LOCUS JOURNAL: Penelitian & Pengabdian
Volume 4 No. 12 Desember 2025
E-ISSN 2829-7334| P-ISSN 2829-5439
Homepage: https://locus.rivierapublishing.id/index.php/jl

Nephroprotective Effect of Neem Leaves Ethanol Extract on NaCl and Hydrocortisone-Induced Hypertensive Rats

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Abstract

Kidney failure is one of the main complications of hypertension and has the potential to cause death. Decreased kidney function can be indicated by increased levels of creatinine in the blood. Neem leaves (*Azadirachta indica*) contain flavonoids, saponins, tannins, phenolics, and terpenoids, which have antihypertensive and nephroprotective potential. This study used 36 male Wistar rats as test animals. The rats were divided into six groups: positive control (captopril 25 mg/kg BW), negative control (0.5% CMC-Na), neem leaf ethanol extract groups at doses of 75, 150, and 300 mg/kg BW, and normal control. All groups except the normal control were induced with 2.5% NaCl solution (2 mL, p.o.) and hydrocortisone (5 mg/kg BW, s.c.) for 10 days, followed by treatment according to each group for 14 days orally. The Kruskal-Wallis test results showed significant differences between groups ($P < 0.05$). In the Mann-Whitney test, the neem leaf ethanol extract groups at doses of 75, 150, and 300 mg/kg BW showed significant differences compared to the negative control. This proves that all three doses of the extract can lower blood pressure and creatinine levels. In kidney histopathology, there was damage to the tubules and interstitium. In the neem leaf ethanol extract group at a dose of 300 mg/kg BW, there was improvement in the renal tubules and interstitium. Based on these results, it can be concluded that the neem leaf ethanol extract, particularly at 300 mg/kg BW, can lower creatinine levels and improve kidney histopathology in hypertensive rats induced by NaCl and hydrocortisone.

Keywords: *Neem, hypertension, creatinine, NaCl, hydrocortisone*

INTRODUCTION

Hypertension is characterized by elevated blood pressure levels, with systolic readings at or above 140 mmHg and diastolic readings at or above 90 mmHg (Nadila, 2014). Globally, approximately 1.04 billion adults—or 31.5% of the population in low-income countries—are affected by hypertension, while in high-income countries, the condition impacts around 349 million individuals, accounting for 28.5% of their adult population (Mills et al., 2020). Individuals diagnosed with hypertension are over 21 times more likely to develop chronic kidney disease than those with normal blood pressure (Nugraha & Utama, 2023). In Indonesia, 2020 data revealed that 61% of individuals diagnosed with hypertension also experienced chronic kidney failure (Afiatin et al., 2023). Kidney failure is a major complication of hypertension that can lead to life-threatening outcomes (Muaddi et al., 2022). One key indicator of impaired kidney function is elevated blood creatinine levels (Kadir, 2016).

Administration of NaCl increases blood pressure in rats (Jannah et al., 2018; Lajania et al., 2018), and rats induced with hydrocortisone also exhibit elevated blood pressure (Laviolle

et al., 2014). Research by Rafiq et al. (2011) shows that administering NaCl and hydrocortisone to rats elevates blood pressure and induces morphological kidney alterations, such as glomerulosclerosis and podocyte damage. Therefore, this study used hypertensive rats induced with NaCl and hydrocortisone as the animal model. Numerous plants can serve as medicine, including the *neem* plant (*Azadirachta indica*).

Several studies have shown that *neem* leaves are rich in active compounds such as gallic acid, catechin, coumarin, rutin, quercitrin, quercetin, kaempferol, nimbin, nimbidin, nimbolide, and limonoids. Reported biological and pharmacological activities of *neem* leaves include antibacterial, antifungal, cardioprotective, hepatoprotective, neuroprotective, *nephroprotective*, and anti-inflammatory effects (Alzohairy, 2016; Cristo et al., 2016; Rahmani et al., 2018).

Methanol extract of *neem* leaves exhibits antihypertensive effects (Omóbòwálé et al., 2020). Additionally, the *nephroprotective* effect of ethanol extract from *neem* leaves has been demonstrated in hyperglycemic rats (Utami et al., 2023) and under oxidative stress conditions (Moneim et al., 2014). It is crucial to investigate the *nephroprotective* activity of *neem* leaves under hypertensive conditions. This study specifically evaluates the *nephroprotective* effects of ethanol extract from *neem* leaves in hypertensive conditions.

