PHARMACOLOGICAL EVALUATION OF HERBAL FORMULATION NEFPRO IN EXPERIMENTALLY INDUCED RENAL DAMAGE IN RATS.

Dissertation

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In partial fulfillment of the requirement for the degree of

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In
Pharmacology

By

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ABBREIVATION

GFR	Glomerular Filtration Rate
AKI	Acute Kidney Injury
ATN	Acute Tubular Necrosis
GN	Glomerulonephritis
GM	Gentamicin
AGs	Aminoglycosides
PCT	Proximal convoluted tubule
KHK	Ketohexokinase
GLUT	Glucose transporter
ADH	Antidiuretic hormone
AIN	Acute interstitial nephritis
TMP	Trimethoprim
VCM	Vancomycin
AmB	Amphotericin
OCT2	Organic Cation Transporter
MTX	Methotrexate
ATP	Adenosine Triphosphate
ER	Endoplasmic Reticulum
UPR	Unfolded Protein Response
CaSR	Calcium Sensing Receptor
ROS	Reactive oxygen species

NO	Nitric Oxide
RBF	Reduced Blood Flow
iNOS	Inducible Nitric Oxide
NHF	Nefpro Herbal Formulation
WHO	World Heath Organisation
ANOVA	Analysis of Variance
BW	Body weight
CAT	Catalase
GSH	Reduced glutathione
Kg	Kilogram
MDA	Malondialdehyde
µg	Microgram
mg	Miligram
ml	Millilitre
µM	Micromole
mg/dl	Milligram per deciliter
p.o	Per oral
s.c	Subcutaneous
SOD	Superoxidismutase
TBARS	Thiobarbituric acid reactive species
CysC	cystatin C
NGAL	Neutrophil gelatinase-associated lipocalin
NAG	N-acetyl-beta-glucosaminidase

ABSTRACT

Objective: To evaluate the effect of Nefpro herbal liquid formulation in gentamicin and High fructose diet induced renal damage in rats.

Methodology: Sprague dawley rats were divided into 6 groups (n=6). Renal damage was induced in animals by administration of 30% fructose in diet and 10% fructose in drinking water for 42 days along with gentamicin (40 mg/day i.p) for last 10 days of induction period.

Group I (normal control) received normal rat feed and water. Group II (disease control) received fructose diet and gentamicin. Group III, IV and V were groups treated with 10mg/kg Simvastatin, 5 and 10 ml/kg Nefpro Herbal formulation (NHF) respectively. Group VI was pretreated with 5ml/kg Nefpro formulation to assess renoprotective activity. Urine creatinine of rats was measured weekly. At the end of the study period, animals were sacrificed and blood was collected. Serum separated from blood was used for estimation of various biochemical parameters i.e Creatinine, urea, albumin, total protein, uric acid. Levels of antioxidant biomarkers in kidney were also estimated. Kidneys dissected out and its histopathology was studied.

Result: Gentamicin and Fructose diet induced renal damage lead to decreased urine creatinine, serum albumin, total protein levels and increased serum creatinine, urea, uric acid and oxidative stress. Treatment of renal damage rats with NHF exhibited a significant dose dependent reduction in serum Creatinine, Urea, Uric Acid and MDA levels. In addition, there was a significant ($p<0.001$) increase in Urine Creatinine, Serum Protein, Albumin and Antioxidant biomarkers viz; SOD, GSH & CAT. Pretreatment group showed non-significant rise in renal damage functions and decreased elevation in oxidative stress.

Conclusion: The experimental results highlighted the antioxidant, improvement in renal function and renoprotective effect of Nefpro herbal formulation. Furthermore, it also exhibited an improvement in Serum Creatinine, Urea, Uric Acid.

Hence, Nefpro herbal liquid formulation could be a promising future herbal treatment for renal damage.

Keywords: Renal damage, Gentamicin, High fructose diet, Nefpro.

