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Evaluation of the efficacy of ethanol extract from black shallot (*Allium ascalonicum*) in reducing oxidative stress and modulating immune response in a paracetamol-induced nephrotoxicity mouse model

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Abstract The protective effects of ethanol extract from black shallot (ESBA) and N-acetylcysteine (NAC) against paracetamol (PARA)-induced nephrotoxicity in mice were investigated. Notable changes in body weight, relative kidney weight, and kidney structure were observed, with the normal group exhibiting the highest body weight (40.34 ± 0.42 g) ($p < 0.05$) and the lowest relative kidney weight (28.61 ± 0.23%) ($p < 0.05$). In contrast, the PARA group showed reduced body weight (27.66 ± 0.46 g) ($p < 0.05$) and increased relative kidney weight (68.99 ± 0.82%) ($p < 0.05$). Treatment with NAC and ESBA improved these parameters, with the PARA+NAC and PARA+ESBA400 groups approaching normal values. Furthermore, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels were significantly elevated in the PARA group ($p < 0.05$), indicating oxidative stress, while NAC and ESBA supplementation effectively reduced these levels across all tissues ($p<0.05$). Analysis of glutathione (GSH) and total antioxidant capacity (TAC) revealed higher levels in the normal group compared to the PARA group ($p < 0.05$), with NAC and ESBA restoring GSH and TAC levels ($p < 0.05$). Antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were also significantly improved with treatment ($p < 0.05$). Additionally, immunomodulatory effects were observed, as evidenced by changes in white blood cell count, nitroblue tetrazolium positivity, and total immunoglobulin levels ($p < 0.05$). ESBA treatment notably restored immune function by decreasing inflammation markers and enhancing phagocytic activity ($p < 0.05$). These findings suggested that NAC and ESBA exhibit protective effects against PARA-induced nephrotoxicity through antioxidant and immunomodulatory mechanisms.

Keywords: Black shallot, Immune modulation, Nephrotoxicity, Oxidative stress, PARA-induced toxicity

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Introduction

Paracetamol (PARA) is a commonly used analgesic and antipyretic for managing headaches, muscle pain, and fever. While safe at recommended doses, misuse or prolonged use can lead to significant toxicity, particularly in the liver and kidneys (Freo *et al*., 2021). PARA toxicity primarily arises from the metabolism of N-acetyl-p-benzoquinone imine (NAPQI) (Luo *et al*., 2023). Normally, NAPQI is detoxified by glutathione. However, in cases of overdose, excess NAPQI accumulates in kidney cells, depleting glutathione and generating free radicals, damaging cell membranes, initiating lipid peroxidation, and harming proteins and DNA, leading to cell death and inflammation (Baponwa *et al*., 2022). In addition to renal toxicity, PARA overdose impairs immune function. Elevated NAPQI induces oxidative stress and renal cell death while also disrupting blood filtration and toxin elimination, triggers systemic inflammation and the release of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, weakening immune defenses (Simião *et al*., 2024). Renal damage further impairs leukocyte production and erythropoietin regulation, reducing white blood cell counts and affecting hematopoiesis. Electrolyte imbalances worsen immune dysfunction, reducing the body's ability to combat infections. This combination of inflammation and decreased renal blood flow exacerbates kidney injury (Khan *et al*., 2021). Untreated PARA toxicity can lead to acute kidney injury and chronic kidney disease, with mortality rates as high as 15% (Saad and Flament, 2024).

Developing safer and more effective therapies to treat PARA-induced nephrotoxicity is crucial, as current treatments primarily address symptoms rather than preventing long-term damage (Rotundo and Pyrsopoulos, 2020). Oxidative stress and immune suppression are key contributors to severe kidney damage and functional decline (Gyurászová *et al*., 2020). Existing treatments, such as N-acetylcysteine (NAC), can neutralize toxins but also cause unwanted side effects (Ye *et al*., 2021). Therefore, new therapies should focus on comprehensively targeting oxidative stress and modulating immune responses. Traditional approaches often utilize herbs and natural compounds rich in antioxidants to neutralize free radicals and alleviate oxidative stress. Extracts from turmeric (*Curcuma longa*), green tea (*Camellia sinensis*), and raspberry (*Rubus alceaefolius*) have shown promise in protecting kidney cells from oxidative damage (Muscolo *et al*., 2024).

Among promising candidates, black shallot (*Allium ascalonicum*) stands out due to its high content of bioactive compounds, such as flavonoids, saponins, and sulfur-containing compounds, which have potent free radical-scavenging properties (Tran and Ngo, 2023). Black shallots have demonstrated

effectiveness in alleviating pain, fever, and inflammation in models induced by formalin, acetic acid, yeast, and carrageenan (Nhung and Quoc, 2024b), and demonstrate anti-breast cancer activity in DMBA models (Tran and Tran, 2024). Notably, they offer protection against PARA-induced liver and kidney toxicity, underscoring their potential as protective agents for these organs (Nhung and Quoc, 2023, 2024a). Furthermore, black shallots possess antioxidant and anti-inflammatory properties that reduce oxidative stress and protect the kidneys. Their bioactive compounds neutralize free radicals and inhibit pro-inflammatory cytokines and enzymes like cyclooxygenase (COX) and lipoxygenase (LOX), thus reducing inflammation, promoting kidney tissue regeneration, and limiting tubular necrosis (Nhung and Quoc, 2023). Building on previous studies of PARA-induced nephrotoxicity and the limitations of current treatments, the research aimed to evaluate the effectiveness of black shallot ethanol extract in reducing oxidative stress and immune dysfunction in PARA-induced kidney injury in mice.

Materials and methods

Chemicals and reagents

To evaluate the efficacy of ethanol extract from black shallot (*A. ascalonicum*) in a PARA-induced nephrotoxicity mouse model, key reagents are required such as paracetamol (Sigma-Aldrich, Merck) induce nephrotoxicity, and ethanol (Sigma-Aldrich, Fisher Scientific) extracts bioactive compounds. Antioxidant activity is measured via DPPH and SOD kits (Sigma-Aldrich), while lipid peroxidation is assessed with MDA assays (Sigma-Aldrich, Abcam). Histology uses formalin and H&E staining (Sigma-Aldrich, Merck). PBS, DMSO, and NaCl are sourced from Gibco (Thermo Fisher) or Merck, ensuring reliable results.

