Ind. J. Pharm. Edu. Res., 2026; 60(4):1730-1735.
https://www.ijper.org

Original Article

Evaluation of the Protective Efficacy of *Nigella sativa* Oil with Some Hematological Parameters and ANAE Positive Lymphocyte Ratio in Bisphenol A Toxicity

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ABSTRACT

Background and Aim: Bisphenol A (BPA) est chemicum endocrinum perturbans, late in variis productis consumptoriis adhibitum, quod per systemata endocrinum et immunologicum nocet valetudini. Huius studii propositum erat explorare effectus expositionis ad BPA in parametris hematologicis et responsione immunitaria in muribus Wistar Albino, simulque aestimare potentiam protectivam olei seminis *Nigella sativa* (*N. sativa*), noti ob proprietates antioxidanticas et antiinflammatorias. **Materials and Methods:** In hoc studio, triginta mures masculi Wistar Albino in quattuor coetus divisi sunt: Control, BPA, N. sativa, et BPA+N. sativa. Per quattuor hebdomadas experimenti, coetibus BPA et BPAN quotidie data est dosis 100 mg/kg BPA, dum oleum essentiale *N. sativa* coetibus NS et BPAN per os datum est ad dosim 5 mL/kg. Post tempus curationis, parametri hematologici quidam et proportio lymphocytorum ANAE-positivorum sunt examinati. **Results and Discussion:** Expositio ad BPA significanter reduxit numerum erythrocytorum, gradum hemoglobini, valorem hematocriticum, numerum thrombocytorum et proportionem lymphocytorum ANAE-positivorum, ducens ad anemia et debilitamentum systematis immunis. In coetu BPAN, hi parametri valores maiores ostenderunt comparati ad coetum BPA, indicantes meliorationem. **Conclusion:** Inventiones studii suggerunt oleum *N. sativa* posse habere effectum protectivum contra toxicitatem inductam a BPA, etiam antequam apparent symptomata clinica. Observatae sunt emendationes in parametris hematologicis, quae indicant potentiam huius plantae in praeventione damni immunologici et hematologici.

Keywords: Bisphenol A (BPA), Endocrine Disrupting Chemicals, *N. sativa*, Hematological Parameters, ANAE-positive.

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Received: 06-04-2026;
Revised: 11-06-2026;
Accepted: 24-07-2026.

INTRODUCTION

BPA, an endocrine-disrupting chemical, is considered an alarming environmental pollutant due to its widespread use worldwide.[1,2] Initially produced as a synthetic estrogen to promote rapid growth in poultry and cattle, BPA is now manufactured in quantities exceeding 2 million tons annually.[3] It is a primary monomer used in the production of fissure sealants and composite resin filling materials in dentistry, Polycarbonate plastics (PC), and epoxy resins, as well as in products such as baby bottles, food storage containers, water bottles, bottle caps, eyeglass lenses, Compact Discs (CDs), Digital Video Discs (DVDs), plastic, nylon, polyester, Polyvinyl Chloride (PVC), and electronic devices.[4] Although contamination can occur through skin contact or inhalation of household dust, BPA primarily enters the body orally through foods contaminated by leaching from food or beverage containers or through industrial wastewater from wastewater treatment plants, petrochemical facilities, and landfill leachate seeping into water bodies.[2,4,5]

BPA is known to have effects on the endocrine system, metabolism, cardiovascular system, nervous system, and immune system, exhibiting both estrogenic and anti-androgenic properties.[5,6] There is a bidirectional and close relationship between the endocrine and immune systems. It has been reported that hormones released from the gonads have a significant impact on the thymus gland and other lymphoid organs. Studies have shown that estrogen administration causes involution of the thymus; removal of the thymus in the neonatal period leads to pathological disorders in the development of the ovaries and testes. Although the biochemical mechanism underlying the bidirectional interaction between these two systems is not yet

DOI: 10.5530/ijper.20263488

fully understood, cytokines are believed to play a crucial role in this interaction.[7,8]

