ORIGINAL ARTICLE

EFFECTS OF *Morus alba* INFUSION ON BIOCHEMICAL, TOXICOLOGICAL, AND BEHAVIORAL PARAMETERS IN OVARIECTOMIZED RATS

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Highlights: (1) *Morus alba* has anxiolytic and antioxidant potential in vitro, with no signs of toxicity. (2) The infusion does not alter body weight gain induced by estrogen deprivation. (3) The infusion did not reverse the biochemical and oxidative changes induced by the model.

PRE-PROOF

(as accepted)

This is a preliminary and unedited version of a manuscript that has been accepted for publication in Revista Contexto & Saúde. As a service to our readers, we are making this initial version of the manuscript available as accepted. The article will still undergo review, formatting, and approval by the authors before being published in its final form.

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ABSTRACT

Morus alba, popularly known as White mulberry, is traditionally used for the treatment of conditions frequently associated with menopause. To investigate its potential effects on these symptoms, this study first aimed to establish the in vitro antioxidant activity of *M. alba* infusion. Subsequently, the aim was to assess whether consumption of the *M. alba* infusion induces biochemical, hematological, antioxidant, and behavioral changes in an animal model of menopause. For this purpose, an experimental study was conducted with 33 Wistar rats, 32 weeks of age. The animals were randomized into two groups: ovariectomized (OVX) and sham surgery (SHAM), and after 12 weeks, began to receive an infusion of *M. alba* leaves (M. alba, OVX+M. alba) or water ad libitum (Control, OVX) for 12 weeks. The *M. alba* leaf infusion exhibited in vitro antioxidant potential. Estrogen deprivation led to increased body weight, regardless of the administration of *M. alba* infusion. In this study, it was shown that even at low concentrations the infusion exhibited in vitro antioxidant activity without evidence of overt toxicity and showed a potential anxiolytic effect. Thus, although many studies show that the plant has therapeutic potential for the treatment of menopause-associated disorders, in this model no changes were observed in biochemical or oxidative parameters.

Keywords: Menopause; Medicinal Plants; Estrogens; Morus; Ovariectomy.

1 INTRODUCTION

Menopause is a physiological event resulting from ovarian follicular insufficiency and the consequent decline in estrogen production; this hormone is involved in the homeostasis of various physiological processes in different organs [1]. It plays a major role in the maintenance of physiological processes, development and functioning of the reproductive system, protection and maintenance of the cardiovascular, immune, and antioxidant systems, regulation of body fat deposition, modulation and protection of the

central nervous system. Thus, after menopause, women are more likely to develop cardiometabolic disorders and cognitive-behavioral changes due to the decline in circulating estrogen levels [2].

In this context, phytotherapy has gained ground and drawn interest due to its therapeutic potential. In Brazil, the Ministry of Health created the National List of Medicinal Plants of Interest to the Unified Health System (RENISUS), which consists of a list of plant species with therapeutic potential for generating products of interest to the Unified Health System [3]. From this, the genus Morus spp. was included in RENISUS due to its therapeutic potential associated with menopause-related disorders. *Morus alba* (*M. alba*), popularly known as White Mulberry, is a plant native to China and widely distributed in regions with warm temperate, tropical, subtropical, and Mediterranean climates [4; 5]. In traditional Chinese medicine, its leaves, fruits, and stems are used in the treatment of hypercholesterolemia, as a sedative, expectorant, analgesic, diuretic, hepatoprotective and renoprotective agent, antihypertensive, and antiepileptic [5; 6]. In Brazil, the plant is traditionally used for the treatment of menopause-associated symptoms, due to its therapeutic potential for controlling hot flashes and hormone replacement during menopause. In addition, in folk medicine, the plant is used to improve cholesterol levels, control diabetes, and promote weight loss in menopausal women [4]. To date, there are no scientific studies describing the effects of consuming *M. alba* infusion in experimental models of menopause.

Previous studies have identified that different extracts from *M. alba* leaves are rich in flavonoids and glycosides including quercetin, astragalin, kaempferol, isoquercetin and rutin, among other phytochemicals [4]. Wei and collaborators [4] demonstrated that treatment with kaempferol from *M. alba* leaves reduces apoptosis in granulosa cells in vitro and in vivo and reverses ovarian insufficiency, promoting an increase in estrogen and progesterone secretion in a menopause model in aged animals. Moreover, consumption of different types of plant extracts showed a reduction in plasma levels of triglycerides, total cholesterol (TC) and very low-density lipoprotein (VLDL), increased plasma concentration of low-density lipoprotein (LDL), and reduced diastolic blood pressure in menopausal women [4]. Considering the possible beneficial effects of *M. alba* for menopausal women, and the documented traditional consumption of this plant

as an infusion in Brazil, this study initially aimed to establish the in vitro antioxidant activity of *M. alba* infusion and subsequently evaluate whether the consumption of *M. alba* infusion induces biochemical, hematological, antioxidant, and behavioral changes in an animal model of menopause.

2 METHODS

This is an experimental study involving an animal model, conducted with 33 female Wistar rats, aged 32 weeks; obtained from the Animal Facility of the Universidade do Noroeste do Rio Grande do Sul (UNIJUÍ). This protocol was approved by the Animal Use Ethics Committee of UNIJUÍ (CEUA 003/2019).

2.1 Experimental Design

The animals were housed in the UNIJUÍ Animal Facility under controlled temperature (22 ± 2 °C), a light-dark cycle (light from 7:00 a.m. to 7:00 p.m.), and relative humidity maintained between 50-60%. The animals received water and food ad libitum.

