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Protective Effects of Astaxanthin on Triple Whammy-Induced Acute Kidney Injury via Antioxidant, Anti-inflammatory and Anti-Apoptotic Mechanisms

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Abstract

The triple whammy combination (diuretics, ACE inhibitors, and NSAIDs) can cause acute kidney injury (AKI). Astaxanthin is known for its antioxidant, anti-inflammatory, and anti-apoptotic properties, which may offer nephroprotection. This study aimed to evaluate the prophylactic effect of astaxanthin in a triple whammy-induced AKI rat model. Thirty rats were divided into 6 groups: Normal control, triple whammy (negative control), N-acetylcysteine (positive control), and 3 astaxanthin-treated groups (0.4, 1.6 and 2.4 mg/kg body weight). Oral treatment was given for 7 days before the administration of the triple whammy combination for 2 days. Blood and urine samples were collected to assess renal biomarkers, and histopathological evaluation was performed. Antioxidant activity was tested using the DPPH radical scavenging assay. Molecular docking was used to analyze astaxanthin's binding affinity to inflammatory (NF-κB) and apoptotic (caspase-3) targets. Triple whammy administration significantly increased serum creatinine, urea, proteinuria, and histological damage. Astaxanthin, especially at 1.6 mg/kg, significantly reduced these biomarkers and improved renal structure compared to both negative and N-acetylcysteine-treated groups. DPPH assay showed strong antioxidant activity for both astaxanthin (IC_{50} = 24.2 µg/mL) and N-acetylcysteine (IC_{50} = 3.7 µg/mL). In silico analysis demonstrated higher binding affinity of astaxanthin to caspase-3 (–7.5 kcal/mol) and NF-κB (–6.6 kcal/mol) than N-acetylcysteine. In conclusion, astaxanthin demonstrates superior nephroprotective efficacy, as evidenced by improved renal biomarkers and histological structure, potent antioxidant capacity, and strong predicted interactions with key inflammatory and apoptotic targets. These findings highlight astaxanthin's potential as a promising prophylactic agent against AKI, warranting further mechanistic and translational studies.

Keywords: Astaxanthin, Triple Whammy, Nephroprotective, Acute Kidney Injury, Molecular Docking, 1,1-diphenyl-2-picrylhydrazyl

Introduction

Acute kidney injury (AKI) remains a significant global health concern, with substantial implications for patient outcomes. AKI affects approximately 13.3 million individuals worldwide each year and is associated with up to 1.7 million deaths [1]. Beyond its acute phase, AKI often leads to long-term complications, including an increased risk of developing chronic kidney disease and elevated cardiovascular morbidity and mortality.

A well-recognized clinical scenario that increases the susceptibility to AKI is the so-called "triple whammy", which is the concurrent use of angiotensin-converting enzyme (ACE) inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), and diuretics. Unlike other nephrotoxic agents that primarily damage renal

cells via toxic metabolites [2], the triple whammy combination induces AKI through a synergistic reduction in renal perfusion and glomerular filtration rate (GFR). Reduction in renal perfusion could lead to ischemia–reperfusion injury (IRI). IRI disrupts mitochondrial homeostasis, leading to the excessive generation of reactive oxygen species (ROS), activation of pro-inflammatory signaling pathways, and cellular apoptosis, all of which contribute to tubular and vascular damage in the kidney [3]. Consequently, the use of triple whammy significantly heightening the risk of AKI, particularly in predisposed patients [4,5].

Since the pathogenesis and high prevalence of AKI have been recognized, the development of effective nephroprotective agents remain critical [6]. Astaxanthin is a naturally occurring carotenoid belonging to the xanthophyll subclass, renowned for its exceptionally strong antioxidant properties. Found abundantly in microalgae, yeast, and marine organisms, astaxanthin plays a pivotal role in cellular defense against oxidative damage. It exerts its antioxidant effect by efficiently neutralizing ROS, interrupting lipid peroxidation chain reactions, and enhancing the activity of endogenous antioxidant enzymes [7]. Furthermore, astaxanthin distinguishes itself from other commonly studied antioxidants, such as β-carotene, vitamin C, and vitamin E, through its superior redox stability and its non-pro-oxidant behavior, even under high oxidative stress conditions [7,8].

Based on these promising characteristics, an *in vivo* rat model was used to evaluate the protective effects of astaxanthin against triple whammy-induced AKI, focusing on renal biomarkers and histopathology. An in vitro study was also conducted to assess the free radical scavenging capacity of astaxanthin. Furthermore, in silico analysis was performed to predict astaxanthin's binding interactions and affinities with AKI-related target proteins. In this study, N-acetylcysteine was selected as a comparative agent because it has been clinically used to prevent AKI.

Materials and Methods

Chemicals and drugs

Astaxanthin supplement (Xantia®) was used as the test compound. The triple whammy combination: ramipril 5 mg tablets (Novell®), furosemide 10 mg/mL injectable solution (Bernofarm®), and ibuprofen 400 mg tablets (Trifa®), N-acetylcysteine (Samparindo®), were procured from a licensed local pharmacy in Makassar, Indonesia.

Analytical-grade reagents including 70% methanol, diethyl ether, and 20% formalin were obtained from an authorized chemical distributor in Makassar, Indonesia. The free radical scavenging agent 2,2-diphenyl-1-picrylhydrazyl (DPPH) and food-grade sodium carboxymethyl cellulose (Na CMC; USP grade) were purchased from Sigma Chemical Co. (USA). Reagent kits for serum creatinine and urea was purchased from Human GmbH, Wiesbaden, Germany.

