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PHARMACOLOGICAL INVESTIGATION OF THE EFFECTS OF CHAENOMELES MAULEI FRUIT JUICE IN AN EXPERIMENTAL MODEL OF METABOLIC SYNDROME

THESIS SUMMARY

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Contents

ABBREVIATIONS

AGEs – advanced glycation end products

BMI – body mass index

EPM – elevated plus maze

FOXO1 – forkhead box protein O1

FST – forced swim test

GAE – gallic acid equivalents

GLUT4 – glucose transporter protein type-4

HDL – high density lipoproteins

HOMA-IR – homeostasis model assessment of insulin resistance

IRS-1 – insulin receptor substrate-1

LDL – low density lipoproteins

LOX – lipoxygenase

MCP-1 – monocyte chemoattractant protein-1

miRNAs – microRNAs

MMP – matrix metalloproteinase

NFkB – nuclear factor kappa B

OFT – open field test

OLT – object location test

PAMPs – pathogen-associated molecular patterns

PCSK9 – proprotein convertase subtilisin/kexin type 9

PEPCK – phosphoenolpyruvate carboxykinase

PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1 alpha

PTP1B – protein tyrosine phosphatase 1B

RAGE – receptor for advanced glycation end products

SIT – social interaction test

SREBP-1c – sterol regulatory element-binding protein 1c

TBARS – thiobarbituric acid reactive substances

TIMP – tissue inhibitor of metalloproteinase

TLRs – toll-like receptors

TNF-α - tumor necrosis factor alpha

VLDL – very low density lipoproteins

I. Introduction

Thousands of years before establishing the science the way we know it today medicinal plants took a major part in prevention and therapy of numerous diseases. Empirical knowledge about their beneficial properties gradually gets confirmation through present laboratory and diagnostic approaches. Development of methods such as high-performance liquid chromatography and liquid chromatography-mass spectrometry significantly expands the horizon of phytochemical studies (Marelli, 2021). Despite the advancement in synthetic methods for drug molecule creation, medicinal plants retain their relevance as a source of drugs that is with a better safety profile and evokes greater trust among the community.

Accumulated data demonstrating polyphenols` useful effects in the prevention of non-communicable diseases and promising results from large epidemiological studies highlight the necessity of conducting additional research on their influence in the context of metabolic syndrome.

In a population where average life expectancy increases, which is the tendency worldwide in the last decades, the importance of non-communicable diseases is also on the rise. An impressive part of them go under the umbrella of metabolic syndrome or are associated with it.

Around 3000 plants of economic and medical value, including apple, strawberry, raspberry, pear, peach, plum, almond, cherry, etc. belong to the family of Rosaceae (Soundararajan et al., 2019). Representatives of the genus *Chaenomeles* are less widely known species of the family that are highly cherished due to decorative and medicinal properties. Parts of the Japanese quince plant have been used for therapeutic purpose for thousands of years in the traditional eastern medicine. Only a few of the biological effects described have already got scientific proof. Nowadays the interest in this climate-resilient and economically viable species and its potential benefits for human health rapidly increases.

The position of fruit juice in the healthy eating recommendations is a trending topic and a controversial issue. Caution is due to the juice being a major source of sugars which in the absence of fibers present in the whole fruit lead to a rapid increase in blood glucose. On the other hand, regular consumption of fruit juice examined in clinical studies with short to medium duration, seems to be a dietary intervention that benefits vascular function, blood pressure and

cognition (Ruxton et al., 2021). A systematic review and meta-analysis of the relation between daily intake of fruit juice and metabolic syndrome demonstrates a U-shaped association, highlighting the protective role of moderate consumption (Ruxton et al., 2021). In a prospective cohort study with 34 560 participants included, drinking up to 7 glasses of juice weekly is associated with significantly reduced risk of cardiovascular diseases, including heart failure, and drinking up to 8 glasses weekly – with reduced risk of stroke (Sheffers et al., 2019). Food consumption is considered a modifiable determinant of cognitive health. Drinking 100 % fruit juice is associated with a 16-19 % decrease in the occurrence of anxiety (Agarwal et al., 2022).

The current study aims to evaluate the biologic effects of Chaenomeles maulei fruit juice in an experimental model of metabolic syndrome and to explore the potential for prophylactic and therapeutic use of the juice.

II. Objective and tasks

Considering the previously established effects of the plants from genus *Chaenomeles*, as well as the investigated activities of the polyphenols that are a major component in their content, we focused on the following **objective**:

Pharmacological evaluation of the effects of Chaenomeles maulei fruit juice in an experimental diet-induced model of metabolic syndrome in animals.

Tasks:

1. To accomplish an experimental model of metabolic syndrome in rats using a high-fat high-fructose diet and to register the presence or absence of the following criteria of metabolic syndrome:

    1.1. Visceral obesity

    1.2. Dyslipidemia

    1.3. Impaired glucose tolerance

2. To evaluate the effects of *Chaenomeles maulei* fruit juice in this experimental model on the following markers:

    2.1. Visceral obesity

    2.2. Biochemical markers of energy metabolism:

        2.2.1. Serum lipids

        2.2.2. Glucose regulation

    2.3. Biochemical markers of antioxidant defence and oxidative stress:

        2.3.1. Superoxide dismuthase

        2.3.2. Thiobarbituric acid reactive substances

    2.4. Behavioural indices of:

        2.4.1. Locomotor activity

2.4.2. Anxiety

2.4.3. Spatial memory

2.4.4. Depressive condition

2.5. Histological changes in:

2.5.1. Myocardium and coronary arteries

2.5.2. Liver

2.5.3. Adipose tissue

III. Materials and methods

1. Materials

1.1. Experimental animals

Adult male Wistar rats with an average beginning weight 270±30 g were used in the experiments. The animals were housed in plastic cages with 5 rats each, located in a well ventilated area at room temperature of 22±1°C. They were subjected to a 12-hour light/dark cycle and consumed food and water ad libitum.

All procedures regarding the experimental animals were conducted in accordance with EU directives and approved by the Bulgarian food safety agency (Document № 177/07.07.2017).

1.2. *Chaenomeles maulei* fruit juice

Chaenomeles maulei fruit juice (CMFJ) in three increasing doses was used for the purposes of the study. It had been produced from fresh ripe handpicked fruits that were crushed, squeezed and filtered after that. The juice was preserved with potassium sorbate (1.0 g/l) and stored at 0°C until the time of the experiment.

The polyphenolic content of CMFJ was established by Valcheva-Kuzmanova et al. (2018) and is graphically presented on **Figure 1**. The total amount of polyphenols was estimated as gallic acid equivalents and was found to be 890 mg/100 ml. Proanthocyanidines were 253.29 mg/100 ml. Procyanidine oligomers were determined as equivalents of catechin. Among phenolic acids the ones with highest concentrations were vanillic, caffeic and chlorogenic, followed by neochlorogenic, p-coumaric, ellagic, ferulic and 2,4-dihydroxybenzoic acid. Among flavonoids epicatechin, catechin, quercetin-3-β-glucoside, quercetin and rutin dominated and naringin, kaempferol and myricetin were found in smaller concentrations. Organic acids were also determined (mg/100ml): malic (3647.0), quinic (1034.0), citric (51.0), shikimic (30.0), ascorbic (22.0), oxalic (17.0). Carbohydrates in the juice are presented by glucose (1713.0), fructose (1237.0), galactose (320.0), sucrose (189.0), xylose (35.0), rhamnose (18.0), arabinose (8.0).

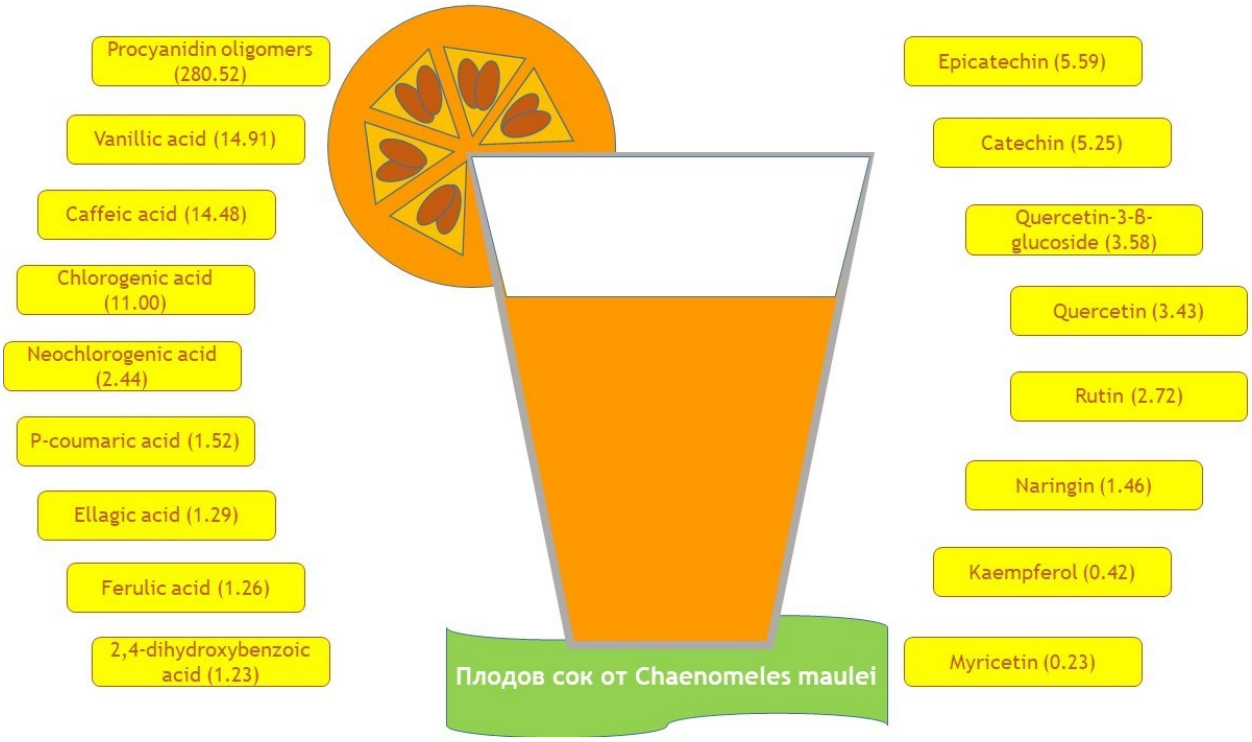


Figure 1. Polyphenolic content of *Chaenomeles maulei* fruit juice as established by Valcheva-Kuzmanova et al. (2018)

The antioxidant activity of CMFJ was also estimated to be 18167.8 ± 938.8 μmol gallic acid equivalents per liter by the hydroxyl radical averting capacity assay and 84401.4±1934.2 μmol Trollox equivalents per liter by the oxygen radical absorbance capacity assay (Valcheva-Kuzmanova et al., 2018).

2. Models and methods

2.1. Experimental model of metabolic syndrome

50 adult male Wistar rats bred in the Vivarium of Medical University – Varna were divided into 5 groups of 10 animals each: Control, MS, MS+CMFJ2.5, MS+CMFJ5 and MS+CMFJ10. For ten weeks, the animals from the Control group received standard laboratory diet and tap water ad libitum, while the other 4 groups were subjected to a high-fat high-fructose diet – 17% lard and 17% fructose added to the standard diet – and 10% fructose solution instead of drinking water in order to induce metabolic syndrome. Given the aforementioned diets, animals from group Control received 279 kkal/100 g food while those on high-fat high-fructose diet – 405 kkal/100 g

food and 40 kkal/100 ml fructose solution. All animals were daily treated with a flexible orogastral tube. Groups Control and MS received destilled water, while the remaining three – CMFJ in increasing dose (2.5 ml/kg, 5 ml/kg and 10 ml/kg, respectively). The choice of doses was made on the basis of the experience of Borisova-Nenova, Eftimov and Valcheva-Kuzmanova (2016, 2017, 2018, 2019, 2023) where the same doses prove their safety and produce biologic activities in healthy rats. The amount of food and liquids consumed by each group was measured on a daily basis. Animals weight was measured once per week.

2.2. Tissue and organ fractionation

2.2.1. Blood serum preparation

At the end of the 10^{th} week of the experiment, animals were anesthetized with diethyl ether and blood was taken from their sublingual veins. The blood tubes were centrifuged at 2000 rpm for 10 minutes. The separated serum was stored at a temperature of -20 ºC until the moment of biochemical tests conduction.

2.2.2. Organ isolation for a histopathological examination

Tissue samples from heart, liver and visceral adipose tissue were fixed in 10 % neutral buffered formalin.

2.3. Biochemical tests

2.3.1. Triglyceride level determination

The levels of triglycerides were evaluated in blood serum by using the kits of BioMaxima S.A., Poland, with all producer`s instructions being strictly followed. The method is based on hydrolysis of triglycerides into glycerol and fatty acids by the enzyme lipoprotein lipase. In result of the following phosphorylation of glycerol with ATP by the glycerol kinase, glycerol-3-phosphate and ADP are produced. The glycerol-3-phosphate is oxidised to dihydroxyacetone phosphate and hydrogen peroxide that upon binding to 4-chlorophenol and 4-aminoantipyrin leads to the production of a coloured complex. Colour intensity is photometrically measured at 500 nm wavelength. Spectrophotometer AURIUS 2021 (Cecil Instruments Ltd., UK) was used.

2.3.2. Total cholesterol determination

Total cholesterol levels were evaluated in blood serum by using the kits of BioMaxima S.A., Poland, with all producer`s instructions being strictly followed. The method is based on hydrolysis of cholesterol esthers to cholesterol and free fatty acids by the enzyme cholesterol esterase. In result of the following oxidation of free cholesterol, hydrogen peroxide is released. It binds to phenol and 4-aminoantipyrin and leads to the production of a coloured complex. Colour intensity is photometrically measured at 500 nm wavelength. Spectrophotometer AURIUS 2021 (Cecil Instruments Ltd., UK) was used.

2.3.3. Glucose levels determination

For the purpose of the intraperitoneal glucose tolerance test (GTT), after a 12-hour period of fasting, the experimental animals were intraperitoneally injected with 40 % glucose solution at a dose of 2 g/kg. Measuring the blood glucose was done with ACCU-CHEK Performa glucomer and ACCU-CHEK Performa test strips. An incision of the distal end of the tail of each animal was performed by the method of Fluttert et al. (2000) immediately before the glucose injection as well as on the 30^{th}, 60^{th} and 90^{th} minute afterwards in order to collect the blood sample.

2.3.4. Superoxide dismutase levels determination

For the superoxide dismutase levels determination, an ELISA kit (Boster Bio, Pleasanton, CA, USA) was used. The method is based on using a tetrazolium salt for the detection of a superoxide radical. The superoxide dismutase catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. The concentration of the enzyme that is necessary for 50 % dismutation of the superoxide radical is defined as one unit of superoxide dismutase. Results are read using spectrophotometer AURIUS 2021 (Cecil Instruments Ltd., UK).

2.3.5. Thiobarbituric acid reactive substances levels determination

For the thiobarbituric acid reactive substances levels determination 0.8% thiobarbituric acid solution was added to the serum. Samples were incubated in a water bath at 95 °C for 2 hours and after that were removed from the water bath and left to cool down at room temperature (Ohkawa et al., 1979). Malondialdehyde was used as a standart. The optical density of the

samples was measured by AURIUS 2021 spectrophotometer (Cecil Instruments Ltd., UK). TBARS are estimated as nmol/ml.

2.4. Adipose tissue indices estimation

Retroperitoneal, mesenterial, paranephral and perigonadal adipose tissues were dissected. Each of the fat depots was separately weighted. For the total fat tissue as well as for each of the fat depots, an index was measured using the following formula:

$$\text{Adipose tissue index} = \frac{\text{Adipose tissue weight}}{\text{Total body weight}} x100$$

2.5. TyG index determination

The triglyceride glucose index is an innovative marker for measuring insuline resistance. It was determined using the following formula:

$$\text{TyG} = \text{Ln}\frac{\text{Fasting triglycerides } \left(\frac{\text{mg}}{\text{dl}}\right) \text{x Fasting glucose } (\frac{\text{mg}}{\text{dl}})}{2}$$

2.6. Liver index determination

Liver index was determined using the following formula:

$$\text{Liver index} = \frac{\text{Liver weight}}{\text{Total body weight}} x100$$

2.7. Histology

The formalin-fixed tissue samples from heart, liver and visceral adipose tissue were embedded in paraffin blocks. 5 μm thick slices were stained with hematoxylin and eosin and examined by light microscopy.

2.8. Behavioral methods

2.8.1. Method of determining the locomotor activity – Open field test (OFT)

The open field test apparatus consists of a wooden arena (100x100 cm) coloured in white with walls that are 40 cm high (Hall et al., 1932). The floor of the arena is divided by blue lines into

25 squares of equal size. Each rat is individually placed in the center of the arena and its behaviour is observed for 5 minutes. The number of horizontal movements (number of squares that are crossed with all 4 paws of the animal), the number of vertical movements (standing up on the hind paws), the time spent in the central area (the inner nine squares of the arena) and the number of entries in the central area (when all 4 paws of the animal have crossed the line). After each test session the arena is thoroughly cleaned with 95 % ethanol and left to dry so that the smell of the previous animal is removed and an alteration of the spontaneous behaviour of the next one is prevented. The number of horizontal and vertical movements stand for the locomotor activity of the rodents, while the time spent in the central area of the apparatus as well as the number of entries in it can be interpreted as a marker of the anxiety level.

2.8.2. Methods of determining the anxiety level

2.8.2.1. Elevated plus maze test (EPM)

The elevated plus maze apparatus (Handley and Mithani, 1984) consists of two open and two closed arms, connected by a central platform. The labyrinth is elevated at a 50 cm height from the floor. Each rat is individually placed on the central platform facing one of the open arms. Its behaviour is observed for 5 minutes. The number of entries in the open arms, the time, spent in the open arms, the number of entries in the closed arms and the time spent in the closed arms are registered. The ratio of number of entries in the open arms versus the number of entries in any of the arms and the ratio of time spent in the open arms versus time spent in any of the arms are registered.

