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# Effects of *Aronia melanocarpa*-Based Fruit Juices on Metabolic Dysfunction-Associated Fatty Liver Disease in Rats

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## Abstract

**Background/Objective**: Metabolic dysfunction-associated fatty liver disease (MAFLD) is defined by the presence of hepatic steatosis, and is associated with obesity, diabetes, and other metabolic alterations. Feeding rats with a high-fat high-fructose (HFHF) diet is a reproducible experimental model of obesity/metabolic syndrome and the related MAFLD. *Aronia melanocarpa*, *Rosa canina*, and *Alchemilla vulgaris* are polyphenol-rich plants with proven health benefits. The aim of this study was to reveal the effects of four *Aronia melanocarpa*-based fruit juices (AMBFJs) in HFHF-fed rats. **Methods**: The AMBFJs were AM20 and AM60 (produced from aronia berries at 20 °C and 60 °C, respectively), AMRC (aronia juice with *Rosa canina*), and AMAV (aronia juice with *Alchemilla vulgaris*). Male Wistar rats were allocated to 6 groups. Except for the Control, the rest of the groups were fed an HFHF diet for 60 days. Throughout the experiment, each of the AMBFJs was administered to one HFHF-fed group. **Results**: HFHF-fed rats had an increased calorie intake on the background of increased liquid and decreased food consumption. At the end of the experiment, they had similar body weights, slightly increased fat indices, increased levels of blood lipids and liver enzymes, as well as typical histopathological changes in liver and adipose tissue. AMBFJs-treated animals showed improvement in most of these parameters, especially in triglyceride levels, liver enzymes, and the histopathological changes in the liver and fat. AMAV, the juice with the highest polyphenolic content, had the highest effect on adiposity. **Conclusion**: In HFHF-fed rats, AMBFJs exerted beneficial effects on MAFLD probably due to their polyphenolic ingredients.

**Keywords:** metabolic dysfunction-associated fatty liver disease; *Aronia melanocarpa*-based fruit juices; rats

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## 1. Introduction

According to WHO [1], overweight is the presence of excessive fat deposits, and obesity is a chronic disease in which excessive fat deposits can imperil health. The same organization reports that in 2022 around 12% of people worldwide suffered obesity. In the same year, 43% of adults had body mass index (BMI) 25 or above (overweight) and 16%

were obese (BMI 30 or above), 37 million children under 5 and 390 million aged 5–19 were overweight. For the last 30 years, obesity has doubled among adults and has quadrupled in adolescents. In 2019, 5 million deaths from noncommunicable diseases such as metabolic syndrome, cardiovascular diseases, cancer, diabetes, chronic respiratory diseases, and neurological and digestive disorders were associated with overweight and obesity [2].

The imbalance between energy intake (diet) and energy expenditure (physical activity) is the basis of overweight and obesity. In most cases, obesity is due to a combination of factors: obesogenic environments and psychosocial and genetic factors. The modern lifestyle—busy daily life, unhealthy diet high in calories and overeating combined with low physical activity results in positive energy balance. These excess calories are stored over time as fat leading to overweight and obesity. BMI shows low sensitivity and other related factors increasing cardiovascular risk should be considered, especially visceral and liver fat, as well as fat/lean mass ratio [3]. The World Obesity Federation emphasizes the fact that the diagnosis of obesity should be focused on health consequences and that anthropological measures like BMI and waist circumference are insufficient and contribute to the development of weight stigma [4].

Metabolic dysfunction-associated fatty liver disease (MAFLD), previously known as non-alcoholic fatty liver disease (NAFLD), represents a condition of hepatic steatosis with concomitant overweight or obesity, or type 2 diabetes, or two or more of the following metabolic alterations: hypertension, hypertriglyceridemia, hypercholesterolemia, hyperglycemia, high levels of homeostasis model assessment of insulin resistance or of plasma high-sensitivity C-reactive protein. The adverse outcomes of MAFLD include cardiovascular diseases, liver complications, and increased incidence of non-liver malignancies [5].

High-fat high-fructose (HFHF) diet in rats is a reproducible experimental model of obesity/metabolic syndrome representing "cafeteria diet" in humans and its negative health consequences [6], including the features of NAFLD [7]. Scientific data reveal the link between oxidative stress and obesity-related complications [8,9]. Chronic low-grade inflammation is present in obesity and is involved in the pathogenesis of its complications such as coronary artery disease, atherosclerosis, and insulin resistance [10]. The conventional drugs used in the treatment of obesity belong to a variety of pharmacological groups and exert multiple adverse effects [11]. Polyphenolic substances found in many fruits, vegetables, herbs, spices, and beverages are an important part of the human diet. The consumption of plant-derived polyphenol-rich products affects human health beneficially [12]. The antioxidant and anti-inflammatory properties of polyphenols have been demonstrated in a number of obesity studies, making these substances a promising alternative of the conventional anti-obesity drugs [13]. The fruits of black chokeberry—*Aronia melanocarpa* (AM) [Michx.] Elliot—are rich in polyphenols, including flavonoids (especially anthocyanins) and phenolic acids with prominent antioxidant [14–16] and anti-inflammatory properties [17,18]. AM products have demonstrated promising effects in models of metabolic syndrome [19,20] and obesity [18,21,22]. The possibility of further increasing the polyphenolic content of AM food products has been a matter of investigation. AM fruit juice is one the most suitable forms for consumption, preserving the high content of polyphenols. It is important to consider the technological aspects of juice production since extraction temperature may have a significant impact on the polyphenol quantity and composition [23]. Another approach to increase the antioxidant activity of the juice is to combine it with other polyphenol-rich plants. Polyphenols with antioxidant and anti-inflammatory activity are abundant in rosehip (*Rosa canina*) fruits [24–26]. In the study of Kratchanova et al. [27], an increased antioxidant activity was observed when rosehip was added to chokeberry, blackberry, blackcurrants or elderflower extracts. Lady's mantle (*Alchemilla vulgaris*) represents another polyphenol-rich plant exhibiting anti-inflammatory, antibacterial, and antioxidant

activity [28] which makes it a suitable supplement, increasing the polyphenol composition and beneficial potential of AMFJ. Although the effects of AM fruit juice on obesity have been widely studied, no data exist regarding the anti-obesity properties of the juice when combined with other polyphenol-enriched plant extracts or taking into account the technological aspects of its preparation. Therefore, the aim of this study was to reveal the effects of two AM fruit juices (AM20 and AM60) obtained at different extraction temperatures (20 °C and 60 °C, respectively) and two polyphenol-enriched AM fruit juices when combined with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV) extracts in rats fed an HFHF diet.