Experimental animals

A total of thirty male Wistar rats, weighing between 180 - 300 g were used in this study. The animals were housed in standard plastic cages under controlled environmental conditions (12-hour light/dark cycle, temperature 22 ± 2 °C, and relative humidity 50% - 60%) and were provided 20 g/day standard laboratory chow and water ad libitum throughout the experimental period. All procedures involving animals were conducted in accordance with internationally accepted ethical guidelines for the use of animals in scientific research. The experimental protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Pharmacy, Hasanuddin University, Indonesia (Approval No. 1085/UN4.17/KP.06.05/2025).

Drug preparation

The triple whammy combination, consisting of ramipril, ibuprofen, and furosemide, was prepared at doses selected based on human therapeutic regimens known to be associated with AKI [9], then converted to rat-equivalent doses using standard dose conversion methods [10]. These doses were validated in a pilot study and successfully induced AKI in rats within 3 days of administration. Ramipril was administered at 0.5 mg/kg body weight (b.w) of rat and ibuprofen at 124 mg/kg b.w. Furosemide was given by intraperitoneal injection at 3.72 mg/kg b.w. Astaxanthin was administered at 3 dosage levels: Low dose (0.4 mg/kg bw), mid dose (1.6 mg/kg b.w), and high dose (2.4 mg/kg b.w); while N-acetylcysteine, used as a reference antioxidant, was given at a dose of 41 mg/kg b.w. All drugs were freshly prepared as suspension in 0.5% Na

CMC before per oral administration to ensure dosing accuracy and chemical stability.

Experimental protocol

The animals were randomly divided into 6 groups (n = 5 per group): Normal control group (received vehicle only), the triple whammy group (received ramipril, ibuprofen, and furosemide), the N-acetylcysteine group (received N-acetylcysteine + triple whammy), and 3 astaxanthin groups (received 0.4, 1.6 or 2.4 mg/kg astaxanthin + triple whammy). The treatment period lasted for 7 days. During the first 5 days, rats in the treatment groups received daily oral administration of either N-acetylcysteine or astaxanthin at their respective doses. Starting on day 6, all groups (except the normal control) were induced with the triple whammy drug combination to simulate AKI. Treatments were continued alongside the triple whammy until day 7.

Blood and urine analysis

On day 8, blood samples were collected via appropriate methods for biochemical analysis. Samples were first centrifuged at 2,000 rpm for 30 min to separate the serum. The final serum samples were analyzed for creatinine and urea levels using a commercial reagent kit (Human GmbH, Germany) following the manufacturer's protocol, and measurements were taken using a Humalyzer 4,000 spectrophotometer. On the same day, urinalysis was performed for all groups. Urine was collected in sterile urine pots, extracted using a syringe, and analysed using Verify U-120 urine analyzer to assess urinary protein.

Renal weight index

At the end of the study, animals were euthanized and final body weight was recorded. Kidneys were excised, freed of adherent fat and connective tissue. Left kidneys were weighed immediately on a calibrated analytical balance. Renal index for each kidney was calculated as:

$$\text{Renal weight index (\%)} = [\text{renal weight (g)} / \text{body weight (g)}] \times 100$$

Histopathological examination

Rat kidneys immediately fixed in 10% neutral-buffered formalin for 48 h to preserve tissue morphology. Following fixation, the kidneys were sectioned vertically and processed in a tissue processor for 12 h. The processed tissues were then embedded in paraffin to form formalin-fixed paraffin-embedded (FFPE) blocks. Thin sections of 4 - 5 µm thickness were cut using a rotary microtome and floated in a warm water bath to smoothen the tissue. After drying for approximately 2 h, the slides were stained using the standard hematoxylin and eosin (H&E) staining protocol to visualize histological structures. Histopathological evaluation was conducted using a light microscope (Olympus®), with representative photomicrographs captured at 40× magnification. Histopathological analyses were independently conducted by 2 biomedical scientists, ensuring accuracy, objectivity, and consistency of the results. Renal damage was scored based on the percentage of affected renal tissue area observed under 10× magnification: score 0 = no or minimal changes; score 1 = mild injury (< 25%); score 2 = moderate damage (26% - 50%); score 3 = severe damage (51% - 75%); and score 4 = extensive damage (> 75%).

Radical scavenging assay

The radical scavenging activity of astaxanthin and N-acetylcysteine was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. A 0.4 mM DPPH stock solution was prepared by dissolving 4 mg of DPPH in 25 mL of absolute methanol in a volumetric flask. Separately, 10 mg each of astaxanthin and N-acetylcysteine were weighed and dissolved. Aliquots of 50, 100, 150, 200 and 250 µL were each diluted with absolute methanol to a total volume of 5 mL to obtain final concentrations of 10, 20, 30, 40 and 50 ppm, respectively.

For the assay, 200 µL of the 0.4 mM DPPH solution was dispensed into each well of a 96-well microplate, followed by the addition of 200 µL of each test solution (astaxanthin or N-acetylcysteine) at the specified concentrations. The absorbance of each well was measured at 517 nm using a microplate reader after incubation at room temperature in the dark for 30 min. The percentage of DPPH radical inhibition was calculated for each concentration and plotted against the

logarithm of concentration to obtain the dose-response curve. The half-maximal inhibitory concentration (IC_{50}) was determined by linear regression analysis of the log concentration versus percentage inhibition using Microsoft Excel.

Molecular docking procedure

Molecular docking was conducted to evaluate the interaction between the antioxidant compounds (astaxanthin and N-acetylcysteine) and target proteins involved in oxidative stress and inflammation. The 3-dimensional (3D) structures of the target protein receptors: Caspase-3 (PDB ID: 3DEI) and NF-κB (PDB ID: 4DN5), were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org). Prior to docking, protein structures were prepared and refined using AutoDock Tools, which included removal of water molecules, addition of polar hydrogens, and assignment of Kollman charges. The 3D ligand structures of astaxanthin (PubChem CID: 5281224) and N-acetylcysteine (CID: 12035) were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Ligand and receptor files were prepared using UCSF Chimera with the dock prep module to optimize structures and convert them to the appropriate format for docking.

