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Supplementation of an Anthocyanin-Rich Elderberry (*Sambucus nigra* L.) Extract in FVB/n Mice: A Healthier Alternative to Synthetic Colorants

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Featured Application: Due to anthocyanins' potential as colorants, this anthocyanin-rich elderberry extract presents a healthier alternative to commercially available colorants and has the potential to be used at industry level.

Abstract: *Sambucus nigra* L., popularly known as elderberry, is renowned for its amazing therapeutic properties, as well as its uses as a food source, in nutraceuticals, and in traditional medicine. This study's aim was to investigate the effects of an elderberry extract (EE) on mice for 29 days, as well as the safety of the extract when used as a natural colorant. Twenty-four FVB/n female mice (n = 6)

were randomly assigned to one of four groups: control, 12 mg/mL EE (EE12), 24 mg/mL EE (EE24), or 48 mg/mL EE (EE48). The predominant anthocyanins detected were cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-glucoside. Food and drink intake were similar between groups, with the exception of EE48, who drank significantly less compared with the Control. Biochemical analysis of the liver showed that the changes observed in histological analysis had no pathological significance. The EE, at doses of 24 and 48 mg/mL, significantly reduced the oxidative DNA damage compared with the non-supplemented group. The *S. nigra* extract showed a favorable toxicological profile, affording it potential to be used in the food industry.

Keywords: *Sambucus nigra*; in vivo; oral administration; colorant

1. Introduction

The genus *Sambucus* belong to the Adoxaceae family and consists of 5 to 30 species, with *Sambucus nigra* L. being the most commonly occurring species [1–3]. This species, which is native to the northern hemisphere and may be found on practically every continent, is sometimes referred to as "black elder, European elder, or elderberry" [4]. It is a 6 m tall deciduous shrub that thrives in locations with direct sunlight. Between the spring and summer, the elderberry produces white hermaphrodite flowers, and in the late summer, the fruits mature [5]. The fruits of *S. nigra* L. are dark purple berries in a cluster with a diameter of up to 6 mm [5], and are a good source of vitamins, sugars, organic acids, fatty acids, protein, and essential oils [3,6–8]. Elderberry is also rich in phenolic compounds, especially anthocyanins. In folk medicine, elderberry has been widely consumed for many years due to its therapeutic effects; the berries have been used for the preparation of juice and tea to treat several illnesses, such as constipation, common cold, and diarrhea [8–10].

In the food industry, elderberry fruits and flowers are used to produce several products, such as liqueurs, jams, and juices [11]. Moreover, *S. nigra* can be used as a functional food for the prevention and the treatment of numerous diseases, as it has demonstrated antioxidant, anti-inflammatory, immune-stimulating, anti-cancer, and atheroprotective properties [12,13].

The largest class of water-soluble pigments, anthocyanins, afford many fruits and vegetables their red, purple, and blue hues [14]. These natural pigments can be found in flowers, roots, and vegetables, and are mostly associated with red fruits [14,15]. Furthermore, the food industry's interest in natural dyes has been increasing in order to replace synthetic dyes, as the latter have demonstrated disadvantages, such as allergenic, toxic, and even carcinogenic effects [16]. Cyanidin-3-glucoside and cyanidin-3-sambubioside are the most abundant anthocyanins found in elderberry juice [11,17]. Furthermore, anthocyanin cyanidin-3-glucoside has demonstrated anti-cancer [18,19], anti-angiogenic [20] and anti-obesity properties [21]. As such, anthocyanins have the added benefit of being used to prevent a variety of diseases, in addition to being of particular interest due to their great colorant properties, and could be used as a potential natural colorant [14,15]. The aim of this research was to evaluate the effect of an anthocyanin-rich elderberry extract (EE) supplementation on mice's physiological parameters.

2. Materials and Methods

2.1. Sample Preparation

Sambucus nigra L. fruits were collected in mid-September 2019 in Braganza, Portugal, when the maturation process was complete (Figure 1). The harvested fruits were immediately separated from the stems, washed, and frozen at −20 °C. The frozen fruits were crushed using a knife mill (model A327R1, Moulinex, Madrid, Spain) with a small amount of water to obtain juice; this process was facilitated by the previous freezing of the fruits, and later centrifuging them (K24OR refrigerated centrifuge, Centurion, West Sussex, UK)

to separate the husks and small seeds. The supernatant obtained was frozen, lyophilized, reduced to a fine, dried powder (35 mesh), and stored for further analysis.

Figure 1. Elderberry tree with the ripe fruit used to obtain the extract.

Stability of the Aqueous Extract

The stability of the aqueous extract was assessed taking into consideration the anthocyanin profile of these fruits and the respective percentage of degradation over four days. The anthocyanin phenolic profile was previously described by other authors [17]; nonetheless, a new identification was performed for this sample following the procedure previously described by Bastos et al. (2015) [22]. Two anthocyanins were identified, cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-glucoside, and, as such, the stability of the aqueous extract was assessed by measuring the percentage of anthocyanin loss by high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) using 520 nm as preferred wavelength.