Macrophages are key cells involved in many inflammatory and infectious diseases, such as asthma, type 2 diabetes, obesity, cardiovascular diseases, and cancer.[9] Opinions regarding the effects of BPA on macrophage activation vary. It has been reported that BPA induces the expression of proinflammatory cytokines, including Interleukin-1 beta (IL-1β), Tumor Necrosis Factor alpha (TNFα), and Interleukin-6 (IL-6), in human macrophages, Tamm-Horsfall Protein-1 (THP-1 cells), and fish macrophages.[10]

Alpha-Naphthyl Acetate Esterase (ANAE) is acquired during the maturation of T lymphocytes and is present in lysosomes. It is used to distinguish between T and B lymphocytes in various animal species, including rats, mice, and chickens.[11-16] Previous experimental studies have demonstrated that BPA decreases the proportion of ANAE-positive peripheral blood lymphocytes in rats and chickens, indicating an immunotoxic effect on T lymphocytes.[17,18]

Traditional or complementary medicine has been witnessing a steady rise in popularity, often being favored over conventional pharmaceuticals. Among these alternatives, *N. sativa*, a plant from the Ranunculaceae family commonly referred to as black seed or black cumin stands out for its therapeutic potential. *N. sativa* is frequently used in culinary and medicinal applications. Its oil and seeds are widely utilized, especially in Eastern medicine, for their diuretic, antihypertensive, antidiabetic, anticancer, immunomodulatory, analgesic, antimicrobial, anthelmintic, anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective, and antioxidant and therapeutic properties.[19,20]

In the present research, the potential impact of *N. sativa* seed oil recognized for its potent antioxidative capacity and pronounced anti-inflammatory activity was evaluated in terms of selected hematological indices and the proportion of ANAE-reactive lymphocytes, in response to Bisphenol A (BPA), a widely studied endocrine disruptor that significantly contributes to metabolic dysregulation and poses substantial threats to both human and ecological health.

Materials and methods

Materials

In this experimental study, a total of 30 healthy male Wistar Albino rats, aged between 6 and 8 weeks and exhibiting comparable body mass, were selected for use. Animal care and maintenance were carried out at the Experimental Medicine Research and Application Center of Selçuk University, where all procedures adhered to ethical and standardized conditions. Throughout the study duration, optimal environmental parameters including regulated temperature, humidity, and lighting were consistently maintained. The rats were individually housed in transparent polycarbonate cages under a controlled 12-hr light/12-hr dark photoperiod, with ambient room temperature stabilized at 23±2°C and relative humidity maintained within %50±10. Standardized pellet-based rat chow and fresh potable water (approximately 50 mL per rat per day) were made available ad libitum to ensure uninterrupted access to nutrition and hydration.

The experimental protocol received official approval from the Experimental Animals Ethics Committee of Selçuk University, affiliated with the Experimental Medicine Research and Application Center, on July 30, 2021, in accordance with ethical decision number 2021-44.

The rats used in the study were divided into four groups: Control (C), BPA, *N. sativa* (NS), and BPA+*N. sativa* (BPAN). The control group received 1 ml of olive oil orally for four weeks during the trial. The rats in the second and fourth groups were administered BPA (Sigma, Catalog No: 133027) at a dose of 100 mg/kg body weight orally for the same period. BPA was initially dissolved in ethanol and then mixed with olive oil to obtain a final ethanol concentration of %0.5 in the vehicle. This preparation method is consistent with previously described BPA administration protocols in experimental studies.[21,22] The rats in the third and fourth groups were given 5 mL/kg *N. sativa* seed oil (Botalife) orally for 30 days. The dose of *N. sativa* seed oil (5 mL/kg) was selected based on previous rodent studies demonstrating its safety and protective efficacy against chemically induced toxicity.[34,47]

Upon completion of the experimental protocol, blood specimens were obtained from each animal under general anesthesia (thiopental sodium, 40 mg/kg) via cardiac puncture and collected into tubes containing the anticoagulant Ethylenediaminetetraacetic Acid (EDTA). The collected samples were analyzed to assess key hematological parameters, including total Leukocyte (WBC), Erythrocyte (RBC), Hemoglobin (HGB), Hematocrit (HCT), Platelet (PLT) counts, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC), along with differential leukocyte distributions. The analyses were performed using a fully automated hematology analyzer (Mindray BC800).

