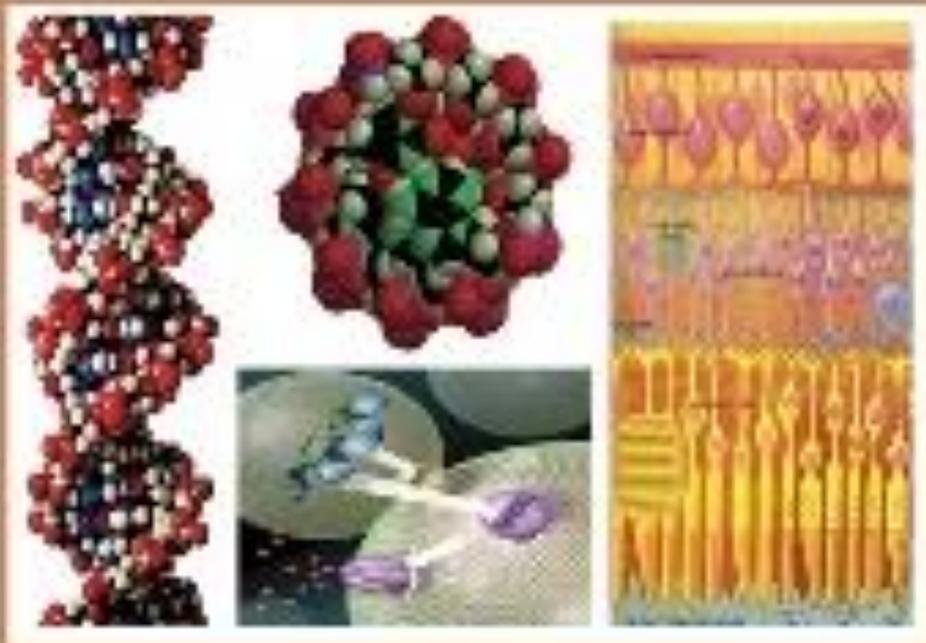




C

EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES

PHYSIOLOGY & MOLECULAR BIOLOGY



ISSN
2090-0767

WWW.EAJBS.ICA.NET

Vol. 16 No. 1 (2024)



***Eucheuma cottonii* Extract Improved The Blood Profile and Kidney Function of Male Albino Rats Induced with Sodium Nitrite (NaNO₂)**

Ida Ayu Ratih Purnama Dewi¹, Ngurah Intan Wiratmini^{2*} and Iriani Setyawati³

¹Master Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Udayana, P.B. Sudirman Street, Denpasar, Bali, Indonesia.

²Pogram Study of Biology, Faculty of Mathematics, and Natural Sciences, Universitas Udayana, Bukit Jimbaran, Badung, Bali, Indonesia.

³Program Study of Biology, Faculty of Mathematics, Natural and Earth Sciences, Universitas Negeri Manado, South Tondano, Minahasa, North Sulawesi, Indonesia.

*E-mail: wiratminiintan@unud.ac.id

ARTICLE INFO

Article History

Received: 7/12/2023

Accepted: 13/1/2024

Available: 17/1/2024

Keywords:

Eucheuma cottonii,
food preservative,
antioxidant, blood,
kidney

ABSTRACT

Sodium nitrite (NaNO₂) in food preservation causes oxidative stress in the body. Antioxidants are widely used to ward off free radicals caused by oxidative stress. This study aimed to determine the potential of *Eucheuma cottonii* seaweed extract as a natural antioxidant to improve blood profiles and kidney damage in male albino rats induced by NaNO₂. This research used a completely randomized design. Thirty animals were divided into five groups, namely negative control, positive control, and three treatment doses of *E. cottonii* extract. NaNO₂ was dissolved in distilled water, and *E. cottonii* extract was dissolved in 0.5% CMC-Na solution. The research was conducted for 45 days. The treatment group was given NaNO₂ for 35 days; on days 16 to 45, *E. cottonii* extract was given at doses of 150, 300, and 450 mg/kg BW. The positive control was given NaNO₂ for 35 days, then given the extract solvent. The negative controls were given the solvent only. At the end of the treatment, the rats were sacrificed to collect blood and kidney organs. The results showed that the extract at doses of 150 and 300 mg/kg BW increased the number of hemoglobin and erythrocytes. The dose of 150 mg/kg BW reduced various kidney damage, such as inflammatory cell infiltration, congestion, hemorrhage, fatty degeneration, necrosis, glomerular abnormalities, and protein deposits in the lumen of the renal tubules. The highest dose of 450 mg/kg BW reduced the number of leukocytes.

INTRODUCTION

Sodium nitrite (NaNO₂) is a food additive that preserves meat. Its preservation mechanism operates by hindering the proliferation of *Clostridium botulinum* bacteria and the synthesis of neurotoxins generated by these bacteria. This action effectively averts poisoning and spoilage (Afiah et al., 2017; Shakil et al., 2022). Following the head regulation No. 36 of 2013 by The National Food and Drug Agency of Indonesia or BPOM, the utilization of NaNO₂ as a food additive remains permissible, with a designated safe limit of 30 mg/kg BW for humans. NaNO₂ can bind with amino or amide groups, forming nitrosamine derivatives, which are known to be both toxic and carcinogenic (Helal et al., 2008). The nitrite ion within sodium nitrite converts hemoglobin into methemoglobin in the blood. This conversion occurs due to the oxidation of Fe²⁺ ions in blood hemoglobin to form Fe³⁺ ions, leading to a condition known as methemoglobinemia (Aita and Faten, 2014).

Research has demonstrated that administering NaNO₂ at doses of 0.5 and 1.5 mg/200 g BW in mice can result in a reduction in the number of erythrocytes and hemoglobin levels in rats (Ambarwati, 2012). Similarly, studies involving rats induced by NaNO₂ at doses of 10 and 20 mg/kg BW revealed that NaNO₂ induced histological damage to the liver and kidneys when compared to control groups (Ozen *et al.*, 2014).

The negative effects of NaNO₂ oxidative stress can be prevented by using antioxidants. Natural antioxidants can be obtained from the seaweed *E. cottonii* (Sambodo, 2019). According to Maharany *et al.* (2017), *E. cottonii* extract contains phenolics, triterpenoids, and flavonoids which act as antioxidants. Furthermore, research on *E. cottonii* extract at a dose of 150 mg/kg BW was reported to be able to reduce levels of Alanine Aminotransferase (ALT) and Aspartate Transaminase (AST), as well as improve liver histology damage (Dewi *et al.*, 2020). However, the effect of *E. cottonii* extract on blood and kidney profiles due to NaNO₂ consumption has not been studied. This study aims to determine the potential of *E. cottonii* extract in improving the blood profile and kidney structure of male albino mice induced by NaNO₂.

MATERIALS AND METHODS

The study used a Completely Randomized Design (CRD) with two controls and three treatments, each replicated six times. The experimental cohort consisted of 30 albino rats (*Rattus norvegicus*) that were 3 to 4 months old and had a body weight of 200 ± 10 g.

E. cottonii Seaweed Extraction:

E. cottonii seaweed was obtained from Geger Beach, Nusa Dua, Bali, Indonesia. The seaweed was undergone washing and a 30-day drying process away from sunlight. It was then cut, grounded, and sifted through a 60-mesh sieve. The maceration method was employed, soaking 200 g of seaweed powder in 1 L of 96% ethanol for 72 hours. The resulting macerate was filtered using Whatman filter paper no.

42. The filtrate was then evaporated at 40°C using a vacuum evaporator, yielding a thick crude extract. Finally, the crude extract was analyzed for its phytochemical content and antioxidant activity using the DPPH method.