The dried lyophilized extract of *S. nigra* was re-hydrated with water (100%) at a final concentration of 5 mg/mL and stored for 96 h at room temperature (~25 °C, mimicking the conditions under which the animals would be supplemented with these extracts, described below) and protected from light (to avoid the maximum degradation of the extracts). An aliquot of the samples was collected at 24, 72, and 96 h, injected into the chromatography system, and quantified using a 7-level calibration curve of the most similar standard compound available in the laboratory, cyanidin-3-*O*-glucoside (y = 105,078x − 12,437; R2: 0.9993; LOD = 0.28 μg/mL; LOQ = 0.84 μg/mL). The results were expressed as the percentage of anthocyanin loss (%).

2.2. Experimental Design

The University of Trás-os-Montes and Alto Douro Ethics Committee (approval no. 10/2013) and the Portuguese Veterinary Authorities (approval no. 0421/000/000/2014) approved this study. The national law (Decree-Law 113/2013) and European Directive 2010/63/EU on the protection of animals used in scientific research were both followed in all animal procedures.

Animals

As this is a preliminary study, to best apply the 3Rs for animal experiments (replacement, reduction, and refinement) and obtain enough data, we estimated the minimum number of animals required using a power analysis. Twenty-four FVB/n 8-week-old female mice (*Mus musculus*), obtained from a colony at University of Trás-os-Montes and Alto

Douro's animal facility, were randomly divided into four different groups (n = 6/group) using a computer-generated randomization sequence.

The control group drank tap water, while EE12, EE24, and EE48 were supplemented with increasing concentrations of a *S. nigra* extract, 12 mg/mL, 24 mg/mL, and 48 mg/mL, respectively, dissolved in normal tap water and changed every 2–3 days due to the compounds' stabilities. Drink and food (Diet Standard 4RF21 Certificate, 4RF21, Mucedola, Milan, Italy) were available *ad libitum* for every group. Under controlled conditions of temperature (19 ± 2 °C), 12 h:12 h light–dark cycle, and relative humidity (50 ± 10%), the animals were housed in separate open polycarbonate cages with bedding made of corn cob and provided with environmental enrichment.

Weekly, several murinometric parameters were evaluated, such as the body weight and temperature of each animal, as well as drink and food consumption by each group. The same researcher examined humane endpoints on a weekly basis using a previously published grading sheet [23]. Animals with a cumulative score of four or higher at any point in time were designated for euthanasia.

Twenty-nine days after the beginning of the experiment, the animals were sacrificed by intraperitoneal administration of ketamine (Imalgene 1000, Vetoquinol, Barcarena, Portugal) and xylazine (Rompun® 2% Bayer, Healthcare S.A., Kiel, Germany), followed by cardiac puncture and exsanguination according to the Federation of European Laboratory Animal Science Associations guidelines [24].

Complete necropsies were performed, and organs were collected and weighed on a precision balance (KERN® PLT 6200-2A, Dias de Sousa S.A., Alcochete, Portugal). These organs were fixed by immersion in 10% neutral-buffered formalin. Liver and kidney samples were stored at −80 °C for further analysis of oxidative stress.

2.3. Hematological Analysis

2.3.1. Microhematocrit

Microhematocrit values were obtained after the blood samples were centrifuged at 4500× *g* for 5 min (PrO-Vet, Centurion Scientific Limited, Chichester, UK) in capillary tubes, and the column of red blood cells was measured with a ruler.

2.3.2. Serum Biochemistry

Blood collected into lithium–heparin tubes (FL MEDICAL, Torreglia, Italy) was centrifuged at 1400× *g* (Heraeus Labofuge™ 400R, Thermo Fischer Scientific, Waltham, MA, USA) for 15 min, at 4 °C, and plasma was stored at −80 °C. Using an autoanalyzer (Prestige 24i, Cormay PZ, Warsaw, Poland), spectrophotometric methods were used to measure the concentrations of creatinine, urea, aspartate aminotransferase (ASAT), and alanine aminotransferase (ALAT).

2.4. Comet Assay

Mononuclear blood cells were used to perform the alkaline (pH > 13) comet assay, following the methods of Collins (2004) [25], and a system of twelve gels per slide, as adopted by Marques et al. (2021) [26], was used to increase the yield. Six slides precoated with normal-melting-point agarose were used for each treatment, with three replicates per animal. One set was used to run the assay with the repair enzyme, formamidopyrimidine DNA glycosylase (Fpg), and the other was used to conduct the assay without it. In order to precisely identify oxidative damage to DNA, particularly 8-oxoguanines and other changed purines, Fpg converted oxidized purines into DNA single-strand breaks. Peripheral blood was diluted in ice-cold phosphate-buffered saline (PBS), and this cell suspension was combined with 1% low-melting-point agarose. Twelve drops were placed on each of the twelve precoated slides, and the slides were refrigerated for solidification. The samples were then incubated in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) and rinsed (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0).

