in the present study is rich in these flavonoids.

There is also evidence of a direct sympatholytic effect of some flavonols. In one study, norepinephrine-mediated cardiomyocyte hypertrophy and apoptosis were prevented by treatment with anthocyanin-rich aqueous bilberry extract *in vitro* (Louis et al., 2014). An antihypertrophic effect on the myocardium was observed after resveratrol administration in another study, with this effect attributed to increased expression of NO and AMPK (Thandapilly et al., 2011).

Another putative mechanism that could protect the myocardium from damage in MS is the suppression of RAAS. Suppression of this system would reduce both the pathological remodeling of the myocardium and the production of ROS induced by this system, which are also involved in myocardial disorders in MS. In their study, Parichatikanond et al. studied the effects of flavonol quercetin and the anthocyanins cyanidin and delfinidin on human embryonic kidney (HEK)-293 cells after treatment with dexamethasone. The corticosteroid increased the activity of ACE, while the mentioned PPs reduced it, regardless of the treatment with dexamethasone (Parichatikanond et al., 2012). Catechin, epicatechin and polymeric proanthocyanidin-rich açai (Euterpe oleracea) seed extract, reduced the inflammatory response and oxidative stress in MS subjects by modulating local (renal) or total RAAS activity (Santos et al., 2020). Although these studies have been performed on other tissues, we can assume that the same effects may occur on myocardial tissue, too.

The vasoprotective effect demonstrated in the present study is very likely to be due to the improvement in endothelial function mediated by the juice polyphenols. There are a large number of studies demonstrating this effect of PPs (including those derived from *Aronia melanocarpa*). For example Kim et al. proved AMFJ was able to induce relaxation of isolated coronary arteries. The authors found that the conjugated cyanidins and chlorogenic acids contained in the juice phosphorylated endothelial nitric oxide synthase and thus increased the production of vasodilatory factor NO (Kim et al., 2013). In another study, the use of anthocyanin-rich chokeberry extract inhibited TNF-alpha-mediated expression of vascular adhesion molecule-1 (VCAM-1). It is thought that this mechanism could underlie the anti-atherogenic effect of Aronia melanocarpa (Iwashima et al., 2019). Similar results are described by Zapolska-Downar et al. - they found that chokeberry extract exerted an anti-inflammatory effect on human aortic endothelial cells by suppressing the expression of VCAM-1, intercellular adhesion molecule-1 (ICAM-1) and NF-κB (Zapolska-Downar et al., 2012). It is important to note that the antioxidant activity of the polyphenolic compounds contained in the juice could be responsible for the described effects on the coronaries. Therefore, again, this mechanism could explain the vascular protective effect observed in this study.

6. Effects of *Aronia melanocarpa* fruit juice on the liver

6.1. Liver histology

The results are shown in **Figure 18**. Normal architecture was observed in the liver of the control rats. In MS group the liver was characterized by degenerative changes, steatosis, presence of apoptotic bodies (hepatocytes) and inflammatory granulomas represented by lymphocytes. In the MS+AMFJ$_5$ and MS+AMFJ$_{10}$ groups the alteration of the liver parenchyma was reduced, and in the MS+AMFJ$_{10}$ group the effect of AMFJ was more pronounced, but inflammatory cells and single hepatocytes with necrotic changes were still found.

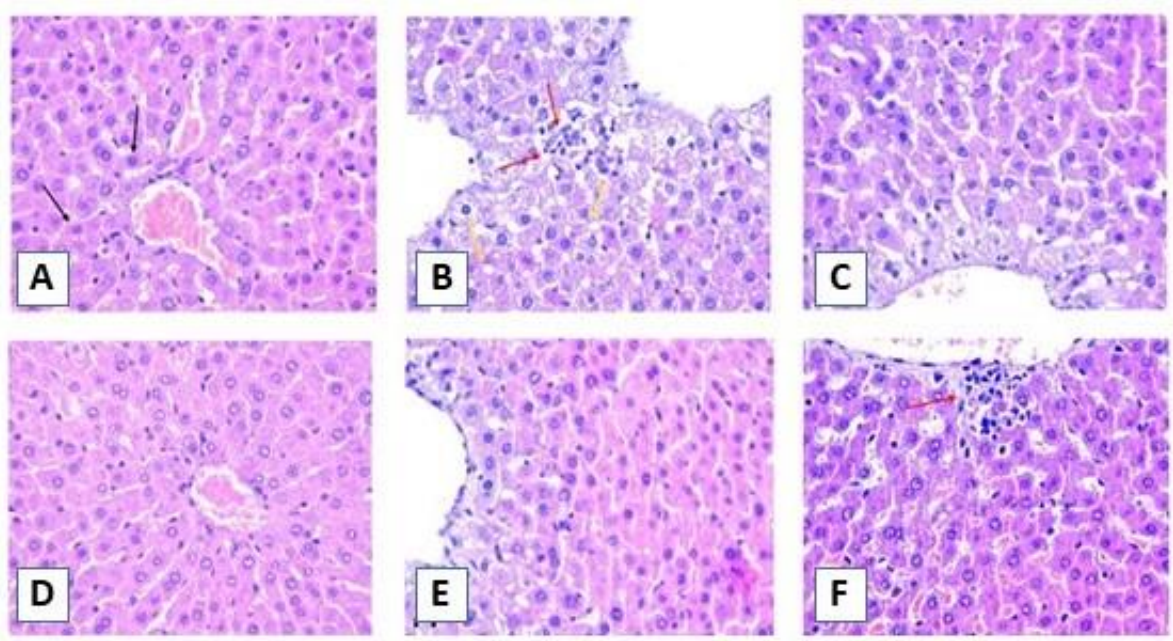

Figure 18. Liver of rats from the control group (A), MS group (B, C), MS+AMFJ$_{2.5}$ group (D), MS+AMFJ$_5$ group (E) and MS+AMFJ$_{10}$ group (F); black arrows - normal hepatocytes, yellow arrows - hepatocytes with lipid inclusions, red arrows - non-specific granulomas around hepatocytes; hematoxylin-eosin staining; magnification x200

6.2. Bax, Bcl-2 and MAC387 expression in the liver

6.2.1. Bax expression

The results are represented in **Table 17** and **Figures 19** and **20**.