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1.0 INTRODUCTION

The kidneys are dynamic organs representing the most important control system maintaining body homeostasis. They are the principal organ which targets to remove toxic effects of drugs, xenobiotics and their metabolites from the body helping in maintaining blood pressure.[1] Nephropathy is observed worldwide regardless of age, race, location and is prevailing as one of the progressive complications. Also it is ranked as topmost among the ten causes of deaths worldwide.[2] Renal injuries are the leading cause of nephrology consultation and have high mortality rates.[3] A cross-sectional survey of adult patients admitted screened in china estimated that 1·4–2·9 million people with AKI were admitted to hospital in China in 2013 highlighted Delayed AKI recognition, under diagnosis and under treatment as the reason behind huge medical burden and mortality.[4] The primary causes of renal damages include ischemia, hypoxia or nephrotoxicity.[5] Renal injuries can be grouped into three primary etiologies: prerenal, renal, and postrenal. Prerenal azotemia is characterized by a decrease in Glomerular filtration rate (GFR) due to a decrease in renal perfusion pressure. Postrenal causes are characterized by acute obstruction to urinary flow. Urinary tract obstruction increases intratubular pressure and thus decreases GFR. Renal etiologies are challenging form of Acute kidney injury (AKI) to evaluate because of the wide variety of injuries that can occur to the kidney. In general damage to the four major structures of the kidney 1) The Tubules, 2) The Glomeruli, 3) The Interstitium, and 4) The Intrarenal blood vessels.

- Tubular Damage—Acute tubular necrosis (ATN)

  1. Ischemic – Resulting from severe or protracted decrease in renal perfusion.

  2.Nephrotoxic – Resulting from a variety of exogenous compounds (e.g. aminoglycosides, amphotericin B, cis-platinum, radiocontrast media) and endogenous compounds (e.g. hemoglobin in hemolysis, myoglobin in rhabdomyolysis) that are potentially toxic to the kidney.

- Glomerular Damage—AKI from glomerular damage occurs in severe cases of acute glomerulonephritis (GN).
- Interstitial Damage—AKI from interstitial damage can result from acute interstitial nephritis due to an allergic reaction to a variety medications (commonly antibiotics such as penicillins, cephalosporins, sulfonamides) or an infection
- Vascular Damage—AKI from vascular damage occurs because injury to intrarenal vessels decreases renal perfusion and diminishes GFR.[6]

Accumulation of toxic substances in kidneys leads to other major complications. Clinicians frequently encounter drug induced AKI. Both prescribed and over-the-counter agents potentially injure all renal compartments and induce AKI. In this era of modern medicines, patients are exposed intentionally or unintentionally to an expanding variety of drugs used for diagnostic or therapeutic purpose that harm the kidney. Certain medications are known to cause renal injury on its frequent usage and high dosage. [7]

KIDNEY'S SUSCEPTIBILITY TO TOXICANT INJURY:

Kidney is susceptible to toxic injury considering various specific and non-specific factors. Major nonspecific factor is that kidney receives 20-25% of cardiac output which ensures that high percent of circulating toxins are delivered to kidney. Other factors include sensitivity to

vasoactive compounds, concentration of drugs or its metabolites through local tubular reabsorption, secretion, and reabsorption of water. Also the large luminal membrane surface area and large biotransformation capacity makes kidney more susceptible to renal damages.[8,9]

Drug induced AKI has been reported in 8% to 60% of all cases of in hospital AKI.[10] Kidney injury is the most important adverse reaction that was reported with antibiotics. The incidence of antibiotic induced renal damages alone may be as high as 36%.[6] It is a global public health concern affecting 13.3 million patients per year. Probably prevalence of AKI is particularly underestimated leading to huge human burden of it. This led the International Society of Nephrology in 2015 to institute the 0by25 initiative, that objectified, zero preventable deaths from AKI by 2025.[3]