The slides with and without Fpg treatment were then incubated for 30 min at 4 °C in an alkaline electrophoresis solution (0.3 M NaOH and 1 mM EDTA, pH > 13) and electrophoresed for 30 min at 25 V and 300 mA. The cells were then neutralized with PBS, followed by distilled water, and then dehydrated in 70% and absolute ethanol. A fluorescent microscope at 400× magnification (Olympus BX41, Olympus America Inc., Hauppauge, NY, USA) was used to view the DNA stained with 4,6-diamidino-2-phenylindole (DAPI).

The comets were then classified visually based on a five-class scale of severity (class 0, no damage, and class 4, highest damage) [25]. Three hundred nucleoids were classified per animal (100 per mini gel) and, using the following formula, the total score was expressed as a genetic damage index (*GDI*) with a range of 0 to 400 arbitrary units:

$$GDI = \sum \%\ \text{nucleoids class}\ i \times i$$

where *I* represents the number of each class.

Untreated *GDI* values were subtracted from the Fpg incubation scores (GDI_{Fpg}) to calculate the net enzyme-sensitive sites (NSSFpg).

2.5. Hepatic and Renal Histology

The organs underwent routine processing for paraffin embedding, including sectioning. Hematoxylin and eosin (H&E)-stained tissue sections that were 3 µm thick were examined under a light microscope for histological analysis.

Liver histological analysis included the recording of the presence or absence of hydropic changes, their distribution, as well as the presence of inflammatory cells. Kidneys were examined to assess the presence of inflammatory lesions.

2.6. Hepatic and Renal Oxidative Stress

Samples of the liver and kidney were homogenized in a cold buffer solution (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, and 0.5 mM phenylmethylsulfonylfluoride (PMSF), pH 7.4) and the supernatants were collected following centrifugation and analyzed as previously described [27]. Briefly, excitation at 485 nm and emission at 530 nm were employed to assess the production of reactive oxygen species (ROS) using the probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The inhibition of nitroblue tetazolium (NBT) reduction was used to assess the superoxide dismutase (SOD) activity at 560 nm. The H_2O_2 decay at 240 nm was used to evaluate the catalase (CAT) activity. The oxidation of NADPH to $NADP^+$ at 340 nm was employed to assess the glutathione peroxidase (GPx) activity. The reaction of glutathione's thiol group with 1-chloro-2,4-dinitrobenzene (CDNB) was used to determine the activity of glutathione S-transferase (GST) at 340 nm. Derivatization with ortho-phthalaldehyde at 320 nm and 420 nm (excitation and emission, respectively) was used to assess the quantities of reducing glutathione (GSH) and oxidized glutathione (GSSG). The ratio of GSH to GSSG was used to calculate the oxidative stress index (OSI). Malondialdehyde (MDA), a lipid peroxidation (LPO) biomarker, was measured at 530 nm using a thiobarbituric acid (TBA)-based technique.

2.7. Statistical Analysis

Statistical analysis was performed using IBM SPSS version 20 (Statistical Package for the Social Sciences, Chicago, IL, USA). The Shapiro–Wilk test was used to ensure that the data followed a normal distribution. A statistical one-way ANOVA was conducted, followed by the Bonferroni's multiple-comparison test. For histological analysis, the Chi-square test was performed. For oxidative stress analysis, statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison test or the Kruskal–Wallis test followed by Dunn's test. At $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), or $p < 0.0001$ (****), data were deemed statistically significant.

3. Results

3.1. General Findings

The anthocyanin extract stability was assessed by HPLC-DAD (*data not shown*), revealing a maximum percentage of anthocyanin loss of 41.7% after 96 h of storage. In the first 48 and 72 h, the anthocyanin losses were 4.2 and 23.1%, respectively. Given these results, replacement of the supplementation water enriched with the anthocyanin-enriched extract occurred every two to three days during the experimental trial.

The animals in this study were monitored daily, and there were no phenotypic and/or behavioral changes in the mice in this study, as well as no deaths during the experiment. The aforementioned humane endpoint table was used to assess animal well-being, with no animal achieving the required score for euthanasia or any other notable behavioral change.

This study lasted for 29 days, during which almost no alteration was observed regarding the murinometric parameters evaluated. However, animals from EE12 ($p = 0.012$) lost a significant amount of body weight when compared with the control group (in weeks 1–4) and EE48 (in weeks 1 and 3), whereas animals from EE24 and EE48 did not show any significant differences when compared with the control group (Figure 2).

