RESEARCH ARTICLE

F.U. Vet. J. Health Sci.
2026; 40 (2): 188 - 197
http://www.fusabil.org

Effects of Morus alba Extract on Antioxidant and Anti-Inflammatory Pathways in Nonylphenol- and Bisphenol A-Induced Neurotoxicity in Rat Brain *

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Received : 01.04.2026
Accepted : 02.06.2026

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Endocrine disruptors cause temporary and permanent effects in animals and humans by disrupting internal hormonal balance and causing oxidative damage to cells. Among endocrine disruptors, Bisphenol A (BPA) and Nonylphenol (NP) are two members with the most significant risk potential for neurotoxicity. White mulberry (*Morus alba* (MA)) is among the fruits consumed worldwide. Scientific research has shown various pharmacological effects of white mulberry fruit extract, including antioxidant, hepatoprotective, hypotensive, anticancer, anti-anxiety, hypolipidemic, and antidiuretic properties. In our study, the antioxidant and anti-inflammatory capacity of MA extract was investigated against the oxidative and inflammatory effects of BPA and NP, administered separately and in combination, on the brain. For this purpose, 48 male Wistar albino rats were experimentally grouped, and all substances (BPA 100 mg/kg, NP 100 mg/kg, MA extract 0.5 mL/kg) were administered orally via gavage. Lipid peroxidation marker MDA levels and antioxidant markers GSH, GPx, and CAT activities were analyzed spectrophotometrically. Inflammation markers IL-1β, IL-6, and TNF-α levels were determined using ELISA. Our results showed that BPA (p=0.009) and NP (p=0.0006), when administered separately and together (p=0.0001), increased MDA levels, while they decreased GSH (p=0.014) and CAT (p=0.002) activity when administered together (BPA+NP). MA extract generally reduced MDA levels (p=0.031) and may positively affect GSH and CAT activities. The BPA+NP group increased the analyzed inflammation parameters (respectively, p=0.01, 0.001, 0.0001), while the extract specifically decreased IL-1β values (p=0.047). In the cerebral cortex, degeneration and perineuronal edema were observed in neuronal and neuroglial cells in the BPA+NP groups, while MA extract somewhat normalized these levels. BPA was more effective in oxidative damage, while NP was more dominant in triggering inflammation. Its synergistic effect resulted in a high level of neurotoxicity. MA extract prevented lipid peroxidation and exhibited more anti-inflammatory properties. It is hypothesized that further investigation of these effects on different or similar intracellular anti-inflammatory and antioxidant pathways through gene and protein expression and behavioral tests could be beneficial.

Key Words: *Bisphenol A, nonylphenol, Morus alba extract, antioxidant, brain, neurotoxicity, oxidative stress, inflammation*

Rat Beyninde Nonilfenol ve Bisfenol A Kaynaklı Nörotoksisitede *Morus alba* Ekstraktının Antioksidan ve Anti-inflamatuvar Yolaklar Üzerindeki Etkileri

Endokrin bozucular, hayvanlarda ve insanlarda iç hormonal dengeyi bozarak, hücreleri oksidatif hasara uğratarak geçici ve kalıcı etkilere neden olmaktadır. Endokrin bozucular arasında Bisfenol A (BPA) ve Nonilfenol (NP) ise nörotoksisite için en önemli risk potansiyeline sahip olan iki üyesidir. Beyaz dut [*Morus alba* (MA)] meyvesi dünya genelinde tüketilen meyveler arasındadır. Bilimsel araştırmalar, beyaz dut meyve ekstraktının antioksidan, hepatoprotektif, hipotansif, antikanser, antianksiyete, hipolipidemik ve antidiüretik gibi çeşitli farmakolojik etkilerini göstermiştir. Çalışmamızda BPA ve NP'nin ayrı ayrı ve birleşik verilmesiyle beyindeki oksidan ve inflamatuar etkisine karşı MA ekstraktının antioksidan ve antiinflamatuar kapasitesi araştırılmıştır. Bu amaçla, 48 adet erkek Wistar albino ırkı ratlar deneysel olarak gruplandırılmıştır ve tüm maddeler (BPA 100 mg/kg, NP 100 mg/kg, MA ekstraktı 0.5 mL/kg) oral gavajla verilmiştir. Lipid peroksidasyon belirteci MDA düzeyleri ile antioksidan belirteç için GSH, GPx ve CAT aktiviteleri spektrofotometrik analiz edilmiştir. İnflamasyon belirteçleri olarak IL-1β, IL-6 ve TNF-a seviyeleri ELİSA yöntemiyle belirlenmiştir. Sonuçlarımız, BPA (p=0.009) ve NP (p=0.0006) ayrı ayrı ve birlikte (p=0.0001) verildiğinde MDA seviyelerini artırmış, GSH (p=0.014) ve CAT (p=0.002) aktivitesini ise birlikte (BPA+NP) verildiğinde azaltmıştır. MA ekstraktı ise MDA düzeylerini genel olarak azaltmış(p=0.031), GSH ve CAT aktivitelerini olumlu yönde etkileyebilmektedir. BPA+NP grubu analiz edilen inflamasyon parametrelerini artırmış (sırasıyla, p=0.01, 0.001, 0.0001) ve ekstrakt ise özellikle IL-1β değerlerini azaltmıştır (p=0.047). Serebral korteksde BPA+NP gruplarında nöron ve nöroglial hücrelerde dejenerasyon ve perinöronal ödem bulguları gözlemlenirken, MA ekstraktı bir miktar normale döndürmüştür. BPA oksidatif hasarda daha etkiliyken, NP inflamasyonu tetiklemekte daha baskın görülmüştür. Sinerjik etkisi ise yüksek düzeyde nörotoksisite meydana getirdi. MA ekstraktı lipid peroksidasyonu önlemiş ve daha çok antinflamatuar özellik göstermiştir. Bu etkisinin hücre içi farklı veya benzer birtakım antinflamatuar ve antioksidan yolaklarının gen, protein ekspresyonu ve davranış testleri üzerinde araştırılmasının faydalı olabileceği tahmin edilmektedir.