2.8.2.2. Social interaction test

The test is conducted in the OFT arena (File and Hyde, 1978). Two rats with similar body weight and with the same treatment, but bred in different plastic cages and not familiar to each other, are simultaneously placed in the opposite corners of the field and their behaviour is observed for five minutes. The time of active social interaction, which includes following, sniffing, wrestling, jumping over or going under the other animal, is registered. Passive stay near the other animal is not registered. The time spent in active social interaction is interpreted as an inverse marker of the anxiety level of the rodents.

2.8.3. Method of spatial memory evaluation – Object location test (OLT)

The object location test is conducted in an arena with the following dimensions: 65x45x40 cm. The experimental setup is presented in a schematic way on **Figure 2**. The test consists of two sessions with a 30-minute interval between them. Two objects (A and B) are placed in the arena. They are with the same shape, volume and colour and are firmly attached to the arena so that it is not possible for the animals to move them. During the first session the rat is allowed to freely explore the arena and the objects for a period of 3 minutes. During the second session (also lasting 3 minutes) one of the objects (B) is located in a different place on the arena. The time spent exploring the relocated (B) object as well as the time spent exploring the object with unchanged place (A) are registered. The discrimination index B/(A+B) is calculated.

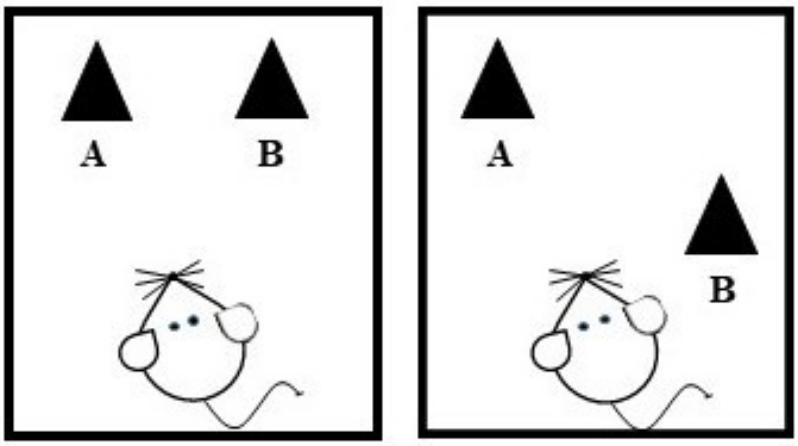


Figure 2. Experimental setup for the object location test and spatial memory evaluation

Higher values of the discrimination index are interpreted as an improvement of the spatial memory.

2.8.4. Method of determining the depressive-like behavior – Forced swim test (FST)

The forced swim test, known also as Porsolt`s test (Porsolt et al., 1977) is conducted in a glass cylinder with a diameter of 17 cm and a height of 50 cm. The cylinder is filled with warm (30°C) water up to the 30^{th} cm which is enough so that the animal is not allowed to reach the bottom with its hind paws or tail and is forced to swim. Two sessions are conducted on two consecutive days with a 24 hour interval between them. Each rat is individually placed in the cylinder for a period of 5 minutes and immobility time during the session is registered. It is interpreted as a

marker of hopelessness/desperation and corresponds to the presence of depressive-like behaviour.

2.9. Statistical methods

Statistical analysis is conducted with GraphPad Prism Software 5.00. Data is presented as Mean±SEM. One-way analysis of variance (ANOVA) with Dunnet`s Multiple Comparison Post Test and student`s t-test were used. Values of $p<0.05$ are considered statistically significant.

IV. Results and discussion

1. Effects of *Chaenomeles maulei* fruit juice on energy metabolism, total body weight and visceral obesity

1.1. Effects of *Chaenomeles maulei* fruit juice on energy metabolism

Biological parameters related to the energy metabolism are presented on **Table 1** and **Figure 3**. Animals from the groups that were on a high-fat high-fructose diet consumed a smaller amount of food compared to group Control ($p<0.001$). One-way ANOVA analysis showed a difference between group MS and the treated groups ($p<0.001$) and Dunnet`s multiple comparison post test revealed that CMFJ intake at doses 5 ml/kg and 10 ml/kg increased the amount of food consumed. Liquids intake was significantly higher in the groups that were on a high-fat high-fructose diet ($p<0.001$) as compared to group Control. One-way ANOVA analysis showed a difference between group MS and the treated groups ($p<0.05$) and Dunnet`s multiple comparison post test revealed that CMFJ intake at a dose of 5 ml/kg reduced the liquids intake. The total caloric intake in all groups on the high-fat high-fructose diet was higher ($p<0.001$) than that in group Control. One-way ANOVA analysis showed a difference between group MS and the treated groups ($p<0.001$) and Dunnet`s multiple comparison post test revealed that CMFJ intake at doses 5 ml/kg and 10 ml/kg increased the caloric intake.

Table 1. Biological parameters regarding energy metabolism in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. ***$p<0.001$ compared to group Control, ##$p<0.01$, #$p<0.05$ compared to group MS.

	Control	**MS**	**MS+CMFJ 2.5**	**MS+CMFJ 5**	**MS+CMFJ 10**
Food intake (g / day / rat)	21.12±0.17	14.24±0.17***	14.14±0.20***	15.15±0.17***##	14.92±0.18***#

Liquids intake (ml / day / rat)	30.9±0.35	45.46±0.69***	45.76±0.79***	42.51±0.91***#	43.98±0.73***
Total caloric intake (kkal/ day / rat)	58.91±0.48	75.87±0.74***	75.45±0.85***	78.16±0.66***##	77.78±0.69***#
Body weight at the beginning of the experiment (g)	266.8±8.08	262.3±5.56	267.8±5.56	267.2±5.88	266.0±5.43
Body weight at the end of the experiment (g)	334.2±8.21	344.0±7.38	330.4± 9.00	336.0± 8.41	341.2± 10.61

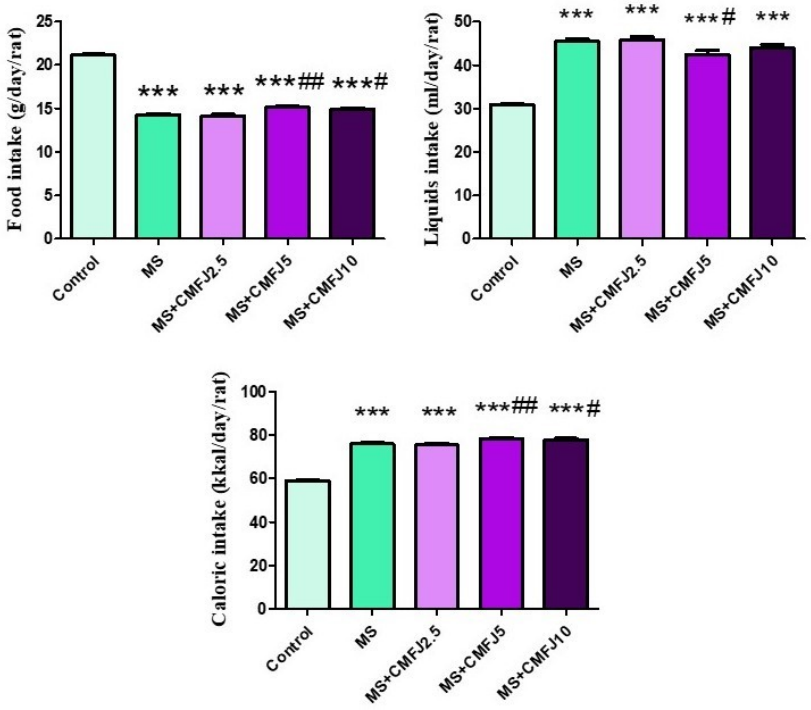


Figure 3. Food, liquids and caloric intake in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. ***p<0.001 compared to group Control, ##p<0.01, #p<0.05 compared to group MS

1.2. Effects of *Chaenomeles maulei* fruit juice on total body weight

Total body weight gain is shown in **Figure 4**, and body weight gain per weeks for each experimental group – on **Figure 5**. Body weights of the animals at the beginning and at the end of the experiment are shown on **Table 1**. Group MS demonstrated only a slight increase in body weight gain compared to group Control. In groups MS+CMFJ2.5 and MS+CMFJ5 there was a non significant decrease in this parameter, while in group MS+CMFJ10 body weight gain was comparable to that in group MS.

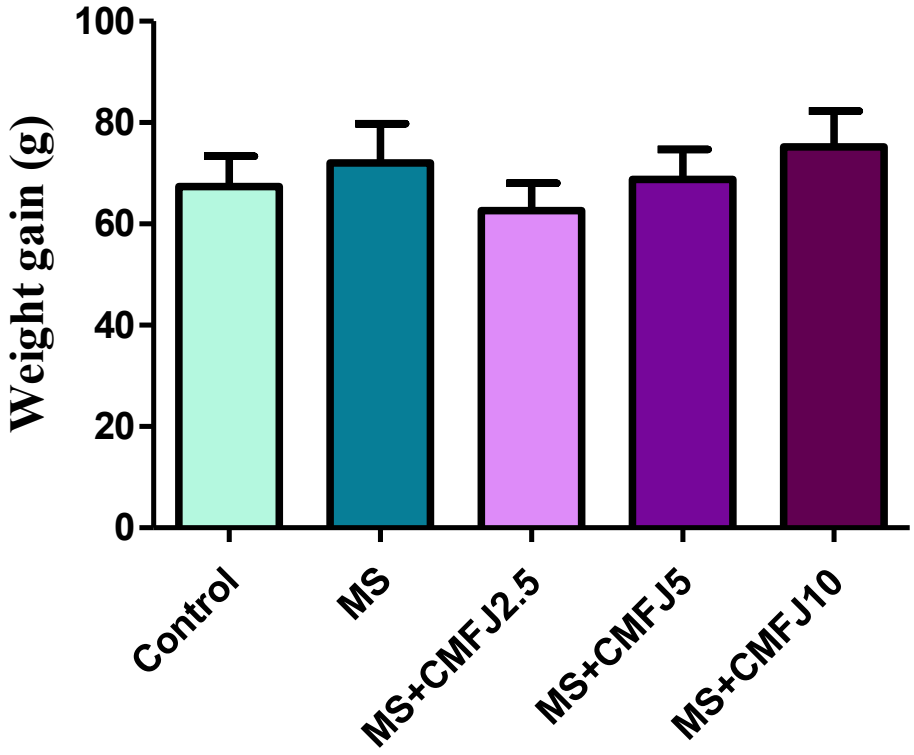


Figure 4. Total weight gain in each group for all of the 10 weeks of the experiment in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

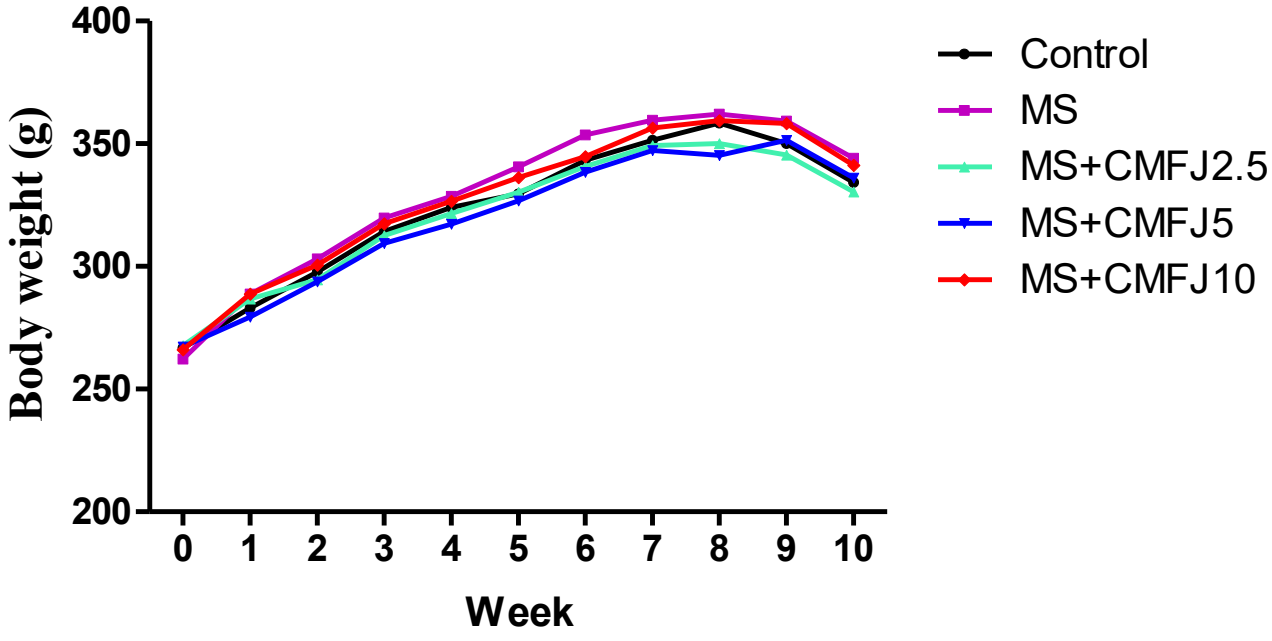


Figure 5. Change in body weight during the 10 weeks of the experiment in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

1.3. Effects of *Chaenomeles maulei* fruit juice on visceral obesity

The weight of total, mesenterial, paranephral, perigonadal and retroperitoneal adipose tissues as well as their respective estimated indexes were significantly increased in group MS in comparison to group Control, which signifies the development of visceral obesity as a consequence of the high-fat high-fructose diet. Total fat tissue index was significantly increased in groups MS, MS+CMFJ2.5 and MS+CMFJ10 compared to the Control, while its values in MS+CMFJ5 remained similar to these of the Control. Dunnet`s post test showed a statistically significant ($p<0.05$) decrease in total fat tissue index in group MS+CMFJ5 in comparison to group MS, as shown in **Figure 6**. One-way ANOVA demonstrated an effect from CMFJ intake ($p=0.0339$) with Dunnet`s post test showing a significant decrease in the mesenterial adipose tissue index of group MS+CMFJ5 in comparison to MS, as depicted in **Figure 7**. The paranephral adipose tissue index was significantly decreased in MS+CMFJ5 compared to MS. The values of the remaining two indices – of the retroperitoneal and the perigonadal adipose

tissues – were also decreased in MS+CMFJ5 compared to MS, although difference did not reach statistical significance, and were similar to these of group Control.

Table 2. Total, mesenterial, retroperitoneal, paranephral and perigonadal adipose tissue indices in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control, #p<0.05 compared to group MS

Group	**Control**	**MS**	**MS+CMFJ 2.5**	**MS+CMFJ 5**	**MS+CMFJ 10**
Total adipose tissue index	2.87±0.22	4.77±0.16***	4.32±0.38**	3.62±0.30#	4.60±0.34**
Mesenterial adipose tissue index	0.63±0.05	1.06±0.06***	0.88±0.06**	0.76±0.09#	0.97±0.06***
Retroperitoneal adipose tissue index	1.01±0.11	1.95±0.09***	1.77±0.16**	1.50±0.16	1.94±0.18***
Paranephral adipose tissue index	0.20±0.02	0.35±0.03**	0.32±0.04*	0.24±0.03#	0.32±0.02*
Perigonadal adipose tissue index	1.02±0.07	1.41±0.08*	1.34±0.15	1.12±0.06	1.38±0.12

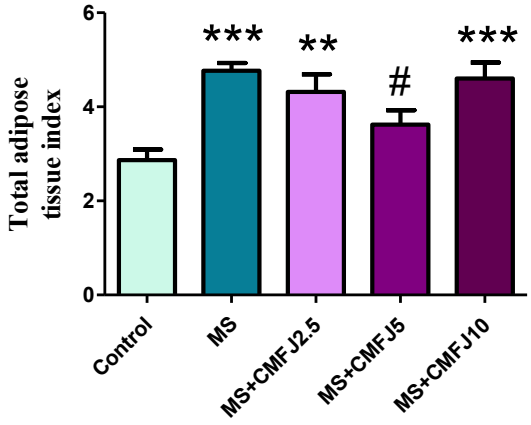


Figure 6. Total adipose tissue index in rats with diet-induced MS, treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control, #p<0.05 compared to group MS

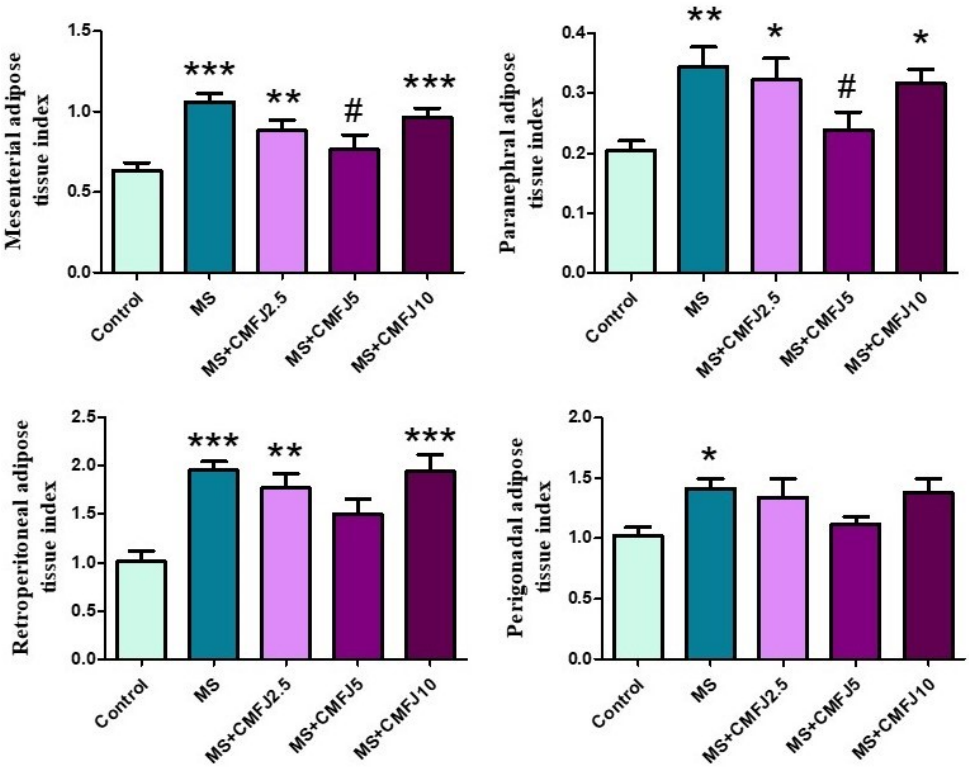


Figure 7. Mesenterial, paranephral, retroperitoneal and perigonadal adipose tissue indices in rats with diet-induced MS, treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control, #p<0.05 compared to group MS

1.4. Discussion

Obesity in people is a natural consequence of reduced physical activity and increased caloric intake combined with genetic factors. In the current study, total body weight of the animals is only slightly increased by the high-fat high-fructose diet, which is a common finding in diet-induced experimental models of MS and resembles clinical experience in humans – visceral adiposity, but not the amount of total adipose tissue – is a more specific marker for the presence of MS. Some authors that observe limited increase in total body weight but significant increase in visceral adipose tissue in experimental models also describe loss of skeletal muscle tissue in the animals (Rodríguez-Correa et al., 2020).

