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ORIGINAL PAPER

Supplementation with *Morus alba* L. leaves alters hematological parameters and reduces spleen antioxidant capacity in spontaneously hypertensive rats*

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Abstract

White mulberry (*Morus alba* L.) leaves are traditionally used to support glycemic control in diabetic and prediabetic individuals; however, their physiological effects in the context of hypertension remain poorly defined. This study investigated the effects of dietary supplementation with dried, powdered mulberry leaves (ML) on hematological indices and splenic redox biomarkers in spontaneously hypertensive rats (SHRs). Middle–aged male SHRs (n=10 rats) were fed either standard rat chow or chow supplemented with dried ML (7% w/w; ~4.0 g kg^{-1} body weight) for eight weeks. Hematological parameters were assessed from the whole blood, and antioxidant and inflammatory markers in spleen homogenates were quantified. ML supplementation increased hematocrit (HCT; 1.05-fold, p=0.0206), mean corpuscular volume (MCV; 1.01-fold, p=0.0013), and total white blood cell count (WBC; 1.56-fold, p=0.0128), while decreasing mean corpuscular hemoglobin concentration (MCHC; 0.99-fold, p=0.0192). In splenic tissue, DPPH scavenging activity and protein levels of PTGS1, NOS2, NOS3, HO-1, and GAPDH remained unchanged, whereas ferric-reducing antioxidant power (FRAP) was significantly reduced (0.75-fold, p=0.0014). No significant alterations were observed in body weight change (p=0.999), feed consumption (p=0.676), or relative spleen weights (p=0.798). Dietary dried, powdered ML supplementation elicits measurable alterations in hematological parameters and attenuates splenic antioxidant capacity in SHRs, indicating that ML may modulate hematological parameters and spleen redox homeostasis in hypertension.

Keywords: alkaloids, flavonoids, hematological parameters, oxidative stress, RBC morphology

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INTRODUCTION

White mulberry (*Morus alba* L.) is a deciduous tree of the *Moraceae* family, widely recognized for its long history in traditional medicine, particularly in Asia, where its leaves have been used to manage diabetes, hyperglycemia and obesity. The leaves are rich in bioactive compounds, including flavonoids, phenolic acids, and 1-deoxynojirimycin (DNJ), which exhibit antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and cardioprotective properties (Bharani et al. 2010, Zhao et al. 2019, Lin et al. 2022, Cheng et al. 2024, Janiak et al. 2025). In several countries, mulberry leaves are commonly consumed as herbal infusions or incorporated into dietary supplements to support glycemic control and overall metabolic health.

Emerging evidence indicates that beyond their metabolic effects, mulberry leaves (ML) may influence hematological and immune functions (Chen et al. 2019, So-In, Sunthamala 2022, Cheng et al. 2024, Putaggio et al. 2025). Studies in animal models have suggested that bioactive constituents of mulberry can modulate leukocyte activity, erythrocyte parameters, and antioxidant defenses, although the findings are inconsistent and often depend on dose, duration, and the form of supplementation (Bharani et al. 2010, So-In, Sunthamala 2022, Cheng et al. 2024, Putaggio et al. 2025). The spleen, as a key organ for erythrocyte turnover, iron recycling, and immune regulation, represents a critical target for evaluating these effects. Understanding how dietary interventions with mulberry leaves affect splenic oxidative status and systemic hematological parameters is important for assessing their safety and potential health benefits.

Given these considerations, the present study aimed to investigate the impact of dietary supplementation with dried, powdered ML on hematological indices and spleen redox balance in spontaneously hypertensive rats, providing novel insights into the organ-specific effects of ML bioactives beyond glycemic control.

MATERIALS AND METHODS

Animals and experimental design

Male spontaneously hypertensive rats (SHR), three weeks old (n=10 per group), were obtained from Janvier Laboratories (France). Animals were confirmed healthy at baseline, with no signs of illness or behavioral abnormalities. Rats were maintained on a standard chow diet for three months. Baseline body weights were similar between groups (in g: control: 259.1±17.94 *vs* ML: 259.2±20.24, p>0.999). Although each experimental run included 10 subjects, the study was performed in duplicate under identical

conditions, yielding an effective sample size of n=20 and improving the reliability of the results.

