Journal of Applied Pharmaceutical Research
Volume 14 Issue 3, Year of Publication 2026, Page 253 – 270
DOI: https://doi.org/10.69857/joapr.v14i3.1885

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Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | JOAPR
www.japtronline.com ***ISSN: 2348 – 0335***

KAEMPFEROL-RICH THESPESIA LAMPAS LEAF EXTRACT MITIGATES GENTAMICIN-INDUCED NEPHROTOXICITY VIA ANTIOXIDANT AND ANTI-INFLAMMATORY MECHANISMS IN WISTAR RATS

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Article Information
Received: 19th January 2026
Revised: 10th March 2026
Accepted: 17th April 2026
Published: 15th May 2026

Keywords
Gentamicin, Nephrotoxicity, Thespesia lampas, Kaempferol, NF-κB signaling.

ABSTRACT

Background: Gentamicin-induced nephrotoxicity (GIN) is a major limitation of aminoglycoside therapy and a common cause of acute kidney injury (AKI). The underlying mechanisms involve excessive oxidative stress and activation of inflammatory signaling pathways, particularly the nuclear factor-κB (NF-κB) pathway. Natural flavonoids such as kaempferol possess strong antioxidant and anti-inflammatory properties. Thespesia lampas, traditionally used in renal disorders, is rich in kaempferol, necessitating scientific validation of its nephroprotective potential. **Methodology:** ALE was characterized using HR-LCMS, confirming kaempferol as the major flavonoid (20.5 ± 2.8 mg/g extract; 2.05 ± 0.28% w/w). Antioxidant activity was assessed by the DPPH assay, demonstrating potent free radical scavenging (IC_{50} = 62.4 µg/mL) comparable to rutin standard (IC_{50} = 48.6 µg/mL). Rats received ALE orally at doses of 100, 200, and 400 mg/kg for 28 days, while nephrotoxicity was induced by gentamicin (50 mg/kg/day, i.p.) during the final 10 days. Renal function markers, oxidative stress parameters, pro-inflammatory cytokines (TNF-α, IL-6), NF-κB (p65/p50) nuclear translocation, and renal histopathology were evaluated. **Results and Discussion:** Gentamicin caused significant renal dysfunction, oxidative imbalance, elevated inflammatory cytokines, and increased NF-κB activation. ALE treatment produced dose-dependent nephroprotection, with the 400 mg/kg dose markedly restoring renal biomarkers, improving antioxidant defenses, suppressing inflammatory mediators, and ameliorating histopathological damage. **Conclusion:** Kaempferol-rich *Thespesia lampas* ALE confers significant protection against gentamicin-induced nephrotoxicity by attenuating oxidative stress and inhibiting NF-κB–mediated inflammation, highlighting its potential as a therapeutic agent for drug-induced renal injury.

INTRODUCTION

Gentamicin (GM), a broad-spectrum aminoglycoside antibiotic, remains a remedy in the treatment of severe Gram-negative bacterial infections owing to its potent bactericidal efficacy, rapid action, and low cost [1]. Despite its clinical importance,

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the therapeutic use of gentamicin is frequently limited by nephrotoxicity, which represents one of its most critical adverse effects. Gentamicin-induced nephrotoxicity (GIN) accounts for a substantial proportion of drug-induced acute kidney injury (AKI), affecting approximately 5–25% of treated patients, depending on dosage, duration, and pre-existing renal function [1]. Clinically, GIN manifests as a non-oliguric form of AKI characterized by elevated serum creatinine and urea levels, reduced glomerular filtration, and proximal tubular epithelial cell injury [2]. Mechanistically, gentamicin accumulates in renal proximal tubular cells via megalin/cubilin-mediated endocytosis, leading to lysosomal rupture, mitochondrial dysfunction, and increased generation of reactive oxygen species (ROS). The resulting oxidative stress triggers lipid peroxidation, glutathione depletion, and activation of pro-inflammatory mediators such as NF-κB, TNF-α, and IL-6, culminating in tubular apoptosis, necrosis, and acute tubular damage [3]. Thus, oxidative stress and inflammation are central and interconnected events in the pathogenesis of gentamicin-induced renal injury.

Given this mechanistic framework, considerable attention has been directed toward the use of antioxidants and anti-inflammatory agents as nephroprotective strategies against GIN. Natural products and phytochemicals have attracted growing scientific attention as potential strategies to mitigate drug-induced nephrotoxicity due to their multitargeted antioxidant and anti-inflammatory actions. Among these, flavonoids, polyphenolic secondary metabolites widely distributed in plants, have been particularly recognized for their renal protective potential [4]. These compounds exert robust free radical scavenging activity, upregulate endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and suppress pro-inflammatory signaling cascades mediated by NF-κB, COX-2, and inducible nitric oxide synthase (iNOS). Preclinical studies have demonstrated that flavonoid supplementation significantly attenuates biochemical and histopathological indices of gentamicin-induced nephrotoxicity by restoring oxidative balance, preserving mitochondrial integrity, and reducing tubular inflammation [5]. Such findings highlight the translational promise of flavonoid-rich botanicals in managing renal oxidative stress and inflammation. *Thespesia lampas* (Cav.), belonging to the family Malvaceae, is a small medicinal shrub traditionally used in the Indian, Unani, and Ayurvedic systems of medicine to treat urinary disorders, inflammation, gonorrhea, and microbial infections. Various parts of the plant, particularly the leaves and roots, are reported to possess anti-inflammatory, antioxidant, antimicrobial, and hepatoprotective properties [6]. Phytochemical investigations have revealed the presence of bioactive constituents, including flavonoids, phenolic acids, tannins, and sterols, with kaempferol identified as one of the major flavonoids in *T. lampas* leaves. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)- 4H-chromen-4-one) is a well-characterized flavonol known to exert potent antioxidant and anti-inflammatory effects by scavenging ROS, modulating the NF-κB and Nrf2 pathways, and preventing lipid peroxidation and cytokine-induced renal injury [7]. The abundance of kaempferol within *T. lampas* leaves, coupled with the plant's ethnomedicinal relevance in renal and inflammatory ailments, provides a strong pharmacological basis for exploring its nephroprotective potential. However, no study has yet correlated kaempferol-enriched *T. lampas* extract with NF-κB inhibition in GIN.

Therefore, the present study was designed to investigate the nephroprotective potential of aqueous leaf extract of *T. lampas* against gentamicin-induced nephrotoxicity in Wistar rats, and to elucidate the mechanistic role of kaempferol in mediating renal protection. The section below outlines the detailed materials and methods used to evaluate the nephroprotective efficacy of *Thespesia lampas* aqueous leaf extract against gentamicin-induced toxicity in Wistar rats. This is the first study to investigate the Kaempferol-rich *T. lampas* extract in a subchronic Gentamicin-induced nephrotoxicity model, linking the protection to the precise mechanism of NF-κB nuclear translocation suppression, thereby establishing the novelty of this research.

MATERIALS AND METHODS

This section outlines the materials, methods, and technical approaches employed in the current study.

Preparation and Characterization of Test Material

Plant Collection, Authentication, and Processing

Fresh leaves of *Thespesia lampas* (Malvaceae family) were systematically collected from authenticated geographical locations and were taxonomically authenticated. Following collection, the leaves were thoroughly washed to remove surface contaminants, shade-dried at ambient temperature, and subsequently ground into a uniform, fine powder using a mechanical blender [8].

Aqueous Leaf Extract (ALE) Preparation

A standardized mass of the above powdered leaf material (100 g) was measured and subjected to exhaustive extraction using distilled water, which is the most polar inorganic solvent. The powdered material was mixed with water at a 1:10 w/v ratio (10 g powder in 100 mL water) and heated at 60 ∘°C while continuously stirred for 30 minutes. This specific extraction method was employed to maximize the yield of polar, water-soluble phytoconstituents, such as flavonoids and phenolics, which were hypothesized to be responsible for the extract's antioxidant activity. The resultant crude mixture was centrifuged at 4,000 g for 15 minutes, and the supernatant was collected. To ensure complete extraction, the exhausted residue pellet was subjected to two further re-extractions using fresh volumes of water until the resultant supernatant appeared colorless. All aqueous supernatants were combined and filtered through Whatman filter paper (No. 1). The filtrate was then concentrated under reduced pressure using a rotary evaporator, with the temperature meticulously maintained below 45 °C to prevent degradation of thermolabile components. The concentrated extract was subsequently transferred to a freeze-dryer (lyophilizer) to obtain the solid powdered aqueous leaf extract (ALE). The percentage yield was calculated as (w/w) from 100 g of powder, based on the initial dry weight of the plant material [9, 10]. The final dry powder was stored in hermetically sealed containers at −20 ℃ until required for animal dosing and phytochemical analysis. That is discussed in the sections below.

Quantitative Determination and Confirmation of Kaempferol by High-Resolution Liquid Chromatography-Mass Spectrometry (HR LCMS) [11]

To confirm the presence of Kaempferol, the active compound, High-Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS) analysis was performed on the prepared Aqueous Leaf Extract (ALE). This method was used to provide robust structural confirmation through exact mass and accurate quantification.

Standard and Sample Preparation

Chromatographic-grade Kaempferol reference standard (Purity ≥98%) was accurately weighed and prepared in methanol to prepare stock solutions for establishing the calibration curve. The prepared ALE sample was accurately weighed, dissolved in the mobile phase, and filtered through a 0.22-µm syringe filter before injection to remove particulate matter.