Collection of material and preparation of the extract

Shallot samples (*Allium ascalonicum*) were collected in March 2024 from Vinh Chau Town, Soc Trang Province, Vietnam, and processed at the Biotechnology Labomiceory of the Industrial University of Ho Chi Minh City under voucher number AS140324VST. Following collection, black shallots were rigorously selected for quality, with only healthy, intact bulbs of uniform size and shape retained. Fermentation was conducted using the KS-10 Fermentation Box (Kenshin, Japan), following the method of Nhung and Quoc (2023). Specifically, shallots were fermented at 80 °C and 70% relative

humidity for 21 days, yielding a characteristic dark brown or black product. After fermentation, the bulbs were dried at 40 °C in a Memmert oven (Germany) under controlled conditions to preserve bioactive compounds, while avoiding direct sunlight to prevent degradation. The final powder was stored in airtight, opaque containers at room temperature, protected from moisture and light to ensure stability and prevent oxidation for future analysis.

The ethanol extraction process of black shallot, referred to as ESBA followed these steps: First, 200 g of finely ground ESBA powder was weighed and soaked in 2000 mL of 70% ethanol to ensure efficient extraction of bioactive compounds. The mixture was then stirred continuously at room temperature for 48 hours to maximize compound solubility. After extraction, the solution was filtered using Whatman No. 4 filter paper to remove solid residues. The filtrate was concentrated under reduced pressure using a rotary evaporite (Rotavapor® R-300, Büchi, Switzerland) at 40 °C to evaporite the ethanol, yielding the crude extract. This ESBA was then stored in airtight containers at 4 °C until further use in experimental analysis.

Qualitative and quantification phytochemical analysis of bioactive compounds in extract

Phytochemical screening was conducted to qualitatively identify bioactive compounds such as alkaloids, flavonoids, saponins, terpenoids, cardiac glycosides, phenolics, and tannins in ESBA. The process followed methods established by Tran *et al*. (2023), detailed in Table 1. The presence or absence of these compounds was determined through specific chemical reactions. Indicators such as color changes, precipitate formation, or fluorescence indicated the presence of a compound. In the absence of such reactions, the compound was considered absent. Results are typically presented in a table, using "+" for presence and "-" for absence.

The quantification of polyphenols, flavonoids, saponins, and tannins in ESBA was carried out using methods outlined by Tran and Tran (2021) and Nhung and Quoc (2024c). The determination of these compounds was based on standard calibration curves, with concentrations calculated using specific equations.

Experimental animals

Swiss albino mice, weighing 30–32 g, were purchased from the Pasteur Institute in Ho Chi Minh City. The mice were housed in glass cages with wood shavings as bedding, which were treated with microbial products to control

odor. Before the experiment, the animals were acclimatized for 7 days at the animal facility in District 12, Ho Chi Minh City, under standard conditions (temperature 25 ± 2 °C, humidity 55–60%, and a 12-hour light/dark cycle). During this period, the mice were provided with specialized rodent feed and RO-filtered drinking water. All experimental procedures were conducted by the Australian Guidelines for the Prevention and Control of Infection in Healthcare (2019).

Experimental design

In this experiment, 30 mice were randomly divided into six groups and treated over 14 days to evaluate the effects of PARA and various treatment interventions. Except for the normal control group, all other groups received a single dose of PARA (3 g/kg) (Nhung and Quoc, 2023). Specifically, the normal control group was administered saline (5 mL/kg) daily without PARA exposure. The negative control group (PARA group) received a dose of PARA (3 g/kg) followed by daily saline (5 mL/kg). The positive control group (PARA+NAC group) was also given PARA (3 g/kg). Still, it was subsequently treated with N-acetylcysteine (NAC) (50 mg/kg) daily to assess the protective effect of NAC. The three treatment groups, ESBA200, ESBA300, and ESBA400, were administered PARA (3 g/kg) followed by daily treatment with ESBA at 200 mg/kg, 300 mg/kg, and 400 mg/kg, respectively, the treatment period lasted 14 days. On the final day of the experiment, all animals were sacrificed to collect samples for biochemical and histological analysis, enabling the evaluation of PARA-induced effects and the efficacy of the different therapeutic interventions.

Body and relative kidney weight

Body and relative kidney weight are key indicators for assessing kidney health and function, particularly in physiological and pharmacological studies. The procedure begins with measuring experimental animals' body weight (B.W) using an electronic scale, Mettler Toledo (Switzerland). After completing experimental protocols, the animals are anatomy, and the kidneys are carefully harvested to prevent tissue damage. The kidneys are then rinsed and weighed using an electronic balance to ensure precise measurements, and the data is recorded. Relative kidney weight (RKW) is calculated as the mission of kidney weight to the animal's total body weight and is expressed as a percentage: $\text{RKW } (\%) = \frac{\text{Kidney weight}}{\text{Body weight}} \times 100$ (Dubiwak *et al*., 2022)

Evaluation of the extract's activity in reducing oxidative stress

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) determination: The determination of MDA and H_2O_2 was conducted as described by Nhung and Quoc (2024d, 2024e). Tissue samples were homogenized, followed by chemical reactions and absorbance measurements at 532 nm for MDA and 525 nm for H_2O_2. The concentrations of both compounds were calculated using their respective calibration curves.

Glutathione (GSH) and total antioxidant capacity (TAC) determination: The determination of glutathione (GSH) and total antioxidant capacity (TAC) was conducted as described by Nhung and Quoc (2024d). GSH was measured after tissue homogenization in sulfosalicylic acid and reaction with DTNB, with absorbance at 412 nm. TAC was extracted using ethanol, followed by filtration, centrifugation, and measurement using a calibration curve.

Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) determination: The determination of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) was performed as described by Nhung and Quoc (2024d, 2024e). Tissue samples were homogenized in phosphate buffer, followed by centrifugation to remove debris. CAT activity was measured by monitoring the decomposition of hydrogen peroxide, SOD by its inhibition of the superoxide anion reaction, and GPx through its reaction with hydrogen peroxide. The concentrations of all three enzymes were calculated using their respective calibration curves.

Evaluation of the extract's ability to modulate the immune response

White blood cell (WBC), nitroblue tetrazolium reduction test (NBT), and T-independent (TI) determination: The determination of white blood cells (WBC), nitroblue tetrazolium (NBT) reduction, and T-independent (TI) immune responses was conducted as outlined by Nhung and Quoc (2024f). WBCs were counted using a Neubauer chamber after staining with Natt-Herrick solution. The NBT assay assessed reactive oxygen species generation in WBCs by measuring absorbance at 620 nm following incubation with NBT solution. TI immune response was quantified using ELISA to measure serum immunoglobulin levels, with absorbance measured at 450 nm and concentrations calculated from a standard curve.