## 2. Materials and Methods

### *2.1. Plant Materials*

A licensed producer supplied the AM berries. The fresh fruits were frozen and stored at −18 °C until the juice was prepared. After defrosting, the berries were homogenized with a laboratory blender.

The *Rosa canina* fruits were harvested and freeze-dried at −18 °C for 96 h. Dried fruits were deseeded and stored at room temperature until preparation of polyphenol-enriched AM–*Rosa canina* fruit juice (AMRC).

Dried herbs of *Alchemilla vulgaris* produced by SD "Sharkovi i Sie" were obtained from a local pharmacy in Bulgaria.

### *2.2. Preparation of Aronia melanocarpa-Based Fruit Juices (AMBFJs)*

Preparation of juices was performed according to a procedure described by Valcheva-Kuzmanova et al. [29].

#### 2.2.1. Preparation of AM20 and AM60

A total of 1 kg of AM berry homogenate was incubated in a thermostatic shaker water bath (NUVE, Akyurt, Turkiye) at 20 °C and at 60 °C for the preparation of AM20 and AM60, respectively. One hour later the mixtures were filtrated and centrifuged for 20 min at 6200× *g* in a benchtop centrifuge Megafuge (Heraerus Instruments, Hanau, Germany). The resulting supernatants were labeled as AM20 and AM60.

#### 2.2.2. Preparation of AMRC

An amount of 200 g of pre-processed rosehips were homogenized with 800 mL ultrapure water, using a laboratory blender. The sample was extracted for 1 h in 60 °C in a thermostatic shaker water bath (NUVE, Turkiye). After centrifugation (20 min at 6200× *g*), the rosehip extract was mixed with AM60 in a ratio of 70:30 (*v*/*v*). The resulting mixture was labeled as AMRC.

#### 2.2.3. Preparation of AMAV

Dried parts of *Alchemilla vulgaris* were pulverized in a laboratory mill; after which 20 g of the powder were mixed with *Aronia melanocarpa* berry homogenate and incubated in a thermostatic shaker water bath (NUVE, Turkiye) for 1 h at 60 °C. The product was centrifuged for 20 min at 6200× *g* and the supernatant was labeled as AMAV.

### *2.3. Polyphenolic Content of Aronia melanocarpa-Based Fruit Juices (AMBFJs)*

The polyphenolic composition of the AMBFJs was HPLC determined and described by Denev et al. [23,29]. An Agilent 1220 HPLC system (Agilent technology, Santa Clara, CA, USA) with a binary pump and UV–VIS detector was used. Total phenolic composition was determined by the method of Singleton and Rossi [30]. Oxygen radical absorbance capacity (ORAC) was measured according to the method of Ou et al. [31]. The data regarding the polyphenolic composition of juices and their antioxidant capacity are presented in Table 1.

**Table 1.** Polyphenolic composition and oxygen radical absorbance activity (ORAC) of *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV) extracts; TE/L (Trolox equivalents per liter) [29].

| Ingredient | Juice AM20 | Juice AM60 | Juice AMRC | Juice AMAV |
|---|---|---|---|---|
| Total polyphenol content (mg/L) | 7772.7 ± 321.1 | 11,237.4 ± 456.2 | 10,802.1 ± 218.3 | 15,929.1 ± 356.7 |
| *Flavonoids* | | | | |
| Quercetin | 90.4 ± 8.7 | 49.6 ± 3.2 | 42.2 ± 2.5 | 76.7 ± 5.2 |
| Rutin | 593.1 ± 21.3 | 446.5 ± 12.5 | 382.2 ± 15.6 | 2614.0 ± 189.5 |
| Catechin | -- | -- | 1230.4 ± 78.9 | 1731.5 ± 95.2 |
| Epicatechin | 251.4 ± 14.2 | 408.2 ± 25.6 | 383.3 ± 29.1 | 858.6 ± 50.2 |
| Total anthocyanins | 863.8 | 2125.0 | 1359.1 | 2148.4 |
| Cyanidin-3-galactoside | 638.5 ± 21.0 | 1498.4 ± 102.3 | 991.5 ± 45.6 | 1507.0 ± 58.2 |
| Cyanidin-3-glucoside | 44.4 ± 4.1 | 120.1 ± 8.7 | 78.9 ± 3.6 | 133.5 ± 9.8 |
| Cyanidin-3-arabinoside | 177.5 ± 13.2 | 501.9 ± 31.8 | 285.4 ± 14.5 | 502.3 ± 45.6 |
| Cyanidin-3-xyloside | 2.73 ± 0.2 | 4.59 ± 0.2 | 3.25 ± 0.3 | 5.51 ± 0.5 |
| *Phenolic acids* | | | | |
| Chlorogenic acid | 1142.9 ± 81.2 | 1375.6 ± 80.3 | 1262.9 ± 56.2 | 1809.7 ± 103.8 |
| Neochlorogenic acid | 1305.2 ± 102.8<br>2027.0 ± 131.8 | 1543.1 ± 111.2 | 1499.2 ± 96.5 | 2027.0 ± 131.8 |
| ORAC, µmol TE/L | 81,256 ± 6545 | 122,545 ± 9849 | 138,569 ± 10,253 | 168,456 ± 10,458 |

### *2.4. Animals*

Male Wistar rats ($n$ = 60) weighing 200 ± 50 g at the beginning of the experiment were used. The animals were kept in plastic cages at a temperature of 22 ± 1 °C, in a well-ventilated room under a 12:12 h light–dark regimen. During the experiment, the rats had free access to food and drinking water.

All experimental procedures were performed in accordance with the requirements of national laws and regulations, as well as international guidelines (European Union Directive, 2010/63/EU for experiments with animals). The procedures with animals were approved by Bulgarian Food Safety Agency (Protocol 204/07.07.2017).

### *2.5. Experimental Setting*

The rats were allocated to six groups with 10 animals in each. The average weight per animal in each group was between 211.8 and 212.6 g. The groups were labeled as Control, HFHF, HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV. The Control group received standard laboratory pellets and plain drinking water. The standard diet provided a total energy value of 279 kcal per 100 g. The HFHF groups were subjected to a 60-day high calorie diet prepared by enriching the standard laboratory chow with 17% lard and 17% fructose plus 10% fructose added to the drinking water. To prepare the HFHF diet, 100 g of lard and 100 g of fructose were placed in a water bath at 90 °C which allowed for the melting of the lard and dissolving the fructose; after which they were mixed and evenly distributed in 400 g of standard chow [6]. The HFHF diet provided 405 kcal per 100 g.

The composition of the diets is presented in Table 2.