Anahtar Kelimeler: *Bisfenol A, nonilfenol, Morus alba ekstraktı, antioksidan, beyin, nörotoksisite, oksidatif stres, inflamasyon*

* This study was supported by the Kastamonu University Scientific Research Projects Unit with project number KÜBAP-01/2024-22.

Introduction

Endocrine disruptors are a term given to all compounds that disrupt the internal hormonal balance in animals and humans, causing temporary and/or permanent effects (1). Xenoestrogens are a type of xenohormone that mimics estrogen. They can be synthetic or natural chemical compounds. Synthetic xenoestrogens include some commonly used industrial compounds such as polychlorinated biphenyls, bisphenol A (BPA), and phthalates, which, although chemically different from estrogenic substances produced internally by the endocrine system of any organism, have estrogenic effects on a living organism (2, 3). BPA and nonylphenol (NP), which are among the chemicals called xenoestrogens, are the most important endocrine disrupting compounds used in the polymer industry to produce various products for human use (4). Prenatal and childhood exposure to NP and BPA can have different effects on the neurodevelopment of young children and may negatively affect intellectual development (5).

NPs are a subset of alkylphenols and belong to the family of organic compounds. Their solubility in water is 6 mg/L (6). Many varieties are widely used in industrial applications. After the use of chemicals such as NP ethoxylates and tris (nonylphenyl) phosphide, they are converted into NPs that contaminate the environment and food (7). Numerous studies have shown that they have many destructive functions in various organs, including the brain. This toxic substance causes oxidative stress in the cortex and hippocampus cells, two key regions in the brain responsible for maintaining memory and learning (8).

BPA is a synthetic crystalline solid compound with a very high melting point and insoluble in water. The most common use of BPA is in the production of polycarbonate plastics, accounting for 60%. This is followed by epoxy resin production at 26%. BPA is used in the manufacture of polyvinyl chloride plastic windows, compact discs, automotive parts, powder coatings, water and milk bottles, baby bottles, and many electrical and electronic components that we use in our daily lives (9). BPA produces free oxygen radicals (ROS) that cause oxidative damage in the brains of rats (10).

Oxidative stress is a condition resulting from an imbalance between the biological activity of the body's antioxidant systems, such as antioxidant enzymes, to reduce and eliminate factors causing oxidative stress, and products such as ROS and reactive nitrogen species at the cellular and tissue levels. ROS molecules are highly reactive substances that can react with other important cellular structures. Free radicals can damage parts of cells, including proteins, DNA, and cell membranes, by stealing their electrons through a process called oxidation. Reactive nitrogen species cause ROS accumulation in the brain and can lead to lipid peroxidation. The brain is susceptible to lipid peroxidation because it is rich in polyunsaturated fatty acids. The rate of lipid peroxidation in the brain can be determined by measuring the level of the malondialdehyde (MDA) enzyme as a marker of lipid peroxidation and oxidative stress. ROS accumulation and the resulting cellular redox can be one of the triggers of the apoptosis process (8). BPA and NP reduced glutathione (GSH) levels in rat brain while increasing MDA levels, the end product of lipid peroxidation (11).

Inflammation is a protective pathophysiological response aimed at restoring tissue homeostasis, involving the activation of non-immune cells in the living organism against harmful agents such as infection, toxic compounds, and damaged cells. The mediators that cause inflammation and inhibit its effects are cytokines. Three cytokines, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), play a role in the main mechanisms of inflammation (12). IL-1β is one of the most important messenger cytokines of the immune system. It is a key protein that the body produces when it perceives a threat, initiating the inflammatory response. An increase above normal levels is usually an indication that there is a fight or damage in the body. Elevated IL-1β levels indicate that the body's immune system is actively working. However, if IL-1β levels remain high for too long or rise uncontrollably (excessively), they can cause serious harm to the body. Continuously high levels lead to tissue destruction (e.g., cartilage erosion in joints). Excessive and sudden increases (as in sepsis or severe viral infections) can lead to organ failure and shock. It also lowers the pain threshold, causing the individual to feel more pain (13, 14).