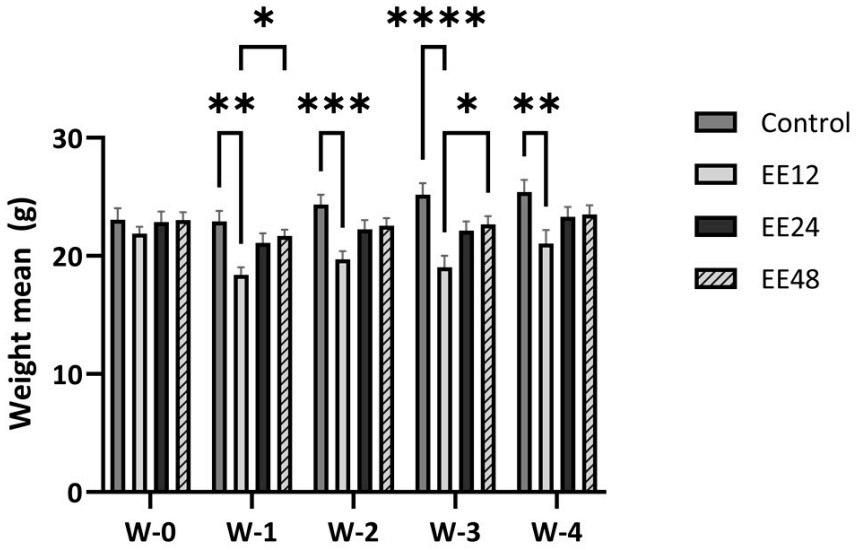


Figure 2. Body weight (g) mean [±Standard error (SE)] throughout the experiment. Statistically significant differences are denoted by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Regarding food and drink intake, all of the groups had relatively similar average daily food and drink intake values (Table 1).

Table 1. Mean daily values of food and drink intake (g) per experimental group during the four weeks.

Groups	Food (g)		Drink (mL)	
	Initial	Final	Initial	Final
Control	22.14	23.02	27.90	29.33
EE12	19.99	25.27	26.12	26.60
EE24	22.51	21.57	27.09	28.12
EE48	21.47	21.60	22.08	27.12

The relative organ weight of the heart (Table 2) was significantly higher in EE12 compared with the control ($p = 0.011$), EE24 ($p = 0.02$), and EE48 ($p = 0.01$). Additionally, the left kidney's relative organ weight was significantly higher in the control than EE12 ($p = 0.013$) and EE24 ($p = 0.013$).

Table 2. Mean (±SE) relative organ weights (organ weight (mg)/body weight of the animal (g)) from the experimental groups.

Group/Organ	Control	EE12	EE24	EE48
Thymus	1.92 ± 0.129	1.71 ± 0.259	2.02 ± 0.194	2.29 ± 0.255
Heart	4.69 ± 0.148 [a]	5.50 ± 0.186 [b]	4.50 ± 0.147 [a]	4.49 ± 0.156 [a]
Lungs	6.92 ± 0.349	7.37 ± 0.314	7.13 ± 0.257	7.78 ± 0.494
Spleen	3.89 ± 0.279	4.15 ± 0.239	4.08 ± 0.245	3.94 ± 0.344
Liver	54.56 ± 1.293	49.61 ± 0.904	50.50 ± 2.287	51.64 ± 2.171
Left Kidney	6.00 ± 0.118 [a]	6.99 ± 0.114 [b]	6.95 ± 0.196 [b]	6.51 ± 0.271 [a,b]
Right Kidney	6.05 ± 0.308	7.36 ± 0.134	6.33 ± 0.456	6.77 ± 0.330
Left Adrenal	0.42 ± 0.102	0.46 ± 0.126	0.47 ± 0.076	0.48 ± 0.101
Right Adrenal	0.32 ± 0.075	0.45 ± 0.064	0.29 ± 0.069	0.34 ± 0.060

Significant statistical differences between groups are indicated by different letters ([a] and [b]) ($p < 0.05$).

3.2. Haematological Analysis

The microhematocrit values per animal group are shown in Figure 3a (Supplementary Table S1), which were not significantly different between the experimental groups. Regarding the serum biochemical parameters (Figure 3c–e), there were no significant differences between the groups, except for the urea values (Figure 3e) in the control group and EE12 ($p = 0.011$).