Table 17. Bax expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***p < 0.001 vs. Control group, $^{###}$p < 0.001 vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Bax expression score (liver) **Mean±SEM**	1.40±0.08	3.27±0.17***	1.81±0.22$^{###}$	1.53±0.07$^{###}$	1.08±0.22$^{###}$

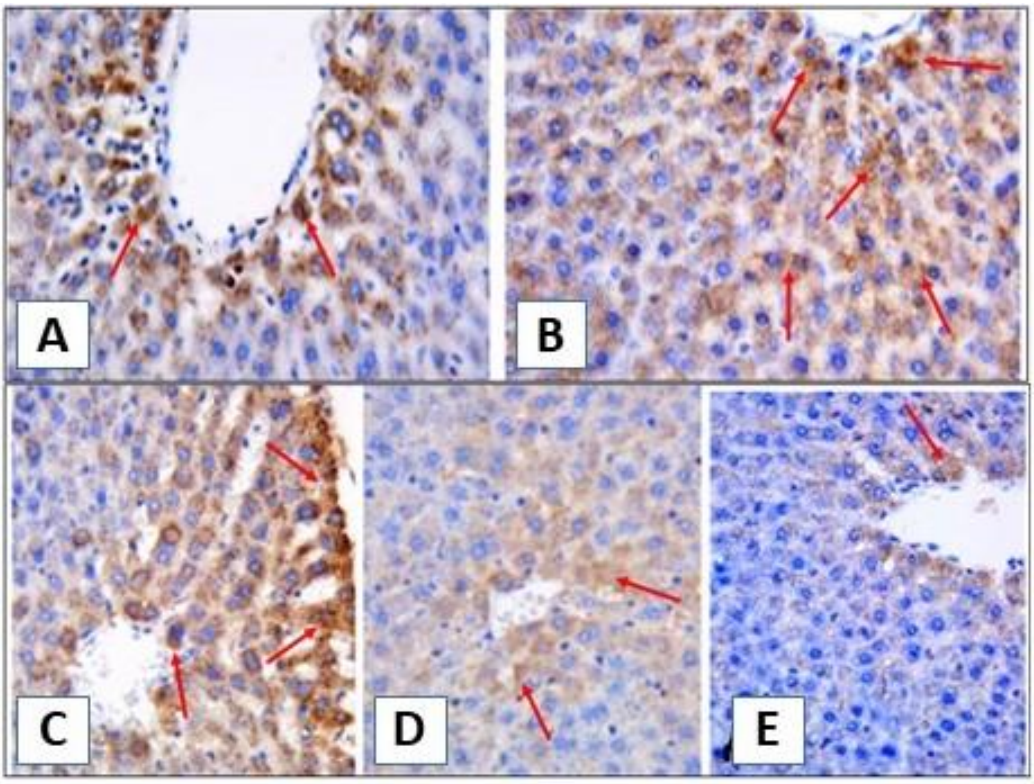

Figure 19. Bax expression (red arrows) in the liver of rats from the control group (A), MS group (B), MS+AMFJ$_{2.5}$ group (C), MS+AMFJ$_5$ group (D) and MS+AMFJ$_{10}$ group (E)

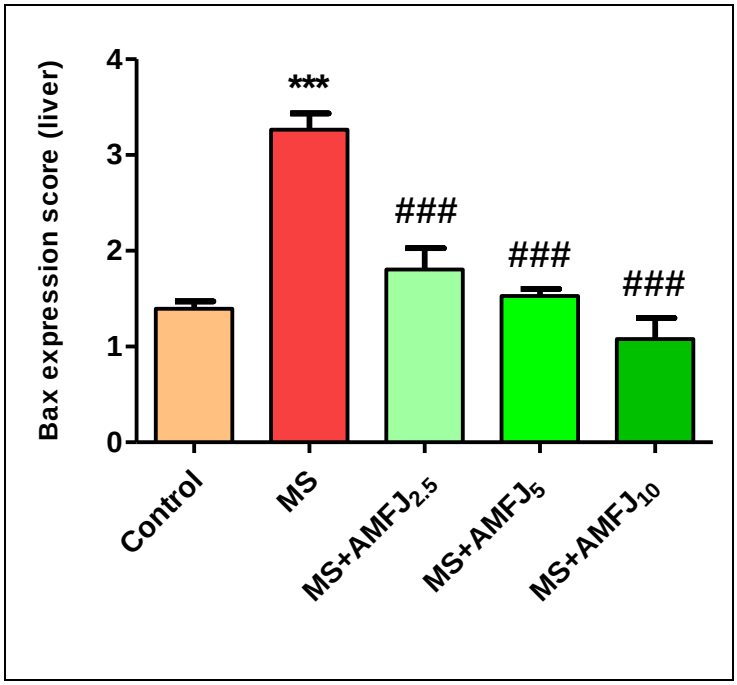

Figure 20. Bax expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***p < 0.001 vs. Control group, $^{###}$p < 0.001 vs. MS group

In the liver parenchyma of the control rats Bax expression was observed predominantly in hepatocytes around *v.centralis*. MS induced diffuse expression of the marker and it was significantly higher (more than 2 times) compared to the control group ($p < 0.001$). AMFJ treatment reduced in a dose-dependent manner the hepatic expression of the marker in all groups ($p < 0.001$ vs. MS group) and this protection was most pronounced at the highest dose administered. The decrease in the expression of Bax in the groups MS+AMFJ$_{2.5}$, MS+AMFJ$_5$ and MS+AMFJ$_{10}$ was 44.6%, 53.2% and 67%, respectively. In all AMFJ-treated groups, the expression of the marker did not differ significantly from that of the control group.

6.2.2. Bcl-2 expression

The results are shown in **Table 18** and **Figures 21** and **22**. In the control group Bcl-2 expression was observed predominantly in hepatocytes around *v.centralis*. Despite the lack of statistical significance, in the MS group the expression of the marker decreased by 65%. Compared to the MS group, in all AMFJ-treated groups the expression of Bcl-2 was increased both in hepatocytes and sinusoidal cells, and this effect was statistically significant in MS+AMFJ$_5$ group (five-fold increase in the value) ($p < 0.01$ MS vs.group).