The Wistar rats were initially evaluated for biometric and glycemic profile and randomized into two groups: ovariectomized (OVX, n = 16) and sham surgery (SHAM, n = 17). After 12 postoperative weeks, the animals were randomized into another four groups: Control (n=7), *M. alba* (n=10), OVX (n=9), and OVX + *M. alba* (n=7), with the animals in the *M. alba* and OVX + *M. alba* groups receiving an infusion of *M. alba* leaves as their exclusive water source for 12 weeks. Throughout the study period, biometric data from all animals were collected weekly, and glycemic data monthly. In the 12th week of experimental treatment, the animals were evaluated for their behavioral profile of anxiety, depression, and short-term memory.

The animals were euthanized 48 hours after the last administration of the *M. alba* infusion to collect biological material for biochemical and toxicological assays. For tissue morphometry evaluation, liver, spleen, kidneys, adipose tissue, gastrocnemius muscle, soleus, and uterus were collected.

2.2 Phytochemical Analysis and Preparation of the *M. alba* Leaf Infusion

2.2.1 Acquisition of Plant Material and Preparation of the Infusion

The dried leaves of the plant were commercially obtained from Kitt Med Laboratórios LTDA – Campo Largo, PR. The *M. alba* leaves were harvested in March 2019 with an expiration date of 03/2020, in the municipality of Mandirituba–PR, and underwent a drying process in an oven. Physicochemical analyses were performed (foreign material: 6.72%, moisture: 7.08%, and ash: 5.12%) as well as microbiological analyses (Salmonella spp: absence in 25g, Total Coliforms at 45°C < 3 MPN/g).

The infusion was prepared daily by considering a concentration of 7.5 mg of dried *M. alba* leaves per ml of potable water at 80 ± 2 ºC; the mixture was left infusing, covered with a watch glass at room temperature for 10 minutes, then filtered using filter paper and offered to the animals orally as the exclusive source of water.

2.2.2 Determination of the 50% Effective Concentration of the Infusion and Total Phenolic Content

The determination of in vitro antioxidant capacity and effective concentration (EC50) was carried out based on the free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by Sousa et al., 20077, using the infusion of the plant leaves. To determine EC50, increasing concentrations of 0.10 g, 0.25 g, 0.50 g, 0.75 g, 1.0 g, 1.25 g, and 1.5 g of leaves were used.

Total phenolic content was determined by the Folin Ciocalteu method as described by Rangel et al., 2013, using a concentration of 0.75g [8]. The standard used was gallic acid (GA), and the result was expressed in GA equivalents (mg/GAE).

2.3 In vivo Experimental Procedure

2.3.1 Surgical, Anesthetic, and Analgesic Procedure

The surgical procedure was performed in a hospital setting and for the OVX group involved bilateral removal of the ovaries accessed by a longitudinal incision of the skin and musculature in the region caudal to the last rib and near the level of the kidney. The SHAM animals had their ovaries identified and surgically exposed, then repositioned for

subsequent suturing of the previously incised muscle and skin layers. For the surgical procedure, animals were anesthetized using pethidine 15 mg/kg IP as a pre-anesthetic; isoflurane at 4% and 2% by inhalation for induction and maintenance, respectively; finally, in the immediate postoperative period and 24 hours later, the animals received a dose of Meloxicam 2mg/kg subcutaneously.

2.3.2 Determination of Biometric Profile

For determination of biometric profile, animals were monitored weekly for body weight, and every two weeks the total body mass, abdominal and thoracic circumferences, and naso-anal length were measured using a non-elastic measuring tape. The Lee Index was determined from the cube root of body weight (g)/naso-anal length (cm), considering 0.30 g/cm as the cutoff point for obesity. The Abdomen–Thorax Ratio (ATR) was calculated using the following formula: ATR = Abdominal circumference/thoracic circumference [9]. The Body Mass Index (BMI) was calculated using the following formula: BMI = Weight (g)/naso-anal length (cm^2). The cutoff point established for this indicator for normal weight ranges between 0.45 and 0.68/cm^2 [10].

2.3.4 Blood Determinations

2.3.4.1 Collection of Biological Material

Blood was collected, following euthanasia by decapitation, into a tube with EDTA anticoagulant. Immediately after collection, the blood was centrifuged in a Minispin Plus Eppendorf microcentrifuge at 3500 rpm for 15 minutes to obtain plasma. Plasma samples were aliquoted and frozen in liquid nitrogen for subsequent analysis.

2.3.4.2 Hematological assessment

Whole blood was collected in a tube with anticoagulant (EDTA) for determination of hematological parameters and hematological and red blood cell indices (5µl of EDTA per 500 µl of blood). Automated determination was performed using the Micros 60 hematology analyzer (Horiba), following the manufacturer's recommendations. Subsequently, blood smears were prepared on slides and stained with panoptic stain

(Newprov); microscopy was then performed, with a count of 100 cells in distinct fields for each slide.

2.3.4.3 Biochemical assessment

The determination of the animals' lipid profile (TC, LDL, VLDL, HDL, and Triglycerides), alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, and gamma-glutamyltransferase (GGT) was performed using colorimetric methods, with commercial Bioclin – Quibasa kits and the BS200-Mindray automated system. Results were expressed in mg/dl, the AST, ALT and GGT were expressed in U/L. Glucose tolerance tests (GTT) were performed in all experimental groups at baseline, and at the 4th, 8th, and 12th week of treatment, with animals fasted for 12 hours. For the test, an 80% (w/v) glucose solution was prepared and administered intraperitoneally at a dose of 1 g/kg, via intraperitoneal injection. To obtain the glycemic curve, fasting blood glucose values were measured at 0, 15, 30, and 120 minutes after glucose injection. Blood glucose measurements were taken from whole blood collected via puncture of the lateral tail vein using a capillary blood glucose meter, On Call Plus. Results were expressed in mg/dl [9].

2.3.4.4 17β Estradiol Assay

The assay for 17β estradiol (E2) was conducted using plasma samples for quantitative measurement using chemiluminescence methodology, with with an analytical measurement range of 11.8–3,000 pg/mL.

