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Posted Date: 5 February 2026

doi: 10.20944/preprints202602.0446.v1

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Effects of *Chaenomeles japonica* Fruit Juice on Energy Balance, Biochemical and Histological Parameters in a Model of Diet-Induced Metabolic Syndrome in Rats

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Abstract

Background/Objectives: Metabolic syndrome (MS) is associated with an increased cardiovascular risk. The aim of this study was to reveal the effects of *Chaenomeles japonica* fruit juice (CJFJ) on energy balance, biochemical and histological parameters in rats with diet-induced MS. **Methods**: Fifty Wistar rats were allocated into 5 groups. For ten weeks, the Control group received a standard laboratory diet and tap water, while the other groups were given a high-fat high-fructose (HFHF) diet. Control and MS groups were treated with distilled water, while the other three groups – with CJFJ at increasing doses. **Results**: Rats on HFHF diet consumed less food, more liquids and had a higher caloric intake than group Control. Among the CJFJ-treated animals, increased food consumption, as well as increased total caloric intake, and no difference in body weight gain were observed in comparison with group MS. CJFJ did not affect glucose tolerance, triglyceride and total cholesterol levels. CJFJ prevented HFHF-induced decrease in superoxide dismutase and caused a decrease in thiobarbituric acid reactive substances in serum. The medium CJFJ dose prevented HFHF-induced increase in adipose tissue indices. Liver and adipose tissue histology revealed a protective effect of CJFJ. **Conclusions**: CJFJ exhibited appetite-stimulating, antioxidant, antiobesogenic and liver-protective properties in rats with diet-induced MS.

Keywords: *Chaenomeles japonica*; Japanese quince; metabolic syndrome; antiobesogenic; hepatoprotective; rats

1. Introduction

Metabolic syndrome (MS) encompasses a cluster of metabolic disturbances, including insulin resistance, visceral obesity, hypertension and dyslipidemia, that increases the risk of cardiovascular disease and diabetes type 2, among others. Unhealthy nutritional patterns are often a major underlying cause. Insulin resistance, chronic low-grade inflammation and oxidative stress are some of the fundamental drivers and contributors to the pathogenesis of MS. One of the earliest sources of this inflammation, also regarded as 'metainflammation' or metabolic-induced inflammation, is suspected to be adipocyte hypertrophy and hyperplasia. This facilitates the occurrence of hypoxia in adipocytes far away from blood vessels, followed by necrosis, phagocyte accumulation and local inflammation that is intended to dispose of damaged cells [1]. Oxidative stress is generated, that drives the inflammatory process further, brings about inflammatory cytokine production and leads

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to cellular injury. Over time, pathological mechanisms unfold in various directions, engaging multiple pathways associated with MS. Despite its increasing prevalence and decreasing age of onset, limited advance is achieved towards its prevention and management.

While dietary interventions remain one of the commonest approaches to MS, whether fruit juice (FJ) has a justifiable place as a part of them is highly controversial. While a valid source of vitamins, minerals and bioactive substances such as polyphenols, FJ is also a source of free sugars that can easily outweigh the health benefits, especially in the higher dose range. When investigating the relation of FJ with MS and with each of its components on its own, results are highly variable based on study design, context, etc. Part of the discrepancies arise from failure to differentiate between FJ with added sugar and 100% FJ with evidence showing that their influence on metabolic outcomes is quite distinct [2]. Another discrepancy comes from the difficulty of properly estimating the exact amount of FJ consumed daily. D'Elia et al. report an inverse relationship between 100% FJ consumption and cardiovascular risk, in particular the risk of stroke [3]. In a prospective cohort study including 34560 participants, Scheffers et al. found that consumption of up to 7 glasses of pure FJ per week was associated with decreased risk of cardiovascular disease, while this benefit was lost with consumption of 8 or more glasses weekly [4]. What is more, consumption of up to 8 glasses of pure FJ weekly was also associated with a lower risk of stroke. The intake of 100% FJ was also associated with a decreased rate of anxiety in adults [5]. A meta-analysis of prospective studies including 49591 participants reported a U-shape dose-response association between 100% FJ consumption and MS with protection at moderate doses [6]. It has been proposed that the inverse association between moderate FJ intake and MS is due to the nutritional content while at higher amounts the damaging properties of excessive sugar outweigh the benefits [7]. Recommendations for FJ intake vary greatly among countries [4,8,9] due to the mixed and inconclusive data.

The abundance of artificially sweetened beverages on the market and their increasing consumption draws the attention to the high consumer interest in similar drinks, while also causing disappointment since they are actually associated with increased risk of obesity [10], even though they provide fewer calories than the sugar-sweetened ones. So it seems like the quest for an alternative continues. Even though whole fruit consumption is generally regarded as the better option than consumption of FJ, juice might offer some advantages such as a better nutrient bioavailability [8].

Plants used in traditional eastern medicine are a focus of attention in the context of metabolic diseases. The genus *Chaenomeles*, belonging to the Rosaceae family, consists primarily of five species: *Chaenomeles speciosa*, *Chaenomeles thibetica*, *Chaenomeles cathayensis*, *Chaenomeles japonica*, and *Chaenomeles sinensis* [11]. While originating in East Asia, the plants have been introduced to all the continents on Earth except for Antarctica [12]. In vitro studies have reported antioxidant, lipid- and glucose-regulating, hepatoprotective, antineoplastic and antimicrobial effects of Chaenomeles [12]. The relatively low fructose content of Chaenomeles fruit juice in combination with the high concentration of polyphenolic compounds makes it a good candidate for use in obesity-related disorders. Fruit extracts from different *Chaenomeles* species have shown protective qualities in experimental models of diabetes mellitus [13], atherosclerosis [14], hyperuricemia [15], depression [16]. *Chaenomeles japonica* (Thunb.) Lindl, also known as Japanese quince, is a species with many potential health benefits, few of which have been thoroughly studied.

The aim of the present study was to reveal the effects of *Chaenomeles japonica* fruit juice (CJFJ) on energy balance, biochemical and histological parameters in rats with diet-induced MS.

2. Results

2.1. Effects of Chaenomeles japonica Fruit Juice on Energy Balance

Parameters related to the energy balance are presented in Figure 1. Animals from the groups that were on a high-fat high-fructose (HFHF) diet consumed a smaller amount of food compared to

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group Control ($p < 0.001$). Furthermore, a difference between group MS and the treated groups ($p < 0.001$) was observed – CJFJ at doses of 5 ml/kg and 10 ml/kg increased the amount of food consumed.

The consumption of the 10% fructose solution by the groups on the HFHF diet was significantly higher ($p < 0.001$) as compared to the consumption of tap water by group Control. The analysis also revealed a difference between group MS and group MS+CJFJ5 – CJFJ at the dose of 5 ml/kg reduced the liquids intake ($p < 0.05$).

The total caloric intake in all groups on the HFHF diet was higher ($p < 0.001$) than that in group Control. CJFJ at doses of 5 ml/kg and 10 ml/kg increased ($p < 0.001$) the caloric intake in comparison to group MS.