Table 18. Bcl-2 expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; $^{\#\#}p < 0.01$ vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Bcl-2 expression score (liver) **Mean±SEM**	0.80±0.21	0.28±0.14	0.81±0.22	1.46±0.17$^{\#\#}$	1.02±0.15

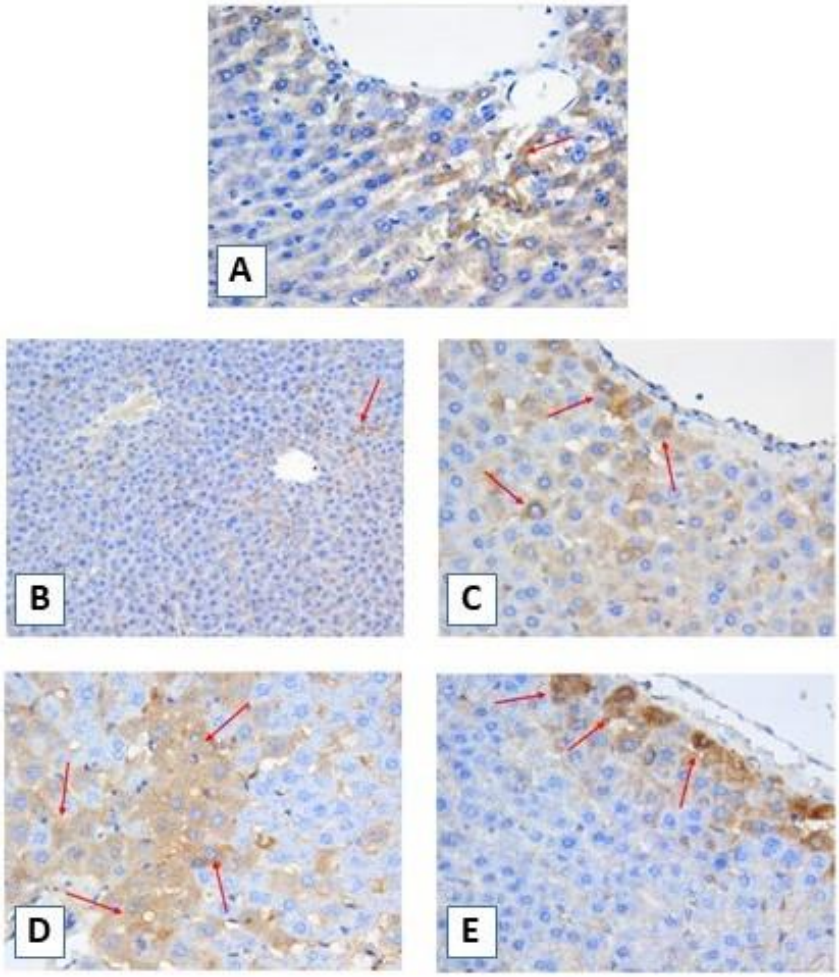

Figure 21. Bcl-2 expression (red arrows) in the liver of rats from the Control group (A), MS group (B), group MS+AMFJ$_{2.5}$ (C), group MS+AMFJ$_{5}$ (D) and MS+AMFJ$_{10}$ (E)

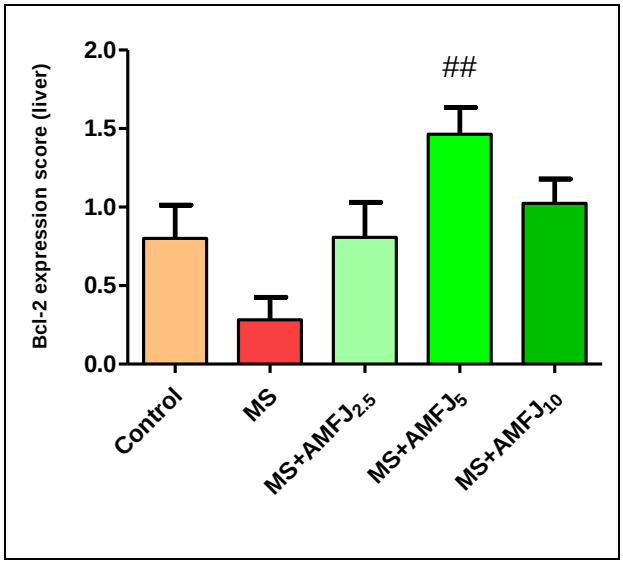

Figure 22. Bcl-2 expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; $^{\#\#}p < 0.01$ vs. MS group

6.2.3. Bax/Bcl-2 ratio

The results are shown in **Table 19** and **Figure 23**. The Bax/Bcl-2 ratio was statistically significantly increased in MS rats compared to the control group ($p < 0.001$). AMFJ administration reduced the ratio in all treated groups to levels, close and even lower than that of the control group.

Table 19. Bax/Bcl-2 ratio in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***$p < 0.001$ vs. Control group, $^{###}p < 0.001$ vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
Bax/Bcl-2 ratio (liver) **Mean±SEM**	3.19±1.03	12.11±0.06***	2.41±0.12$^{###}$	0.97±0.09$^{###}$	0.96±0.16$^{###}$

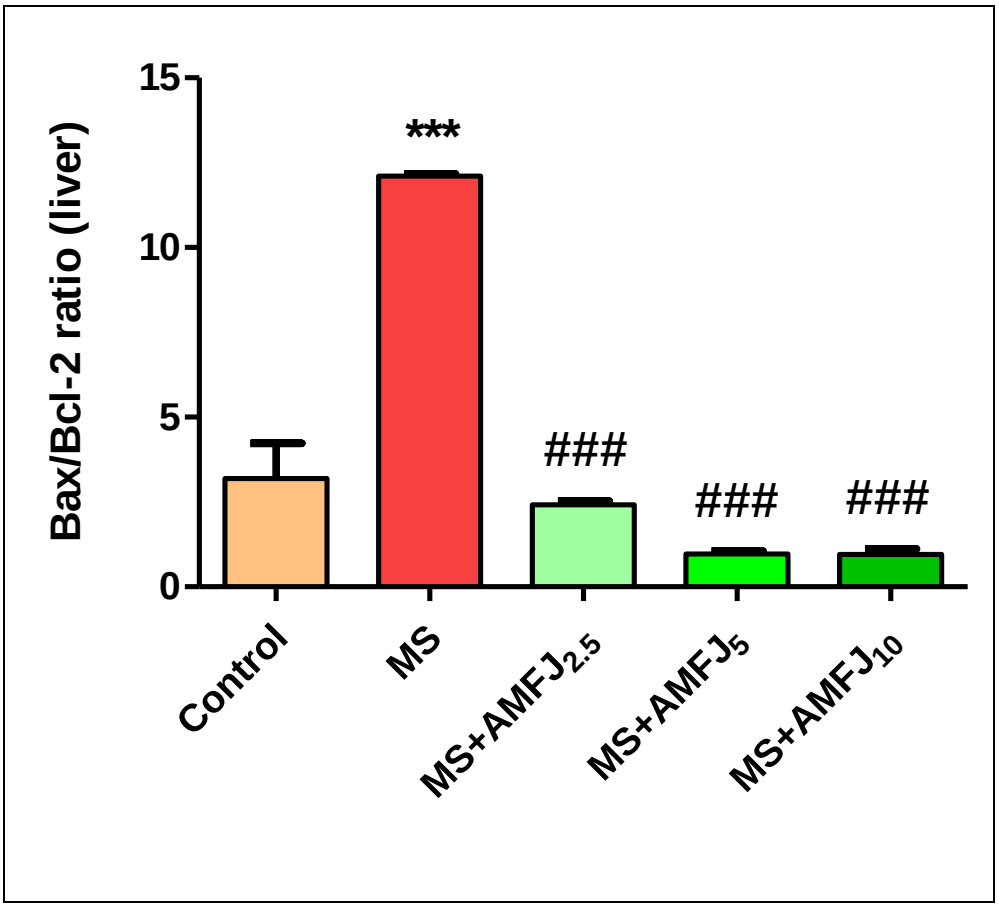

Figure 23. Bax/Bcl-2 ratio in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***$p < 0.001$ vs. Control group, $^{###}p < 0.001$ vs. MS group