IL-6, like IL-1β, is one of the most critical messenger molecules (cytokines) of the immune system. However, IL-6 has a very interesting and "dual" nature: it can both initiate and stop inflammation. An increase in blood IL-6 levels indicates that the body has entered emergency mode. It rises rapidly in acute infections, tissue damage, autoimmune activity, metabolic stress, and exercise. Its temporary increase stimulates C-reactive protein production in the liver and increases body temperature, exhibiting antimicrobial effects. It also increases fat breakdown and improves insulin sensitivity, which can be considered positive aspects. Chronically high levels can lead to muscle wasting in the elderly and those with chronic diseases, cardiovascular diseases, anemia due to impaired iron absorption, growth of certain tumors, and depression by affecting the brain (15-17).

TNF-α is one of the effective cytokines of the immune system. It is known to be the cytokine that primarily coordinates inflammation in the body, with the ability to kill tumor cells (necrosis). By triggering the production of IL-1 and IL-6, it initiates the inflammation chain and causes severe inflammation. It is the main molecule that attacks our own tissues in diseases such as rheumatoid arthritis and psoriasis (autoimmune diseases). In cancer, it prevents the nourishment of tumor cells and directs immune cells to the area of inflammation. It is the main culprit behind the extreme weight loss (muscle and fat wasting) in patients with cancer or chronic diseases. It suppresses appetite and

destroys muscles. It directly erodes bone and cartilage in joints (rheumatic diseases). It disrupts insulin signaling in cells, which is why it is a significant factor in obesity and Type 2 diabetes. It disrupts brain chemistry, causing severe fatigue (18-20).

Morus alba (MA) L. (a species of Moraceae), commonly known as mulberry, is native to Asia and found worldwide. MA berries are considered a nutritious food; in addition to the production of natural dyes, medicines, and cosmetics, it is widely used in the production of jams, wines, and canned foods. Traditionally, MA berries have been used as a cooling agent, laxative, diuretic, anthelmintic, brain tonic, and antibacterial. They are used to nourish blood tissue, strengthen joints, and improve eye and kidney function. They are also used to treat weakness, fatigue, anemia, and premature graying of hair. MA berries are reported to be a rich source of phenolic compounds, including phenolic acids (caffeic acid, chlorogenic acid, cinnamic acid, p-coumaric acid, ferulic acid) and flavonoids (quercetin). Modern scientific research has demonstrated and reported various pharmacological effects of MA fruit extract, including antioxidant, hepatoprotective, hypotensive, anticancer, anti-anxiety, hypolipidemic, and antidiuretic properties. Recently, the neuroprotective potential of MA fruit extract (MAE) has been reported to improve memory impairment and cognitive abilities in Parkinson's disease and vascular dementia (21).

Nowadays, the exposure rate of humans and animals to oxidizing agents such as BPA and NP is increasing. Information on the protective effect of white mulberry fruit extract against oxidative damage and inflammation in the brain caused by the intake of these two substances, both individually and together, has not been found. The hypothesis of this study is to investigate the protective effect of MA extract against neurotoxicity caused by inflammation and oxidative damage in the brain by BPA and NP, both individually and in combination, through its anti-inflammatory and antioxidant properties.

Materials and Methods

Research and Publication Ethics: In our study, 48 male (An a priori sample size calculation performed via G*Power (v3.1.9.7) indicated that a total of 48 animals (6 per group) was sufficient to provide a statistical power >0.80.) Wistar albino rats (average 200-250 g, 10-12 weeks old) were used. The animals were obtained from the Experimental Medicine and Research Center of Kastamonu University, and their care was provided at the Experimental Research Center of Kastamonu University. The rats were kept in standard cages at a temperature of 24±2°C, humidity of 45±5%, and a 12-hour light/12-hour dark cycle. During the experiment, the rats were fed ad libitum with a standard diet and water. Gavages were administered once a day for 30 days. All study and animal care procedures were carried out with the ethical approval of the Kastamonu University Local Animal Ethics Committee dated 11.03.2024 (Number: 2, Decision No: 2) and numbered E-16498365-050.99-2400034726. The procedures in the study were carried out in accordance with the principles of the "Guide for the Care and Use of Laboratory Animals". The animals were divided into 8 groups in total, with 6 animals in each group.

Study Groups: Control: 0.5 mL/kg corn oil was given by oral gavage. BPA: 100 mg/kg bisphenol A (Sigma, 239658) was given by oral gavage (22). NP: 100 mg/kg NP (Acros, 41624001) was given by oral gavage (23). BPA+MA Extract: 100 mg/kg bisphenol A+0.5 mL/kg Mulberry Extract was given by oral gavage. NP+MA Extract: 100 mg/kg NP+0.5 mL/kg Mulberry Extract was given by oral gavage. BPA+NP: 100 mg/kg bisphenol A and 100 mg/kg NP were administered orally via gavage. BPA+NP+MA Extract: 100 mg/kg bisphenol A and 100 mg/kg NP+0.5 mL/kg Mulberry Extract were administered orally via gavage. MA Extract: 0.5 mL/kg Mulberry Extract was administered orally via gavage to each animal in the group. BPA and NP were dissolved in corn oil (24). In animals, white mulberry (*Morus alba*) extract was used, which was collected from Yeşilçay village in Erzincan province for previous research purposes, extracted and subjected to content analysis (GC-MS). The content analysis of white mulberry extract was shown in the study by Inanc et al. (25).