Antioxidant Activity Analysis of *E. cottonii* Seaweed Extract:

Analysis of the antioxidant activity of *E. cottonii* extracts using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. A total of 3 mL of *E. cottonii* extract was put into a test tube wrapped in aluminum foil. One mL of 0.004% DPPH solution was added to the tube. The mixture was homogenized and kept in a dark place for 30 minutes at room temperature. The absorbance value was measured using a UV-Vis spectrophotometer (Shimadzu type 2450) at a wavelength of 517 nm. The inhibition percentage value was calculated based on the formula:

$$\frac{\text{DPPH Absorbant} - \text{Extract Absorbant}}{\text{DPPH Absorbant}} \times 100 \%$$

To obtain the IC₅₀ value (Inhibitory Concentration 50%, a concentration that can reduce 50% of DPPH free radicals), percentage calculation inhibition was substituted into a linear equation: $Y = aX + b$, where $Y = \% \text{ inhibition}$, $a = \text{gradient}$, $X = \text{concentration (mg/L)}$, and $b = \text{constant}$ (Podungge *et al.*, 2018).

Phytochemical Analysis of *E. cottonii* Seaweed Extract:

The thick extract was dissolved in 5 mL of 96% ethanol and then mixed with a 25 mL combination of chloroform and distilled water (1:1). It was left for approximately 5 minutes before analyzing the content of flavonoid, saponin, alkaloid, phenolic, steroid, and triterpenoid compounds following the guidelines of Podungge *et al.* (2018).

Animal Treatment:

Animals were acclimatized in experimental cages for 7 days. During acclimatization and treatment, rats were given standard CP 551 food and water ad libitum. Sodium nitrite (NaNO₂) was dissolved in distilled water while the crude extract of *E. cottonii* extract was dissolved in 0.5%

Carboxymethyl Cellulose Sodium (CMC-Na) solution. Animals were divided into five groups (six rats each) namely two controls (negative and positive control), and three treatments (T1, T2, and T3). Table 1 shows the procedure of the animals for 45 days

Table 1. Animal Treatment Procedure

Days	Groups (Rats were given...)				
	Negative Control (NC)	Positive Control (PC)	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)
1-15	Distilled water	NaNO ₂	NaNO ₂	NaNO ₂	NaNO ₂
16-35	Distilled water 0.5% CMC-Na	NaNO ₂	NaNO ₂ and <i>E.cottonii</i> extract dose 150 mg/kg BW	NaNO ₂ and <i>E.cottonii</i> extract dose 300 mg/kg BW	NaNO ₂ and <i>E.cottonii</i> extract dose 450 mg/kg BW
36-45	0.5% CMC-Na	0.5% CMC-Na	<i>E. cottonii</i> extract dose 150 mg/kg BW	<i>E. cottonii</i> extract dose 300 mg/kg BW	<i>E. cottonii</i> extract dose 450 mg/kg BW

Sample Collection:

At the end of the study, the rats were initially anesthetized using a combination of xylazine (20 mg/kg BW) and ketamine (10 mg/kg BW) administered through an intramuscular injection. Once the animal became unconscious, blood was drawn directly from the heart using a syringe, collecting 4 mL of blood in an EDTA capillary tube. After blood sampling, surgical procedures were performed to remove the kidneys. The kidneys were preserved in a 10% Neutral buffered formalin solution for one day in preparation for subsequent histological incisions. The execution of these procedures followed the ethical standards set by the Animal Ethics Committee at the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia (ID No.4/UN14.2.9/PT.01.04/2020).

Blood Profile Examination:

The blood profile examination included assessments of erythrocytes, hemoglobin, and leukocytes (each type of

leukocyte). The examinations were conducted at the Bali Regional Health Laboratory in Denpasar, Bali, following the operational standards of the Bali Regional Health Laboratory.

Histology Preparations:

The histology preparations were conducted at the Denpasar Veterinary Center in Bali. The kidney organs were immersed in 10% Neutral Buffer Formalin for 24 hours. Then, they were processed into histological sections using the embedding method in a paraffin block. Afterward, the sections were stained with Harris-Hematoxylin Eosin dye.

Observation of Kidney Histology:

The preparations were observed under an electric microscope (Olympus®) with 400x magnification encompassing five fields of view. From each field of view, the percentage of cells experiencing damage and cell death (fatty degeneration and necrosis, respectively) was observed and calculated using the formula (Indriana and Titrawani, 2022):

$$\frac{\text{The number of cells that have died or been damaged}}{\text{Total number of cell}} \times 100\%$$

The percentage of abnormalities in the glomerulus (atrophy and edema) and the presence or absence of protein deposits in the renal tubules were also calculated. Observations were made with 100x magnification with five fields of view. The percentage of abnormalities in the glomerulus was calculated using the formula (Wientarsih

et al., 2014):

$$\frac{\text{Abnormal glomeruli (atrophy and edema)}}{\text{Total number of glomeruli}} \times 100\%$$

Meanwhile, the percentage of protein deposits in the tubules was calculated using the formula:

$$\frac{\text{Tubules containing protein deposits}}{\text{Total number of tubules}} \times 100\%$$

Data Analysis:

Data analysis was carried out using the ANOVA statistical test with the SPSS version 23 for Windows program, followed by the Duncan Multiple Range Test if there were significant differences with a level of $P < 0.05$ in all groups.

RESULTS**Qualitative Phytochemical Analysis of *E. cottonii* Extract:**

A phytochemical qualitative analysis showed that *E. cottonii* extract contains flavonoid, triterpenoid, phenolic, saponin, and alkaloid compounds. However, the test for steroid compounds showed negative results (Table 2).

Table 2. Phytochemical test results of *E. cottonii* extract.

Compound Classes	Test Results
Flavonoids	+
Phenolic	+
Alkaloids	+
Triterpenoids	+
Saponins	+
Steroids	-

The positive symbol (+) indicates that this compound was contained in the *E. cottonii* extract. The negative symbol (-) indicates that this compound was not contained in the *E. cottonii* extract.

Antioxidant Activity of *E. cottonii* Extract:

The antioxidant activity test in this study was carried out using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The inhibition percentage (%IC, Inhibitory Concentration) of *E. cottonii* extract was obtained by comparing four concentrations (420, 630, 840, and 1050 mg/L) of *E. cottonii*

extract. Figure 1 showed that increasing the concentration of *E. cottonii* extract caused an increase in inhibiting DPPH free radicals.

Based on the inhibition percentage calculation (Fig. 1), it is then substituted into the linear equation $Y = aX + b$, to obtain a linear equation as in Figure 2.

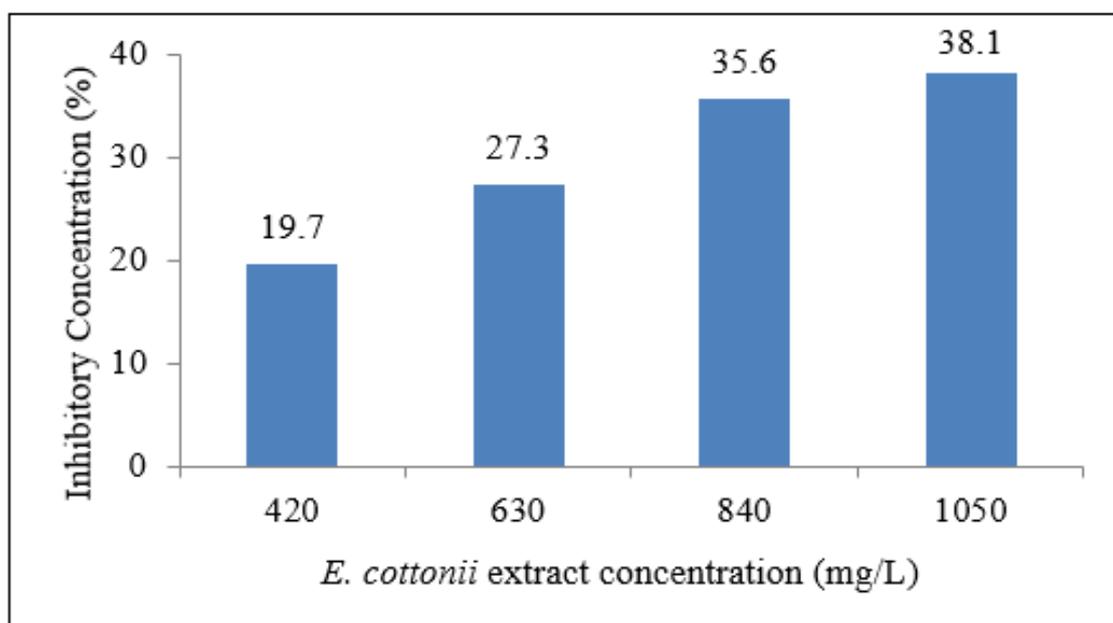


Fig.1: The percentage of inhibition of *E. cottonii* extract in counteracting DPPH free radicals.