6.2.4. MAC387 expression

The results are shown in **Table 20** and **Figures 24** and **25**. In the control group poor expression of the macrophage marker was observed, mainly in single hepatocytes around *v.centralis*. Significantly increased expression was detected in the MS group, both in hepatocytes and in sinusoidal cells ($p < 0.001$ vs. Control group). AMFJ treatment significantly suppressed the expression of the marker at all used doses ($p < 0.001$ vs. MS group), with the decrease being most pronounced at a dose of 10 ml/kg.

Table 20. MAC387 expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***$p < 0.001$ vs. Control group, $^{###}p < 0.001$ vs. MS group

	Control	**MS**	**MS+AMFJ$_{2.5}$**	**MS+AMFJ$_5$**	**MS+AMFJ$_{10}$**
MAC387 expression score (liver) **Mean±SEM**	0.12±0.07	2.45±0.15***	1.02±0.07$^{###}$	1.07±0.13$^{###}$	0.72±0.02$^{###}$

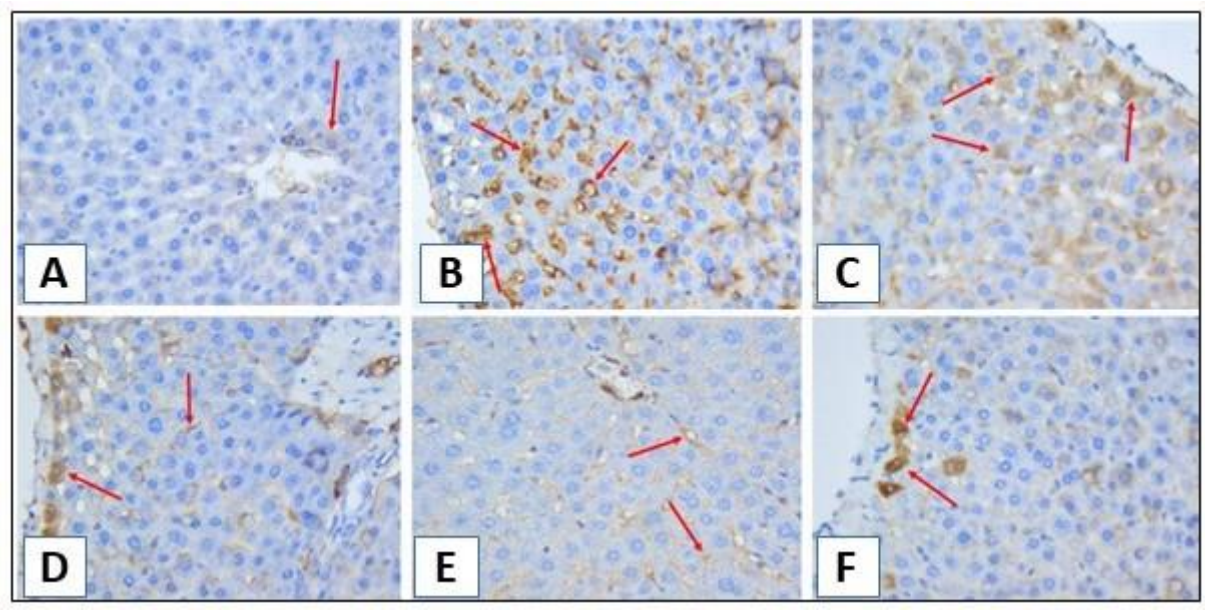

Figure 24. MAC387 expression (red arrows) in the liver of rats from the control group (A), MS group (B and C), MS+AMFJ$_{2.5}$ (D), MS+AMFJ$_5$ (E) and MS+AMFJ$_{10}$ group (F)

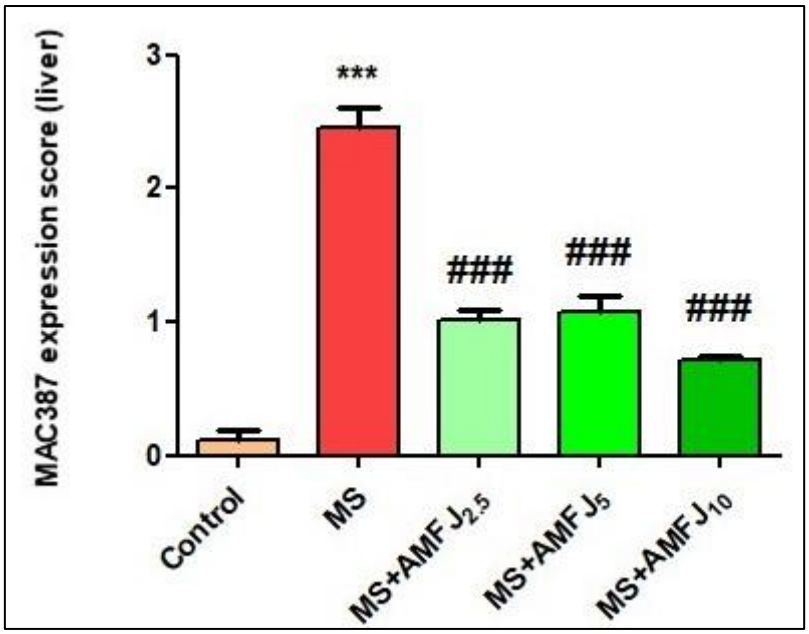

Figure 25. MAC387 expression score in the liver of rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; ***$p < 0.001$ vs. Control group, $^{###}p < 0.001$ vs. MS group

6.3. Discussion

Liver disorders associated with MS are among the most common causes of liver failure and hepatocellular carcinoma worldwide. The increasing frequency of NAFLD correlates directly with the rising incidence of obesity and MS. NAFLD is considered a hepatic manifestation of MS. In Europe, the incidence of NAFLD varies between 26% and 30%, in Asia - 11.8-30%, in USA - about 39% (Goyal et al., 2020). Epidemiological data indicate that this condition is among the second leading cause of liver transplantation in the United States. Despite the slow progression, given the huge number of people affected, the health and economic consequences are significant (Golabi et al., 2018). These data prove the need for early diagnosis and timely measures for its prevention.

