Biocatalysis and Agricultural Biotechnology 74 (2026) 104070

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Preserved organ function and redox homeostasis following repeated oral exposure to *Quercus suber* acorn shell extract in female FVB/n mice

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ARTICLE INFO

Keywords:
By-products
Kidney
Liver
Oxidative stress
Polyphenols
Quercus suber

ABSTRACT

Acorn shells (AS), a by-product of *Quercus suber* acorn processing, are rich in phenolic compounds with recognized bioactivities. However, their incorporation into food or nutraceutical formulations requires *in vivo* safety assessment under physiologically relevant exposure conditions. This study evaluated the effects of a hydroethanolic AS extract following repeated oral intake in female FVB/n mice over 25 days. The extract's phenolic profile was characterized by HPLC-DAD-ESI-MS/MS. Nineteen animals (8-12 weeks old) were randomly assigned to four groups receiving drinking water containing 0, 100, 200 and 500 μg/mL of extract. Animal welfare, food and water intake, and body weight were monitored throughout the study. At the end, animals were euthanized via anesthetic overdose, after which blood was collected for biochemical analysis, organs were weighed, and liver and kidney processed for histology and oxidative stress analysis. The extract was dominated by phenolic acids and hydrolysable tannins, primarily gallic/ellagic acid derivatives. No toxicity signs or mortality were recorded, and food and water intake remained similar across groups. Body weight and food and water intakes were unaffected, whereas an increase in liver, spleen, and left kidney relative weight was observed in the 200 μg/mL dose ($p < 0.05$). Biochemical parameters were also preserved between groups, though the 200 μg/mL group showed reduced renal lipid peroxidation ($p < 0.05$). Histological examination revealed no treatment-related hepatic or renal lesions. Overall, these findings indicate that repeated oral exposure to AS extract does not compromise systemic homeostasis under the tested conditions,

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https://doi.org/10.1016/j.bcab.2026.104070
Received 16 February 2026; Received in revised form 10 April 2026; Accepted 1 May 2026
Available online 4 May 2026

supporting further exploration as a sustainable source of bioactive compounds as functional dietary ingredients.

1. Introduction

Quercus suber L., commonly known as the cork oak, is as an evergreen tree widely distributed across the western Mediterranean basin (Touati et al., 2015). Its main economic value lies in cork production, primarily used for wine stoppers and insulation materials (Custódio et al., 2015; Touati et al., 2015). Beyond cork, the species also produces acorns represent a traditional food resource increasingly revisited for their nutritional and functional potential (Inácio et al., 2024). Acorn kernels are rich in carbohydrates and protein, low in lipids, and a source of essential minerals and unsaturated fatty acids (Haddada et al., 2025). Acorns also contain bioactive compounds such as phenolic compounds (Castro et al., 2022; Oliveira et al., 2023), which grant them strong antioxidant activity and have been associated with anti-inflammatory, anti-diabetic, anti-obesity, and cardioprotective activities (Custódio et al., 2015; Oliveira et al., 2023; Youn et al., 2016). Although the acorn kernel has been the main focus of nutritional and phytochemical research, its processing (peeling, roasting, milling, oil extraction) also generates substantial residues, namely acorn shell (AS) which represents approximately 20% of total fruit weight (Araújo-Rodrigues et al., 2024; Mateus et al., 2025).

Currently, AS has mostly been incorporated into industrial processes, such as a bio-adsorbent for dye removal (Ghaedi et al., 2011; Saka et al., 2012), as a precursor for activated carbon capable of removing pharmaceutical pollutants from water (Nourmoradi et al., 2018), as a natural coagulant in wastewater treatment (Šćiban et al., 2009) or even as natural pigments in textiles and as tannin-rich agents in leather tanning (Ozdemir and Karadag, 2023). Recent phytochemical analyses have demonstrated that acorn shells contain hydrolysable tannins and phenolic acids (Mateus et al., 2025; Youn et al., 2016), compounds widely recognized for their redox-modulating and bioactive properties. Such composition positions acorn shells as promising candidates for incorporation into functional foods, nutraceutical formulations, or natural antioxidant systems. However, the translational feasibility of these applications critically depends on establishing systemic tolerance following oral exposure. To date, most research on AS extracts has focused on in vitro bioactivity, with the limited available *in vivo* evidence restricted to a disease model of atopic dermatitis that did not assess systemic effects beyond disease-related parameters (Lee et al., 2019; Yarani et al., 2013). Data addressing repeated oral exposure under physiological conditions, simulating daily consumption rather than acute pharmacological dosing, has yet to be addressed.

Therefore, the present study aimed to evaluate the effect of repeated oral intake an AS extract in female FVB/n mice, with a focus on physiological, biochemical, and histological assessments relevant to systemic safety, thereby providing a basis for its prospective dietary incorporation.

2. Materials and methods

2.1. Acorn shell extract

The sample from *Quercus suber* L. was collected in November 2024, at Herdade da Corisca, Santiago do Cacém, Portugal (37.943496, −8.661962). The sample analysed underwent a manual peeling procedure to separate the shell from the acorn body, sourced from batches of no less than 100 kg of mature acorns, meaning those that had fallen to the ground and had >90% fully ripened fruits, gathered and processed by the Landratech company (Azambuja, Portugal) in November 2024. The sample was dehydrated in an oven with air ventilation at a set temperature of 50 °C, while the actual chamber temperature varied from 35 to 55 °C. Lastly, once dried, the shells were milled with a disc mill (Oukaning, China). The AS sample studied was subjected to a hydroethanolic maceration (60:40, v/v) with a solid/liquid ratio of 25 g/L for 1 h at room temperature with constant stirring; the extraction process was performed twice to maximize compound extraction. These conditions were selected to obtain a representative phenolic-rich extract while minimizing potential degradation or oxidation associated with prolonged extraction times, as reported in the literature (Cacace and Mazza, 2003; Naczk and Shahidi, 2004). After filtration, the samples were freeze-dried (FreeZone 4.5, Labconco, Kansas City, MO, USA). The AS extract obtained was stored under protection from the light and froze at −20 °C until further analysis.

2.2. Mice

Nineteen FVB/n female mice (*Mus musculus*) aged 8-12 weeks old were used in this study, obtained from a colony maintained the at University of Trás-os-Montes and Alto Douro's animal facilities. FVB/n is an inbred strain, providing a genetically homogeneous background that reduces inter-individual variability and supports consistent phenotypic assessment across experimental assays (Fernandes et al., 2024). The experimental assay was carried out at the animal facilities of the University of Trás-os-Montes and Alto Douro with approval from the Ethics Committee (approval no. 852-e-CITAB-202_A_1-e−122CITAB-2021) and the *Direção Geral de Alimentação e Veterinária* (approval no. 014139). 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. The animals were maintained under controlled conditions of temperature (23 ± 2 °C), 12 h:12 h light-dark cycle and relative humidity (50 ± 10%). The mice were housed in transparent cages lined with corn cob and provided environmental enrichment (e.g., toilet paper rolls, reused cardboard boxes, and shredded paper). The cages were cleaned once a week.