Experimental Termination: The experiment was terminated at the end of the 30th day, and the rats were euthanized using cervical dislocation technique under Ketamine (90 mg/kg, ip) / Xylazine (10 mg/kg, ip) anesthesia. Brain tissues were dissected, washed with saline, and then stored at -80°C for the measurement of oxidant-antioxidant and inflammatory parameters.

Oxidant and Antioxidant Determination

Preparation of Homogenates: Brain tissues removed from the deep freezer were transferred to glass tubes while maintaining their cold temperature. A buffer containing 1.15% potassium chloride at a 1/10 dilution was added to the tissues, and the tissues were homogenized while maintaining their cold temperature. Total protein determination was performed in these prepared homogenates using bicinchoninic acid assay (BCA Protein assay kits). The homogenate was centrifuged at 5000 g for 1 hour in a centrifuge cooled at +4°C to obtain the supernatant. Glutathione peroxidase (GSH-Px), catalase (CAT) and GSH enzyme activities and MDA levels were measured in the separated supernatants.

Measurement of Tissue MDA Levels: MDA levels in tissues are based on the reaction of MDA, one of the aldehyde products of lipid peroxidation, with thiobarbituric acid (26). MDA forms a pink complex with thiobarbituric acid, and the degree of lipid peroxidation was determined by spectrophotometric measurement of the absorbance of this solution at 532 nm. MDA was expressed as mmol/g protein (unit).

Measurement of Tissue GSH-Px Activity: Tissue GSH-Px activities were determined spectrophotometrically according to the method described by Lawrence and Burk (27). Essentially, GSH-

Px catalyzes the oxidation of reduced GSH to oxidized glutathione (GSSG) via hydrogen peroxide. GSSG, formed by GSH-Px in the presence of hydrogen peroxide t-butylhydroperoxide, is reduced to GSH with the help of glutathione reductase and NADPH. GSH-Px activity was determined by spectrophotometrically reading the absorbance difference at 340 nm during the oxidation of NADPH to NADP. GSH-Px was expressed as IU/g protein (unit).

Measurement of Tissue CAT Activity: Tissue CAT activities were determined spectrophotometrically according to the method described by Aebi (28). In enzyme activity determination, the enzyme activity is determined by measuring either the decreasing amount of substrate or the amount of product formed. Catalase catalyzes the breakdown of hydrogen peroxide (H_2O_2). The rate of breakdown of H_2O_2 by catalase was measured spectrophotometrically by taking advantage of the fact that H_2O_2 absorbs light at a wavelength of 240 nm. Catalase activity was calculated by measuring the absorbance difference within 30 seconds after setting the blind to zero at 240 nm. CAT activity was expressed as KU/g protein (unit).

Measurement of Tissue GSH Levels: GSH level was determined by the method of Sedlak and Linsay (29). The methods used in this determination were; 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) is a disulfide compound reduced by sulfhydryl compounds. It forms a highly yellow anion. The color intensity of the yellow complex formed by DTNB and the supernatant obtained from the sample is directly proportional to the GSH concentration in the medium, measured spectrophotometrically at 412 nm. GSH is expressed as mmol/g protein (unit).

Analysis of Inflammation Parameters: One lobe of the brain taken from rats was stored at -80°C. Tissues were homogenized by adding PBS before the study. Then centrifuging was performed and the supernatants were transferred to microtubes. TNF-α (E0764Ra; BT LAB), IL-1β (E0119Ra; BT LAB) and IL-6 (E0135Ra; BT LAB) levels were measured in 96-well plates using the enzyme-linked immunosorbent assay (ELISA) method. The measurement was performed at 450 nm with an ELISA reader (EPOCH Take3Plate, BioTek) following the supplier's procedure.

Histopathological Examination: The study was carried out in the Histology-Embryology Laboratory of the Faculty of Veterinary Medicine, Kastamonu University. Brain tissue samples were fixed in 10% neutral formalin (NBF) solution, then processed through routine tissue handling steps and embedded in paraffin blocks (Paraplast Plus, Sigma: P3683). Serial sections of 5 µm thickness were taken from the prepared paraffin blocks using a microtome (Leica RM 2125). Hematoxylin-Eosin staining was performed on the serial sections taken from each animal to investigate the general histopathological appearance (30), and the preparations were examined under a light microscope (Zeiss Axiolab 5, Jena, Germany).

Cerebral cortex H&E sections were morphologically evaluated at 20× magnification. Areas selected from the same anatomical region for each animal and free of artifacts/marginal tears were examined. Histopathological evaluation was performed blindly by evaluators independently of groups, after coding the preparations. During the examination, the presence and extent of degenerative changes such as hyperchromatic/pyknotic neurons and perineuronal halo/pericellular spacing were considered. Findings were reported descriptively using images that best represented the cerebral cortex histomorphology of each group.