2.3.4.5 Oxidative Parameters

The analysis of superoxide dismutase (SOD) activity was performed using the pyrogallol auto-oxidation inhibition technique at 420 nm for 120 seconds [11]. Results were expressed in U of SOD/ml. Catalase (CAT) activity was measured according to the method described by Aebi (1984), with results expressed in U of CAT/ml [12]. The SOD/CAT ratio was calculated using the following formula: ([USOD/ml] /[UCAT/ml] [13]. Lipid peroxidation was determined by the thiobarbituric acid reactive substances (TBARS) technique using plasma [14]. To measure TBARS, plasma proteins were

precipitated with 10% trichloroacetic acid, centrifuged, and incubated with thiobarbituric acid (1:1) (Sigma, Chem. Co.) for 15 minutes at 100 °C. TBARS were extracted using butanol (1:1). After centrifugation, the absorbance of the butanol layer was measured by spectrophotometry at 535 nm, and TBARS concentration was expressed in mmol of MDA/mg of protein [15].

2.3.5 Behavioral Assessments

For all behavioral tests, the animals were acclimated to the experimental laboratory for a prior period of 1 hour before testing and kept out of the researcher's presence. During the experiments, a constant temperature of 22±2 ºC, and controlled lighting, noise, and sound were maintained; in addition, the researcher remained out of the animals' line of sight throughout the testing period. Apparatus were cleaned between animals with 70% alcohol, all tests were recorded using a digital camera and later analyzed using Any Maze software.

2.3.5.1 Open Field Anxiety Assessment

The test was performed in a device made of MDF measuring 60x40cm (width/height) and covered with white adhesive, with the floor divided into 25 quadrants measuring 12x12 cm each. The behaviors evaluated were: distance traveled, time spent in motion, grooming frequency, and time spent in the center of the apparatus during 5 minutes of testing. The animals underwent a 24-hour adaptation period prior to testing, with 5 minutes of free exploration of the apparatus [16].

2.3.5.2 Elevated Plus Maze

The animals were placed in a wooden cross-shaped apparatus with two open arms (10x50cm), two closed arms (10x50cm), and elevated 50cm from the floor. During a 5-minute period, the frequency of entries and time spent in the open arms, closed arms, and neutral (central) area were observed, as well as rearings, head dips, and grooming. Entry was considered when the animal placed all four paws within the arm [16].

2.3.5.3 Light–Dark Box

The test was performed by placing the animals in an MDF apparatus covered with adhesive, consisting of a light side (45 x 41 cm) and a dark side (35 x 41 cm), with a central opening (12x14 cm) between compartments allowing free access to both sides. Over 5 minutes, the number of transitions, latency to enter the dark side, and time spent in each compartment were recorded [16].

2.3.5.4 Object Recognition Test

The test was conducted in two phases: in the first, the animal was placed in the apparatus with two identical objects for familiarization, then returned to its cage for 30 minutes. In the second phase, the animal was returned/reintroduced to the apparatus with two objects: one familiar (old) and one new, and the time spent exploring each object was recorded. The animals underwent a 24-hour pretest session before testing, spending 5 minutes in the apparatus for free exploration without objects. The test apparatus was constructed from MDF measuring 60x40 cm (width/height) covered with white adhesive; the selected objects were fixed to the background and made of plastic [16].

2.3.5.5 Depressive State Forced Swimming Assessment

The forced swimming test was performed by placing the animal in a cylindrical plastic tube (25x51cm) filled with water (25±1ºC) to a depth of 30 cm for 5 minutes. For this test, animals underwent a prior 15-minute adaptation period, 24 hours before testing [16].

2.4 Statistical Analysis

Results were expressed as mean ± standard error or standard deviation. Data were tested for normality using the Shapiro-Wilk test and groups were compared using the Student's t-test and one-way analysis of variance followed by Tukey's post hoc test and their non-parametric equivalents. All statistical analyses were performed using GraphPad Prism, version 5.0. The significance level was set at $P < 0.05$.

3 RESULTS

Increasing concentrations of *M. alba* leaf infusion were evaluated by the DPPH radical reduction method, through which it was observed that the concentration of 0.75g/100mL yields an EC50% of 52.87% and contains a total phenol content of 159.39 mg/GAE. This demonstrates that even at low concentrations, the plant exhibits in vitro antioxidant potential.

Regarding the animals, the groups showed no difference in body weight before the experimental procedures (P = 0.742); after 12 weeks post-surgery, a difference in body weight was observed between the OVX and SHAM rats (P = 0.026) (Fig. 1A). Following the experimental treatment, differences were observed in the ATR (P = 0.014) *M. alba* vs OVX (Fig. 1B); BMI (P < 0.0007) *M. alba* vs OVX + *M. alba* (Fig. 1C); and in the Lee Index (P = 0.049) *M. alba* vs OVX + *M. alba* (Fig. 1D).