Phagocytic activity (PA) determination: Phagocytic activity (PA) was evaluated as per Nhung and Quoc (2024f). Blood samples were collected, and phagocytes were isolated via density gradient centrifugation. After incubating with opsonized fluorescent particles at 37 °C, non-phagocytosed particles were

removed, and cells were stained with trypan blue. The phagocytic index (PI) and phagocytic ratio (PR) are calculated using the formulas:

$$\text{PI (particles/cell)} = \frac{Total\ number\ of\ ingested\ particles}{Total\ number\ of\ phagocytic\ cells}$$

$$\text{PR (\%)} = \frac{Number\ of\ phagocytic\ cells\ with\ ingested\ particles}{Total\ number\ of\ phagocytic\ cells} \times 100$$

Statistical analysis

The results of this study are presented as the mean ± standard deviation (SD) to provide a clear representation of the data variability. A one-way analysis of variance (ANOVA) was employed to assess the differences between the various groups, which allows for the comparison of means across multiple groups. Following the ANOVA, Fisher's Least Significant Difference (LSD) test was applied to identify specific group differences and determine their statistical significance. A p-value of less than 0.05 was considered indicative of statistical significance. All statistical analyses were performed using Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, Virginia, USA), ensuring the robustness and reliability of the findings.

Results

Qualitative and quantitative phytochemical analysis of bioactive compounds in extracts

Result demonstrated that the ethanol extract from ESBA contains several notable phytochemical compounds, including alkaloids, flavonoids, phenolics, steroids, tannins, terpenoids, and saponins, while cardiac glycosides were undetected (Table1). The presence of compounds such as tannins (7.44 ± 0.19 mg TE/g), flavonoids (42.26 ± 1.08 mg QE/g), polyphenols (70.77 ± 1.37 mg GAE/g), and saponins (13.63 ± 0.42 mg SE/g) were significantly potential for ESBA in antioxidant activity and immune modulation.

Body and relative kidney weight

The changes in body weight, relative kidney weight, and kidney structure across groups showed the effects of PARA toxicity and the protective role of NAC and ESBA (Table 2 and Figure 1). The normal group had the highest body weight (40.34 ± 0.42 g) ($p < 0.05$) and the lowest relative kidney weight (28.61 ± 0.23%) ($p < 0.05$), indicating healthy kidneys. The PARA group had reduced body weight (27.66 ± 0.46 g) ($p < 0.05$) and increased kidney weight

(68.99 ± 0.82%) ($p < 0.05$), showing severe damage. NAC and ESBA treatments improved body weight and RKW, with PARA+NAC and PARA+ESBA400 groups nearing normal values (38.59 ± 0.37 g and 37.42 ± 0.42 g) ($p < 0.05$) and reduced RKW (31.17 ± 0.17% and 32.82 ± 0.24%) ($p < 0.05$). Kidney color, surface texture, and structure also improved, suggesting NAC and ESBA (especially at higher doses), help protect and restore kidneys affected by PARA toxicity.

Table 1. Qualitative and quantitative assessment of phytochemicals in ESBA

Plant constituents	Test	Examination	Present in ESBA	Measurement of plant compounds
Tannins	2 mL ESBA + 2 mL H_2O + 2-3 drops $FeCl_3$ (5%)	Green sediment	+	7.44 ± 0.19 (mg TE/g)
Flavonoids	1 mL ESBA + 1 mL $Pb(OAc)_4$ (10%)	Yellow hue	+	42.26 ± 1.08 (mg QE/g)
Terpenoids	2 mL ESBA + 2 mL $(CH_3CO)_2O$ + 2-3 drops conc. H_2SO_4	Dark red hue	+	-
Polyphenol	2 mL ESBA + 2 mL $FeCl_3$	Blue-green look	+	70.77 ± 1.37 (mg GAE/g)
Saponins	5 mL ESBA + 5 mL H_2O + heat	Foam forms	+	13.63 ± 0.42 (mg SE/g)
Steroids	2 mL ESBA + 2 mL $CHCl_3$ + 2 mL H_2SO_4 (conc.)	The rust-colored ring at the interface	+	-
Cardiac glycosides	2 mL ESBA + 2 mL $CHCl_3$ + 2 mL CH_3COOH	Purple to blue to green hue	-	-
Alkaloids	2 mL ESBA + a few drops of Hager's reagent	Yellow sediment	+	-

Note: The existence of phytochemicals in ESBA is indicated by (+) for present and (-) for absent.

Table 2. Effect of ESBA on body weight and relative kidney weight

Parameters	Normal group	PARA group	PARA+NAC group	PARA+ESBA200 group	PARA+ESBA300 group	PARA+ESBA400 group
B.W (g)	$40{,}34 \pm 0{,}42^{f}$	$27{,}66 \pm 0{,}46^{a}$	$38{,}59 \pm 0{,}37^{e}$	$32{,}42 \pm 0{,}42^{b}$	$34{,}71 \pm 0{,}39^{c}$	$37{,}42 \pm 0{,}42^{d}$
RKW (%)	$28{,}61 \pm 0{,}23^{a}$	$68{,}99 \pm 0{,}82^{f}$	$31{,}17 \pm 0{,}17^{b}$	$42{,}07 \pm 0{,}33^{e}$	$37{,}78 \pm 0{,}25^{d}$	$32{,}82 \pm 0{,}24^{c}$