Statistics: IBM SPSS 23 and GraphPad Prism 8 were used for the statistical analysis of the data obtained from the study. Prior to the analysis, the assumption of normality was evaluated using the Shapiro-Wilk test, and the homogeneity of variances was confirmed using Levene's test. The sample size of n= 6 per group was determined in accordance with the 3Rs (Replacement, Reduction, Refinement) principle of animal ethics, consistent with previously published models (68, 69), and a retrospective power analysis confirmed that this sample size provided adequate statistical power (Power >0.80). Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to identify specific differences between the eight experimental groups. Furthermore, to account for the arrangement of multiple tests for various biochemical and inflammatory parameters, p-values were checked using the Benjamini-Hochberg False Detection Rate (FDR) method. All data are expressed as mean ± standard deviation (SD). Adjusted differences with $p<0.05$ were considered statistically significant.

Results

MDA values were increased by BPA ($p=0.009$), NP ($p=0.0006$), and BPA+NP ($p=0.0001$) compared to the control. Significant differences were observed between the NP and NP+Extract ($p=0.023$), and BPA+NP and BPA+NP+Extract ($p=0.031$) groups, with MDA values approaching the control in the extract group. Significant differences were observed in GSH values between the Control and BPA+NP ($p=0.014$) and Control and BPA+NP+Extract ($p=0.026$) groups, with GSH activity decreasing compared to the control. No significant difference was found between the groups in GPx levels. Significant differences were observed in CAT levels between the Control and BPA ($p=0.033$), Control and BPA+NP ($p=0.002$), and Control and BPA+NP+Extract ($p=0.007$) groups, with CAT activity decreasing compared to the control. According to these values, both BPA and NP, when administered separately and together, significantly increased MDA levels compared to the control. While the MA extract decreased MDA levels compared to the BPA+NP group, it was not yet at control levels. However, no significant difference was found between the NP+Extract group and the control group. When BPA and NP were administered separately, they did not cause any significant change in GSH levels, but together (BPA+NP group) they reduced exposure

compared to the control group. Although the BPA+NP+Extract group brought GSH levels closer to control levels, the significant difference between the control group and the BPA+NP+Extract group did not disappear. For CAT values, the decrease in the BPA group returned to control levels in the BPA + Extract group (Figure 1).

Regarding IL-1β levels; Significant differences were found between the Control and NP (p=0.0057), Control and BPA+NP (p=0.010), NP and NP+Extract (p=0.007), and BPA+NP and BPA+NP+Extract (p=0.047) groups, with MA extract strongly reducing IL-1β levels. Only the BPA+NP group (p=0.001) showed significantly higher IL-6 levels compared to the Control group. TNF-alpha levels were significantly higher in the NP (p=0.008) and BPA+NP (p=0.0001) groups compared to the Control group (Figure 2).

In the Control group, the general histological structure of the cerebral cortex was preserved, with neurons and neuroglial cells exhibiting normal morphology; no significant perineuronal space widening (edema) was detected. In the extract group, tissue organization was largely preserved, similar to the control group, and degenerative changes were minimal. In the BPA group, degeneration in neurons and neuroglial cells became more pronounced, while areas of increased perineuronal space/edema were observed more frequently. In the NP group, similar to BPA, degenerative cellular changes and perineuronal edema were detected. In the BPA+NP (mix) group, the severity and extent of the findings increased, and degenerated cells and perineuronal space enlargement became more prominent. In the BPA+Extract and NP+Extract groups, compared to the respective toxic agent groups, a tendency towards a decrease in degeneration and perineuronal edema findings and partial improvement in tissue integrity were observed. In the BPA+NP+Extract group, degenerative changes and perineuronal space enlargement were more limited compared to the mix group, but findings persisted in some areas (Figure 3).