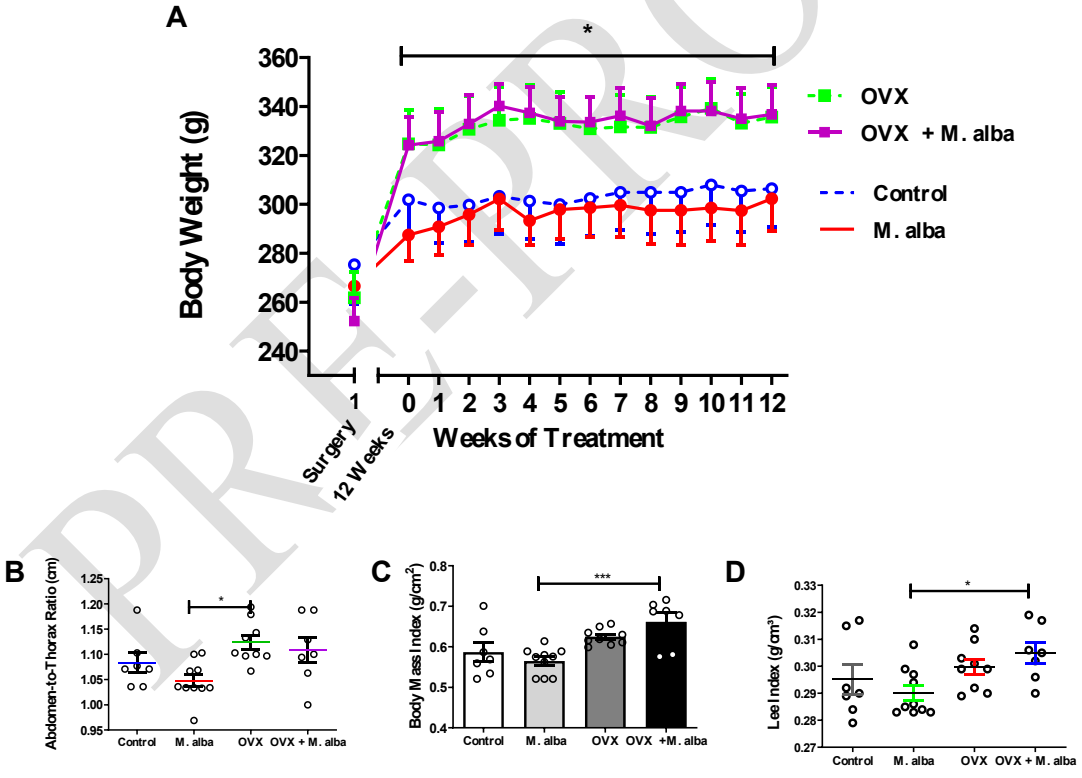

Figure 1. Biometric profile of animals subjected to OVX and treated with *M. alba* infusion. A= Body weight of the animals, B= Abdomen-Thorax Ratio, C= Body Mass Index, and D= Lee Index. Data are presented as mean ± standard error, n= 6-9 per group, one-way ANOVA followed by Tukey's post hoc test, * = P<0.05, *** = P<0.001. Source: The authors.

No change in GTT was observed during the treatment period. Figure 2 illustrates glucose tolerance before the start of treatment (P = 0.323) (Fig. 2A), at the 4th week of treatment (P = 0.458) (Fig. 2B), at the 8th week of treatment (P = 0.227) (Fig. 2C), and at the end of the study in the 12th week of treatment (P = 0.7783) (Fig. 2D). When analyzing graphs A and D, no difference was observed between the groups before and after treatment.

As for the biochemical profile, no changes were observed in fasting blood glucose, triglycerides, TC, and fractions (HDL, VLDL, LDL). With respect to hepatic and renal function (Table 1), among the parameters analyzed, only a slight increase in Alanine Transaminase was observed in the OVX + *M. alba* group vs Control. At the end of the study, OVX rats exhibited lower levels of 17-β Estradiol compared to the SHAM groups, with no effect from the *M. alba* treatment (Table 1). Furthermore, no differences were observed in the plasma oxidative profile of the animals treated and not treated with the

M. alba infusion.

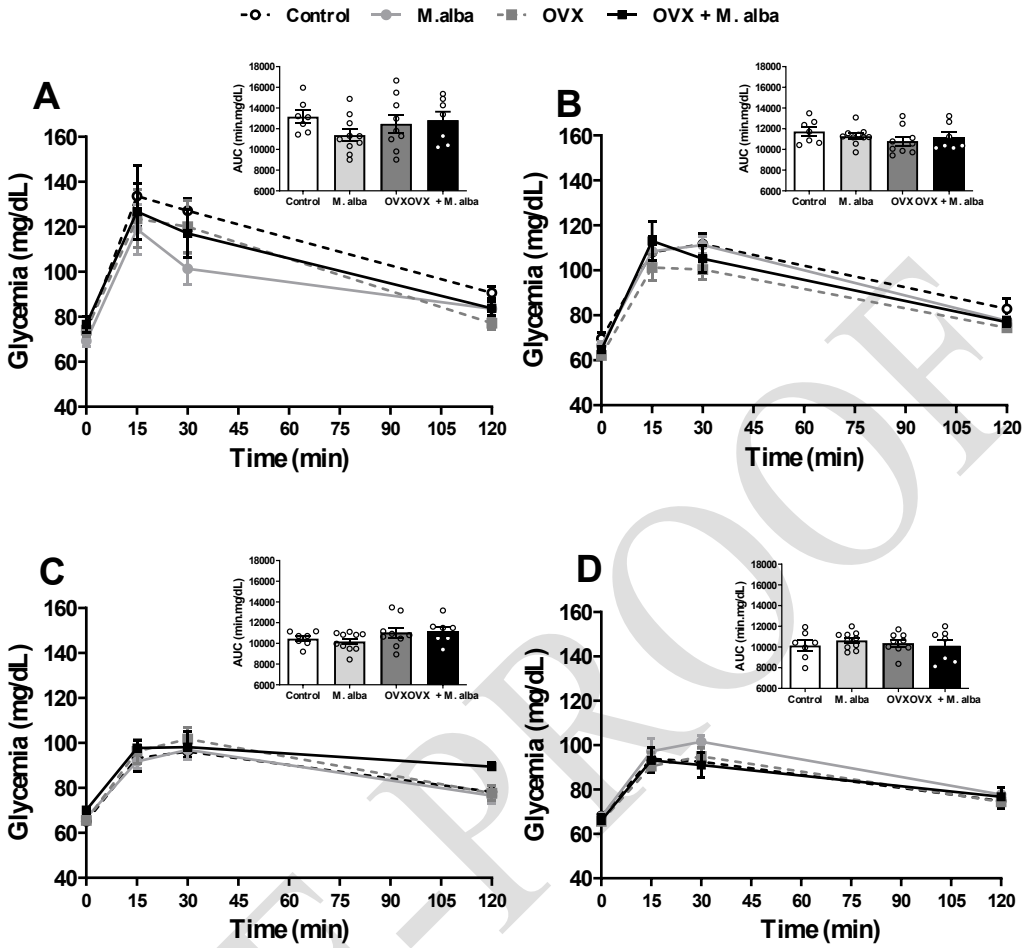

Figure 2. Glucose tolerance test response in ovariectomized animals treated with infusion of *M. alba* leaves. A= Glycemia before treatments, B= Glycemia at week 4 of treatment, C= Glycemia at week 8 of treatment, and D= Glycemia at week 12 of treatment. Data are presented as mean ± standard error, n= 6-9 per group, one-way ANOVA followed by Tukey's post hoc test. Source: The authors.