**Table 2.** Composition of the standard and high-fat high-fructose (HFHF) diet.

| Diet | Proteins (Per 100 g) | Fat (Per 100 g) | Carbohydrates | |
|---|---|---|---|---|
| | | | Starch (Per 100 g) | Sugars (Per 100 g) |
| Standard diet | 20.48 g | 3 g | 38.3 g | 3 g |
| HFHF diet | 13.65 g | 18.67 g | 25.5 g | 19.55 g |

Throughout the whole experimental period, animals from groups Control and HFHF were treated once daily orally with distilled water at a dose of 10 mL/kg, while groups HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV received 10 mL/kg of the respective AM-based fruit juice (AMBFJ). This dose of the four AMBFJs proved to be efficacious to exert a good gastroprotective effect in a rat model of indomethacin-induced ulcerogenesis [29]. The body weight was measured once a week.

At the end of the experimental period, animals were sacrificed under ether anesthesia and blood from sublingual veins was collected. The blood-derived serum was stored at −20 °C until biochemical tests were performed.

The liver, retroperitoneal, perigonadal, and mesenteric fat pads were dissected on ice. The organs were weighed and fat tissue indices calculated using the formula: fat pad weight/body weight × 100. Samples of liver and retroperitoneal fat were fixed in 10% neutral buffered formalin and stored for histopathological examination. Pieces of these tissues were frozen at −20 °C for biochemical testing.

### *2.6. Histopathological Examination of Liver and Adipose Tissue*

Formalin-fixed liver and fat tissues were dehydrated in graded ethanol concentration, embedded in paraffin, and cut into 4 µm sections. The sections were stained with hematoxylin–eosin and the degree of histopathological changes was assessed microscopically.

Liver steatosis was evaluated semi-quantitively in 5–6 liver lobes of each liver sample according to the following scale:

0—<5% of the lobule affected;

1—5–33% of the lobule affected;

2—34–66% of the lobule affected;

3—>66% of the lobule affected.

The liver lobular inflammation score was evaluated semi-quantitively in 5–6 liver lobes of each liver sample according to the following scale:

0—no inflammation;

1—1 focus in a lobule;

2—2–4 foci in a lobule;

3—>4 foci in a lobule.

Tissue sections from visceral fat were photographed using a Leica DM 1000 LED camera (Leica Microsystems, Wetzlar, Germany) at 200× magnification. The diameter in mm of 10 consecutive adipocytes from fat tissue of each animal was measured using Leica Application Suite software, version 4.13.0 (Leica microsystems, Heerbrugg, Switzerland).

### *2.7. Biochemical Tests*

The serum levels of cholesterol, triglycerides, and liver enzymes alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined with colorimetric kits (Biomaxima-Poland, Lublin, Poland) using a spectrophotometer AURIUS 2021 (Cecil Instruments Ltd., Cambridge, UK).

### *2.8. Statistics*

To analyze the data, GraphPad Prism 5 statistical software was used. The Control group was compared with the HFHF group by Student's *t*-test. One-way ANOVA with Dunnet's multiple comparison test was performed between the HFHF group and the groups treated with AMBFJs. The results were presented as mean ± standard error of the mean (SEM). A significant difference was defined as $p$ value lower than 0.05.

## 3. Results

### *3.1. Body Weight*

The weight gain of HFHF rats did not differ from that of the Control group (76.22 ± 6.99 vs. 91.2 ± 6.96 g). The weight gain of AMBFJs-treated animals was not significantly different from that of the HFHF group (73.8 ± 4.46, 68.4 ± 10.69, 82.67 ± 7.65, and 71.6 ± 6.09 g for HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively; Figure 1a,b).

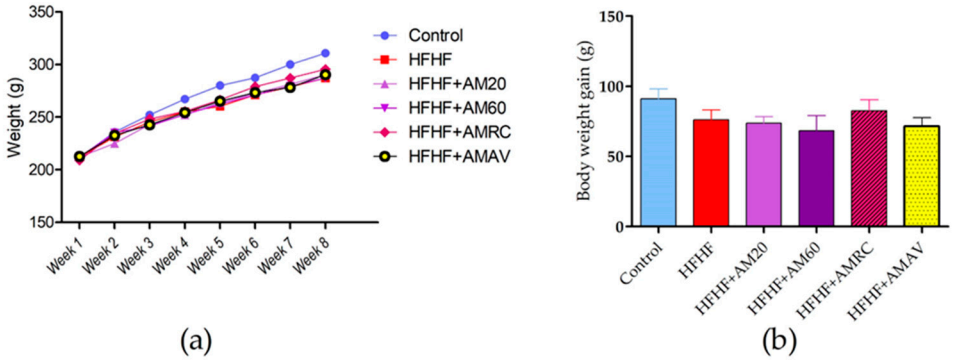



**Figure 1.** Body weight during the experimental period of 8 weeks (**a**) and body weight gain (**b**) of rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV).

The final body weight of HFHF rats did not differ from that of the Control group (290.4 ± 9.72 vs. 303.0 ± 7.28 g). The final body weight of AMBFJs-treated animals was not significantly different from that of the HFHF group (285.8 ± 7.05, 280.6 ± 9.88, 291.8 ± 6.65, and 284.2 ± 6.50 g for HFHF+AM20, HFHF+AM60, HFHF+AMRC, and HFHF+AMAV, respectively; Figure 2).

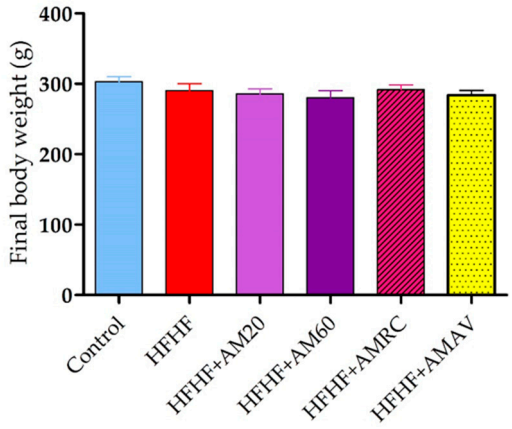



**Figure 2.** Final body weight of rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV).

### 3.2. Food, Liquid, and Calories Consumption

The weekly food consumption of the control rats during the testing period (149.0 ± 1.45 g) was significantly higher ($p < 0.001$) in comparison with animals fed the HFHF diet (87.38 ± 1.40 g). Treatment with all AMBFJs did not significantly change the rats' food consumption compared to the HFHF group (85.76 ± 2.01, 80.27 ± 2.22, 92.90 ± 3.07, and 83.56 ± 2.53 g for HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively) (Figure 3a).