HR LCMS Conditions and Analysis

The analysis was conducted using an integrated HRLC-MS system comprising a liquid chromatograph coupled with a high-resolution mass spectrometer. Chromatographic separation was achieved using a C18 reversed-phase column (100×2.1 mm, 1.7 µm). A gradient mobile phase, typically consisting of distilled water (Solvent A, modified with 0.1% formic acid) and acetonitrile (Solvent B), was employed at a fixed flow rate (0.3 mL/min). The injection volume was typically set at 5 µL. Mass spectrometry detection was performed using an electrospray ionization (ESI) source in negative ion mode, which optimizes ionization efficiency for flavonoids. Mass acquisition was performed in full-scan mode (m/z 100–1,000) with high mass resolving power (≥30,000). Major phytochemicals, including kaempferol, quercetin, rutin, gossypol, and β-sitosterol, were conclusively identified by matching their retention times and exact deprotonated ions $[M-H]^-$ against authentic analytical standards (purity ≥98%). For quantification, seven-point calibration curves (25–2500 ng/mL) were constructed for each compound from 1 mg/mL stock solutions in methanol, yielding correlation coefficients (R^2) of 0.9996–0.9998. For sample preparation, 10.0 mg of lyophilized ALE was sonicated in 10.0 mL methanol for 15 minutes, centrifuged (10,000 rpm, 10 min), and the supernatant collected as primary stock (1000 µg/mL). This was diluted 1:10 with mobile phase to obtain a working solution (100 µg/mL), filtered (0.22 µm), and injected in triplicate (5 µL). Phytochemical concentrations were calculated from integrated peak areas using calibration equations and expressed as mg/g dried extract (mean ± SD, n=3).

C) In Vitro DPPH Radical Scavenging Assay [12, 13]

The intrinsic free radical scavenging capacity of the *T. lampas* Aqueous Leaf Extract (ALE) was evaluated using the stable 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical method. This assay assesses the extract's ability to act as a hydrogen atom donor. A stock solution of DPPH radical (0.1 mM) was prepared by dissolving the powder in methanol. The assay was performed by mixing 2 mL of the prepared ALE or Rutin (positive standard) at various concentrations ranging from 10 µg/mL to 200 µg/mL with 2 mL of the 0.1 mM DPPH solution. The reaction mixtures were immediately vortexed and incubated in the dark at ambient room temperature for 30 minutes. The absorbance was subsequently measured at 517 nm using a UV-Vis spectrophotometer. Control samples contained 2 mL of 0.1 mM DPPH and 2 mL of methanol. The percentage inhibition (% Inhibition) was calculated using the formula:

$$\% \, \boldsymbol{Inhibition} = \frac{\boldsymbol{A\ control\ -\ A\ sample}}{\boldsymbol{A\ control}} \times \mathbf{100}$$

Where $A_{control}$ is the absorbance of the control reaction, and A_{sample} is the absorbance in the presence of the extract or standard. The inhibitory concentration required to scavenge 50% of DPPH radicals (IC50) was determined by plotting extract/standard concentration against the corresponding percentage inhibition and fitting a linear regression.

Acute Oral Toxicity Assessment (OECD 423)

Before efficacy testing, the acute oral toxicity of the *T. lampas* Aqueous Leaf Extract (ALE) was evaluated in accordance with the internationally recognized Organization for Economic Co-operation and Development (OECD) Guideline 423 (Acute Toxic Class Method). A single dose of 2000 mg/kg of the ALE was administered orally to female rats. All animals survived without exhibiting clinical signs of toxicity, gross behavioral changes, or mortality throughout the observation period. Based on these results, the ALE was determined to be safe at doses up to 2000 mg/kg body weight, thereby justifying the selection of the therapeutic doses (100, 200, and 400 mg/kg) used in the nephroprotective study [14].

ANIMALS AND EXPERIMENTAL DESIGN

Ethical Approval and Animal Management

The animal study protocols were meticulously reviewed and approved by the Institutional Animal Ethics Committee (IAEC Approval No: ARCMR/2023/04), in strict accordance with national and international regulations governing the ethical care and use of laboratory animals. Healthy Adult Wistar rats of either sex, weighing 200–300 gm upon arrival, were selected for the study. Animals were acclimatized for a minimum of one week before the start of treatment and housed in standard cages under controlled laboratory conditions (ambient temperature of 22±2 °C and a 12-hour light/dark cycle). Food (standard rodent chow pellets) and water were provided *ad libitum.*

Experimental Grouping

Rats were randomly assigned to six experimental groups (n=6 per group, total N=36) using stratified body-weight randomization to ensure uniformity across groups. Animals were first weighed and ranked by body weight, then systematically allocated to groups using Microsoft Excel (Microsoft 365) to ensure that each group had a comparable mean body weight (±5% variance).

This stratification method minimized baseline physiological variability and enhanced statistical power for detecting treatment effects. The total study duration spanned 28 consecutive days of treatment, a period significantly longer than typical acute models, which often run only 4–7 days. This extended duration was selected specifically to assess the extract's ability to maintain protection against the chronic, cumulative nephrotoxicity characteristic of clinical aminoglycoside use, which relies on prolonged intracellular drug accumulation in the renal tubules. Table 1 lists the experimental grouping and treatment protocol for 28 Days.

Table 1: Experimental Grouping and Treatment Protocol for 28 Days

Group Designation	Treatment Regime (Daily)	Route	Duration
NC (Normal Control)	Distilled water (1mL/day)	Oral (P.O.)	28 Days
GM (Nephrotoxicity Control)	Gentamicin (50 mg/kg)	Intraperitoneal (I.P.)	10 Days
PC (Standard)	Rutin (50 mg/kg/day)	Oral (P.O)	28 Days
ALE-L (Test I) [Oral (P.O)]+ GM [Intraperitoneal (I.P.)]	ALE (100 mg/kg/day)	Oral (P.O)	28 Days
ALE-H (Test II) [Oral (P.O)]+ GM [Intraperitoneal (I.P.)]	ALE (200 mg/kg/day)	Oral (P.O)	28 Days
ALE-HH (Test III) [Oral (P.O)]+ GM [Intraperitoneal (I.P.)]	ALE (400 mg/kg/day)	Oral (P.O)	28 Days

Treatment Protocol

A subchronic gentamicin model was selected to mimic clinically relevant nephrotoxic exposure. The 28-day protocol, comprising 18 days of prophylactic pre-treatment followed by 10 days of concurrent gentamicin exposure, was designed to ensure adequate tissue accumulation of bioactive flavonoids, to upregulate endogenous antioxidant defenses (SOD, CAT, GSH, Nrf2/ARE pathway), and to precondition cellular stress-response mechanisms before the nephrotoxic insult. This prophylactic approach allows kaempferol and other flavonoids to exert genomic effects, including modulation of NF-κB, enhancement of mitochondrial biogenesis, and membrane stabilization, thereby maximizing renoprotective capacity. This strategy mirrors clinical preventive therapy in high-risk patients. It is supported by evidence demonstrating the superior efficacy of flavonoid pre-treatment compared with concurrent or post-treatment regimens in models of drug-induced and ischemia-reperfusion renal injury. Gentamicin dosing and duration were

chosen based on previously published rodent studies that reproducibly produced biochemical and histopathological indices of nephrotoxicity: repeated intraperitoneal injections of gentamicin at 50–100 mg/kg/day induce measurable renal injury within days to weeks, depending on dose and schedule.

Hence, Gentamicin was administered via intraperitoneal (i.p.) injection at a dose of 50 mg/kg per day for 10 days. This divided-daily dosing strategy represents a rigorous model for studying renoprotective agents by ensuring consistent drug exposure. The normal control group received only distilled water vehicle (1 mL/day). The standard positive control received Rutin (50 mg/kg), and the ALE groups received their respective dose daily [15].

Morphological studies

Animal body and kidney weights were meticulously recorded weekly over 28 days. Maintenance of body weight, or inhibition of body weight loss, was evaluated as a preliminary macroscopic indicator of systemic stress and illness progression induced by the GM.

Biological Sample Collection and Preparation

On Day 29, following 24 hours after the final treatment administration, all animals underwent sacrifice under deep anesthesia (induced via a ketamine). Blood was collected via cardiac puncture into plain serum separator tubes. Serum was subsequently isolated by centrifugation (3,000 rpm for 15 minutes at 4 °C). The serum was used immediately for routine clinical chemistry analysis or flash-frozen and stored at -80 °C for subsequent analysis. Both kidneys were rapidly excised, meticulously cleaned, and weighed to determine relative kidney weight (kidney weight normalized to body weight), a crucial marker of edema and inflammation caused by GM exposure. The right kidney was immediately fixed in 10% neutral buffered formalin for histopathological examination. The left kidney was divided: a small section was flash-frozen for future molecular assays, and the remaining tissue was used to prepare tissue homogenates and Post-Mitochondrial Supernatant (PMS) immediately for oxidative stress assays [16, 17].

ASSESSMENT OF RENAL FUNCTION & INJURY [18]

Determination of Serum Biochemical Indicators

Renal function was assessed by measuring the concentration of key nitrogenous waste products in the serum. All analyses were conducted using standard enzymatic and colorimetric diagnostic kits and in accordance with the manufacturer's instructions.