METHOD

Materials used: neem leaves, ethanol, neem leaves, sulfanilic acid, NaNO2, NaOH, HCl, 25% ammonia, chloroform, distilled water, Mg powder, amyl alcohol, FeCl3, NaCl, gelatin, reagan Dragendorff, reagent Mayer, reagen Bouchardat, anhydrous acetic acid, H2SO4, hydrocortisone injection (Fartison®), captopril tablets, CMC-Na 0.5% and creatinine reagent (Dyasis®), the test animals were male white Wistar rats aged 2-3 months with a body weight of 150-250 grams. Equipment used: glassware, analytical scales,silica gel plate 60 F254, UV lamps 254 and 366 nm, microhematocrit, eppendorf, probe, centrifuge, micropipet, CODA And Microlab 300.

Neem leaves were obtained from Rembang, Central Java. The harvested neem leaves were wet sorted and washed. Furthermore, the neem leaves were dried in a drying cabinet. The dried neem leaves were then ground with a blender and sieved (Hayong et al., 2019; Rohmawaty & Yunivita, 2016). Neem leaves were extracted using the remaceration method using 96% ethanol (1:10) at room temperature for 3 x 24 hours. The macerate was concentrated using a rotary vacuum evaporator at 40°C and evaporated over a water bath until a thick extract was formed (Soraya et al., 2019; Suhartono et al., 2023).

The prepared neem leaf ethanol extract was then subjected to phytochemical screening tests with preliminary test procedures and TLC to evaluate the presence of phytochemical compounds such as alkaloids, flavonoids, tannins, saponins, and terpenoids. The animals used were male Wistar rats weighing 150-250 g. Animals were acclimatized for 7 days. The procedures and methods in this study have been approved by the ethics committee of the

Semarang Pharmacy Foundation College of Pharmacy with number 654 / EVN-NA / KEPK / STIFAR / EC / VII / 2024.

Animal testing was carried out in the Integrated Biomedical Laboratories (IBL) FK UNISSULA animal test laboratory. 36 male Wistar rats were adapted for 7 days, then 30 rats were induced with 2.5% NaCl (2 ml, p.o.) and 5 mg/kgbb hydrocortisone (s.c.) for 10 days (Azyenela et al., 2021; Rafiq et al., 2011). The test animals were grouped into 6 groups, namely positive control (captopril 25 mg/kgbb), negative control (CMC-Na 0.5%), neem leaf ethanol extract group with doses of 75, 150, 300 mg/kgbb, and normal control. Then continued with the provision of treatment according to each group for 14 days orally. Blood pressure and creatinine levels were measured on day 1 (before induction), day 11 (after induction) and day 25 (after treatment). On the 25th day, the rats were sacrificed and their kidneys were taken to observe kidney histopathology.

Blood pressure was measured using the non-invasive direct tail-cuff method with the CODA Instrument. The rat was placed in the holder. The rat's tail was inserted into the "Cuff Occlusion" as close as possible to the base of the tail, and the rat's tail was inserted into the "VPR Cuff" within 2 mm of the "Occlusion Cuff". Open the CODA software, then measure the rat's blood pressure. The measured blood pressure is in mmHg.

Blood was taken through the rat's eye vein, the blood was centrifuged at 3000 rpm for 20 minutes and the serum was taken. The serum obtained was then measured for creatinine levels using Microlab 300 with Dyasis® reagent. Creatinine levels were measured by reacting 1000 µl of R1 reagent and 250 µl of R2 reagent with 50 µl of serum. Creatinine levels were measured in mg/dL.

Tissuenbiopsies werekpreserved in 10% Neutral Buffer Formalin (NBF) in order to quantify histological alterations. Each sample was stained with H&E following tissue processing and sectioning. The samples were interpreted by a pathologist. Each sample was assigned a score according to the tubular, endothelial, glomerular, and interstitial sections of the EGTI renal histology scoring system. Data were analyzed using SPSS 23 with non-parametric tests Kruskal Wallis and Mann Whitney.