The *in vivo* safety study of this extract was conducted according to Azevedo et al. (2022) with minor modifications. Briefly, female

FVB/n mice were divided into four groups: control group drank tap water (CTRL, $n = 4$), while the remaining groups were administrated a *Quercus suber* shell extract dissolved in tap water at 100 μg/mL (AS100, $n = 5$), 200 μg/mL (AS200, $n = 5$), and 500 μg/mL (AS500, $n = 5$); extract solutions were replaced every 3-4 days to avoid compound degradation. Food (Standard 4RF21 GLP, Mucedola, Italy) and drink was available *ad libitum* for every group. The selected concentration range (100-500 μg/mL) was defined to encompass low, intermediate, and high exposure levels, in line with concentrations commonly reported for plant-derived phenolic extracts in biological assays (Emsen et al., 2026; Mascoloti Spréa et al., 2022). Given the absence of standardized dosing for such complex mixtures, a range-based approach is typically adopted to capture potential dose-dependent effects. Using standard allometric scaling based on mouse water intake (Nair and Jacob, 2016), the selected concentration range corresponds approximately to human-equivalent intakes of 107, 214, and 535 mg of AS extract per day, respectively, for a 60 kg adult. The experimental assay had a duration of 25 days. Drink and food consumption and body weight were recorded on a weekly basis. Ponderal weight gain (%) was calculated with the following formula (Medeiros-Fonseca et al., 2024b):

$$Ponderal\ weight\ gain = \frac{Final\ weight - Initial\ weight}{Initial\ weight} \times 100$$

Additionally, humane endpoints, which refer to pre-established criteria for prematurely end an animal study to avoid unnecessary pain, suffering, or distress without compromising the scientific objectives of the study (Williams and Baneux, 2022), were assessed weekly by the same researcher, based on clinical parameters recorded on a previously published score sheet (Oliveira et al., 2017). After each assessment, the scores were totalled, and if the total score was 4 or greater, the animal would be euthanized.

Twenty-five days after the beginning of the experiment, all animals were sacrificed by intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by exsanguination by cardiac puncture, following the guidelines of FELASA (Federation of European Laboratory Animal Science Associations) (Forbes et al., 2007). Complete necropsies were then performed on all animals, where internal organs (heart, lung, spleen, kidneys, and liver) were collected and weighed individually using a precision scale (KERN® PLT 6200-2A, Dias de Sousa S.A., Alcochete, Portugal). Relative organ weight was calculated by dividing the organ weight (g) by the body weight at necropsy (g). The right kidney and a section of the liver were frozen at −80 °C for subsequent oxidative stress studies. The remaining tissues collected for histopathology were fixed in 10% neutral buffered formalin.

2.3. Microhematocrit and biochemical parameters

The blood was collected into lithium-heparin tubes (FL MEDICAL, Torreglia, Italy), centrifuged at 1500×*g* for 10 min at 4 °C (Heraeus Labofug™ 400R, Thermo Fischer Scientific, Waltham, MA, USA). The resulting plasma supernatant was stored at −80 °C until use. Creatinine and alanine aminotransferase (ALT) were determined as markers of renal and hepatic function, respectively, at an external clinical laboratory using validated automated methods in accordance with routine internal and external quality control procedures.

Microhematocrit was determined by centrifuging capillary tubes containing whole blood at 12,000×*g* for 5 min and measuring the red cell column with a ruler to calculate the microhematocrit value (%).

2.4. Hepatic and renal histology

Following fixation, liver and kidneys were processed for routine histological analysis. The organs were embedded in paraffin, and histological sections, cut with a microtome (3 μm) for haematoxylin and eosin (H&E) staining, and analysed under a light microscope.

2.5. Hepatic and renal oxidative stress

Kidney and liver samples were homogenized in a cold buffer solution (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, and 0.5 mM PMSF, pH 7.4) (Deng et al., 2009), centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatant was removed for subsequent analysis on a PowerWave X2S microplate reading spectrophotometer (Bio-Tek Instruments, USA). Total protein content was determined at 280 nm in the same instrument and used for data normalization.

Reactive oxygen species were evaluated using DCFH-DA (2′,7′-dichlorofluorescein diacetate), with excitation at 485 nm and emission at 530 nm, quantified using dichlorofluorescein (DCF) standards (0-500 μM) and expressed in μmol DCF/mg protein (Deng et al., 2009). Superoxide dismutase (SOD, 560 nm) activity was evaluated by the reduction of nitroblue tetrazolium (NBT), generated by the xanthine/xanthine oxidase system, and quantified using standards (0-150 U/mL), with results expressed in U/mg protein (Durak et al., 1993). Catalase activity (240 nm) was assessed by the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen, quantified using standards (0-480 U/mL) and expressed in U/mg protein (Claiborne, 1985). The activity of glutathione peroxidase (GPx) and glutathione reductase (GR) was evaluated at 340 nm by the extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}) and is expressed in μmol NADPH/min/mg protein (Massarsky et al., 2017). Glutathione S-transferase activity (340 nm) was evaluated by the reaction of the thiol group of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB), considering a molar extinction coefficient of 9.60 mM^{-1} cm^{-1}, and the results were presented as μmol CDNB/min/mg protein (Habig and Jakoby, 1981). The levels of reduced glutathione (expressed in μmol GSH/mg protein) and oxidized glutathione (expressed in μmol GSSG/mg protein) were determined by ortho-phthalaldehyde derivatization (excitation 320 nm/emission 420 nm) using standards (0-1000 μM) (Gartaganis et al., 2007). The oxidative stress index (OSI) was determined by the ratio of GSH to GSSG (Gartaganis et al., 2007). Malondialdehyde (MDA, 530 nm),

an indicator of lipid peroxidation, was assessed by the thiobarbituric acid-based method and quantified using malondialdehyde (MDA) standards (0-100 μM), with values expressed in μmol MDA/mg protein (Wallin et al., 1993). Protein carbonylation (450 nm) was measured by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), and levels were expressed as μmol DNPH/min/mg protein (Mesquita et al., 2014). Lactate dehydrogenase activity (340 nm) was determined by the molar extinction coefficient of 6.22 mm^{-1} cm^{-1}, expressed as μmol NADH/min/mg protein (Domingues et al., 2010). DNA strand breaks (excitation at 360 nm/emission at 450 nm) were assessed by measuring the fluorescence intensity of the Hoechst dye, which preferentially binds to intact regions of the DNA double helix, using 0-10 mM dsDNA standards expressed as dsDNA/mg protein (Olive, 1988).