Adipose tissue distribution is considered a cornerstone in the pathogenesis of MS. A longitudinal cohort study with 1964 patients included finds out that while visceral adipose tissue is certainly associated with an increased risk of MS, subcutaneous adipose tissue might even possess a protective effect (Kwon et al., 2017). This is one of the possible explanations why obesity, measured in body mass index (BMI), does not always directly correlate to the metabolic status – around 30% of obese people are metabolically normal, while 5-45 % of people with BMI in the reference range manifest the same metabolic disturbances that are typical for obese patients (Bremer et al., 2012). In the current study, total as well as mesenterial and paranephral adipose tissues were influenced by CMFJ intake – the medium dose of 5 ml/kg significantly reduces them. It has been observed that mesenterial fat tissue that is drained into the portal circulation is more metabolically active in comparison with other fat depots that are drained elsewhere (Liu et al., 2006). In a clinical study the thickness of the mesenterial fat tissue turns out to be an independent determinant of MS and is associated with increased intima-media thickness ratio in carotid vessels (Liu et al., 2006). Mesenterial adipose tissue is considered a possible prognostic factor for the development of nonalcoholic fatty liver disease as well as polycystic ovaries (Perelas et al., 2012) – both of these conditions being narrowly intertwined with MS. A significant correlation is observed between mesenterial adipose tissue and atherogenic LDL apoB particles, as well as apoAII levels, the latter being relevant to the role of mesenterial adipose tissue as a source of triglycerides in the fasting state (Perelas et al., 2012). The prospect of exogenous manipulation of mesenterial fat tissue could be studied in the setting of Crohn`s disease, where the typical phenomenon “creeping fat” originates from mesenterial adipose tissue

depots and is confirmed to be an important player in intestinal inflammation and a possible driver of disease progression. Targeting pharmacologically the mesenterial adipose tissue is also considered a possible approach towards MS (Tchkonia et al., 2013).

Despite the existence of the obese phenotype without metabolic disturbances, it should be noted that such people also have a cardiovascular risk that is higher than that of their leaner counterparts (Aires et al., 2019). Data is consistent that the loss of visceral fat tissue leads to a number of beneficial consequences for the body. In a clinical trial with 117 overweight or obese patients included, systemic inflammation was significantly reduced following visceral adipose tissue reduction (Castro-Barquero et al., 2023). Another clinical trial shows that visceral adipose tissue loss correlates to a higher extent with improvement of indices such as fasting blood glucose, triglycerides and HOMA-index than does subcutaneous fat tissue loss (Park and Lee, 2005). Another study with 172 adolescents with obesity included, considers visceral fat tissue reduction to be an independent predictor of ameliorating insuline resistance, hyperleptinemia and other metabolic disturbances (Campos et al., 2019). Based on these data, the observed reduction of visceral adipose tissue by CMFJ suggests that it could be a potentially appropriate diet intervention in patients with MS.

Central effects of polyphenols on the feeling of satiety and on the regulation of food intake have been described in literature. Some of these effects are probably typical for the combination of polyphenols in the CMFJ content since the amount of food consumed and the total caloric intake are significantly higher in groups MS+CMFJ5 and MS+CMFJ10. The liquids intake, fructose solution in particular, is decreased in group MS+CMFJ5. The decrease in the visceral adipose tissue in group MS+CMFJ5 is observed even in light of an increased energy intake.

The antiobesity effects of polyphenols are well studied. In an experimental model of MS in rats on cafeteria diet for 16 weeks, adding biscuits enriched with hesperidin and naringenin to the diet of the animals leads to reduction of visceral adipose tissue and of total body weight as well as to improvement of the markers of blood pressure, insuline resistance, lipid profile and oxidative stress (Mayneris-Perxachs et al., 2019). One of the described mechanisms is the browning of the white adipose tissue. In a double-blind placebo-controlled clinical trial with healthy women, Nirengi et al (2016) find out that the intake of catechin-rich drink for 12 weeks significantly increases brown adipose tissue.

2. Effects of *Chaenomeles maulei* fruit juice on the lipid profile

2.1. Effects of *Chaenomeles maulei* fruit juice on the triglycerides levels

One-way ANOVA analysis shows that in group MS the serum triglycerides levels were significantly increased ($p<0.001$) in comparison to the Control group (1.23±0.15 compared to 0.67±0.04). Subjecting the animals to high-fat high-fructose diet led to almost doubling the values compared to the rats receiving the standart laboratory diet. CMFJ intake reduced, but not significantly, the triglycerides levels and these changes were most pronounced in group MS+CMFJ5 (0.98±0.07 compared to 1.23±0.15 in group MS). Results are shown on **Table 3** and **Figure 8**.

Table 3. Serum triglycerides and total cholesterol levels (mmol/l) in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control.

Group	**Triglycerides (mmol/l)**	**Total cholesterol (mmol/l)**
Control	0.67±0.04	1.77±0.11
MS	1.23±0.15***	2.00±0.22
MS+CMFJ2.5	1.09±0.06**	2.01±0.11
MS+CMFJ5	0.98±0.07*	1.77±0.11
MS+CMFJ10	1.09±0.09**	1.77±0.08

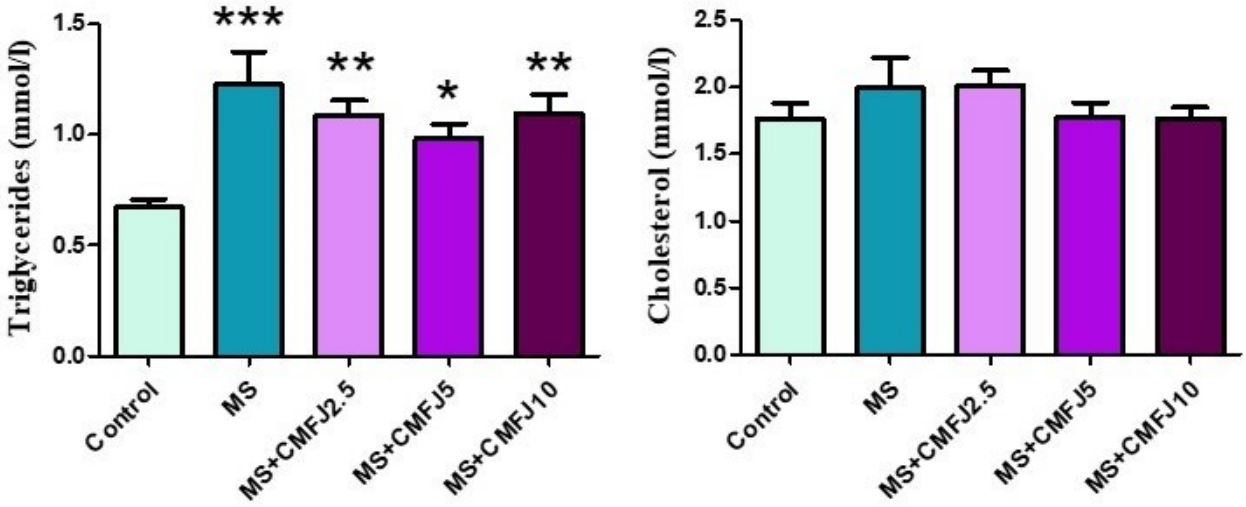


Figure 8. Serum triglycerides and total cholesterol levels (mmol/l) in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control

2.2. Effects of Chaenomeles maulei fruit juice on total cholesterol levels

As shown on **Table 3** and **Figure 8**, total cholesterol levels in group MS are increased (2.00±0.22) compared to group Control (1.77±0.11) without reaching statistical significance of the result. CMFJ at doses 5 and 10 ml/kg tends to prevent the increase in cholesterol (1.77±0.11 and 1.77±0.08, respectively) and preserve the values seen in the Control group, while CMFJ at the lowest dose used – 2.5 ml/kg – does not alter the value (2.01±0.11) in comparison to group MS.

2.3. Discussion

Dyslipidemia is a main component of MS and is associated with many pathological conditions. It is a major risk factor for the development of atherosclerotic disease, ischemic heart disease, ischemic stroke, peripheral vascular disease, heart failure and sudden cardiac death (Rygiel, 2018). Cardiovascular diseases due to atherosclerosis and thrombosis are the most common reason for premature morbidity, mortality and disability worldwide (Rygiel, 2018). Increased levels of oxidative stress and systemic inflammation in the context of MS are important factors potentiating the development of dyslipidemia (Khongrum et al., 2022).

It is not a coincidence that fructose is a carbohydrate often preferred for use in experimental models of obesity and MS. Its metabolism leads to the production of unregulated amounts of lipogenic substrates that are directly delivered to the mitochondria, stimulate the de novo lipogenesis in the liver, the intrahepatic lipid accumulation and steatosis development as well as hepatic insulin resistance (Bremer et al., 2012). A long lasting postprandial dyslipidemia is observed while at the same time the expected supression of the "hunger hormone" ghrelin that has orexigenic function is lacking (Bremer et al., 2012, Galderisi et al., 2019). The lack of supression of ghrelin is even more pronounced in obesity than in normal body weight which signifies that the obesogenic effect of fructose is enhanced in the context of MS (Galderisi et al., 2019). Leptin secretion in response to the fructose intake is also low, therefore it is unable to sufficiently inhibit the feeling of hunger as expected by the calories ingested (Basciano et al., 2005). Fructose does not directly stimulate insulin secretion since there are no GLUT-5 transporters on the pancreatic beta cells (Galderisi et al., 2019). Clinical data also confirms the development of dyslipidemia in response to the overconsumption of fructose (Faeh et al., 2005). In the diet that was used in the present study a significant part of the daily caloric intake is provided by fructose. The treatment with CMFJ reduced but not significantly the triglyceride levels with the biggest effect seen in group MS+CMFJ5. In the group receiving the highest dose of CMFJ – 10 ml/kg – there was an increase in the triglyceride levels compared to group MS+CMFJ5, although they remained lower than those observed in group MS. The small triglyceride reduction was probably caused by the polyphenolic content of the juice, while the raise seen in group MS+CMFJ10 in comparison to MS+CMFJ5 could probably be associated with the higher concentration of fructose in the higher dose of the juice used. The total cholesterol levels did not vary significantly between the experimental groups although there was a small increase in groups MS and MS+CMFJ2.5. Data is consistent with the prevailing scientific literature – since LDL levels in MS are usually increased and those of HDL – decreased – the total cholesterol levels often remain largely unchanged. Lipid-lowering effects are reported for many of the polyphenols found in CMFJ (Cheung et al., 2023) and improvement of the lipid profile was observed in some clinical trials testing the activities of fruit juices with similar content. In an 8-week double blind placebo-controlled study including adult people with dyslipidemia, the consumption of jelly-like fruit drink with a high polyphenolic content leads to a significant improvement of the lipid profile, including the levels of HDL and triglycerides

(Khongrum et al., 2022). Regardless of that, in the present study the tendency of the juice to decrease both triglycerides and total cholesterol did not succeed in reaching statistical significance. It would be interesting to compare these results with the effects of Chaenomeles maulei whole fruit consumption, where the pectin content is higher and, as it was already discussed, pectin also possesses some lipid-lowering qualities.

3. Effects of Chaenomeles maulei fruit juice on glucose tolerance and on insulin resistance

3.1. Glucose tolerance test (GTT)

Results of the GTT are presented on **Table 4** and **Figure 9**. Fasting blood glucose levels did not differ significantly among the experimental groups (4.46±0.09 in Control, 4.48±0.14 in MS, 4.44±0.09 in MS+CMFJ2.5, 4.38±0.15 in MS+CMFJ5 и 4.34±0.15 in MS+CMFJ10). One way ANOVA analysis of the values on the 30th minute after the glucose load showed significance of the result (p=0.0020) with Dunnet`s Multiple Comparison Test registering a significant difference between groups Control and MS. One-way ANOVA analysis of the values on the 60$^{th}$ minute showed significance of the result (p=0.0260) of the difference between group Control and group MS. Student`s t-test showed a significant increase in the glucose values in the Control group on the 30th (p=0.0109), 60th (p=0.0066) and 90th (p=0.0031) minute, as well as in the values presented as a percentage of the initial values (p<0.05). Treatment with CMFJ did not significantly influence glucose tolerance as compared to group MS in the doses used.

Table 4. Plasma glucose levels during a glucose tolerance test, presented as absolute values (mmol/l) as well as a percentage (%) of the initial values on the 30th, 60th and 90th minute after the glucose load in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05 as compared to Control according to one-way ANOVA, **p<0.01 as compared to Control according to one-way ANOVA, $^{\&}$p<0.05 as compared to Control according to student`s t-test analysis, $^{\&\&}$p<0.01 as compared to Control according to student`s t-test analysis.

		Control	MS	MS+CMFJ 2.5	MS+CMFJ 5	MS+CMFJ 10
0 min (mmol/l)		4.46±0.09	4.48±0.14	4.44±0.09	4.38±0.15	4.34±0.15
30th minute	**(mmol/l)**	11.97±0.78	15.92±1.14**	17.27±0.72***	16.64±1.26**	15.58±0.51*
	(%)	169±17.3	257±27.95**	289±15.62***	280.4±26.77**	261.1±14.34*
60th minute	**(mmol/l)**	8.13±0.26	9.93±0.53$^{\&\&}$	10.82±0.74	9.83±0.63	10.05±0.56
	(%)	83.25±7.341	136±16.5$^{\&\&}$	145±18.46	163±38.68	157.4±27.43

90th minute	**(mmol/l)**	7.41±0.25	8.73±0.28&	8.95±0.35	8.69±1.07	8.34±0.28
	(%)	66.82±6.5	96.23±7.68&	102.6±9.78	100.3±24.67	102±12.56

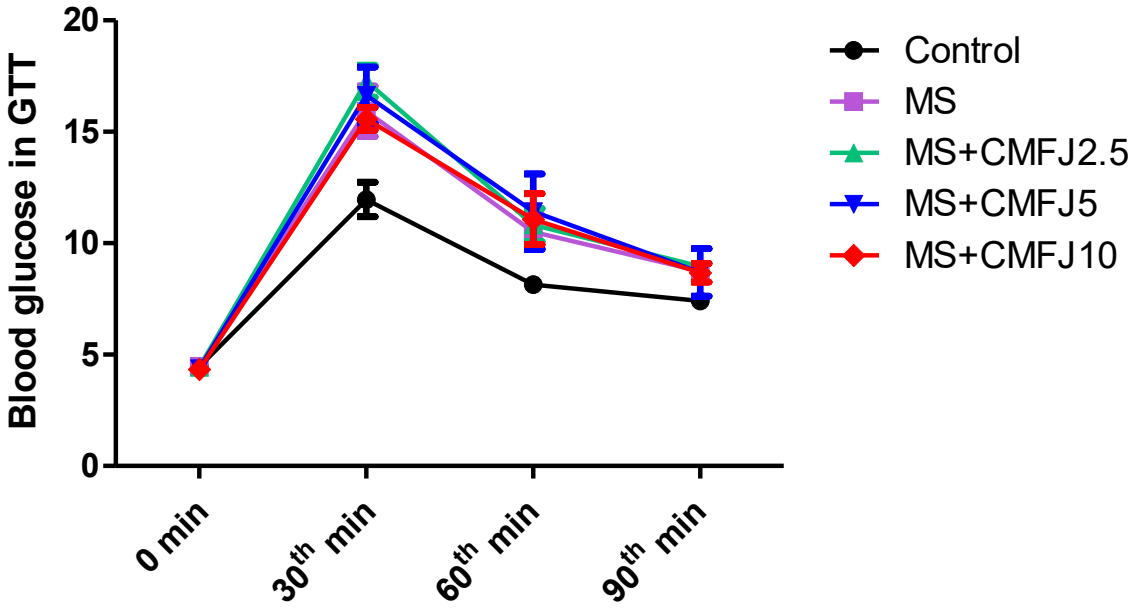


Figure 9. Plasma glucose levels during a glucose tolerance test, presented as absolute values (mmol/l) in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

3.2. TyG index as a marker of insulin resistance

The triglyceride/glucose index (TyG) was significantly increased in group MS compared to group Control (p=0.0002) which suggests the development of insulin resistance. CMFJ intake did not significantly influence the TyG index in the concentrations used. The results are presented on **Table 5** and **Figure 10**.

Table 5. TyG index in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control.