From an etiopathogenetic point of view, NAFLD is a multifactorial condition, with IR playing a central role in its development. IR correlates with the degree of abdominal obesity. Visceral adipose tissue acts as a source of secretion of adipocytokines, reducing the metabolic effects of insulin. These events result in the inability to suppress lipolysis in adipose tissue, release CMC in the bloodstream and their accumulation in hepatocytes, where lipogenesis is suppressed. In addition to IR, other factors such as oxidative stress, low-grade inflammation, mitochondrial dysfunction, and intestinal dysbiosis are involved in its pathophysiology. Lipid

overload of the liver leads to the production of ROS, which cause DNA, cell membranes and proteins damage on the one hand, and mitochondrial damage and dysfunction on the other. The first effect directly damages hepatocytes. Mitochondrial dysfunction manifests as increased production of free radicals, cytokines (TNF-α, FAS-ligand) and stimulation of the internal (mitochondrial) apoptotic pathway mediated by BAX (Rinaldi et al., 2021). On the other hand, the cytokines produced induce a low-grade state and activate the external (death-receptor) proapoptotic pathway. Thus, both apoptotic pathways are involved in the pathogenesis of NALFD (Alkhouri et al., 2011). Intestinal dysbiosis and disturbance of the intestinal-liver axis amplify low-grade inflammation and insulin resistance (Rinaldi et al., 2021).

NAFLD includes a range of diseases such as hepatic steatosis, steatohepatitis, liver fibrosis or cirrhosis (Goyal et al., 2020). Histologically it manifests as steatosis, with / without lobular / portal inflammation, balloon degeneration of hepatocytes, fibrosis with varying degrees of distribution or cirrhosis. Like other chronic liver diseases, patients are at higher risk of developing hepatocellular carcinoma and other primary liver tumors (Kleiner and Makhlouf, 2016).

In the current study, to assess the hepatic status of the experimental rats, histological sections from the liver were examined and immunohistochemical staining of the same tissues was further performed to determine the degree of programmed cell death and the inflammatory response. In the histological samples of the animals from MS group, degenerative and necrotic changes in the hepatocytes, inflammatory infiltrate, as well as steatosis were detected. These data are consistent with available publications on hepatic manifestations in MS. In the groups treated with AMFJ, improvement in the histological picture of the liver samples was detected, and this effect was dose-dependent. These results are consistent with the findings described by other authors regarding the effect of Aronia melanocarpa on the liver. Valcheva-Kuzmanova et al. investigated the effect of 5, 10 and 20 ml/kg AMFJ in a model of CCl4-induced acute hepatotoxicity. The application of the juice reduced the transaminase (ALT, AST) activity, dose-dependently reduced the level of MDA in the liver, improved the hepatic histopathological changes and prevented the reduction of hepatic glutathione (Valcheva-Kuzmanova et al., 2004). In another study, where AMFJ was administered prior to induction of hepatotoxicity by CCl4, these results were confirmed (Valcheva-Kuzmanova et al., 2006). The effect was even comparable to the hepatoprotective agent sylimarin (Kondeva-Burdina et al., 2015). The hepatoprotective effects of Aronia

melanocarpa are attributed to the high content of antioxidant PPs capable of inactivating ROS. In addition, the role of Nrf2 (nuclear factor erythroid 2-like 2) in hepatoprotection has been demonstrated in a model of chronic alcohol-induced hepatotoxicity. Activation of this factor suppresses key pro-inflammatory pathways and reduces free radical levels (Wang Z et al., 2020). The same factor is known to modulate the activity of genes that regulate lipid metabolism and reduce lipid accumulation in the liver (Chambel et al., 2015). Thus, the induction of the Nrf2 signaling pathway (including by AMFJ) is an important pathway for influencing NAFLD. In another study, in a model of rat liver fibrosis, although no anti-fibrotic effect was found, a beneficial effect of AMFJ on liver histology (reduction in vacuolated hepatocytes and lymphocytic infiltration in the liver) was confirmed (Piotrowska et-al., 2020).

In the current experiment, programmed cell death was assessed by examining the apoptotic marker Bax and the anti-apoptotic marker Bcl-2 and calculating their ratio (Bax/Bcl-2). The expression of Bax was significantly higher in MS group compared to Control group. Treatment with AMFJ dose-dependently reduced Bax expression in all three groups. The expression of Bcl-2 tended to decrease in MS group compared to the Control group. AMFJ treatment increased the expression of this marker in all three groups, with a significant effect reported for the second treated group (MS+AMFJ$_5$). The Bax/Bcl-2 ratio, reflecting the susceptibility of cells to programmed cell death, was significantly increased in MS group compared to all other groups and this finding indicated the active hepatocyte apoptosis in MS. AMFJ administration significantly reduced this ratio in all three treated groups. These results are consistent with the anti-apoptotic effects of a number of phenolic compounds and polyphenolic extracts with a content close to the juice used in this study. For example, flavanol-rich and phenolic acid-rich Hibiscus sabdarifa L.l extract prevented paracetamol-induced hepatic steatosis by stimulating cellular antioxidant defenses, improving the mitochondrial function, and reducing Bax level. A dose-dependent reduction in steatosis has been reported (Lee CH et al., 2012). The hepatoprotective effect of curcumin is mainly due to the suppression of apoptosis and this effect was demonstrated in a study from 2016, which found that it down-regulated Bax (Fadda et al., 2017).

MAC387 (macrophage marker antibody 387) is a macrophage immunohistochemical marker that differentiates local tissue macrophages from those that have migrated from the bloodstream (Subimerb et al., 2010). Our results show that in the MS group there is a pronounced reactive inflammation, as expressed by the significantly higher number of

MAC387 (+) cells. This proinflammatory response was suppressed by the administration of AMFJ at all three doses, where marker expression (and therefore macrophage infiltration in the liver) was significantly suppressed. These data confirm the presence of hepatic pathology in MS and once again the anti-inflammatory effects of Aronia melanocarpa, but for the first time a hepatoprotective effect of fruit juice is established in a model of metabolic syndrome. These data could be used for the use of this product in clinical practice as a therapeutic functional food in this hepatic complication of MS.