Alpha-Naphthyl Acetate Esterase Demonstration

The proportion of ANAE-positive lymphocytes was quantified from peripheral blood smears using previously described cytochemical methods.[13] For cytochemical evaluation, two blood film preparations were generated from each specimen. These films were left to air dry at room temperature and subsequently stabilized in a cold glutaraldehyde–acetone fixative (−10°C, pH 4.8) for 3 min. Post-fixation, the slides were incubated in 80 mL of phosphate buffer (pH 5.0) supplemented with 0.8 mL of acetone (Merck) and 20 mg of fully solubilized alpha-naphthyl acetate (N-8505, Sigma). Following this, a reagent mixture composed of 2.4 mL of %4 sodium nitrite (S-3421, Merck) and 2.4 mL of

pararosaniline solution (prepared by combining 1 g pararosaniline (P-3750, Merck), 20 mL distilled water, and 5 mL concentrated HCl) was reacted for 2 minutes to yield the hexazotized pararosaniline complex, which was then added to the incubation medium. The final working solution was pH-adjusted to 5.8 using 1N NaOH and filtered prior to a controlled 2-hr incubation. The presence of reddish-brown cytoplasmic granules marked a positive enzymatic reaction. After stopping the reaction, the slides were washed thrice with distilled water and counterstained using Giemsa. ANAE-reactive lymphocyte percentages were quantified by examining 200 cells per slide under ×100 magnification with a Leica DM2500 light microscope (Switzerland).

All hematological and ANAE-related parameters were measured only at the end of the 4 week exposure period because blood sampling was done by terminal cardiac puncture under general anesthesia. To avoid repeated invasive procedures and in accordance with Ethics Committee approval and the 3R principles, no intermediate time points were included.

Statistical Analysis

Statistical evaluation of the experimental data was performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL). To compare differences among groups, one-way Analysis of Variance (ANOVA) was employed, followed by Duncan's *post hoc* test for multiple comparisons. A p-value of ≤ 0.05 was considered statistically significant in all analyses.

RESULTS

Tables 1 and 2 display the evaluated hematological profiles, including leukocyte subpopulation distributions and the proportion of ANAE-reactive lymphocytes, as assessed from blood samples collected upon completion of the experimental procedures in the respective study groups.

DISCUSSION

The haematopoietic system in animals and humans is the most sensitive system for evaluating environmental toxins and drugs. Due to their versatility and high sensitivity, haematological and biochemical analyses are often used to assess human and animal health for prognosis or diagnosis purposes. Blood parameters serve as important targets for any chemical exposure and can predict toxicity as the earliest clinical signs.[15,23]

One of the most prominent hematotoxic consequences of Bisphenol A (BPA) exposure is its potential to induce anaemia. Experimental studies in animal models have demonstrated that BPA-related toxicity triggers oxidative disturbances within the bone marrow environment, resulting in profound alterations in both cellular morphology and physiological function.[24] According to Tiwari and Vanage, BPA exposure elevates oxidative pressure in marrow tissue, which adversely affects hematopoietic processes by interfering with core metabolic pathways and inflicting structural damage at the cellular level. As a result, erythroblastic precursors exhibit reduced membrane integrity and diminished lifespan.[25] BPA-induced alterations in bone marrow hematopoietic dynamics have been associated with suppressed proliferation and distribution of erythroid precursors.[26] This condition ultimately manifests as anaemia, attributed to a marked decline in erythrocyte concentration and volume, primarily due to impaired maturation of red blood cells an effect likely linked to the suppressed synthesis of erythropoietin, predominantly secreted by renal tissue.[27]

In the present study, it was observed that the erythrocyte count, haemoglobin level, haematocrit value, and platelet count were significantly lower in rats subjected to BPA toxicity compared to the C and N groups, this decrease was less in the BPAN group (Table 1). In the BPAN group, Hb increased from 11.18 g/dl in the BPA group to 12.75 g/dl, moving towards the control level of 14.11 g/dL. This shows that roughly half of the Hb reduction caused by BPA alone was reversed. Consistent with the findings of previous studies, the data obtained from this study indicate that BPA significantly reduces haematological parameters and causes anaemia.[3,24,28-30]