Group	**TyG index**
Control	6.18±0.07
MS	6.80±0.12***
MS+CMFJ2.5	6.65±0.06**
MS+CMFJ5	6.50±0.07*
MS+CMFJ10	6.62±0.11**

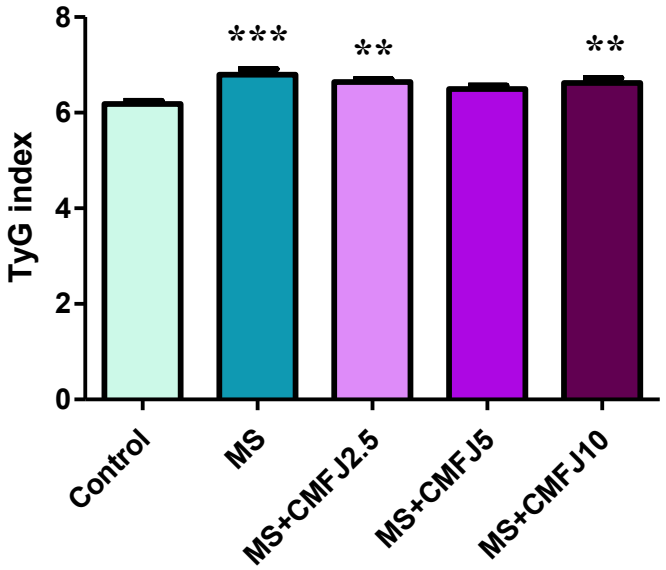


Figure 10. TyG index in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control

3.3. Discussion

In the current study, fasting blood glucose levels were comparable in all experimental groups, while on the 30th, 60th and 90th minute of the GTT they were significantly increased in all groups

on high-fat high-fructose diet. Together with the observed insulin resistance, these changes correspond to the condition of prediabetes. The impairments of glycaemic control that are observed in prediabetes are considered reversible (Tuso, 2014), which highlights the significance of timely diagnosis and the use of appropriate interventions to reduce the risk of progression of the condition to the full range of manifestations of diabetes type 2. It has been estimated that, in the absence of such an intervention, 37 % of patients with prediabetes reach diabetes type 2 in a period of 4 years (Tuso, 2014).

While insulin resistance takes a central place in the pathogenesis of MS, its direct measurement and monitoring turns out to be not that easily accomplished. For this purpose, indirect markers are developed that are widely used in clinical practice. One of the most popular is the HOMA-IR, but the search continues and leads to the use of new markers such as the TyG index. It is an innovative surogate marker for estimation of the level of insulin resistance (Lopez-Jaramillo et al., 2023). In the current study the TyG index is significantly increased in response to the high-fat high-fructose diet, being an evidence for the development of insulin resistance.

A major driver of insulin resistance are the free fatty acids in blood that decrease GLUT-4 translocation to the surface of the muscle cells, promote gluconeogenesis and lipogenesis in the liver and display lipotoxic effect in many organs and tissues, including pancreatic beta cells (Fahed et al., 2022).

Although demonstrated insulin resistance undoubtedly plays a role in the development of decreased glucose tolerance in the GTT, a number of studies point out that decreased sensitivity of the pancreatic beta cells towards glucose is the leading factor in the pathogenesis of this impairment (Ferrannini et al., 2003; Mari et al., 2010). Based on this and the results from the current study, the possibility that the high-fat high-fructose diet may compromise pancreatic beta cell sensitivity towards glucose should be considered. This sensitivity is largely dependent on the glucose transporter GLUT-2 and the enzyme glucokinase – both of them being inhibited by the intake of a high-fat diet (Cerf, 2007).

Clinical data confirms that the high content of fructose in the diet leads to both liver and adipose tissue insulin resistance (Faeh et al., 2005).

The intake of CMFJ did not succeed in preventing the development of impaired glucose tolerance and insulin resistance in the experimental animals in the doses used. A meta-analysis of 18 randomised clinical studies shows that the consumption of 100 % fruit juice does not lead to a change in the glucose levels and in the insulin resistance (Murphy et al., 2017). The studies included consider the use of apple, pomegranate, grapefruit, cranberry and other fruit juices (Murphy et al., 2017). Another study shows that the direct effect of citrus fruits on the postprandial glycemic response is limited, although there is a large interindividual variance, which the authors suggest could be largely due to differences in gut microbiota (Visvanathan and Williamson, 2021).

4. Effects of *Chaenomeles maulei* fruit juice on biochemical markers of antioxidant defense and oxidative stress

4.1. Effects on the serum levels of superoxide dismutase

Results from the serum activity of the antioxidant enzyme superoxide dismutase (SOD) are presented on **Table 6** and **Figure 11**. One-way ANOVA registered a statistically significant difference between the groups (p=0.0173) with Dunnet`s multiple comparison post test showing a significantly lower activity of the enzyme in group MS as well as in the group treated with the highest dose of CMFJ – MS+CMFJ10 – in comparison with group Control. The treatment with CMFJ in doses 2.5 ml/kg and 5 ml/kg prevented the high-fat high-fructose diet-induced reduction of the enzyme activity, illustrated by the fact that there was no statistically significant difference between group Control and groups MS+CMFJ2.5 and MS+CMFJ5. SOD activity in the experimental group receiving the highest dose of CMFJ was reduced to a value similar to that in group MS.

Table 6. Activity of the enzyme superoxide dismutase (SOD) in the serum of rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01 compared to group Control.

Group	**Superoxide dismutase activity (E/ml)**
Control	0.305±0.013
MS	0.233±0.023**
MS+CMFJ2.5	0.262±0.011
MS+CMFJ5	0.262±0.012
MS+CMFJ10	0.238±0.018*

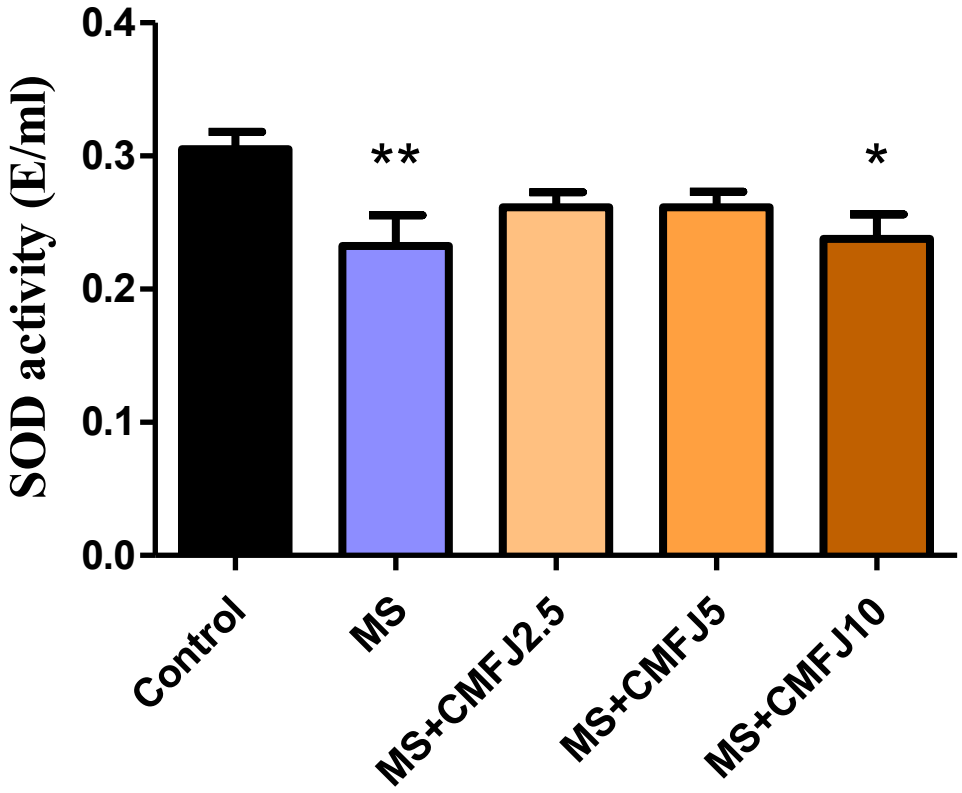


Figure 11. Activity of the enzyme superoxide dismutase (SOD) in the serum of rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01 compared to group Control

4.2. Effects on the serum levels of thiobarbituric acid reactive substances

The values of the thiobarbituric acid reactive substances (TBARS) in the serum of animals from group MS were higher than those of group Control (130.5±21.09 спрямо 85.32±7.73), but without reaching statistical significance of the difference. CMFJ intake caused a linear trend (p=0.0105) directed at a decrease of the TBARS serum levels. In the group treated with the highest dose of the juice – MS+CMFJ10 – they were significantly reduced (p<0.05) compared to those in group MS and were comparable to the values of group Control. Results are expressed on **Table 7** and **Figure 12**.

Table 7. Serum thiobarbituric acid reactive substances (TBARS) in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Group	**Serum TBARS (nmol/ml)**
Control	85.32±7.73
MS	130.5±21.09
MS+CMFJ2.5	113±11.76
MS+CMFJ5	102.2±11.04
MS+CMFJ10	79.07±8.55$^{\#}$

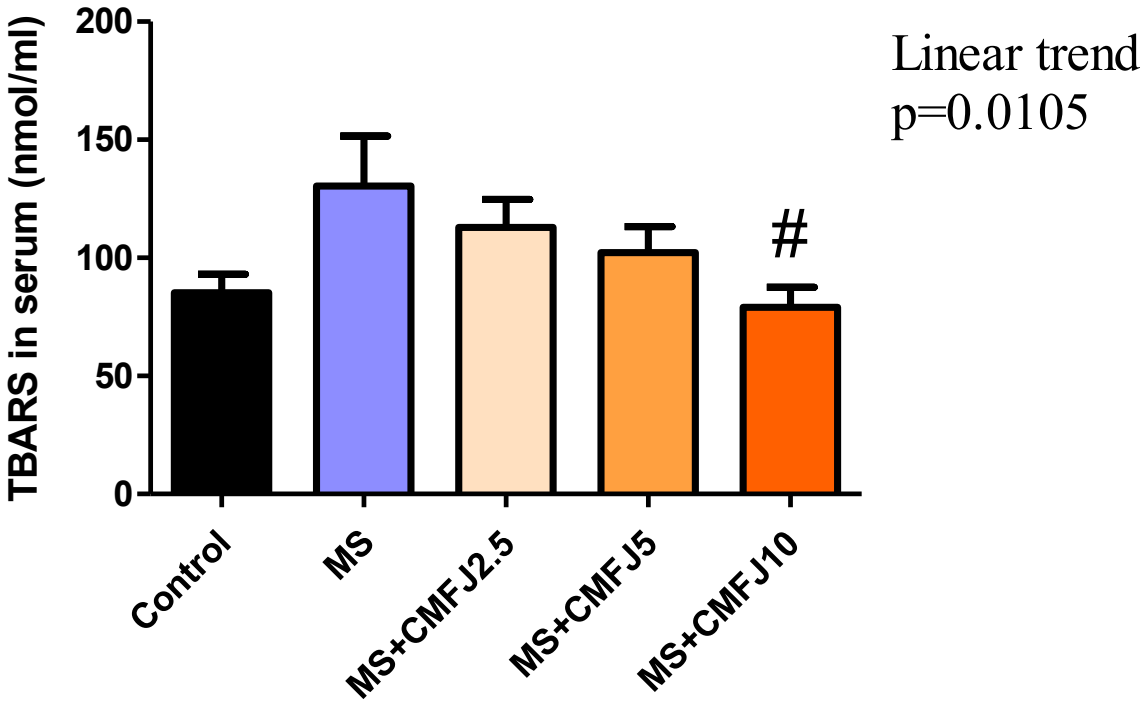


Figure 12. Serum thiobarbituric acid reactive substances (TBARS) (nmol/ml) in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

4.3. Discussion

Not only increased oxidative stress, but also decreased endogenous abilities antioxidant defense mechanisms of the body are a key factor in MS pathogenesis and a linkage with many of its associated conditions. Superoxide dismutases are a family of metaloenzymes that are responsible for the break down of free superoxide radicals. The current study shows a statistically significant decrease in the serum SOD activity in rats with diet-induced MS which is consistent with the prevailing scientific literature. The decreased activity of SOD in the serum is a distinctive feature of patients with obesity and MS (Catalán et al., 2018). MS diminishes the natural antioxidant abilities of the body which is a prerequisite for aggravating the tissue damage induced by the syndrome. In a clinical study with 3234 people included, a negative correlation was was observed between the serum levels of SOD and BMI, systolic and diastolic blood pressure, serum levels of triglycerides and glucose as well as the intima-media thickness of the carotid artery (Isogawa et al., 2009).

In the current study, treatment of the experimental animals with CMFJ at doses 2.5 ml/kg and 5 ml/kg succeeded in preventing the high-fat high-fructose-induced decrease of the activity of the antioxidant enzyme superoxide dismutase. An increase in the SOD, catalase and glutathione activity were also observed after oral treatment with *Chaenomeles sinensis* of rats with inhibited antioxidant defense as a response to diabetes type 2 induction (Watychowicz et al., 2017). Antioxidant properties have been described for other species belonging to the *Chaenomeles* genus as well – *Chaenomeles speciosa* – which improves the activity of the enzyme glutathione peroxidase in an in vivo study (Watychowicz et al., 2017). Similar results have also been described for other fruit juices that are rich in polyphenols. In a 9-week placebo-controlled clinical study in healthy volunteers the activity of SOD was significantly increased following the intake of anthocyanin-rich fruit juice (Bakuradze et al., 2019).

The highest dose of CMFJ used in the current study – 10 ml/kg – did not succeed in preventing the decrease of the antioxidant enzyme activity, induced by the high-fat high-fructose diet. The observed effects of the CMFJ intake on SOD activity correlate with the results of its use on the visceral obesity – in both markers an improvement was noted with the low and medium dose of the juice (in the case of the visceral fat tissue the effect was only significant with the medium dose), while with the highest dose used the values are comparable to those in group MS. This

data could be explained by the prominent interrelationship between obesity and oxidative stress. A possible factor for the lack of antioxidant effect in group MS+CMFJ10 is also the potential prooxidative activities of polyphenols when used in high doses.

Since reactive oxygen species (ROS) are with an extremely short half-life, their direct measurement is difficult and expensive and this is why more practical methods for oxidative stress examination have been developed that rely on registering some end products of their metabolism or other biomarkers (Catalán et al., 2018). Thiobarbituric acid reactive substances (TBARS) evaluation as a marker of the lipid peroxidation in the serum of rats in the current study demonstrates increased values in group MS as compared to group Control. CMFJ intake dose-dependently prevents the lipid peroxidation and the levels of TBARS in the group treated with the highest dose – MS+CMFJ10 – are comparable with those in group Control.

Increased plasma levels of TBARS have been desribed in leptin-deficient mice with obesity, as well as in humans with obesity and with MS (Catalán et al., 2018). In a prospective study with 1945 people included, Tanaka et al. (2011) discover that serum levels of TBARS are a strong and independent predictor of coronary disease, which is in support of the hypothesis that lipid peroxidation is a serious risk factor for the development of coronary artery disease. Serum levels of TBARS are also associated with vascular events incidence, including fatal and non-fatal infarction and stroke and with the need of vascular interventions in patients with a stable coronary disease (Walter et al., 2004).

5. Effects of *Chaenomeles maulei* fruit juice in a histopathological examination of myocardium and coronary arteries

5.1. Results

In the coronary vessel of the experimental animals from group Control, a continuous endothelium lined the vessel wall. In group MS there were focal regions of absent endothelium and the observed endothelial cell were necrotic. The basal membrane was left exposed. In group MS+CMFJ2.5, focal regions of absent endothelium were still found and the endothelial cells were activated. In groups MS+CMFJ5 and MS+CMFJ10 endothelial lining of coronary arteries was preserved. Histological results are presented on **Figure 13**.

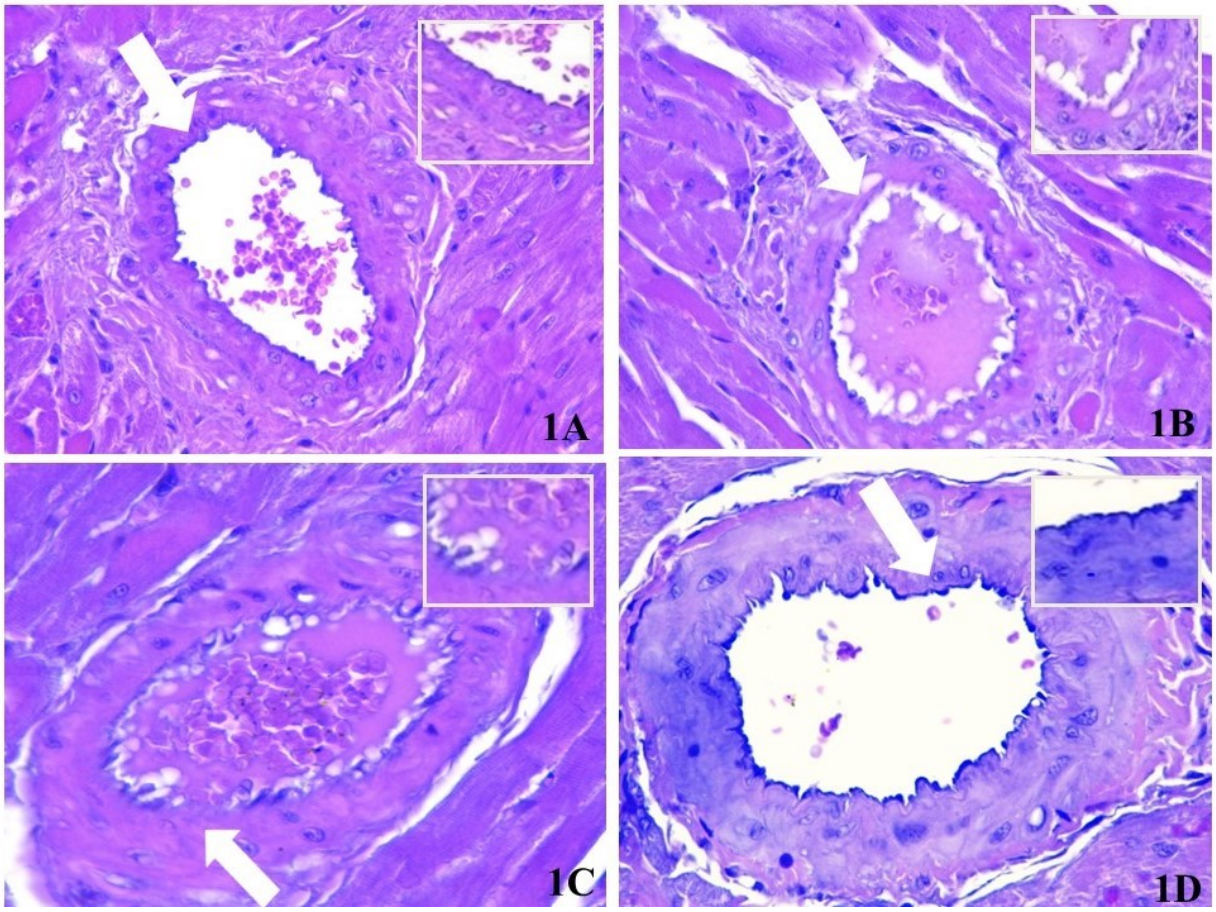


Figure 13. Microscopic appearance of coronary vessels of groups: Control (panel 1A), MS (panel 1B), MS+CMFJ2.5 (panel 1C), and MS+CMFJ10 (panel 1D); Hematoxylin-eosin staining, magnification x 400

Results from the histological evaluation of the myocardium are presented on **Figure 14.** In the animals from group Control, a normal structure of the myocardium was observed. In group MS, there were degenerational changes and increased distance between the cardiomyocytes. In group MS+CMFJ2.5, the impairment was partly prevented – along with zones of degeneration and increased distance between cardiomyocytes, there were also zones of normal structure. In groups MS+CMFJ5 and MS+CMFJ10 the treatment with CMFJ prevented the development of the changes induced by the high-fat high-fructose diet and the myocardium did not show any histological alterations.