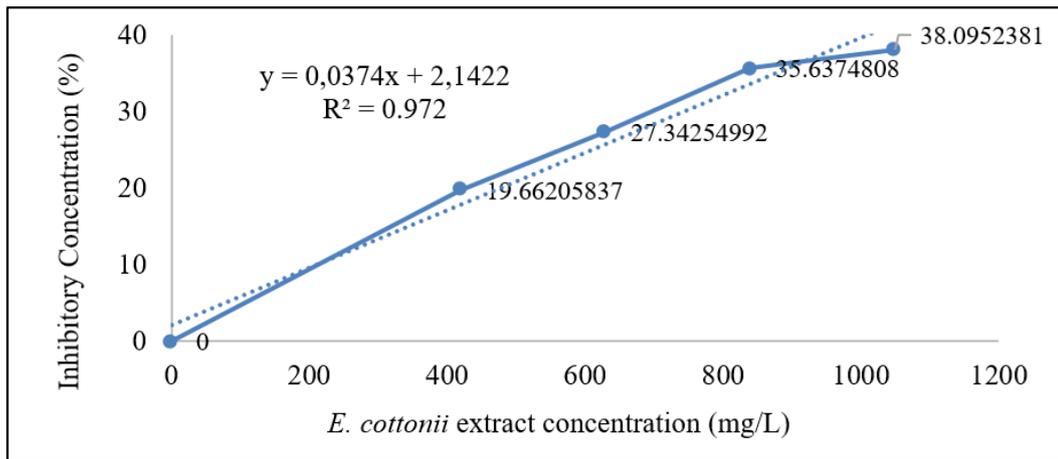


Fig.2: Linear equation of percentage inhibition of *E. cottonii* extract in counteracting DPPH free radicals.

Using the linear equation $Y = 0.0374X + 2.1422$, the IC_{50} value (concentration that can reduce 50% of DPPH free radicals) for *E. cottonii* extract is 1279.62 mg/L. These results indicate that the antioxidant activity of *E. cottonii* extract in warding off free radicals is categorized as very weak (Molyneux, 2004).

Blood Profile of Albino Rats Treated with NaNO₂ and *E. cottonii* Extract:

The comprehensive blood profile analysis involved assessing the count of erythrocytes, hemoglobin levels, and the various types of leukocytes, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The NaNO₂-induced rats without any treated with *E. cottonii* extract (the positive control) exhibited a notable reduction in hemoglobin levels and the number of erythrocytes. Contrarily, there was a significant increase in the number of leukocytes.

The results of statistical tests revealed that the number of erythrocytes and hemoglobin levels in the negative control (rats without NaNO₂ nor *E. cottonii* extract) were significantly different ($P < 0.05$) from those in the positive control and T3. In

contrast, there was no significant difference between the negative control, and treatments T1 and T2; so was between the positive control and T3 (Fig. 3).

With an increase in the dose of seaweed extract, there was a noticeable decrease in the number of erythrocytes and hemoglobin levels in the animals. The T1 treatment recorded the highest number of erythrocytes ($8.30 \times 10^6/\mu\text{L}$), while the positive control had the lowest ($5.96 \times 10^6/\mu\text{L}$). Similarly, the highest hemoglobin levels were found in the T1 (15.30 g/dL), while the positive control had the lowest (11.02 g/dL) (Fig. 3).

The number of leukocytes in the negative control and T3 treatment exhibited statistically significant differences ($P < 0.05$) compared to the positive control and T1 and T2. Meanwhile, the number of leukocytes in the positive control and T1 treatment did not demonstrate a significant difference. With an increase in the dose of extract, there was a consistent trend of decreasing leukocyte counts in albino rats. The positive control recorded the highest leukocyte count ($9.11 \times 10^3/\mu\text{L}$), while the negative control had the lowest ($2.56 \times 10^3/\mu\text{L}$) (Fig. 3).

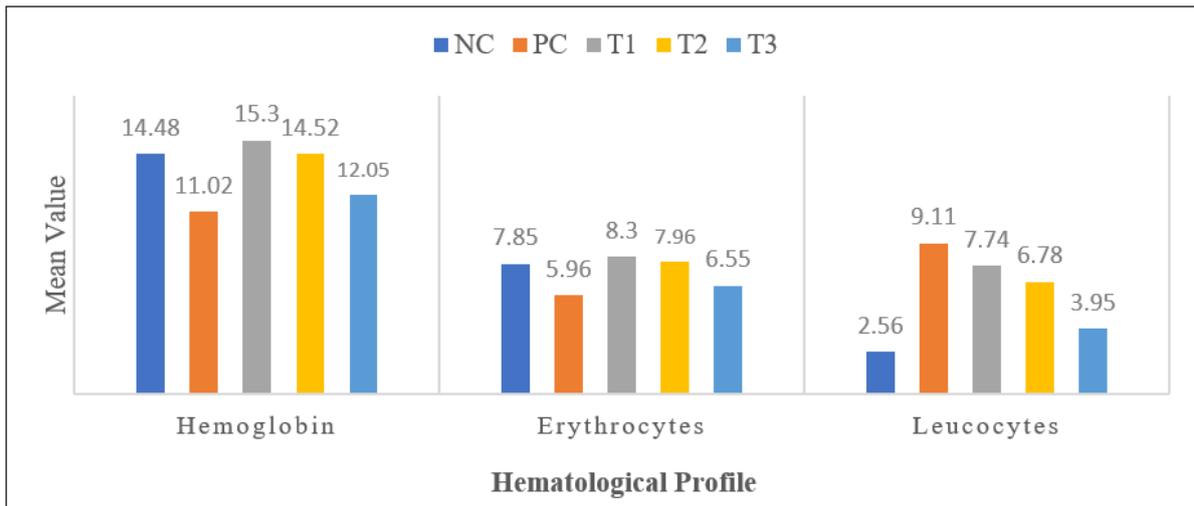


Fig.3: The number of erythrocytes, hemoglobin (Hb), and leukocytes in all subject groups. Values (mean) followed by different letters in the same parameter indicate significant differences ($P < 0.05$). NC, negative control (distilled water, CMC-Na 0.5%). PC, positive control (NaNO₂, CMC-Na 0.5%). Treatment groups of NaNO₂ followed by therapy of *E. cottonii* extract dose 150 mg/kg BW (T1), 300 mg/kg BW (T2), and 450 mg/kg BW (T3). Number of erythrocytes (x10⁶/μL); hemoglobin (g/dL); and leukocytes (x10³/μL).

The number of each type of leukocyte (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) is shown in Table 3. Based on statistical tests, the number of neutrophils and basophils in all groups did not show a significant difference. The positive control shows an increased number of lymphocytes, monocytes, and eosinophils significantly ($P < 0.05$) when

compared to the negative control. The highest number of neutrophils, lymphocytes, monocytes, and eosinophils was in the positive control. In contrast, the lowest number of lymphocytes, monocytes, and eosinophils each was in the negative control. The number of lymphocytes in the negative control and T3 treatment did not show a significant difference.