EFFECTS OF *MORUS ALBA* INFUSION ON BIOCHEMICAL, TOXICOLOGICAL, AND BEHAVIORAL PARAMETERS IN OVARIECTOMIZED RATS

Table 1: Effects of treatment with *M. alba* infusion on biochemical, oxidative, and tissue morphometry parameters in ovariectomized Wistar rats.

	Control	***M. alba***	**OVX**	**OVX + *M. alba***	**P**
Cholesterol (mg/dl)	131.1 ± 47.96	129.0 ± 51.85	113.1 ± 17.29	114.6 ± 9.57	0.905
HDL-Cholesterol (mg/dl)	30.86 ± 12.43	22.60 ± 10.04	28.33 ± 10.82	28.29 ± 14.40	0.362
VLDL- Cholesterol (mg/dl)	34.97 ± 10.64	31.88 ± 8.50	29.18 ± 4.76	35.11 ± 11.91	0.729
LDL- Cholesterol (mg/dl)	65.31 ± 42.89	74.52 ± 37.28	55.20 ± 10.30	51.17 ± 12.98	0.415
Triglycerides (mg/dl)	174.9 ± 53.19	159.4 ± 42.51	180.60 ± 106.4	175.60 ± 59.52	0.934
Alanine Transaminase (U/L)	16.86 ± 8.39	20.90 ± 3.75	15.11 ± 3.18	19.57 ± 9.71*	0.043
Albumin	5.314 ± 1.83	4.620 ± 1.78	3.956 ± 1.61	3.956 ± 1.61	0.107
Aspartate Aminotransferase (U/L)	19.43 ± 7.98	25.00 ± 5.08	17.78 ± 3.53	21.57 ± 4.89	0.058
Creatinine (mg/dl)	0.40 ± 0.23	0.28 ± 0.10	0.23 ± 0.50	0.17 ± 0.57	0.638
17-β estradiol (pg/ml)	30.86 ± 12.43	22.60 ± 10.04	Below the limit of detection***	Below the limit of detection***	< 0.001
Alkaline Phosphatase (U/L)	3.29 ± 3.68	1.30 ± 0.48	2.89 ± 2.26	1.29 ± 0.49	0.367
Gamma-glutamyl transpeptidase (U/L)	1.57 ± 0.53	1.50 ± 1.08	1.56 ± 1.01	1.43 ± 1.13	0.568
Glycemia (mg/dl)	67.14 ± 6.15	67.8 ± 4.96	66.44 ± 4.28	66 ± 7.70	0.920
Urea (mg/dl)	32.57 ± 13.40	22.8 ± 6.55	25.33 ± 6.0	24.29 ± 3.90	0.104
Superoxide Dismutase (SOD)	15.03 ± 3.85	16.95 ± 7.98	16.67 ± 8.12	17.89 ± 7.9	0.799
Catalase (CAT)	24.37 ± 9.28	33.23 ± 24.78	28.46 ± 18.44	18.62 ± 9.45	0.808
SOD/CAT Ratio	0.76 ± 0.54	0.73 ± 0.51	0.79 ± 0.6	1.31 ± 1.31	0.694
TBARS	12.93 ±4.55	10.93 ± 7.49	15.5 ± 3.17	15.55 ± 3.69	0.514
Tissue morphometry					
Spleen (g)	0.273 ± 0.05	0.264 ± 0.02	0.242 ± 0.02	0.236 ± 0.04	0.126
Liver (g)	3.069 ± 0.47	3.005 ± 0.22	2.502 ± 0.29**	2.508 ± 0.32*	< 0.001
Pancreas (g)	0.437 ± 0.14	0.359 ± 0.16	0.288 ± 0.04*	0.292 ± 0.03*	0.016
Kidneys (g)	0.695 ± 0.09	0.672 ± 0.06	0.579 ± 0.02*	0.644 ± 0.10	0.017
Gastrocnemius (g)	1.464 ± 0.08	1.460 ± 0.22	1.282 ± 0.10	1.376 ± 0.35	0.252
Soleus (g)	0.105 ± 0.02	0.096 ± 0.01	0.082 ± 0.01**	0.088 ± 0.01	< 0.001
Adipose Tissue (g)	2.719 ± 2.22	2.388 ± 0.89	2.881 ± 1.32	2.760 ± 1.02	0.887
Uterus (g)	0.319 ± 0.20	0.347 ± 0.09	0.115 ± 0.06**	0.142 ± 0.07*	< 0.001

Data expressed as mean ± standard deviation. *P<0.05 versus M. alba group, ** P<0.01 versus control, *** P<0.001 versus control. One-way ANOVA followed by Tukey's post hoc test (Alanine Transaminase, Glycemia, Urea, SOD, CAT, SOD/CAT Ratio, TBARS, Pancreas, Soleus Muscle). Kruskal-Wallis test followed by Dunn's post hoc test (Cholesterol, HDL, VLDL, LDL, Albumin, Aspartate Aminotransferase, Creatinine, 17-β estradiol, Alkaline Phosphatase, Gamma-glutamyl transpeptidase, Spleen, Liver, Kidneys, Gastrocnemius Muscle, Adipose Tissue, Uterus). 17-β estradiol Undetectable = 11.8 pg/ml. Source: The authors.