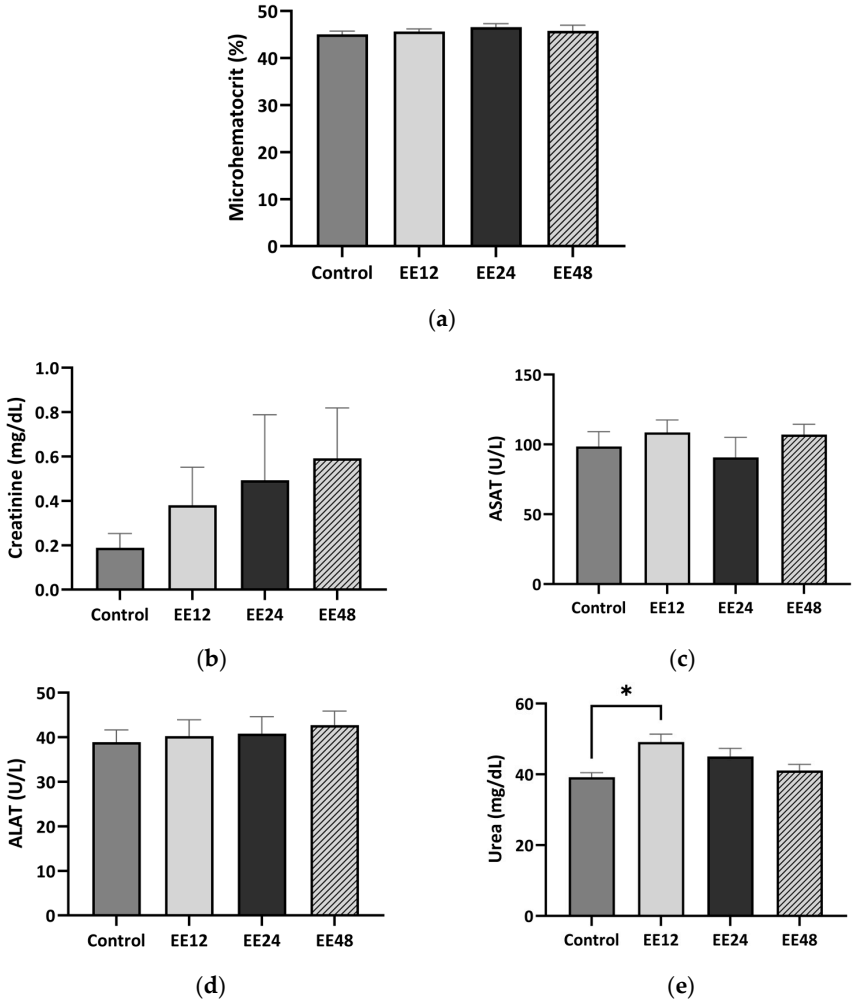


Figure 3. Hematological parameters evaluated (mean ± SE): (**a**) microhematocrit, (**b**) creatinine, (**c**) ASAT (aspartate aminotransferase), (**d**) ALAT (alanine aminotransferase), and (**e**) urea. * Statistically significant differences ($p < 0.05$).

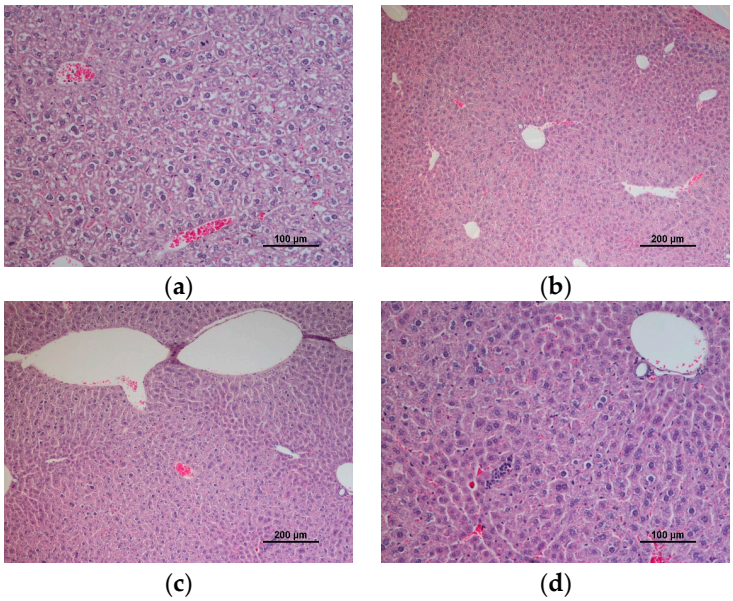
3.3. Hepatic and Renal Histology

A significant difference between the control group and EE12 was seen in the liver's histology ($p = 0.036$), with the control group's histology displaying overall hydropic changes (Table 3). When compared with the other groups, the control mice showed a generalized distribution of these changes ($p = 0.006$), while the other groups showed hydropic changes at the centrilobular zone. With at least one animal in each group displaying chronic focal hepatitis, liver inflammation was also evaluated; the findings did not statistically differ between groups. Except for one instance of chronic interstitial nephritis in EE24, no histological alterations were seen in the experimental animals (Figure 4).

Table 3. Numbers of animals with hepatic and renal histological lesions per experimental group (%).

	Control	EE12	EE24	EE48
Liver				
Normal	1/5 (20.0%) [a]	5/6 (83.3%) [b]	4/6 (66.7%) [a,b]	4/6 (66.7%) [a,b]
Hydropic changes (HC)	4/5 (80.0%) [a]	1/6 (16.7%) [b]	2/6 (33.3%) [a,b]	2/6 (33.3%) [a,b]
HC general	4/5 (80.0%) [a]	0/6 (0.0%) [b]	0/6 (0.0%) [b]	0/6 (0.0%) [b]
HC centrilobular	0/5 (0.0%)	1/6 (16.7%)	2/6 (33.3%)	2/6 (33.3%)
Inflammation				
Absent	3/5 (60.0%)	5/6 (83.3%)	3/6 (50.0%)	3/6 (50.0%)
Chronic focal hepatitis	2/5 (40.0%)	1/6 (16.7%)	3/6 (50.0%)	3/6 (50.0%)
Kidney				
Normal	5/5 (100.0%)	6/6 (100.0%)	5/6 (83.3%)	6/6 (100.0%)
Chronic interstitial nephritis	0/0 (0.0%)	0/0 (0.0%)	1/6 (16.7%)	0/0 (0.0%)

Significant statistical differences between groups are indicated by different letters ([a] and [b]) ($p < 0.05$). HC: hydropic changes.