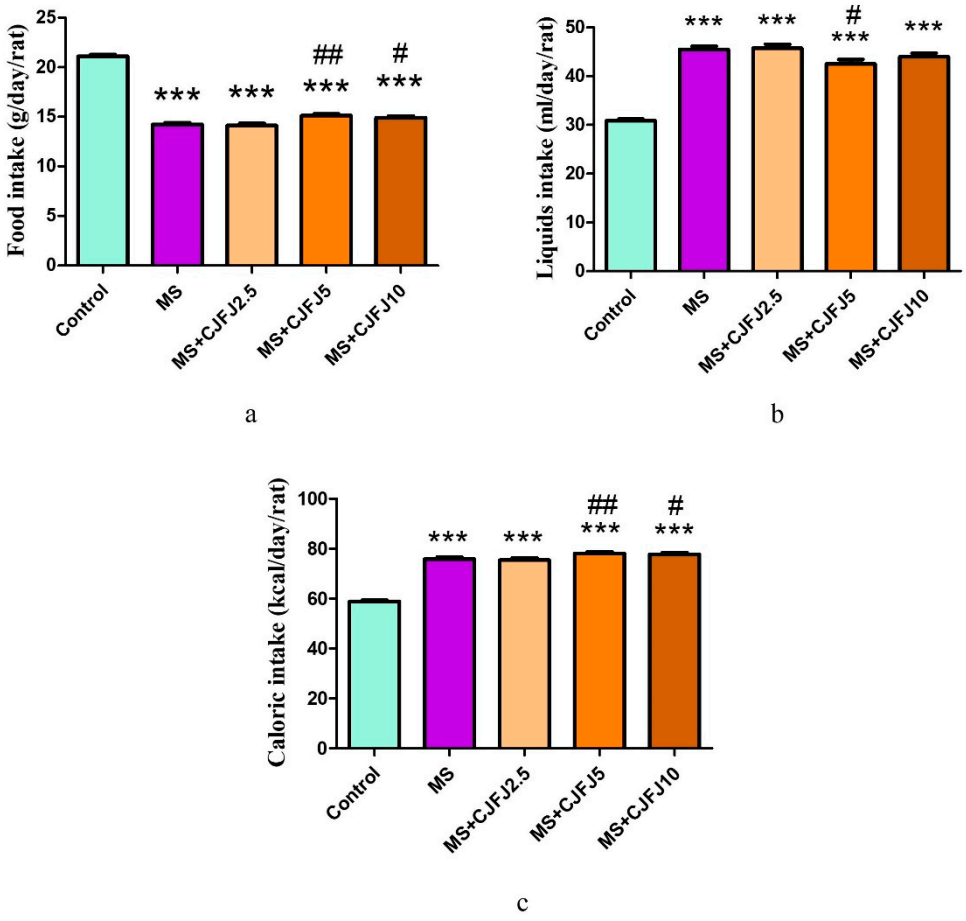

Figure 1. Food (a), liquids (b) and caloric intake (c) in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg. ***p<0.001 compared to group Control, ##p<0.01, #p<0.05 compared to group MS.

2.2. Effects of Chaenomeles japonica Fruit Juice on Total Body Weight

Body weights throughout the experiment are shown in Figure 2a. Table 1 presenting body weights of the animals at the beginning and at the end of the experiment. Mean body weight gain did not differ among the animals from all experimental groups (Figure 2b). What is more, as depicted in Figure 2a, the body weight gain in all HFHF-diet groups followed the same variation pattern as the Control group throughout the entire experiment.

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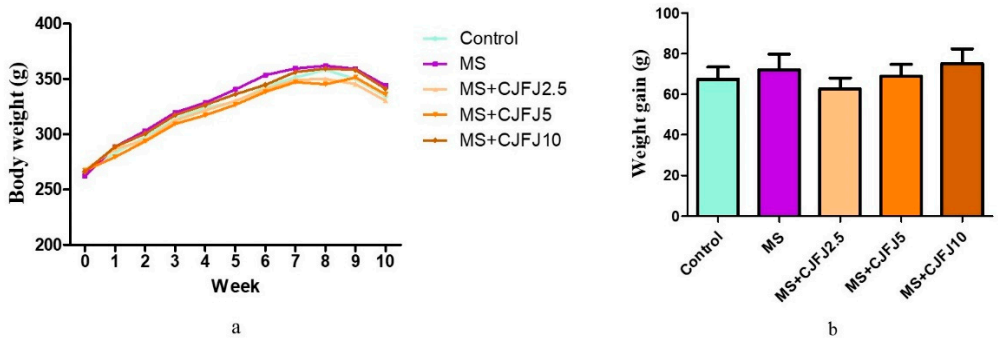

Figure 2. Body weight (a) and mean body weight gain (b) during the 10 weeks of the experiment in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

Table 1. Initial and final body weight in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg.

	Control	MS	MS+CJFJ2.5	MS+CJFJ5	MS+CJFJ10
Initial body weight (g)	266.8±8.08	262.3±5.56	267.8±5.56	267.2±5.88	266.0±5.43
Final body weight (g)	334.2±8.21	344.0±7.38	330.4± 9.00	336.0± 8.41	341.2± 10.61

2.3. Effects of Chaenomeles japonica Fruit Juice on Glucose Tolerance Test (GTT)

The results from the GTT are presented in Table 2. 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+CJFJ2.5, 4.38±0.15 in MS+CJFJ5 и 4.34±0.15 in MS+CJFJ10). At the 30th minute, a significant increase in glucose in MS group compared to Control was observed ($p<0.05$). CJFJ administration did not significantly affect glucose tolerance as compared to group MS on any minute in the doses used.

Table 2. Plasma glucose levels during a glucose tolerance test, presented as absolute values (mmol/l) before as well as on the 30th, 60th and 90th minute after the glucose load in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05 as compared to Control, **p<0.01 as compared to Control.

	Control	MS	MS+CJFJ2.5	MS+CJFJ5	MS+CJFJ10
0 min	4.46±0.09	4.48±0.14	4.44±0.09	4.38±0.15	4.34±0.15
30th minute	11.97±0.78	15.92±1.14**	17.27±0.72***	16.64±1.26**	15.58±0.51*
60th minute	8.13±0.26	9.93±0.53	10.82±0.74	9.83±0.63	10.05±0.56
90th minute	7.41±0.25	8.73±0.28	8.95±0.35	8.69±1.07	8.34±0.28

2.4. Effects of Chaenomeles japonica Fruit Juice on Fat Indices

The weight of total, mesenteric, paranephral, perigonadal and retroperitoneal fat pads as well as their respective estimated indices were significantly increased in group MS in comparison with group Control. Total fat tissue index was significantly increased in groups MS, MS+CJFJ2.5 and MS+CJFJ10 compared to the Control, while its values in MS+CJFJ5 remained similar to those of the Control (Figure 3). A significant decrease in total fat tissue index in group MS+CJFJ5 as compared to group MS was observed. CJFJ intake also affected ($p=0.0339$) mesenteric adipose tissue index in group MS+CJFJ5 as compared to group MS (Figure 4a). The same dose of CJFJ also decreased paranephral adipose tissue index in comparison to group MS (Figure 4b).

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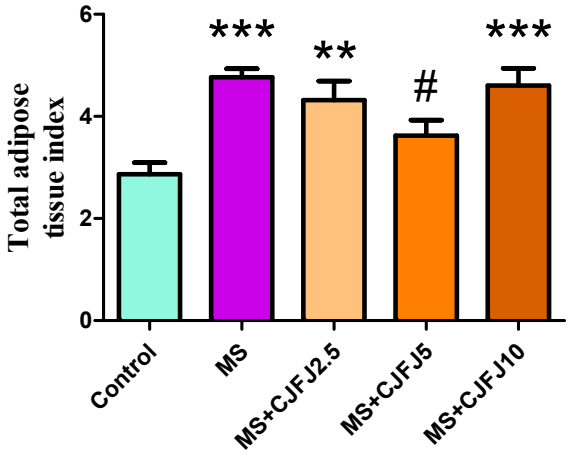

Figure 3. Total adipose tissue index in rats with diet-induced metabolic syndrome (MS), treated with *Chaenomeles japonica* fruit juice (CMFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg. *p<0.05, **p<0.01, ***p<0.001 compared to group Control, #p<0.05 compared to group MS.