7. Effect of *Aronia melanocarpa* fruit juice on carrageenan-induced paw edema

7.1. Results

The results are shown in **Table 21** and **22** and **Figure 26**. Subplantar carrageenan injection induced acute inflammation of the left hind paw of rats from all experimental groups. In the MS group, the edema increased gradually to 0.87 ± 0.09 ml at the fifth hour. Significant reduction in the edema was achieved with a dose of 5 ml/kg AMFJ on the second (37% edema inhibition) and third hour after injection (41% edema inhibition) ($p < 0.05$ compared to the MS group) and 10 ml/kg on the third hour (44% inhibition of edema) ($p < 0.05$ compared to the MS group). Despite the registered 54% and 49% inhibition of edema in the first hour in the MS+AMFJ$_{2.5}$ and MS+AMFJ$_{10}$ groups compared to group MS, there is no significance in the statistical analysis of the absolute values of the edema volume in these groups.

Table 21. Left hind paw eedema (ml) on the 30th minute as well as on the 1st, 2nd, 3rd, 4th and 5th hour after carrageenan injection to rats with diet-induced MS, treated with doses of 2.5, 5.0 and 10 ml/kg; $^{\#}p < 0.05$ vs. MS group

Group	**Paw edema – 30th minute**	**Paw edema – 1st hour**	**Paw edema – – 2nd hour**	**Paw edema – – 3rd hour**	**Paw edema – 4th hour**	**Paw edema – 5th hour**
MS	0.16 ± 0.07	0.37 ± 0.08	0.57 ± 0.07	0.57 ± 0.07	0.75 ± 0.10	0.87 ± 0.09
MS+AMFJ$_{2.5}$	0.12 ± 0.05	0.17 ± 0.06	0.53 ± 0.04	0.65 ± 0.04	0.78 ± 0.06	0.70 ± 0.12
MS+AMFJ$_{5}$	0.20 ± 0.01	0.24 ± 0.03	0.36 ± 0.03$^{\#}$	0.40 ± 0.06$^{\#}$	0.62 ± 0.08	0.70 ± 0.09
MS+AMFJ$_{10}$	0.14 ± 0.06	0.19 ± 0.06	0.41 ± 0.06	0.40 ± 0.10$^{\#}$	0.56 ± 0.09	0.73 ± 0.07

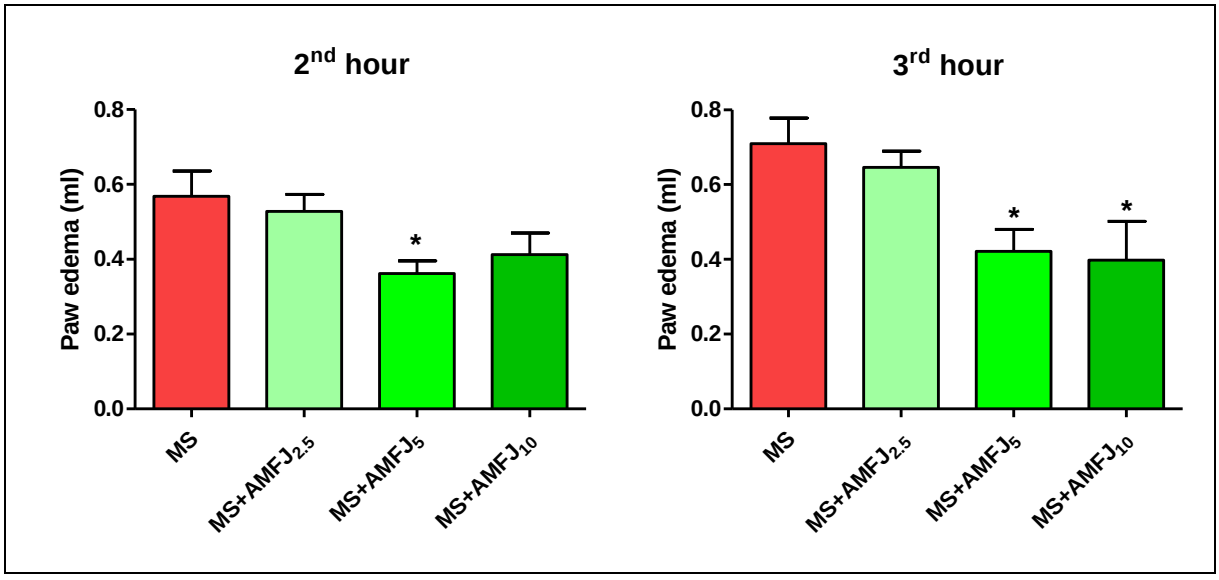

Figure 26. Left hind paw edema (ml) on the 2nd (left) and 3rd (right) hour after carrageenan injection to rats with diet-induced MS, treated with AMFJ in doses of 2.5, 5.0 and 10 ml/kg; #p < 0.05 vs. MS group

Table 22. Paw edema inhibition (%) in rats with diet-induced MS, treated with AMFJ at doses of 2.5, 5.0 and 10.0 ml/kg

	Paw edema inhibition – 30th minute	**Paw edema inhibition – 1st hour**	**Paw edema inhibition – 2nd hour**	**Paw edema inhibition – 3rd hour**	**Paw edema inhibition – 4th hour**	**Paw edema – 5th hour**
MS+AMFJ2.5	Absent	54%	7%	8%	9%	19.5%
MS+AMFJ5	25%	35%	37%	41%	18.5%	19.5%
MS+AMFJ10	12.5%	49%	28%	44%	18.5%	16%

7.2. Discussion

Metabolic syndrome is considered as a low-grade inflammatory state. This is associated with the visceral adipose tissue which produces pro-inflammatory mediators such as CRP, IL-1 and IL-6. (Eckel et al., 2005).

Carrageenan is a mucopolysaccharide extract isolated from the British pharmacist Stanford in 1862 (Morris, 2003). Carrageenan-induced paw edema is a commonly used model of acute inflammation. It consists of 2 phases: first phase (1–2 h after carrageenan injection), characterized by an increased release of serotonin, bradykinin, histamine from mast cells and second phase (3–6 h after carrageenan injection), characterized by neutrophil infiltration, production of reactive oxygen species (ROS), as well as release of arachidonate metabolites

such as prostaglandins, leukotrienes and cytokine release (IL-1β, IL-6, IL-10, and TNF-α) (Vinegar et al., 1987; Crunkhorn and Meacock, 1971).

Aronia melanocarpa fruits are among the plant sources with the highest content of polyphenolic ingredients. Currently there are no data about the effects of Aronia melanocarpa fruit juice on carrageenan-induced edema in an experimental model of MS. This study examined the anti-inflammatory potential of polyphenol-rich AMFJ on carrageenan-induced paw inflammation in rats with MS. AMFJ caused a suppression in the acute inflammatory response during the six time intervals, having its highest values on the 2nd (in MS+AMFJ5) and 3rd hour (in MS+AMFJ10 group) after carrageenan injection.