Note: The values are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

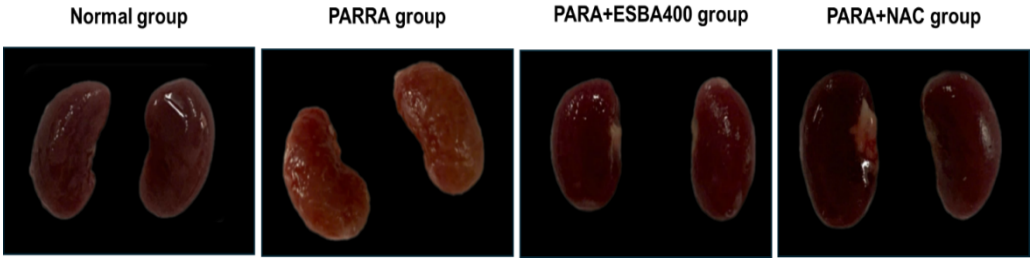

Figure 1. Effect of ESBA on the external morphology of the kidneys

Evaluation of the extract's activity in reducing oxidative stress

Results showed malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels in the liver, kidneys, and spleen, highlighting key trends as seen in Table 3. The normal group had the lowest MDA and H_2O_2 levels, indicating minimal oxidative stress, with liver values of 2.05 ± 0.06 nmol/mg protein and 1.18 ± 0.04 nmol/g tissue, kidney values of 1.37 ± 0.05 nmol/mg protein and 0.54 ± 0.05 nmol/g tissue, and spleen values of 0.48 ± 0.02 nmol/mg protein and 0.48 ± 0.02 nmol/g tissue ($p < 0.05$). In contrast, the PARA group showed significantly higher MDA and H_2O_2 levels, indicating severe oxidative stress, with liver values of 3.91 ± 0.03 and 3.33 ± 0.07, kidney values of 2.61 ± 0.01 and 2.16 ± 0.04, and spleen values of 0.91 ± 0.09 and 0.91 ± 0.09 ($p < 0.05$). NAC supplementation in the PARA+NAC group reduced MDA and H_2O_2 levels across all tissues ($p < 0.05$), with liver values of 2.26 ± 0.05 and 1.31 ± 0.07, kidney values of 1.51 ± 0.02 and 0.59 ± 0.02, and spleen values of 0.53 ± 0.05 and 0.53 ± 0.05. Similarly, the PARA+ESBA400 group showed significant reductions, particularly in the liver and kidneys, further supporting the protective effects of ESBA ($p < 0.05$).

Analysis of glutathione (GSH) and total antioxidant capacity (TAC) in the liver, kidneys, and spleen (Figures 2 and 3) revealed significant trends. The normal group had the highest levels: GSH at 8.27 ± 0.06 µmol/g (liver), 4.56 ± 0.03 µmol/g (kidneys), and 2.48 ± 0.06 µmol/g (spleen), and TAC at 70.97 ± 0.18 µmol Trolox equivalent/g (liver), 54.99 ± 0.14 µmol/g (kidneys), and 41.88 ± 0.30 µmol/g (spleen) ($p < 0.05$), indicating strong antioxidant defense. In contrast, the PARA group showed significantly lower GSH and TAC levels: GSH at 4.87 ± 0.07 µmol/g (liver) and TAC at 37.35 ± 0.07 µmol/g (liver) ($p < 0.05$), indicating oxidative stress. NAC supplementation (PARA+NAC group) significantly restored GSH (7.52 ± 0.06 µmol/g) and TAC (64.52 ± 0.43 µmol/g) in the liver ($p < 0.05$). The PARA+ESBA400 group also exhibited similar improvements, highlighting the protective effects of NAC and ESBA against oxidative stress.

Table 3. Effect of ESBA on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels

Groups	Malondialdehyde (MDA) (nmol/mg protein)			Hydrogen peroxide (H_2O_2) (nmol/g tissue)		
	Livers	**Kidneys**	**Spleens**	**Livers**	**Kidneys**	**Spleens**
Normal group	2.05 ± 0.06[a]	1.37 ± 0.05[a]	0.48 ± 0.02[a]	1.18 ± 0.04[a]	0.54 ± 0.05[a]	0.48 ± 0.02[a]
PARA group	3.91 ± 0.03[f]	2.61 ± 0.01[f]	0.91 ± 0.09[d]	3.33 ± 0.07[f]	2.16 ± 0.04[e]	0.91 ± 0.09[d]
PARA+NAC group	2.26 ± 0.05[b]	1.51 ± 0.02[b]	0.53 ± 0.05[ab]	1.31 ± 0.07[b]	0.59 ± 0.02[a]	0.53 ± 0.05[ab]
PARA+ESBA200 group	3.28 ± 0.06[e]	2.19 ± 0.06[e]	0.77 ± 0.02[c]	1.89 ± 0.01[e]	0.86 ± 0.02[d]	0.77 ± 0.02[c]
PARA+ESBA300 group	3.08 ± 0.06[d]	2.06 ± 0.05[d]	0.72 ± 0.05[c]	1.77 ± 0.04[d]	0.81 ± 0.04[c]	0.72 ± 0.05[c]
PARA+ESBA400 group	2.46 ± 0.04[c]	1.64 ± 0.06[c]	0.58 ± 0.01[b]	1.42 ± 0.05[c]	0.65 ± 0.04[b]	0.58 ± 0.01[b]

Note: The values are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

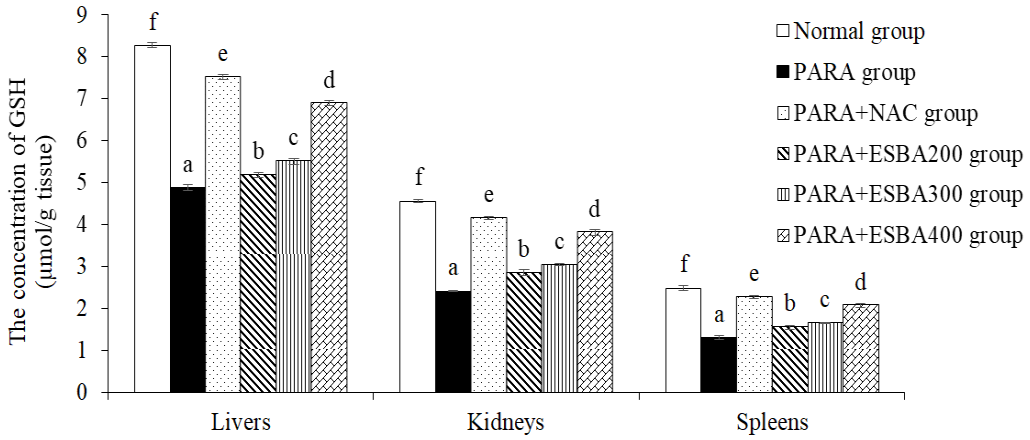

Figure 2. Effect of ESBA on glutathione (GSH) levels, Results are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