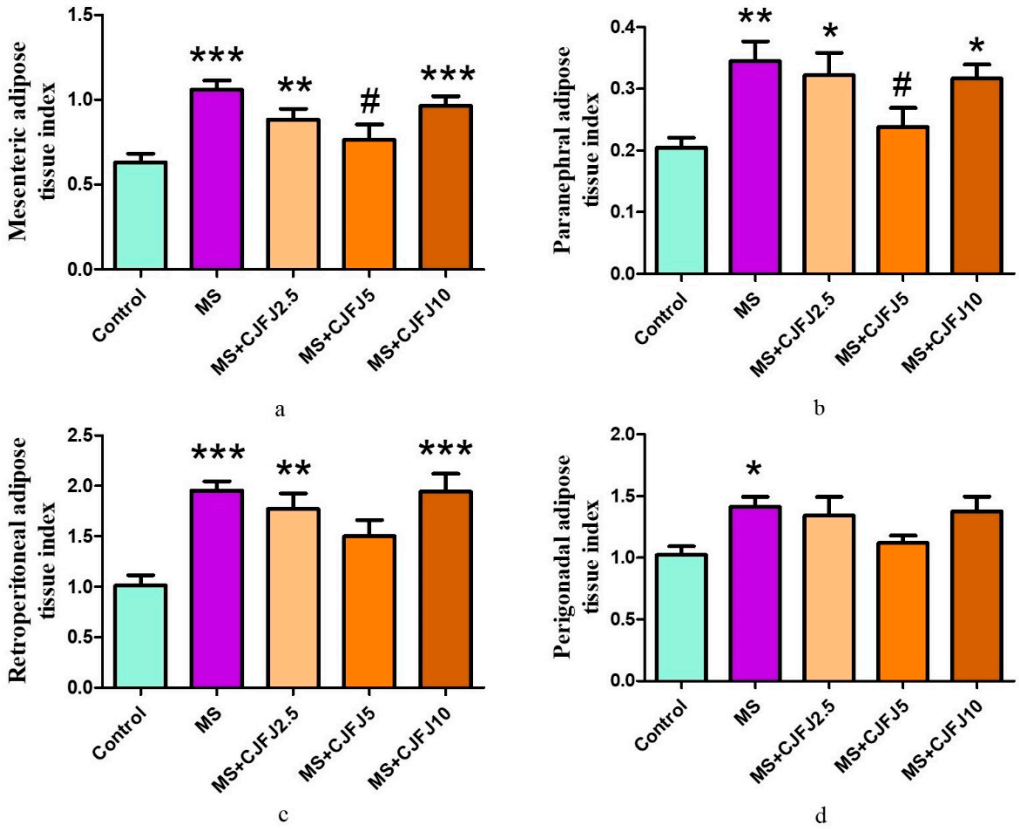

Figure 4. Mesenteric (a), paranephral (b), retroperitoneal (c) and perigonadal (d) adipose tissue indices in rats with diet-induced metabolic syndrome (MS), treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05, **p<0.01, ***p<0.001 compared to group Control, #p<0.05 compared to group MS.

2.5. Effects of Chaenomeles japonica Fruit Juice on Liver Index

Liver index results are presented on Figure 5. As a result of the HFHF diet, liver index values were significantly increased in groups MS and MS+CJFJ2.5 (p=0.0343). The values observed in groups MS+CJFJ5 and MS+CJFJ10 were comparable with those in group Control.

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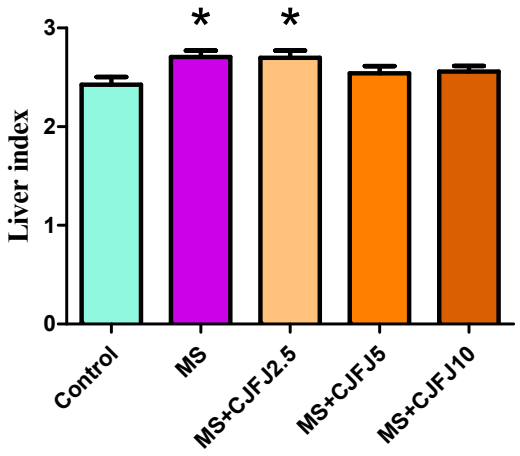

Figure 5. Liver index in rats with diet-induced metabolic syndrome (MS) treated with Chaenomeles japonica fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05 compared to group Control.

2.6. Effects of Chaenomeles japonica Fruit Juice on the Lipid Profile

Serum triglyceride levels in group MS were significantly increased (p<0.001) in comparison with the Control group (1.23±0.15 compared to 0.67±0.04) (Figure 6a). Subjecting the animals to HFHF diet led to almost doubling the values compared to the rats receiving the standard laboratory diet. CJFJ intake reduced, but not significantly, the triglycerides levels and these changes were most pronounced in group MS+CJFJ5 (0.98±0.07 compared to 1.23±0.15 in group MS). As shown in Figure 6b, total cholesterol levels in group MS were increased (2.00±0.22) compared to group Control (1.77±0.11) without reaching statistical significance. CJFJ at any of the doses used did not significantly affect total cholesterol levels.

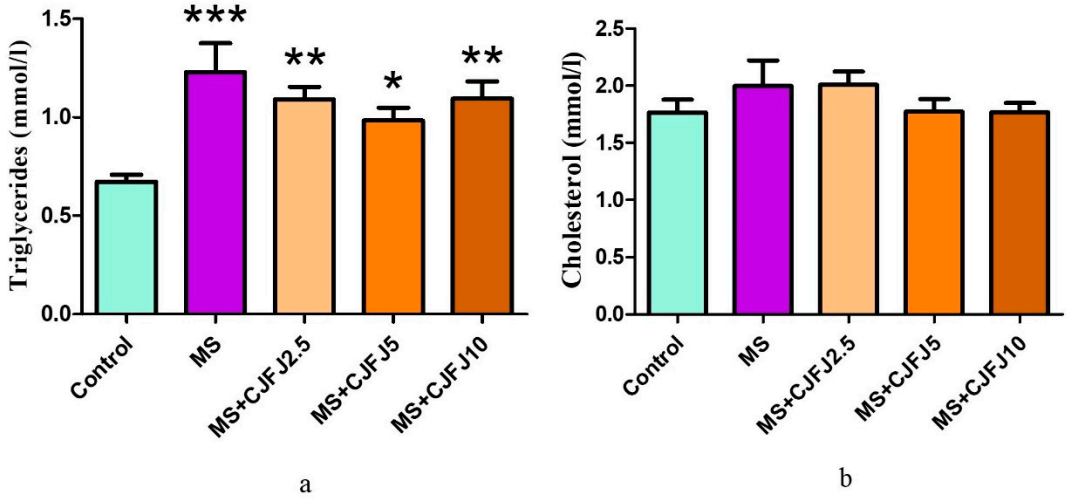

Figure 6. Serum triglycerides (a) and total cholesterol levels (b) in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05, **p<0.01, ***p<0.001 compared to group Control.

2.7. Effects of Chaenomeles japonica Fruit Juice on Triglyceride/Glucose (TyG) Index

The triglyceride/glucose (TyG) index was significantly increased in all groups of animals consuming HFHF diet compared to group Control (p=0.0002), as observed in Figure 7. Administration of CJFJ did not significantly influence the TyG index.