Regarding tissue morphometry, a lower hepatic, pancreatic, and uterine weight was observed when comparing the OVX + M. alba and control groups. Between the OVX and control groups, there was a reduction in renal weight and in soleus muscle weight (Table 1).

Regarding the hematological profile (Table 2), no changes were observed when comparing the M. alba and OVX + M. alba groups to the Control and OVX groups.

Table 2: Effects of treatment with *M. alba* infusion on the hematological profile of ovariectomized Wistar rats.

	Control	***M. alba***	**OVX**	**OVX + *M. alba***	**P**
Erythrocytes 10^6/mm^3	10.4 ± 4.05	11.51 ± 3.00	11.43 ± 3.13	12.42 ± 4.26	0.771
Leukocytes 10^3/mm^3	3.43 ± 2.09	5.16 ± 2.20	5.67 ± 2.88	3.81 ± 1.42	0.173
Lymphocytes (%)	82.43 ± 6.99	74.80 ± 6.01	77.0 ± 8.99	80.57 ± 4.43	0.129
Neutrophils (%)	14.43 ± 6.85	22.40 ± 5.78	21.11 ± 10.79	16.43 ± 4.20	0.125
Monocytes (%)	3.14 ± 0.38	2.80 ± 0.63	3.00 ± 0.50	3.00 ± 0.82	0.676
Platelets 10^3/mm^3	1022 ± 701.2	772.80 ± 152.9	734.70 ± 89.03	748.3 ± 224.3	0.940
Hemoglobin g/dl (Hb)	19.29 ± 6.73	21.0 ± 4.99	21.03 ± 5.36	22.76 ± 7.46	0.777
Hematocrit % (Hc)	57.13 ± 21.56	60.42 ± 15.56	61.17 ± 17.64	65.91 ± 21.83	0.854
Mean Corpuscular Volume fL (MCV)	53.29 ± 1.11	52.60 ± 1.27	53.22 ± 1.09	52.86 ± 1.22	0.534
Mean Corpuscular Hemoglobin pg (MCH)	18.74 ± 0.72	18.12 ± 1.19	18.52 ± 0.71	18.39 ± 0.55	0.519
Mean Corpuscular Hemoglobin Concentration % (MCHC)	35.19 ± 1.69	35.03 ± 1.59	34.76 ± 1.57	34.61 ± 1.14	0.859
Red Blood Cell Distribution Width % (RDW)	12.99 ± 0.51	12.74 ± 0.53	13.17 ± 0.33	13.01 ± 0.36	0.237
Mean Platelet Volume (fL)	6.14 ± 0.33	6.11 ± 0.28	6.26 ± 0.33	6.49 ± 0.60	0.507
Platelet Distribution Width (PDW)	10.93 ± 0.66	10.6 ± 1.03	11.17 ± 1.37	9.76 ± 3.97	0.853
Neutrophil/Lymphocyte Ratio (NLR)	0.14 ± 0.07	0.22 ± 0.06	0.21 ± 0.11	0.16 ± 0.04	0.125
Platelet/Lymphocyte Ratio (PLR)	304.7 ± 166.1	180.2 ± 84.32	164.3 ± 87.14	226.6 ± 115.7	0.087

Data expressed as mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test (Erythrocytes, Leukocytes, Lymphocytes, Monocytes, Hc, MCH, RDW, NLR, PLR), Kruskal-Wallis test followed by Dunn's post hoc test (Monocytes, Platelets, Hb, Hc, MCV, MCH, MPV, PDW). Source: The authors.

Regarding the behavioral profile of anxiety response, of the tests performed, an increase in time spent in the periphery in the Open Field test was observed between the OVX vs Control groups (Table 3) (P = 0.022).

EFFECTS OF *MORUS ALBA* INFUSION ON BIOCHEMICAL, TOXICOLOGICAL, AND BEHAVIORAL PARAMETERS IN OVARIECTOMIZED RATS

Table 3: Effects of treatment with M. alba infusion on the behavioral profile of anxiety, depression, and memory response in ovariectomized Wistar rats.