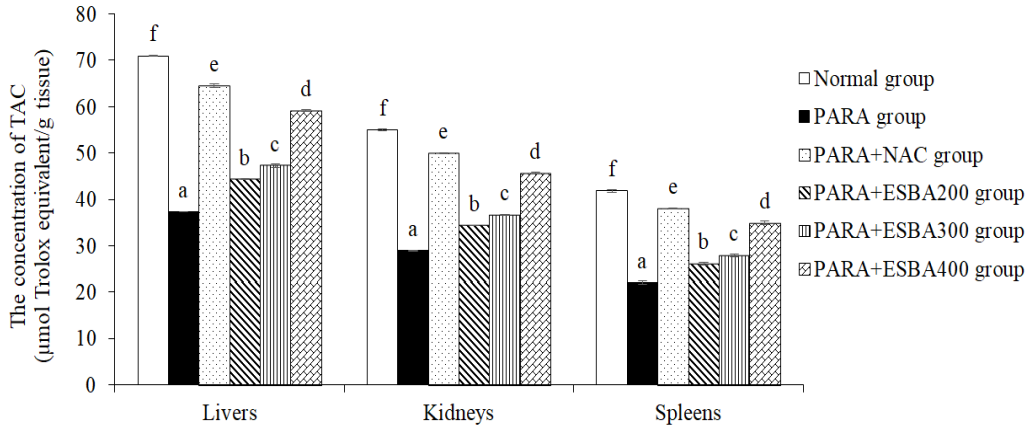

Figure 3. Effect of ESBA on total antioxidant capacity (TAC) levels, Results are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

Table 4. Effect of ESBA on superoxide dismutase (SOD), and catalase (CAT) levels

Groups	Catalase (CAT) (U/mg protein)			Superoxide dismutase (SOD) (U/mg protein)		
	Livers	Kidneys	Spleens	Livers	Kidneys	Spleens
Normal group	87.62 ± 0.03^{f}	40.75 ± 0.03^{f}	48.59 ± 0.17^{f}	34.77 ± 0.31^{f}	24.96 ± 0.16^{f}	15.89 ± 0.16^{f}
PARA group	46.12 ± 0.12^{a}	22.02 ± 0.12^{a}	25.57 ± 0.14^{a}	18.31 ± 0.07^{a}	13.14 ± 0.07^{a}	8.36 ± 0.08^{a}
PARA+NAC group	79.65 ± 0.16^{e}	38.03 ± 0.35^{e}	44.17 ± 0.09^{e}	31.61 ± 0.07^{e}	22.69 ± 0.18^{e}	14.45 ± 0.18^{e}
PARA+ESBA200 group	54.76 ± 0.17^{b}	25.47 ± 0.34^{b}	30.37 ± 0.09^{b}	21.73 ± 0.02^{b}	15.61 ± 0.09^{b}	9.93 ± 0.19^{b}
PARA+ESBA300 group	58.41 ± 0.03^{c}	27.17 ± 0.24^{c}	32.39 ± 0.03^{c}	23.18 ± 0.12^{c}	16.64 ± 0.03^{c}	10.59 ± 0.05^{c}
PARA+ESBA400 group	73.02 ± 0.30^{d}	33.96 ± 0.21^{d}	40.49 ± 0.16^{d}	28.98 ± 0.13^{d}	20.81 ± 0.11^{d}	13.24 ± 0.14^{d}

Note: The values are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

Assessment of antioxidant enzyme activities, specifically superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in the liver, kidneys, and spleen of mice showed significant differences among treatment groups (Table 4 and Figure 4). The normal group exhibited high CAT activity: 87.62 ± 0.03 U/mg (liver), 40.75 ± 0.03 U/mg (kidneys), and 48.59 ± 0.17 U/mg (spleen), indicating strong antioxidant defenses. SOD activity was also effective, measuring 34.77 ± 0.31 U/mg (liver), 24.96 ± 0.16 U/mg (kidneys), and 15.89 ± 0.16 U/mg (spleen). In contrast, the PARA group

showed significantly reduced CAT (46.12 ± 0.12 U/mg in the liver) and SOD (18.31 ± 0.07 U/mg in the liver) activities, reflecting oxidative stress from PARA. NAC supplementation in the PARA+NAC group restored CAT (79.65 ± 0.16 U/mg) and SOD (31.61 ± 0.07 U/mg) levels ($p < 0.05$). The PARA+ESBA400 group also showed improvements, with CAT at 73.02 ± 0.30 U/mg and SOD at 28.98 ± 0.13 U/mg ($p < 0.05$). GPx activity mirrored these trends, with the normal group at 48.59 U/mg (liver) and the PARA group at 25.57 U/mg (liver, $p < 0.05$). The PARA+NAC group recorded GPx levels of 44.17 U/mg (liver), while the PARA+ESBA400 group showed 40.49 U/mg (liver), indicating potential mitigation of oxidative stress.

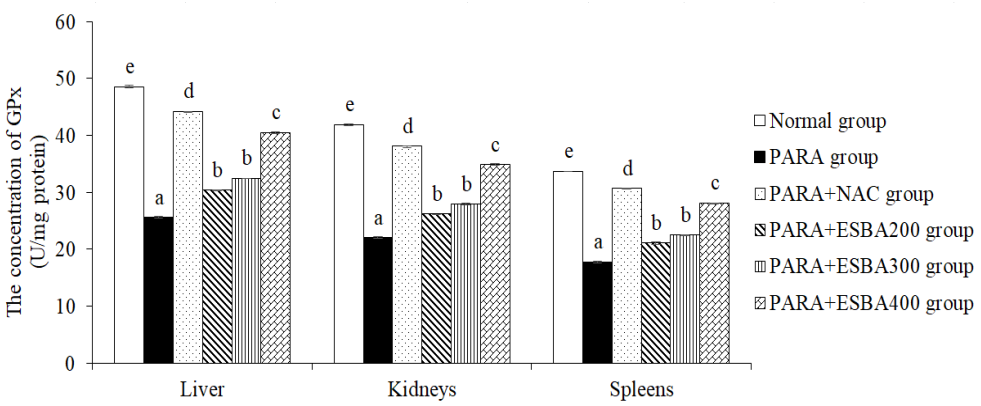

Figure 4. Effect of ESBA on glutathione peroxidase (GPx) levels, Results are presented as Mean ± SD, with the letters (a – e) denoting significant differences between groups ($p < 0.05$).