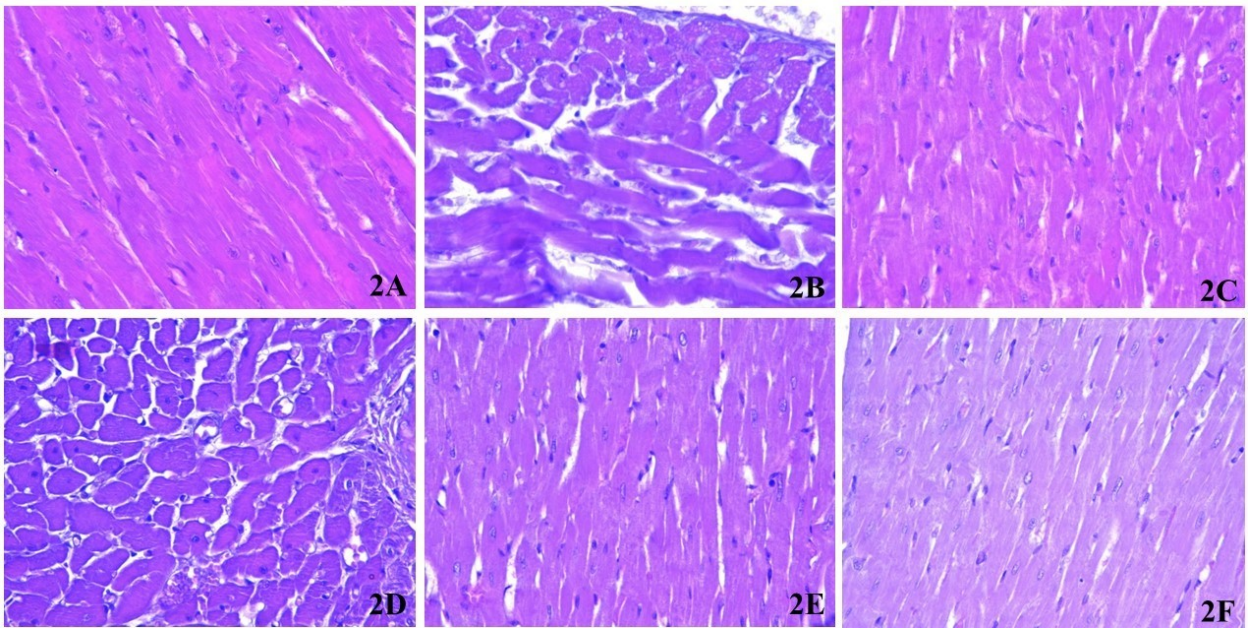


Figure 14. Microscopic appearance of the myocardium of groups: Control (panel 2A), MS (panel 2B), MS+CMFJ2.5 (panels 2C and 2D), MS+CMFJ5 (panel 2E) and MS+CMFJ10 (panel 2F); Hematoxylin-eosin staining, magnification x 200

5.2. Discussion

The consumption of a high-fat high-fructose diet in the current experiment led to an endothelial impairment. Apart from the diet intervention itself, the observed consequences such as the visceral adiposity, insulin resistance and increased oxidative stress are also important mediators of endothelial damage. It has been established that endothelial dysfunction is an early marker of vessel damage, a foundation of impaired regulation of vascular resistance and tissue perfusion and determines the initiation and progression of vascular atherogenesis, developing at later stages of MS (Tune et al., 2017). Endothelial dysfunction leads to impairment of the

endothelium-dependent vasodilation, further contributing to the complex pathogenesis of the syndrome. The bidirectional relationship between endothelial activation and endothelial dysfunction also initiates smooth muscle cells proliferation, a proinflammatory condition with leukocyte adhesion, platelet aggregation, lipid oxidation and activation of matrix metalloproteinases (Hayden, 2023). Cardiomyocytes also responded with degenerative changes to the HFHF diet. This is in consistence with existing literature on experiments conducted with similar nutritional interventions (Prassartthong et al., 2022).

In the current study, CMFJ intake led to dose-dependent cardio- and vasoprotective effects. The histological data correlates with the levels of TBARS in the serum of the experimental animals – the decrease in lipid peroxidation is probably important in the cardio- and vasoprotective activities of the juice. Antiatherogenic and cardioprotective effects have also been reported for other fruit juices – black chokeberry (Daskalova et al., 2015), pomegranate (Basu et al., 2009), grapes (Svezia et al., 2020), orange (Martinez et al., 2021) and many others. It has long been known that consumption of plant foods correlates with a lower cardiovascular risk (Daskalova et al., 2015; Basu A et al., 2009). Polyphenols have a significant contribution to these health-beneficial effects. CMFJ is a rich source of polyphenols. MS components and the subsequent cardiovascular consequences are influenced by polyphenols in various ways. Polyphenols improve glycemic control probably by inhibition of α-amylase and α-glucosidase (thus blunting postprandial glucose spikes), sodium-dependent glucose transporter 1 as well as stimulation of insulin secretion and a reduction in hepatic glucose output (Kim et al., 2016). Quercetin has been reported to have antioxidant and anti-inflammatory properties, to enhance glucose uptake in muscles and adipocytes and to induce autophagy in the setting of MS and associated disorders (Gasmi et al., 2022). A major phenomenon in MS is the chronic low-grade inflammation. Inhibition of cyclooxygenase, lipoxygenase, inducible nitric oxide synthase, nuclear factor kappa-B and activating protein-1 (AP-1), as well as MAPK, protein kinase-C and nuclear factor erythroid 2-related factor activation are some of the polyphenols` molecular activities leading to a decrease in inflammation (Hussain et al., 2016). The anti-inflammatory properties of polyphenols are partially mediated by epigenetic modifications (Ramos-Lopez et al., 2021). Another cornerstone in MS pathogenesis is mitochondrial dysfunction. Correct functioning of cellular mitochondria depends on the selective degradation of impaired ones through autophagy – a process, called mitophagy. Mitophagy impairments are an important part of MS genesis and

progression and its corresponding conditions, including cardiovascular implications (Miao et al., 2023). Polyphenol-mediated mitophagy presents an opportunity for modulation of mitochondrial health through dietary manipulation (Tan et al., 2017). Polyphenols also influence mitochondrial biogenesis. They are considered calory restriction mimetics since they activate some of the molecular mechanisms, stimulated during limited caloric intake – one of the most effective exogenous strategies of improving mitochondrial condition (Davinelli et al., 2020). Polyphenols are also known to modulate gut microbiota, including acting as prebiotics (Kim et al., 2016). Another, usually less discussed, mechanism implicated in MS pathogenesis, is circadian dysrhythmia. Polyphenols have been shown to interact with circadian clocks by modulating the transcription and expression of clock genes (Man et al., 2020). There is data that toll-like receptors, especially TLR4 which are expressed in the myocardium to a larger extent than the other members of the TLR family, mediate some of the unfavorable effects of the high-fat diet on the cardiovascular system, including hypertrophy and fibrosis (Tian et al., 2023). Polyphenols influence the TLR4 signaling pathways (Rahimifard et al., 2017), so they are one of the potential targets of CMFJ.

Among the polyphenols found in JQFJ, the olygomeric proanthocyanidins (Rathinavel et al., 2018), vanillic (Yalameha B et al., 2023), caffeic, chlorogenic (Agunloye et al., 2019), p-coumaric (Fuentes et al., 2014) and ellagic acid (Sharifi-Rad et al., 2022) as well as the flavonoids epicatechin, catechin, quercetin, rutin, naringin, kaempferol and myricetin (Ciumărnean et al., 2020) have been reported to have cardio- and/or vasoprotective properties. A large prospective cohort study with almost 100 000 people included, demonstrates that flavonoid consumption is associated with a decrease in mortality risk from cardiovascular diseases (McCullough et al., 2012).

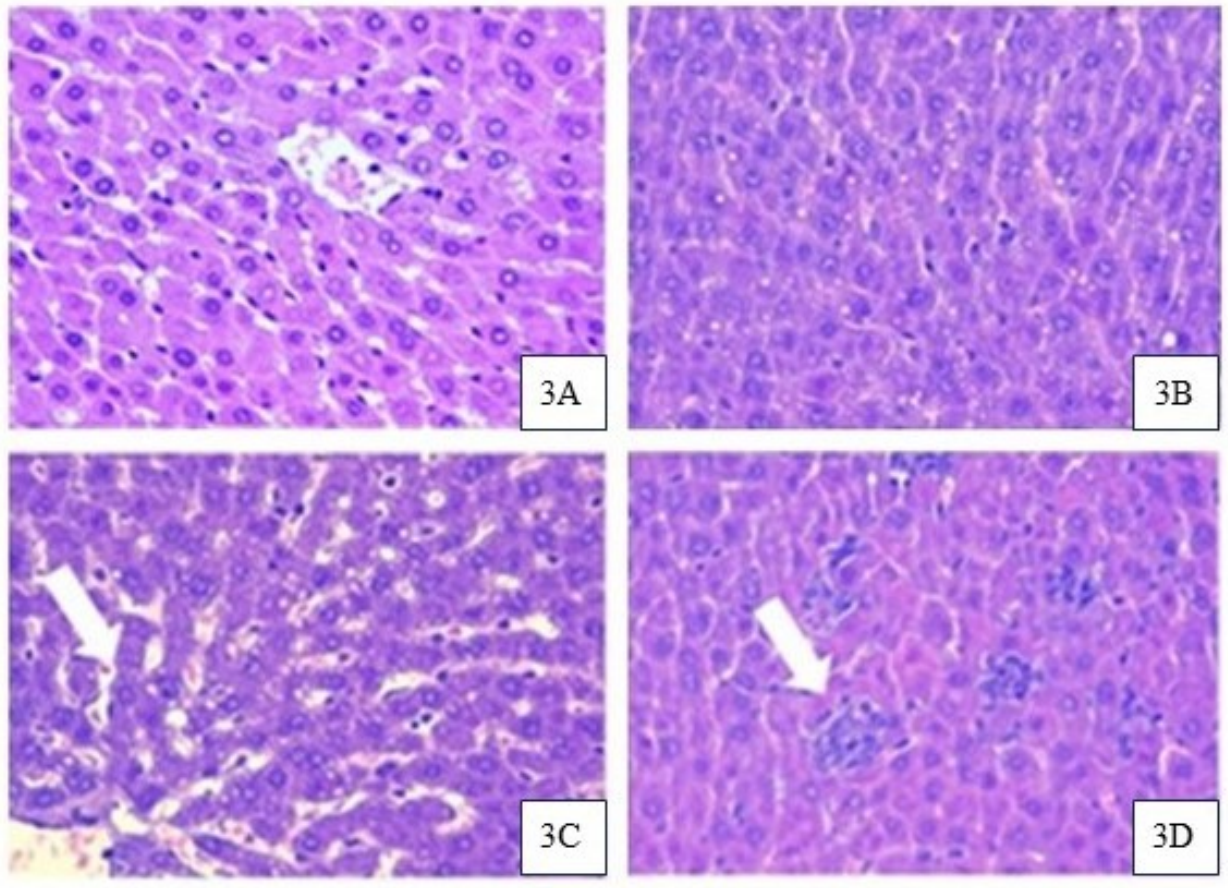
Experimental data for other fruit juices with a rich polyphenolic content and pronounced antioxidant properties demonstrates that they reduce the serum levels of leptin and increase those of adiponectin in animals subjected to a high-fat diet (Wu et al., 2013). Leptin signaling impairments lead to a metabolic switch of the main energy source for the myocardium from glucose to fatty acids and stimulate lipid accumulation in cardiomyocytes, lipotoxicity, mitochondrial dysfunction and increased reactive oxygen species production (Senesi et al.,

2020). Prevention of leptin dysfunction could be another potential mechanism of the cardioprotective effects of CMFJ.

6. Effects of *Chaenomeles maulei* fruit juice in a histopathological examination of liver and on the liver index

6.1. Histopathology results

Results from the histopathological examination are presented on **Figure 15**. In group Control a normal structure of the liver was observed. In the animals from group MS a microvesical steatosis, liver necrosis and non-specific granulomas were observed. In group MS+CMFJ2.5 a smaller number of hepatocytes were affected by the microvesicular steatosis. In groups MS+CMFJ5 and MS+CMFJ10 single non-specific granulomas and single hepatocytes with fatty degeneration were observed. CMFJ treatment led to a dose-dependent reduction in the liver damage induced by the high-fat high-fructose diet without completely preventing the occurrence of degenerative changes.



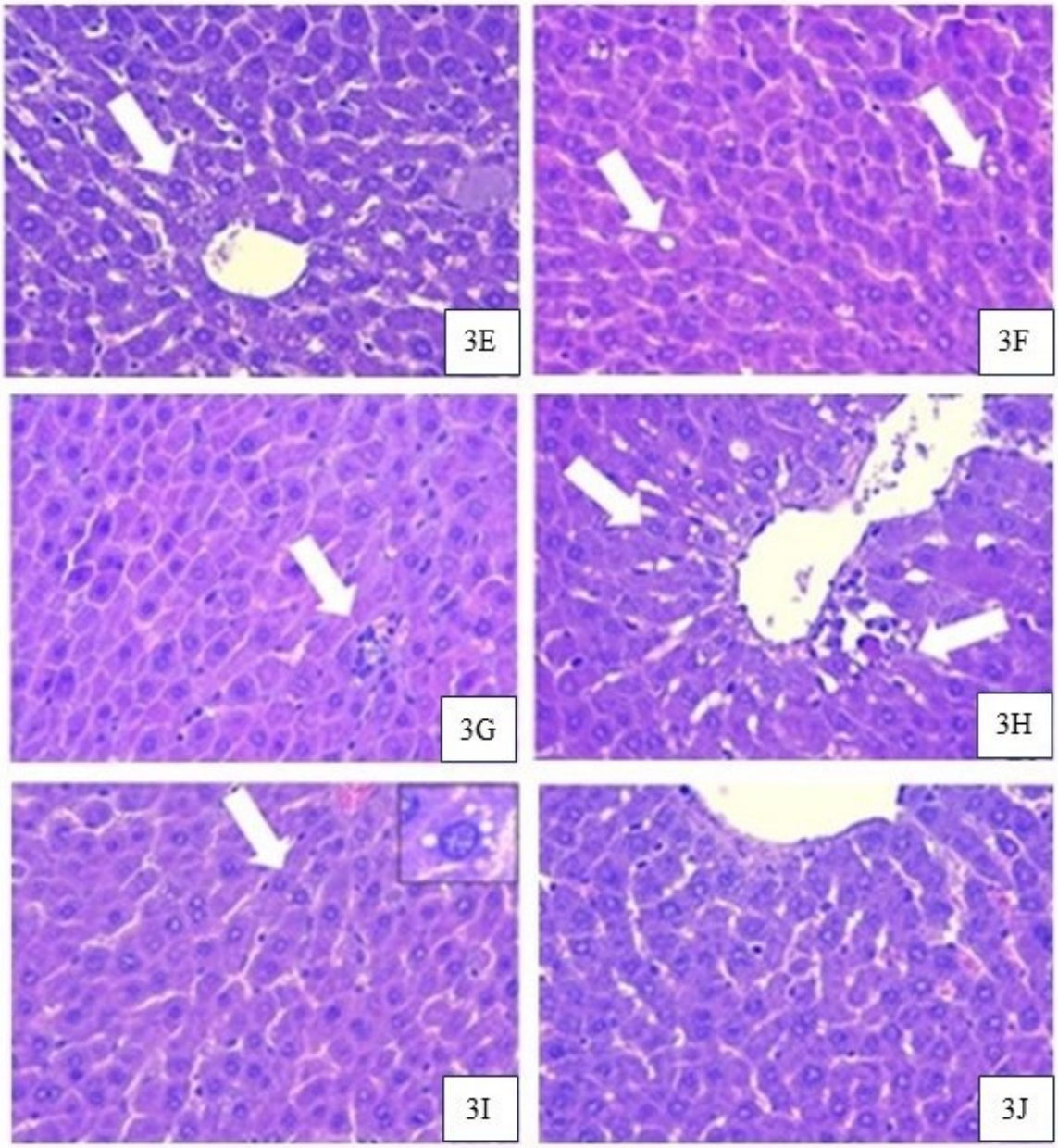


Figure 15. Microscopic appearance of the liver of groups: Control (panel 3A), MS (panel 3B, 3C and 3D), MS+CMFJ2.5 (panel 3E and 3F), MS+CMFJ5 (panel 3G and 3H) and MS+CMFJ10 (panel 3I and 3J); Hematoxylin-eosin staining, magnification x 200

6.2. Liver index results

Liver index results are presented on **Table 8** and **Figure 16**. As a consequence of the high-fat high-fructose diet, liver index values were significantly increased in groups MS and MS+CMFJ2.5 as shown by One-way ANOVA analysis (p=0.0343). The values observed in

groups MS+CMFJ5 and MS+CMFJ10 were comparable with those in group Control – the medium and the high dose prevented the development of the changes, induced by the high-fat high-fructose diet, in the liver index.

Table 8. Liver index demonstrating the ratio between liver weight and body weight of each animal x 10^2 in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05 compared to group Control.