2.6. Phenolic composition

The identification of phenolic compounds in the AS extract was performed using high-performance liquid chromatography (HPLC) with a Thermo Scientific HPLC system (Dionex UltiMate™ 3000, Thermo Fisher Scientific, San Jose, CA, USA) that includes a diode-array detector (DAD) and is linked in series to an Orbitrap Exploris™ 120 mass spectrometer. Phenolic compounds were separated using a Spherisorb S3 ODS-2 C18 column (3 μm, 4.6 × 150 mm; Waters, Milford, CT, USA) held at 35 °C. The mobile phases included 0.1% (v/v) formic acid in ultrapure water (A) and acetonitrile (B), employing a gradient that commenced at 85:15 (A:B, %) for 5 min, transitioned to 80:20 for the next 5 min, 75:25 for 10 min, 65:35 for another 10 m, and reached 50:50 for 10 min. The system subsequently reverted to the starting conditions over 10 min and was maintained for another 10 min for column re-equilibration. The injection volume was 10 μL and the flow rate was 0.5 mL/min. UV-Vis spectra were obtained from 180 to 700 nm, and chromatograms were observed at 280, 330, and 370 nm based on the specific phenolic categories. High-resolution MS and MS/MS data were acquired using an OptaMax NG electrospray ionization (ESI) source functioning in negative mode. Instrument settings comprised a spray voltage of 2.5 kV, a temperature of 325 °C for the ion transfer tube, and a vaporizer temperature of 350 °C. Sheath (50 arb), auxiliary (10 arb), and sweep gas (1 arb) were all nitrogen. Full MS and MS/MS spectra were acquired in the range from 110 to 1800 charge-to-mass ratio (m/z) at a resolution of 15,000, with the RF lens adjusted to 70%. Data-dependent acquisition was executed using a top-4 $ddMS^2$ approach, implementing stepped HCD fragmentation (30, 50, and 150%; normalized to 30%). Dynamic exclusion was enabled when required. Data acquisition and processing were performed with Xcalibur™ software. Compound identification was based on retention behaviour, UV-Vis characteristics, deprotonated molecular ions ($[M–H]^-$), and MS/MS fragmentation patterns, and was supported by comparison with available standards, literature data, and spectral libraries (NIST™, MZ Vault™, and MZCloud™) using the Freestyle™ 1.7 platform.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10 (Boston, Massachusetts, USA). Data normality was assessed using the Shapiro-Wilk test, while homogeneity of variance was assessed using the Levene test. For data that were normally distributed, analysis of variance (ANOVA) was used, followed by the Bonferroni test for multiple comparisons and data were presented as mean ± standard error of the mean (SEM). In cases where the data were not normally distributed, the nonparametric Kruskal-Wallis test was applied, followed by Dunn's test and data were presented as median (25th percentile - 75th percentile). Statistical significance was established at p-value <0.05.

3. Results

3.1. General findings

During the entire experiment, no behavioural changes or clinical signs indicative of disease were observed, and no animal deaths occurred. Additionally, no animal reached a score of 4 or higher, predefined as the humane endpoint, since the extract did not induce any alterations in the animals' well-being.

Body mass was evaluated over the 25-day exposure period and results regarding initial and final body weight, as well as ponderal weight gain, are presented in Table 1. At the beginning of the assay, body mass in groups supplemented with the AS extract did not differ significantly from CTRL ($p > 0.05$) and, similarly, at the end of study, body mass remained comparable to CTRL in all supplemented groups ($p > 0.05$). Ponderal weight gain did not differ from CTRL in any AS-supplemented group ($p > 0.05$) and showed no consistent trend toward gain or loss. However, AS200 and AS500 represented the lowest and highest gains, respectively, and differed significantly from each other ($p < 0.05$).

Table 1
Initial and final body weight (g) per animal and ponderal weight gain (%). Data are presented as mean ± SEM for parametrical data or median (25th percentile - 75th percentile) for non-parametric data.

	Initial weight (g)	Final weight (g)	Ponderal weight gain (%)
CTRL ($n = 4$)	24.88 (22.94 - 26.26) ^{a,b}	24.53 ± 0.72	−0.5032 ± 2.646 ^{a,b}
AS100 ($n = 5$)	23.1 (22.94 - 24.76) ^{a,b}	24.43 ± 0.69	3.093 ± 1.793 ^{a,b}
AS200 ($n = 5$)	26.07 (25.51 - 29.82) ^{a}	25.83 ± 1.00	−5.206 ± 2.881 ^{b}
AS500 ($n = 5$)	21.89 (20.14 - 22.28) ^{b}	22.82 ± 0.66	6.946 ± 1.759 ^{a}

Different letters denote statistically significant differences ($p < 0.05$).

Table 2
Estimated daily food (g) and drink (mL) consumption per animal.

	Food (g)		Drink (mL)	
	Initial	Final	Initial	Final
CTRL	3.27	3.35	5.30	5.16
AS100	3.08	3.24	5.69	5.78
AS200	3.70	3.36	5.69	5.75
AS500	3.48	3.79	5.62	5.60

Initial and final food and drink consumption are presented in Table 2. Across the experimental groups, estimated intake was overall similar between groups at both timepoints and between the beginning and the end of the study, indicating no supplementation-related changes.

Relative organ weights are presented in Table 3. For the heart and lungs, relative mass did not differ from CTRL in any supplemented groups ($p < 0.05$). Spleen and liver relative weight was significantly higher in AS200 when compared with CTRL ($p < 0.05$), whereas other AS-supplemented groups did not differ from CTRL ($p > 0.05$); in the liver, AS200 was also higher than AS100 ($p < 0.05$). Regarding the right kidney, no AS-supplemented group differed from CTRL ($p > 0.05$), whereas in the left kidney AS200 was higher compared with the remaining groups ($p < 0.05$).

3.2. Haematological analysis

Microhematocrit and serum markers of renal (creatinine) and hepatic (ALT) function are presented in Table 4. Microhematocrit was comparable with CTRL across all supplemented groups ($p > 0.05$). Creatinine was higher in AS500 than in CTRL ($p < 0.05$), whereas the remaining AS-supplemented groups did not differ significantly from CTRL ($p > 0.05$). ALT levels were also similar across all experimental groups ($p > 0.05$).