Molecular docking was performed using AutoDock Vina integrated within UCSF Chimera. For caspase-3, the grid box was set to 30×30×30 points (XYZ dimensions) with a center at coordinates −46.62, 15.37, −22.20 and a grid spacing of 0.375 Å. For NF-κB, the grid box was set to 28×12×20 points, centered at −9.33, 29.08, −4.44, also with a spacing of 0.375 Å. The file directories for receptor and ligand input and output were defined within the AutoDock Vina interface. The docking simulations yielded binding affinity values (in kcal/mol), with more negative values indicating stronger predicted binding interactions between the ligand and receptor. Visualization and analysis of docking poses, including hydrogen bonding and hydrophobic interactions, were performed using Discovery Studio Visualizer to interpret the potential biological relevance of the binding interactions. The validation results indicate that the docking protocol used was quite reliable. With RMSD values consistently falling between 2.0 and 3.0 Å, the predicted binding poses for NF-κB and Caspase-3 are considered acceptably accurate. This suggests that the protocol can reliably predict how these molecules interact [11].

Statistical analysis

All data are expressed as mean ± standard deviation (SD), and statistical significance was defined as $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 10.4.2. The normality of the distribution for biochemical parameters (serum creatinine and urea levels) was assessed using the Shapiro-Wilk test. For normally distributed data, one-way analysis of variance (ANOVA) was conducted, followed by Fisher's Least Significant Difference (LSD) post hoc test to identify pairwise differences between groups. Non-parametric data, including urinalysis parameters, were analyzed using the Kruskal-Wallis test, followed by the Mann–Whitney U test for post hoc comparisons when appropriate.

Results and discussion

Biomarker analysis and renal weight index

Throughout the experimental period, rats in all groups maintained relatively stable body weight trajectories without significant loss (**Figure 1**). The normal control and treatment groups showed a steady increase, consistent with physiological growth.

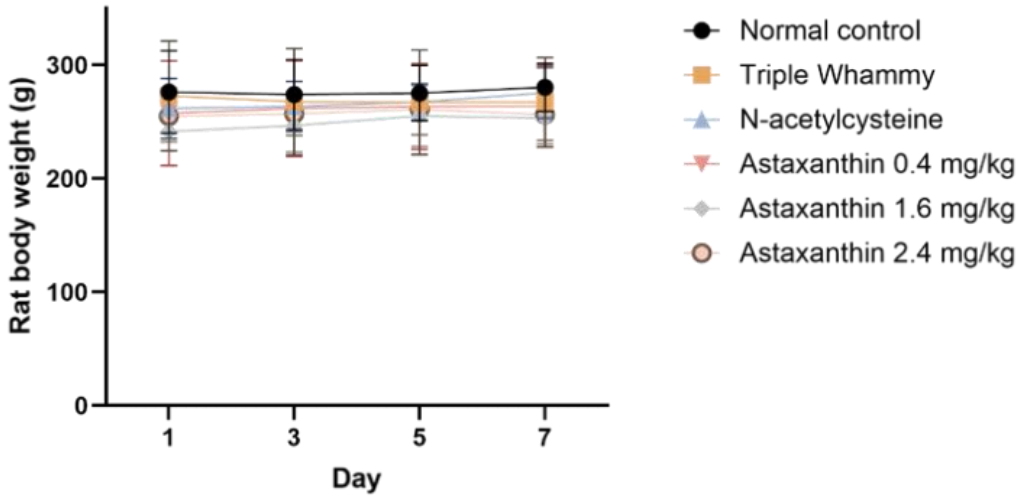

Figure 1 Comparison of rat bodyweight among treatment groups.

Serum creatinine, blood urea, and urinary protein levels were assessed as primary biomarkers for confirming AKI induced by the triple whammy drug combination. In the normal control group, mean serum creatinine and urea levels were 1.08 ± 0.23 and 51.5 ± 7.18 mg/dL, respectively (**Figures 2(a)** and **2(b)**). In contrast, the triple whammy group exhibited a significant elevation in both biomarkers compared to the normal control group ($p < 0.05$), confirming successful induction of AKI.

These findings align with previous studies demonstrating that the concurrent use of ACE inhibitors, NSAIDs, and diuretics, referred to as the "triple whammy" combination, disrupts renal autoregulation, resulting in impaired glomerular filtration and reduced renal perfusion [4,5]. This drug combination exerts synergistic hemodynamic effects that compromise both systemic and intrarenal blood flow regulation. The observed elevations in serum creatinine and urea reflect a decline in glomerular filtration rate (GFR), which is a hallmark of AKI [6]. Mechanistically, NSAIDs inhibit the synthesis of vasodilatory prostaglandins such as PGE2 and prostacyclin, leading to vasoconstriction of afferent arterioles and decreased renal blood flow. Concurrently, ACE inhibitors cause efferent arteriolar vasodilation, reducing glomerular capillary pressure and further lowering GFR [12]. The addition of diuretics exacerbates this effect by inducing volume depletion and hypovolemia, further impairing renal perfusion [13]. Moreover, NSAIDs may contribute to direct tubular injury via acute interstitial nephritis or acute tubular necrosis [14]. Collectively, these pharmacodynamic interactions explain the significant renal biomarker elevations observed in the triple whammy group, supporting the successful induction of AKI in this model.