The effect on the 2nd hour might be attributed to the ability of AMFJ to antagonize the effects of histamine, serotonin, and bradykinins. Such an effect of AMFJ has been well documented in a model of histamine-induced and serotonin-induced rat paw inflammation (Borissova et al., 1994). In this study, the anti-inflammatory effect remained high during the 3rd hour and was less pronounced thereafter. During the second phase of carrageenan-induced inflammation, AMFJ antagonized the effect of arachidonate metabolites and various cytokines. Such activities of Aronia PPs have been demonstrated in other studies. In a mouse model of sodium dextran sulfate-induced ulcerative colitis, chokeberry extract administered orally inhibited prostaglandin E2 production and reduced nitric oxide, IL-6, and TNF-α levels in lipopolysaccharide-stimulated macrophages (Kang SH et al., 2017). In a rat model of amiodarone-induced pulmonary toxicity, AMFJ administration decreased IL-6 (Valcheva-Kuzmanova et al., 2014). Dry Aronia melanocarpa extract (containing at least 25% anthocyanins) was found to inhibit markers of inflammation (IL-1β, TNF-α) and lipid peroxidation (malondialdehyde, MDA) in lipopolysaccharide-stimulated RAW 264 cells (Banach et al., 2020). Similarly, in a model of MS in rats induced by fructose-rich diet, Aronia extract supplementation resulted in a decrease of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and increase of adiponectin (Qin and Anderson, 2012). Athletes, supplemented with chokeberry juice, showed a decrease in the level of TNF-α and higher total antioxidant capacity (Qin and Anderson, 2012). Black chokeberry supplementation in patients after myocardial infarction resulted in a decrease in the level of monocyte-chemoattractant protein-1 (MCP-1), C-reactive protein (CRP), IL-6, ICAM and VCAM and an increase of the level of the anti-inflammatory adiponectin (Naruszewicz et al., 2007). Cyanidin 3-arabinoside has pronounced antioxidant activity due to blockade of pro-oxidant enzymes such as 15-lipoxygenase and xanthine oxidase (Bräunlich et al., 2013). In the juice we used, this

anthocyanin was present in a relatively high concentration, which probably contributed to the observed acute anti-inflammatory effect of AMFJ.

As described, carrageenan-induced paw inflammation is associated with oxidative stress. Based on that fact, we could assume that substances having an antioxidant effect would be beneficial. There are number of studies which demonstrate the antioxidant activity of Aronia melanocarpa polyphenols. Valcheva-Kuzmanova et al. found that AMFJ and its polyphenolic substances have catalase-like and superoxide dismutase-like effects and radical scavenging activity (Valcheva-Kuzmanova et al., 2007; Valcheva-Kuzmanova et al., 2012). The juice used in this experiment was demonstrated to possess a high oxygen radical absorbance capacity (ORAC) and hydroxyl radical averting capacity (HORAC) in vitro (Valcheva-Kuzmanova et al., 2014). Polyphenol-rich Aronia melanocarpa extracts significantly and dose-dependently inhibited the superoxide radical formation in patients at high cardiovascular risk (arterial hypertension, hypercholesterolemia, smoking and diabetes mellitus) (Ryszawa et al., 2006). In a model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, AMFJ improved the macroscopic and microscopic signs of colitis and prevented the increase in the concentrations of thiobarbirturic acid reactive substances (TBARS; marker for oxidative stress) in the colon (Valcheva-Kuzmanova et al., 2018). In a study of Coiocoiu et al. the antioxidant activity of polyphenol-rich Aronia melanocarpa extract was examined in a L-NAME-induced experimental model of arterial hypertension. A significant increase in the glutathione-peroxidase activity, total antioxidant capacity as well as a significant decrease in the serum level of MDA was observed in Aronia-treated groups (Ciocoiu et al., 2013).

Therefore, we could assume that the observed effects of AMFJ in a model of acute carrageenan-induced inflammation are very likely due to the antioxidant and anti-inflammatory effects of the phenolic compounds contained in the juice.

V. CONCLUSIONS

1. Administration of high-fat, high-fructose diet to male Wistar rats for 10 weeks induced metabolic syndrome. The following changes were observed in the experimental animals:

1.1. Behavioral changes occured: spatial memory was deteriorated, anxiety developed.

1.2. Biochemical abnormalities, characteristic of metabolic syndrome, were observed – elevated serum glucose and triglycerides.

1.3. Biochemical changes, reflecting the development of oxidative stress, were observed - compensatory increase in serum SOD activity.

1.4. Visceral obesity developed – a clinical sign of metabolic syndrome.

1.5. Histologically:

1.5.1. Adipocyte hypertrophy was observed in the adipose tissue.

1.5.2. Inflammatory (lymphocytic infiltration) and degenerative changes, steatosis and hepatocyte necrosis ocurred in the liver.

1.5.3. Degenerative changes, expressed as distancing of cardiomyocytes, were observed in the myocardium.

1.5.4. In the coronary blood vessels, activation or necrosis of endothelial cells developed and the basement membrane was exposed.

1.6. Immunohistochemically:

1.6.1. In adipose tissue, the expressions of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 were increased. The Bax/Bcl-2 ratio was also elevated, a sign of a predominant apoptotic phenotype.

1.6.2. In the liver, Bax expression was diffuse and significant and Bcl-2 expression was reduced. MS increased the Bax/Bcl-2 ratio in the hepatocytes and induced a pro-apoptotic phenotype. The expression of pro-inflammatory

MAC387 was significant and was observed both in the hepatocytes and sinusoidal cells – a sign of inflammatory activity.

1.7. Subplantar administration of 1 mg carrageenan in rats with metabolis syndrome induced acute inflammation and increased the volume of the left hind paw.

2. In the experimental animals, oral administration of *Aronia melanocarpa* fruit juice in three different doses (2.5 ml/kg, 5.0 ml/kg and 10 ml/kg) resulted in an improvement of a significant proportion of the behavioral, biochemical, histological and immunohistochemical abnormalities associated with metabolic syndrome:

2.1. The spatial memory was improved at all doses and this effect was most pronounced at the dose of 5.0 ml/kg.

2.2. Metabolic syndrome-induced anxiety was reduced and this effect was most pronounced at the dose of 2.5 ml/kg.

2.3. The blood glucose was decresead at the 30^{th} and 60^{th} minute during glucose-tolerance test to initial (control) values at doses of 2.5 and 5.0 ml/kg

2.4. Serum triglycerides were reduced dose-dependently in all treated groups.

2.5. The oxidative stress was decreased through reduction of the compensatory elevated serum SOD activity.

2.6. The retroperitoneal and total adipose tissue indices were reduced, with the effect being most pronounced at doses of 2.5 and 5.0 ml/kg for the first index and at a dose of 2.5 ml/kg for the second one.