- **Serum Creatinine (SCR):** SCR concentration was determined spectrophotometrically using the kinetic alkaline picrate method (Jaffe's reaction). SCR elevation is a well-established indicator of decreased glomerular filtration rate (GFR), a common outcome detected after 4 days of GM injections.
- **Blood Urea Nitrogen (BUN):** BUN (or urea) concentration was quantified using the Urease-Berthelot method, which measures the release of ammonia after urea hydrolysis. The elevated BUN and SCR together indicate acute azotemia and impaired renal clearance.
- **Uric Acid:** Serum uric acid levels were quantified using an enzymatic colorimetric assay (Uricase method). Measurement of uric acid provided an additional marker reflecting the overall metabolic clearance status associated with renal function decline

Electrolyte Homeostasis Analysis

Electrolyte status was evaluated as a specific indicator of tubular integrity. Gentamicin primarily targets the proximal convoluted tubules, and subsequent cellular damage impairs the kidney's ability to reabsorb and secrete ions selectively. Serum concentrations of sodium (Na^+), potassium (K^+), and chloride (Cl^-) were measured using an automated electrolyte analyzer. Disturbances in these concentrations, particularly hyperkalemia or dysregulation of sodium/chloride, serve as specific, sensitive markers of proximal tubular dysfunction, providing morphological evidence of injury beyond general GFR decline.

Measurement of Urinary Parameters

To assess the functional integrity of both the filtration barrier and the renal tubules, 24-hour urine samples were meticulously collected from all groups on day 28 of the study using individual metabolic cages. Following collection, key urinary parameters, including urine volume, pH, protein, glucose, urea, creatinine, and specific gravity, were quantitatively estimated. Estimation of these parameters was performed using semi-automated urine analyzer kits (ERBA, India) in accordance with the manufacturer's specifications. The specific measurement of urinary protein and glucose excretion provided direct markers of glomerular and proximal tubular dysfunction, respectively, allowing for a comprehensive assessment of the extent of gentamicin-induced kidney damage.

EVALUATION OF RENAL OXIDATIVE STRESS BIOMARKERS [19, 20]

Gentamicin induces nephrotoxicity primarily by increasing reactive oxygen species (ROS) production in the renal cortex. Therefore, the quantification of lipid peroxidation and the activity of endogenous antioxidant enzymes were essential for assessing the extract's cytoprotective mechanism.

Preparation of Kidney Tissue Homogenates and Post-Mitochondrial Supernatant (PMS)

A portion of the frozen left kidney tissue was accurately weighed and homogenized in ice-cold homogenizing buffer (50 mM Tris HCl, 1.15% KCl, pH 7.4) using a motorized Teflon homogenizer. This homogenization step was performed immediately to prevent enzyme degradation. The resulting homogenate was subjected to differential centrifugation. First, it was centrifuged at 9,000 g for 20 minutes at 4 °C to remove cellular debris and nuclei. The supernatant was then transferred and subjected to further high-speed centrifugation (15,000 g for 20 minutes at 4 °C) to isolate the Post-Mitochondrial Supernatant (PMS), which contains the soluble enzymes used in the subsequent biochemical assays. Protein concentration in the PMS was standardized using the Lowry method, with bovine serum albumin (BSA) as the standard, ensuring that all enzyme activities and biomarker concentrations could be accurately normalized per unit protein mass.

Assessment of Lipid Peroxidation (Malondialdehyde, MDA)

Lipid peroxidation, a measure of membrane damage caused by oxidative stress, was quantified by measuring the production of thiobarbituric acid reactive substances (TBARS), predominantly Malondialdehyde (MDA), following the standardized method of Nichans and Samuelson. 0.1 mL of kidney PMS was treated with 2 mL of TBA-TCA-HCl reagent (a 1:1:1 ratio mixture containing 0.37% thiobarbituric acid, 0.25 N HCl, and 15% TCA). The mixture was then heated in a boiling water bath for 15 minutes to facilitate the reaction, cooled rapidly, and centrifuged at 3,000 g for 10 minutes to obtain a clear supernatant. The absorbance of this supernatant was measured spectrophotometrically against a reference blank at 535 nm. MDA concentration was calculated using a standard curve of 1, 1, 3, 3-tetraethoxypropane (TEP) and expressed as nmol/mg protein. A successful protective effect of the ALE was defined as a significant reduction in MDA levels relative to the GM-only control group.

MEASUREMENT OF ANTIOXIDANT ENZYME ACTIVITIES

A. Superoxide Dismutase (SOD) Activity Assay

SOD activity, which catalyzes the conversion of the highly reactive superoxide radical into less reactive hydrogen peroxide and molecular oxygen, was estimated by its ability to inhibit the photoreduction of Nitro Blue Tetrazolium (NBT), according to the method established by Beauchamp and Fridovich. The reaction mixture consisted of 0.5 mL of renal PMS, 1.0 mL of 50 mM sodium carbonate, 0.4 mL of 25 µM NBT, and 0.2 mL of 0.1 mM EDTA. The enzymatic reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. The resulting change in absorbance, indicative of NBT reduction, was recorded spectrophotometrically at 560 nm. Units of SOD activity were quantified as the amount of enzyme required to inhibit NBT reduction by 50% and were reported as U/mg protein.

B. Catalase (CAT) Activity Assay

CAT activity, which converts hydrogen peroxide into water and oxygen, was determined using Aebi's standard method. The assay monitors the decomposition rate of hydrogen peroxide (H_2O_2). The reaction was initiated by adding 0.1 mL of PMS to a reaction mixture containing phosphate buffer (pH 7.0) and 10 mM H_2O_2. The decrease in H_2O_2 concentration was continuously monitored spectrophotometrically by recording the reduction in absorbance at 240 nm over a designated time period (e.g., 1 minute). Activity was calculated using the molar extinction coefficient of H_2O_2 (ϵ=43.6 M^{-1} cm^{-1}) and expressed as U/mg. Comprehensive measurement of SOD, CAT, and the non-enzymatic defenses provides crucial insight, as previous studies suggest that while Kaempferol protects SOD activity, it may not significantly affect CAT activity. Therefore, assessing all parameters allows evaluation of whether the entire ALE exhibits a broader protective profile than pure Kaempferol.

C. Quantification of Non-Enzymatic Antioxidant Status (Reduced Glutathione, GSH)

The concentration of reduced glutathione (GSH), a pivotal non-protein thiol and major component of the cellular defense system, was determined using the method described by Moren. This method relies on GSH's ability to react with 5, 5 ′-Dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent) to form a yellow-colored complex. 500 µL of the tissue homogenate was first precipitated by adding 100 µL of 25%

TCA to remove proteins. The mixture was centrifuged (3,000 g for 10 minutes) to settle the precipitate. 100 μL of the resulting clear supernatant was added to a test tube containing 2 mL of 0.6 mM DTNB and 0.9 mL of 0.2 mM sodium phosphate buffer (pH 7.4).

The resulting yellow color was measured spectrophotometrically at 412 nm against a reagent blank. Sulphydryl content, which represents GSH, was calculated utilizing the DTNB molar extinction coefficient of 13,100 $M^{-1} cm^{-1}$ and expressed as mg/g. A robust protective agent should inhibit the GM-induced depletion of this crucial endogenous antioxidant.

ASSESSMENT OF RENAL INFLAMMATORY SIGNALING (MOLECULAR MECHANISM) [21]

Oxidative stress induced by gentamicin activates transcription factors that drive the inflammatory response. The anti-inflammatory action of the ALE was therefore evaluated by measuring key inflammatory mediators and the state of the NF-κB pathway activation.

Determination of Cytokines via Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of the key pro-inflammatory cytokines, Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6), were quantified in both the collected serum and the kidney tissue homogenates. Commercial rat-specific ELISA kits (vendor-required) were employed for both cytokines, following the manufacturer's protocols. Samples (serum or tissue supernatant) and standards were added to pre-coated microtiter plates.

After appropriate incubation periods, washes, and the addition of enzyme-linked detection antibodies and substrate solution, the reaction was halted, and the optical density was measured spectrophotometrically at 450 nm. Concentrations were determined by reference to the standard curve and expressed as pg/mL for serum samples or pg/mg protein for tissue samples. Reduced levels of these cytokines in the ALE-treated groups would demonstrate successful mitigation of the systemic and local inflammatory cascade.

Analysis of Nuclear Factor-kappa B (NF-κB) Signaling Pathway (Western Blotting)

The activation state of the NF-κB pathway, a canonical regulator of inflammatory gene expression, was analyzed via Western Blotting. NF-κB activation involves the phosphorylation and degradation of its inhibitor, IκBα, thereby allowing the p65 and p50 subunits to translocate from the cytoplasm to the nucleus to initiate transcription.

a. Cytosolic and Nuclear Fractionation

To accurately assess the translocation state, kidney tissue was subjected to differential centrifugation using a commercially available nuclear & cytoplasmic extraction kit (vendor required). This process yields purified cytosolic & nuclear protein fractions. The purity & efficacy of this fractionation were rigorously confirmed post-Western Blotting by probing for known exclusive cytosolic markers (β-actin) and nuclear markers (Lamin B1).

b. Western Blotting Protocol

Equal amounts of protein (30–50 μg) from both the nuclear and cytoplasmic fractions were separated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) under reducing conditions. Separated proteins were then transferred by electrophoresis onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked (e.g., 5% non-fat milk dissolved in Tris-Buffered Saline with Tween 20) and incubated overnight at 4 °C with primary antibodies targeting key components: NF-κB p65, NF-κB p50, total IκBα, and phosphorylated IκBα. After washing, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, and signals were visualized using enhanced chemiluminescence (ECL) detection reagents.

c. Densitometric Quantification

Band intensity was quantified using specialized densitometry software. Normalization was performed relative to the appropriate housekeeping proteins:

- Cytosolic p65 and p50 densities were normalized against the cytosolic loading control, β-actin.
- Nuclear p65 and p50 densities (the critical measure of functional activation) were normalized against the nuclear loading control, Lamin B1.
- The activation status of the upstream pathway was confirmed by calculating the ratio of phosphorylated IκBα to total IκBα in the cytosolic fraction.