RESULTS AND DISCUSSION

Ethanol extract of neem leaves obtained as much as 184.79 grams with a yield of 18.46%. The results of the phytochemical screening test listed in table 2 show that the ethanol extract of neem leaves contains flavonoids, tannins, saponins and terpenoids.

Table 1. Phytochemical Screening of Neem Leaves Ethanol Extract

Phytochemical	Observation
Flavonoids	+
Alkaloids	-
Tannins	+
Saponins	+
Terpenoids	+

Description: (+) present; (-) absent

The nephroprotective activity of the extract was tested in a rat model of hypertension induced by NaCl and hydrocortisone solutions. Administration of NaCl and hydrocortisone induction has been shown to increase blood pressure and cause morphological changes in the kidneys of rats (Rafiq et al., 2011). In high salt conditions (due to administration of NaCl solution), dendritic cells or macrophages become active due to increasednintracellular sodium entering through the epithelial sodium channel (ENaC). Sodiumkactivates NADPHhoxidase and inflammation which causes the formation of additional products of the Isolevuglandin protein (IsoLGs) which are processed into major histocompatibility molecules and presented to T cells. Activated T cells will trigger the production of inflammatory cytokines IFN-γ, TNF-α and IL-17A which cause hypertension (Masenga & Kirabo, 2023).

Hydrocortisone (cortisol) can increase blood pressure accompanied by changes in the Renin Angiotensin Aldosterone System (RAAS), and the activity of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) (Bailey, 2017; Buning et al., 2016). Hydrocortisone stimulates mineralocorticoid receptors in the kidney by increasing the transcription of two mineralocorticoid receptor target genes, serum and glucocorticoid-regulated kinases-1 (Sgk-1) and Na+/H+ exchanger isoform-1 (NHE1). Increased Sgk-1 activity is thought to be a risk factor for accelerating kidney damage (Buning et al., 2016; Rafiq et al., 2011; Sierra-Ramos et al., 2021).

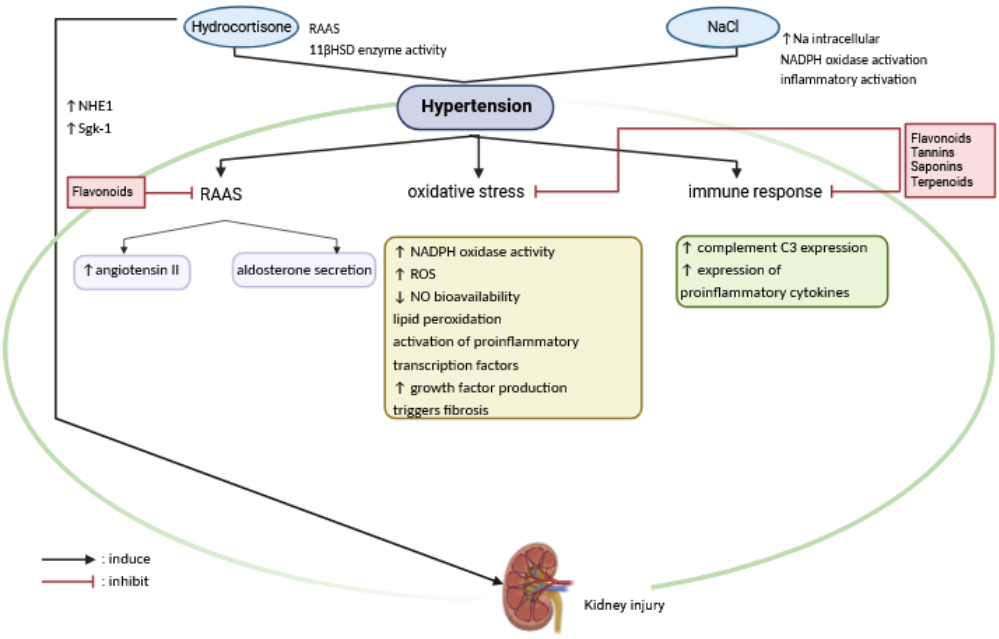

Figure 1. Induction mechanism of NaCl and hydrocortisone and inhibition mechanism of compounds in ethanol extract of neem leaves. (Created in https://BioRender.com)

The results of the observation showed an increase in systolic and diastolic blood pressure in mice after induction for 10 days (Table 3). After statistical analysis using the Paired T-test, it was found that there was a significant difference ($p < 0.05$) between the initial blood pressure and blood pressure on the 11th day (after induction). This means that the induction carried out has conditioned the test animals to experience hypertension.