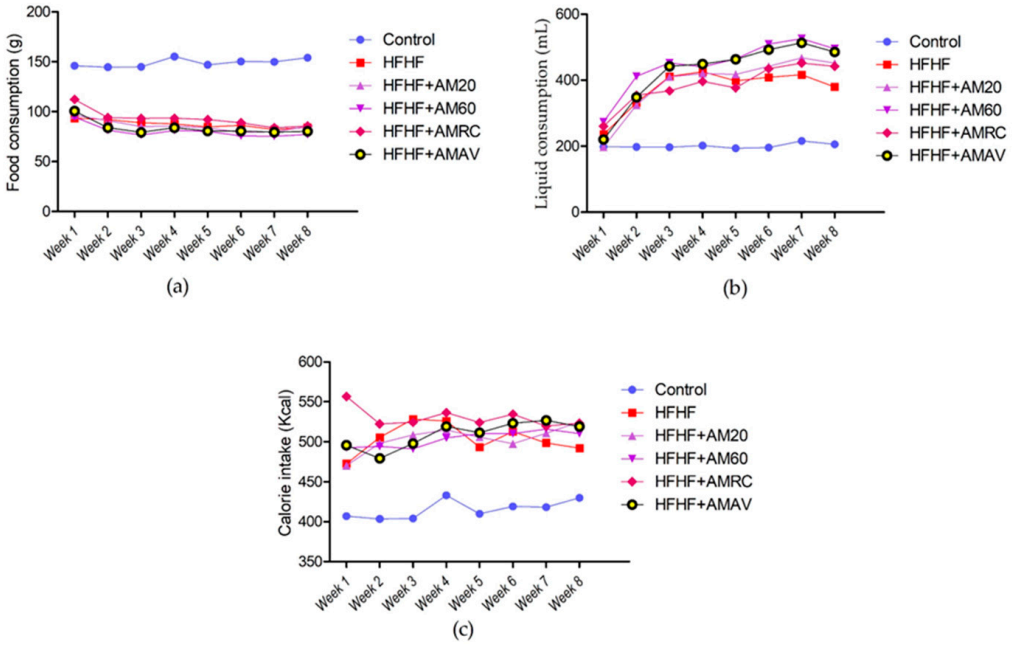



**Figure 3.** Changes in the consumption of food (**a**), liquid (**b**), and calories (**c**) during the experimental period of 8 weeks in rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV).

Rats fed the HFHF diet had a significantly higher ($p < 0.001$) weekly consumption of liquid during the testing period (375.9 ± 22.6 mL) compared to the control animals (200.7 ± 2.49 mL). Treatment of rats with all AMBFJs did not significantly change the weekly liquid consumption compared to the HFHF group (391.3 ± 31.51, 446.5 ± 28.04, 385.5 ± 22.04, and 426.6 ± 34.35 mL for HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively) (Figure 3b).

HFHF diet-fed rats consumed significantly more ($p < 0.001$) calories weekly (503.6 ± 6.59) than controls (415.6 ± 4.04 kcal). HFHF + AMRC rodents had even higher ($p < 0.01$) weekly calorie intake (530.2 ± 4.35 kcal) than HFHF. The weekly calorie intake of other AMBFJ-treated groups did not differ significantly in comparison with the HFHF group (503.9 ± 5.71, 503.7 ± 3.42, and 509.0 ± 5.85 kcal for HFHF + AM20, HFHF + AM60, and HFHF + AMAV, respectively) (Figure 3c).

### 3.3. Fat Indices

Although insignificant, the retroperitoneal, mesenteric, and total fat indices tended to be higher in the HFHF group (1.12 ± 0.14, 0.62 ± 0.08, and 2.89 ± 0.29, respectively) when compared to the Control group (0.84 ± 0.06, 0.51 ± 0.04, and 2.6 ± 0.13, respectively) (Figure 4a,b,d). Perigonadal fat index did not differ in the HFHF group in comparison with the Control (1.00 ± 0.07 vs. 1.01 ± 0.05) (Figure 4c). Treatment with AMBFJ led to a decrease in the indices for retroperitoneal (0.89 ± 0.08 for HFHF + AM20, 1.02 ± 0.07 for

HFHF + AM60, 0.97 ± 0.06 for HFHF + AMRC, and 0.73 ± 0.08 for HFHF + AMAV) (Figure 4a), mesenteric (0.56 ± 0.05 for HFHF + AM20, 0.62 ± 0.07 for HFHF + AM60, 0.52 ± 0.04 for HFHF + AMRC, and 0.53 ± 0.09 for HFHF + AMAV) (Figure 4b), perigonadal (0.94 ± 0.05 for HFHF + AM20, 0.96 ± 0.05 for HFHF + AM60, 0.95 ± 0.03 for HFHF + AMRC, and 0.93 ± 0.12 for HFHF + AMAV) (Figure 4c), and total fat (2.57 ± 0.17 for HFHF + AM20, 2.77 ± 0.17 for HFHF + AM60, 2.59 ± 0.11 for HFHF + AMRC, and 2.44 ± 0.36 for HFHF + AMAV) (Figure 4d). In group HFHF + AMAV, the retroperitoneal and total fat indices were significantly lower in comparison with group HFHF ($p < 0.05$) (Figure 4a,d).

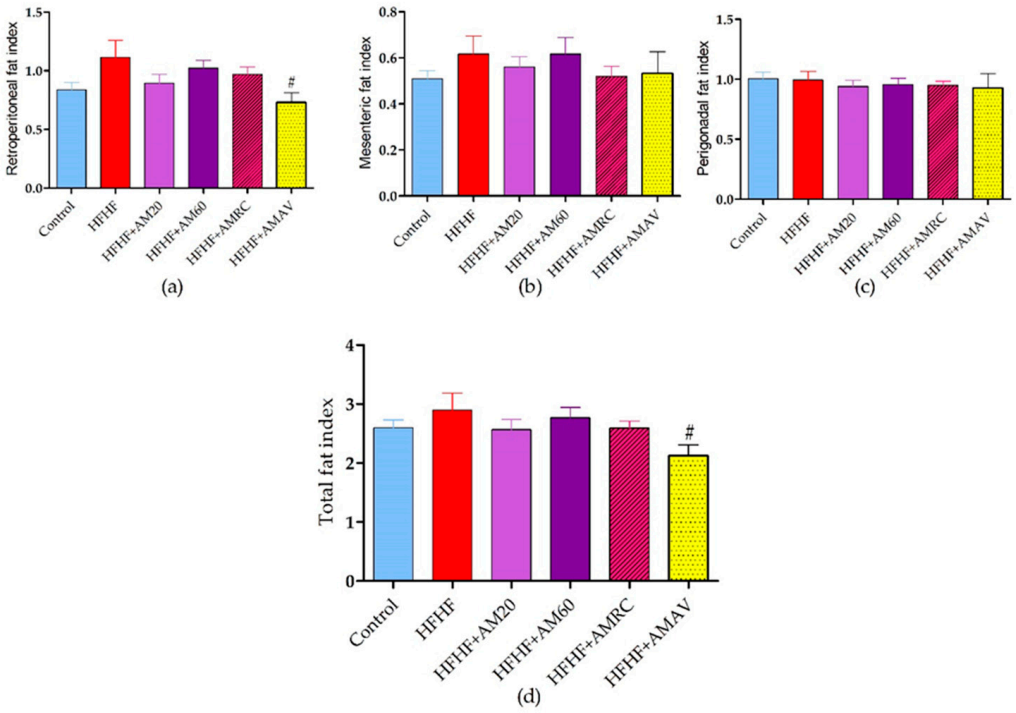



**Figure 4.** Retroperitoneal (**a**), mesenteric (**b**), perigonadal (**c**), and total fat (**d**) indices in rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV); # $p < 0.05$ vs. HFHF group.