Pre-treatment with astaxanthin attenuated the elevation of renal biomarkers in a dose-dependent manner. Particularly, the mid dose (1.6 mg/kg) of astaxanthin group showed a significant reduction in serum creatinine compared to the triple whammy and N-acetylcysteine groups ($p < 0.05$). Urea levels were also significantly lower in all astaxanthin-treated groups (low, mid and high doses) compared to the triple whammy and N-acetylcysteine groups ($p < 0.05$), and not significantly different from the normal control group.

Urinary protein, a sensitive indicator of glomerular damage, was significantly elevated in the triple whammy group ($p < 0.05$) but remained undetectable in the normal control group (**Figure 2(c)**). Treatment with astaxanthin at mid and high doses reduced urinary protein levels (0.113 ± 0.075 g/L) compared to 0.475 ± 0.35 g/L in the triple whammy group. Although this reduction did not reach statistical significance, the trend suggests a potential protective effect of astaxanthin on glomerular and tubular structures, possibly through preservation of renal barrier integrity.

Changes in the renal index (kidney weight-to-body weight ratio) may reflect structural and pathological adaptations to renal injury. In our study, the normal control value (0.38% ± 0.04%) did not differ significantly from triple-whammy–exposed rats, with or without treatment (**Figure 2(d)**). Over a 2-day exposure, the triple-whammy combination is expected to produce predominantly hemodynamic effects, reduced renal perfusion and glomerular filtration, which may not sufficient to cause changes in kidney mass (enlargement or atrophy). Accordingly, the renal index remained stable across groups, while blood and urine biomarkers captured the functional impact of reduced filtration.

Based on creatinine levels, the greater protection observed at the mid dose (1.6 mg/kg) versus the high dose (2.4 mg/kg), which may reflect a hormesis or biphasic dose–response commonly seen with phytochemicals [15]. Protective pathways can become saturated, producing a plateau in benefit despite increased dosing. Since astaxanthin is lipophilic, higher administered doses may not proportionally increase tissue bioavailability and could favor aggregation or altered distribution. Finally, at higher concentrations some antioxidants can exert pro-oxidant or off-target effects that counteract protection. These mechanisms are consistent with prior studies showing antioxidant enzyme suppression with high dose of astaxanthin in mice [16].

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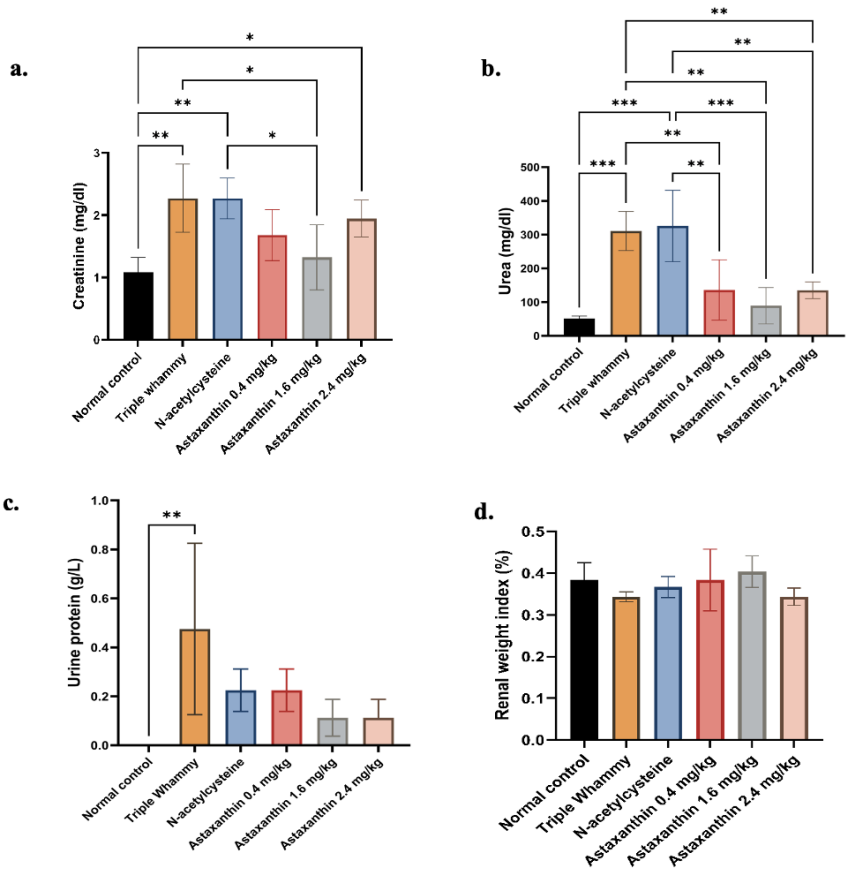

Figure 2 Comparison of renal biomarkers among treatment groups: (a) serum creatinine, (b) serum urea, (c) urine protein levels, and (d) renal weight index**.** Statistical significance: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

Astaxanthin has been shown to exert significant antioxidant and renoprotective effects in various preclinical models. A previous study demonstrated that a 20 mg dose of astaxanthin significantly enhanced the activity of the antioxidant enzyme superoxide dismutase (SOD), supporting its role in mitigating oxidative stress [17]. Furthermore, astaxanthin, when used as an adjunct therapy, provided protection against lithium-induced

nephrotoxicity by attenuating oxidative damage, modulating apoptotic pathways, suppressing inflammatory cytokines, and preserving renal histoarchitecture [18]. These protective effects are attributed to its potent antioxidant, anti-inflammatory, and anti-apoptotic properties. At the molecular level, astaxanthin exerts anti-inflammatory effects primarily by modulating the NF-κB signaling pathway. It inhibits IκB kinase (IKK), thereby preventing the phosphorylation and degradation of IκB, which in turn sequesters NF-κB in the cytoplasm and prevents its nuclear translocation and activation of pro-inflammatory genes [19]. This mechanism leads to reduced expression of key inflammatory mediators such as TNF-α and IL-1β [20].