Table 3. Mean \pm SD values of each type of leukocyte in all subject groups.

Group	Types of Leukocytes (x10 ³ /μL)				
	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
NC	0.79 \pm 0.39 ^a	1.55 \pm 0.38 ^a	0.10 \pm 0.05 ^a	0.05 \pm 0.04 ^a	0.07 \pm 0.12 ^a
PC	1.13 \pm 0.44 ^a	7.13 \pm 3.25 ^b	0.36 \pm 0.34 ^b	0.38 \pm 0.35 ^b	0.01 \pm 0.01 ^a
T1	1.11 \pm 0.42 ^a	6.10 \pm 1.98 ^b	0.21 \pm 0.11 ^{ab}	0.27 \pm 0.16 ^{ab}	0.01 \pm 0.01 ^a
T2	0.89 \pm 0.33 ^a	5.45 \pm 0.11 ^b	0.17 \pm 0.13 ^{ab}	0.25 \pm 0.02 ^{ab}	0.01 \pm 0.01 ^a
T3	0.76 \pm 0.44 ^a	2.94 \pm 1.23 ^a	0.13 \pm 0.01 ^a	0.09 \pm 0.12 ^a	0.03 \pm 0.03 ^a

Values (mean \pm standard deviation) followed by different superscript letters (^a or ^b) in the same column indicate significant differences ($P < 0.05$). NC, negative control (distilled water, CMC-Na 0.5%). PC, positive control (NaNO₂, CMC-Na 0.5%). Treatment groups of NaNO₂ and therapy of *E. cottonii* extract dose 150 mg/kg BW (T1), 300 mg/kg BW (T2), and 450 mg/kg BW (T3).

Kidney Histopathology of Albino Rats:

The administration of 22.5 mg/kg BW NaNO₂ without *E. cottonii* extract (positive control) significantly increased the

percentage of kidney histological damage. Animals administered a dose of 22.5 mg/kg BW of NaNO₂ and then treated with *E. cottonii* extract at doses of 150, 300, and 450

mg/BW (T1, T2, and T3, respectively) showed lower histological damage compared to the positive control. However, with the increase in the dose of seaweed extract, there was a corresponding rise in histological damage to the kidneys (Table 4, Figs. 4-6).

Table 4 shows the statistical test results of kidney histopathology parameters of rats. The percentage of inflammatory cell infiltration and the presence of protein deposits in the renal tubules revealed a significant difference ($P < 0.05$) between the negative control and the other groups, except between the T2 and T3, where no significant difference was observed. The negative control

exhibited the lowest percentage of inflammatory cell infiltration (3.93%), whereas the positive control had the highest (21.60%). The lowest percentage of protein deposition in the tubules (7%) was found in the negative control, while the positive control recorded the highest (35.33%). The statistical test results for average congestion, hemorrhage, glomerular abnormalities (atrophy and edema), fatty degeneration, and necrosis showed significant differences ($P < 0.05$) among all treatments. The positive control demonstrated the highest damage, whereas the negative control exhibited the lowest damage.

Table 4. Kidney Histopathology of Albino Rats

Parameters (%)	Treatments				
	NC	PC	T1	T2	T3
Inflammatory Cell Infiltration	3.93 ± 0.97 ^a	21.60 ± 1.92 ^b	10.40 ± 1.40 ^c	14.37 ± 2.06 ^d	16.40 ± 2.22 ^d
Congestion	1.40 ± 0.13 ^a	10.27 ± 0.27 ^b	3.13 ± 0.50 ^c	5.17 ± 0.51 ^d	8.00 ± 0.33 ^e
Hemorrhage	4.60 ± 0.33 ^a	20.87 ± 0.81 ^b	9.60 ± 0.52 ^c	14.77 ± 0.68 ^d	16.13 ± 0.82 ^e
Necrosis	4.28 ± 1.04 ^a	34.67 ± 2.57 ^b	11.74 ± 2.15 ^c	22.70 ± 2.45 ^d	27.06 ± 2.45 ^e
Fatty Degeneration	2.49 ± 0.33 ^a	19.34 ± 0.81 ^b	6.19 ± 0.27 ^c	13.43 ± 0.69 ^d	17.60 ± 0.67 ^e
Glomerular Edema	9.31 ± 2.11 ^a	38.87 ± 3.10 ^b	14.07 ± 2.33 ^c	29.10 ± 4.21 ^d	33.98 ± 3.78 ^e
Glomerular Atrophy	6.76 ± 0.61 ^a	22.40 ± 1.23 ^b	12.30 ± 0.77 ^c	18.77 ± 0.94 ^d	20.56 ± 0.52 ^e
Protein Deposits	7.00 ± 2.52 ^a	47.17 ± 4.58 ^b	15.67 ± 3.99 ^c	31.00 ± 5.14 ^d	35.33 ± 6.25 ^d

Values (mean ± standard deviation) followed by different superscript letters (^a or ^b) in the same row indicate significant differences ($P < 0.05$). NC, negative control (distilled water, CMC-Na 0.5%). PC, positive control (NaNO₂, CMC-Na 0.5%). Treatment groups of NaNO₂ and therapy of *E. cottonii* extract dose 150 mg/kg BW (T1), 300 mg/kg BW (T2), and 450 mg/kg BW (T3).

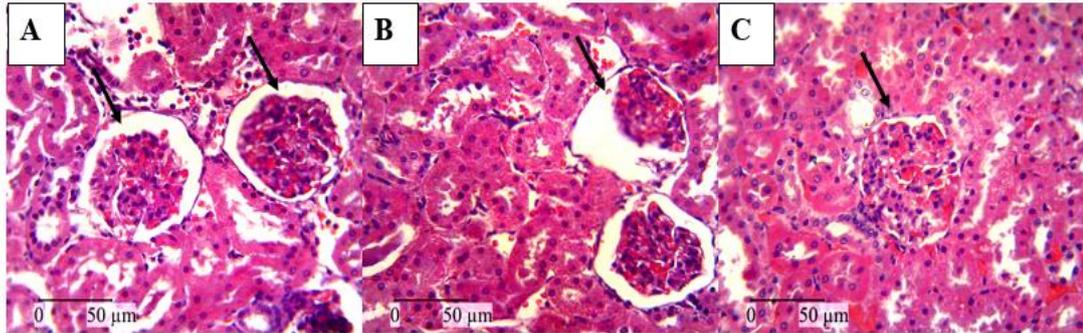


Fig.4: Histopathological abnormalities in the rat glomerulus. Normal glomerulus (A), atrophic glomerulus (B), and edema in the glomerulus (C) (Hematoxylin-Eosin staining, Magnification 400x, Bar = 50 µm).