	Control Mean ± SD (n=7)	***M. alba* Mean ± SD (n=10)**	**OVX Mean ± SD (n=7)**	**OVX + *M. alba* Mean ± SD (n=7)**	**P**
Anxiety					
Open Field					
Time in Center (s)	31.54 ± 30.53	15.83 ± 16.18	8.57 ± 8.24	15.09 ± 6.02	0.085
Time in Periphery (s)	262.2 ± 29.33	282.4 ± 17.21	289.9 ± 7.33 *	282.6 ± 2.53	0.022
Distance Traveled (mm)	15671 ± 10184	15548 ± 9050	12870 ± 4938	10348 ± 8861	0.251
Immobility Time (s)	220.1 ± 53.54	191.3 ± 75.25	188.9 ± 47.37	212.9 ± 42.06	0.634
Light and Dark					
Latency to Dark side (s)	15.29 ± 9.39	18.80 ± 17.52	26.44 ± 18.64	22.57 ± 18.61	0.562
Time in Dark side (s)	257.3 ± 34.54	272.2 ± 26.74	257.8 ± 40.34	274.9 ± 20.38	0.658
Time in Light side (s)	42.71 ± 34.54	27.80 ± 26.74	42.22 ± 40.34	25.14 ± 20.38	0.658
Transitions to Dark side	2.29 ± 1.60	1.50 ± 1.08	1.56 ± 0.73	1.14 ± 0.39	0.256
Rearing	3.29 ± 2.29	3.60 ± 3.56	3.12 ± 1.76	2.00 ± 1.15	0.475
Elevated Plus Maze					
Distance Traveled (mm)	6996 ± 2949	6420 ± 2906	4190 ± 2730	6542 ± 4294	0.285
Total Immobility Time (s)	236.1 ± 21.79	237.0 ± 21.87	257.4 ± 22.41	235.5 ± 26.11	0.163
Time in Center (s)	30.93 ± 40.18	24.33 ± 25.00	16.19 ± 24.22	17.62 ± 17.39	0.552
Entries into OA	1.286 ± 2.215	0.900 ± 1.101	1.667 ± 1.936	2.500 ± 1.871	0.303
Time in OA (s)	19.31 ± 40.15	11.74 ± 17.51	5.067 ± 6.27	10.53 ± 14.70	0.898
Entries into CA	8.429 ± 4.28	8.200 ± 5.84	5.889 ± 4.75	7.833 ± 5.34	0.726
Time in CA (s)	249.8 ± 64.96	264.0 ± 33.89	278.7 ± 28.85	271.9 ± 24.03	0.552
Grooming	2.0 ± 1.91	2.6 ± 1.71	4.0 ± 1.66	2.167 ± 1.72	0.108
Rearing	8.286 ± 2.81	9.60 ± 2.99	10.11 ± 3.01	9.833 ± 5.23	0.754
Depression					
Forced Swim					
Immobility (s)	93.57 ± 73.87	106.9 ± 35.95	146.4 ± 79.77	103.4 ± 86.37	0.432
Climbing (s)	145.1 ± 77.36	62.5 ± 43.47	141.4 ± 75.03	135.7 ± 103.2	0.073
Memory					
Object Recognition					
Novel object interaction (s)	20.86 ± 7.31	19.1 ± 11.04	15.38 ± 7.48	18.17 ± 10.36	0.711
Familiar object interaction (s)	4.286 ± 4.39	3.5 ± 3.69	6.625 ± 4.50	4.667 ± 4.97	0.424

Data expressed as mean ± standard deviation. * P<0.05 versus control, one-way ANOVA followed by Tukey's post hoc test [Open Field (Time in Periphery), Elevated Plus Maze (Distance Traveled, Total Immobility Time, grooming, hearing), Forced Swim (Immobility, Mobility), Object Recognition (Interaction with ON)], # Kruskal-Wallis test

followed by Dunn's post hoc test [Open Field (Time in Center, Distance Traveled, Immobility Time), Light and Dark (Latency to LD, Time in LD, Time in DC, Transitions to LD, rearing), Elevated Plus Maze (Time in Center, Time in OA, Time in CA, Entries into CA), Object Recognition (Interaction with novel object)]. Source: The authors.

4 DISCUSSION

In this study, we demonstrated that the infusion of *M. alba* leaves exhibits in vitro antioxidant potential and did not show evidence of overt toxicity under the experimental conditions used. Furthermore, the administration of *M. alba* infusion at this concentration for 12 weeks of treatment is not capable of reversing the weight gain induced by estrogen deprivation in the model. Regarding tissue morphometry, a reduction in hepatic, renal, pancreatic, and muscle weight was found in the OVX animals compared to the control group, suggesting that low circulating estrogen levels interfere with the maintenance of organ weight. Another noteworthy aspect of the results is that OVX animals appear to exhibit higher anxiety levels compared to the Control group, which is not observed in OVX + *M. alba* animals.

The difference in weight and adiposity when comparing the OVX and control groups corroborates the findings of Burch et al. [17], who describe an increase in body weight resulting from estrogen deprivation due to surgical removal of the ovaries in Wistar rats. The association between the absence of circulating estrogen and increased body adiposity arises mainly from its ability to modulate energy metabolism and interact with target receptors in tissues involved in lipogenesis. Studies have shown that OVX rats exhibit greater weight gain than non-ovariectomized rats from the second week post-OVX, with a proportional increase up to the ninth week, at which point body weight stabilizes. Thus, it is suggested that the animals in this study reached a weight gain plateau, which was not reversed by treatment with the plant [9; 15; 18]. The importance of these results regarding body fat in the absence of ovaries reinforces that estrogen deprivation increases the risk of cardiometabolic diseases [2]. Insulin resistance in menopause is observed in human studies and is also reflected in animal models. This occurs because, in adipose tissue, insulin stimulates triglyceride storage through increased uptake of glucose and fatty acids derived from circulating lipoproteins, promoting lipogenesis in mature adipocytes and inhibiting lipolysis [17; 19].

In this scenario, studies involving different types of extracts from *M. alba* leaves have been conducted, utilizing various experimental models that mostly employ cholesterol-enriched diets [20]. These have demonstrated the plant's potential biological effect on weight loss and improvement in lipid, glycemic, and oxidative profiles in animals treated with a hypercholesterolemic diet. Metwally et al. [21] demonstrated that treatment for eight weeks with the ethanolic extract of *M. alba* leaves (100 mg/kg) reduced body weight gain, hypercholesterolemia, hypertriglyceridemia, atherogenic indices, glucose level, and insulin resistance index in female rats treated with a cholesterol-rich diet. Supporting the literature and the present study, Zeni and Dall'Molin [6] evaluated the effects of the aqueous extract of *M. alba* leaves (150 mg/kg) for fourteen days in Wistar rats treated and untreated with a hyperlipidemic diet and showed that treatment with the plant was able to reduce plasma triglyceride levels only in the group treated with both a cholesterol-rich diet and extract.

Regarding changes in the morphometry of hepatic, renal, and muscle tissue, Sucupira [22] clarifies that low circulating estrogen levels can promote cellular and tissue damage in tissues metabolically stimulated by this hormone. In addition, there was an alteration in ALT, indicating possible dysfunction in the organ. Goettems-Fiorin et al.[18], using the same experimental menopause model, found similar results, showing that the absence of estrogen during menopause can promote a reduction in tissue weight. It is also worth highlighting the role of estrogen in maintaining skeletal muscle mass, by inducing protein synthesis for tissue maintenance [23]; after menopause, women have an increased risk of developing sarcopenia and osteopenia, resulting from metabolic-functional changes caused by reduced circulating estrogen levels in tissues [24].