Histopathological analysis

Histopathological analysis revealed no signs of AKI in the normal control group, with intact glomerular and tubular architecture (**Figures 3(a)** and **3(b)**). In contrast, the triple whammy-induced group exhibited characteristic features of AKI, including Bowman's capsule dilation (red arrow), inflammatory cell infiltration (blue arrow) and prominent proteinaceous casts within the renal tubules (green arrow), indicating glomerular and tubular damage (**Figures 3(c)** and **3(d)**). Treatment with N-acetylcysteine partially mitigated these changes; although Bowman's capsule dilation, tubular protein casts, and inflammatory cells were still observed (red, green, and blue arrow, respectively), the overall tubular architecture appeared better preserved compared to the triple whammy group (**Figures 3(e)** to **3(f)**).

In the low dose astaxanthin group (0.4 mg/kg), the kidney sections demonstrated absence of Bowman's capsule dilation and protein casts (orange arrow), though focal hemorrhage (black arrow) was still present (**Figure 3(g)** to **3(h)**). Interestingly, the administration of 1.6 and 2.4 mg/kg doses of astaxanthin led to near-normal histological features, with no visible Bowman's capsule dilation or tubular protein casts, suggesting significant structural recovery and nephron preservation (**Figures 3(i)** to **3(j)**, **Figures 3(k)** to **3(l)**).

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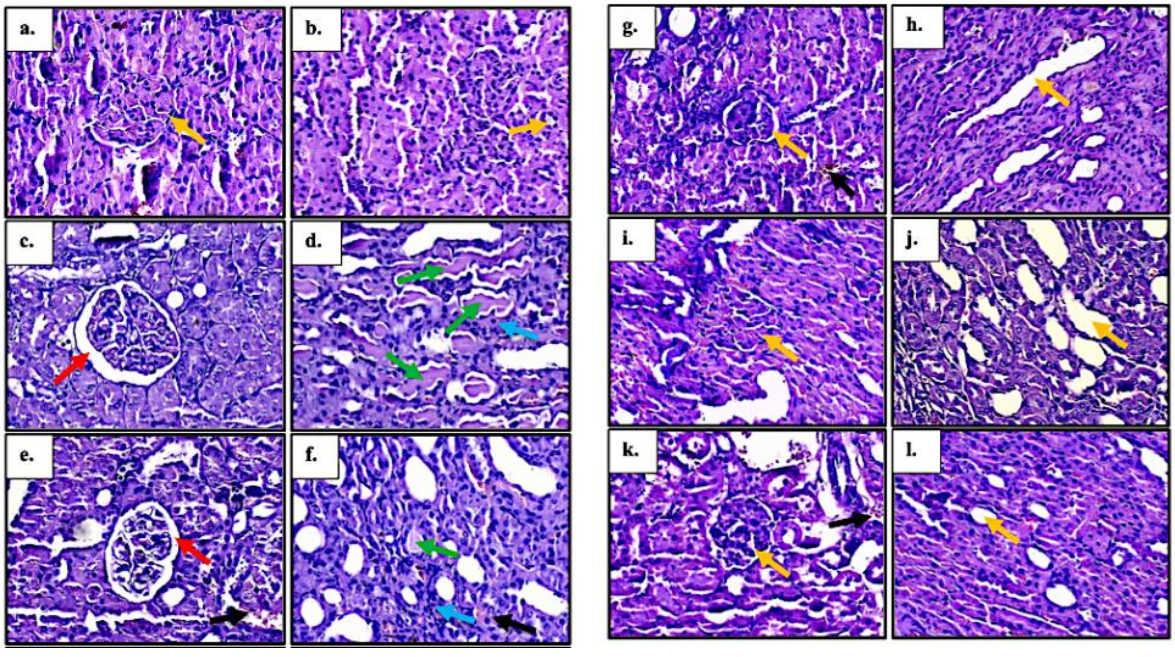

Figure 3 Representative histopathological images of rat kidney tissue at 40× magnification: (a - b) normal control; (c - d) triple whammy; (e - f) N-acetylcysteine; (g - h) astaxanthin 0.4 mg/kg; (i - j) astaxanthin 1.6 mg/kg; (k - l) astaxanthin 2.4 mg/kg. Orange arrow: normal architecture of glomerulus and tubulus; red arrow: Bowman's capsule dilation; blue arrow: Inflammatory cell infiltration; green arrow: proteinaceous casts in renal tubules; black arrow: Haemorrhage.

The histopathological scoring results are summarized in **Table 1**. The triple whammy group exhibited the most prominent renal alterations, characterized by marked glomerular dilation (2.6 ± 0.5)

and protein casts within the tubular lumen (2.2 ± 0.8). These findings reflect impaired glomerular filtration and tubular reabsorption, which are consistent with the biomarker evidence of renal dysfunction. Mild inflammatory infiltrates and hemorrhage were also observed in this group, although the scores did not differ significantly from other treatment groups. Nevertheless, rats treated with astaxanthin generally displayed lower injury scores, which support its nephroprotective effect.

Table 1 The score of renal tissue damage.