The goal of this analysis was to demonstrate that the extract's antioxidant activity (Section IV) led to a corresponding reduction in the phosphorylation and degradation of IκBα, thereby significantly reducing the nuclear translocation ratio of

p65/p50 compared to the GM group. This confirms that renoprotection operates mechanistically by suppressing the oxidative stress-driven inflammatory transcriptional pathway.

HISTOPATHOLOGICAL EXAMINATION AND SCORING

Tissue Fixation, Processing, and Staining

The right kidney tissue, fixed in 10% neutral-buffered formalin, was maintained in this solution for at least 48 hours. Following fixation, the tissues were processed using standard histological techniques: dehydration through an ascending series of ethanol concentrations, clearing in xylene & final embedding in paraffin wax blocks. Tissue sections were cut at a uniform thickness of 4 μm using a rotary microtome. The sections were then deparaffinized, rehydrated & stained with Hematoxylin and Eosin (H&E) for standard morphological evaluation [22, 23].

Histopathological Evaluation and Injury Scoring

H&E-stained sections were evaluated under a light microscope by a board-certified veterinary pathologist. To ensure methodological objectivity, the pathologist was completely blinded to the experimental treatment groups during the evaluation process. The assessment focused primarily on the renal cortex, examining key features of Gentamicin-induced acute tubular necrosis (ATN), including tubular epithelial necrosis, loss of the tubular brush border, tubular dilation, granular and hyaline casts, interstitial edema, and inflammatory cell infiltration [24].

Semi-Quantitative Scoring

The extent of renal injury was rigorously quantified using a standardized semi-quantitative scoring system (Table 2), based on the percentage of cortical tubules exhibiting pathological damage. Ten random high-power fields (HPFs) were examined per section, and the average score for each animal was calculated. This numerical scoring system provided a statistically analyzable, objective endpoint that allowed direct correlation between the severity of morphological damage and the observed biochemical functional markers (SCR, BUN, Proteinuria) and oxidative stress parameters [24].

Table 2: Semi-Quantitative Scoring Criteria for Histopathological Evaluation of Renal Cortex.

Histopathological Parameter	Score 0	Score 1 (Mild)	Score 2 (Moderate)	Score 3 (Severe)
Tubular necrosis	None	Focal necrosis involving <25% tubules	25–50% tubules affected	>50% tubules affected
Interstitial inflammation	Absent	Mild infiltration	Moderate infiltration	Severe infiltration
Cast formation	Absent	Few hyaline/granular casts	Moderate number	Numerous casts throughout the cortex

Statistical Analysis

All quantitative data collected across Sections II through VI were analyzed using the dedicated statistical software GraphPad Prism v10 and one-way ANOVA. Results were uniformly expressed as the Mean ± Standard Error of the Mean (SEM). Statistical comparisons among the six experimental groups were performed using a one-way analysis of variance (ANOVA), followed by an appropriate post-hoc test (Tukey's) to identify specific differences between the treatment groups and the nephrotoxicity control group (GM).

For the Western Blot densitometry results, the normalized ratios (e.g., nuclear p65/Lamin B1) were used as input for the ANOVA. Differences were considered statistically significant if the calculated p-value was less than 0.05 ($p<0.05$). Higher levels of statistical confidence were noted where applicable ($p<0.01$ or $p<0.001$). This rigorous approach ensured the statistical validity of the findings linking the prophylactic ALE treatment to the observed protective effects across functional, oxidative, and inflammatory endpoints [25].

RESULT AND DISCUSSION

Preparation and Characterization of Test Material

Fresh leaves of *Thespesia lampas* (Malvaceae) were collected from authenticated geographical sites, taxonomically verified, and a voucher specimen was deposited in the institutional herbarium. The shade-dried leaf powder of *Thespesia lampas* was subjected to exhaustive aqueous extraction (60°C for 30 minutes) followed by filtration, concentration under reduced pressure, and subsequent lyophilization. This specific protocol, designed to optimize the recovery of polar phytochemicals, including phenolic acids and flavonoids, yielded a dark brown, solid, powdered Aqueous Leaf Extract (ALE). The calculated percentage yield of the ALE, based on the initial dry weight of the plant material (100 g), was 2.5% (w/w). The concentrated extract was stored appropriately for subsequent chemical and biological analyses

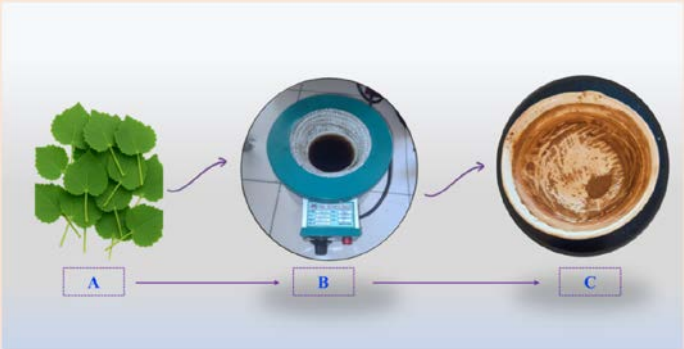

Figure 1: (A) Fresh leaves of *T. lampas*, (B) Powdered material under extraction, (C) Aqueous extract (ALE) powder

Quantitative Determination and Confirmation of Kaempferol by High-Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS).

High-Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS) analysis was employed to profile and confirm the major bioactive flavonoids present in the aqueous leaf extract of Thespesia lampas. The HR-LCMS system, integrating ultra-high-performance liquid chromatography (UHPLC) with a high-resolution accurate-mass analyzer, provided excellent chromatographic resolution and precise mass detection for unambiguous compound identification. The analysis revealed multiple flavonoid constituents, including kaempferol, quercetin, rutin, and gossypol, as well as β-sitosterol, as the principal phytochemicals. Among these, kaempferol exhibited the highest abundance, as indicated by its dominant chromatographic peak and most intense mass spectral signal. Kaempferol was detected at a retention time of 17.36 min with a molecular ion peak at m/z 285.04 ($[M-H]^-$), consistent with its theoretical mass ($C_{15}H_{12}O_6$). The compound identity was further verified by accurate mass measurement (within ±5 ppm), isotopic distribution, and characteristic MS/MS fragmentation patterns, thereby confirming the presence of kaempferol as the major flavonoid in the extract (Figure 2). Quantitative determination of all identified phytochemicals was performed using external calibration methodology with authentic reference standards (purity ≥98%). Seven-point calibration curves (25–2500 ng/mL) were established for each compound, demonstrating excellent linearity with correlation coefficients (R^2) ranging from 0.9996 to 0.9998. Based on these validated methods, the phytochemical composition of the ALE was quantified as follows: kaempferol 20.8 ± 2.4 mg/g (2.08 ± 0.24% w/w), quercetin 3.1 ± 0.4 mg/g (0.31 ± 0.04% w/w), rutin 1.8 ± 0.3 mg/g (0.18 ± 0.03% w/w), gossypol 1.4 ± 0.2 mg/g (0.14 ± 0.02% w/w), and β-sitosterol 2.6 ± 0.3 mg/g (0.26 ± 0.03% w/w). The predominance of kaempferol (2.08% w/w) confirms the extract's designation as "kaempferol-rich" and, along with the synergistic presence of other bioactive flavonoids, provides a robust phytochemical basis for the observed antioxidant and nephroprotective pharmacological effects.

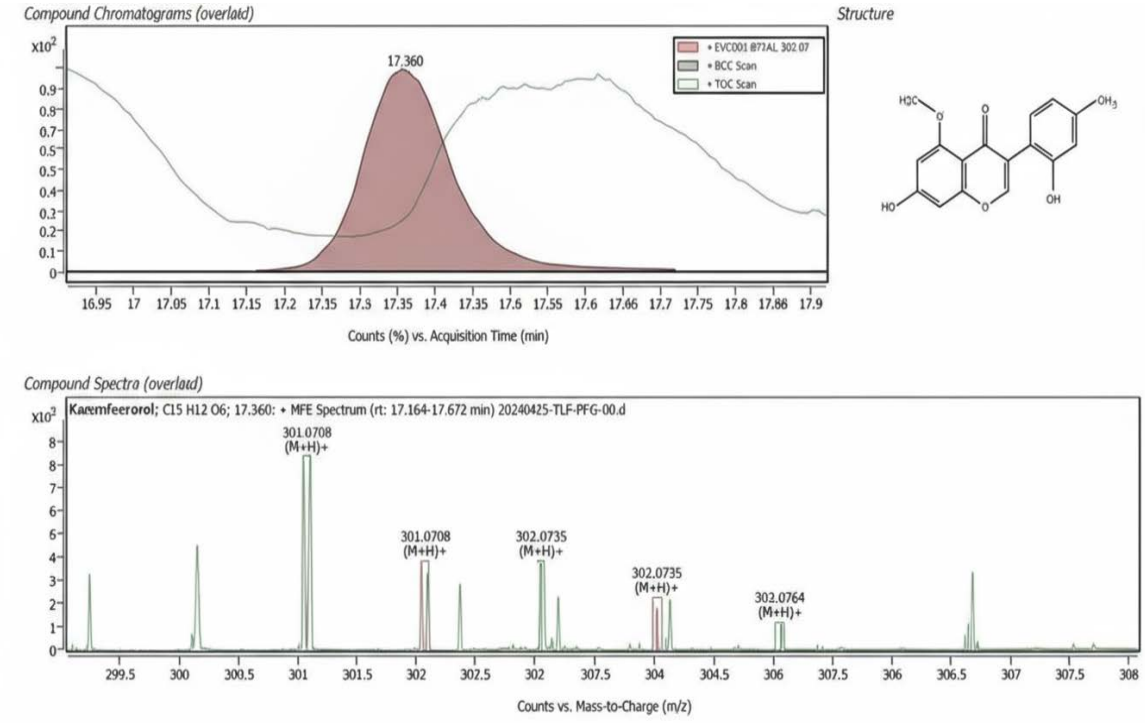

Figure 2: High-Resolution LC–MS chromatogram and mass spectrum of kaempferol detected in the aqueous leaf extract of *Thespesia lampas*.