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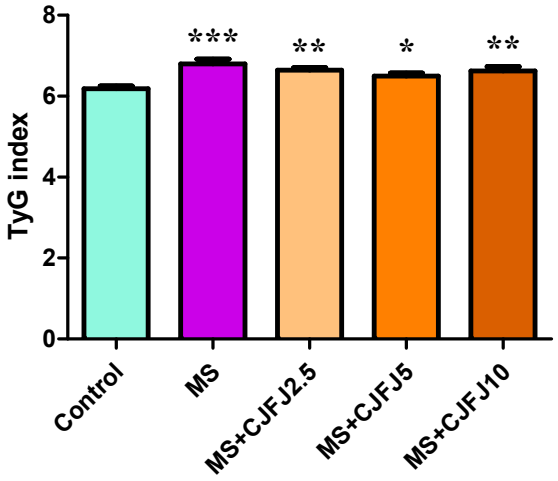

Figure 7. TyG index in rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05, **p<0.01, ***p<0.001 compared to group Control.

2.8. Effects of Chaenomeles japonica Fruit Juice on Biochemical Markers of Antioxidant Defense and Oxidative Stress

Results from the serum activity of the antioxidant enzyme superoxide dismutase (SOD) are illustrated in Figure 8a. A statistically significant difference between the groups (p=0.0173) was observed: there was a lower activity of the enzyme in group MS as well as in group MS+CJFJ10 in comparison with group Control. The treatment with CJFJ at doses of 2.5 ml/kg and 5 ml/kg prevented the HFHF diet-induced reduction of the enzyme activity, illustrated by the fact that there was no statistically significant difference between group Control and groups MS+CJFJ2.5 and MS+CJFJ5.

In the serum of animals from group MS, higher values of the thiobarbituric acid reactive substances (TBARS) were observed as compared to group Control (130.5±21.09 versus 85.32±7.73), but the difference did not reach statistical significance (Figure 8b). CJFJ intake caused a linear trend (p=0.0105) towards a decrease of the TBARS serum levels. In MS+CJFJ10 group they were significantly reduced (p<0.05) compared to those in group MS and were comparable to the values of group Control.

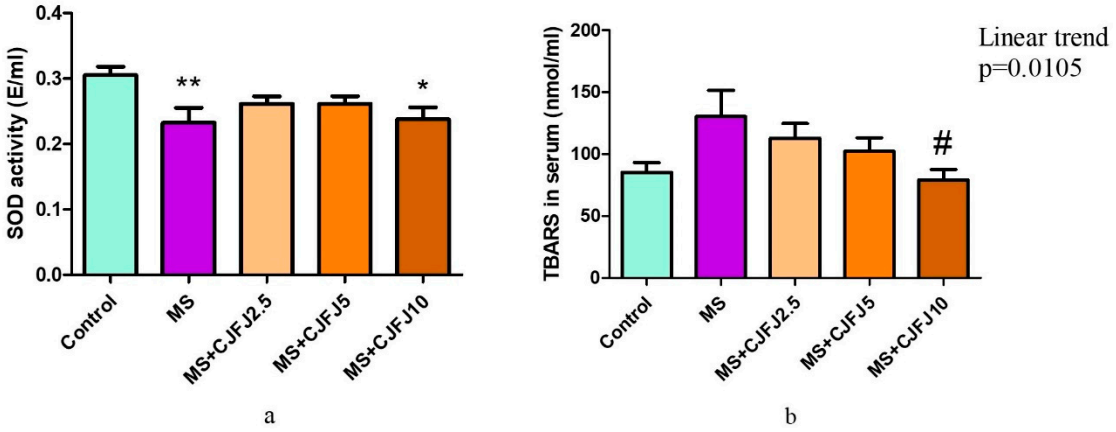

Figure 8. Activity of the enzyme superoxide dismutase (SOD) (a) and levels of thiobarbituric acid reactive substances (TBARS) (b) in the serum of rats with diet-induced metabolic syndrome (MS) treated with *Chaenomeles japonica* fruit juice (CJFJ) at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg; *p<0.05, **p<0.01 compared to group Control.

2.9. Correlations of SOD and TBARS with Other Biochemical Parameters and with Fat Indices

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As shown on Figure 9, SOD negatively correlated with triglycerides (r = -0.473, P=0.0006), TyG index (r = -0.481, P=0.0008), mesenteric (r = -0.454, P=0.0010) and retroperitoneal (r = -0.415, P=0.0030) fat indices, as well as with glucose levels on the 30th min of GTT (r = -0.365, P=0.0128). TBARS positively correlated with triglycerides (r = 0.408, P=0.0040) and with TyG index (r = 0.423, P=0.0042). No correlation was found between both SOD and TBARS and the rest of the parameters evaluated.

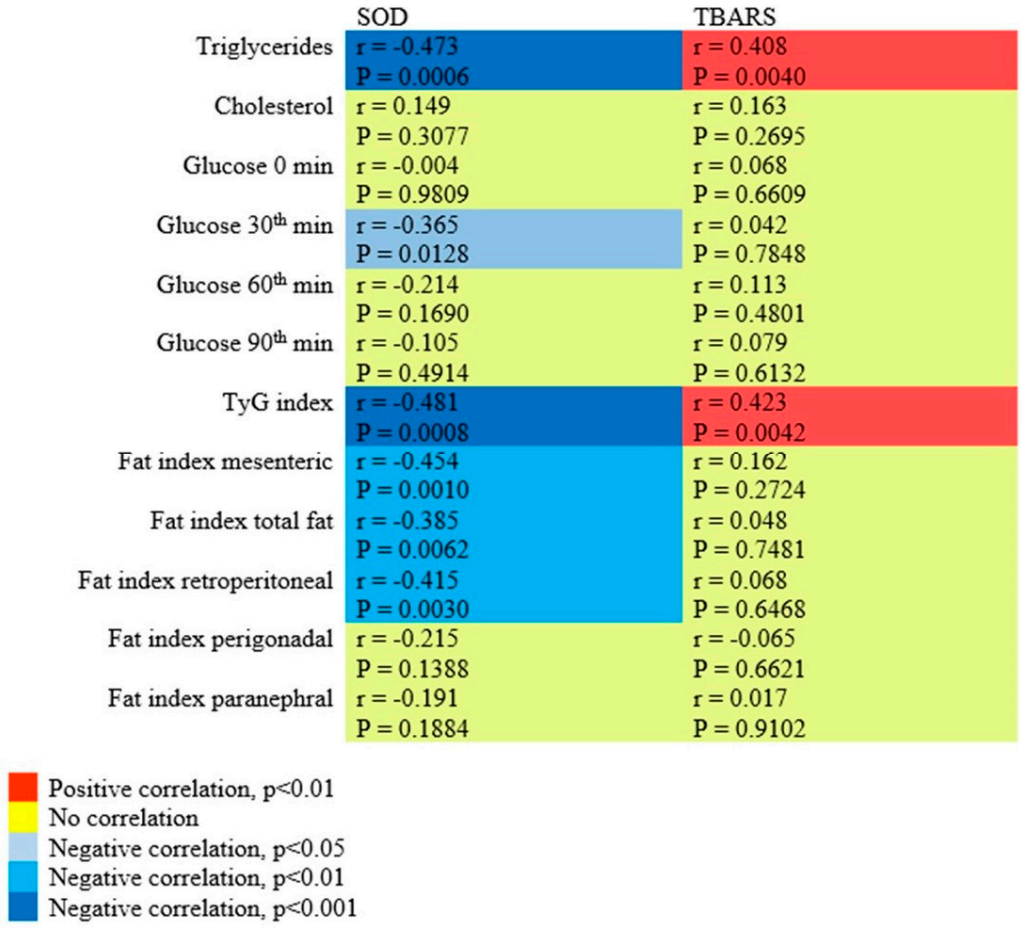

Figure 9. Pearson correlation heat map between SOD and TBARS and other biochemical parameters and fat indices. The red colour represents a positive correlation and the blue colour represents a negative correlation. The depth of the colour stands for the size of the correlation coefficient. Darker colour visualizes a stronger correlation, while lighter colour visualizes a weaker correlation. The yellow colour represents no significant correlation.