Group	Glomerular dilation	Protein cast	Inflammation	Haemorrhage
Normal control	0.2 ± 0.4	0.0 ± 0.0	0.2 ± 0.4	0.2 ± 0.4
Triple Whammy	2.6 ± 0.5 **	2.2 ± 0.8 **	1.8 ± 1.0	0.8 ± 0.8
N-acetylcysteine	1.8 ± 0.8	1.2 ± 0.4	1.8 ± 0.4	0.4 ± 0.5
Astaxanthin 0.4 mg/kg	0.6 ± 0.8	0.6 ± 0.8	0.8 ± 0.8	0.6 ± 0.8
Astaxanthin 1.6 mg/kg	0.6 ± 0.5	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.4
Astaxanthin 2.4 mg/kg	0.8 ± 0.8	0.4 ± 0.5	0.4 ± 0.8	0.2 ± 0.4

Score 0: No or minimal changes; score 1: a mild injury (6 < 25%); score 2: Moderate damage (26% - 50%); score 3: severe damage (51%-75%); score 4: massive damage (>75% - 100%). Statistical significance: (**) $p < 0.01$ compared to normal control

Measurement of antioxidant activity

The antioxidant capacities of astaxanthin and N-acetylcysteine were assessed using the DPPH radical scavenging assay. This method has been widely used for evaluating the ability of compounds to donate hydrogen atoms or electrons to neutralize stable free radicals. In this study, both astaxanthin and N-acetylcysteine exhibited very strong antioxidant activity, with IC_{50} values of 24.2 and 3.7 µg/mL, respectively (**Figure 4**). Based on established classifications, compounds with IC_{50} values below 50 µg/mL are categorized as having very strong radical scavenging activity [21]. Therefore, both test compounds fall within this highest category of antioxidant strength.

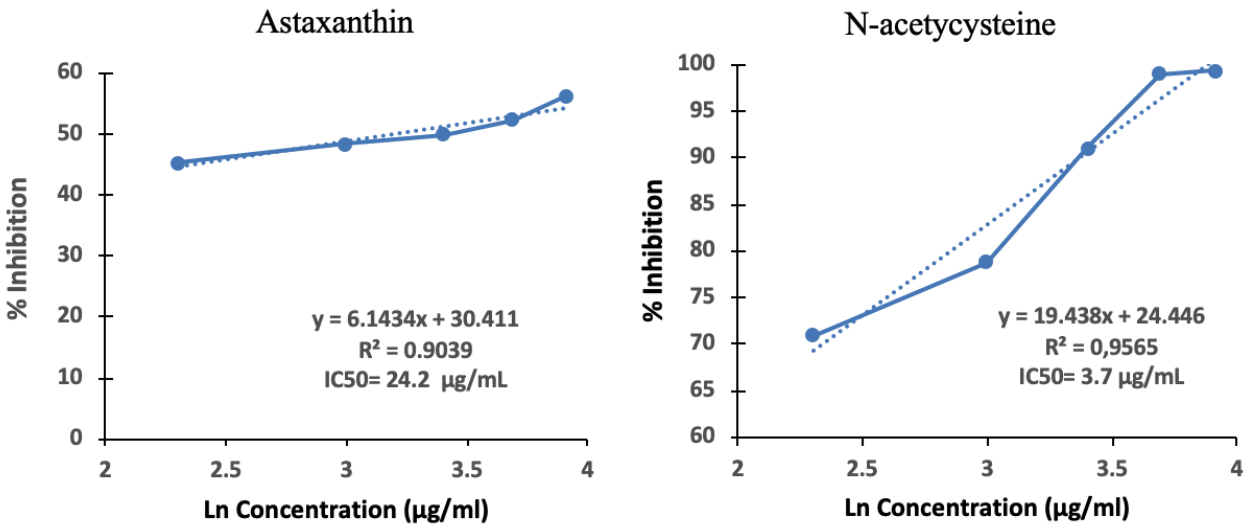

Figure 4 Determination of IC_{50} values of astaxanthin and N-acetylcysteine.

N-acetylcysteine exhibited the lower IC_{50}, indicating higher potency in scavenging DPPH radicals under the same experimental conditions. The strong activity of N-acetylcysteine may be attributed to its free thiol (-SH) group, which can directly donate electrons or hydrogen atoms, making it highly effective in the rapid

electron-transfer environment of the DPPH assay. Nevertheless, with IC_{50} values of 24.2 μg/mL, astaxanthin also reflects substantial radical-scavenging capacity. Astaxanthin possesses an extended conjugated double bond system and lipophilic properties that generally favor antioxidant activity. Previous studies have shown astaxanthin administration contributes to increased SOD, total antioxidant capacity (T-AOC), and reduced lipid peroxidation markers [8,22].

Molecular docking

Residue-ligand interactions and affinity energy

Molecular docking analysis was performed to assess the interaction profiles of astaxanthin and N-acetylcysteine with key apoptotic and inflammatory targets. Binding affinities and interacting amino acid residues of astaxanthin, N-acetylcysteine, and the native ligands of caspase-3 [RXB: (1S)-2-oxo-1-phenyl-2-((1,3,4-trioxo-1,2,3,4-tetrahydroisoquinolin-5-yl)amino)ethyl acetate] and NF-κB [AGS: phosphothiophosphoric acid-adenylate ester] with their respective receptors are summarized in Table 2.

For caspase-3, astaxanthin exhibited a binding affinity of −7.5 kcal/mol, which is considered a strong binding interaction (ΔG < −7.0 kcal/mol) [23]. It formed interactions with 5 amino acid residues: ASN208, LEU168, PHE256, TYR204, and TRP206. These interactions include aromatic residues such as TYR204 and TRP206, which are often associated with stabilizing ligand binding through π–π stacking or hydrophobic interactions [24]. In comparison, N-acetylcysteine demonstrated a weaker binding affinity of −4.6 kcal/mol to caspase-3, suggesting a lower binding stability. It interacted with HIS121, GLY122, GLY165, SER205, and TYR204, forming a total of 5 interactions as well, but mainly involving small or polar residues that may contribute less to strong binding stabilization compared to the more hydrophobic and aromatic contacts of astaxanthin.