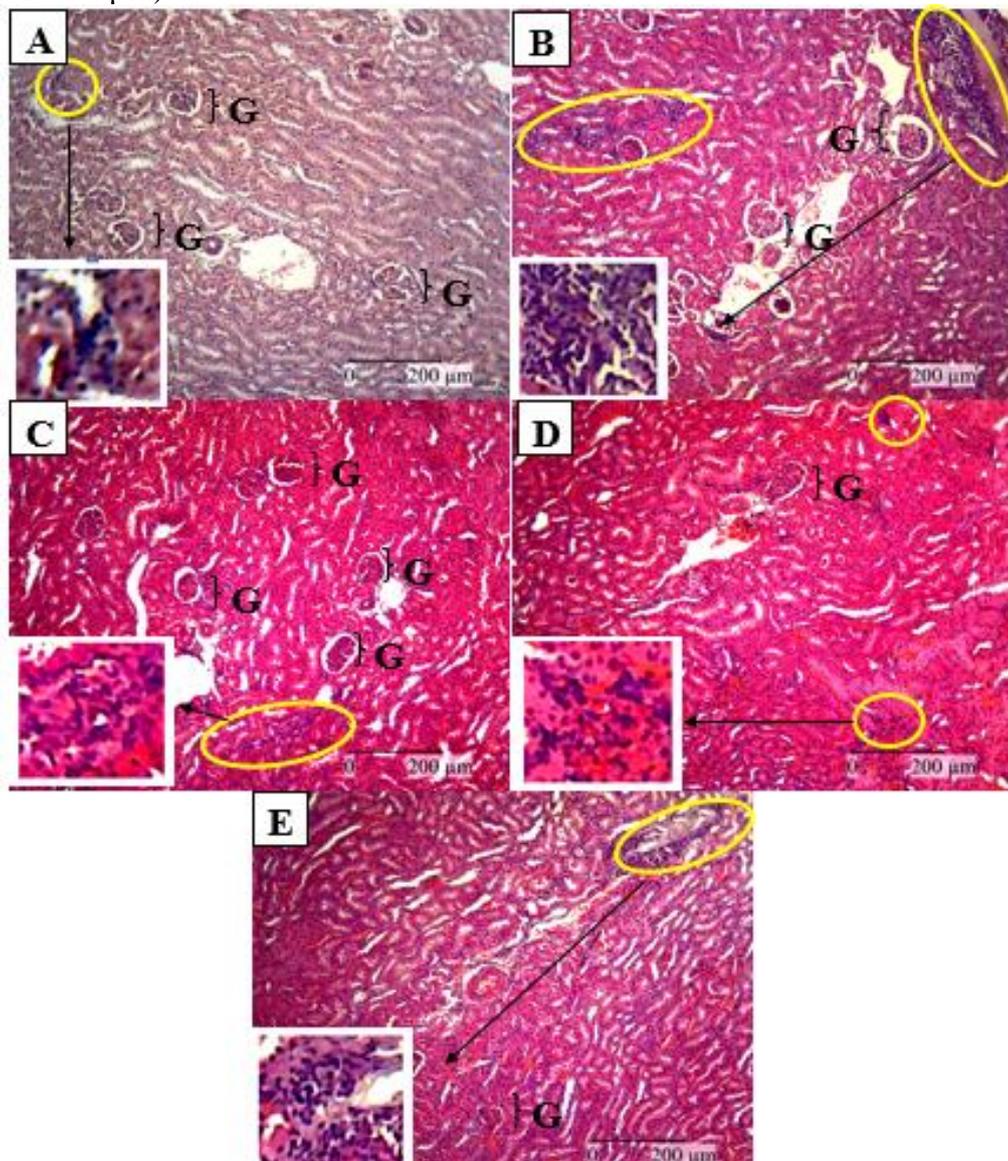


Fig.5: Kidney histopathology of inflammatory cell infiltration in albino rats. A = NC, negative control (distilled water, CMC-Na 0.5%); B = PC, positive control (NaNO₂, CMC-Na 0.5%); C = T1 (NaNO₂ + *E. cottonii* extract 150 mg/kg BW); D = T2 (NaNO₂ + *E. cottonii* extract 300 mg/kg BW); E = T3 (NaNO₂ + *E. cottonii* extract 450 mg/kg BW); G = Glomerulus, yellow circle = inflammatory cell infiltration (Hematoxylin-Eosin staining, 100x magnification, Bar = 200 µm).

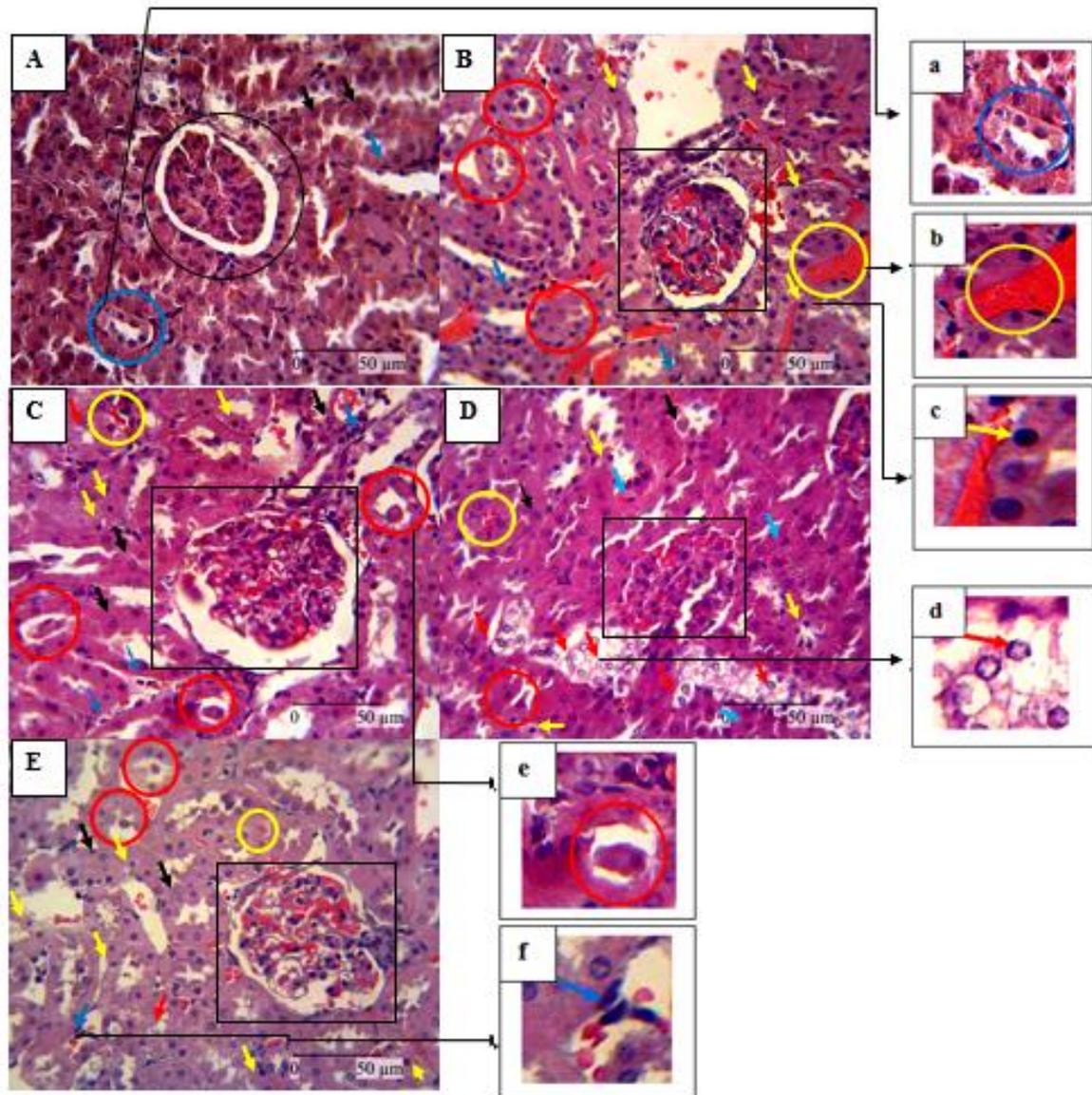


Fig.6: Kidney histopathology of albino rats. A = NC, negative control (distilled water, CMC-Na 0.5%); B = PC, positive control (NaNO₂, CMC-Na 0.5%); C = T1 (NaNO₂ + *E. cottonii* extract 150 mg/kg BW); D = T2 (NaNO₂ + *E. cottonii* extract 300 mg/kg BW); E = T3 (NaNO₂ + *E. cottonii* extract 450 mg/kg BW). Normal glomerulus (black circle), visible congestion in the glomerulus (black box), normal cells (black arrow), necrosis (yellow arrow), fatty degeneration (red arrow), inflammatory cell infiltration (blue arrow), hemorrhage (yellow circle), normal tubules (blue circle), protein deposits (red circle), hemorrhage (yellow circle). Insert normal tubules (a), hemorrhage (b), necrosis characterized by pyknotic nuclei (c), fatty degeneration (d), protein deposits in the tubules (e), inflammatory cell infiltration (f) (Hematoxylin-Eosin staining, 400x magnification, Bar = 200 µm).