For the subsequent eight weeks, rats were randomly assigned to either continue the control diet or receive chow supplemented with 7% (w/w) ground ML, providing approximately 4.0 g kg^{-1} body weight per rat per day. Diets were offered *ad libitum*. Rats were housed individually in stainless-steel cages under controlled environmental conditions (22±1°C, 60±5% relative humidity, 12 h light-dark cycle, 15 air changes per hour) with free access to tap water. Housing enrichment included nesting material, wooden chew sticks, and small toys to promote natural behaviors and reduce stress. Diets were prepared in advance, stored at 4°C in airtight containers, and provided as needed. Investigators were blinded to group allocation. The experiment was independently repeated twice to ensure reproducibility. Sample size (n=10) was determined based on previous studies to detect biologically relevant differences with sufficient statistical power (Borkowska-Sztachańska et al. 2024).

The 7% supplementation of ground ML was selected based on extensive previous studies in our laboratory and elsewhere, where this concentration consistently showed beneficial effects on metabolic and hematological parameters in rodents without toxicity (Majewski et al. 2020, Nwokocha et al. 2014). The leaves contained 1.15 ± 0.01 mg DNJ g^{-1} dry weight, and the flavonoid content was also analyzed (data not presented). The plant material was obtained from a consistent source and processed under controlled conditions.

Ethical approval

All procedures complied with Directive 2010/63/EU on the protection of animals used for scientific purposes and the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 86-26, revised 2014). The study adhered to ARRIVE guidelines and the principles of the 3Rs (Replacement, Reduction, Refinement). The experimental protocol was approved by the Ethics Committee for Animal Experiments in Olsztyn, Poland (protocol code 72/2021, approved 17 November 2021). Animals were monitored daily for general health, behavior, and body weight throughout the study. Humane endpoints were predefined, including criteria for early euthanasia in case of severe illness, distress, or weight loss exceeding 15% of baseline.

Sample collection and anesthesia

Rats were fasted for 12 hours prior to sample collection. Anesthesia was induced by intraperitoneal injection of ketamine (100 mg kg^{-1} body weight) combined with xylazine (10 mg kg^{-1} body weight). Blood samples were collected from the caudal *vena cava* of anesthetized animals. All animals in each group (n=10) were sampled. Anesthesia depth and recovery were carefully

monitored to minimize distress. Animals were handled gently, and all procedures were performed by trained personnel to reduce stress.

Blood analysis

Peripheral venous blood samples were collected from SHRs into microtubes containing dipotassium ethylenediaminetetraacetic acid (K_2EDTA) as an anticoagulant. Samples were gently mixed immediately aft er collection to prevent clotting and were analyzed within 2-4 h of sampling. Complete blood counts (CBC) and leukocyte differentials were performed using the Yumizen H500 hematology analyzer (Horiba, Kyoto, Japan) according to the manufacturer's instructions. Quality control procedures were conducted daily to ensure instrument accuracy and precision.

Tissue homogenate preparation

Tissues were rinsed in ice-cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4) to remove residual blood, and weighed, and minced into small pieces (~1-2 mm^3). Minced tissue (1 g) was homogenized in 9 mL PBS (1:9 w v^{-1}) using a glass homogenizer (10-20 strokes on ice). Homogenates were centrifuged at 5000 × g for 5 min at 4°C, and the supernatant was collected. Homogenates were stored at -80°C and subjected to a maximum of one freeze-thaw cycle to minimize degradation. Total protein concentration was quantified using a BCA protein assay kit (Pierce, cat. no. 23225) according to the manufacturer's instructions and stored aliquots at -80°C. Assays were performed in triplicate, and quality control measures, including standard curves, blanks, and internal controls, were implemented to ensure reliability and accuracy of the results.

Enzyme-linked immunosorbent assay (ELISA)

The levels of PTGS1, NOS2, NOS3, HO-1, and GAPDH in samples were quantified using commercially available rat ELISA kits (Biorbyt, Cambridge, UK) according to the manufacturers' protocols. Briefly, tissue samples were preprocessed by homogenization and centrifuged at 5000×g for 5 min at 4°C. Supernatants were collected and stored at -80°C until analysis. Samples and standards were added to pre-coated 96-well plates. The plates were incubated at room temperature for the recommended duration. After washing, detection antibodies were added, followed by horseradish peroxidase (HRP), a conjugated secondary antibody. Substrate solution was added to develop color, and the reaction was stopped with the provided stop solution. Absorbance was measured at 450 nm using a microplate reader. Concentrations were calculated from standard curves generated using known standards.

GAPDH was used as an internal control to normalize protein expression levels. All samples were analyzed in duplicate or triplicate to ensure reproducibility.