The anti-apoptotic mechanism of astaxanthin can be explained through several pathways. Astaxanthin is thought to stabilize mitochondria, organelles that play a central role in the initiation of intrinsic apoptosis. By maintaining mitochondrial membrane integrity, astaxanthin prevents the release of cytochrome c into the cytoplasm. Cytochrome c release is a powerful signal that activates the caspase cascade, leading to caspase-3 activation. In this way, astaxanthin effectively inhibits the caspase cascade, protecting kidney cells from unwanted death and maintaining the structural and functional integrity of kidney tissue [25].

For NF-κB, astaxanthin again showed stronger binding with an affinity of −6.6 kcal/mol, categorized as moderate to strong binding [26]. The interaction involved 2 key residues: GLU560 and ARG416, which may contribute through electrostatic interactions or hydrogen bonding. N-acetylcysteine, in contrast, bound NF-κB with a lower affinity of −4.2 kcal/mol, forming contacts with LYS429, ASP534 and ASN520, which are all polar or charged residues but with overall weaker predicted interaction strength. The number and type of interacting residues also support the observed differences in binding affinities. Astaxanthin, while engaging with fewer residues in NF-κB (2 vs 3 for N-acetylcysteine), showed more energetically favorable interactions, emphasizing that quality of binding (residue type and binding pocket compatibility) may outweigh the quantity of residue contacts.

Several studies have shown that astaxanthin can inhibit the phosphorylation and degradation of IκB, the inhibitory protein that sequesters NF-κB in the cytoplasm. By stabilizing IκB, astaxanthin prevents NF-κB translocation into the nucleus and subsequent activation of pro-inflammatory genes such as TNF-α and IL-6 [27]. Previously, in vivo studies show that astaxanthin attenuates inflammatory and apoptotic responses in bisphenol A (BPA)-exposed rat kidneys, by reversing biomarkers associated with oxidative stress, inflammation, and apoptosis [28]. These results support that astaxanthin offers a triple protective strategy that is particularly relevant in oxidative stress-mediated renal injury.

Table 2 The binding affinity and residues of Astaxhantin and N-acetylcysteine on Caspase-3 and NF-κB receptor.

Protein	Compound	Binding affinity (kcal/mol)	Hydrogen bonds	Number of residue	Amino acid residue
Caspase-3	Astaxanthin	–7.5	1	5	ASN 208, LEU 168, PHE 256, TYR 204, TRP 206
	N-acetylcysteine	–4.6	2	3	HIS 121, GLY 122, GLY 165, SER 205, TYR 204
	Native ligand RXB	–8.1	2	5	ARG207, SER 209, TYR 204, PHE 256, TRP 206
NF-κB	Astaxanthin	–6.6	1	2	GLU 560, ARG 416
	N-acetylcysteine	–4.2	2	3	LYS 429, ASP 534, ASN 520
	Native ligand AGS	–8.9	7	16	LEU 406, ALA 427, LEU 472, GLU 470, LEU 522, MET 469, VAL 414, CYS 533, ASP 519, PHE 411, GLY 409, GLY 412, LYS 517, SER 410, LYS 429, ASP 534

Protein-ligand interaction

The molecular interactions between ligands and target proteins were further analyzed through 2D visualization using Discovery Studio Visualizer to gain insight into the binding mode and interaction types. As shown in **Figure 5**, astaxanthin binding to caspase-3 was stabilized through one hydrogen bond, supported by interactions involving 5 amino acid residues. In contrast, N-acetylcysteine formed 2 hydrogen bonds with caspase-3, involving 3 residues. While N-acetylcysteine exhibited a greater number of hydrogen bonds, the broader residue engagement by astaxanthin suggests a potentially more stable or distributed interaction within the binding pocket.

Similarly, for the NF-κB protein, astaxanthin generated one hydrogen bond and interacted with 3 residues, while N-acetylcysteine formed 2 hydrogen bonds, also involving 3 residues. Although N-acetylcysteine engaged in more hydrogen bonding, the type and spatial configuration of the interacting residues, as well as the binding energy, should be considered when evaluating overall interaction strength.

These visualizations emphasize that both ligands form stabilizing hydrogen bonds with their respective target proteins; however, the interaction profile of astaxanthin, which combines the hydrogen bonding with hydrophobic and aromatic contacts, may contribute to its stronger predicted binding affinity compared to N-acetylcysteine, as previously demonstrated in the docking score analysis.