DISCUSSION

The results of phytochemical tests in this study revealed the presence of flavonoid, triterpenoid, phenolic, saponin, and alkaloid compounds in the extract of *E. cottonii* (refer to Table 2). These findings align with previous research indicating that the methanol

extract of *E. cottonii* contains flavonoids, steroids, triterpenoids, and alkaloids (Andriani *et al.*, 2015). Another study reported that the methanol extract of *E. cottonii* only contained flavonoid, phenolic, and triterpenoid compounds (Maharany *et al.*, 2017). However, only the steroid compound

was found in the ethanol extract of *E. cottonii* (Arisanti, 2023). These differences can be attributed to various factors, including the choice of solvent during extraction and the environmental conditions in which the seaweed grows.

The extraction method employed in this research was the maceration method. The duration of maceration time plays a crucial role. The longer the maceration time, the greater the opportunity for contact between the material and the solvent, leading to continuous increases in results until the solvent reaches saturation. The solvent extraction method follows the like-dissolves-like principle, where compounds are attracted based on the polarity of the solvent (Zhang *et al.*, 2018).

The IC₅₀ value of *E. cottonii* extract in this study was determined to be 1279.62 mg/L. This value indicates that the antioxidant activity of *E. cottonii* extract is very weak. The ability to neutralize free radicals is classified as very strong if the IC₅₀ value is below 50 mg/L, strong if it falls between 50-100 mg/L, moderate if between 101-150 mg/L, and weak if between 150-200 mg/L. Antioxidant activity is considered very weak if the IC₅₀ value exceeds 200 mg/L (Molyneux, 2004). *E. cottonii* belongs to the Rhodophyceae class (red seaweed), which contains pigments such as phycoerythrin, carotenoids (especially beta carotene), and chlorophyll-a. These pigments possess the potential to function as natural antioxidants (Pereira *et al.*, 2012).

Normal hemoglobin levels in albino rats typically range from 11.6 to 16.1 g/dL (Douglas and Wardrop, 2010). Consequently, the hemoglobin levels of all groups in this study remained within the normal range, except for the positive control. This study revealed a significant decrease of 23.89% in hemoglobin levels in the positive control.

Sodium nitrite (NaNO₂) reduces hemoglobin levels in albino rats because the nitrite content in NaNO₂, upon entering the circulatory system, initiates an oxidative reaction that transforms hemoglobin into methemoglobin. Methemoglobin is a variant

of hemoglobin containing iron in the form of Fe³⁺ (Aita and Faten, 2014). As the nitrite ion (NO₂⁻) enters erythrocytes, it binds with Hb and O₂, creating bonds between nitrogen dioxide (NO₂[•]), Hb₂+NO₂⁻, and hydrogen peroxide (H₂O₂). The resulting nitrogen dioxide oxidizes the Fe²⁺ ions present in blood hemoglobin, converting them into Fe³⁺ ions, thus altering the iron form in hemoglobin to Fe³⁺ (methemoglobin) (Spagnuolo *et al.*, 1987). Methemoglobin cannot bind oxygen, unlike the normal function of hemoglobin. Consequently, elevated levels of methemoglobin and reduced levels of hemoglobin in the blood gradually induce tissue hypoxia. This condition is referred to as methemoglobinemia (Astuti and Rini, 2006).

In the first pathway, nitrite ions that have entered the bloodstream will convert hemoglobin into methemoglobin and decompose into O₂^{-•} (superoxide anion radical and NO⁻ or nitric oxide). The reaction of O₂^{-•} and NO⁻ will produce ONOO⁻ (peroxynitrite anion), with ONOO⁻ being produced three times faster than the SOD (superoxide dismutase) enzyme catalyzing O₂^{-•} to H₂O₂ (hydrogen peroxide). Increased ONOO⁻ production will cause damage to proteins in the erythrocyte membrane resulting in hemolysis. In the pathway, the H₂O₂ produced will be catalyzed by the catalase enzyme, transforming it into H₂O and O₂. However, the presence of nitrite ions inhibits the catalase enzyme's function, preventing the breakdown of H₂O₂ into H₂O and O₂. Oxidative stress caused by nitrite occurs due to the accumulation of H₂O₂ into OH[•] (hydroxyl radicals). Furthermore, OH[•] oxidizes polyunsaturated fatty acids in cell membranes, inducing the formation of ROOH (hydroperoxide), and producing malondialdehyde (MDA) as a sign of lipid peroxidation. This triggers disruption of the erythrocyte membrane resulting in hemolysis (Campos *et al.*, 2018).

E. cottonii extract doses of 150 and 300 mg/kg BW (T1 and T2) significantly increased hemoglobin levels (38.83% and 31.76%, respectively), and the number of

erythrocytes (39.26% and 33.55%, respectively), compared to the positive control. This increase is attributed to the *E. cottonii* extract containing flavonoids which act as antioxidants. Flavonoids can minimize the oxidation of hemoglobin, which binds to oxygen (oxyhemoglobin), preventing it from being converted to methemoglobin. This allows hemoglobin to continue carrying out its role in oxygen binding (Gebicka and Banasiak, 2009). *E. cottonii* also contains Fe and vitamin C. The vitamin C content speeds up the absorption process of iron. In the gastrointestinal tract, iron (Fe) will be reduced from ferric (Fe^{3+}) to ferrous (Fe^{2+}) which is more easily absorbed and needed in hemoglobin synthesis, thereby increasing blood hemoglobin levels (Rahmadani *et al.*, 2021).

Lipophilic flavonoids can bind to cell membranes, in this case, erythrocytes, and protect against cell membrane damage due to oxidative stress, preventing the erythrocyte membrane from experiencing damage and hemolysis (Kitagawa *et al.*, 2004). Flavonoids, which act as natural antioxidants, can donate H^+ (hydrogen) atoms to radical compounds that have the property of damaging erythrocyte membranes (Banjarnahor and Artanti, 2014). According to Sundaryono (2011), the flavonoid compounds contained in *Gynura segetum* leaf extract can increase the number of erythrocytes in mice.

The higher dose of *E. cottonii* extract administered was followed by a decrease in the number of erythrocytes and hemoglobin levels in albino rats. This is attributed to the saponin content of *E. cottonii* extract. Saponin content in certain amounts can trigger hemolysis. Saponin diffuses into the erythrocyte membrane and binds to cholesterol, disrupting and reducing the stability of the erythrocyte membrane. This causes the cytoplasm to leak out of the cell and results in hemolysis (Netala *et al.*, 2014). The decrease in the number of erythrocytes and hemoglobin was significant in the T3 treatment (extract dose of 450 mg/kg BW). Therefore, it is suspected that the amount of

saponin at this dose had a negative impact through the mechanism of damaging the permeability of the red blood cell membrane resulting in hemolysis.

The administration of NaNO_2 without any therapy of *E. cottonii* extract to the animals (the positive control) showed a significant increase in the number of leukocytes compared to the negative control. The highest leukocyte count was observed in the positive control ($9.11 \times 10^3/\mu\text{L}$), while the lowest was in the negative control ($2.56 \times 10^3/\mu\text{L}$). The normal range for albino rat leukocyte counts is $2-10 \times 10^3/\mu\text{L}$ (Douglas and Wardorp, 2010). Therefore, the leukocyte counts of all groups were within the normal range. These results align with research that reported the administration of 50 mg/kg BW NaNO_2 for 20 days caused a significant increase in the number of leukocytes compared to controls (Ivanov *et al.*, 2016).