DPPH Assay

A 100 μM DPPH solution (Sigma Aldrich, USA) was made by dissolving 4 mg of the compound in 100 mL of methanol (POCH, Poland). Each Eppendorf tube was filled with 1 mL of this DPPH solution plus 250 μL of centrifuged homogenate. The mixtures were vortexed, then kept in the dark at room temperature for 60 min. Prior to analysis, tubes were spun at 1500 × g for 2 min (room temperature). Absorbance readings were taken at 517 nm, using methanol as the blank. The control was a 100 μM DPPH solution in methanol, measured at the start and end of the experiment. For calibration, homogenate was swapped with Trolox standards.

For the calibration curve, Trolox (TE; (+)–6–hydroxy–2,5,7,8–tetramethylchromane–2–carboxylic acid; Sigma Aldrich, USA) standards ranged from 2.5 to 80 mg L^{-1} across seven concentrations. Antioxidant capacity was reported as Trolox equivalents (mg TE 1000 mL^{-1}). DPPH inhibition (%) by human milk samples followed this formula:

$$\%\ \text{inhbition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where: $A_{control}$ – control absorbance and A_{sample} – the sample's mean absorbance.

FRAP assay

Reagents included (a) 300 mM acetate buffer (pH 3.6); (b) 10 mM TPTZ in 40 mM HCl; (c) 20 mM $FeCl_3 \cdot 6H_2O$. The FRAP working solution combined (a):(b):(c) at 10:1:1 (v/v/v). Blanks used FRAP reagent alone or with 50 μL homogenate per standards. Samples (50 μL) were mixed with 1.5 mL FRAP reagent, vortexed, and read at 593 nm right away (t = 0 min). Mixtures incubated at 37°C for 4 min in a water bath, were re–vortexed, and re–read at 593 nm.

For the calibration curve ascorbic acid (Chempur, Poland) standards spanned 100-1000 μM (six levels). Results were given as ascorbic acid equivalents (μM). FRAP values were determined as:

$$\text{FRAP}\ (\mu M) = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{std}}} \times C_{\text{std}} \times 2,$$

where: ΔA_{sample} – the sample's absorbance change (0-4 min), ΔA_{std} – the standard's change (0-4 min), C_{std} – standard concentration, and ascorbic acid's stoichiometric factor is 2.0.

Data analysis

Data are expressed as mean ± standard deviation (SD). Normality was assessed using the Shapiro–Wilk test. For variables that followed normal distribution, differences between two groups were evaluated using the Stu-

dent's *t*-test, with $p<3*0.05$ considered statistically significant. Skewed data were log-transformed prior to analysis. When variables remained non-normally distributed after transformation, comparisons were performed using the nonparametric Mann-Whitney test. All statistical analyses were conducted using GraphPad Prism 10.6.1 (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

No significant alterations were observed in body weight (in g: control: 328.0±20.70 *vs* ML: 330.1±25.71, $p=0.999$), feed consumption (in g: control: 109.9±25.33 *vs* ML: 117.0±16.61, $p=0.676$), or relative spleen weights (in g: control: 0.656±0.103 *vs* ML: 0.628±0.069, $p=0.798$). These findings indicate that dietary supplementation with dried ML is well tolerated and does not compromise growth, appetite, or organ development, aligning with the findings of Cai et al. (2019) and Ntalouka and Tsirivakou (2024).

Hematological parameters are among the most sensitive indicators in pharmacological studies, reflecting the effects of xenobiotics or their metabolites on cellular components. Consequently, alterations in these parameters provide a highly predictive measure of potential toxicity in both humans and mammals. In the present study, supplementation with dried ML resulted in modest increases in HCT and MCV, accompanied by a slight decrease in MCHC, while all other hematological parameters remained within normal ranges, consistent with Cai et al. (2019) and Jayswal et al. (2024) – Table 1. These changes suggest subtle alterations in red blood cell morphology rather than a pathological condition. Specifically, the elevated MCV indicates a minor increase in red blood cell size, while the reduced MCHC reflects a corresponding decrease in hemoglobin concentration per unit cell volume. The increased HCT may reflect either a slight rise in red blood cell number or a mild reduction in plasma volume. Collectively, these findings may point to a physiological adaptation of erythrocytes potentially mediated by bioactive compounds in ML, without evidence of anemia or hematological toxicity. Thus, dietary supplementation with ML appears to be hematologically safe and does not compromise erythrocyte function or systemic blood parameters, consistent with Chen et al. (2019) and Jayswal et al. (2024). Siddiqui et al. (2022) also reported non-significant differences in blood parameters such as PCV, TEC, TLC, MCV, MCH, and MCHC of 28-week-old White Leghorn laying hens when supplemented with mulberry leaf powder.