Evaluation of the extract's ability to modulate the immune response

The ethanol extract from ESBA exerted significant immunomodulatory effects on rats with PARA-induced nephrotoxicity, as indicated by changes in white blood cell (WBC) count, nitroblue tetrazolium (NBT) positivity, and total immunoglobulin (TI) levels (Table 5). In the PARA-treated group, WBC count markedly increased to 8.68 ± 0.56 (× 10^3 cells/mm^3) compared to the normal group (4.57 ± 0.17 (× 10^3 cells/mm^3), $p < 0.05$), and the percentage of NBT-positive cells (PNPC) rose sharply to 24.66 ± 0.44% ($p < 0.05$), reflecting significant inflammation and oxidative stress. Additionally, TI levels dropped significantly to 8.81 ± 0.38 mg/mL ($p < 0.05$), indicating impaired immune function. Treatment with ESBA significantly ameliorated these parameters. WBC counts progressively decreased in a dose-dependent manner ($p < 0.05$), with the 400 mg/kg dose reducing WBC levels to 5.48 ± 0.15 (× 10^3 cells/mm^3), approaching normal values. Similarly, PNPC decreased across

ESBA doses ($p < 0.05$), with the highest reduction observed at 400 mg/kg (15.58 ± 0.13%), indicating a reduction in oxidative stress and better neutrophil regulation. TI levels increased significantly with ESBA treatment, reaching 13.95 ± 0.31 mg/mL at the highest dose ($p < 0.05$), partially restoring immune function compared to the PARA-damaged group.

Table 5. Effect of ESBA on white blood cell (WBC) count, nitroblue tetrazolium (NBT) positivity, and total immunoglobulin (TI) levels

Groups	Blood cell (WBC) ($\times 10^3$cells/mm^3)	Percent NBT-positive cells (PNPC) (%)	Total immunoglobulin (TI) (mg/mL)
Normal group	4.57 ± 0.17^{a}	12.98 ± 0.84^{a}	16.74 ± 0.24^{f}
PARA group	8.68 ± 0.56^{f}	24.66 ± 0.44^{f}	8.81 ± 0.38^{a}
PARA+NAC group	5.03 ± 0.21^{b}	14.28 ± 0.13^{b}	15.22 ± 0.29^{e}
PARA+ESBA200 group	6.87 ± 0.25^{e}	19.47 ± 0.17^{e}	10.46 ± 0.13^{b}
PARA+ESBA300 group	6.39 ± 0.23^{d}	18.17 ± 0.15^{d}	11.96 ± 0.32^{c}
PARA+ESBA400 group	5.48 ± 0.15^{c}	15.58 ± 0.13^{c}	13.95 ± 0.31^{d}

Note: The values are presented as Mean ± SD, with the letters (a – f) denoting significant differences between groups ($p < 0.05$).

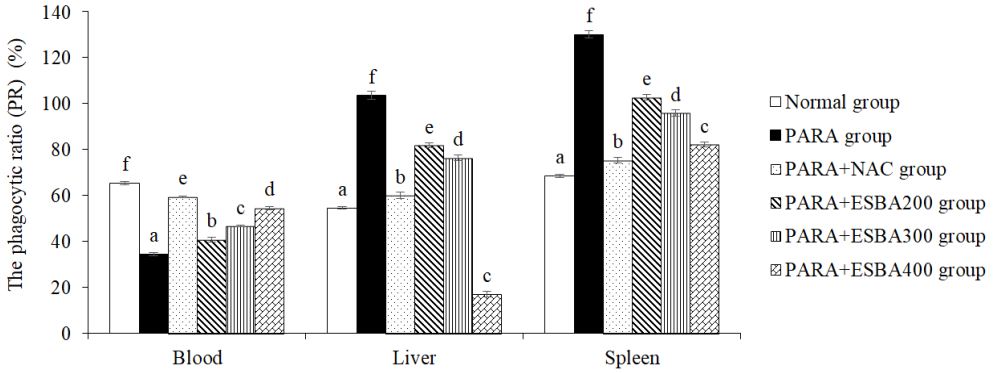

Figure 5. Effect of ESBA on phagocytic ratio (PR), Results are presented as Mean ± SD, with the letters (a–e) denoting significant differences between groups ($p < 0.05$).

The ethanol extract from ESBA significantly modulates the immune response in mice with PARA-induced nephrotoxicity, as shown in Figures 5 and 6, which indicate alterations in the phagocytic ratio (PR) and phagocytic index (PI) across blood, liver, and spleen. In the PARA group, PR was significantly reduced in the blood (34.35 ± 0.58%) compared to the normal group (65.26 ± 0.65%) ($p < 0.05$), while PR in the liver and spleen increased significantly (103.53 ± 1.62% and 129.98 ± 1.59%, respectively) ($p < 0.05$),

reflecting immune dysfunction and inflammation. Treatment with ESBA, particularly at a dose of 400 mg/kg, improved these imbalances, restoring blood PR to a level close to normal (54.37 ± 0.85%) ($p < 0.05$). Additionally, the PARA group exhibited suppressed PI values, indicating diminished phagocytic activity; however, ESBA treatment at 400 mg/kg significantly enhanced PI in the blood (10.71 ± 0.13 particles/cell), liver (128.86 ± 1.26 particles/cell), and spleen (363.28 ± 2.76 particles/cell) ($p < 0.05$), bringing these values closer to normal ranges.

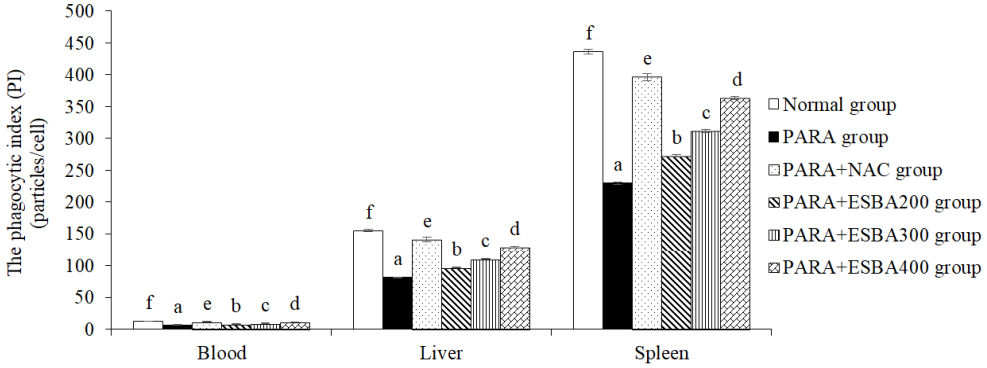

Figure 6. Effect of ESBA on phagocytic index (PI), Results are presented as Mean ± SD, with the letters (a – e) denoting significant differences between groups ($p < 0.05$).

Disscusion

Natural herbs are gaining recognition for their therapeutic potential, particularly in mitigating oxidative stress and modulating immune responses—key mechanisms in managing kidney toxicity caused by PARA overdose (Agbor *et al*., 2023). PARA overdose induces excessive oxidative stress in renal tissues, leading to cellular damage and impaired kidney function (Nhung and Quoc, 2023). Antioxidant-rich herbs, such as flavonoids, polyphenols, tannins, and saponins, help neutralize free radicals, enhance the body's antioxidant defenses, reduce inflammation, and support kidney recovery (Michalak, 2022), highlighting their potential role in protecting against PARA-induced nephrotoxicity.