It has also been reported in other studies[31-33] that BPA causes a significant decrease in RBC count, Hb, and PCV levels. Pal *et al.* (2017) state that BPA leads to a significant reduction in RBC count due to hemolysis in peripheral blood and an increase in the incidence of heart disorders in rats. The researchers also note a decrease in hemoglobin levels, indicating the progression of anemia. Snarska *et al.* (2018) also report similar findings in their studies, highlighting that BPA causes anemia by reducing HGB, MCH, and RBC concentrations, which in turn leads to hypoxia. Similarly, Hoque *et al.* (2020) found a decrease in Hb, PCV, RBC, and lymphocyte counts in mice exposed to BPA.

A decline in circulating erythrocytes following BPA toxicity may reflect compromised erythropoietic activity. In a study where *N. sativa* oil was administered to mitigate the toxic effects of BPA, it was reported that black cumin oil significantly increased Hb, RBC, and PCV levels and decreased MCH and MCHC levels.[34] In another study where BPA and *N. sativa* oil were administered together, *N. sativa* oil was found to improve Hb, RBC, PCV, MCV, MCHC, and MCH in the BPA group.[35] In our study, it was also observed that *N. sativa* oil ameliorated some hematological parameters compared to the BPA group (Table 1). The positive effect of N. *sativa* oil on RBC, Hb, and PCV is thought to be due to its potent antioxidant properties. N. *sativa* is considered a strong systemic radical scavenger. It has also been reported that N. *sativa oil* can increase erythrocyte count and hemoglobin concentration by stimulating the bone marrow and enhancing cellular respiration mechanisms.[36,37]

It is known that estrogen receptors are present in many cells of the immune system, especially in lymphocytes.[38] Estrogen exerts

its biological effects through Estrogen Receptor-α (ERα) and -β (ERβ).[39] BPA has a low affinity for binding to ERβ in target cells. It has the ability to mimic, enhance, or inhibit the effects of estrogen.[40,41]

Environmental xenoestrogens, such as BPA, are hypothesized to modulate immune responses by disrupting cytokine signaling pathways. A substantial body of research has been devoted to elucidating the immunomodulatory and estrogen-mimicking properties of bisphenol A. It has been reported that estrogen plays an important role in the immune system, exhibiting a stimulatory effect on humoral immunity while having a suppressive effect on cellular immunity, and also influencing cytokine production and the distribution of T lymphocyte subtypes.[39] It has also been reported that significant morphological and functional changes are observed in peripheral blood lymphocytes during BPA toxicity, leading to weakened immune processes under certain conditions.[42,43] In a study, it was noted that WBC and neutrophil counts increased significantly, while lymphocyte count decreased in rats exposed to BPA.[44] Similarly, Nduonofit *et al.* (2023) reported an increase in WBC and leukocyte subtypes in rats exposed to BPA for 6 weeks. The researchers explained the observed increase in BPA-exposed groups as a defense mechanism initiated in response to stress caused by this chemical. In our study, an increase in total WBC and neutrophil counts was observed in the BPA-treated group, while lymphocyte count decreased (Tables 1 and 2). Our findings support these previous studies. The increase in white blood cells and neutrophil percentage detected may be related to stress conditions induced by the inflammatory response to BPA. Thus, the increase in WBC and neutrophils in the BPA group most likely reflects a non-specific inflammatory or stress response. At the same time, the reduction in ANAE-positive T lymphocytes points to impaired adaptive immune function, showing that higher neutrophil counts and reduced immune competence can coexist. In the BPAN group, it was observed that the data improved and approached the control group values due to the anti-inflammatory and antioxidant effects of NS (Tables 1 and 2).

It has been observed that cytokine and antibody levels produced in cells decrease as a result of BPA exposure.[45] Various studies have reported that the different effects of BPA on the immune system depend on several factors such as gender, dose, duration, age, and the methods used in BPA administration.[46] In their study, Huang *et al.* (2019) reported that BPA stimulates the production of IL-1β, TNFα, and IL-6. Based on their findings, the researchers suggest that BPA is a strong activator or enhancer of inflammation. In a study conducted on humans exposed to endocrine-disrupting chemical-containing pesticides, an increase in CD4+ and CD8+ lymphocyte ratios and the formation of autoantibodies was reported.[47] Sugita-Konishi *et al.* (2003) reported in their study on mice that BPA has an immunotoxic effect, leading to a decrease in macrophages and T cells in the spleen. Tian *et al.* (2003) also suggested that BPA may induce allergic diseases by stimulating IL-4 production from Th2 cells.