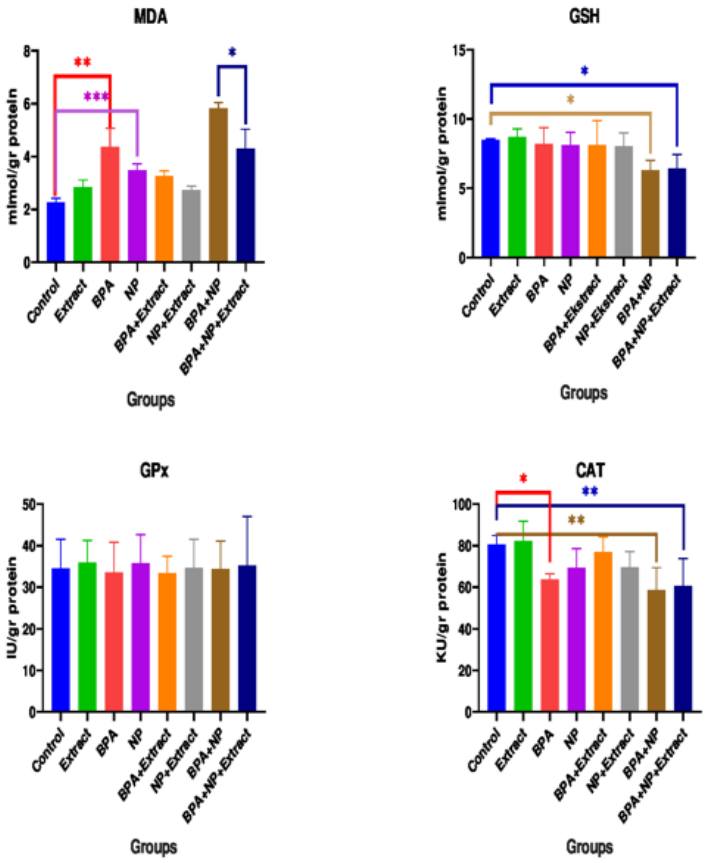

Figure 1. Statistical graphs of MDA, GSH, GPx, and CAT levels in brain tissue across all experimental groups. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. Graphs were generated using GraphPad Prism 8.0.2. Statistical significance is indicated as follows: *p<0.05, **p<0.01, and ***p<0.001.

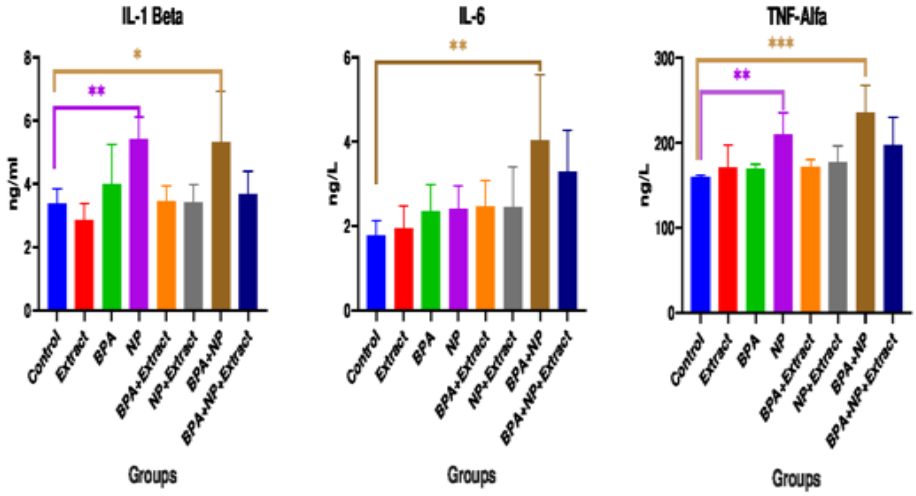

Figure 2. Statistical graphs of IL-1β, IL-6, and TNF-α levels in brain tissue across all experimental groups. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. Graphs were generated using GraphPad Prism 8.0.2. Statistical significance is indicated as follows: *p<0.05, **p<0.01, and ***p<0.001.