(**a**) (**b**) (**c**) (**d**)

Figure 4. Microscopic images (staining with H&E) of liver sections from the different groups under study. (**a**) Cellular tumefaction and hydropic changes in a mouse's liver from the control group, 200×. (**b**) Hydropic changes observable only in the centrilobular region of an animal's liver from EE12, 100×. (**c**) Cellular tumefaction and centrilobular hydropic changes in a mouse's liver from EE24, 100×. (**d**) Cellular tumefaction, centrilobular hydropic changes, and multifocal inflammatory infiltration in an animal from EE48, 200×.

3.4. Comet Assay

The parameters evaluated with the comet assay (GDI, GDI_{Fpg}, and NSS_{Fpg}) were measured in peripheral blood mononuclear cells (Figure 5). Regarding the GDI values, there were no statistically significant differences between the experimental groups. However, the GDI_{Fpg} values were significantly greater in the control group than EE24 ($p = 0.037$) and EE48 ($p = 0.047$). Regarding NSS_{Fpg}, the value in the control group was also significantly higher compared with those in EE24 ($p = 0.043$) and EE48 ($p = 0.033$).

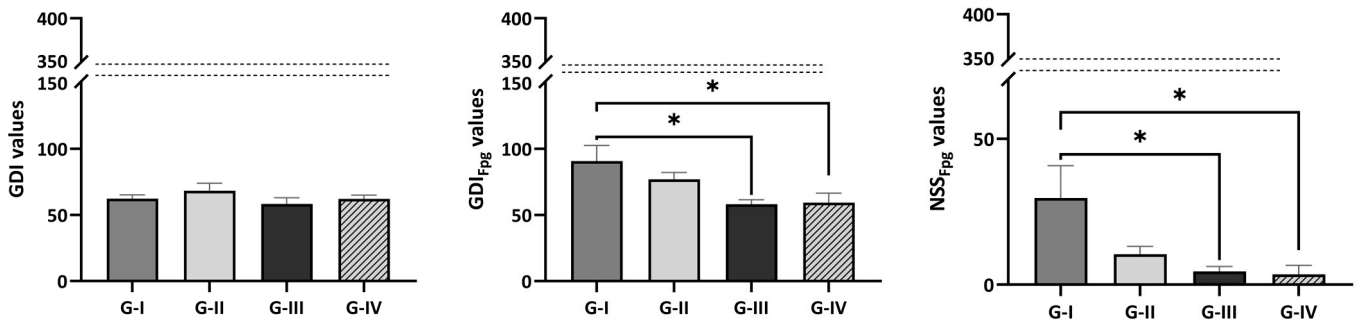


Figure 5. Genetic damage index values (mean ± SE and expressed as arbitrary units) determined by the comet assay, corresponding to untreated slides (**a**) and slides treated (**b**) with Fpg and the resulting NSS_{Fpg} (**c**). * Statistically significant differences ($p < 0.05$).

3.5. Liver and Kidney Oxidative Stress

The liver and kidney oxidative stress analysis (Table 4) showed significant differences in the antioxidant enzymes SOD and CAT. The liver SOD activity increased in the EE48 group in comparison with the control and EE12 groups ($p < 0.01$). In the opposite direction, liver CAT activity decreased in the EE48 group in comparison with the control group ($p < 0.01$). Concerning kidney oxidative parameters, the only significant differences observed between groups were the increased activity of SOD in the EE48 group in comparison with the control ($p < 0.01$).

Table 4. Values of liver and kidney oxidative parameters. Data are expressed as mean ± standard deviation for parametric data distribution or median (25th–75th quartile) for non-parametric data.