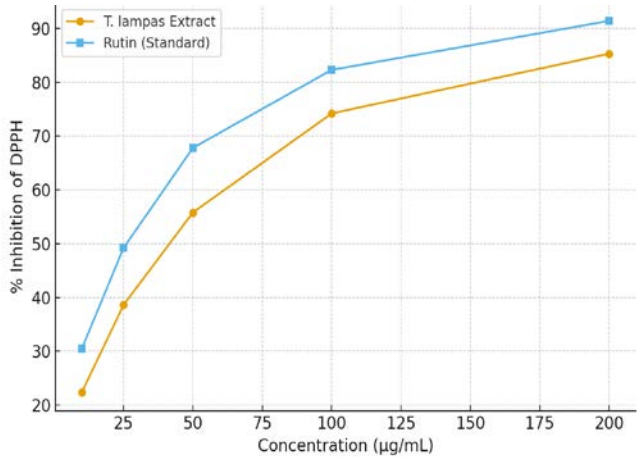

Figure 3: DPPH radical scavenging activity of *Thespesia lampas*aqueous leaf extract compared with rutin.

The extract exhibited concentration-dependent antioxidant activity, with an IC_{50} of 62.4 µg/mL, while rutin showed higher activity (IC_{50} = 48.6 µg/mL). Values are expressed as mean ± SEM (n = 3).

In Vitro DPPH Radical Scavenging Assay

The antioxidant potential of the *Thespesia lampas* aqueous leaf extract (ALE) was evaluated using the DPPH radical scavenging assay. The extract exhibited a marked, concentration-dependent increase in free radical-scavenging activity, confirming its potent hydrogen-donating ability. At the highest tested concentration (200 µg/mL), the extract exhibited 85.32 ± 1.2% inhibition of DPPH radicals, corresponding to an IC_{50} value of 62.4 µg/mL. In comparison, the standard flavonoid rutin achieved 91.45 ± 1.0% inhibition with an IC_{50} of 48.6 µg/mL under identical conditions. Although slightly less potent than rutin, the ALE displayed substantial antioxidant efficacy, indicative of its richness in phenolic and flavonoid constituents. These findings validate the extract's strong free-radical scavenging capacity, supporting its proposed role in attenuating gentamicin-induced oxidative stress & nephrotoxicity (Figure 3).

Acute Oral Toxicity Assessment (OECD 423)

The acute oral toxicity evaluation of the *Thespesia lampas* aqueous leaf extract (ALE), conducted in accordance with OECD Guideline 423, revealed no signs of toxicity or mortality at the highest dose of 2000 mg/kg body weight. Throughout the 14-day observation period, all treated animals remained healthy, exhibiting normal behavioral, neurological, and autonomic responses, with no alterations in food and water intake or body weight gain. No gross pathological abnormalities were observed upon necropsy. These findings confirm that the ALE is non-toxic up to 2000 mg/kg, indicating a wide margin of safety and supporting the selection of 100, 200 & 400 mg/kg as the therapeutic dose range for subsequent nephroprotective evaluation.

Animals and Experimental Design

The animal study, conducted over 28 consecutive days following ethical approval (IAEC Approval No: ARCMR/2023/04), utilized six groups of Wistar rats (n=6) to evaluate the protective effects of the *T. lampas* extract against gentamicin (GM) nephrotoxicity model. The very first step was to determine the GM-induced body weight reduction in a dose-dependent manner, along with other parameters, which are discussed in the sections below.

Morphological studies

Gentamicin administration (Group II) resulted in a marked decline in body weight gain and a significant increase in relative kidney weight compared to the normal control group, indicating renal hypertrophy and systemic toxicity. In contrast, co-administration of *Thespesia lampas* aqueous leaf extract (ALE) mitigated these alterations in a dose-dependent manner. Notably, the 400 mg/kg dose demonstrated the most prominent normalization of both body and kidney weights, comparable to the standard flavonoid rutin-treated group. These findings suggest that *T. lampas* ALE confers a protective effect against gentamicin-induced renal stress and weight loss, likely through its antioxidant and anti-inflammatory bioactivity (Table 3). And (Figure 4). After Biological Sample Preparation, the Assessment of Renal Function was carried out, and Biochemical Parameters (Serum Creatinine, Urea, Uric Acid, and Electrolytes) were measured, as discussed below.

Table 3: Effect of *T. lampas*aqueous leaf extract on body weight and kidney weight in gentamicin-induced nephrotoxic rats.

Parameter	Normal Control	Gentamicin Control	Standard (Rutin)	*T. lampas* 100 mg/kg	*T. lampas* 200 mg/kg	*T. lampas* 400 mg/kg
Change in body weight (g)	4.36 ± 0.34	3.35 ± 0.21	4.13 ± 0.21	3.44 ± 0.45	3.52 ± 0.34*	3.92 ± 0.27**
Kidney weight (g)	1.76 ± 0.23	2.63 ± 0.34	1.60 ± 0.21***	1.35 ± 0.24*	1.51 ± 0.33*	1.56 ± 0.32***

*Values are expressed as mean ± SEM (n = 6). Statistical significance: *p < 0.05, **p < 0.01, **p < 0.001 vs. gentamicin control*

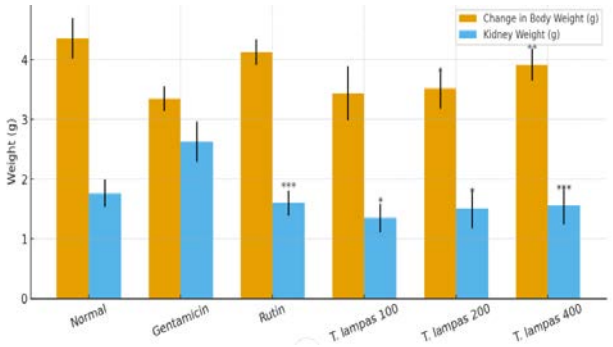

Figure 4: Effect of *T. lampas* aqueous leaf extract on body and kidney weight in gentamicin-induced nephrotoxicity.

Assessment of Renal Function and Electrolyte Homeostasis Analysis

Gentamicin administration produced a pronounced nephrotoxic effect, as evidenced by significant elevations in serum creatinine, urea, uric acid, sodium (Na^+), potassium (K^+), and chloride (Cl^-) levels compared to the normal control group, reflecting renal dysfunction and electrolyte imbalance. Oral co-administration of *T. lampas* aqueous leaf extract (ALE) markedly restored these biochemical parameters toward normal values in a dose-dependent manner. The high-dose group (400 mg/kg) showed the most pronounced normalization of renal biomarkers, with values comparable to those in the standard rutin-treated group. Specifically, *T. lampas* ALE (400 mg/kg) reduced serum creatinine & urea levels from 1.13 ± 0.40 mg/dL & 26.33 ± 0.30 mg/dL in the gentamicin group to 0.30 ± 0.20 mg/dL & 16.16 ± 0.30 mg/dL, respectively ($p < 0.001$). Similarly, serum uric acid, Na^+, K^+ & Cl^- levels were significantly normalized, indicating restoration of renal excretory and ionic homeostasis. These results corroborate the nephroprotective efficacy of *T. lampas*, likely mediated through its antioxidant and anti-inflammatory bioactive constituents (Table 4, Figure 5).

Measurement of Urinary Parameters

Gentamicin administration (Group II) produced marked alterations in urinary indices consistent with acute tubular injury. Compared to the normal control group, gentamicin-treated rats exhibited a significant reduction in urine output (7.5 ± 0.3 mL/24h) and urinary pH (5.6 ± 0.2), accompanied by pronounced proteinuria (38.5 ± 2.3 mg/dL) & glycosuria (16.8 ± 0.4 mg/dL). Urinary creatinine levels were markedly decreased (0.85 ± 0.08 mg/dL), while specific gravity increased (1.032 ± 0.01), reflecting impaired glomerular filtration & tubular reabsorption due to gentamicin-induced nephrotoxicity. Treatment with *T. lampas* aqueous leaf extract (ALE) significantly ameliorated these abnormalities in a dose-dependent manner. The medium- and high-dose group (200 & 400mg/kg) notably restored urinary output, pH & creatinine levels, while markedly reducing protein and glucose excretion compared to the gentamicin control. At the highest dose (400 mg/kg), urinary protein (12.3 ± 1.1 mg/dL), glucose (4.3 ± 0.2 mg/dL), and pH (6.8 ± 0.3) values were comparable to the standard rutin-treated group, indicating near normalization of renal excretory function. Microscopic examination of urine sediments corroborated these findings, showing diminished epithelial cell shedding, absence of casts, and overall improved urine clarity in extract-treated groups. These results confirm that *T. lampas* ALE effectively preserved tubular integrity and glomerular function, mitigating gentamicin-induced alterations in urinary parameters through its nephroprotective mechanism (Table 5).