In this context, medicinal plants have been widely used as functional foods and alternative treatments for the prevention of various diseases, mainly due to their bioactive compounds with biological potential [4]. The leaves of *M. alba* contain important compounds responsible for the plant's therapeutic properties, among them: alkaloids, flavonoids (quercetin, rutin, and isoquercetin), tannins, and terpenes [4]. Although studies involving *M. alba* have indicated potential improvement of lipid, oxidative, glycemic, and neuroprotective metabolism [5], we did not observe changes in these parameters in this

study. This can be explained, at least in part, by the extraction method used, duration of treatment, or the estrogen deprivation condition studied.

Among the extraction methods used in traditional medicine to obtain plant extracts, maceration, infusion, decoction, among others, can be highlighted. In this study, infusion was used, in which in vitro antioxidant potential was indicated; in addition, infusion is the form in which the plant is commonly consumed. It is known that the choice of extraction method, as well as the solvent used, concentration, and temperature, directly influence the concentration of substances extracted from a plant and consequently its biological and pharmacological activity. Although widely used in traditional medicine, infusion is known to have low extraction potential, which may explain the absence of effects on the biochemical markers evaluated [25].

In this study, the enzymatic activity of the SOD/CAT antioxidant system and lipid peroxidation through TBARS measurement were assessed; however, no differences were observed between the groups in any oxidative parameter. It is important to highlight the role of estrogen in the redox homeostasis of biological systems, acting through different signaling pathways to maintain oxidative balance. Estrogen can neutralize reactive species (RS), acting as a direct antioxidant, due to its chemical characteristics. Additionally, it acts indirectly by modulating antioxidant enzymes [26]. Thus, estrogen deprivation may lead to alterations in the redox balance of various biological systems, as the imbalance between RS generation and neutralization results in a pro-oxidative state, a condition that may be linked to the genesis of cardiometabolic disorders [9; 27]. Understanding the role of estrogen in redox homeostasis makes it clear that, in the context of this study, there is a strong intrinsic factor that by itself leads to increased oxidative stress. Although the antioxidant potential of different types of *M. alba* extracts has been described in other studies [5; 28] at different dosages, this is the first study to evaluate the effects of the plant infusion. At least at this dose and treatment duration, the infusion did not demonstrate an in vivo antioxidant effect.

As for the toxicity analyses, it is noteworthy that in our study no hematological or renal profile alterations were observed; we noted only a slight change in a hepatic enzyme. These findings confirm that at this concentration and treatment duration, the plant infusion did not produce toxic effects. Although the popular use of medicinal plants is

mainly based on the principle of naturalness and low toxicity, it is known that, just like conventional treatments, medicinal plants may have toxic potential. Therefore, the evaluation of hematological parameters and hepatic and renal function can provide indications of local and systemic toxic manifestations and the safety of use. Alterations, for example, in the number of circulating blood cells or in liver and kidney enzymes, may indicate impairment of hematopoiesis and injury to target organs responsible for detoxification of the organism [29]. It is emphasized that until now, there were no reports in the literature of toxicity assessment associated with the chronic use of *M. alba* infusion; toxicity of different extraction forms of the plant extract has also not been observed [4].

Regarding animal behavior in the open field test analysis, it was observed that the OVX group showed a longer duration in the periphery compared to the control groups; this difference was not observed in the OVX + *M. alba* group, indicating a potential anxiolytic effect of the plant. In the central nervous system, there are numerous estrogen-responsive areas that interfere with the synthesis and modulation of neurotransmitter function, such as acetylcholine, catecholamines, and serotonin, which are involved in behavioral and memory modulation [30]. Studies have shown that in OVX animals there are alterations in the regulation of serotonin transmission, binding, and metabolism in brain areas implicated in affect and cognition regulation. Raj et al. [31] highlight that flavonoids such as quercetin, present in high amounts in *M. alba* leaves, have the potential to enhance the brain's enzymatic antioxidant system by inhibiting enzymes such as Catechol-O-methyltransferase and Monoamine oxidase, culminating in improved redox balance in the brain and reduced deleterious effects of oxidative imbalance in this tissue. Furthermore, Raj et al. [31] demonstrate the anxiolytic effect of different preparations of *M. alba* leaves in animal models of anxiety, thus corroborating the findings of this study, where animals treated with the infusion showed reduced time spent in the periphery of the open field test apparatus compared to untreated animals.

Based on the above, this work suggests that estrogen is a determining factor in the modulation of body weight, increased anxiety, and loss of tissue mass, which, at this dose, preparation, and treatment duration, was not reversed by the administration of the *M. alba* leaf infusion. Given the traditional use of this plant, further studies are necessary to clarify whether higher doses, longer treatment periods, and other forms of preparation may have

different metabolic effects in conditions of cardiometabolic disorders associated with menopause. The use of a normolipidemic diet can be cited as a limitation of this study, since OVX alone was not able to produce significant changes, especially in terms of oxidative and biochemical markers, that could be reversed by treatment. Moreover, the age of the animals may also be a limiting factor, since by the end of treatment all animals were in an acyclic state.

5 CONCLUSION

In this study, it was shown that *M. alba* leaf infusion, even at low concentration, exhibits antioxidant potential in vitro and its chronic administration does not induce hepatic, renal, or hematological toxicity. Furthermore, it is suggested that the *M. alba* infusion suggested a potential anxiolytic effect. Meanwhile, estrogen deprivation leads to increased body weight, which is not reversed by ingestion of the *M. alba* infusion.

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EFFECTS OF *MORUS ALBA* INFUSION ON BIOCHEMICAL, TOXICOLOGICAL, AND BEHAVIORAL PARAMETERS IN OVARIECTOMIZED RATS

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