Biochemical Parameters	Control	EE12	EE24	EE48
Liver				
ROS	1212 (1115–1623)	1649 (1330–1729)	2051 (1678–3046)	1896 (1408–2308)
SOD	231 (169–271) [a]	250 (239–255) [a]	274 (233–322) [a,b]	333 (290–382) [b]
CAT	5956 (5047–6608) [a]	4001 (3197–4482) [a,b]	3906 (2960–5081) [a,b]	2925 (2241–3612) [b]
GPx	0.41 ± 0.2	0.46 ± 0.13	0.61 ± 0.19	0.3 ± 0.27
GST	0.17 ± 0.01	0.18 ± 0.02	0.2 ± 0.03	0.19 ± 0.02
GSH	14.58 ± 3.4	14.71 ± 1.86	13.33 ± 5.55	12.95 ± 2.68
GSSG	270 ± 63.6	272.9 ± 138.5	339.7 ± 109	318.8 ± 141.6
OSI	0.05 ± 0.01	0.03 ± 0.02	0.04 ± 0.004	0.04 ± 0.01
MDA	205.1 ± 45.1	189.8 ± 23.8	207.0 ± 60.3	176.0 ± 28.6
Kidney				
ROS	1460 ± 323	1545 ± 338	1429 ± 443	1337 ± 322
SOD	161 (139–188) [a]	203 (178–271) [a,b]	333 (267–432) [a,b]	341 (305–374) [b]
CAT	78.55 ± 7.19	76.05 ± 10.12	77.37 ± 12.21	77.89 ± 13.25
GPx	0.15 (0.13–0.22)	0.08 (0.04–0.13)	0.07 (0.05–0.1)	0.08 (0.04–0.11)
GST	0.058 ± 0.01	0.045 ± 0.002	0.058 ± 0.01	0.045 ± 0.002
GSH	148.1 ± 31.60	131.4 ± 16.69	146.2 ± 35.49	132.8 ± 21.63
GSSG	18.18 ± 13.02	23.07 ± 14.43	18.54 ± 8.78	14.93 ± 6.07
OSI	5.83 ± 1.92	8.01 ± 2.75	9.57 ± 5.12	10.6 ± 5.21
MDA	5600 ± 1189	4960 ± 728	5501 ± 1378	5040 ± 864

Significant statistical differences between groups are indicated by different letters ([a] and [b]) ($p < 0.05$). ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GST: glutathione S-transferase; GSH: reducing glutathione; GSSG: oxidized glutathione (GSSG); OSI: oxidative stress index; MDA: malondialdehyde.

4. Discussion

A previous study evaluated the extract's cytotoxicity in five different cell lines: human breast adenocarcinoma (MCF-7), human non-small lung carcinoma (NCI-H460), human cervical carcinoma (HeLa), human hepatocellular carcinoma (HepG2), and porcine liver cells (PLP2) [17]. These authors reported that the extract had significant capacity to inhibit the proliferation of these tumor cell lines, while low concentrations of EE had no effect on the normal cell line. After cell studies, a correct approach is to switch to an experimental animal protocol. To the best of our knowledge, this is the first study to assess the safety of different concentrations of an elderberry colorant extract in mice. Although the FVB/n strain is normally used for genetic and transgenic experiments, wild-type FVB/n mice have also been employed in metabolic and toxicology studies [28–31].

Animals supplemented with EE had considerably lower mean body weight values, particularly EE12, which was significantly inferior to the control group. *In vitro* assays have revealed that the primary components of this extract have an anti-obesity activity [32]. In a before-and-after clinical trial, Chrubasik et al. (2008) reported that a combination of *S. nigra* and *Asparagus officinalis* was significantly effective in lowering the mean body weight and blood pressure while improving the participants' physical and emotional well-being and quality of life [33]. The same occurrence appeared to happen to the EE-supplemented animals, especially at the lowest dose. Given that these animals consumed identical amounts of food and that the humane endpoint analysis revealed no changes in behavior associated with toxicity, the inclusion of anthocyanins in their diet appeared to influence their body weight gain. Another study discovered that dietary supplementation with purified mulberry (*Morus australis* Poir) anthocyanins suppressed body weight gain in high-fat-diet-fed C57BL/6 mice by reducing the epididymal fat weight and adipocyte size [34]. A future study should explore this extract's effect on adipose tissue and how it can affect body weight gain. Food consumption was standard compared with other studies developed by our group with the same diet [27,30]. The mice's initial reactions to the extract were not very positive, but, over time, the overall average consumption by the supplemented groups became similar to that by the control group. As this particularly occurred for the group supplemented with the highest concentration of EE, this difference may be due to the extract's flavor, which might have been too strong or overbearing. The values regarding drink intake were, however, consistent with those of the aforementioned works.

The relative weight of the heart in EE12 significantly increased when compared with the other groups, probably due to residual blood after cardiac puncture. Regarding hydropic changes, animals from the control group exhibited a generalized distribution of these changes. According to the literature, these represent acute and reversible, sublethal cell injuries [35], and in the absence of biochemical evidence of liver damage in these animals, the changes observed in the control group were not regarded as having pathological significance. In the present study, the animals supplemented with EE at the lowest concentration showed minor and zone-restricted hydropic changes when compared with the control group. This effect may be related to the anthocyanins present in the EE, because, in chronic ethanol-induced liver injury in male Wistar rats and CCl4-induced Kunming mice [36,37], these substances have been reported to have an hepatoprotective effect.