2.10. Effects of Chaenomeles japonica Fruit Juice on Liver and Adipose Tissue Histology

2.10.1. Effects of *Chaenomeles japonica* Fruit Juice on Liver Histology

Results from the histopathological examination are presented in Figure 10. In group Control, a normal structure of the liver was observed. In the animals from group MS a microvesicular steatosis, liver necrosis and non-specific granulomas were observed. In group MS+CJFJ2.5, a smaller number of hepatocytes were affected by the microvesicular steatosis. In groups MS+CJFJ5 and MS+CJFJ10, single non-specific granulomas and single hepatocytes with fatty degeneration were observed. Thus, CJFJ treatment led to a dose-dependent reduction in the liver damage induced by the HFHF diet without completely preventing the occurrence of degenerative changes.

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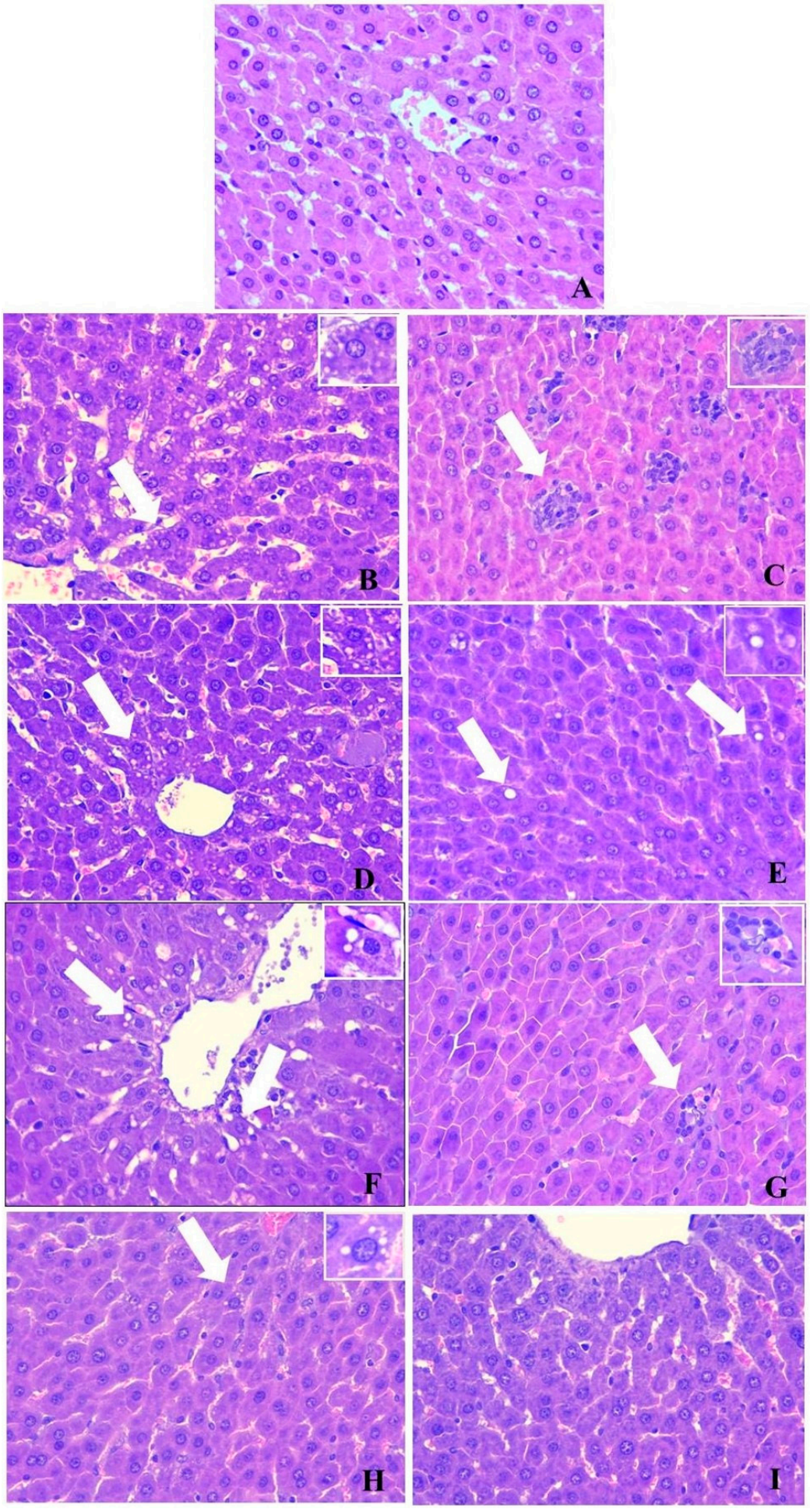

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Figure 10. Microscopic appearance of the liver of groups: Control (panel A): normal structure of liver tissue; MS: microvesicular steatosis around v. centralis (panel B), nonspecific granulomas (panel C); MS+CJFJ2.5: microvesicular steatosis around v. centralis (panel D), single hepatocytes with fatty degeneration (panel E); MS+CJFJ5: fatty degeneration around vena centralis (panel F), single nonspecific granulomas (panel G); MS+CJFJ10: single hepatocytes with fatty degeneration (panel H), normal structure of liver tissue (panel I); Hematoxylin-eosin staining, magnification x 200.

2.10.2. Effects of *Chaenomeles japonica* Fruit Juice on Adipose Tissue Histology

The microscopic appearance of the adipose tissue of the experimental animals is presented in Figure 11. Group Control demonstrated a normal structure of adipose tissue with small and medium-size adipocytes prevailing and single large-size adipocytes also observed. In animals from group MS, adipocytes were with a larger size compared to group Control. Treatment with CJFJ dose-dependently prevented the development of the HFHF diet-induced adipocyte enlargement. In MS+CJFJ2.5 group, large-size adipocytes were still prevailing, while in MS+CJFJ5 and MS+CJFJ10 groups, the histological picture was similar to that observed in group Control.