Giving treatment according to each group for 14 days appeared to cause a decrease in blood pressure in several groups. In the negative control group, blood pressure that increased due to induction did not change much even though the induction had been stopped. In the positive control group, mice treated with 25 mg/kgbw captopril experienced a decrease in systolic and diastolic blood pressure. Captopril is used as a positive control in this case, in addition to being an antihypertensive, it is also a nephroprotective, so the dose used is 25 mg/kgbw. Captopril is a group of ACE-inhibitors that are used as the first line in the management of hypertension (Kemenkes, 2021). The blood pressure of the positive control group when compared to the negative control showed a significant difference ($p<0.05$). This means that the method used is correct and the way of working is correct.

The results of the statistical analysis of the group with the administration of neem leaf ethanol extract at doses of 75, 150 and 300 mg/kgbb, there was a significant difference ($p<0.05$) compared to the negative control group. The three groups of neem leaf ethanol extract doses when compared to the positive control group showed no significant difference ($p>0.05$). In addition, the same significance value ($p>0.05$) was also shown in the comparison of blood pressure in mice between the three groups of neem leaf ethanol extract doses. The meaning of the results of these analyses is that neem leaf ethanol extract can lower blood pressure, where its activity is comparable to captopril and this activity does not change even if the extract dose is increased.

Table 2. Systolic-Diastolic Blood Pressure of Rats during Nephroprotective Activity Testing of Ethanol Extract of Neem Leaves (*Azadirachta indica*) in NaCl and Hydrocortisone Induced Hypertensive Rats

Group	T1	T11	T25	%Decrease
		Systolic		
Positive control	124,11±6,95	151,50±8,29*	120,56±6,90[a]	20,20±6,16
Negative control	122,11±8,66	149,06±5,03*	166,78±16,46	-12,18±13,30
E75	123,56±5,73	155,17±9,44*	119,61±8,79[ab]	22,73±6,24
E150	127,11±3,35	157,94±7,43*	122,72±3,81[ab]	22,06±5,36
E300	124,11±6,60	153,50±6,58*	121,00±7,36[ab]	21,14±4,15
Normal control	113,72±12,98	114,72±11,85	117,89±5,96[ab]	-3,57±9,00
		Diastolic		
Positive control	88,39±10,92	107,56±6,48*	88,33±6,24[a]	17,63±7,22
Negative control	88,89±3,65	105,17±9,95*	123,11±19,33	-18,89±26,02
E75	90,56±4,95	111,06±9,52*	85,78±8,08[ab]	22,25±9,48
E150	85,89±4,85	114,61±9,69*	90,06±6,85[ab]	20,84±9,04
E300	84,78±4,66	104,89±10,27*	82,56±6,95[ab]	20,72±9,21
Normal control	82,83±9,69	82,94±11,96	84,44±4,74[ab]	-3,48±13,07

Note : Values are expressed as mean ± SD n=6; *p<0.05 when compared to T1; [a]p<0.05 when compared with the negative control group; [b]p>0.05 when compared with the positive control group.

High blood pressure can narrow blood vessels and eventually damage and weaken blood vessels throughout the body, including the kidneys. If the renal blood vessels experience problems, it will subsequently lead to kidney damage (De Bhailis & Kalra, 2022). Induction using NaCl and hydrocortisone in this study not only caused an increase in blood pressure but also proved to have caused kidney damage. This condition is evident from the increase in serum creatinine levels after induction, as shown in Figure 2. The results of the statistical analysis using the Paired T-test showed a significant difference (p<0.05) between the initial serum creatinine levels and the levels on day 11 (after induction), proving that the kidney damage occurred due to the induction.