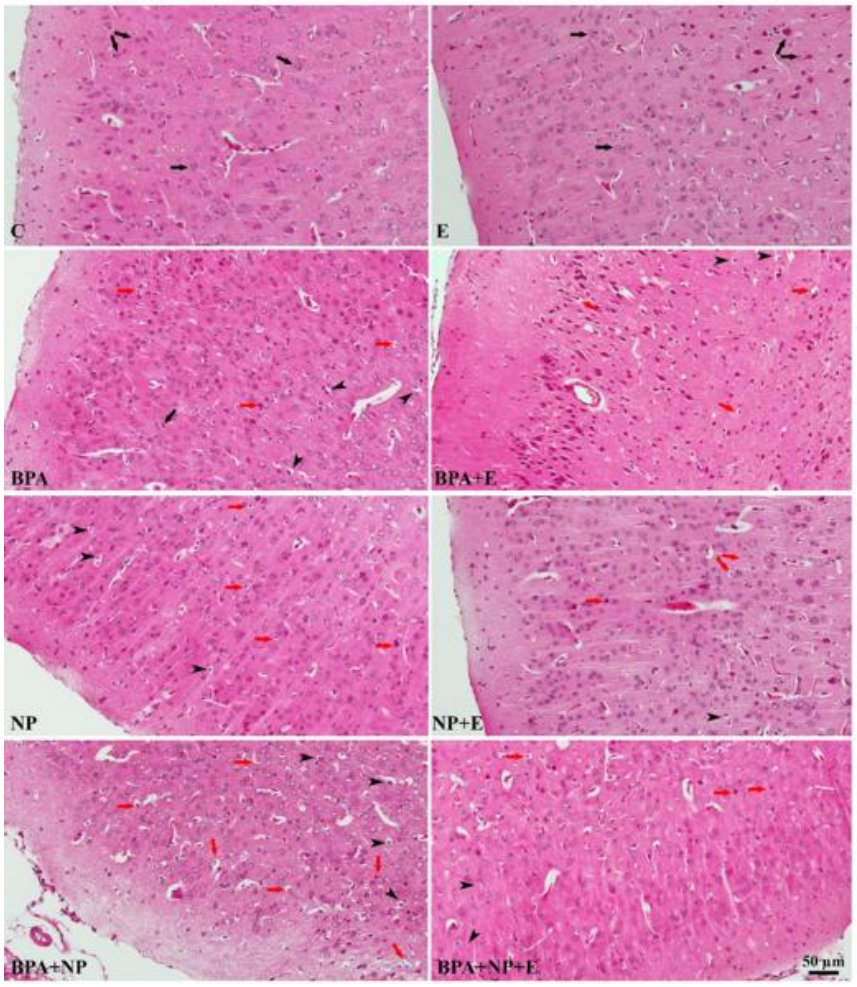

Figure 3. Hematoxylin–eosin (H&E) staining of the rat cerebral cortex. C: control; E: extract; BPA: bisphenol A; NP: nonylphenol. Scale bar: 50 µm. Black arrows indicate healthy neurons and neuroglial cells with normal morphology; black arrowheads indicate perineuronal and perineuroglial vacuolization; and red arrows indicate degenerated neurons and neuroglial cells.

Discussion

EDCs currently pose a serious threat to human and animal health, as seen in polycyclic aromatic hydrocarbon, dioxin, polychlorinated diphenyl, nitrate, and nitrite poisonings (31, 32). BPA and NP are common EDCs in the environment. Exposure of mice to EDCs during brain development increases the risk of neurodevelopmental disorders (33, 34). It is suggested that BPA and bisphenol analogs can cross the blood-brain barrier and affect nerve tissues, thus impacting the development and function of the nervous system and increasing the risk of neuropsychiatric diseases, and BPA can increase neurotoxicity (35, 36). As a result, conditions such as severe memory loss, cognitive impairment, tremor, and rigidity occur (37). BPA generally appears to cause higher oxidative damage in the brain compared to nonylphenol. However, NP can exert its effects in different ways, particularly in the hippocampus and cortex, through activation of inflammatory factors, disruption of the cell cycle, changes in neuron, dendrite, and synapse morphology, disruption of extracellular and intracellular calcium ion balance, and memory and learning impairments (8). This may be attributed to differences in the absorption processes of both substances in brain tissue or by different transport pathways. The extract of *Morus alba* fruit has a very rich content, containing nearly 50 antioxidant and anti-inflammatory compounds (38). It is also known to contain melatonin (39). Hormones like melatonin can reduce neurotoxicity (35). Thymoquinone has shown a neuroprotective effect against NP-induced neurotoxicity by increasing GSH and total antioxidant capacity (40).

Oxidative stress is a state of disruption between oxidants and antioxidants in favor of oxidants due to the induction of free radicals. Free radicals act as a critical factor in cellular destruction resulting from the use of toxic chemicals that induce cell necrosis (41). Oxidative stress also occurs due to the rise of ROS, which can cause significant cell destruction by reacting with many molecules, including proteins, fats, and DNA, and controlling lipid peroxidation. ROS can lead to lipid peroxidation of mitochondrial and plasma membranes, trigger the opening of mitochondrial permeability transition pores, and activate apoptosis pathways. Therefore, it has a critical effect on the pathogenesis of many human health disorders (42-45). MDA formation is a key marker of oxidative stress, which can destroy the cell membrane with a decrease in the cell's anti-oxidative activity (23). MDA has also been shown to reduce mitochondrial membrane potential and lead to mitochondrial dysfunction (46).

In rats exposed to BPA or NP, significant adverse effects on antioxidants, immune-inflammatory mediators, and many different tissues are known. The combined use of these two substances can also produce a synergistic negative effect on many parameters. Different studies have shown that BPA and NP may have potential interactions (23, 47). These toxic substances have been shown to suppress the Nrf2 (Nuclear factor erythroid 2-related factor 2) pathway, the cell's main antioxidant defense regulator (48). In addition, it has been determined that the toxins activate the NLRP3 inflammasome in the brain, initiating neuroinflammation and causing an increase in cytokines (49). Chronic exposure to BPA, NP, and BPA&NP has been shown to affect lipid metabolism and the production of protein secondary structures over multiple generations (50). The use of a mixture of BPA and NP at both high and low doses significantly increases some oxidative stress markers such as MDA, protein carbonyl (PC), and 4-hydroxyononenal (4-HNE) in rat sera (23). A study conducted in the early 2020s showed that high MDA levels and decreased activity of antioxidant enzymes in serum, liver, and kidneys indicate that oxidative stress plays a role in BPA and NP-induced toxicity (47). NP is a well-known endocrine-disrupting chemical with many harmful effects on the central nervous system, including memory impairment, neuroendocrine disruption, cognitive impairment, and neurotoxicity (40). NP exposure triggers microglial activation and disrupts the myelination of oligodendrocytes. Mice can exhibit anxiety and depressive-like behaviors under the influence of NP (51). Studies in the literature show that BPA and NP significantly increase MDA levels in the brain and some tissues (23, 35, 40, 52). Compared to the data we obtained in our study, this is supported by the oxidant-antioxidant reactions occurring in the brain. We can say that this increase is even more effective than parasitic infections such as coccidiosis when compared to control groups (53). Exposure to both BPA and NP, individually and in combination, significantly increased MDA levels in brain tissue (Figure 1).