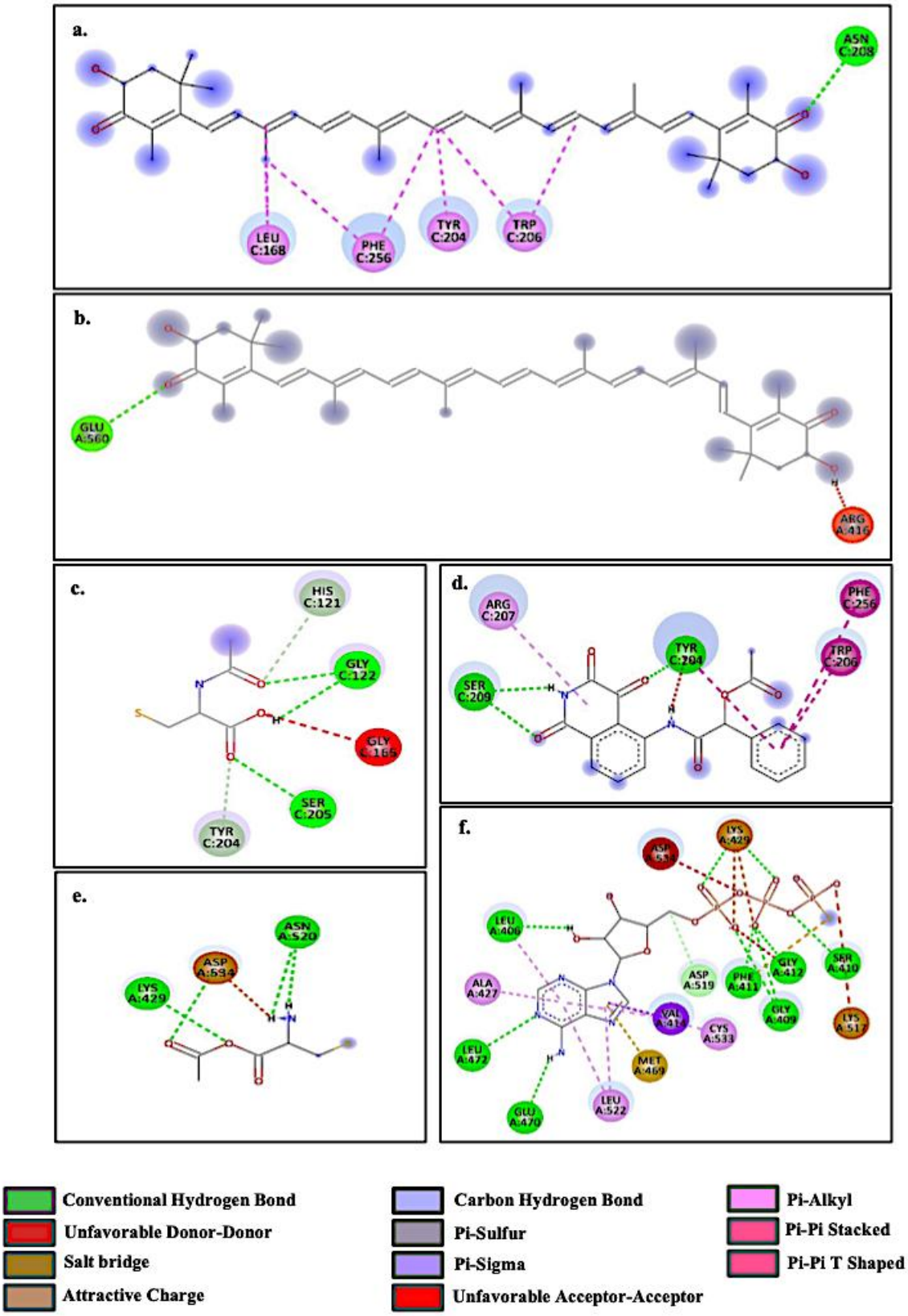

Figure 5 Two-dimensional visualization of molecular docking interactions. (a) Astaxanthin with caspase-3 receptor; (b) Astaxanthin with NF-κB receptor; (c) N-acetylcysteine with caspase-3 receptor; (d) Native ligand RXB with caspase-3 receptor; (e) N-acetylcysteine with NF-κB receptor; (f) Native ligand AGS with NF-κB receptor.

Limitations

While this study provides evidence of astaxanthin's nephroprotective potential against triple whammy-induced renal injury, some limitations should be acknowledged. The experiment was limited to an acute rat model; hence, the long-term effects and safety profile of astaxanthin require further investigation. Additionally, although improvements were observed in biochemical markers and histopathology analysis, the antioxidant capacity was assessed solely using the DPPH radical-scavenging assay, which primarily reflects electron-transfer capacity in a simplified chemical system. While useful for comparative purposes, DPPH does not fully capture the complexity of biological antioxidant mechanisms, particularly those occurring in lipid-rich environments or involving enzymatic systems. Further studies employing serum antioxidant enzyme analysis are warranted to provide a more robust evaluation for astaxanthin's mechanism of action.

The *in-silico* findings suggest a potential mechanistic basis for the renoprotective effects observed in vivo. However, it should be noted that molecular docking has inherent limitations, as it relies on static protein and ligand structures and does not fully account for conformational flexibility or dynamic biological interactions. Therefore, docking results should be interpreted as predictive rather than definitive. Experimental validation, such as gene expression profiling, protein quantification, or functional assays, would be required to substantiate pathway involvement.

Another limitation is that this study did not assess food and water intake or behavioral changes, which could provide additional information regarding the general health status of the animals. Moreover, systemic biochemical parameters beyond BUN and creatinine were not evaluated, as the primary focus was to investigate acute kidney injury. While body weight data have been included to partly address this concern, future studies should incorporate a broader assessment of animal health and systemic toxicity to strengthen the interpretation of protective effects.

Conclusions

This study demonstrates that both astaxanthin and N-acetylcysteine offer renoprotective effects against triple whammy-induced nephrotoxicity, supported by improvements across multiple parameters. Among the tested doses, astaxanthin at a dose of 1.6 mg/kg showed the most prominent effect in improving blood biomarkers, particularly in reducing serum creatinine levels. Additionally, urinary protein excretion was notably improved across all astaxanthin doses, suggesting restoration of glomerular integrity. Histopathological analysis further confirmed structural protection of renal tissue, with reduced signs of glomerular damage, tubular injury and inflammation. In the DPPH assay, astaxanthin showing very strong antioxidant activity although the IC_{50} value was still higher than N-acetylcysteine. Molecular docking analysis revealed that astaxanthin exhibited strong and stable interactions with NF-κB and caspase-3, suggesting a potential role in modulating inflammatory and apoptotic pathways. While in vitro and docking results are predictive, these findings strengthen the hypothesis that astaxanthin may exert nephroprotective effects through synergistic antioxidant, anti-inflammatory, and anti-apoptotic mechanisms.

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