3.3. Hepatic and renal histology

No major histological changes were observed in the liver (Fig. 1) or kidney (Fig. 2) of the CTRL and AS-supplemented groups. Across all experimental groups, mild hydropic change in centrilobular hepatocytes was observed and, occasionally, isolated hepatocytes showed vacuolar change, karyomegaly, and/or hyperchromatic nuclei. In the kidney, small, isolated foci of non-suppurative interstitial nephritis were noted in two animals in the A200 group.

3.4. Hepatic and renal oxidative stress

Oxidative stress and antioxidant parameters in the liver and kidney are summarized in Tables 5 and 6, respectively. No significant differences were found between AS-supplemented groups and the CTRL ($p > 0.05$) in both organs, except renal MDA content in AS200 which was significantly lower than the CTRL ($p < 0.05$).

Table 3
Relative weight of organs in experimental groups. Data are presented as mean ± SEM for parametrical data or median (25th percentile - 75th percentile) for non-parametric data.

Organs	CTRL ($n = 4$)	AS100 ($n = 5$)	AS200 ($n = 5$)	AS500 ($n = 5$)
Heart	0.0050 (0.0041 - 0.0060)	0.0045 (0.0043 - 0.0045)	0.0058 (0.0050 - 0.0064)	0.0060 (0.0050 - 0.0069)
Lungs	0.0066 ± 0.0002	0.0072 ± 0.0005	0.0073 ± 0.0001	0.0075 ± 0.0001
Spleen	0.0044 ± 0.0002 ^{b}	0.0052 ± 0.0002 ^{a,b}	0.0056 ± 0.0002 ^{a}	0.0048 ± 0.0003 ^{a,b}
Liver	0.0479 ± 0.0017 ^{b}	0.0476 ± 0.0006 ^{b}	0.0575 ± 0.0021 ^{a}	0.0520 ± 0.0016 ^{a,b}
Right kidney	0.0053 ± 0.0003	0.0055 ± 0.0004	0.0062 ± 0.0004	0.0061 ± 0.0002
Left kidney	0.0059 ± 0.0002 ^{b}	0.0060 ± 0.0002 ^{b}	0.0070 ± 0.0002 ^{a}	0.0061 ± 0.0002 ^{b}

Different letters denote statistically significant differences ($p < 0.05$).

Table 4
Microhematocrit and serum markers of renal (creatinine) and liver function (ALT) in experimental groups. Data are presented as mean ± SEM.

	Microhematocrit (%)	Creatine (mg/dL)	ALT (U/L)
CTRL ($n = 4$)	45.73 ± 1.11 ^{a}	0.320 ± 0.004 ^{b}	38.25 ± 5.66 ^{a}
AS100 ($n = 5$)	45.63 ± 0.40 ^{a}	0.333 ± 0.008 ^{a,b}	43.00 ± 6.87 ^{a}
AS200 ($n = 5$)	44.2 ± 1.41 ^{a}	0.338 ± 0.008 ^{a,b}	42.50 ± 4.13 ^{a}
AS500 ($n = 5$)	47.23 ± 1.14 ^{a}	0.358 ± 0.013 ^{a}	42.80 ± 3.01 ^{a}

Different letters denote statistically significant differences ($p < 0.05$). ALT, alanine aminotransferase.

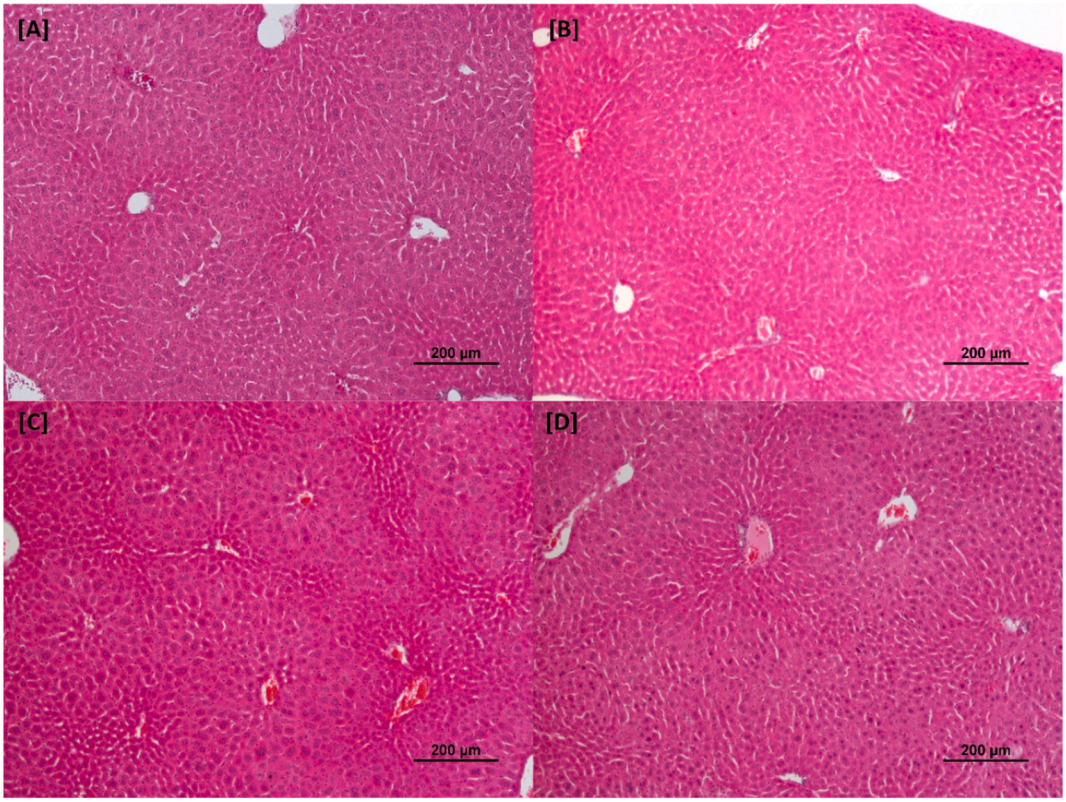

Fig. 1. Representative H&E-stained liver sections from CTRL (A), AS100 (B), AS200 (C), and AS500 (D) groups (100×). All images show preserved hepatic architecture with no treatment-related lesions.

Table 5
Oxidative stress parameters evaluated on the liver of all experimental groups. Data are presented as mean ± SEM for parametrical data or median (25th percentile - 75th percentile) for non-parametric data.