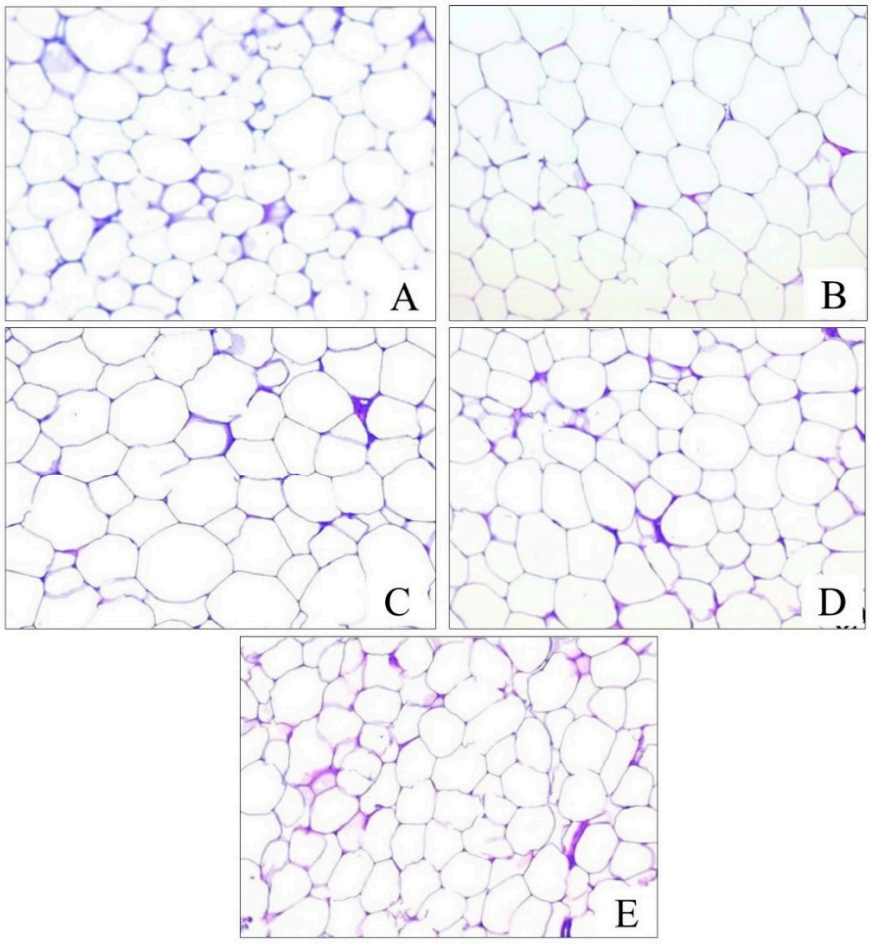

Figure 11. Histological structure of adipose tissue from groups: Control (panel A), MS (panel B), MS+CJFJ2.5 (panel C), MS+CJFJ5 (panel D) and MS+CJFJ10 (panel E); Hematoxylin-eosin staining, magnification x 200.

3. Discussion

All animals on HFHF diet consumed a smaller amount of food compared to group Control. Probably, the elevated carbohydrate and lipid content of the HFHF diet contributed to a high energy density and caused a reduced food intake. What is more, part of the daily caloric intake was provided by the fructose solution. It has been continuously observed that calories ingested from fructose, especially in a liquid form, are not completely compensated for by reducing the amount of other food

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in order to maintain the usual daily caloric intake [17]. Instead, what is often observed is an increased energy consumption, which is consistent with our data – energy intake was expectedly higher in all animals consuming HFHF diet. Liquids intake was higher in all groups fed a HFHF diet compared to group Control. This is most probably due to the palatability of the fructose solution. The design of the current diet, most of all positioning fructose as the main carbohydrate, allows the reproduction of the hedonistic features of the pathological feeding patterns often contributing to the human MS condition. Additionally, fructose is also known to induce thirst, possibly by increasing serum osmolality due to a shifting of water into the cell for the purpose of glycogenesis [18]. CJFJ at doses of 5 ml/kg and 10 ml/kg increased the amount of food consumed as well as the energy intake. This finding is consistent with one of the popular uses of *Chaenomeles* species in traditional medicine as an appetite stimulant [19]. According to the authors' knowledge, this is the first scientific report in support of this traditional use. Liquids intake was reduced by CJFJ at a dose of 5ml/kg. This is suggestive of a possible modulatory effect of the polyphenols found in CJFJ on fructose-induced thirst.

Body weight of animals did not appear to be affected by the HFHF diet or by CJFJ. This is a common finding in diet-induced models of MS [20–22], and resembles the clinical experience with humans as visceral adiposity and not total body adiposity is a more accurate marker of MS [23]. In fact, distribution of body fat is considered a cornerstone in the pathogenesis of MS. Lipid accumulation in visceral depots, as well as in insulin-sensitive organs such as the liver and the skeletal muscles, plays a pivotal role in development and continuous reinforcement of insulin resistance as well as the typical low-grade inflammation [23,24]. At the same time, 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 [24]. These are exactly the reasons why the body mass index is no longer considered as the most appropriate way to estimate the obesity criteria as a part of MS, but rather waist circumference or other markers are preferred for being more accurate. The increase in total, mesenteric, paranephral, perigonadal and retroperitoneal fat tissue weights, as well as in their estimated indices, in group MS in comparison to group Control, signifies the development of visceral obesity as a consequence of the HFHF diet. Treatment with CJFJ with the medium dose of 5 ml/kg led to a decrease in total, mesenteric and paranephral fat tissue indices in comparison to group MS, suggestive of anti-obesity properties. The loss of visceral fat tissue has been clinically verified to lead to a number of beneficial metabolic effects, including decreased systemic inflammation [25], improvement of indices such as fasting blood glucose, triglycerides and HOMA-index [26], etc. A study with 172 obese adolescent participants considers visceral fat tissue reduction to be an independent predictor of ameliorating insulin resistance, hyperleptinemia and other metabolic disturbances [27]. Based on these data, the observed reduction of visceral adipose tissue by CJFJ suggests that it could be a potentially appropriate diet intervention in patients with MS.

It is established that mesenteric fat, drained by the portal circulation, is metabolically more active than other nonportal adipose tissues; it was also found to be an independent determinant of MS and associated with increased carotid intima-media thickness [28]. Mesenteric fat is considered a possible prognostic factor for fatty liver and polycystic ovary syndrome [29] – both conditions being deeply intertwined with MS. A significant correlation is observed between mesenteric fat and both atherogenic LDL apoB particles and apoAII levels, the latter being relevant to the role of mesenteric fat as a source of triglycerides in the fasting state [29]. While noticing anti-obesity properties in an appetite stimulant may seem somewhat surprising, such a combination is plausible if thermogenesis is affected or digestive enzymes (involved in the digestion of carbohydrates and fats) are inhibited. Thermogenesis in rodents is considered to contribute to 15-20 % of daily energy expenditure [30]. Therefore, influencing this process can have a significant impact on the energy metabolism and body weight of animals. Several of the predominating polyphenols in CJFJ have been reported to have thermogenic properties: epicatechin, chlorogenic acid, ellagic acid [31], quercetin [32], vanillic acid [33], p-coumaric [34,35]. At the same time, *Chaenomeles japonica* extract has already been shown to

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inhibit pancreatic lipase and α-amylase [36], so both of the aforementioned mechanisms could be involved in the anti-obesity properties we observed.