Group	**Liver index**
Control	2.427±0.076
MS	2.707±0.064*
MS+CMFJ2.5	2.758±0.087*
MS+CMFJ5	2.540±0.075
MS+CMFJ10	2.560±0.056

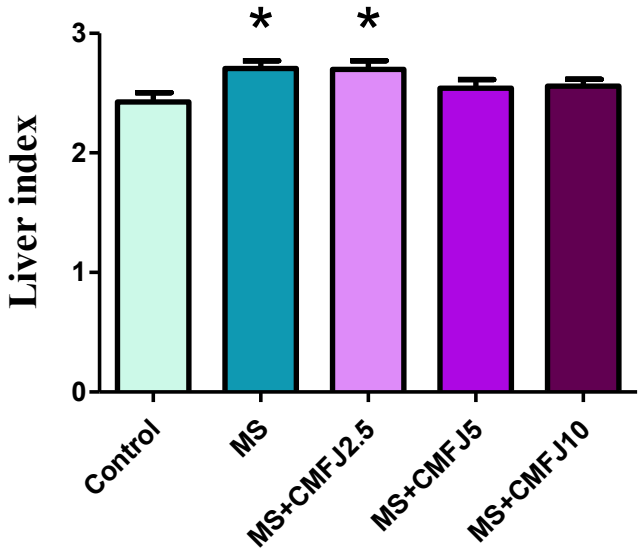


Figure 16. Liver index demonstrating the ratio between liver weight and body weight of each animal x 10^2 in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05 compared to group Control

6.3. Discussion

Non-alcoholic fatty liver disease (NAFLD) is considered the liver manifestation of MS. It is a spectrum of pathological conditions varying from steatosis to steatohepatitis, cirrhosis and even hepatocellular carcinoma. On average around 25.24% of the population worldwide are affected by NAFLD and morbidity reaches its peak in the Middle East (31.79 %) and in South America (30.45 %)(Yuan et al., 2022). The incidence in Europe is 23.71 % (Yuan et al., 2022). NAFLD is a major risk factor for the development of diabetes type 2 as well as for an increase in the cardiovascular morbidity and mortality (Lozano et al., 2016). The classic hypothesis of the NAFLD pathophysiology explains the genesis of the condition with to consequent hits – first, lipid accumulation in the liver, and after that, the detrimental influence of oxidative stress and inflammation. This hypothesis is currently replaced by the multiple-hit hypothesis. It becomes clear that insulin resistance, oxidative stress, genetic and epigenetic factors, the gut microbiota are only some of the many factors responsible for the appearance and aggravation of this pathological condition (Abenavoli et al., 2021).

During insulin resistance, processes are activated that potentiate the intrahepatic lipid accumulation. Increased lypolysis leads to a higher serum concentration of free fatty acids; hyperglycaemia stimulates the synthesis of fatty acids in the liver; hyperinsulinemia and hyperglycaemia inhibit the beta-oxidation of fatty acids and potentiate lypogenesis in the liver (Kucera and Cervinkova, 2014). NAFLD and non-alcoholic steatohepatitis are also considered mitochondrial diseases. Convincing data suggests that mitochondrial dysfunction actually preceeds the development of insulin resistance and NAFLD (Dornas and Schuppan, 2020). Damages on the mitochondrial level can compromise the fatty acids oxidation and generate exceptional amounts of reactive oxygen species as well as toxic lipid intermediate products (Dornas and Schuppan, 2020).

Next mechanism on the subcellular level of liver damage in steatosis and also a driver of the disease progression to non-alcoholic steatohepatitis is the increased endoplasmic reticulum stress. The exceptional amounts of reactive oxygen species, first generated from the food overload and after that multiplied by the many pathological pathways activated in MS, cannot be regulated by the peroxisomes and spread to the endoplasmic reticulum where they change the proper environment needed for protein folding. Chronic endoplasmic reticulum stress is

associated with lipotoxicity, insulin resistance (not only in the liver, but also in the adipose tissue) and inflammation (Zhang et al., 2014). Considering that, the hepatoprotective effect of CMFJ that was demonstrated in the current study is probably at least partly due to the antioxidant properties of the juice.

For many of the polyphenols in the CMFJ content hepatoprotective properties have been reported. As a result of its antiinflammatory, antioxidant, antiangiogenic and antimetastatic activities, vanillic acid displays hepatoprotective effects – it can target almost any of the metabolic anomalies in NAFLD such as liver steatosis, inflammation and liver damage (Shekari et al., 2021). Adenosine-monophosphate activated protein kinase (AMPK) activation is considered to be one of the major mechanisms behind these effects (Shekari et al., 2021). AMPK is a major regulator of energy homeostasis in the body and is thought to be a possible therapeutic target in chronic diseases, including in MS. The same mechanism of action is shared by other polyphenols in CMFJ with significant data about quercetin (Kim et al., 2016). Naringin modulates signaling pathways, implicated in the metabolism of fatty acids – it stimulates their oxidation, prevents lipid accumulation in the liver and sensitizes hepatocytes to the action of insulin (Salehi et al., 2019).

For the majority of the polyphenols in CMFJ it is known that they increase the expression of PPARα in the liver (Domínguez-Avila et al., 2016) – a transcriptional factor that modulates energy metabolism in the liver and is considered a possible target for influencing NAFLD, non-alcoholic steatohepatitis, MS, neurodegenerative and cardiovascular diseases (Todisco et al., 2022; Lin et al., 2022). Adipokine levels are also important for the development, progression and severity of NAFLD. The increase in serum levels of adiponectin for example is considered a possible intervention that improves the course of the condition (Shabalala et al., 2020). Some polyphenols influence adiponectin by increasing its expression or the expression of the receptors AdipoR1 and AdipoR2 – such preclinical data exists for chlorogenic acid and for quercetin (Shabalala et al., 2020).

The positive influence of CMFJ on the liver manifestations of MS could also be attributed to the carotenoids in its content. They have a pronounced antiinflammatory and antioxidant activities and modulate a number of intracellular signaling pathways. In an experiment with mice subjected to a high-cholesterol high-fat diet, beta cryptoxanthine intake leads to inhibition of

steatosis by a change in the gene expression of genes implicated in inflammatory processes, infiltration and activation of macrophages and other leucocytes, regulation of the number of T-cells as well as scavenging free radicals (Yilmaz et al., 2015).

In a cohort study with 2017 participants included and followed for a period of 4 years, a longitudinal association between the volume of the visceral adipose tissue and the NAFLD incidence was discovered (Kim et al., 2016). The observed effect of CMFJ in the current study of reduction of the visceral adiposity induced by the high-fat high-fructose diet is probably one of the mechanisms, implicated in the hepatoprotective activity of the juice.

Fructose, which is a major component of the diet in the current model of MS, is one of the four food substrates that are not susceptible to insulin regulation, along with trans fats, branched chain aminoacids and ethanol (Bremer et al., 2012). In this way, unregulated amounts of intermediate metabolites are led to the mitochondria of the liver, stimulating the ectopic accumulation of lipids in this organ (Bremer et al., 2012). So accumulating data suggests that these are the major dietary factors that potentiate the genesis and the progression of NAFLD and MS.

7. Effects of *Chaenomeles maulei* fruit juice in a histopathological examination of adipose tissue

7.1. Results

Results from the histological examination of adipose tissue are presented on **Figure 17**. In group Control a normal structure of the adipose tissue and a normal size of the adipocytes were observed. In rats from group MS adipocytes with a larger size compared to group Control were found. Treatment with CMFJ in all doses used prevented the development of the high-fat high-fructose diet-induced changes and in the highest dose preserved the normal size of the adipocytes as observed in group Control.

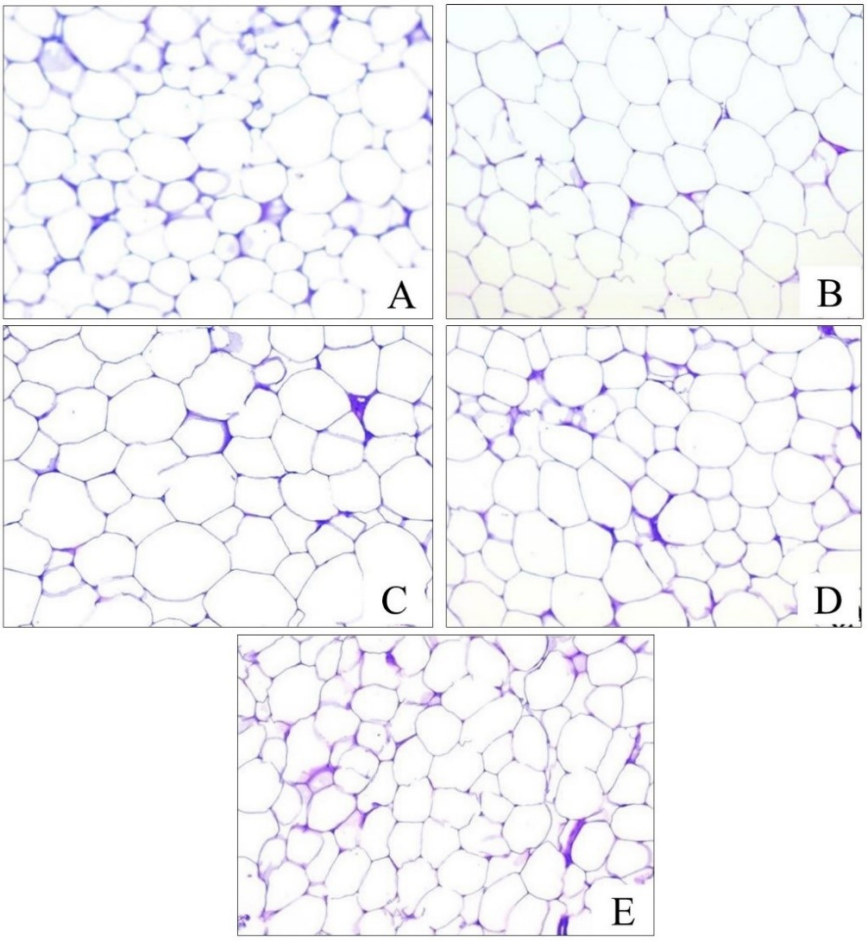


Figure 17. Microscopic appearance of adipose tissue from groups: Control (panel A), MS (panel B), MS+CMFJ2.5 (panel C), MS+CMFJ5 (panel D) and MS+CMFJ10 (panel E); Hematoxylin-eosin staining, magnification x 200

7.2. Discussion

Obesity is defined as a body mass index ≥ 30 kg/m^2 while overweight is observed when the index is between 25 and 29.9 kg/m^2. Nowadays obesity stands out as one of the most serious health challenges globally. It is both an initial trigger and a driver for MS, its components and its associated diseases, including an increased risk of certain neoplastic diseases such as liver, pancreatic, renal, colorectal, breast and endometrial cancer, etc. For every 5 units increase in the BMI above 25 kg/m^2, total mortality is increased with 29 %, cardiovascular mortality – with 41 %, and mortality related to diabetes type 2 – with 210 % (Apovian, 2016). Adipocyte hypertrophy that is observed during obesity is followed by local accumulation of immune cells in the adipose tissue which are a major driver of metainflammation (Catalán et al., 2018). The latter in combination with the increased oxidative stress and hypoxia of the adipocytes that are further away from the blood vessels exert a detrimental effect on metabolic health (Catalán et al., 2018). Reduction of body weight is a major strategy in the combat with MS and significantly improves the prognosis of the patients affected by the syndrome. Obesity also brings about impairments in glucose and lipid metabolism, oxidative stress and inflammation. In the current study, CMFJ exerts an antiobesity effect, preventing the high-fat high-fructose diet-induced enlargement of the adipocytes, as seen on the histological examination, which is in consistence with the already discussed total, mesenterial and paranephral adipose tissue indices reduction. The antiobesity effects of polyphenols include mechanisms such as enzyme inhibition, inhibition of neurohormones related to food intake and the feeling of satiety, and induction of mitochondrial biogenesis (Aloo et al., 2023). They decrease lipogenesis, accelerate lipolysis, influence energy intake, energy expenditure and differentiation and proliferation of preadipocytes (Siddiqui et al., 2023). An in vivo experiment with mice on a high-fat high-fructose diet demonstrates that vanillic acid contributes to an improved glucose tolerance and reduced insulin resistance by promoting thermogenesis in the brown adipose tissue (Han et al., 2018). In a 9-week placebo-controlled clinical trial in healthy men the intake of fruit juice that is rich in anthocyanins leads to a reduction of the adipose tissue, but an increase in the fat-free body mass (Bakuradze et al., 2019). As it was already discussed, one of the many mechanisms involved in obesity pathogenesis is the central energy regulation impairment in the hypothalamus. The high-fat high-fructose diet is a direct source of AGEs, as well as a prerequisite for their increased endogenous production. It has been observed that polyphenols decrease the levels of AGEs, therefore

prevention of the AGEs-stimulated inflammation of the hypothalamus could be one of the possible mechanisms of their antiobesity effects (Xie and Chen, 2013; Sergi et al., 2020).

Recently, scientific literature often considers „obesity, induced by the gut microbiota“ (Liu et al., 2021). The abnormal gut microbiota could have an obesogenic effect due to its regulating properties on the caloric intake, feeling of hunger, circadian rhythms and chronic inflammation (Liu et al., 2021). In obesity the most common finding is an increased ratio of *Firmicutes/ Bacteroidetes* species. Normal gut microbiota impairments might also compromise the barrier function of the gut, which leads to bacterial fragments leakage in the blood, which provoke inflammation and become a prerequisite for the development of obesity and insulin resistance (Patterson et al., 2016). As it was already discussed, gut microbiota modulation is one of the mechanisms of action of polyphenols. Considering the limited absorption of polyphenols in the intestines and their accumulation in the large intestine, where they are being metabolised by the gut microbiota, it is not surprising that the content of the microbiota is also important for polyphenols to exert their biological effects and the relationship is bidirectional. It has been established that proanthocyanidins stimulate the proliferation of *Akkermansia muciniphila* which is known for its antiinflammatory effects and favorable properties in the context of obesity and diabetes (Redondo-Castillejo et al., 2023). They also support the proliferation of a number of butyrate-producing bacteria (Redondo-Castillejo et al., 2023). Butyrate is a short-chain fatty acid with established beneficial properties in MS due to receptor activity and epigenetic modulation (Bridgeman et al., 2020). In a 6-week experiment in mice the use of chlorogenic acid reduces dysbiosis, induced by a high-fat diet, body weight, plasma lipids and influences mRNA expression of genes related to lipogenesis and lipolysis (Wang et al., 2019).

8. Effects of *Chaenomeles maulei* fruit juice on the behaviour of rats with diet-induced metabolic syndrome

8.1. Effects of *Chaenomeles maulei* fruit juice in the Open field test

Locomotor activity of experimental animals was evaluated with the Open field test. Results from the test are presented on **Table 9** and **Figures 18** and **19**. They show that the number of horizontal movements was slightly increased in all the groups that were on a high-fat high-fructose diet, but only reached statistical significance in group MS+CMFJ2.5 as compared to group Control ($p=0.0361$). In the locomotor activity of the animals taking CMFJ no significant difference was registered compared to group MS. The number of vertical movements remained unaffected by the treatment with CMFJ. The time spent in the central area of the apparatus as well as the number of entries in the central area were comparable in all of the experimental groups.

Table 9. Number of horizontal and vertical movements, time spent in the central area of the apparatus and number of entries in the central area in Open field test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *$p<0.05$ compared to group Control.

Group	Horizontal movements	Vertical movements	Time spent in the central area	Number of entries in the central area
Control	69.4±5.916	18.5±2.592	21.3±4.088	2.8±0.6289
MS	85.3±8.099	22.4±1.368	16.79±3.006	2.2±0.5735
MS+CMFJ2.5	116.2±7.724*	23.6±2.713	13.23±1.708	3.7±1.033
MS+CMFJ5	92.6±13.85	22.8±2.936	14.4±1.812	2.4±0.5812
MS+CMFJ10	84.4±11.66	22.3±3.303	12.7±1.836	2.4±0.6700

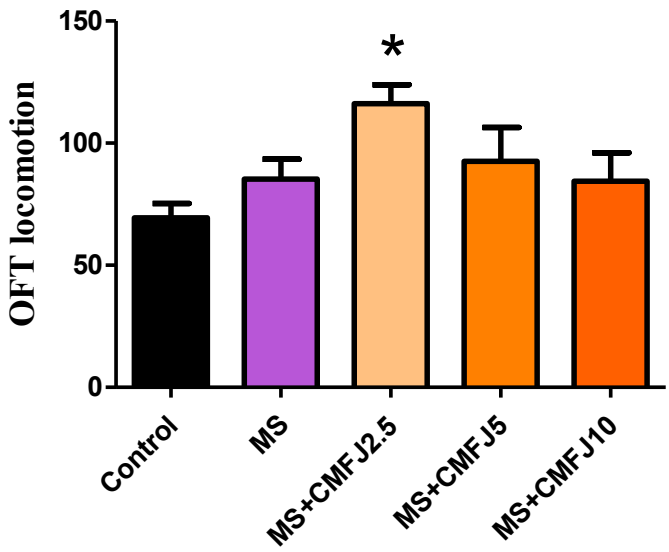


Figure 18. Number of horizontal movements in Open field test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05 in comparison to group Control

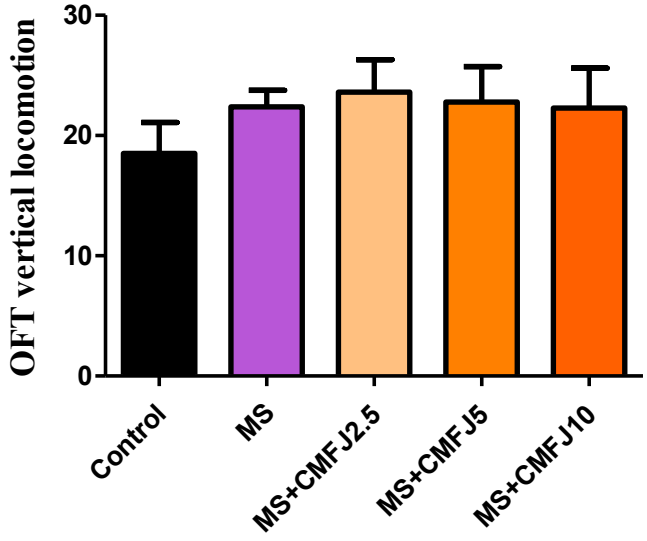


Figure 19. Number of vertical movements in Open field test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

8.2. Effects of *Chaenomeles maulei* fruit juice on anxiety levels

8.2.1. Effects of *Chaenomeles maulei* fruit juice in the Elevated Plus Maze

Results from the Elevated Plus Maze test are presented on **Table 10**. CMFJ treatment led to an increase in the time, spent in the open arms of the apparatus. One-way ANOVA with post test for linear trend showed a positive linear trend (p=0.0354), as demonstrated on **Figure 20.** The number of entries in the open arms did not significantly differ among the experimental groups, as demonstrated on **Figure 21.**

The ratio between time spent in the open arms and total time spent in any of the arms was increased by the CMFJ treatment. Post test for linear trend of this ratio showed a positive linear trend (p=0.0335), as demonstrated on **Figure 22.**

The ratio between the number of entries in the open arms and the total number of entries in any of the arms showed a certain tendency to increase, but results did not reach statistical significance. Data is presented on **Figure 23.**

Locomotor activity of the animals, measured as the total number of entries in any of the arms, did not significantly differ among the experimental groups.