The ESBA contains many phytochemicals, including alkaloids, flavonoids, phenolics, steroids, tannins, terpenoids, and saponins. These compounds play a key role in reducing oxidative stress and regulating immune responses, especially in cases of nephrotoxicity induced by PARA (Nhung and Quoc, 2023). Tannins (7.44 ± 0.19 mg TE/g) exhibit strong antioxidant properties, neutralizing free radicals and protecting renal cells from damage.

Flavonoids (42.26 ± 1.08 mg QE/g) boost antioxidant capacity and exert anti-inflammatory effects, reducing PARA-induced kidney damage. Polyphenols (70.77 ± 1.37 mg GAE/g) provide protective effects through their potent antioxidant activity and ability to reduce inflammation. Saponins (13.63 ± 0.42 mg SE/g) modulate immune cell activity and inhibit pro-inflammatory cytokines, promoting tissue recovery. Alkaloids, terpenoids, and steroids in ESBA support kidney protection by reducing oxidative stress, and inflammation, and modulating immune responses. Many studies have demonstrated the effectiveness of plant-derived compounds in reducing oxidative stress and inflammation in PARA-induced nephrotoxicity (Nhung and Quoc, 2024d) and confirm that these compounds, due to their antioxidant properties, help counteract oxidative damage in renal tissues (Avila-Carrasco *et al*., 2021). Nithiyanandam and Prince (2023) showed that polyphenols, alkaloids, and terpenoids from *Caesalpinia bonducella* seed extract protect against PARA-induced kidney toxicity in rodents. Similarly, extracts from *Petroselinum crispum* roots and *Trifolium repens* leaves prevent PARA-induced kidney damage in mice (Nouioura *et al*., 2023; Ahmad and Zeb, 2020). Nhung and Quoc (2023) demonstrated that flavonoids and polyphenols in ESBA extract regulate inflammatory pathways, which are essential for protecting the kidneys in murine models of PARA toxicity. These findings underscore the potential of plant-derived compounds in mitigating inflammation and reinforce the role of natural products in managing nephrotoxicity.

PARA induces oxidative stress in the kidneys, leading to the production of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). As glutathione stores are depleted, NAPQI accumulates, generating reactive oxygen species (ROS) that cause kidney cell damage. This oxidative stress triggers inflammation, releasing pro-inflammatory cytokines, which further harm tissues and impair kidney function (Kanchanasurakit *et al*., 2020). This condition results in weight loss (28.61 ± 0.23%) and kidney hypertrophy, increasing relative kidney weight (RKW) (68.99 ± 0.82%). ESBA protects against PARA-induced nephrotoxicity by reducing oxidative stress and modulating immune responses through improvements in body weight, RKW, and kidney structure in treated groups. The PARA+ESBA400 group demonstrated recovery in body weight (37.42 ± 0.42 g) compared to the significant weight loss in the PARA group (27.66 ± 0.46 g), suggesting ESBA mitigates PARA-induced damage. This effect is linked to ESBA's antioxidant properties, which reduce ROS and enhance endogenous antioxidant activity. ESBA also inhibits pro-inflammatory cytokines, protecting kidney cells and promoting tissue repair (Nhung and Quoc, 2023). The reduction in RKW (32.82 ± 0.24% in the PARA+ESBA400 group) indicates that ESBA prevents kidney

hypertrophy and aids in functional recovery. Morphological assessments revealed that ESBA-treated kidneys had a more normal surface and coloration than the rough, discolored kidneys in the PARA group. The current findings on the nephroprotective effects of ethanol extract from ESBA against PARA-induced toxicity align with previous research demonstrating the efficacy of natural extracts in reducing oxidative stress and modulating immune responses. Chinnappan *et al*. (2019) reported that antioxidant-rich *Eurycoma longifolia* extract significantly reduced reactive oxygen species (ROS) generated during PARA metabolism, thereby preventing kidney cell damage. Similarly, Rabani *et al*. (2021) showed that *Stachys pilifera* extract treatment improved body weight and reduced relative kidney weight (RKW) after PARA exposure, mirroring the results observed in the PARA+ESBA group in our study. Additionally, the immunomodulatory effects of ESBA are consistent with research on the anti-inflammatory properties of other herbal extracts. Dkhil *et al*. (2019) demonstrated that *Myristica fragrans* extract reduced pro-inflammatory cytokines in a PARA-induced kidney injury model, facilitating kidney recovery. Our study also observed decreased RKW and improved kidney morphology after ESBA treatment, supporting the hypothesis that ESBA effectively inhibits inflammation pathways associated with oxidative stress. These findings are consistent with those of Nhung and Quoc (2023), who used ESBA extract to treat PARA-induced nephrotoxicity.

The investigation of PARA's effects on biochemical parameters in mice revealed significant changes in markers of oxidative stress. Mice treated with PARA showed notable increases in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), indicative of heightened lipid peroxidation and severe oxidative damage in renal tissues, with MDA levels at 3.91 nmol/mg protein and H_2O_2 at 3.33 nmol/g tissue. PARA metabolism produces toxic intermediates, particularly N-acetyl-p-benzoquinone imine (NAPQI), which exacerbates lipid peroxidation and reactive oxygen species (ROS) production (Ramachandran and Jaeschke, 2021). Additionally, there was a marked reduction in glutathione (GSH), with renal levels declining to 4.87 µmol/g, reflecting compromised antioxidant defenses. The antioxidant parameters such as total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were significantly diminished, indicating impaired mechanisms for combating oxidative stress. The reductions in GSH, TAC, SOD, CAT, and GPx across liver, kidney, and spleen tissues were linked to increased free radical generation, as NAPQI depletes GSH in the liver, triggering oxidative stress that damages cell membranes and inhibits antioxidant enzyme function (Luo *et al*., 2023). In contrast, treatment with N-acetylcysteine (NAC) led to significant improvements in oxidative stress parameters. GSH levels increased to 6.89 µmol/g, indicating restored antioxidant capacity, while TAC also improved, demonstrating NAC's