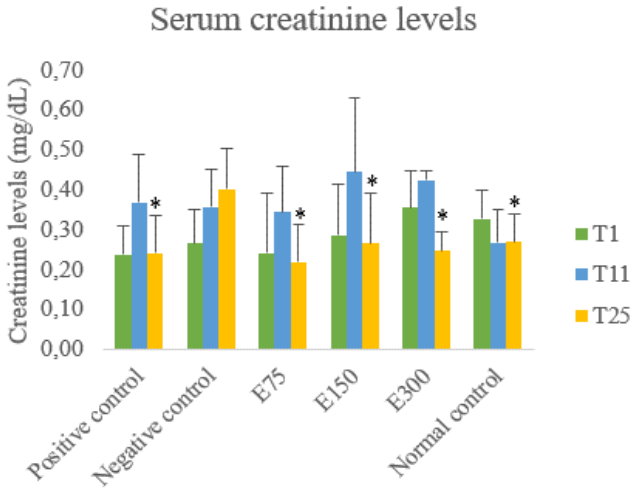

Figure 2. Serum Creatinine Levels Rats during the Nephroprotective Activity Test of Ethanol Extract of Neem Leaves (Azadirachta indica) on Hypertensive Rats Induced by NaCl and Hydrocortisone. The values are mean ± SD; n = 6; *p < 0.05 when compared with the negative control group.

The treatment given for 14 days appeared to cause a decrease in serum creatinine in several groups. In the negative control group, creatinine levels continued to increase due to induction even after the induction had been stopped. In the positive control group, rats that received 25 mg/kg body weight of captopril experienced a decrease in serum creatinine. In chronic kidney disease, captopril is used as the first line (Kemenkes, 2021). According to Zhang et al., (2020) ACEI is superior to Angiotensin Receptor Blockers (ARB) and other antihypertensive drugs, which have the highest benefits in preventing kidney and cardiovascular disorders.

In the group with the administration of neem leaf ethanol extract at doses of 75, 150, and 300 mg/kg body weight, compared to the negative control group, there were significant

differences ($p<0.05$). The group receiving the ethanol extract of neem leaves at the three doses, when compared to the positive control group, showed no significant difference ($p>0.05$). In addition, the same significance value ($p>0.05$) was also shown in the comparison of the percentage decrease in serum creatinine levels of rats among the three groups of ethanol extract of neem leaves. The significance of these analysis results is that the ethanol extract of neem leaves can lower serum creatinine levels, with its activity comparable to captopril, and this activity does not change even with increased doses of the extract.