Studies have been conducted on the effects of BPA, considered an estrogen receptor binder, on antigen-specific antibody production, lymphoid cells, and the development of Th1 and

Table 1: Hematological parameters compared among experimental groups, reported as arithmetic means along with Standard Error Margins (± SEM).

Parameters	C	N	BPA	BPAN
WBC(x10^3/mm^3)	5.26 ±0.40^b	5.68 ±0.26^b	8.25 ± 0.54^a	7.02 ± 0.43^a
RBC (x10^6/mm^3)	7.63±0.16^a	7.60±0,33^a	5.85 ± 0.60^b	6.77 ± 0.40^{ab}
HGB (g/dl)	14,11 ±0,35^a	14,50 ±0,53^a	11,18 ±1,12^b	12,75 ± 0,63^{ab}
HCT (%)	43.80 ±1.55^a	45.40 ±0.85^a	34.88 ±3.87^b	39.33 ± 2.07^{ab}
PLT (L)	736.00±40.15^a	712.12±16.86^a	507.66±78.51^b	609.16±45.64^{ab}
MCV (μ^3)	57.33 ±1.33	60.08 ±1.80	59.18 ±2.28	58.23 ±1.41
MCH (pg)	18.48 ±0.48	19.30 ±1.34	19.76 ±2.02	19.06 ± 1.25
MCHC (%)	32.35± 1.33	31.93 ±1.26	33.53 ± 3.51	32.60±1.007

a, b: Different superscript letters within a row indicate statistically significant differences between groups ($p< 0.05$). C: control; N: *N. sativa*; BPA: bisphenol A; BPAN: BPA + *N. sativa*.

Table 2: Distribution of leukocyte subtypes and proportion of ANAE positive lymphocytes (%) across experimental groups, presented as Mean ± SE.

Parameters (%)	C	N	BPA	BPAN
Lymphocyte	56.68 ± 3.17^a	57.03 ± 3.14^a	34.51 ± 4.66^b	42.38 ± 4.46^b
Monocyte	5.08 ± 1.13^a	4.21 ± 0.55^{ab}	2.53 ± 0.23^b	3.70 ± 0.42^{ab}
Neutrophile	35.87 ± 2.35^c	36.35 ± 2.03^c	60.54 ± 2.30^a	51.91 ± 3.76^b
Eosinophile	1.53 ± 0.44	1.55 ± 0.35	1.35 ± 0.42	1.05 ± 0.21
Basophile	0.85 ± 0.63	0.86 ± 0.48	1.05 ± 0.15	1.03 ± 0.14
ANAE positivity	60.00 ± 3.20^a	58.16 ± 2.05^a	47.66 ± 2.44^b	57.50 ±1.08^a

a, b, c: Different superscript letters within a row indicate statistically significant differences between groups ($p < 0.05$).

Th2 responses in mice, as well as on thymus development and peripheral blood ANAE-positive lymphocyte ratios in chickens administered BPA in eggs.[48,49] Yoshino *et al.* (2003) reported that increased duration and dose of BPA administration in mice led to an increase in the production of IgG1, IgG2a, IFN-γ, and IL-4. Yılmaz and Öznurlu (2019) found that BPA injected into eggs decreased the lymphoid cell density in the thymus and caused significant reductions in peripheral blood ANAE-positive lymphocyte ratios in chickens. In this study, BPA administration was also found to significantly ($p<0.05$) reduce ANAE-positive lymphocyte ratios. This reduction may be attributed to a decrease in relative T lymphocyte counts resulting from B lymphocyte activation. Talebi *et al.* (2021) demonstrated that *N. sativa* oil administration enhances the humoral immune response in broilers. In this study, it was found that *N. sativa oil* alone (%58.16) had no effect on ANAE positivity; however, *N. sativa* oil administered together with BPA (%57.5) significantly ($p<0.05$) increased ANAE-positive lymphocyte counts compared to the BPA group, with ANAE-positive lymphocyte ratios similar to those in the control group (%60) (Table 2). The similarity of ANAE-positive lymphocyte ratios in the BPAN group to the control group may suggest that *N. sativa* oil mitigates the negative effects of BPA on T lymphocytes.[50]