The observed increase in white blood cell (WBC) counts following supplementation with dried ML may indicate a modulatory effect on immune cell dynamics, see Table 1. Although the precise mechanism remains unclear, bioactive compounds in ML, such as flavonoids and polysaccharides, have been reported to influence leukocyte proliferation and function in animal

Table 1

Hematological parameters in blood of SHRs supplemented with *Morus alba* L. leaves

Assay	Units	Control (K group)		*Morus alba* L. leaves (B group)		x–fold	*p**
		means	SD	means	SD		
RBC	$\times 10^{12}\ L^{-1}$	9.58	0.61	9.84	0.23	1.03	0.2716
HGB	$g\ L^{-1}$	14.11	0.91	14.45	0.38	1.02	0.5028
HCT	%	47.23	3.16	49.56	1.34	1.05	0.0206
MCV	fL	49.77	0.49	50.36	0.50	1.01	0.0013
MCH	pg	14.71	0.17	14.68	0.16	1.00	>0.9999
MCHC	$g\ dL^{-1}$	29.56	0.26	29.16	0.32	0.99	0.0192
RDW–CV	%	10.86	0.19	10.77	0.18	0.99	>0.9999
RDW–SD	fL	23.43	0.44	23.33	0.41	1.00	>0.9999
WBC	$\times 10^{9}\ L^{-1}$	3.40	1.02	5.30	1.83	1.56	0.0128
PLT	$\times 10^{9}\ L^{-1}$	770.0	141.4	819.5	66.89	1.06	0.5310
PCT	%	0.56	0.10	0.60	0.05	1.07	0.4798
MPV	fL	7.24	0.13	7.25	0.14	1.00	>0.9999
PDI	fL*	9.69	0.47	9.78	0.52	1.01	>0.9999
P–LCC	$\times 10^{9}\ L^{-1}$	69.12	12.37	74.17	10.71	1.07	0.7131
P–LCR	%	9.11	1.20	9.04	0.86	0.99	>0.9999

Data are means ± SD; * $p<0.05$

Supplementation did not significantly affect most hematological indices. In contrast, HCT (hematocrit), MCV (mean corpuscular volume), MCHC (mean corpuscular hemoglobin concentration), and WBC (white blood cells) were significantly altered, suggesting selective modulation of erythrocyte morphology and leukocyte populations.

models (Bharani et al. 2010, Zhao et al. 2019, Cheng et al. 2024). Since remaining hematological parameters were unaffected, this finding suggests a selective effect on immune cells rather than a broad hematological disturbance. Mohamed and Ashour (2018) reported that ethanolic mulberry leaf (ML) extract significantly ameliorated irradiation-induced biochemical and hematological alterations in male rats, restoring red blood cell count, hemoglobin, hematocrit, platelet count, leukocyte count, lymphocytes, neutrophils, glucose-6-phosphate dehydrogenase, and insulin levels. Similarly, a 30% ethanolic extract of ML flavonoids alleviated clinical symptoms, suppressed pro-inflammatory cytokine secretion, and inhibited inflammatory pathway activation in dextran sulfate sodium-induced colitis mice (Lin et al. 2022). Bharani et al. (2010) demonstrated that methanolic ML extract exerted immunomodulatory effects in mice by regulating immune cell populations and leukocyte and neutrophil counts, consistent with observed changes in white blood cell profiles. In addition, Venkatachalam et al. (2009) showed that aqueous ML extract stimulated the innate immune system in a dose-

dependent manner. Dietary supplementation with ML polysaccharides enhanced immune organ development and systemic immune markers in weanling pigs (Zhao et al. 2019) and improved growth performance while significantly increasing cytokine secretion, immunoglobulin levels, and antioxidant enzyme activity in the serum of immunosuppressed chicks (Cheng et al. 2024).

Further studies are needed to clarify whether this increase corresponds to enhanced immune activity or represents a transient physiological adaptation.

Although the analyzed levels of DPPH, PTGS1, NOS2, NOS3, HO-1, and GAPDH in spleen homogenates did not change significantly, FRAP values decreased by approximately 0.75-fold following supplementation with dried ML (Table 2). This reduction indicates a moderate decrease in overall

Table 2

Antioxidant and oxidative stress-related parameters in spleen homogenates of SHRs supplemented with *Morus alba* L. leaves