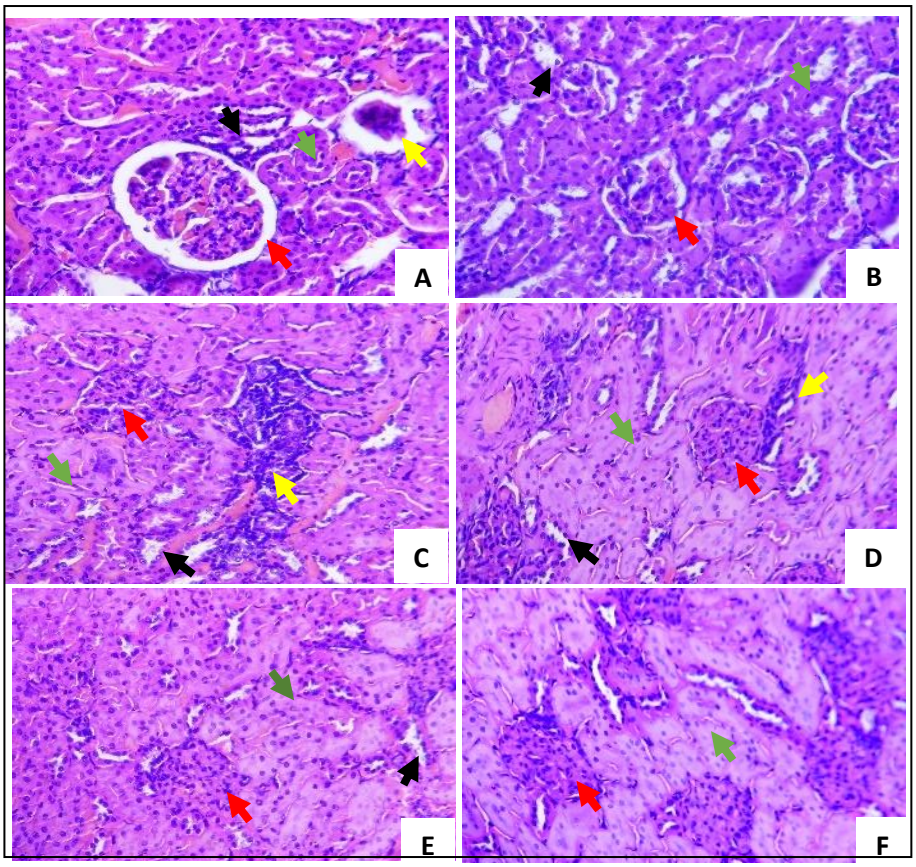

Figure 1. Microscopic Image of Rat Kidneys during Nephroprotective Activity Testing of Ethanol Extract of Neem Leaves (*Azadirachta indica*) in NaCl and Hydrocortisone Induced Hypertensive Rats. A (negative control), B (positive control), C (E75), D (E150), E (E300) and F (normal control); tubular cells (green arrows), glomerulus (red arrows), damaged cells (black arrows), inflammatory cells (yellow arrows) with Hematoxylin-Eosin (HE) staining at 400x magnification.

Kidney damage will increase the level of creatinine in the blood, and the accumulation of creatinine in the blood occurs when there is a decrease in GFR in kidney disorders (Xue et al., 2014). According to Romi et al., (2021), tubular injury is directly proportional to serum creatinine levels. Therefore, another parameter observed to strengthen the activity of the extract on kidney damage is the microscopic appearance of the kidneys.

Histopathological observation of the kidneys was conducted to determine the damage occurring in the kidneys. Based on Figure 3, in all groups except the normal control group, damaged cells were found. Next, to determine which part of the kidney is damaged, EGTI scoring was performed. The EGTI scoring system is used because it is a more reliable, simple, informative, and detailed system for assessing the level of kidney tissue damage. This scoring system consists of histological injuries in 4 parts, namely: endothelium, glomerulus, tubular, and interstitial.

The EGTI scoring results shown in Table 4 indicate that kidney damage occurs in the tubules and interstitium, while the glomerular and endothelial regions do not experience damage. The damage that occurs in the tubules is likely because in hypertensive conditions, there is continuous retention of sodium and water, causing the tubules to work excessively and leading to damage. Additionally, in this condition, the immune system produces pro-inflammatory cytokines that ultimately cause damage to the interstitium.

Table 4. Results Scoring EGTIHistopathology of Rat Kidney during Nephroprotective Activity Test of Ethanol Extract of Neem Leaves (*Azadirachta indica*) in NaCl and Hydrocortisone Induced Hypertensive Rats

Group	Left Kidney			
	Tubule	**Endothelial**	**Glomerular**	**Interstitial**
Positive control	6,7±0,6	0,0±0,0	0,0±0,0	1,7±0,6
Negative control	7,0±2,0	0,0±0,0	0,0±0,0	1,7±0,6
E75	4,0±2,6	0,0±0,0	0,0±0,0	1,7±1,2
E150	3,7±1,5[a]	0,0±0,0	0,0±0,0	1,0±0,0
E300	2,7±0,6[a]	0,0±0,0	0,0±0,0	0,0±0,0[a]
Normal control	2,0±1,7[a]	0,0±0,0	0,0±0,0	0,0±0,0[a]
Group	**Right Kidney**			
	Tubule	Endothelial	Glomerular	Interstitial
Positive control	8,0±1,0	0,0±0,0	0,0±0,0	1,3±0,6
Negative control	8,3±0,6	0,0±0,0	0,0±0,0	1,3±0,6
E75	2,7±1,5[a]	0,0±0,0	0,0±0,0	1,3±0,6
E150	3,3±2,1[a]	0,0±0,0	0,0±0,0	1,3±0,6
E300	2,0±1,7[a]	0,0±0,0	0,0±0,0	0,0±0,0[a]
Normal control	2,7±2,3[a]	0,0±0,0	0,0±0,0	0,0±0,0[a]

Note: Values are expressed as mean ± SD; n=18; a (p<0.05) significantly different values compared to negative controls

In the positive control group, no sig. difference (p>0.05) was observed compared to the negative group, suggesting that the damage occurring in the renal tubules and interstitial tissue may not have been effectively reversed. This outcome could be attributed to an insufficient dosage or a treatment period that was too short to induce notable recovery. Based on research by Gan et al., (2018) administration of captopril at a dose of 34 mg/kg body weight over a 13-week period has been shown to reduce blood pressure, alleviate renal tissue damage, and inhibit kidney inflammation along with NF-κB activation in spontaneously hypertensive rats (SHR).