Morus Alba extract reduced lipid peroxidation against rotenone-induced oxidative damage in the brain, increased antioxidant capacity by increasing CAT and GSH activity (54). In the brains of experimental animals with streptococcus-induced dementia, high doses of white mulberry extract for 13 days reduced methanolic and lipid peroxidation and increased GSH activity (55). The extract of white mulberry leaves reduced MDA levels in the brain against glyphosate-induced neurotoxicity but was notable for reducing CAT and peroxidase activity (56). Neither BPA nor NP alone had any effect on GSH levels, but when administered together, they significantly reduced this level (Figure 1).

BPA increases oxidant products and suppresses antioxidant activity in the brain, while also increasing pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (57). It also increases TNF-α levels in the embryonic brain (58). Its increase not only in the brain but also in the serum can cause inflammation in many tissues (59). Furthermore, some in vitro studies have indicated that it triggers these cytokines, particularly in microglial cells (60). Similarly, NP has increased microglial activation (61). NP toxicity activates the NF-κB signaling pathway by causing oxidative damage in tissues. This leads to a significant increase in TNF-α, IL-1β, and IL-6 levels. It has been reported that early life exposure, in particular, increases these cytokines in the prefrontal cortex via the gut-brain axis (62). White mulberry is rich in bioactive

compounds such as rutin, quercetin, chlorogenic acid, and morucin. Pharmacological studies have revealed that these compounds reduce the gene expression (at the mRNA level) of TNF-α and IL-6 by blocking the NF-κB (Nuclear factor kappa B) signaling pathway (63, 64). In our study, BPA exposure alone did not affect cytokines. However, when administered with NP, it increased all three types of cytokines. NP alone showed a result parallel to previous studies, particularly increasing TNF-α and IL-1β cytokines in the brain. In our study, white mulberry confirmed its anti-inflammatory properties against BPA+NP by significantly inhibiting the cytokine storm (Figure 2).

In our study, the combined administration of BPA and NP triggered a pronounced synergistic neurotoxicity, evidenced by severe lipid peroxidation and a robust cytokine storm characterized by elevated TNF-α, IL-1β, and IL-6 levels. While both substances individually disrupt redox homeostasis, their co-exposure likely exerts converging destructive effects on intracellular targets, leading to exacerbated neuronal degeneration and perineuronal edema. The original contribution of the present study lies in demonstrating the specific protective capacity of Morus alba extract against this synergistic endocrine-disruptor exposure in brain. While previous literature highlights the extract's efficacy against single toxic agents, our findings uniquely reveal that its rich bioactive profile is potent enough to partially reverse the amplified neuroinflammatory cascade and oxidative stress caused by the specific combination of BPA and NP.

Studies on the histopathological effects of BPA and NP in brain tissue have revealed some damaging properties of these two substances. BPA can cause significant pathological changes, particularly in neurons, such as hydropic degeneration, single-cell necrosis (apoptosis), increased glial cell count, neuronophagia, perineuronal/neuropil edema, endothelial cell swelling in blood vessels, hypersemia, hemorrhage in meninges and neuropil tissues, infiltration of mononuclear cells (MNCs), and marked vascular hyperemia (65). Rats exposed to NP may show areas of neuronal degeneration, vacuolation, and cytoplasmic shrinkage in the brain cortex (66). MA extract is known to reduce vacuolation areas and increase regular neuronal areas in response to streptozotocin-induced brain damage (67). It can also prevent loss of midbrain cells and reduce neurodegeneration in rotenone-induced damage (54). Overall, our results support the literature, showing that while BPA and NP damage brain histology, MA extract can relatively normalize this situation (Figure 3).

In conclusion, our preclinical findings demonstrate that white mulberry extract has a mitigating effect on MDA levels induced by BPA and NP exposure. The absence of significant changes in GPx levels may be related to the exposure duration; a 30-day period might be insufficient to observe major alterations in this specific enzyme. The combined exposure to BPA and NP exacerbated neurotoxicity by synergistically increasing inflammatory markers (TNF-α, IL-1β, IL-6) and causing tissue damage, while simultaneously suppressing the antioxidant activity of GSH and CAT.

In response, *Morus alba* extract exhibited a promising neuroprotective potential by attenuating neurodegeneration, largely driven by its anti-inflammatory properties. Although its restorative impact on antioxidant enzymes (GSH, GPx, CAT) was not fully realized within this study's timeframe, the significant reduction in lipid peroxidation is an encouraging outcome. It is important to interpret these findings with caution; while these results suggest a therapeutic potential for MA extract against environmental EDC exposure, direct clinical applications cannot be extrapolated without further evidence. Future studies focusing on extended exposure times, specific intracellular pathways, gene/protein expressions, and long-term behavioral tests are essential to fully elucidate its mechanism of action and translational clinical viability.

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