Protein Quantification for Normalization

Total protein content in renal tissue homogenates was determined by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin (BSA; Sigma-Aldrich) as the reference standard. A seven-point calibration curve was generated using BSA concentrations of 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/mL prepared in phosphate buffer. The Lowry reagent mixture (alkaline copper sulfate and Folin-Ciocalteu reagent) was added sequentially, and absorbance was measured at 660 nm using a UV-Vis spectrophotometer after 30 min of color development at room temp. The calibration equation obtained was $y = 0.425x + 0.012$ ($R^2 = 0.9985$), where y represents absorbance and x represents protein concentration in mg/mL. Sample protein concentrations were interpolated from this standard curve, and all oxidative stress biomarkers (MDA, SOD, GSH, CAT) were normalized per mg of protein to ensure accurate quantitative assessment.

Evaluation of Renal Oxidative Stress Biomarkers:

Gentamicin administration resulted in a pronounced oxidative imbalance in renal tissue, as evidenced by a significant increase in malondialdehyde (MDA) levels and a marked depletion of endogenous antioxidant defenses, including superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) activities (Table 6). The substantial rise in MDA (80.76 ± 0.21 nmol/mg protein) in the gentamicin control group confirmed enhanced lipid peroxidation and membrane damage resulting from the accumulation of reactive oxygen species

(ROS). Treatment with *Thespesia lampas* aqueous leaf extract (ALE) significantly mitigated these oxidative disturbances in a dose-dependent manner. The medium and high doses (200 and 400 mg/kg) notably restored renal antioxidant enzyme levels, reflected by increased SOD, GSH, and CAT activities, while markedly reducing MDA concentrations. At 400 mg/kg, MDA levels (15.14 ± 0.21 nmol/mg protein) and antioxidant markers (SOD: 164.32 ± 0.20 U/mg protein; GSH: 149.22 ± 0.30 mg/g; CAT: 3.92 ± 0.11 U/mg) were comparable to those observed in the rutin-treated standard group, indicating near-complete normalization of oxidative parameters. These findings clearly demonstrate that *T. lampas* ALE confers potent antioxidant protection against gentamicin-induced renal oxidative damage by attenuating lipid peroxidation and enhancing the enzymatic and non-enzymatic antioxidant defense systems, thereby maintaining redox homeostasis in renal tissues (Table 6).

Table 4: Effect of *Thespesia lampas*aqueous leaf extract on renal biomarkers and electrolyte balance in gentamicin-induced nephrotoxic rats.

Group	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)
Normal	0.31 ± 0.03	16.05 ± 0.30	0.03 ± 0.02	140.7 ± 0.1	4.06 ± 0.3	101.6 ± 0.2
Gentamicin	1.13 ± 0.40	26.33 ± 0.30	0.05 ± 0.03	165.5 ± 0.3	6.60 ± 0.3	111.6 ± 0.3
Standard (Rutin)	0.30 ± 0.2***	16.12 ± 0.2***	0.03 ± 0.02***	142.5 ± 0.4***	4.02 ± 0.3***	109 ± 0.2***
T. lampas 100 mg/kg	0.30 ± 0.1**	18.83 ± 0.3**	0.09 ± 0.4***	147.5 ± 0.2***	3.96 ± 0.3**	105.8 ± 0.3*
T. lampas 200 mg/kg	0.36 ± 0.3**	24.5 ± 0.5***	0.04 ± 0.1***	147.3 ± 0.3**	4.31 ± 0.2**	105.8 ± 0.2**
T. lampas 400 mg/kg	0.30 ± 0.2***	16.16 ± 0.3***	0.02 ± 0.3***	144.8 ± 0.3***	3.66 ± 0.3***	101 ± 0.2***

*Values are expressed as mean ± SEM (n = 6). Statistical significance: p < 0.05, *p < 0.01, *p < 0.001 vs. gentamicin control*

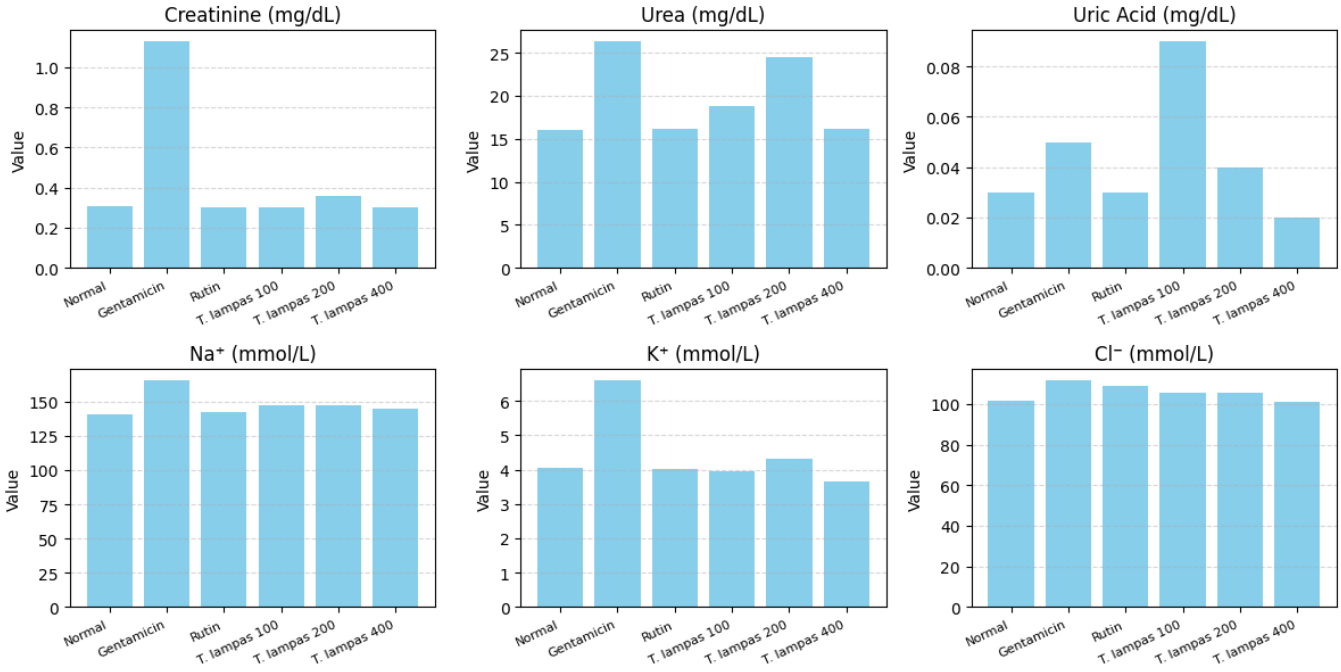

Figure 5: Effect of *Thespesia lampas*Extract on Renal Biomarkers and Electrolyte Balance in Gentamicin-Induced Nephrotoxicity

Group	Urine Volume (mL/24 h)	pH	Protein (mg/dL)	Glucose (mg/dL)	Creatinine (mg/dL)	Specific Gravity
Normal Control	12.3 ± 0.4	6.9 ± 0.2	8.4 ± 0.3	2.3 ± 0.2	2.15 ± 0.12	1.020 ± 0.01
Gentamicin Control	7.5 ± 0.3	5.6 ± 0.2	38.5 ± 2.3	16.8 ± 0.4	0.85 ± 0.08	1.032 ± 0.01
Standard (Rutin 50 mg/kg)	11.6 ± 0.5***	6.8±0.1***	10.2±0.5***	3.0±0.2***	2.10 ±0.11***	1.022± 0.01***
T. lampas 100 mg/kg	8.4 ± 0.4*	6.1 ± 0.2**	21.6 ± 1.2*	10.5 ± 0.3*	1.56 ± 0.10*	1.028 ± 0.01*
T. lampas 200 mg/kg	9.8 ± 0.5***	6.5±0.2***	16.4 ± 1.0*	7.2 ± 0.3**	1.86 ± 0.09**	1.025 ± 0.01**
T. lampas 400 mg/kg	11.2 ± 0.6***	6.8±0.3***	12.3 ± 1.1***	4.3 ± 0.2***	2.06± 0.10***	1.021±0.01***

*Values represent mean ± SEM (n=6 per group). Statistical analysis: One-way ANOVA followed by post-hoc pairwise comparisons. **p < 0.001 vs. Gentamicin Control group.*

Evaluation of Renal Oxidative Stress Biomarkers:

Gentamicin administration resulted in a pronounced oxidative imbalance in renal tissue, as evidenced by a significant increase in malondialdehyde (MDA) levels and a marked depletion of endogenous antioxidant defenses, including superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) activities (Table 6). The substantial rise in MDA (80.76 ± 0.21 nmol/mg protein) in the gentamicin control group confirmed enhanced lipid peroxidation and membrane damage resulting from the accumulation of reactive oxygen species (ROS). Treatment with *Thespesia lampas* aqueous leaf extract (ALE) significantly mitigated these oxidative disturbances in a dose-dependent manner. The medium and high doses (200 and 400 mg/kg) notably restored renal antioxidant enzyme levels, reflected by increased SOD, GSH, and CAT activities, while markedly reducing MDA concentrations. At 400 mg/kg, MDA levels (15.14 ± 0.21 nmol/mg protein) and antioxidant markers (SOD: 164.32 ± 0.20 U/mg protein; GSH: 149.22 ± 0.30 mg/g; CAT: 3.92 ± 0.11 U/mg) were comparable to those observed in the rutin-treated standard group, indicating near-complete normalization of oxidative parameters. These findings clearly demonstrate that *T. lampas* ALE confers potent antioxidant protection against gentamicin-induced renal oxidative damage by attenuating lipid peroxidation and enhancing the enzymatic and non-enzymatic antioxidant defense systems, thereby maintaining redox homeostasis in renal tissues (Table 6).