Non-alcoholic fatty liver disease is considered the liver manifestation of MS. Nowadays, it is even referred to as metabolic dysfunction-associated fatty liver disease (MAFLD) [37]. It is a spectrum of pathological conditions, including steatosis, steatohepatitis, cirrhosis and hepatocellular carcinoma. Fructose-related stimulation of de novo lipogenesis and fatty liver has been observed both in animal and in human studies [17]. Results from both liver index and liver histology in this experiment are suggestive of a dose-dependent hepatoprotective effect of CJFJ. On the subcellular level, increased endoplasmic reticulum stress is a key process involved in mediating the liver damage in MAFLD. The exceptional amounts of reactive oxygen species, initially originating from the food overload and later amplified by the many pathological pathways activated in MS, surpass the peroxisomes' capacity to regulate them and spread to the endoplasmic reticulum where they alter the proper environment needed for protein folding. Chronic endoplasmic reticulum stress is associated with lipotoxicity, insulin resistance and inflammation [38]. Therefore, we can presume that the demonstrated hepatoprotective effects of CJFJ are at least partly mediated by the antioxidant properties of the juice. Many of the polyphenols in CJFJ are reported to increase the liver expression of PPARα [39] – a transcriptional factor responsible for energy metabolism modulation and considered a possible target for MAFLD, MS and cardiovascular diseases alleviation [40,41]. The hepatoprotective activities of CJFJ in this experiment could also be attributed to its high carotenoid content. Carotenoids express pronounced anti-inflammatory and antioxidant effects and modulate a number of intracellular pathways. They have been reported to inhibit steatosis by a change in inflammatory genes' expression, regulation of T-cells number and scavenging free radicals in similar experiments with rodents [42].

Data from the glucose tolerance tests suggested normoglycaemia, but impaired glucose tolerance as a result of the HFHF diet. CJFJ did not affect glucose tolerance in the doses used. While hypoglycemic effects of *Chaenomeles japonica* polyphenolic extract have been reported in an in vitro study [43], according to our knowledge this activity of the species has not yet been demonstrated in vivo.

Dyslipidemia is one of the central features of MS. 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 [44]. Dyslipidemia develops partly in response to insulin resistance. The impairment of physiological insulin suppression of lipolysis in adipocytes results in increased levels of free fatty acids that act as a substrate for triglycerides synthesis in the liver [45]. Increased levels of oxidative stress and systemic inflammation also contribute to the development of dyslipidemia [46]. In this study, serum triglycerides were increased in all groups receiving HFHF diet. This could be explained by the particular metabolic properties of fructose – a major component of the HFHF diet that is responsible for providing a significant part of the daily caloric intake of the experimental animals. It is known that fructose 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 [24]. As a response to fructose intake, a long-lasting postprandial dyslipidemia is usually observed, ghrelin secretion is not adequately suppressed and leptin secretion is low, contributing to a continuous feeling of hunger despite the calories ingested [24,47,48]. The development of dyslipidemia in response to overconsumption of fructose has also been confirmed by clinical data [49]. In this study, no significant difference in the total cholesterol levels was observed among the experimental groups. These findings are consistent with available literature showing largely unchanged total cholesterol levels resulting from low HDL cholesterol levels and increased LDL levels – key features of the atherogenic dyslipidemia found in MS [45]. Despite lipid-lowering effects being reported for many of the polyphenols found in CJFJ [50], triglyceride levels were not significantly affected in any of the doses used. Still, the triglyceride values in all CJFJ-treated groups remained lower than those in group MS. This may be related to the fact that some of the effects of

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polyphenols in the body, such as modulation of gut microbiota – one of the mechanisms potentially involved in insulin resistance and dyslipidemia amelioration [51], may require longer-term exposure in order to be manifested.

The TyG index has been recently used as a surrogate marker of insulin resistance [52]. In the present study it was shown to be significantly increased in response to the HFHF diet, suggesting the development of insulin resistance. Treatment with CJFJ did not significantly affect the TyG index in the doses used, even though all estimated values in the treated groups were lower than those in group MS.

Oxidative stress is not only a central feature of visceral obesity and MS but also one of the common denominators between MS and cardiovascular disease [53]. Decreased endogenous antioxidant abilities are a distinctive trait of MS and further contribute to aggravation of MS-induced tissue damage. SOD is a major endogenous free radical scavenger responsible for the breakdown of superoxide radicals [53]. The current study reveals a significant decrease in the serum SOD activity in rats with diet-induced MS, which is consistent with existing literature [54]. The treatment with CJFJ at doses of 2.5 ml/kg and 5 ml/kg prevented the HFHF diet-induced reduction of SOD activity in the serum of animals, suggestive of improvement of the endogenous cellular antioxidant capacity. Restoration of physiological antioxidant capacities via serum SOD elevation was also shown for *Chaenomeles speciosa* fruit powder in an in vivo experiment with oxidative stress induced by exhaustive exercise in rats [55]. In this study, SOD negatively correlated with triglycerides, TyG index and glucose levels on the 30th min of GTT, indicating a close relationship between oxidative stress and lipid and glucose metabolism. Such correlations have been previously observed [54].

Levels of thiobarbituric acid reactive substances are evaluated as a marker of lipid peroxidation. In the current study, increased values were observed in group MS as compared to group Control. Such findings have been described in rodent models of obesity as well as in humans with obesity and MS [56]. Serum levels of TBARS have also been shown to be a strong and independent predictor of coronary artery disease [57]; they are further associated with vascular events incidence, including fatal and non-fatal infarction and stroke [58]. CJFJ dose-dependently prevented the lipid peroxidation. The levels of TBARS in group MS+CJFJ10 were comparable with those in group Control. These results provide a further evidence of the antioxidant properties of CJFJ.

4. Materials and Methods

4.1. Preparation and Storage of Chaenomeles japonica Fruit Juice

Chaenomeles japonica plants were grown in the Balkan mountains, Bulgaria, in the region of Troyan. The fresh fruits were handpicked, ground, crushed and squeezed. The juice was filtered, preserved with potassium sorbate (1.0 g/l) and stored at 0°C until the experiment.

4.2. Chemical Composition and Antioxidant Activity of Chaenomeles japonica Fruit Juice

The chemical composition of CJFJ was determined by Valcheva-Kuzmanova et al., 2018 [59]. Total content of phenolic substances was spectrophotometrically estimated to be 890.00 mg GAE/l. High-performance liquid chromatography was performed and revealed a high procyanidin oligomers level and the presence of several phenolic acids and flavonoids. Among flavonoids epicatechin, catechin and quercetin-3-β-glucoside were most abundant (Figure 12). Among the phenolic acids, vanillic, caffeic and chlorogenic had the highest concentrations, followed by neochlorogenic, p-coumaric, ellagic, ferulic and 2,4-dihydroxybenzoic. Six organic acids, namely malic, quinic, citric, shikimic, ascorbic and oxalic acid, were also detected. Several carbohydrates were found in the juice: the predominant glucose (1713 mg/100 ml) and fructose (1237 mg/100 ml) as well as sucrose, xylose, rhamnose and arabinose. The antioxidant activity of CJFJ was 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 [59].

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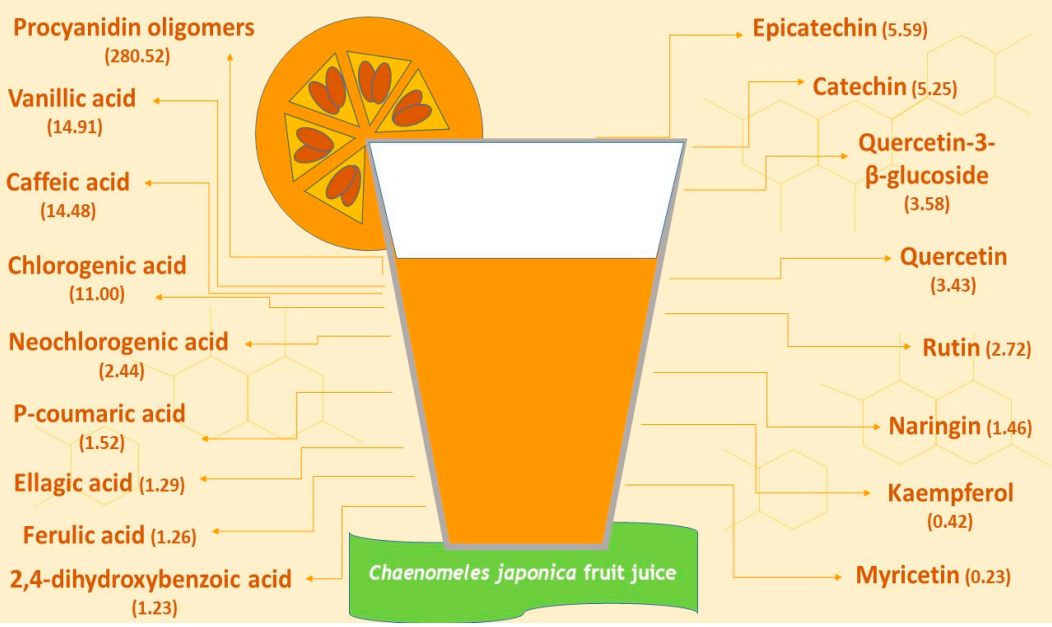