### *3.4. Histopathological Investigation*

#### 3.4.1. Liver

The HFHF diet caused degenerative changes in hepatocytes, small-droplet steatosis, necrosis of hepatocytes, and inflammatory reaction. In groups HFHF + AM20 and HFHF + AM60, the alteration of the liver parenchyma was greatly reduced. Hepatocytes with small-droplet steatosis and inflammatory cells were still found. In groups HFHF + AMRC and HFHF + AMAV, no damage of the liver parenchyma was detected histopathologically (Figure 5a).

HFHF diet-fed animals had a significantly higher ($p < 0.001$) liver steatosis score compared to the Control group (1.77 ± 0.16 vs. 0.034 ± 0.034). The liver steatosis score in all AMBFJ-treated rats was significantly ($p < 0.001$ vs. HFHF) reduced (0.65 ± 0.14, 0.27 ± 0.09, 0.15 ± 0.07, and 0.11 ± 0.06 for groups HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively) (Figure 5b).

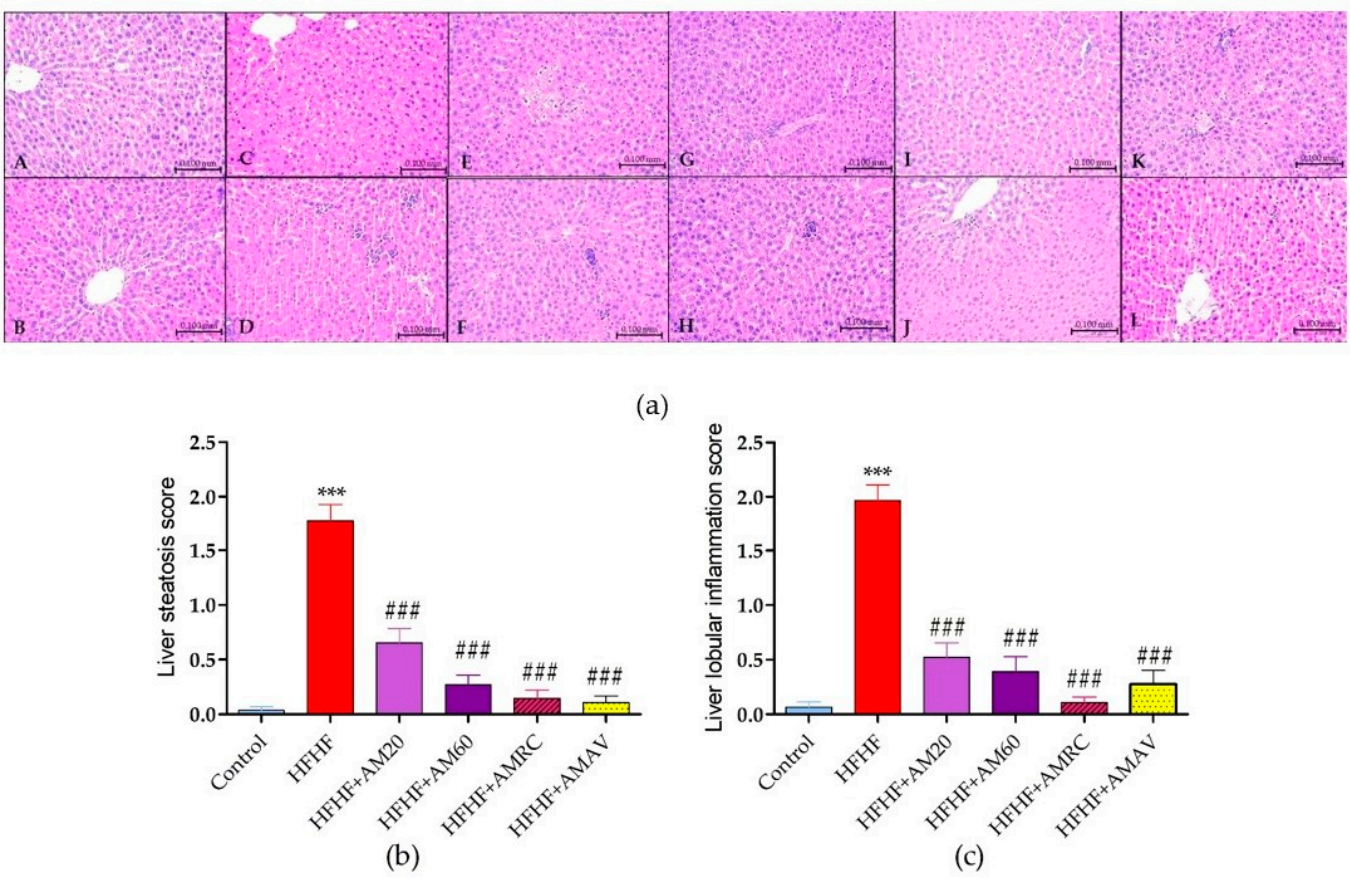



**Figure 5.** Liver changes in rats fed a high-fat high-fructose (HFHF) diet treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV). (**a**) Microscopic appearance of hepatocytes: panels A, B: Control group—normal architectonics of hepatocytes; panels C, D: HFHF group—fatty degeneration and inflammatory granulomas; panels E, F: HFHF + AM20 group—small droplet steatosis in the cytoplasm of hepatocytes and inflammatory granulomas; panels G, H: HFHF + AM60 group—small droplet steatosis in the cytoplasm of hepatocytes and inflammatory granulomas; panels I, J: HFHF + AMRC group—normal architectonics of hepatocytes; panels K, L: HFHF + AMAV group—normal architectonics of hepatocytes; hematoxylin and eosin staining; bar = 0.1 mm; (**b**) liver steatosis score; (**c**) liver lobular inflammation score; *** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. HFHF.