Table 6: Effect of *T. lampas* Extract on Oxidative Stress Biomarkers in Gentamicin-Induced Nephrotoxicity

Group	MDA (nmol/mg protein)	SOD (U/mg protein)	GSH (mg/g tissue)	CAT (U/mg protein)
Normal Control	16.26 ± 0.23	178.55 ± 0.34	153.69 ± 0.21	4.35 ± 0.15
Gentamicin Control	80.76 ± 0.21	59.82 ± 0.23	53.21 ± 0.23	1.53 ± 0.10
Std. (Rutin 50 mg/kg)	17.34 ± 0.52***	172.90 ± 0.43***	151.78 ± 0.34***	4.10 ± 0.11***
T. lampas 100 mg/kg	12.27 ± 0.70*	137.00 ± 0.10*	129.53 ± 0.10*	2.61 ± 0.21*
T. lampas 200 mg/kg	13.35 ± 0.23**	148.90 ± 0.20*	138.26 ± 0.23*	3.20 ± 0.12**
T. lampas 400 mg/kg	15.14 ± 0.21***	164.32 ± 0.20***	149.22 ± 0.30***	3.92 ± 0.11***

*Values represent mean ± SEM (n=6 per group). MDA: malondialdehyde (lipid peroxidation marker); SOD: superoxide dismutase; GSH: reduced glutathione; CAT: catalase. Statistical analysis: One-way ANOVA followed by post-hoc pairwise comparisons. **p < 0.001 vs. Gentamicin Control group.*

Assessment of Renal Inflammatory Signaling (Molecular Mechanism)

Gentamicin administration markedly upregulated renal inflammatory mediators, as evidenced by significant elevations in serum and renal tissue levels of TNF-α and IL. In the nephrotoxic control group, TNF-α and IL-6 concentrations increased nearly threefold compared with the normal control group, confirming the establishment of a robust inflammatory response. Treatment with *Thespesia lampas* aqueous leaf extract (ALE) produced a dose-dependent suppression of these pro-inflammatory cytokines. The high-dose group (400 mg/kg) demonstrated cytokine levels comparable to those observed in the rutin-treated group, indicating effective inhibition of inflammatory cascades. The reduction in systemic and local cytokine release suggests that the extract mitigated oxidative stress–driven inflammatory signaling, consistent with the proposed kaempferol-mediated mechanism. Western blot analysis further substantiated these findings by delineating the modulation of the NF-κB signaling pathway, the principal transcriptional regulator of inflammation. In the gentamicin-only group, densitometric analysis revealed a pronounced increase in phosphorylated IκBα levels along with nuclear accumulation of NF-κB subunits (p65 and p50), indicating canonical pathway activation. Conversely, ALE treatment effectively downregulated IκBα phosphorylation and significantly curtailed the nuclear translocation of NF-κB p65/p50 subunits in a dose-dependent manner. The high-dose extract (400 mg/kg) nearly restored NF-κB activity to basal control levels, paralleling the standard rutin-treated group. These data collectively demonstrate that the nephroprotective efficacy of *Thespesia lampas* extract is mechanistically mediated through the suppression of oxidative stress-induced NF-κB activation and subsequent attenuation of downstream pro-inflammatory cytokines (TNF-α, IL-6). This dual antioxidant and anti-inflammatory modulation underscores the pivotal role of kaempferol and other flavonoids in restoring renal homeostasis in gentamicin-induced nephrotoxicity.

Histopathological Examination and Scoring

Microscopic evaluation of hematoxylin–eosin (H&E)-stained kidney sections revealed marked differences in renal cortical architecture among the experimental groups (Figure 7A–F). The normal control group (Figure 7A) exhibited well-preserved renal histoarchitecture characterized by normal glomeruli, intact tubular epithelium, and clear Bowman's spaces without any evidence of necrosis or inflammation. In contrast, the

gentamicin-treated group (Figure 7B) demonstrated extensive histopathological alterations, including severe tubular epithelial necrosis, desquamation, vacuolar degeneration, interstitial edema, hemorrhage, and marked leukocyte infiltration consistent with acute tubular necrosis (ATN) typically induced by aminoglycoside nephrotoxicity. Treatment with rutin (standard, Figure 7C) significantly mitigated these lesions, showing regeneration of the tubular epithelium, reduction in necrosis, and preservation of glomerular structure, indicating its effective antioxidant and anti-inflammatory protective effects. Animals treated with *T. lampas* aqueous leaf extract displayed a dose-dependent improvement in renal histoarchitecture. The 100 mg/kg group (Figure 7D) showed mild tubular degeneration and limited inflammatory infiltration. The 200 mg/kg group (Figure 7E) exhibited notable restoration of tubular morphology, reduced necrosis, and minimal infiltration. Remarkably, the 400 mg/kg group (Figure 7F) showed near-complete normalization of renal structure, including intact glomeruli, absence of inflammatory foci, and fully regenerated tubular epithelium, comparable to the standard group.

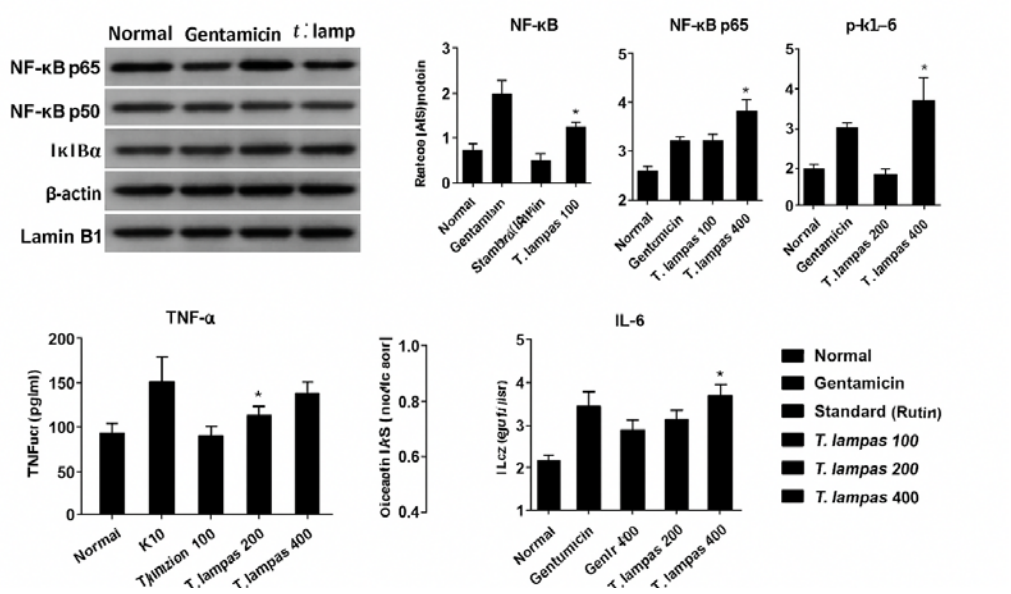

Figure 6: Western blot layout showing bands for p65, p50, IκBα, p-IκBα, β-actin, Lamin B1 and TNF-α and IL-6 concs.

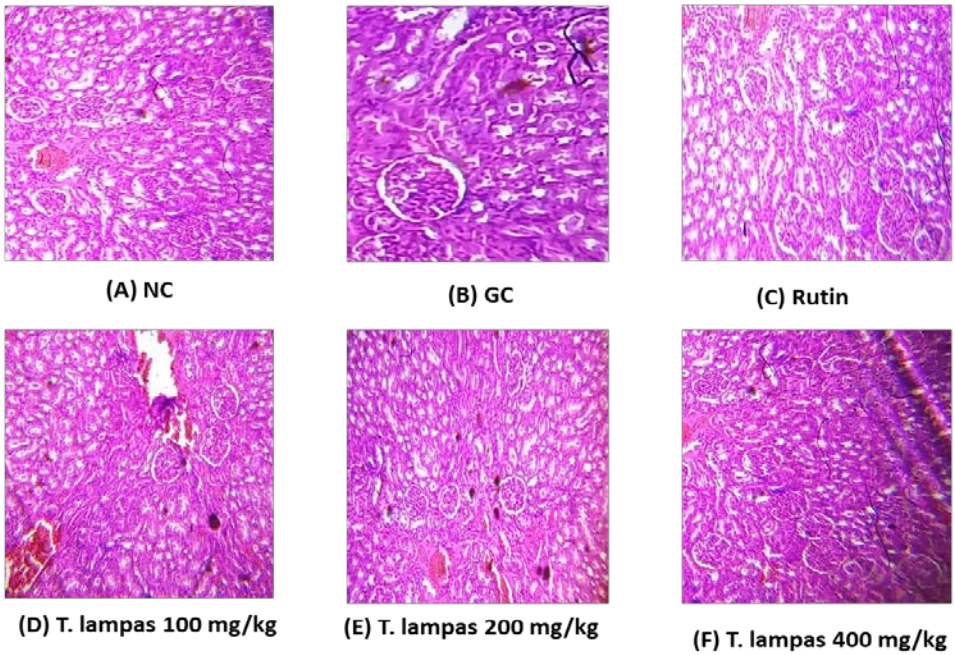

Figure 7: Representative photomicrographs of renal cortex (H&E, ×400)

(A) Normal control showing intact renal architecture; (B) Gentamicin control showing severe tubular necrosis and inflammation; (C) Rutin (standard) showing marked recovery of renal tubules; (D) T. lampas 100 mg/kg showing mild necrosis; (E) T. lampas 200 mg/kg showing substantial tubular regeneration; (F) T. lampas 400 mg/kg showing nearly complete restoration of normal histology.