An increase in the number of leukocytes in the positive control due to long-term administration of NaNO_2 causes tissue hypoxia. Tissue hypoxia occurs as a result of a decrease in hemoglobin levels and the number of erythrocytes (Astiti and Rini, 2006). Tissue hypoxia leads to cell injury and even cell death (Guo *et al.*, 2019). The increase in the number of cells experiencing damage and death triggers an inflammatory response. Inflammation is a physiological response to stimulation in the form of infection or tissue injury (Chen *et al.*, 2018). Leukocytes serve as the first line of defense or the main immune cells, along with plasma cells and mast cells. When tissue damage occurs, leukocytes migrate through the blood capillary walls to the damaged tissue and phagocytose damaged and dead cells (cell debris).

Administration of *E. cottonii* extracts at doses of 150, 300, and 450 mg/kg BW after NaNO_2 administration can reduce the number of leukocytes by 15.03%, 25.57%, and 56.64%, respectively. This reduction is attributed to the flavonoid content in the extract, acting as an antioxidant that can mitigate the effects of free radicals caused by NaNO_2 . Consequently, the inflammatory

response due to cell damage and death is diminished. Additionally, flavonoids function as anti-inflammatory compounds, inhibiting the accumulation of leukocytes in inflammatory areas (Tasya and Kustiawan, 2023). Flavonoids in *Gynura segetum* leaf extract have been reported to reduce the number of leukocytes in mice (Sundryono, 2011).

The administration of sodium nitrite (NaNO_2) at a dose of 22.5 mg/kg BW for 35 days significantly increased renal histology damage in this study when compared with the negative control, and the most severe kidney histological damage was found in the positive control (rats were given NaNO_2 without any therapy of *E. cottonii* extract). Kidney histological damage was assessed through parameters such as the percentage of congestion, inflammatory cell infiltration, hemorrhage, cells undergoing fatty degeneration, pyknotic nuclear necrosis, glomerular abnormalities, and protein deposits in the kidneys' tubules (Figure 5-7).

The results of this research align with studies in which rats given a dose of 80 mg/kg BW of NaNO_2 experienced an increase in kidney damage. NaNO_2 causes serious damage to kidney tissue, leading to a significant increase in the level of pro-inflammatory cytokines in the tissue, thereby triggering inflammation. The toxic effect of NaNO_2 when it binds to amino or amide groups, results in the formation of nitrosamine and nitrosamide derivatives, known to be carcinogenic and cause cell damage due to inflammation and oxidative stress (Uslu *et al.*, 2019).

Kidney damage due to administration of NaNO_2 can also occur as a result of the accumulation of NaNO_2 , leading to the formation of NO (nitric oxide), where the excretion of NO metabolites always undergoes a filtration mechanism in the renal glomerulus (Carlstrom, 2021). This leads to an accumulation of NO in the kidneys, resulting in kidney damage, which in this study was characterized by damage to the abnormal tubular and glomerular epithelial cells. The entry of toxic compounds into the

kidney can cause damage to the renal tubular epithelial cells (Fahrimal *et al.*, 2016). Treatment T1, involving the administration of *E. cottonii* extracts at a dose of 150 mg/kg BW, significantly reduces the average percentage of kidney histology damage. This reduction is evidenced by decreases in the percentage of inflammatory cell infiltration (51.85%), congestion (69.52%), hemorrhage (54%), cells experiencing fatty degeneration (67.99%), necrosis with pyknotic nuclei (66.13%), protein deposits in the tubules (66.77%), and glomerular abnormalities in the form of atrophy and edema (45.08% and 63.80%, respectively).

E. cottonii extract demonstrated the ability to reduce kidney histological damage in this study due to the presence of phytochemicals that can act as an antioxidant. Alkaloids found in the extract, efficiently halt free radical chain reactions by counteracting hydroxyl radicals. Alkaloids are often found in polar solvents because the class of alkaloid compounds that have the potential to act as antioxidants are polar compounds that will be extracted in polar solvents (Maryuni *et al.*, 2022). The *E. cottonii* extract also contains phenolic compounds, which also possess antioxidant capacity similar to flavonoid compounds, directly reducing radical compounds such as hydroxyl, peroxy, and superoxide radicals, and can increase the activity of antioxidant enzymes (Walter and Marchesan, 2011).

As the dose of *E. cottonii* extract increased, the level of kidney damage in albino rats in this study also increased. The saponin content in *E. cottonii* extract has toxic properties. At high concentrations, saponin content can cause hemolysis (Ambarwati, 2012). Hemolysis results in an increase in the amount of free hemoglobin released into the blood plasma. This free hemoglobin is unstable and will later break down into alpha-beta dimers, which bind to haptoglobin and then phagocytosed by macrophages. Haptoglobin, a protein found in plasma, plays a role in binding free hemoglobin that is released from erythrocytes into plasma during hemolysis. The binding of hemoglobin alpha-

beta dimer to haptoglobin prevents hemoglobin from being excreted through the kidneys. During hemolysis, haptoglobin can bind 1.25 g/L of free hemoglobin in plasma. If hemolysis is continuous, it will cause the amount of haptoglobin in the blood to decrease continually. When haptoglobin is depleted, unbound hemoglobin dimers are excreted by the kidneys as free hemoglobin, leading to the deposition of hemoglobin dimers in the kidneys. It can cause kidney damage (Lim *et al.*, 2000).

CONCLUSIONS

In albino rats induced by NaNO₂, *E. cottonii* seaweed extract doses of 150 and 300 mg/kg BW increased hemoglobin levels and erythrocyte counts significantly, while the dose of 450 mg/kg BW reduced the number of leukocytes. The *E. cottonii* extract dose of 150 mg/kg BW reduced the level of kidney damage based on kidney histology.

Declarations:

Ethical Approval

This research protocol was approved by Animal Ethics Committee at the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia (ID. No.4/UN14.2.9/PT.01.04/2020).

Competing interests: The authors declare no conflict of interest.

Contributions:

I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

Funding: No funding was received.

Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

REFERENCES

- Afiqoh, A.N., Ika, F., and Ety, S.H. (2017). The effect of pegagan leaf (*Centella asiatica*) ethanol extract on spleen histopathology in rat (*Rattus norvegicus*) induced by sub-acute sodium nitrite. *Jurnal Kedokteran Unila*, 1(3):457-462.
- Aita, N.A., and Faten, F.M. (2014). Effect of marjoram oil on the clinicopathological, cytogenetic, and histopathological alterations induced by sodium nitrite toxicity in rats. *Global Veterinaria*, 12(5):606-616.
- Ambarwati, R. (2012). Effect of sodium nitrite (NaNO₂) on erythrocyte and hemoglobin profile in white rat (*Rattus norvegicus*). *Folia Medica Indonesiana*, 48(1):1-5.
- Andriani, Z., Fasya, G.A., and Hanapi, A. (2015). Antibacterial activity of the red algae *Eucheuma cottonii* extract from Tanjung coast, Sumenep Madura. *Alchemy*, 4(2):93-100.
- Arisanti, A. (2023). Phytochemical constituent and antioxidant activity evaluation of red seaweed *Eucheuma* sp. *Indonesian Journal of Medical Chemistry and Bioinformatics*, 2(1):1-10.
- Astiti, Y., and Rini, H. (2006). Comparison of methemoglobin and hemoglobin concentration between individuals living in the malaria-endemic area and non-malaria-endemic area. *Mutiara Medika*, 6(2):106-112.
- Banjarnahor, S.D.S., and Artanti, N. (2014). Antioxidant properties of flavonoids. *Medical Journal of Indonesia*, 23(4):239-244.
- Campos, E.S., Solana, M.A.V., Almazan, M.I.C., Dominguez, C.A., Rivera, J.L.H., Sanchez, D.L., Arenas, G.G., and Valencia, A.T. (2018). Nitrate and nitrite in drinking water affect antioxidant enzymes in erythrocytes of rats. *The Ukrainian Biochemical Journal*, 90(4):90-101.
- Carlstrom, M. (2021). Nitric oxide signaling in kidney regulation and cardiometabolic health. *Nature Reviews Nephrology*, 1-16. <https://www.nature.com/articles/s41581-021-00429-z>
- Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., and Zhao,