Our results regarding the renal function markers, particularly the urea levels in EE12, which were considerably greater than those of the control group, were in conflict with the literature, as anthocyanins are usually associated with a reduction in urea levels [38,39]. Our values are, however, in accordance with those of other studies regarding this parameter in FVB/n mice and do not raise suspicion of renal impairment [28,40]. As an increase in urea levels can occur in settings unrelated to renal disease, creatinine is seen as a more reliable marker of renal function [41]. Although there was a slight increase in creatinine levels, there was no statistically significant difference between the groups. The left kidney's relative weight was significantly higher in EE12 and EE24 compared with that of the control group, but, as no kidney lesions were registered by the histological analysis, this increase in the relative weight and biochemical parameters may be unrelated to renal function impairment.

It is also crucial to note that the EE supplementation was chronic, i.e., the animals consumed the extract in their drinking water for a lengthy period of time without interruption, which may not occur if it is incorporated into a product that is not the consumer's whole diet.

As mentioned above, elderberry extract supplementation did not cause hepatotoxicity at the biochemical level, as the hepatic transaminases (ALAT and ASAT) showed no significant statistical differences. These results are corroborated by another study that used an extract of *S. nigra* [42].

In terms of the comet assay results, our findings show that this extract did not appear to be genotoxic. Interestingly, when the Fpg enzyme was added, there were statistically significant differences between the control group and the two highest concentrations. As such, this leads us to believe that this extract, although not antigenotoxic, may protect against oxidative DNA damage. These findings are consistent with the literature [13,43], which indicates that *S. nigra* has antimutagenic and antigenotoxic effects, mainly due to its antioxidant properties. Olejnik et al. (2016) reported that, in a non-transformed, non-tumorigenic colon cell line (NCM460), an *S. nigra* extract was capable of protecting these cells against oxidative stress's detrimental effects to DNA [44]. In another study, Ferreira-Santos and collaborators employed an *S. nigra* aqueous extract for treating human colon carcinoma cells (RKO) and observed that this extract at concentrations of 200 and 400 μg/mL neither protected RKO cells from the oxidative activity induced by H_2O_2 nor triggered DNA repair activity [45]. The authors only observed a reduction in DNA damage of around 20% when the cells were treated for 24 h. The same was not observed when the cells were treated for 48 h; however, the results were not shown to be significantly different from those of the control group at both times [45]. Our findings regarding NSS_{Fpg} indicate that this extract, particularly the two highest concentrations (EE24 and EE48), had a strong capacity for repairing oxidative DNA damage.

The group treated with a higher concentration of extract (EE48) also exhibited increased SOD activity, the primary intrinsic antioxidant enzyme, in the hepatic and renal tissue, confirming the stimulation of antioxidant activity previously reported for anthocyanins in the extracts of *S. nigra* [46,47]. However, CAT activity decreased in the same group only at the hepatic level. This discrepancy in activities recorded for SOD and CAT has been previously reported between tissues for the effect of anthocyanins [48] and in the same tissue attributed to different forms of modulation [49] that need further studies to understand the antioxidant mechanisms of these compounds. Moreover, the remaining parameters of oxidative stress did not vary significantly, which confirms the safety of the use of this extract at the tested doses.

Although synthetic food colorants are frequently used in the food industry, consuming them continuously could be toxic and have several side effects [50]. For this reason, natural origin colorants have gradually taken their place, adding potential health benefits as well [51]. Given their numerous reported medicinal properties, including antioxidant activity and anti-carcinogenic and anti-inflammatory properties, these natural dyes, particularly anthocyanins, have a significant potential to replace synthetic colorants in the food industry [52].

5. Conclusions

As a result of the decreased liver lesions and the potent defense against oxidative DNA damage, our research shows that this anthocyanin-rich elderberry extract has a favorable toxicological profile. The two highest concentrations (EE24 and EE48) appeared to perform exactly as intended, which was to create a natural colorant that had no negative effects on the health of individuals who consumed it and might even have some positive effects. In the future, further research may be needed to confirm our findings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122311928/s1, Table S1. Hematological parameters evaluated (mean ± SE).

Author Contributions: Methodology and writing—original draft: T.A. and T.F.; Conceptualization: T.A., T.F., R.M.G.d.C., M.M.S.M.B., R.M., L.B. and P.A.O.; Supervision and project administration: R.M.G.d.C., M.M.S.M.B., R.M., L.B. and P.A.O.; Sample preparation and HPLC: M.I.D. and L.B.; Conducted the experiments with live animals and preparation of the drink: T.A., T.F. and R.S.-R.; Participated in the sacrifice of the animals: T.A., T.F., R.S.-R., M.J.P. and P.A.O.; Participated in biochemical analysis and microhematocrit: M.J.N. and M.J.P.; Performed the comet assay: T.A., J.F. and I.G.; Prepared the histology samples, histological analysis, and image capture: T.A., T.F. and M.d.L.P.; Participated in oxidative stress: F.T., D.F., L.F. and C.V.; Statistical analysis: T.A., T.F., R.M.G.d.C., M.M.S.M.B., R.M. and P.A.O. All authors have read and agreed to the published version of the manuscript.

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