The liver lobular inflammation score was also significantly higher ($p < 0.001$) in the HFHF group compared to the Control (1.97 ± 0.14 vs. 0.07 ± 0.05). All AMBFJ-treated groups demonstrated a significantly ($p < 0.001$ vs. HFHF) reduced lobular inflammation score (0.52 ± 0.13, 0.39 ± 0.14, 0.11 ± 0.05, and 0.28 ± 0.13 for groups HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively) (Figure 5c).

#### 3.4.2. Adipose Tissue

The histopathological investigation of retroperitoneal fat showed that in the Control group the adipocytes were small- and medium-size, while in group HFHF the prevailing were the large-size adipocytes. In the HFHF + AM20 group, most of the adipocytes were small- and medium-size, but there were rare large-size adipocytes. Groups HFHF + AM60, HFHF + AMRC, and HFHF + AMAV presented with small- and medium-size adipocytes characteristic for the normal architectonics of fat tissue as that of the Control group (Figure 6a).

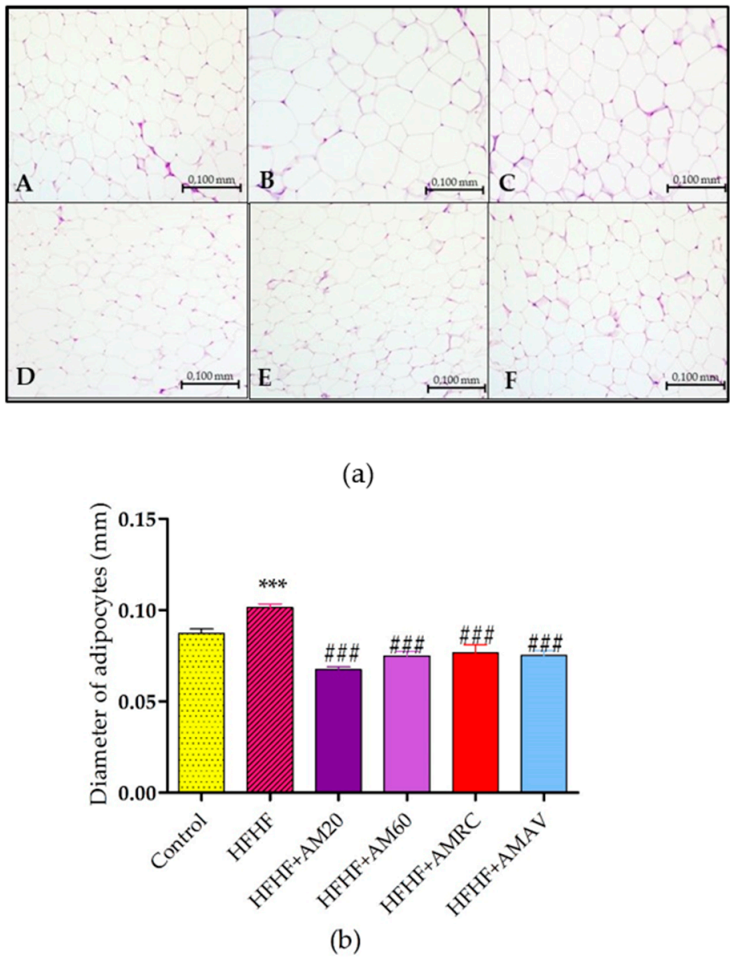



**Figure 6.** Adipocyte changes in rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV). (**a**) Microscopic pictures of retroperitoneal fat tissue: panel A: Control group—small- and medium-size adipocytes; panel B: HFHF group—large-size adipocytes prevailing; panel C: HFHF + AM20 group—small-, medium- and rare large-size adipocytes; panel D: HFHF + AM60—small- and medium-size adipocytes; panel E: HFHF + AMRC—small- and medium-size adipocytes; panel F: HFHF + AMAV—small- and medium-size adipocytes. Hematoxylin and eosin staining; bar = 0.1 mm. (**b**) Adipocyte diameter in retroperitoneal fat; *** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. HFHF.

Adipocyte diameter was significantly higher in the HFHF group ($p < 0.001$) compared to the Control (0.101 ± 0.002 vs. 0.087 ± 0.002 mm). Restoration to normal size was observed in all AMBFJ-treated groups (0.067 ± 0.002, 0.075 ± 0.002, 0.077 ± 0.005, and 0.075 ± 0.003 mm for groups HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively; $p < 0.001$ vs. HFHF) (Figure 6b).

### *3.5. Biochemical Tests*

#### 3.5.1. Serum Lipids

The levels of serum cholesterol and triglycerides are presented in Figure 7. The results showed significantly elevated serum levels of cholesterol ($p < 0.05$) (Figure 7a) and triglycerides ($p < 0.01$, Figure 7b) in the HFHF group compared to the Control group (2.22 ± 0.16 vs. 1.76 ± 0.10 mmol/L for cholesterol and 1.19 ± 0.09 vs. 0.81 ± 0.07 mmol/L for triglycerides). Treatment with AMBFJs antagonized this tendency, lowering the levels of cholesterol (1.85 ± 0.22, 1.76 ± 0.16, 1.93 ± 0.29, and 2.08 ± 0.11 mmol/L for HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively) (Figure 7a) and triglyc-

erides (0.75 ± 0.08, 0.74 ± 0.13, 0.84 ± 0.11, and 0.80 ± 0.09 mmol/L for HFHF + AM20, HFHF + AM60, HFHF + AMRC, and HFHF + AMAV, respectively, Figure 7b) nearly to control levels. A statistically significant reduction in triglyceride levels was observed in groups HFHF + AM20, HFHF + AM60, and HFHF + AMAV in comparison to group HFHF ($p < 0.05$, Figure 7b).

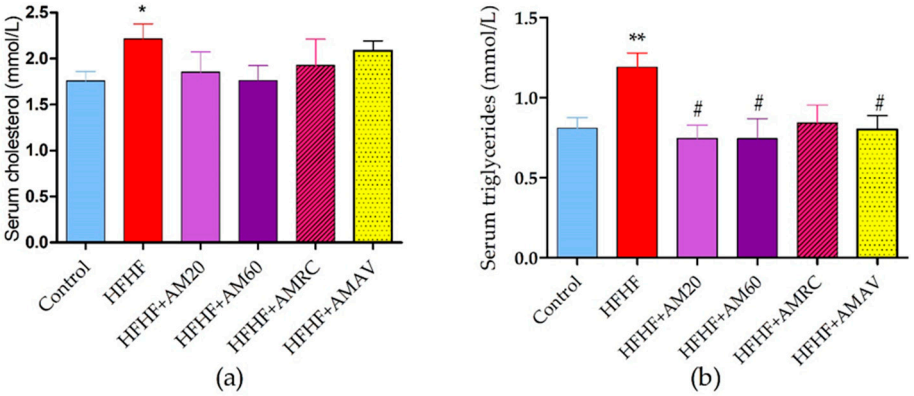



**Figure 7.** Levels of serum cholesterol (**a**) and triglycerides (**b**) in rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV); * $p < 0.05$, ** $p < 0.01$ vs. Control group; # $p < 0.05$ vs. HFHF group.