Table 10. Time spent in open arms, number of entries in open arms, ratio time spent in open arms to total time spent in any arm, ratio number of entries in open arms to total number of entries in any arm and locomotor activity, measuring the total number of entries in any arm in the Elevated plus maze test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Group	**Time in open arms**	**Number of entries in open arms**	**Ratio number of entries in open arms/ total number of entries in any arm**	**Ratio time spent in open arms/ total time spent in any arm**	**Locomotor activity**
Control	10.21±4.297	1.3±0.518	0.067±0.023	0.044±0.018	11.90±0.888
MS	8.89±3.054	1.3±0.668	0.068±0.025	0.040±0.013	11.60±1.392

MS+CMFJ2.5	24.77±7.543	1.5±0.453	0.116±0.039	0.097±0.030	9.444±1.529
MS+CMFJ5	33.14±7.771	1.7±0.396	0.148±0.039	0.126±0.028	9.200±1.298
MS+CMFJ10	33.06±11.32	1.5±0.477	0.140±0.041	0.139±0.047	10.30±1.407

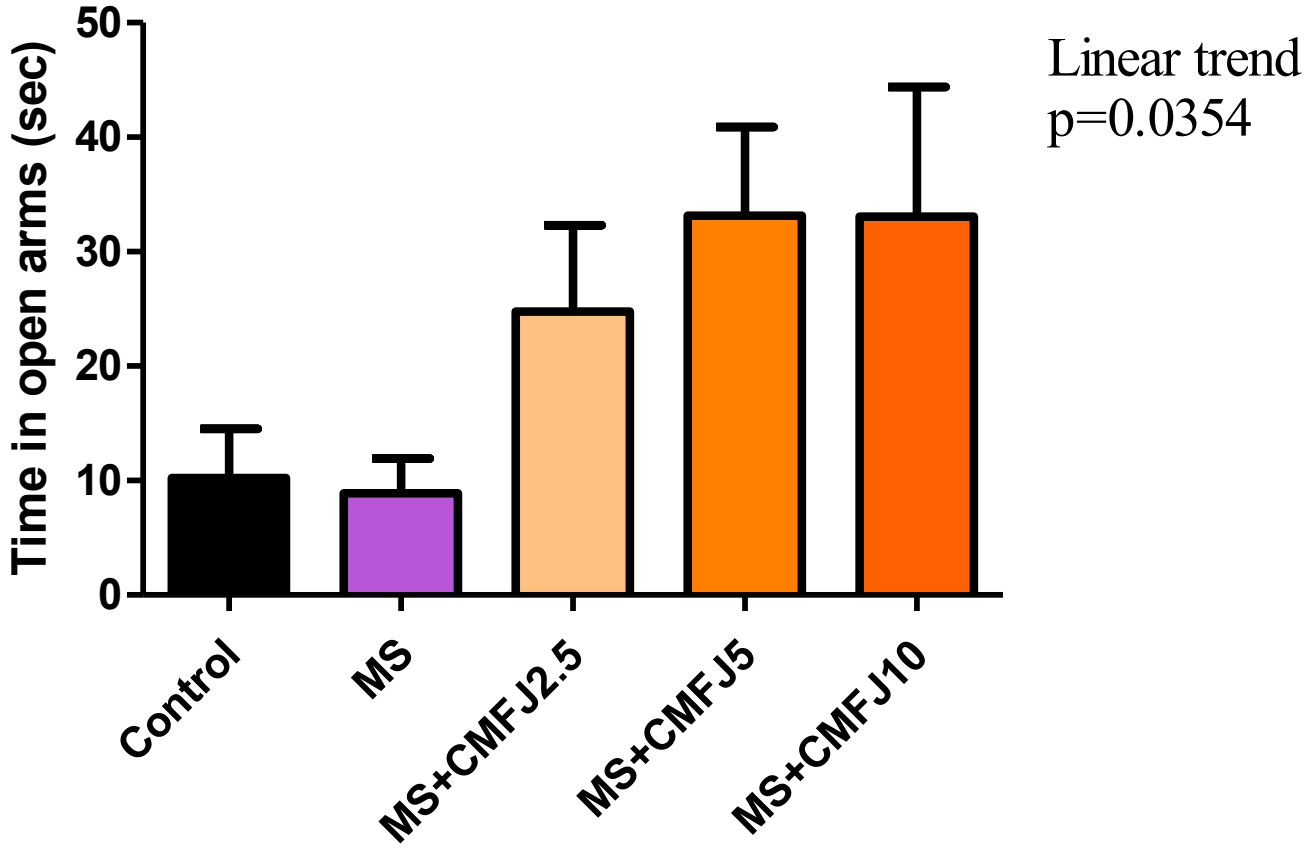


Figure 20. Time spent in the open arms of the apparatus in the Elevated plus maze test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. Positive linear trend: $p<0.04$.

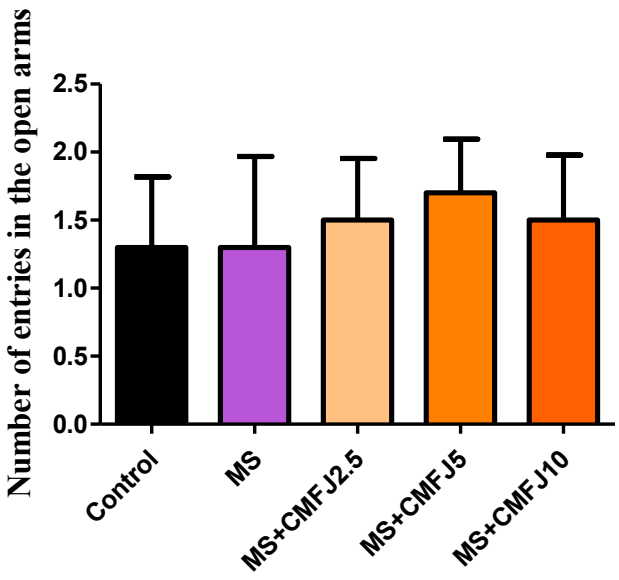


Figure 21. Number of entries in the open arms of the apparatus in the Elevated plus maze test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

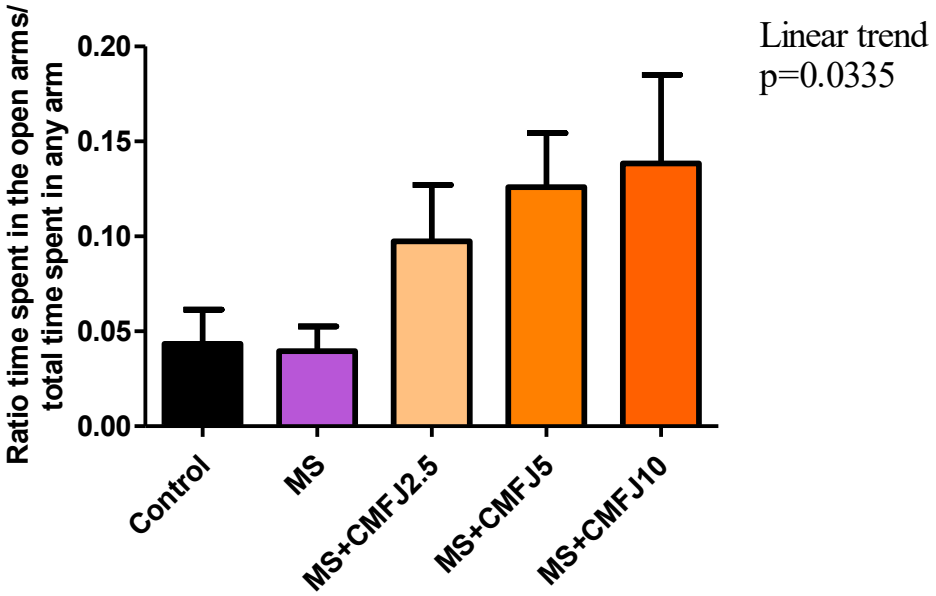


Figure 22. Ratio time spent in the open arms to total time spent in any of the arms of the apparatus in the Elevated plus maze test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg. Positive linear trend: $p<0.04$

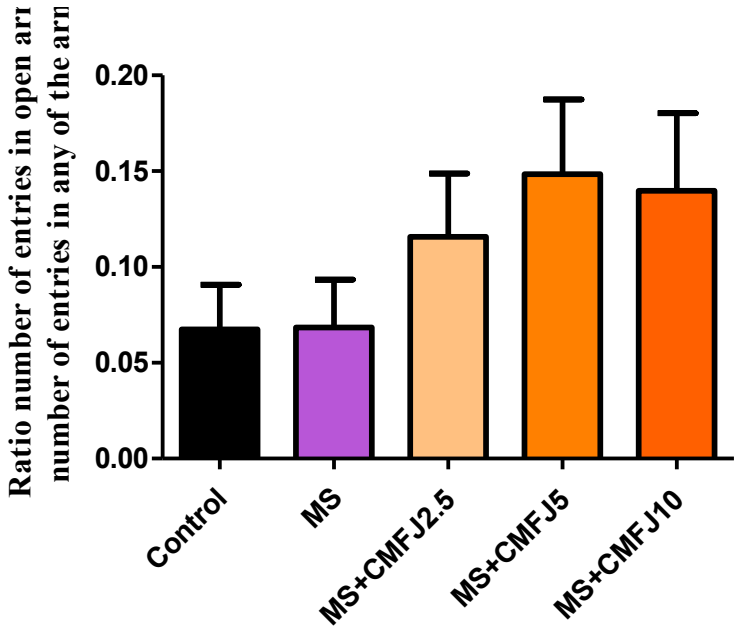


Figure 23. Ratio number of entries in the open arms to total number of entries in any of the arms of the apparatus in the Elevated plus maze test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

8.2.2. Effects of *Chaenomeles maulei* fruit juice in the Social interaction test

By conducting the social interaction test, social contact duration between the experimental animals is measured and an increase in it is interpreted as a decrease in anxiety. Results from the test are visualised on **Table 11** and **Figure 24** and show that the values in group MS were only slightly decreased compared to group Control (22.16±1.596 in comparison to 27.83±2.818), but without reaching statistical significance. Time of social interaction in the groups that were treated with CMFJ did not differ significantly from the one in group MS.

Table 11. Time of social interaction in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Group	**Time of social interaction (sec)**
Control	27.83±2.818

MS	22.16±1.596
MS+CMFJ2.5	22.33±3.415
MS+CMFJ5	23.94±3.413
MS+CMFJ10	23.23±2.048

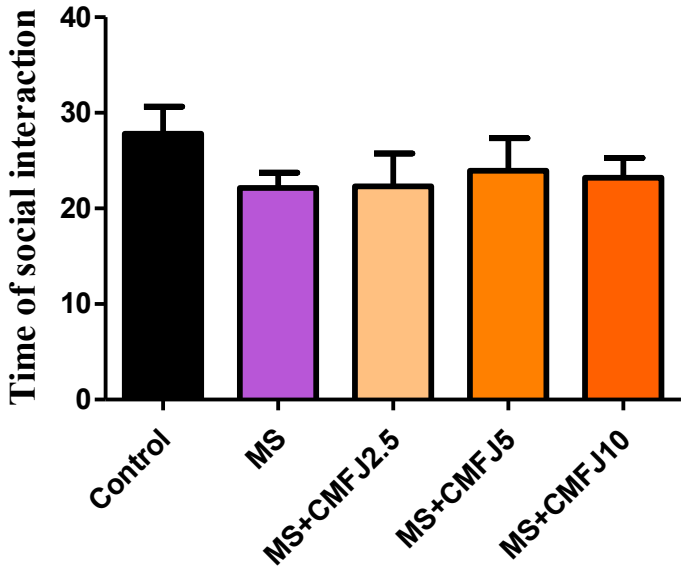


Figure 24. Time of social interaction in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

8.3. Effects of Chaenomeles maulei fruit juice on the spatial memory in the object location test

Spatial memory is examined by conducting the object location test. Higher values of the discrimination index (B/(A+B)) are interpreted as an improvement of the spatial memory. Results from the test are visualised on **Table 12** and **Figure 25**. They show that the discrimination index was slightly decreased in group MS as compared to the Control group, although difference did not reach statistical significance. The intake of CMFJ prevented this

decrease and in the three groups – MS+CMFJ2.5, MS+CMFJ5 and MS+CMFJ10 – values of the index were comparable with those in the Control group.

Table 12. Discrimination index B/(A+B) in the object location test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Group	Discrimination index B/(A+B)
Control	0.581±0.048
MS	0.452±0.050
MS+CMFJ2.5	0.566±0.048
MS+CMFJ5	0.538±0.040
MS+CMFJ10	0.518±0.053

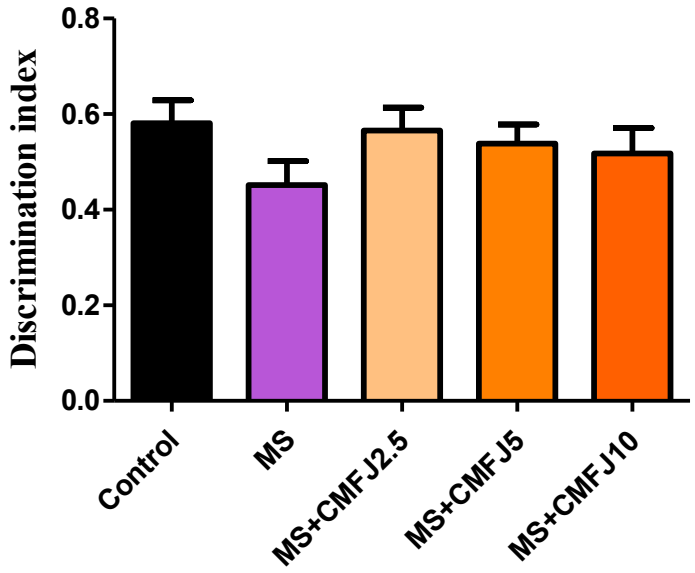


Figure 25. Discrimination index B/(A+B) in the object location test in rats with diet-induced MS treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

8.4. Effects of *Chaenomeles maulei* fruit juice on the depressive behaviour in the Forced swim test

Results from the forced swim test are presented on **Table 13** and **Figure 26**. The statistical analysis did not show any significant difference in the immobility time of the animal groups examined. In group Control the time spent in immobility was 181.6±11.23 sec. In group MS a non-significant dicrease in time (170.2±13.21) in comparison to the Control groups was observed. In the groups treated with CMFJ a non-significant increase in comparison to group MS was registered.

Table 13. Immobility time measured in forced swim test in rats with diet-induced MS, treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Group	Immobility time (sec)
Control	181.6±11.23
MS	170.2±13.21
MS+CMFJ2.5	198.8±12.70
MS+CMFJ5	183.6±5.237
MS+CMFJ10	185.8±11.06

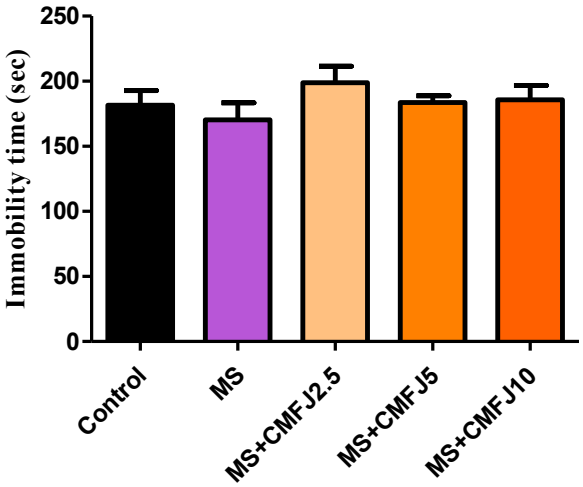


Figure 26. Immobility time measured in forced swim test in rats with diet-induced MS, treated with CMFJ in doses 2.5 ml/kg, 5 ml/kg and 10 ml/kg

8.5. Discussion

Behavioural tests are an important part of experimental studies of cognitive effects of physiological and pathological changes in the body and allow the evaluation of neuronal function in specific brain areas by analysing the behaviour in a controlled environment (Denninger et al., 2018). Processing, storage and expression of behaviours associated with fear and anxiety are mediated by several brain regions, including the amygdala and its connections to the thalamus, the medial prefrontal cortex, the hippocampus, the hypothalamus, the bed nucleus of the stria terminalis, the brainstem and the periaqueductal grey (Sartori et al., 2019). MS is often associated with anxiety (Ji et al., 2023), depression and other cognitive and neuropsychiatric disorders and the latter could also become a prerequisite for the development of MS, influencing the eating habits and the physical activity of the individual as well as their affinity to stick to a prescribed therapeutic regimen. Many aspects of the cognitive function could be compromised due to MS – the rate of information processing, memory, semantic skills, visual-spatial abilities, the ability to focus attention and executive functions (Guicciardi et al., 2019).