The group receiving 75 and 150 mg/kgbw doses of neem leaf ethanol extract showed no significant difference ($p>0.05$) from the negative group in the left tubules and left-right interstitium, but a significant difference ($p<0.05$) from the negative group in the right tubules. If compared to the normal group, there is no significant difference ($p>0.05$) in the left-right tubules, but a significant difference ($p<0.05$) with the negative group in the left-right interstitium. This indicates that the 75 and 150 mg/kg body weight doses of ethanol extract from neem leaves can only repair damage to the tubules, but cannot repair the damage occurring in the interstitial.

In the group receiving 300 mg/kg body weight of neem leaf ethanol extract, there was a significant difference ($p<0.05$) compared to the negative group. In addition, when compared to the normal group, there was no significant difference ($p>0.05$). This indicates that the 300 mg/kgbw dose of neem leaf ethanol extract has been proven to repair kidney damage occurring in both the left and right tubules as well as in the left and right interstitials.

Overall, the pharmacological activity is influenced by the compounds contained in the extract. The compounds contained in the ethanol extract of neem leaves are polyphenols, flavonoids, tannins, saponins, and terpenoids. Among these, polyphenolic substances—especially flavonoids and tannins are known to suppress the expression of key pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, and NF-κB, thereby exerting anti-inflammatory effects. Additionally, polyphenols help preserve mitochondrial integrity by limiting the production of ROS and preventing mitochondrial dysfunction. Their protective mechanism includes inhibiting the opening of MPT pores, this mechanism inhibits the release of cytochrome C, stabilizes mitochondrial membrane potential to prevent swelling, and interferes with the downstream activation of caspase enzymes, thereby halting the progression of apoptosis. Furthermore, polyphenols contribute to cellular defense by normalizing antioxidant enzyme levels such as SOD and catalase (CAT), improving the overall antioxidant capacity, and neutralizing excess free radicals (Ashkar et al., 2022)

According to Vargas et al., (2018) flavonoids have shown potential in preventing or alleviating kidney damage linked to hypertension. While part of their effect may stem from their ability to lower blood pressure and enhance vascular function, flavonoids also exert direct actions on kidney tissues. These compounds modulate critical molecular pathways involved in the progression of renal injury, including oxidative stress responses, inflammatory mediators, protein kinase activity, and matrix metalloproteinase expression independent of their influence on systemic blood pressure. Moreover, flavonoids support renal protection by enhancing endothelial function, inhibiting the RAAS, and activating the Nrf2 pathway, which plays a key role in regulating antioxidant defenses (Cao et al., 2022).

Terpen compounds will suppress the proliferation of mesangial cells caused by angiotensin II, reduce the excessive expression of aldose reductase in the kidneys, and have antioxidant and anti-inflammatory properties (Kulkarni et al., 2024). Saponin compounds will

lower blood pressure, inhibit the expression of TGF-β1, collagen I, and prorenin receptor (PRR) (Chen et al., 2013)

The mechanisms mentioned above are suspected to originate from the major compounds contained in neem leaves. The mechanism is an estimation based on literature review. Further research is needed to confirm the mechanism of bioactive compounds in neem leaf ethanol extract for repairing kidney damage due to hypertension.

CONCLUSION

Neem leaf ethanol extract, administered at doses of 75, 150, and 300 mg/kg body weight, significantly reduced serum creatinine levels in hypertensive rats induced by NaCl and hydrocortisone, with the highest dose (300 mg/kg) also effectively repairing damage to renal tubules and interstitium. These findings highlight the extract's nephroprotective potential against hypertension-induced kidney injury. For future research, investigators could explore the underlying molecular mechanisms, such as antioxidant pathways or anti-inflammatory effects, and evaluate long-term efficacy and safety in higher animal models or clinical trials to support its therapeutic application.

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