2.7. Histological changes associated with metabolic syndrome were improved at all doses:

2.7.1. In the adipose tissue, the adipocyte size was reduced to a level similar to that of control rats.

2.7.2. In the myocardium, the cardiomyocyte structure was restored.

2.7.3. In the coronary blood vessels, the endothelium and the basement membrane were preserved.

2.7.4. In the liver, the inflammatory infiltrate, the degenerative and steatotic changes were reduced.

2.8. Most of the metabolic syndrome-induced immunohistochemical changes were improved:

2.8.1. In the adipose tissue:

2.8.1.1. Bax expression was reduced and this effect was most pronounced at the dose of 2.5 ml/kg.

2.8.1.2. Bcl-2 expression was increased significantly at doses of 2.5 and 5.0 ml/kg and reduced significantly at the dose of 10 ml/kg.

2.8.1.3. The Bax/Bcl-2 ratio decreased at the lower doses (2.5 and 5.0 ml/kg) and increased at 10 ml/kg.

2.8.2. In the liver:

2.8.2.1. Bax expression decreased significantly at all doses.

2.8.2.2. Bcl-2 expression increased at all doses and significant effect was observed at a dose of 5.0 ml/kg.

2.8.2.3. Bax/Bcl-2 was reduced significantly at all doses.

2.8.2.4. The expression of MAC387 decreased significantly at all doses.

2.9. AMFJ administration at all doses and time intervals decreased the left hind paw edema of the rats and this effect was significant at the 2^{nd} hour at the dose of 5.0 ml/kg and at the 3^{rd} hour at doses of 5.0 and 10.0 ml/kg.

VI. CONTRIBUTIONS

1. For the first time the behavioral effects of *Aronia melanocarpa* fruit juice in rats with an experimental model of diet-induced metabolic syndrome were studied and the following findings were established:

1.1. Anxiolytic-like effect;

1.2. Improvement of the spatial memory.

2. For the first time the metabolic effects of *Aronia melanocarpa* fruit juice in rats with an experimental model of diet-induced metabolic syndrome were studied and the following findings were demonstrated:

2.1. Glucose-lowering effect.

2.2. Antihypertriglyceridemic effect.

3. For the first time it was found that *Aronia melanocarpa* fruit juice suppressed oxidative stress in rats with an experimental model of diet-induced metabolic syndrome.

5. For the first time the protective effects of *Aronia melanocarpa* fruit juice against the histological alterations in the adipose tissue, myocardium, coronary vessels and the liver were demonstrated in rats with an experimental model of diet-induced metabolic syndrome.

6. For the first time the effects of *Aronia melanocarpa* fruit juice on the programmed cell death in the adipose tissue and in the liver of rats with an experimental model of diet-induced metabolic syndrome were studied and the following findings were established:

6.1. The juice suppressed the programmed cell death in the adipose tissue at doses of 2.5 and 5.0 ml/kg. For the first time it was found that *Aronia melanocarpa* fruit juice at the highest dose (10 ml/kg) could induce adipocyte apoptosis. This requires more in-depth studies on the effect of juice on adipocyte apoptosis at different doses and with different durations of treatment in order to identify the possible consequences of such an effect.

6.2. The juice suppressed hepatocyte apoptosis at all doses.

7. For the first time an anti-inflammatory effect of *Aronia melanocarpa* fruit juice after carrageenan-induced hind paw edema was demonstrated in rats with an experimental model of diet-induced metabolic syndrome.

VII. LIST OF PUBLICATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION

1. List of publications related to the dissertation

1.**Abtulov M**, Valcheva-Kuzmanova S. Beneficial effects of polyphenols in metabolic syndrome – a review. Scripta Scientifica Medica 2021; 53(3): 9-20.

2.**Abtulov M**, Kuzmanova V, Kuzmanov A, Todorov S, Pavlov D, Kuzmanov K, Todorova M, Eftimov M, Gancheva S, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Effect of Aronia melanocarpa fruit juice on carrageenan-induced paw edema in metabolic syndrome rats. Scripta Scientifica Medica 2021; 53(3): 31-36.

3.**Abtulov M**, Gancheva S, Todorova M, Eftimov M, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Effect of Aronia melanocarpa fruit juice on the antioxidant defense system in rats with diet-induced metabolic syndrome. Scripta Scientifica Medica 2021; 53(4): 47-53.

4.**Reyzov M**, Eftimov M, Gancheva S, Todorova M, Zhelyazkova-Savova M, Tsaneva M, Valcheva-Kuzmanova S. Effect of Aronia melanocarpa fruit juice on glucose tolerance, lipid metabolism and obesity in a rat model of metabolic syndrome. Acta Alimentaria 2022 **(IF 2020: 0.650)** [In print]

2. List of participations related to the dissertation

1.Todorova M, Eftimov M, Gancheva S, **Reyzov M**, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Behavioral effects of the polyphenol-rich Aronia melanocarpa fruit jucie in rats with diet-induced metabolic syndrome. 32nd ECNP Congress 7-10 September 2019, Copenhagen, Denmark.

2.**Reyzov M**, Todorova M, Gancheva S, Eftimov M, Valcheva-Kuzmanova S, Zhelyazkova-Savova M. Diet-induced metabolic syndrome in rats is associated with anxiety and impairment of spatial memory. Jubilee Scientific Conference "45 years Medical university – Pleven" 31 October – 02 November 2019, Pleven, Bulgaria.

3.Цанева М, **Абтулов М**, Ефтимов М, Ганчева С, Тодорова М, Желязкова-Савова М, Вълчева-Кузманова С. Плодов сок от Aronia melanocarpa подобрява хистопатологичните промени в черен дроб в експериментален модел на метаболитен синдром. XIII национален конгрес по патология, 10-12 септември 2021, Бургас, България. Сборник резюмета: стр. 55-56.

4.**Abtulov M**, Gancheva S, Todorova M, Eftimov M, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Effect of polyphenol-rich Aronia melanocarpa fruit juice on antioxidant defense system in rats with diet-induced metabolic syndrome. Seventh Pharmaceutical Business Forum and Scientific and Practical Conference "Digital solutions and innovation in pharmaceutical practice and education – challenges and opportunities", 22-23 October 2021, Varna, Bulgaria. Scripta Scientifica Pharmaceutica 2021; 8(1): 46.

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