### 3.5.2. Liver Enzymes

Serum ALT levels in the HFHF group (19.19 ± 0.66 U/L) were significantly higher ($p < 0.001$) in comparison with the Control group (15.86 ± 0.26 U/L) (Figure 8a). ALP levels were elevated in the HFHF group as well ($p < 0.01$), being 159.6 ± 18.47 U/L for HFHF vs. 89.72 ± 3.66 U/L for Control (Figure 8b). In all AMBFJ-treated groups, ALT levels were decreased ($p < 0.001$ vs. HFHF) in comparison to the HFHF-treated group (16.66 ± 0.48 U/L for HFHF + AM20, 16.12 ± 0.32 U/L for HFHF + AM60, 16.30 ± 0.44 U/L for HFHF + AMRC, and 16.08 ± 0.33 U/L for HFHF + AMAV, respectively; Figure 8a). ALP levels were not different from HFHF in all juice-treated groups—168 ± 21.86 U/L for HFHF + AM20, 147.4 ± 15.2 U/L for HFHF + AM60, 140.7 ± 15.48 U/L for HFHF + AMRC, and 119.1 ± 9.92 U/L for HFHF + AMAV, respectively (Figure 8b).

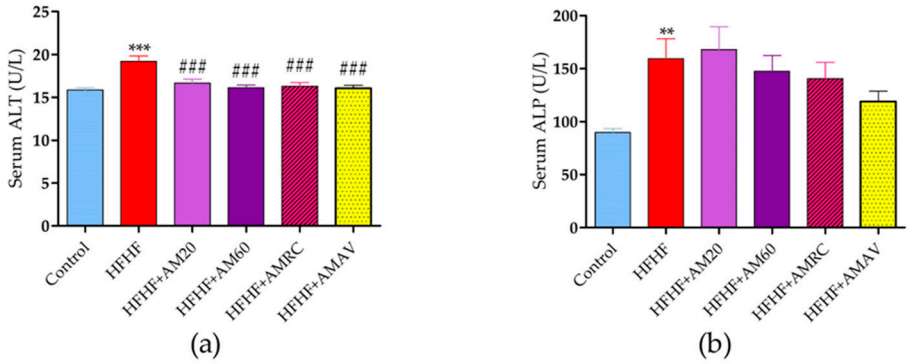



**Figure 8.** Serum levels of alanine aminotransferase (ALT) (**a**) and alkaline phosphatase (ALP) (**b**) in rats fed a high-fat high-fructose (HFHF) diet and treated with *Aronia melanocarpa* fruit juices produced at 20 °C (AM20) and at 60 °C (AM60) and *Aronia melanocarpa* fruit juices enriched with *Rosa canina* (AMRC) and *Alchemilla vulgaris* (AMAV); ** $p < 0.01$, *** $p < 0.001$ vs. Control group; ### $p < 0.001$ vs. HFHF group.

## 4. Discussion

High accessibility of food rich in calories and poor in nutritional density is one of the main factors contributing to the positive energy balance seen in overweight and obesity. Unnecessary energy is stored in the form of triglycerides in adipose tissue causing its expansion [3]. The HFHF used in this study was chosen to reflect the levels of animal fats and simple carbohydrates typically found in the unhealthy "Western" diet [6]. Obesity is a major risk factor for cardiovascular disease, diabetes type 2, certain cancers, and premature death [32]. Obesity disrupts normal metabolism and results in prominent changes in liver histology. Expansion of fat vacuoles in the hepatocytes corresponds with the BMI and results in liver steatosis and cell death. There is consequent elevation of liver enzymes and focal non-specific inflammation. Steatosis may proceed to steatohepatitis, the typical features of which include perisinusoidal, periportal, and bridging fibrosis proceeding to cirrhosis [33].

In this experiment, the body weight of HFHF-fed rats was not increased in comparison with the Control group, nor was the weight of rats treated with AMBFJs. The results from the application of the HFHF diet in this study and other studies of ours were highly and reproducibly linked to unchanged normal body weight and increased visceral adiposity in rats. These findings are consistent with data from the available literature. In a comparable study, male Wistar rats fed a high-fat, high-sugar diet for 20 weeks did not exhibit an increase in body weight compared to the control group until week 16 [34]. The reason for the lack of body weight change may be that rats fed HFHF received more calories which led to appetite suppression and consumption of less food. All HFHF diet-fed animals had significantly higher liquid consumption. The same changes were observed in other similar models [6,35]. A possible explanation for the higher liquid intake in HFHF diet-fed groups may lie probably in the high palatability of the fructose solution as well as the addictive properties of dietary sugars, fructose being one of the most potent in this regard [36]. Total, retroperitoneal, and mesenteric fat indices showed a tendency to increase in HFHF-fed rats. Serum triglycerides, total cholesterol levels, as well as the markers for liver damage (ALT, ALP) were significantly increased in the HFHF group. Such changes are typical for metabolic-syndrome models and were observed by other authors as well [6,7,20]. In rats treated with AMBFJs, total and retroperitoneal fat indices demonstrated some decrease in comparison with HFHF-fed animals. Similar tendencies were observed by us in previous studies with AM fruit juice [20]. The decrease in fat indices was most pronounced in the HFHF + AMAV group, correlating with the highest polyphenol concentration in AMAV. Total cholesterol levels were slightly decreased in AMBFJ-treated groups, and triglyceride levels were significantly lower in most of the treated groups than in the HFHF group. AM fruit juices demonstrated a capacity for lipid profile improvement in a number of studies, including those from our group [20,37]. All AMBFJ-treated groups exhibited decreased levels of ALT, similar to other authors' findings [38]. The histopathological evaluation showed that HFHF caused typical changes in liver and fat tissue. These changes were attenuated in groups HFHF + AM20 and HFHF + AM60. Liver and fat in groups HFHF + AMRC and HFHF + AMAV did not demonstrate any histological changes. All these results correspond with the total polyphenolic content of *Aronia melanocarpa*-based juices—lowest in juice AM20 and highest in juice AMAV, which showed the highest potential to reverse the consequences of the HFHF.