Parameter	CTRL ($n = 4$)	AS100 ($n = 5$)	AS200 ($n = 5$)	AS500 ($n = 5$)
ROS	34.56 ± 1.42	42.75 ± 5.28	41.75 ± 3.57	35.74 ± 1.93
SOD	694.10 (655.70-970.50)	1061.00 (639.40 - 1185.00)	920.00 (610.10 - 1086.00)	668.8 (630.80 - 799.90)
CAT	487.00 ± 13.04	565.40 ± 62.39	723.60 ± 188.90	679.40 ± 106.50
GPx	84.89 ± 7.97	98.38 ± 11.98	80.67 ± 5.00	78.35 ± 5.84
GR	20.51 ± 7.33	10.60 ± 1.57	10.77 ± 1.77	9.18 ± 0.69
GST	97.76 ± 7.67	134.70 ± 12.92	124.00 ± 8.70	111.40 ± 3.51
GSH	25.98 (15.52 - 26.69)	32.53 (22.22 - 35.49)	29.71 (25.74 - 32.69)	33.38 (22.45 - 35.65)
GSSG	13.48 (4.73 - 15.88)	5.55 (5.31 - 13.6)	9.24 (5.15 - 13.55)	8.20 (7.24 - 20.34)
LDH	3.98 (2.65 - 8.23)	10.92 (1.72 - 16.77)	8.26 (4.84 - 21.39)	4.55 (2.72 - 10.35)
MDA	6.77 ± 0.86	6.20 ± 0.52	4.22 ± 0.53	5.76 ± 0.76
PC	7.67 (4.04 - 13.30)	10.11 (3.15 - 13.02)	10.63 (7.34 - 13.37)	10.23 (9.63 - 10.34)
DNA damage	0.58 ± 0.08	0.58 ± 0.07	0.76 ± 0.06	0.72 ± 0.06
OSI	1.65 (0.82 - 2.21)	3.70 (2.39 - 6.87)	2.82 (2.40 - 5.95)	2.48 (1.48 - 4.81)

ROS, reactive oxygen species (µmol DCF/mg protein); SOD, superoxide dismutase (U/mg protein); CAT, catalase (U/mg protein); GPx, glutathione peroxidases (µmol NADPH/min/mg protein); GR, glutathione reductase (µmol NADPH/min/mg protein); GST, glutathione S-transferase (µmol CDNB/min/mg protein); GSH, glutathione (µmol GSH/mg protein); GSSG, oxidized glutathione (µmol GSSG/mg protein); LDH, lactate dehydrogenase (µmol NADH/min/mg protein); MDA, malondialdehyde (µmol MDA/mg protein); PC, protein carbonyls (µmol DNPH/min/mg protein); OSI, oxidative stress index (GSH/GSSG).

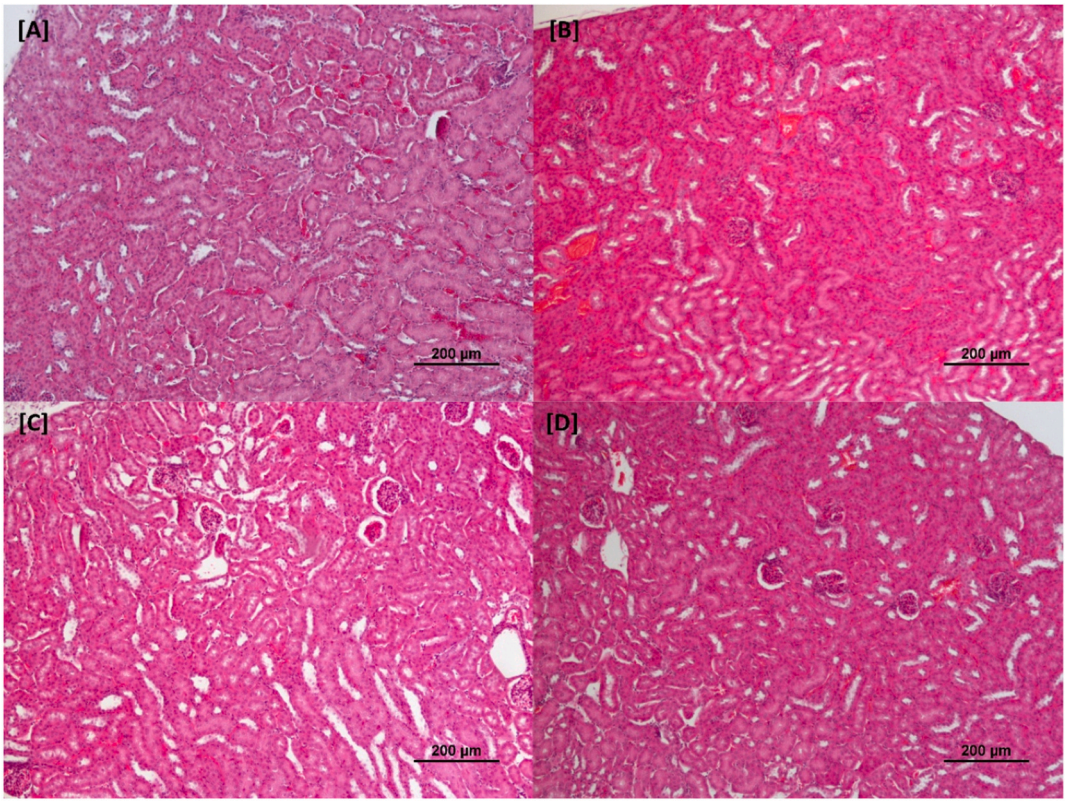

Fig. 2. Representative H&E-stained kidney sections from CTRL (A), AS100 (B), AS200 (C), and AS500 (D) groups (100×). Normal glomerular and tubular structures were observed in all groups, with no inflammatory or degenerative changes.

Table 6
Oxidative stress parameters evaluated on the kidney of all experimental groups. Data are presented as mean ± SEM for parametrical data or median (25th percentile - 75th percentile) for non-parametric data.