Assay	Units	Control (K group)		*Morus alba* L. leaves (B group)		*x*–fold	*p**
		means	SD	means	SD		
FRAP	μM	1795.0	348.7	1343.0	126.1	0.75	0.0014*
DPPH	mg L^{-1}	32.77	0.99	33.11	1.15	1.01	>0.9999
PTGS1	ng mL^{-1}	3.75	0.59	3.67	0.66	0.98	>0.9999
NOS2	pg mL^{-1}	58.55	14.94	58.22	19.37	0.99	>0.9999
NOS3	pg mL^{-1}	348.5	34.63	380.7	39.01	1.09	0.2172
HO–1	ng mL^{-1}	4.34	0.46	5.01	0.64	1.15	0.3177
GAPDH	ng mL^{-1}	0.73	0.14	0.53	0.13	0.72	0.6938

Data are means ± SD; * $p<0.05$

In spleen homogenates of SHRs supplemented with *Morus alba* L. leaves, DPPH, PTGS1, NOS2, NOS3, HO–1, and GAPDH levels were not significantly modified. However, FRAP decreased, indicating reduced overall antioxidant capacity.

non-enzymatic antioxidant capacity, suggesting that while individual oxidative stress markers were stable, the total antioxidant potential of the spleen was somewhat diminished. These results may reflect a tissue-specific or selective modulation of redox balance by ML bioactive compounds. Since the spleen is responsible for erythrocyte turnover and iron re-cycling, the observed changes could reflect mild redox or membrane adaptations rather than overt hematological toxicity.

Future research should focus on isolating either DNJ or the specific flavonoids responsible for the observed hematological effects and evaluating their impact in additional animal models or human subjects.

Several limitations should be considered when interpreting these findings. Spleen oxidative status was evaluated primarily using FRAP and

DPPH assays, along with protein markers PTGS1, NOS2, NOS3, and HO-1. FRAP and DPPH are nonspecific and may not fully reflect enzymatic antioxidant defenses or *in vivo* redox dynamics. PTGS1, NOS2, and NOS3 indicate basal and inducible inflammatory signaling and nitric oxide-mediated responses, while HO-1 reflects heme catabolism and oxidative stress adaptation. Although relevant to spleen physiology in spontaneously hypertensive rats, these markers alone do not provide a comprehensive assessment of redox balance. Classical oxidative stress markers such as GSH/GSSG, MDA, SOD, CAT, and GPx should be included in future studies. Similarly, modest increases in WBC and minor shifts in HCT, MCV, and MCHC were not supported by functional analyses of cytokines, leukocyte subsets, hemolysis, or reticulocytes. In addition, only a single high dose of *Morus alba* leaf powder was tested, without time-course analyses, spleen histology, or evaluation of iron metabolism. Collectively, these limitations indicate that the observed changes should be interpreted as preliminary indications of potential antioxidant or immunomodulatory effects rather than conclusive evidence of altered redox balance, spleen function, or immune modulation. Future studies using dose-response designs, validated tissue-specific assays, and functional hematological and immunological endpoints are warranted to clarify these effects.

CONCLUSIONS

Dietary supplementation with dried *Morus alba* leaf in spontaneously hypertensive rats resulted in modest increases in HCT, MCV, and WBC, with a slight decrease in MCHC, while other hematological parameters remained unchanged. In spleen homogenates, most oxidative stress markers were unaltered, although FRAP decreased by approximately 0.75-fold, indicating a limited reduction in non-enzymatic antioxidant capacity. These findings indicate potential effects of *Morus alba* on selected hematological and redox parameters; however, given the limited dose, assays, and functional validation, definitive conclusions regarding modulation of erythrocyte morphology, immune function, or redox balance cannot be drawn. Further studies with comprehensive, validated measures are needed to clarify the physiological impact of *M. alba* leaves.

Supplementary materials

Not applicable.

Author contributions

Conceptualization – A.G.–R., M.S.M, methodology – M.S.M., validation – M.S.M., formal analysis, M.S.M., investigation – A.C., M.S.M., data

curation – M.S.M., writing – original draft preparation – M.S.M., writing – review and editing, M.S.M., visualization – L.G., A.G.–R. and M.S.M., supervision, M.S.M.; funding acquisition – L.G., M.S.M. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

None.

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
ELISA	enzyme-like immunosorbent assay
FRAP	ferric reducing antioxidant power
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCT	hematocrit
HGB	hemoglobin
HO–1	heme oxygenase-1
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MPV	mean platelet volume
NOS	nitric oxide synthase
PCT	platelet hematocrit
PDI	platelet distribution index
P–LCC	large platelet cell count
P–LCR	large platelet ratio
PLT	platelets
PTGS	prostaglandin-endoperoxide synthase
RBC	red blood cells
RDW–CV	red cell distribution width coefficient of variation
RDW–SD	red cell distribution width standard deviation
SHR	spontaneously hypertensive rat
WBC	white blood cells

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