AM extract has been found to prevent the accumulation of total and visceral adipose tissue, and to attenuate the changes in liver and serum lipids in rats subjected to high-fat diet (HFD). These beneficial effects were attributed to modulation of gut microbiota in favor of anti-obesogenic species. These changes led to improvement of lipid metabolism by modulation of FXR and TGR-5 signaling pathway and to activation of thermogenesis

and improvement of energy balance in the brown adipose tissue [37]. AM extract was able to stimulate the lipolysis and fatty acids expenditure in the visceral fat of mice fed HFD [39]. Another study demonstrated inhibition of fatty acid synthesis and stimulation of lipolysis in HFD-fed mice, as well as in vitro inhibition of the activity of the fat-degrading enzyme lipase [40]. AM extract was able to prevent lipid accumulation in a pre-adipocyte cell line, concomitantly increasing the secretion of adiponectin and decreasing the secretion of leptin by the adipocytes. These changes finally led to a halt of pre-adipocyte differentiation into mature adipocytes [41]. In another study, AM extract succeeded in attenuating HFD-induced changes in body weight, hepatic lipid accumulation, triglycerides, ALT, and aspartate aminotransferase levels by inhibition of the PPARγ2 pathway [38]. The beneficial effect of AM extract on lipid profile (decreased levels of total and LDL-cholesterol and triglycerides) was confirmed in a clinical trial with metabolic syndrome patients [42]. A study performed by our team demonstrated that AM fruit juice prevented the histopathological changes in the liver of rats fed HFHF diet by anti-inflammatory and anti-apoptotic mechanisms [19]. Another possible mechanism of AM-induced hepatoprotection in models of metabolic alterations is the prevention of liver fat accumulation, oxidative stress and inflammation by activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [43].

The application of *Rosa canina* to HFD-fed mice was able to alleviate the weight gain, subcutaneous adipose tissue, and liver adipogenesis by inhibition of PPARγ [44]. The anti-obesity potential of this plant has been confirmed in a clinical trial with overweight subjects. Other possible anti-obesity mechanisms of rosehip include stimulation of liver and muscular oxidation of fatty acids, as well as prevention of lipid accumulation in adipose tissue [45].

*Alchemilla vulgaris* extract was able to dramatically decrease lipid accumulation and differentiation into mature adipocytes in a pre-adipocyte cell line [46].

There are data suggesting a hepatoprotective potential of *Rosa canina* and *Alchemilla vulgaris*, but the underlying mechanisms are not fully elucidated [47–49]. On the other hand, the organ-protective features of polyphenol-rich plants like the aforementioned may be attributed to their well-known antioxidant properties [28,50,51]. The AMBFJs utilized in this study revealed their high antioxidant capacity in the ORAC essay [29].

The group of anthocyanins and rutin are the flavonoids with the highest concentrations in the tested juices. When applied to mice with either genetically, or diet-induced obesity, rutin was able to decrease the adipose tissue accumulation, and to stimulate energy expenditure by stimulation of the formation of beige (brown-like) cells in the subcutaneous fat. These changes were conditioned by stabilization of SIRT1 and increased number of mitochondria in the brown adipose tissue [52]. In rats fed HFD, rutin supplementation was able to prevent obesity (reduced total, retroperitoneal, and epididymal fat) by diminution of fat adipogenesis, stimulation of mitochondria formation in the skeletal muscle, and activation of AMPK [53]. Rutin was able to decrease the triglyceride accumulation in the liver of mice fed HFD and to prevent the development of fatty liver by stimulation of lipolysis and inhibition of lipogenesis, as well as by stimulation of liver cell autophagy [54].

Anthocyanins have demonstrated the potential to improve the dyslipidemia, adipogenesis, and low-grade inflammation in various in vitro and in vivo obesity models, as well as in some clinical trials [55]. Applied in different rodent models of obesity, this class of flavonoids was able to decrease the food intake and the absorption of fat, to improve the regulation of lipid metabolism, to stimulate energy expenditure, and to regulate gut microbiota. They were able to ameliorate obesity-related oxidative stress and low-grade inflammation as well [56]. Anthocyanins applied in different in vivo models of non-alcoholic fatty liver disease (NAFLD) demonstrated a reduction of hepatic steatosis by antioxidant

(increased levels of antioxidant enzymes and molecules like superoxide dismutase, catalase, and glutathione) and anti-inflammatory (decreased levels of inflammatory cytokines like TNF-α, IL-6, and IL-1β) action [57].

Chlorogenic (CGA) and neochlorogenic acids are the most abundant phenolic acids in the tested juices. In mice fed HFD, CGA was able to prevent the increase in body weight and the expansion of visceral fat, and to decrease the triglyceride and cholesterol accumulation in liver, heart, and fat. During another experiment with mice fed HFD, CGA prevented weight gain and liver steatosis. CGA applied to obese mice did not manage to decrease the body weight but reduced the hepatic fat accumulation. Similar results of total and visceral fat reduction were observed in rats fed HFD. CGA has demonstrated effectiveness in clinical trials as well. A CGA-containing coffee drink was able to reduce the body weight of overweight patients. In healthy men, CGA was able to stimulate energy expenditure and the utilization of fat. The underlying mechanisms include antioxidant and anti-inflammatory activity, increased liver uptake of fatty acids due to inhibition of PPARγ, and regulation of lipid metabolism [58]. CGA has been shown to ameliorate the liver damage in various liver disease models, including NAFLD associated with obesity or metabolic syndrome. The mechanisms involved are again antioxidant, anti-inflammatory, and regulation of lipid metabolism, together with autophagy inhibition, strengthening of the intestinal barrier, and regulation of gut microbiota. A CGA-containing food supplement was able to alleviate the severity of liver damage in patients with MAFLD [59]. Neochlorogenic acid successfully ameliorated the changes in serum lipids and prevented the fat accumulation in liver and the consecutive steatosis in HFD-fed mice with spontaneous hyperglycemia [60].

This conducted study possesses a number of strengths. For the first time, the effects of four different AMBFJs on MAFLD were investigated. Strict quality control was maintained while preparing the HFHF diet and AMBFJs as well as during the conduction of the experimental protocol. This study was carried out in rats which are excellent models for human biology and diseases. Rats' physiological characteristics are similar enough to the human ones and findings in rats often translate well to human conditions. However, additional clinical studies might be necessary to confirm the possible beneficial effects of the AMBFJs in humans.

Limitations, however, exist in this study. AMBFJs were administered in a single dose due to which there was no investigation of the dose–effect relationship for each of the juices. However, the dose was justified on the basis of the previous experience of the team with the juices. Apart from this, we did not determine the effects of the AMBFJs on the intracellular signaling molecules which could further elucidate the exact mechanisms underlying the observed changes. That could be a matter of additional investigation.

## 5. Conclusions

*Aronia melanocarpa*-based fruit juices were able to ameliorate the high-fat high-fructose diet-induced adipose tissue expansion, hyperlipidemia, and liver damage in rats. These effects can be attributed to the high polyphenol content of the juices.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

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

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