Semi-quantitative scoring of renal cortical injury further supported these observations, demonstrating significant reductions in scores for tubular necrosis, interstitial inflammation, and cast formation across all treated groups relative to the gentamicin control (Table 7). Gentamicin exposure resulted in severe renal cortical injury, reflected by markedly elevated necrosis, inflammation, and cast formation scores. Treatment with *Thespesia lampas* aqueous leaf extract (ALE) significantly and dose-dependently ameliorated histopathological damage, with the 400.

Table 7: Semi-Quantitative Histopathological Scores of Renal Cortex in Gentamicin-Induced Nephrotoxicity

Group	Tubular Necrosis	Interstitial Inflammation	Cast Formation	Total Injury Score (Mean ± SEM)
Normal Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Gentamicin Control	2.90 ± 0.10	2.80 ± 0.12	2.75 ± 0.11	8.45 ± 0.25
Std. (Rutin 50 mg/kg)	0.60 ± 0.12	0.50 ± 0.10	0.40 ± 0.10	1.50 ± 0.15***
T. lampas 100 mg/kg	1.80 ± 0.15	1.60 ± 0.10	1.40 ± 0.10	4.80 ± 0.20**
T. lampas 200 mg/kg	1.10 ± 0.10	0.90 ± 0.10	0.80 ± 0.12	2.80 ± 0.18**
T. lampas 400 mg/kg	0.50 ± 0.10	0.40 ± 0.10	0.30 ± 0.08	1.20 ± 0.12***

*Values represent mean ± SEM (n=6 per group). Histopathological scoring scale: 0 = none, 1 = mild (<25% cortical area affected), 2 = moderate (25-50% affected), 3 = severe (>50% affected). Total injury score = sum of tubular necrosis, interstitial inflammation, and cast formation scores. Statistical analysis: One-way ANOVA followed by post-hoc pairwise comparisons. **p < 0.001 vs. Gentamicin Control group.*

The sustained clinical reliance on aminoglycoside antibiotics like gentamicin (GM) is critically undermined by the risk of GM-induced nephrotoxicity (GIN), a significant contributor to acquired acute kidney injury (AKI). Given that the pathogenesis of GIN is driven by the generation of Reactive Oxygen Species (ROS) and subsequent activation of pro-inflammatory pathways in renal proximal tubular cells, identifying agents with potent antioxidant and anti-inflammatory properties is a major research objective. This study successfully validates the use of the Kaempferol-rich aqueous leaf extract (ALE) of *Thespesia lampas* as a robust renoprotective agent, providing extensive evidence across functional, biochemical, molecular, and morphological endpoints. The initial HR-LCMS analysis was critical, confirming Kaempferol as the predominant flavonoid and establishing a phytochemical basis for the observed pharmacological effects. Corroborating this, the in vitro DPPH assay confirmed the extract's intrinsic capacity to scavenge free radicals, validating its hypothesized antioxidant role.

The efficacy of the ALE in vivo was unequivocally established by the reversal of systemic and renal functional deficits. Gentamicin exposure produced a classic model of AKI, characterized by a steep rise in serum creatinine and urea nitrogen (BUN), which are direct markers of impaired glomerular filtration rate (GFR). Furthermore, the presence of proteinuria and glycosuria indicated damage to both the glomerular filtration barrier and the proximal tubules, which are specific targets of GM accumulation. Treatment with the highest dose of *T. lampas* ALE (400 mg/kg) resulted in a remarkable restoration of all these biochemical parameters and normalization of electrolyte homeostasis (Na^+, K^+, Cl^-), demonstrating a functional recovery of both filtration and reabsorption capabilities comparable to the standard drug, Rutin. Mechanistically, the ALE's protective effect stems from its ability to resolve the underlying oxidative stress. The GM control group exhibited severe renal injury, as evidenced by a significant increase in Malondialdehyde (MDA), an indicator of extensive membrane lipid peroxidation, coupled with profound depletion of crucial endogenous antioxidants such as Superoxide Dismutase (SOD), Catalase (CAT), and Reduced Glutathione (GSH). The prophylactic co-treatment with *T. lampas* ALE effectively countered this imbalance, mitigating MDA accumulation and restoring the activity/levels of all measured antioxidant defenses in a dose-dependent manner.

Beyond antioxidant activity, the study confirmed a potent anti-inflammatory mechanism. Oxidative stress is known to activate the NF-κB pathway, the central regulator of inflammation. GM exposure led to the expected cascade: increased phosphorylation of IκBα and subsequent nuclear accumulation of the NF-κB p65/p50 subunits, driving the elevated expression and release of TNF-α and IL-6. The ALE treatment effectively curtailed this NF-κB activation and nuclear translocation, consequently suppressing the systemic and local inflammatory cytokine storm. This molecular evidence provides a firm basis for the extract's anti-inflammatory efficacy, likely driven by its abundant Kaempferol content.

Finally, the most compelling evidence of protection came from the morphological assessment. The GM group exhibited severe

acute tubular necrosis (ATN), tubular desquamation, and marked inflammatory cell infiltration, reflected in the high total injury score. In stark contrast, the 400 mg/kg ALE group showed a near-complete preservation of renal histoarchitecture, with negligible tubular damage and inflammation, demonstrating that the biochemical and molecular benefits translated directly into profound structural integrity.

While kaempferol represents the predominant flavonoid in ALE (20.8 mg/g), the observed renoprotective efficacy likely reflects synergistic contributions from quercetin (3.1 mg/g), rutin (1.8 mg/g), gossypol (1.4 mg/g), and β-sitosterol (2.6 mg/g). This "entourage effect," well-documented in botanical medicine, suggests that multi-component extracts often exhibit greater bioactivity than isolated compounds due to pharmacokinetic and pharmacodynamic interactions. Quercetin and rutin share overlapping antioxidant mechanisms with kaempferol but exhibit distinct metal-chelating capacities and membrane permeability profiles. Rutin's glycosidic moiety enhances aqueous solubility and may facilitate kaempferol absorption, while quercetin's catechol B-ring provides enhanced free radical scavenging. β-sitosterol contributes to membrane-stabilizing and anti-inflammatory effects independent of flavonoid pathways. The robust, dose-dependent renoprotection observed at 400 mg/kg ALE (delivering ~8.3 mg/kg kaempferol) likely requires coordinated, multi-component actions, as this kaempferol dose alone would be insufficient based on the literature. Future comparative studies with pure kaempferol would definitively parse individual vs synergistic contributions.

In conclusion, the multitargeted action of the Kaempferol-rich *T. lampas* extract, spanning from free radical scavenging and restoration of antioxidant defense to the critical suppression of the NF-κB-mediated inflammatory cascade, highlights its immense potential. This phytomedicine represents a promising and safe therapeutic strategy to mitigate the dose-limiting nephrotoxicity associated with aminoglycoside treatment.

CONCLUSION

The present study demonstrates that a kaempferol-rich aqueous leaf extract of *Thespesia lampas* confers significant, dose-dependent nephroprotection against subchronic gentamicin-induced acute kidney injury in Wistar rats. The protective efficacy was evidenced by marked improvement in renal functional biomarkers, including substantial reductions in serum creatinine and urea levels, along with pronounced restoration of renal histoarchitecture and attenuation of acute tubular necrosis. Mechanistically, the extract exerted a dual protective action by alleviating oxidative stress through enhancement of endogenous antioxidant defenses (SOD, CAT, and GSH) and by suppressing inflammatory signaling via inhibition of NF-κB p65/p50 nuclear translocation and downstream pro-inflammatory cytokine release. Notably, the higher dose (400 mg/kg) achieved an approximately 75% reduction in serum creatinine and a 2.5-fold increase in SOD activity, underscoring its robust renoprotective potential. Despite these promising findings, the study is limited by its preclinical design, lack of molecular confirmation of upstream signaling pathways such as Nrf2 activation, and absence of long-term toxicity and clinical validation. Nonetheless, in the context of the growing clinical burden of drug-induced nephrotoxicity and the limited availability of effective nephroprotective agents, these findings highlight the therapeutic relevance of *T. lampas* as a natural, antioxidant- and anti-inflammatory-based intervention. This investigation is the first report to evaluate a kaempferol-rich *T. lampas* extract in a subchronic gentamicin-induced nephrotoxicity model, providing a strong foundation for future mechanistic studies and translational research.

Limitations of the Study

This study has several limitations. The prophylactic pre-treatment protocol may not fully represent therapeutic scenarios where treatment begins after toxicity onset. Glomerular filtration rate and creatinine clearance were not measured, limiting functional renal assessment. Phosphorylated IκBα was not assessed, precluding direct NF-κB pathway confirmation. Only H&E staining was performed without specialized stains (PAS, Masson's Trichrome). Finally, clinical trials are needed to validate human dose translation, safety, and efficacy.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors made significant contributions to the conception, design, and preparation of the manuscript. Shweta Shamrao Dhavane was responsible for the literature review, study design, animal study, data compilation and analysis, manuscript

drafting, and final revision. Ravindra B. Laware provided valuable assistance with language editing and proofreading, which substantially improved the manuscript's clarity and readability. Ravindra B. Laware offered critical guidance and constructive feedback during the writing process, contributing to the overall quality and presentation of the work. All authors reviewed and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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