Parameter	CTRL ($n = 4$)	AS100 ($n = 5$)	AS200 ($n = 5$)	AS500 ($n = 5$)
ROS	112.10 ± 3.59	109.40 ± 9.79	98.98 ± 7.73	101.30 ± 5.35
SOD	413.30 ± 65.56	429.60 ± 56.18	381.30 ± 39.22	559.10 ± 71.02
CAT	727.60 ± 85.17	581.60 ± 123.10	620.90 ± 66.94	693.60 ± 107.00
GPx	68.97 ± 9.38	55.38 ± 8.40	61.29 ± 6.24	86.85 ± 8.14
GR	14.35 ± 4.63	12.52 ± 0.92	8.94 ± 1.79	6.60 ± 1.72
GST	3.82 ± 0.39	3.94 ± 0.58	4.75 ± 0.56	7.34 ± 0.97
GSH	5.84 ± 1.02	5.36 ± 1.26	5.70 ± 0.88	5.27 ± 0.56
GSSG	4.25 ± 0.66	4.28 ± 0.55	3.96 ± 0.57	3.36 ± 0.82
LDH	15.66 ± 4.05	16.93 ± 4.06	37.96 ± 7.75	25.58 ± 3.40
MDA	9.61 ± 1.98 [a]	8.02 ± 1.25 [a,b]	4.76 ± 0.38 [b]	7.94 ± 0.80 [a,b]
PC	4.69 ± 1.35	6.12 ± 1.81	6.28 ± 0.98	6.21 ± 0.16
DNA damage	0.62 ± 0.14	0.54 ± 0.04	0.48 ± 0.06	0.47 ± 0.04
OSI	1.19 (0.88 - 2.60)	1.08 (0.72 - 2.09)	1.58 (0.90 - 2.35)	1.26 (1.08 - 3.02)

Different letters denote statistically significant differences ($p < 0.05$). ROS, reactive oxygen species (µmol DCF/mg protein); SOD, superoxide dismutase (U/mg protein); CAT, catalase (U/mg protein); GPx, glutathione peroxidases (µmol NADPH/min/mg protein); GR, glutathione reductase (µmol NADPH/min/mg protein); GST, glutathione S-transferase (µmol CDNB/min/mg protein); GSH, glutathione (µmol GSH/mg protein); GSSG, oxidized glutathione (µmol GSSG/mg protein); LDH, lactate dehydrogenase (µmol NADH/min/mg protein); MDA, malondialdehyde (µmol MDA/mg protein); PC, protein carbonyls (µmol DNPH/min/mg protein); OSI, oxidative stress index (GSH/GSSG).

3.5. Phenolic composition

The obtained chromatographic data and the quantification of the individual compounds detected in the studied AS extract are shown in Table 7, and the corresponding chromatogram is presented in Fig. 3.

A total of thirteen phenolic compounds were tentatively identified in the analysed extract (Table 7). These compounds comprised phenolic acids (peaks **1, 3, 4, 6**, and **13**) and hydrolysable tannins (peaks **2, 5, 7**–**12**), reflecting a profile dominated by gallic/ellagic acid derivatives. Among the phenolic acids, gallic acid (peak **1**), quinic acid (peak **3**), valoneic acid dilactone (peak **4**), hydroxybenzoic

Table 7
Tentative identified phenolic compounds and their quantification (mg/g) in AS extract.

Peak	Retention time (min)	λ_{max} (nm)	[M-H]- (m/z)	MS2 (m/z)	Tentative Identification	Quantification (mg/g extract)
1	4.27	277	169	125(100)	Gallic acid	0.305 ± 0.001
2	4.73	293	613	313(16), 169(100)	Dehydrated tergallagic-C-glucoside	0.0797 ± 0.0003
3	5.13	305	191	163(100), 135(21)	Quinic acid	0.0239 ± 0.0004
4	5.48	357	469	169(100)	Valoneic acid dilactone	1.0362 ± 0.0003
5	8.28	280	631	451(100), 301(23)	Castalin/Vescalin	0.0738 ± 0.0006
6	9.31	280	137	117(100)	Hydroxybenzoic acid	0.016 ± 0.001
7	11.38	280	635	483(100), 465(33), 313(20), 169(11)	Tri-O-galloyl-β-D-glucose	0.0642 ± 0.0001
8	11.94	361	463	301(100)	Ellagic acid hexoside	1.0388 ± 0.0003
9	12.86	280	785	635(100), 483(34), 447(21), 313(31)	Digalloyl HHDP glucoside	0.0739 ± 0.0004
10	15.04	280	937	631(100), 469(33), 169(11)	Trigalloyl-HHDP-glucoside	0.0675 ± 0.0004
11	16.11	356	433	301(100)	Ellagic acid pentoside	1.0556 ± 0.0002
12	17.35	356	447	301(100)	Ellagic acid rhamnoside	1.093 ± 0.002
13	18.56	361	301	177(100)	Ellagic acid	1.366 ± 0.005
					Total phenolic acids	4.98 ± 0.01
					Total hydrolysable tannins	1.316 ± 0.002
					Total phenolic compounds	6.29 ± 0.01

The quantification of the tentatively identified compounds was based on the calibration curves of authentic standards: gallic acid (y = 23857x - 13524, R^2 = 0.9979, LOD = 1.05 μg/mL and LOQ = 0.41 μg/mL) for compounds 1,2,4,5,7,9 and 10; quinic acid (y = 23857x - 13524, R^2= 0.9979, LOD = 1.25 μg/mL and LOQ = 0.32 μg/mL) for compound 3; hydroxybenzoic acid (y = 19485x + 4004.4, R^2 = 0.9999, LOD = 1.28 μg/mL and LOQ = 0.35 μg/mL) for compound 6; ellagic acid (y = 14803x - 151526, R^2 = 0.9956, LOD = 1.30 μg/mL and LOQ = 0.48 μg/mL) for compounds 8, 11,12 and 13.

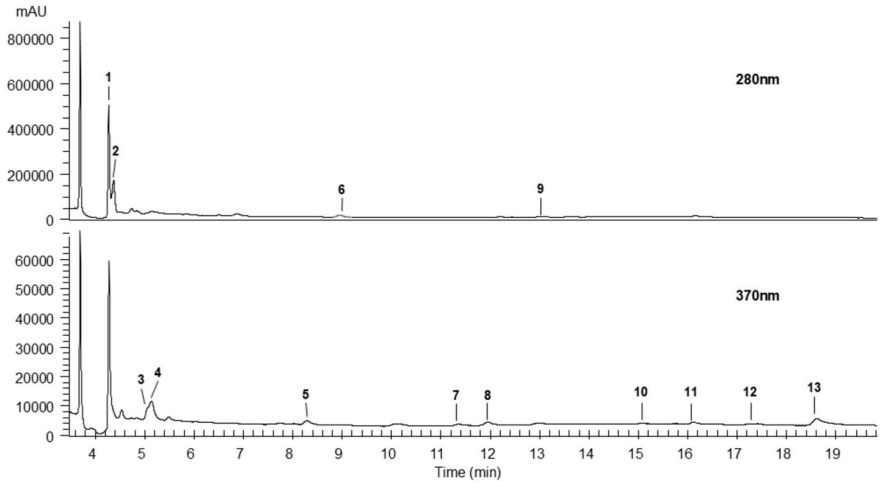

Fig. 3. Chromatogram of the AS extract. The numbered peaks correspond to the individual compounds quantified in Table 7.