- L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9(6):7204-7218.
- Dewi, I.A.R.P., Wiratmini, N.I., and Setyawati, I. (2020). Hepatoprotector of *Eucheuma cottonii* seaweed on male white rat (*Rattus norvegicus* L.) T that induced by natrium nitrite (NaNO₂). *Jurnal Metamorfosa*, 7(2):86-96.
- Douglas, J.W., and Wardrop, K.J. (2010). Schalm's veterinary hematology. 6th edition. Blackwell Publishing, USA.
- Fahrimal, Y., Rahmiwati, and Aliza, D. (2016). Histopathology of male rat (*Rattus norvegicus*) kidney infected with *Trypanosoma evansi* and treated with sernai leaves extract (*Wedelia biflora*). *Jurnal Medika Veterinaria*, 10(2):166-170.
- Gebicka, L., and Banasiak, E. (2009). Flavonoids as reductants of ferric hemoglobin. *Acta Biochimica Polonica*, 55(3):509-513.
- Guo, Y., Tan, J., Miao, Y., Sun, Z., and Zhang, Q. (2019) Effects of microvesicles on cell apoptosis under hypoxia. *Oxidative Medicine and Cellular Longevity* Vol. 2019, Article ID 5972152, 11 pages. <https://doi.org/10.1155/2019/5972152>.
- Helal, E., Zahkok, S., Ghada, Z.A., Al-Kassas, M., and Abde, W.H. (2008). Biochemical studies on the effect of sodium nitrite and/or glutathione treatment on male rats. *The Egyptian Journal of Hospital Medicine*, 30(1):25-38.
- Indriana, E. and Titrawani. (2022). Effects of natural extract of celery leaves on the kidneys of white rats (*Rattus norvegicus*) after aspirin induction. *Jurnal Pendidikan Matematika dan IPA*, 13(2):277-284.
- Ivanov, I., Gluhcheva, Y., Petrova, E., and Gikova, M. (2016). Food preservatives and sport. sodium nitrite (E250)'s influence on hematometrical parameters of white blood cells and platelets in rats. *Research in Kinesiology*, 44(1):67-70.
- Kitagawa, S., Sakamoto, H., and Tano, H. (2004). Inhibitory effect of flavonoids on free radical-induced hemolysis and their oxidative effect on hemoglobin. *Chemical and Pharmaceutical Bulletin*, 52(8):999-1001.
- Lim, Y.K., Jenner, A., Ali, A.B., Wang, Y., Hsu, S.I., Chong, S.M., Bauman, H., Halliwell, B., and Lim, S.K. (2000). Haptoglobin reduces renal oxidative DNA and tissue damage during phenylhydrazine-induced hemolysis. *Kidney International*, 58(3):1033-1044.
- Maharany, F., Nurjanah, Suwandi, R., Anwar, E., and Hidayat, T. (2017). Bioactive compounds of seaweed *Padina australis* and *Eucheuma cottonii* as sunscreen raw materials. *Jurnal Pengolahan Hasil Perikanan Indonesia*, (20)1:10-17.
- Maryuni, D.R., Prameswari, D.A., Astari, S.D., Sari, S.P., and Putri, D.N. (2022). Identification of active compounds in red onion (*Allium ascalonicum* L.) peel extract by Lc-Esi-Qtof-Ms/Ms and determination of its antioxidant activity. *Jurnal Teknologi Hasil Pertanian*. 15(1): 20-33.
- Molyneux, P. (2004). The use of stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarinn Journal Science Technology*, 26(2):211-219.
- Netala, V.R., Ghosh, S.B., Bobbu, P., Anitha, D., and Tarte, V. (2014). Triterpenoid saponins: a review on biosynthesis, applications and mechanism of their action. *Journal of Pharmaceutical Sciences*, 7(1):24-28.
- Ozen, H., Ufuk, K., Musa, K., Suleyman, G., Emine A., Kadir, O., and Onur, A. (2014). Histopathologic,

- biochemical, and genotoxic investigation on chronic sodium nitrite toxicity in mice. *Experimental and Toxicologic Pathology*, 66(1):367-375.
- Pereira, D.C., Trigueiro, T.G., Colepicolo, P., and Marinho-Soriano, E. (2012). Seasonal changes in the pigment composition of natural population of *Gracilaria dumingensis* (Graciliales. Rhodophyta). *Brazilian Journal of Pharmacognosy*, 22:874-880.
- Podungge, A., Damongilala, L.J., and Mewengkang, H.W. (2018). Antioxidant content in seaweed *Eucheuma spinosum* extracted with methanol and ethanol. *Jurnal Media Teknologi Hasil Perikanan*, 6(1):1-5.
- Rahmadani, G., Almurdi, and Endrinaldi. (2021). The effect of vitamin C consumption on increasing hemoglobin and ferritin levels of pregnant anemia who receive blood supplements. *Science Midwifery*, 10(1):1-5.
- Sambodo, D.K. (2019). Antioxidant activity of Sumbawa red seaweed (*Eucheuma cottonii*) extract and lemon peel (*Citrus limon* L) extract combination. *Jurnal Ilmiah Farmasi* 15(2):86-91.
- Shakil, M.H., Trisha, A.T., Rahman, M., Talukdar, S., Kobun, R., Huda, N., and Zaman, W. (2022). Nitrites in cured meats, health risk issues, alternatives to nitrites: a review. *Foods* 11: 3355.
- Spagnuolo, C., Rinelli, P., Coletta, M., Chiancone, E., and Ascoli, F. (1987). Oxidation reaction of human oxyhemoglobin with nitrite: a reexamination. *Biochimica et Biophysica Acta*, 911:59-65.
- Sundayono, A. (2011). Testing the activity of total flavonoid compounds from *Gynura segetum* (Lour) on increasing erythrocytes and decreasing leukocytes in mice (*Mus musculus*). *Jurnal Exacta*, 9(2):8-16.
- Tasya, S.C., and Kustiawan, P.M. (2023). Bioactivity of purple sweet potato (*Ipomoea batatas*) as an anti-inflammatory agent: a review. *Journal Syifa Sciences and Clinical Research (JSSCR)*, 5(1):91-100
- Uslu, G.A., Uslu, H., and Adali, Y. (2019). Hepatoprotective and nephroprotective effects of *Trigonella foenum-graecum* L. (Fenugreek) seed extract against sodium nitrite toxicity in rats. *Biomedical Research and Therapy*, 6(5):3142-3150.
- Walter, M., and Marchesan, E. (2011). Phenolic compounds and antioxidant activity of rice. *Journal of Brazilian Archives of Biology and Technology*, 54(1):371-377.
- Wientarsih, I., Harlina, E., Purwono, R.M., and Utami, I.T. (2014). Activities study of ethanol extracts of avocado leaves (*Persea americana* Mill) to nephrotoxic compound of rat's kidney. *Jurnal Veteriner*, 15(2):246-251.
- Zhang, Q.W., Lin, L.G., and Ye, W.C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine*, 13(20):26pages. doi: 10.1186/s13020-018-0177-x. PMID: 29692864; PMCID: PMC5905184.