The results from the open field test did not demonstrate a significant influence of CMFJ intake on the locomotor activity of the animals. Although in the group treated with the lowest dose of CMFJ a certain increase in the number of horizontal movements was observed as compared to group Control, when compared to group MS the effect was not statistically significant. The lack of change in the locomotor activity was also confirmed by the results of the elevated plus-maze test.

Elevated plus-maze is a well-established unconditioned test for measuring anxiety levels in rodents. It is based on the natural aversive behavior of rats in novel open spaces in combination with fear of balancing on a narrow, raised platform as opposed to the spontaneous exploratory behavior of the animal. The present study demonstrated that chronic administration of CMFJ in rats with MS caused an increase in the time spent in the open arms in a dose-dependent manner which indicated an anxiolytic-like effect. These results correspond with the anxiolytic effect reported for CMFJ administered to healthy rats (Borisova et al., 2018) as well as to rats subjected to mild stress induced by impaired circadian rhythm (Borisova et al., 2023). In both mentioned experiments with subchronic administration of the juice no dose-dependancy was established while with the chronic treatment in the present study a clear dose-dependent effect was evident.

Many of the phenolic substances found in CMFJ have extensive neuropsychiatric impact. Anxiolytic effect has been reported for vanillic (Vestegani et al., 2022), caffeic (Monteiro et al., 2020), chlorogenic (Chen et al., 2021) and p-coumaric acid (Sheepens et al., 2014). Reported possible mechanisms of action include a GABA-A interaction. Some of the flavonols present in the juice, such as quercetin, likely share the same mechanism (Islam et al., 2022), while others like kaempferol have been described to inhibit the fatty acid amide hydrolase in vitro, therefore affecting the endocannabinoid system (Silva Dos Santos et al., 2021). Quercetin also demonstrates a reversible inhibitory activity towards monoaminooxidase – an enzyme participating in the degradation of serotonin (Grabska-Kobyłecka et al., 2023).

An important feature that currently effective pharmacological (such as selective serotonin reuptake inhibitors) and non-pharmacological (cognitive-behavioral therapy) treatments of anxiety share, is the ability to modulate synaptic plasticity (Sartori et al., 2019). Studies show that dietary flavonoids are able to promote the expression of neuroplasticity-related genes and proteins. Adult hippocampal neurogenesis has been outlined as at least partly responsible for the beneficial effects of polyphenols on anxiety and depression (Dias et al., 2012). Quercetin has been reported to increase neurogenesis and synaptogenesis (Tchantchou et al., 2009). Activation of the brain-derived neurotrophic factor – BDNF – is one of the suggested mechanisms of a beneficial neurocognitive influence of some polyphenols (Grabska-Kobyłecka et al., 2023).

Inflammation is considered one of the possible links between anxiety and metabolic syndrome. Higher level of pro-inflammatory cytokines is observed in patients with anxiety (Kumar et al., 2023). Since in humans it is more difficult to directly measure neuroinflammation, often peripheral inflammation and coagulation markers are evaluated. Large human cohort studies reveal a positive correlation between anxiety and C-reactive protein, interleukin-6, homocysteine, fibrinogen, tumor necrosis factor-alpha and white blood cell counts (Pitsavos et al., 2006; Vogelzangs et al., 2013). Polyphenols exert anti-inflammatory actions by a variety of mechanisms, including modulation of immune cell populations, cytokine production and the expression of inflammatory genes (Yahfoufi et al., 2018).

With the social interaction test the time of active social contact between the experimental animals is being measured and its prolongation is interpreted as a reduction in anxiety level. In the present study, the time of social interaction in the animals subjected to a high-fat high-

fructose diet showed lower values than the rats from group Control, although results did not reach statistical significance. This could be attributed to an increase in the level of anxiety in the animals with MS. The time of social interaction in the animals treated with CMFJ did not significantly differ from the time in group MS. Therefore, in this test no anxiolytic effect was observed following the intake of CMFJ in the doses used.

Object location test offers an approach for evaluation of the spatial memory and the major brain structure, involved in the examined processes with this test, is the hippocampus (Denninger et al., 2018). The test relies on the natural preference of rats to novelty and is based on the fact that if an animal remembers the location of the objects at the moment of initial exposition, it tends to spend more time examining the relocated object (Denninger et al., 2018). In the current study, experimental animals from group MS spent less time examining the relocated object as demonstrated by the values of the dicrimination index, which signifies an impairment of the spatial memory – a component often affected by the development of MS. CMFJ intake prevented this pathological influence of the dietary manipulation and in all three of the doses used led to values of the discrimination index comparable with those of group Control.

The forced swim test is often used in experimental pharmacology for evaluation of the depressive-like behaviour in rodents. It is based on the natural initial attempts of the rat to escape the water container, followed by a state of immobility. The latter could be interpreted as a sign of desperation. The test is used for the evaluation of pharmacologically active substances on the depressive-like behaviour. In the present study, no antidepressant activity was observed following the treatment with CMFJ. These data is similar to the findings in healthy male rats, treated with CMFJ (Borisova et al., 2019). On the other hand, Borisova-Nenova et al. (2023) observe a reduction in the immobility time in a forced swim test of rats treated with CMFJ and subjected to impaired circadian rhythm. These data probably suggest that the polyphenolic content of CMFJ contributes to prevention of the development of depressive-like behaviour as a response to isolated circadian disturbance, but does not significantly influence other pathogenic mechanisms with depresogenic effects in the context of MS.

9. General discussion

Manipulation of the diet is a major tool for the development of experimental models of MS in rodents. The current study confirms that the high-fat high-fructose diet is appropriate for provoking visceral obesity, insulin resistance, impaired glucose tolerance, hypertriglyceridemia, inhibition of the natural antioxidant defences and tissue damage in myocardium, coronary vessels and liver in a model of MS. While studying NAFLD - the major manifestation of MS in the liver - experimental models in animals possess even higher importance since the golden standard in diagnosis and tracking the disease progression is the liver biopsy - a test with limited applicability in the clinical environment since its invasive character. As it was already discussed, there is a considerable overlapping between experimental models of MS and NAFLD. Among the NAFLD models, though, histological data is not always backed up by the development of all the components, driving the pathogenesis of the disease in humans, therefore the accuracy and relevancy of the model are submaximal. Considering the fact that in the present study the histological findings in the liver are combined with the development of insulin resistance, visceral obesity, dyslipidemia and impaired glucose tolerance, there are reasons to believe that this model could be successfully used not only for MS, but also for NAFLD.

The contribution of disbalanced diet to the genesis of MS is long known and the exceptional amounts of saturated fats is a widely known risk factor not only among health specialists, but also in the community. Foods content and the official dietary recommendations during the last decades reflect this knowledge and the consumption of saturated fats actually does not peak during this time period. At the same time, MS incidence is rapidly increasing and the apparent discrepancy could be attributed to the enormous intake of refined carbohydrates, especially fructose. Considering this, the diet used in the present experiment reflects the latest trends behind the increased MS morbidity.

A major contribution of the favorable effects of CMFJ, demonstrated in the experimental model of MS, is probably owed to the rich polyphenolic content of the juice. Polyphenols` biological activity is a popular and controversial topic with some persistent discrepancies between their in vitro, in vivo and clinical effects. It is noticeable that the metabolic net of adipokines, myokines and cardiokines that takes a key position in the pathogenesis of MS, is significantly influenced both by eating habits and by physical activity. This is probably one of the biological features

responsible for some of the mentioned differences among the experimental and clinical data of polyphenol intake – while in the case of rodents the possibilities of physical activity are relatively standardised because of the equal laboratory conditions, individual variations in humans are big enough to turn the scales in one or the opposite direction. Another discrepancy is due to the bioavailability of polyphenols. Although it is variable and often low for the majority of the polyphenols, increasing data suggests that bioavailability is actually much higher once metabolites produced under the influence of the gut microbiota are considered.

Oxidative stress and chronic inflammation lead to mitochondrial dysfunction and cellular damage. Both pathological processes could be modulated by polyphenolic intake. Emerging data suggest the beneficial influence of polyphenols on the adipokines balance. And while adipokines are a topic widely discussed in the scientific literature in recent years, the effect of myokines in the context of metabolic disturbances has just started to get some attention. They are secreted by muscle cells during physical activity and are some of the factors responsible for the undisputable favorable health effects of sport, but they could also be affected by different dietary interventions, including the polyphenolic intake. This is one of the possible future directions for examination of the interrelationship between MS and polyphenols.

Obesity has acquired an epidemic dimension during the last decades. The relatively disappointing historical experience with drugs against obesity either due to limited efficacy or non-tolerable side effects, focuses the attention on natural approaches in the prevention and therapy of this disease. The consumption of functional foods has provoked a major interest in the society because of its appropriateness and accessibility. The *Chaenomeles maulei* fruit juice that demonstrated antiobesity properties in the present study could also be classified as a functional food. The prevention of adipose tissue accumulation both in visceral depots and ectopically – in the liver for example – is an effect that could be very useful in the context of the increasing health, economic and social burden of the diet-induced chronic diseases. In combination with the cardioprotective activity and the antioxidant properties, CMFJ intake seems to be an appropriate intervention to limit the detrimental effects of disbalanced eating patterns on the metabolic health.

When considering the beneficial influence of polyphenols in the central nervous system, improving the blood-brain barrier, the brain bloodflow, the cognitive abilities and the mood

regulation are some of the effects described in scientific literature. They could be directly exerted – in the case of those polyphenols that can pass the blood-brain barrier – or indirectly, influencing the gut microbiota and affecting central nervous system processess in this way. The combination of polyphenols found in CMFJ confirms the potential of these phytonutrients to regulate mood by demonstrating an anxiolytic-like effect in the present study with a diet-induced model of MS. It is intriguing that hippocampal neurogenesis in the adult person, that is involved in mediating the beneficial effects of polyphenols in mood and cognition disorders, depends not only on the content of food, but also on its texture, and according to some of the hypothesis the process of chewing is associated with regulation of the hippocampal neurogenesis (Stangl and Thuret., 2009). What is more, a variety of data suggests that bioavailability and biologic activity of polyphenols are strongly associated with the food matrix in which they are consumed. Considering that, it would be interesting to compare the behavioral effects of CMFJ to those following the whole fruits consumption with the same macro- and micronutrient and polyphenolic content.

Despite the relatively safe profile of action of polyphenols, some considerations related to potential toxic effects are found in the literature and they usually regard isolated polyphenols in the form of extract, separated from the food matrix. In light of this, the fruit juice that is rich in polyphenols, seems to be a more favourable form of providing a healthy amount of these bioactive phytonutrients when the benefit/risk ratio is considered. The mutual coexistence of several or many polyphenols in a natural product such as the fruit juice probably provides not only a safer profile of action, but also possible additive and/or supraadditive effects when considering potential health benefits, some of which were demonstrated in the present study.

V. Conclusions

1. The use of high-fat high-fructose diet for 10 weeks in male Wistar rats led to the development of metabolic syndrome.

1.1. Visceral obesity was developed.

1.2. Biochemical impairments, typical of the metabolic syndrome, were found – increased levels of blood glucose during the glucose-tolerance test and hypertriglyceridemia.

1.3. Insulin resistance was observed, demonstrated by the increase values of the TyG index.

1.4. The endogenous mechanisms of antioxidant defense were supressed as demonstrated by the decreased activity of superoxide dismutase in the serum.

1.5. There was increased lipid peroxidation as demonstrated by the increased values of thiobarbituric acid reactive substances in the serum.

1.6. A tendency for spatial memory impairment was observed.

1.7. Histopathologically:

1.7.1. In the adipose tissue a hypertrophy of the adipocytes was observed;

1.7.2. In the liver degenerational changes were noted, including microvesicular steatosis, liver hepatic necrosis and non-specific granulomas;

1.7.3. In the myocardium myocytes were found at a larger distance from each other and with degenerative changes;

1.7.4. In the coronary vessels a focal absence of endothelium was observed, the endothelial cells were necrotic and the basal membrane was exposed.

2. The oral intake of *Chaenomeles maulei* fruit juice in rats during the induction of metabolic syndrome:

2.1. Increased the amount of food consumed at doses 5 ml/kg and 10 ml/kg.

2.2. Decreased the fructose solution intake at a dose of 5 ml/kg.

2.3. Increased the total caloric intake at doses 5 ml/kg and 10 ml/kg.

3. The oral intake of *Chaenomeles maulei* fruit juice at three different doses (2.5 ml/kg, 5 ml/kg and 10 ml/kg) in rats during the induction of metabolic syndrome prevented a large part of the morphometric, biochemical, histopathological and behavioural impairments:

3.1. Lowered the total, mesenterial and paranephral adipose tissue indices at a dose of 5 ml/kg.

3.2. Provoked a weak tendency in a direction of lowering the triglycerides and total cholesterol levels.

3.3. Did not influence glucose tolerance.

3.4. Provoked a weak tendency in a direction of lowering the TyG index and insulin resistance, respectively.

3.5. Improved endogenous antioxidant defense by opposing the high-fat high-fructose diet-induced decrease in serum superoxide dismutase levels, most pronounced at doses 2.5 and 5 ml/kg.

3.6. Dose-dependently decreased the lipid peroxidation in the serum of the animals, demonstrated by lowering the thiobarbituric acid reactive substances levels, most pronounced at a dose of 10 ml/kg.

3.7. At all doses used, histological changes typical of metabolic syndrome were decreased and with the highest dose of Chaenomeles maulei fruit juice used:

3.7.1. Structural damage of cardiomyocytes in the myocardium was prevented;

3.7.2. Endothelial and basal membrane integrity in coronary vessels were preserved;

3.7.3. Degenerative and steatotic changes in the liver were reduced;

3.7.4. Adipocyte hypertrophy was prevented and the size of adipocytes remained similar to that of control animals.

3.8. Did not influence locomotor activity when compared to high-fat high-fructose diet-fed animals that were not treated with the juice.

3.9. Dose-dependently reduced the anxiety levels in high-fat high-fructose diet-fed experimental animals as demonstrated by the increase in time spent in the open arms and in the ratio of time spent in the open arms to total time spent in any of the arms of the elevated plus maze test apparatus.

3.10. Prevented the high-fat high-fructose diet-induced tendency of spatial memory impairment.

3.11. Did not influence depressive-like behaviour in Forced swim test.

VI. Contributions

1. For the first time, Chaenomeles maulei fruit juice effects in an experimental model of metabolic syndrome were evaluated.

2. For the first time, Chaenomeles maulei fruit juice effects on energy metabolism in rats in a model of diet-induced metabolic syndrome were evaluated and it was established that it:

2.1. Increased food consumption;

2.2. Decreased fructose solution consumption;

2.3. Increased total caloric intake of animals.

3. For the first time, Chaenomeles maulei fruit juice effects on visceral adiposity in rats in a model of diet-induced metabolic syndrome were evaluated and it was established that it:

3.1. Decreased visceral adipose tissue, even in light of the increased caloric intake;

3.2. Decreased mesenterial adipose tissue;

3.3. Decreased paranephral adipose tissue.

4. For the first time, data was gathered about stimulation of the endogenous antioxidant defenses and inhibition of lipid peroxidation by Chaenomeles maulei fruit juice in rats in a model of diet-induced metabolic syndrome.

5. For the first time, Chaenomeles maulei fruit juice effects on the histology of the myocardium, coronary vessels, liver and adipose tissue in rats in a model of diet-induced metabolic syndrome were evaluated. It was established that the juice:

5.1. Exerted a dose-dependent cardio- and vasoprotective effect;

5.2. Prevented the high-fat high-fructose diet-induced changes in adipose tissue and preserved the normal size of adipocytes in all doses used;

5.3. Led to a dose-dependent prevention of the high-fat high-fructose diet-induced liver impairment.

6. For the first time the effects of Chaenomeles maulei fruit juice on the behaviour of rats in a model of diet-induced metabolic syndrome were evaluated, and it was established that the juice:

6.1. Did not influence locomotor activity;

6.2. Exerted a dose-dependent anxiolytic effect;

6.3. Showed a tendency for improving the spatial memory;

6.4. Did not influence the depressive-like behaviour.

7. The conducted research on Chaenomeles maulei fruit juice contributed to the better understanding of the effects of the juice and of the polyphenols in its content in the context of metabolic syndrome.

VII. List of publications and congress participations related to the dissertation

1. List of publications related to the dissertation

1.1. Moneva K, Gancheva S, Valcheva-Kuzmanova S. Chemical composition and biologic activities of different preparations of Japanese quince (Chaenomeles japonica). ASN 2023; 10(2): 39-54. DOI: 10.2478/asn-2023-0013.

1.2. Moneva-Marinova K, Valcheva-Kuzmanova S. Caloric restriction mimetics as a therapeutic approach to metabolic syndrome. VMF 2023.

1.3. Moneva-Marinova K, Tzaneva M, Gasanzadeeva E, Abtulov M, Salbashyan M, Eftimov M, Marinova S, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Japanese quince fruit extract exerts a cardioprotective effect in a model of diet-induced metabolic syndrome in rats. JBCR 2023: 16(2): 118-123.

1.4. Moneva-Marinova K, Rafailova E, Reyzov M, Todorova M, Eftimov M, Gancheva S, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Behavioral effects of chronic Japanese quince fruit juice administration to rats with diet-induced metabolic syndrome. Acta Medica Bulgarica 2024 **(IF 2023: 0.12)** [in print]

2. List of congress participations rellated to the dissertation

2.1. K. Moneva-Marinova, M. Tsaneva, M. Todorova, S. Gancheva, M. Eftimov, M. Reyzov, E. Rafailova, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova. Histopathological evaluation of the effects of Chaenomeles maulei fruit juice on the liver in rats with diet-induced metabolic syndrome. 8th Congress of Pharmacy with International Participation, 27-30 April, Borovets, Bulgaria; p.201.

2.2. Moneva-Marinova K , Eftimov M, Todorova M , Gancheva S , Reyzov M , Rafailova E , Zhelyazkova-Savova M , Valcheva-Kuzmanova S. Evaluation of the effects of Chaenomeles

maulei fruit juice on spatial memory and social behavior in rats with metabolic syndrome. 36th ECNP Congress, 7-10 October 2023, Barcelona, Spain; p.2014.