CONCLUSION

The immunological effects of N. sativa have been demonstrated in numerous studies. It is known that N. sativa increases the weight of lymphoid organs, enhances WBC production, and improves cell-mediated immune responses and antibody titers.[43] The positive effects of N. sativa on the parameters examined in rats treated with BPA are likely due to its potent antioxidant and anti-inflammatory properties. The beneficial effects of *N. sativa* oil on hematological parameters may be attributed to its ability to block oxidative damage in DNA, reduce glycation of membrane proteins, decrease lipid peroxidation, and inhibit certain enzymes, thereby preventing free radical formation.[51] The findings of this study also suggest that exposure to BPA poses a potential threat to the immune system by decreasing Hb concentration, RBC, and Hct levels and leading to the development of anemia, while *N. sativa* oil administration appears to exhibit protective or mitigating effects against the adverse effects of BPA toxicity.

ABBREVIATIONS

BPA: Bisphenol A; **N. sativa:** *Nigella sativa*; **BPAN:** BPA+*N. Sativa*; **NS:** *Nigella sativa*; **PC:** Polycarbonate; **CDs:** Compact Discs; **DVDs:** Digital Video Discs; **PVC:** Polyvinyl Chloride; **IL-1β:** Interleukin-1 beta; **TNFα:** Tumor Necrosis Factor alpha; **IL-6:** Interleukin-6; **THP-1 cells:** Tamm-Horsfall Protein-1; **ANAE:** Alpha-naphthyl acetate esterase; **C:** Control, **EDTA:** Ethylenediaminetetraacetic acid; **WBC:** White Blood Cell (Leukocyte); **RBC:** Red Blood Cell (Erythrocyte); **HGB:** Hemoglobin; **HCT:** Hematocrit; **PLT:** Platelet; **MCV:** Mean Corpuscular Volume; **MCH:** Mean Corpuscular Hemoglobin; **MCHC:** Mean Corpuscular Hemoglobin Concentration; **Erα:** Estrogen receptor-α; **Erβ:** Estrogen receptor-β; **IL-4:** Interleukin-4; **Th1:** Type 1 T helper cells; **Th2:** Type 2 T helper cells; **IgG1:** Immunoglobulin G1; **IgG2a:** Immunoglobulin G2a; **IFN-γ:** Interferon gamma.

CONFLICT OF INTEREST

No actual or potential conflicts of interest have been identified by the authors with respect to the research, authorship, or publication of this article.

SUMMARY

The objective of this study was to assess the hematotoxic and immunosuppressive effects of BPA and to evaluate the protective efficacy of *N. sativa* seed oil in Wistar Albino rats. BPA was administered orally at 100 mg/kg/day for 4 weeks, either alone or in combination with N. sativa oil (5 mL/kg/day). Hematological indices (RBC, Hb, HCT, PLT) and ANAE-positive lymphocyte ratios were measured to determine toxicological and immunological effects. BPA exposure significantly reduced erythrocyte count, hemoglobin concentration, hematocrit, platelet count, and ANAE-positive lymphocyte levels. These alterations were partially restored in the group co-treated with N. sativa oil, indicating a potential mitigating effect. This study suggests that N. sativa may provide hematoprotective and immunomodulatory support against BPA-induced toxicity. Further studies with standardized oil composition and extended immunological assays are recommended to confirm these findings.

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Cite this article: Özsan M, Tekdemir I, Dönmez N, Aydın E, Dönmez HH. Evaluation of the Protective Efficacy of *Nigella sativa* Oil with Some Hematological Parameters and ANAE Positive Lymphocyte Ratio in Bisphenol A Toxicity. Indian J of Pharmaceutical Education and Research. 2026;60(4):1730-5.