acid (peak **6**), and ellagic acid (peak **13**) were detected, with ellagic acid being the predominant compound within this group (1.366 ± 0.005 mg/g extract). In addition, several ellagic acid derivatives, namely, ellagic acid hexoside (peak **8**), pentoside (peak **11**), and rhamnoside (peak **12**), were also detected, each occurring at relatively high concentrations (>1.0 mg/g extract), indicating extensive glycosylation of ellagic acid in the sample. Tannin-related structures accounted for a significant portion of the chromatographic profile. These included dehydrated tergallagic-C-glucoside (peak **2**), castalin/vescalin (peak **5**), tri-O-galloyl-β-D-glucose (peak **7**), digalloyl-HHDP-glucoside (peak **9**), and trigalloyl-HHDP-glucoside (peak **10**). Although present at lower individual concentrations than the major ellagic derivatives, these compounds collectively contributed to the tannin fraction (1.316 ± 0.002 mg/g extract), corresponding to complex galloyl- and HHDP-containing structures commonly reported in oak-derived matrices (Fernandes et al., 2022; Szabłowska and Tańska, 2024). Overall, ellagic acid and its glycosylated derivatives were the most abundant compounds detected in the sample, followed by galloyl- and HHDP-based tannins. This pattern closely resembles previously reported profiles of *Quercus* tissues, where ellagitannins and ellagic acid derivatives are typically prevalent (Liu et al., 2023). The total phenolic content of the extract reached 6.29 ± 0.01 mg/g extract, with phenolic acids representing the major proportion (4.98 ± 0.01 mg/g extract). The

predominance of ellagic-type structures suggests extensive hydrolysis or transformation of higher-molecular-mass ellagitannins, which is consistent with observations in other studies describing thermal, oxidative, or storage-related depolymerization processes in *Quercus* materials (Chira et al., 2020).

4. Discussion

Acorn shells represent an abundant and underexplored by-product of acorn processing, rich in phenolic compounds such as tannins and phenolic acids, which are known for their antioxidant and bioactive potential (Mateus et al., 2025). While their valorisation aligns with circular economy principles, assessing their toxicological safety is essential before their incorporation into food, nutraceutical, or pharmaceutical products. To the best of our knowledge, this is the first study to evaluate the oral administration of *Q. suber* AS extract, providing a comprehensive assessment of its toxicological effects. Importantly, the concentrations assessed (100-500 μg/mL) were selected to approximate prolonged dietary intake levels compatible with moderate human consumption, supporting their translational relevance within a functional food scenario.

Body weight, food and water intake, and general clinical condition are among the earliest and most sensitive indicators of systemic toxicity (Niyomchan et al., 2023). In the present study, the absence of any significant alteration in these parameters demonstrates that the AS extract was well tolerated by female FVB/n mice throughout the experimental period. Animals maintained normal behaviour, with no signs of distress or mortality. Food and water intake remained stable across all treatment groups, indicating that the extract did not affect palatability, while body-weight gain was comparable between treated and control animals, confirming the absence of evident toxicity. Comparable safety profiles have been reported for other Quercus-derived extracts with a similar phenolic composition, where gall-derived *Quercus* extracts administered to ICR mice and Wistar rats (Iminjan et al., 2014; Shourmij et al., 2022) and acorn extracts evaluated in FVB/n mice with papillomavirus-induced lesions (Medeiros-Fonseca et al., 2024a) similarly reported no adverse clinical or physiological effects, supporting the general tolerability of *Quercus* phenolic matrices. Notably, a significant increase in the relative weight of the liver, spleen, and left kidney was observed in the AS200 group. In toxicological studies, changes in organ weight may reflect either early adverse effects or adaptive physiological responses, depending on their association with functional or structural alterations (Piao et al., 2013). In the present study, these changes were not accompanied by histopathological lesions, nor by alterations in the biochemical markers of hepatic or renal function, suggesting that they are unlikely to reflect organ toxicity but instead an adaptive response to the bioactive compounds present in the extract. Nevertheless, the absence of a dose-dependent pattern and the lack of corroborating functional or oxidative stress alterations further support the interpretation that these differences are not biologically adverse under the tested conditions. Future studies should further explore these observations through the assessment of inflammatory markers (TNF-α, IL-6), proliferative and hypertrophy-associated pathways (Ki-67, PCNA), and other liver and kidney-specific functional biomarkers (AST, urea, cystatin C), as well as transcriptomic or proteomic approaches to better characterise potential adaptive responses at the molecular level.

Given the importance of liver and kidney function as primary endpoints in toxicological assessment, the combined biochemical, histological, and oxidative stress results of this study indicate that both organs remained functionally and structurally intact following AS administration. Both organs play central roles in xenobiotic metabolism and are often the first to exhibit signs of toxic injury (Jomova et al., 2025). The lack of differences in the values of ALT and the slight increase in creatinine confirm that the extract did not compromise hepatic or renal function, respectively. Oxidative stress analyses further supported the absence of deleterious effects, where the activities of SOD, CAT, GPx, GR, and GST, as well as the contents in GSH and GSSG, remained stable in both organs, confirming the maintenance of redox homeostasis (Aranda-Rivera et al., 2021; Videla and Valenzuela, 2022). Additionally, neither organ showed evidence of oxidative stress accumulation, which could manifest as elevated ROS or MDA levels (Rashid et al., 2024). The stability of glutathione-dependent pathways (GSH/GSSG and GST) suggests that AS exposure did not impose an oxidative burden requiring activation of detoxification pathway, which is consistent with the low systemic reactivity and predominantly gut-mediated metabolism described for ellagitannin-rich matrices (Raya-Morquecho et al., 2025). Interestingly, the intermediate dose (AS200) was associated with reduced renal MDA levels without parallel activation of antioxidant defenses, suggesting a subtle shift in lipid oxidative dynamics at the membrane interface rather than induction of a stress response. This effect may be linked to the phenolic composition of the extract, particularly the predominance of ellagic acid and its glycosylated derivatives, which are known to undergo extensive transformation in the gastrointestinal tract into bioactive metabolites such as urolithins (Zhang et al., 2023). These metabolites have been reported to exert lipid peroxidation-inhibiting and membrane-stabilising effects, even at low systemic concentrations, which could contribute to the observed reduction in renal MDA levels without requiring upregulation of endogenous antioxidant defenses (Cásedas et al., 2020; Djedjibegovic et al., 2020). Although no metabolite profiling was performed in the present study, this mechanistic pathway is consistent with previous reports describing the bioavailability, radical-scavenging capacity and lipid peroxidation-inhibiting properties of AS-derived phenolic compounds (Lee et al., 2019; Mateus et al., 2025; Shon et al., 2016). Histological evaluation confirmed the biochemical evidence of preserved organ function; hepatic tissue maintained normal architecture, with only sporadic, mild hydropic changes in centrilobular hepatocytes, while the kidneys showed intact glomerular and tubular structures without degenerative or necrotic lesions. These results are in line with previous reports indicating that, in general, *Quercus* phenolic extracts exert cytoprotective, rather than cytotoxic, effects on mammalian tissues (Oliveira et al., 2023; Vinha et al., 2016). The present findings provide the first *in vivo* evidence that repeated oral exposure to *Q. suber* AS extract preserves hepatic and renal tissue integrity without inducing functional or oxidative injury, reinforcing its potential for dietary and nutraceutical applications.