Figure 12. Polyphenolic content (mg/ 100ml) of *Chaenomeles japonica* fruit juice as estimated by Valcheva-Kuzmanova et al., 2018 [17].

4.3. Experimental Animals, Induction of MS and Treatment Protocol

Fifty adult male Wistar rats with an average initial weight of 270±30 g bred in the Animal Centre of Medical University of Varna were used for the experiment. The animals were housed in plastic cages at a temperature of 22±1°C, in a well-ventilated room, at a 12-hour light/dark cycle.

All procedures regarding the experimental animals were conducted in accordance with European Union Directive, 2010/63/EU for experiments with animals and approved by the Bulgarian food safety agency (Document № 177/07.07.2017).

The rats were divided into five groups of ten animals each: Control, MS, MS+CJFJ2.5, MS+CJFJ5 and MS+CJFJ10. For ten weeks, rats from the Control group received standard laboratory diet and tap water ad libitum. For the induction of MS, the other four groups were given a HFHF diet – 17% of lard and 17% fructose added to the standard diet as well as 10% fructose solution instead of drinking water. Given the aforementioned diets, animals from group Control received 279 kcal/100 g food while those on HFHF diet – 405 kcal/100 g food and 40 kcal/100 ml fructose solution. While the standard diet provided 20.48 g of protein, 3 g of fat, 38.3 g of starch and 3 g of sugars per 100 g, the HFHF diet provided 13.65 g of proteins, 18.67 g of fat, 25.5 g of starch and 19.55 g of sugars per 100 g. All animals were orally treated on a daily basis with a flexible orogastric tube. Control and MS groups received distilled water (10 ml/kg), while groups MS+CJFJ2.5, MS+CJFJ5 and MS+CJFJ10 were treated with CJFJ at increasing doses – 2.5 ml/kg (diluted with distilled water to 10 ml/kg), 5 ml/kg (diluted with distilled water to 10 ml/kg), and 10 ml/kg, respectively. Food and liquids consumption was registered on a daily basis. The body weight of the animals was assessed once per week.

A glucose tolerance test was performed at the end of the 10th week.

At the end of the experiment, under ether anesthesia, blood was collected from sublingual veins, centrifuged at 2000 r.p.m. for 10 min and stored at -20° C until biochemical tests were performed.

After decapitation of the anesthetized animals, fat deposits and livers were removed.

4.4. Glucose Tolerance Test (GTT)

After 12 h of fasting, rats were intraperitoneally injected with glucose at a dose of 2g/kg body weight. A blood sample from the distal end of the tail was collected. Blood glucose was measured with ACCU-CHEK Performa glucometer by using ACCU-CHECK Performa test strips immediately

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before injection (0 min) as well as on the 30th, 60th and 90th minute after that. The results were presented in mmol/l.

4.5. Calculation of Tissue Indices

4.5.1. Calculation of Fat Indices

Mesenteric, perigonadal, paranephral, and retroperitoneal fat depots were separately weighed. The total visceral adipose tissue was calculated. For each of the fat depots as well as for the total fat tissue, an index was measured using the following formula:

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

4.5.2. Calculation of Liver Index

Liver index was determined using the following formula:

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

4.6. Biochemical Measurements

4.6.1. Estimation of Serum Triglycerides and Calculation of Triglyceride Glucose (TyG) Index

Triglycerides were measured in the serum by a colorimetric kit (Bio Maxima, Poland) using a spectrophotometer AURIUS 2021 (Cecil Instruments Ltd.). The method is based on hydrolysis of triglycerides to glycerol and fatty acids by the enzyme lipoprotein lipase. As a result of the subsequent 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.

Triglyceride glucose (TyG) index was determined in order to assess insulin resistance. The index was calculated using the formula of Lopez-Jaramillo et al., 2023 [18].

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

4.6.2. Estimation of Serum Total Cholesterol

Total cholesterol levels were determined in blood serum using the kits of BioMaxima S.A., Poland, and strictly following the instructions of the producer. The method is based on hydrolysis of cholesterol esters to cholesterol and free fatty acids by the enzyme cholesterol esterase. As a result of the subsequent 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.

4.6.3. Superoxide Dismutase (SOD) Level Determination

An ELISA kit (Boster Bio, Pleasanton, CA, USA) was used for the superoxide dismutase level determination. 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).

 doi:10.20944/preprints202602.0446.v1

4.6.4. Thiobarbituric Acid Reactive Substances (TBARS) 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 [60]. Malondialdehyde was used as a standard. The optical density of the samples was measured by AURIUS 2021 spectrophotometer (Cecil Instruments Ltd., UK). TBARS are estimated as nmol/ml.

4.7. Histological Examination of Liver Tissue and Adipose Tissue

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

4.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 Software. The results were presented as mean ± SEM. One-way analysis of variance (ANOVA) with Dunnet`s Multiple Comparison Post Test was used. The correlations of SOD and TBARS with other biochemical parameters and with fat indices were tested by correlation analysis using the Pearson test, two-tailed. Values of $P<0.05$ were considered statistically significant.

5. Conclusions

The current study offers some insights into *Chaenomeles japonica* fruit juice intake in a model of metabolic syndrome. The juice showed nutritional and medicinal value, based on appetite-stimulating, antioxidant, antiobesogenic and hepatoprotective properties with potential application in metabolic syndrome.

Author Contributions: Conceptualization, S.V. and M.Zh.; methodology, S.V., M.Zh., M. Tz..; data curation, K.M., S.G., M.E., M.T., M.R., E.R.; investigation, K.M., S.G., M.E., M.T., M.R., E.R.; writing—original draft preparation, K.M.; writing—review and editing, S.V., K.M.; visualization, K.M.; supervision, S.V., M.Zh.; project administration, S.V.; funding acquisition, S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Fund for Supporting the Publication of Articles and Scientific Works in Impact Factor (IF) Journals by Researchers from the Structure of the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna [No. P-100-415, 2025].

Institutional Review Board Statement: All procedures concerning animal treatment and experimentation were conducted in conformity with the national and international laws and policies (EU Directive 2010/63/EU for animal experiments) and were approved by the Bulgarian Food Safety Agency (Document № 177/07.07.2017).

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MS	Metabolic syndrome
CJFJ	*Chaenomeles japonica* fruit juice
HFHF	High-fat high-fructose
FJ	Fruit juice
SOD	Superoxide dismutase

 doi:10.20944/preprints202602.0446.v1

TBARS	Thiobarbituric acid reactive substances
TyG	Triglyceride/glucose index
MAFLD	Metabolic dysfunction-associated fatty liver disease

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