effectiveness in mitigating PARA-induced oxidative stress. Enhanced activities of antioxidant enzymes like SOD and CAT reflected the recovery of renal defenses, supported by NAC's role in providing cysteine for GSH synthesis. The elevated GSH levels neutralized ROS, reducing lipid peroxidation and lowering MDA and H_2O_2 levels (Sahasrabudhe *et al*., 2023). Additionally, NAC stimulates antioxidant enzyme activity, promoting detoxification and protecting cells from oxidative stress (Raghu *et al*., 2021). NAC also has anti-inflammatory effects, contributing to reduced tissue damage and improved liver and kidney function (Tenório *et al*., 2021). Similarly, treatment with black onion extract (ESBA) led to varying degrees of recovery based on dosage. In the PARA+ESBA400 group, MDA levels decreased to 2.46 nmol/mg protein, and H_2O_2 levels fell to 1.42 nmol/g tissue, demonstrating the protective effects of ESBA. GSH levels increased to 40.49 U/mg, accompanied by improvements in TAC, indicating that ESBA helped restore the body's antioxidant capacity. Additionally, SOD and CAT enzymatic activities significantly increased, with SOD at 28.98 U/mg and CAT at 73.02 U/mg. The antioxidant properties of ESBA are attributed to its phenolic and flavonoid compounds (Tran and Ngo, 2023), which neutralize ROS, resulting in decreased MDA and H_2O_2 levels in tissues such as the liver, kidneys, and spleen (Nhung and Quoc, 2024a). Moreover, ESBA enhances GSH synthesis, boosting the body's total antioxidant capacity. The increased GSH levels not only provide cellular protection against oxidative damage but also support the activity of key antioxidant enzymes like SOD, CAT, and GPx (Nhung and Quoc, 2023). Through these mechanisms, ESBA effectively protects cells from PARA-induced oxidative stress, thereby improving liver and kidney function and overall health. Research on PARA-induced oxidative stress and the protective roles of antioxidants like NAC and ESBA reveals critical cellular defense mechanisms. PARA significantly increases oxidative stress markers, leading to tissue damage and overwhelming the body's natural antioxidant defenses. Mondal *et al*. (2022) found that PARA elevated markers like MDA and H_2O_2 in the liver, kidneys, and spleen, causing substantial cellular damage. Li *et al*. (2023) reported that PARA depletes GSH and TAC, weakening the body's defenses. Mokhtari *et al*. (2023) further demonstrated that PARA reduces the activity of key antioxidant enzymes such as SOD, CAT, and GPx, exacerbating oxidative damage. In contrast, plant-based antioxidants have shown promise in alleviating PARA-induced oxidative stress. Nhung and Quoc (2024f) observed that *Gardenia stenophylla* extract, rich in phenolic and flavonoid compounds, effectively reduced MDA and H_2O_2 levels while enhancing antioxidant enzyme activity in tissues. Similarly, Papaefthimiou *et al*. (2023) confirmed that *Stevia rebaudiana* extract lowers oxidative stress markers and boosts antioxidant enzyme activity, highlighting the therapeutic potential of plant compounds in neutralizing reactive oxygen species (ROS) and minimizing cellular damage. Additionally, Nhung and Quoc (2023) demonstrated that ESBA combats

oxidative stress and reduces inflammation, thereby protecting the kidneys from PARA-induced damage.

The immunomodulatory effects of black garlic extract (ESBA) on PARA-induced nephrotoxicity were assessed through white blood cell (WBC) count, nitroblue tetrazolium positivity (NBT), immunoglobulin concentration (TI), phagocytosis ratio (PR), and phagocytic index (PI). In the PARA-treated group, WBC counts significantly increased to 8.68 ± 0.56 (× 10^3 cells/mm^3), indicating an acute inflammatory response linked to tissue damage caused by oxidative stress. The elevated WBC count serves as a marker of PARA-induced inflammatory toxicity (Khan *et al*., 2021). Post-ESBA treatment, WBC counts decreased to 5.48 ± 0.15 (× 10^3 cells/mm^3) at the highest dose (400 mg/kg), suggesting ESBA's role in modulating the immune response and reducing inflammation. This reduction indicates a restoration of immune balance, contributing to decreased tissue damage and improved renal function (Nhung and Quoc, 2023). NBT levels were elevated in the PARA group (24.66 ± 0.44%), reflecting increased oxidative stress. ESBA treatment significantly reduced NBT levels to 15.58 ± 0.13%, highlighting its antioxidant properties. Additionally, TI decreased to 8.81 ± 0.38 mg/mL in the PARA group, indicating impaired immune function. Following ESBA treatment, TI increased to 13.95 ± 0.31 mg/mL, suggesting enhanced antibody production and immune response. Changes in PR and PI further emphasized ESBA's ability to normalize phagocytic activity; PR improved from 34.35 ± 0.58% in the PARA group to 54.37 ± 0.85% after treatment, and PI increased from 6.76 ± 0.09 particles/cell to 10.71 ± 0.13 particles/cell. The elevated NBT positivity in the PARA group was linked to reactive oxygen species (ROS) generation, activating inflammatory pathways and damaging immune function (Pharmacy *et al*., 2021). ESBA effectively reduced NBT levels and increased TI, promoting immune balance through its antioxidant properties. The extract enhanced macrophage activity, reflected in improved PR and PI, thereby boosting the immune system's ability to eliminate pathogens (Nhung and Quoc, 2024d). These findings align with previous studies indicating that PARA exposure leads to increased WBC counts and impaired immune function due to oxidative stress (Nouioura *et al*., 2023). The reduction in WBC counts after ESBA treatment corroborates the immunoregulatory potential of herbal extracts (Hooda *et al*., 2024). The increased NBT levels in the PARA group support previous findings linking PARA to oxidative damage and neutrophil activity (Ishida *et al*., 2023). The improvement in TI after ESBA treatment supports the hypothesis that antioxidant-rich plant extracts can enhance immune function (Gasmi *et al*., 2023). Finally, the observed decreases in PR and PI in the PARA group echo earlier research linking PARA-induced oxidative stress to diminished phagocytic activity (Nhung and Quoc, 2024a).

In conclusion, this study is demonstrated that black shallot extract (ESBA) is effective in mitigating the harmful effects of oxidative stress and in

modulating immune response. ESBA improves body weight, reduces relative kidney weight (RKW), and enhances kidney morphology. Furthermore, it decreases levels of MDA and H_2O_2, as well as WBC counts, while increasing concentrations of GSH, TAC, SOD, CAT, GPx, and immunological factors such as the TI, PR, and PI. These changes help to restore immune balance and enhance renal function, highlighting the potential of ESBA as a therapeutic agent against oxidative stress and nephrotoxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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