The toxicological findings observed in this study can be further contextualized by the phenolic composition of the AS extract, in which 13 phenolic compounds were tentatively identified by HPLC-DAD-ESI-MS/MS. The profile was dominated by ellagic acid and its

glycosylated derivatives, together with galloyl- and HHDP-based structures, yielding a total phenolic content of 6.29 ± 0.01 mg/g extract, of which 4.98 ± 0.01 mg/g extract corresponded to phenolic acids and 1.316 ± 0.002 mg/g extract to hydrolysable tannins. This pattern, marked by the predominance of ellagic acid derivatives, is consistent with that described for other *Quercus* tissues rich in ellagitannins and has been associated with a high antioxidant capacity, offering a plausible explanation for the absence of toxicity and the antioxidant effects observed. Recent evidence confirms that oak-derived phenolics exhibit strong antioxidant properties and can protect tissues against oxidative damage (Othón-Díaz et al., 2023). In particular, tannins from oak by-products have been proposed as natural antioxidants capable of inhibiting lipid peroxidation and preserving overall redox balance (Othón-Díaz et al., 2023). The presence of high-molecular-weight tannins and predominance of ellagic acid derivatives, which show limited systemic absorption, may contribute further to the good tolerability of the extract when administered orally (Djedjibegovic et al., 2020). Additionally, the predominance of ellagic-type structures in partially hydrolysed forms suggests partial depolymerization of higher-molecular-weight ellagitannins, which may favour some local antioxidant bioactivity without markedly increasing systemic toxicity, given the generally low intestinal permeability and restricted bioavailability described for these compounds (Cosme et al., 2025). Taken together, the results demonstrate that the AS extract is toxicologically safe and well tolerated under the tested conditions. The absence of hepatic or renal lesions, coupled with preserved antioxidant profile, supports the safe valorisation of these by-products for food, nutraceutical, or pharmaceutical applications.

Nonetheless, some limitations of the present study should be acknowledged. Although the sample size is consistent with exploratory *in vivo* studies and the results were internally consistent across multiple endpoints, it may limit the detection of subtle effects. In addition, the use of female FVB/n mice and a 25-day exposure period provided a controlled model to assess tolerability but does not account for potential sex-dependent responses or longer-term effects associated with chronic intake. Furthermore, as with any single-strain study, the generalisability of the findings may be influenced by strain-specific physiological characteristics. Future studies should evaluate longer exposure periods and a broader concentration range to better define safety margins and dose-response relationships. In addition, it remains to be clarified whether the reduction in MDA observed at the intermediate concentration reflects a true mechanistic redox-modulating effect with potential relevance for oxidative stress-associated conditions. Importantly, the bioactivity of *Quercus* by-products has been shown to depend strongly on harvest conditions and processing parameters (Mateus et al., 2025), highlighting the need for compositional standardization and batch consistency prior to translational or industrial application.

5. Conclusion

The *Q. suber* AS extract resulted in no signs of systemic toxicity or biochemical and/or histopathological changes in female FVB/n mice following 25 days of oral exposure at the concentrations under study. Hepatic and renal function remained within normal physiological limits, redox balance was preserved, and a reduction in renal lipid peroxidation at the intermediate dose suggested antioxidant activity without adverse effects. Collectively, the results support this by-product as a safe and sustainable source of phenolic compounds suitable for inclusion in food, nutraceutical, and pharmaceutical applications within a circular bioeconomy framework. Moreover, this study addresses a notable gap in the literature, providing the first *in vivo* oral safety assessments of AS extract and establishing an important basis for future valorisation and application strategies.

CRediT authorship contribution statement

Pedro Correia: Writing – original draft, Methodology. **Cristiano Mateus:** Writing – original draft, Methodology. **João Pedro Pinto:** Writing – original draft, Methodology. **Tiago Azevedo:** Writing – review & editing, Writing – original draft, Methodology. **Tiane Finimundy:** Writing – original draft, Methodology. **Pedro Babo:** Resources, Methodology. **Elisabete Nascimento-Gonçalves:** Writing – review & editing, Writing – original draft, Methodology. **Catarina Medeiros:** Writing – review & editing, Writing – original draft, Methodology. **Luís Félix:** Writing – review & editing, Methodology. **Fernanda Seixas:** Writing – review & editing, Methodology. **Lillian Barros:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Paula A. Oliveira:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Funding

This work received financial support from PRIMA/0005/2022 within the scope of the MEDACORNET project – Rescuing acorns as a Mediterranean traditional superfood. Additional support was provided by national funds through the Portuguese Foundation for Science and Technology (FCT): UID/04033/2025 (10.54499/UID/04033/2025), Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), and LA/P/0126/2020 (10.54499/LA/P/0126/2020), Institute for Innovation, Capacity Building and Sustainability of Agri-Food Production (Inov4Agro). Funding was also provided by FCT to CIMO through UID/00690/2025 (10.54499/UID/00690/2025) and UID/PRR/00690/2025 (10.54499/UID/PRR/00690/2025), to SusTEC through LA/P/0007/2020 (10.54499/LA/P/0007/2020), to CECAV through UIDB/00772/2020 (10.54499/UIDB/00772/2020) and UID/00772/2025 (10.54499/UID/00772/2025), and to AL4AnimalS through LA/P/0059/2020 (10.54499/LA/P/0059/2020). National funding from FCT also supported the doctoral grant of T.A. (2023.01329.BD) and the Institutional Scientific Employment Program for L.F. (2021.00458.CEECIND/CP1690/CT0001) and L.B. (CEECINST/00107/2021/CP2793/CT0002). E.N.-G. and C.M. acknowledge PRR and European Funds NextGeneration EU for their fellowship grants (BI/UTAD/41/2025 and BI/UTAD/17/2025).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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