The use of potentially nephrotoxic medications is often unavoidable; however, the contribution of treatment induced renal injury is overlooked as a preventable cause of AKI. Categorizations of drug induced AKI are based on the underlying mechanism of renal injury. Direct nephrotoxicity occurs from Renally excreted drugs causing tubuloepithelial injury, interstitial nephritis, glomerular injury, or obstructive nephropathy, whereas indirect nephrotoxicity develops from decreased intrarenal blood flow.[12]

Renal damage is frequently induced by a wide spectrum of therapeutic agents, including antibiotics like Cephaloridine, Gentamicin, Vancomycin, Kanamycin, Tobramycin. Immunosuppressive drugs Cyclosporine, Tacrolimus, Sirolimus. Chemotherapeutics like Cisplatin, Doxorubicin, Ifosfamide, Cyclophosphamides. Also NSAIDS including COX-2 selective inhibitors, potent antifungal agents as Amphoterecin B, Rifampin, Sulphonamide, Acyclovir, Idinavir, Cimetidine, Omeprazole, Ciprofloxacin are other drugs in the list of nephrotoxins.[13, 14]

AMINOGLYCOSIDES (AGs) are potent bactericidal antibiotics that are highly effective against gram-negative bacterial infections.[15] In fact, they were recently found to be prescribed in 12.1% of the cases in which an antibiotic was necessary in the intensive care unit. Gentamicin (GM) is a potent broad-spectrum aminoglycoside that has long been and still is commonly used, especially in the treatment of life-threatening infections. They are non–protein bound drugs that are not metabolized and are primarily excreted by glomerular filtration and reabsorbed by the proximal tubule. The cationic properties of these agents facilitate binding to the tubuloepithelial membrane in the proximal tubule, resulting in rapid intracellular transport. They undergo proximal convoluted tubule (PCT) reabsorption by megalin-mediated endocytosis. This complex is responsible for transport of GM by endocytosis. The drug is then transported to lysosomes, Goldzi apparatus and endoplasmic reticulum. GM binds to membrane phospholipids, alters its function and lead to a condition known as phospholipidosis. This leads to a preferential accumulation of the drug in the cortical tubular cells, which results in tubular cytotoxicity.[16, 17]

Risk factors for AG nephrotoxicity include the type of AG, high peak serum levels, cumulative dose, the duration and frequency of administration, and patient-related factors such as age, pre-existing renal dysfunction, hypoalbuminemia, liver dysfunction, decreased renal perfusion, and the use of concomitant nephrotoxic drugs.[18]

Several investigations demonstrated that patients with metabolic syndrome are at higher risks of chronic kidney diseases. This might be because of higher accumulation of drug in renal cortex.[19] High fructose intake causes metabolic syndrome and contributes to increase risk of renal damage and tubulointerstitial injury. High fructose diet leads to hyperuricemia with decrease renal uric acid excretion, hyperglycemia, dyslipidemia, renal hypertrophy, glomerular hypertension and arteriolopathy. Fructose is used extensively in our modern diet in carbonated beverages, dairy products, canned fruits and baked goods.[20, 21]

Fructose undergoes a unique metabolism in which it is taken up by specific transporters and then phosphorylated by ketohexokinase (KHK). KHK is most heavily expressed in the intestinal epithelium, the liver, and the kidney where it is expressed only in the proximal tubule, but not other segments of the nephron. Fructose transporters are located in the brush border. Glucose trasporter 2 (GLUT2) is a main transporter of fructose in the S1 and S2 segments of the proximal tubule, whereas glucose transporter 5 (GLUT5) is expressed only in the S3 segment of the normal kidney which is a potential mechanism involve in direct stimulation of proximal tubular cell proliferation by fructose.[22]

Reducing or protecting against gentamicin nephrotoxicity is the goal most attracted for research in last decades. Despite only the treatment approaches practiced include reduction of nephrotoxin dose, duration of therapy, and dosing frequency. Renoprotection is mostly focused with the use of many naturally available antioxidant studied considering oxidative stress as one of the main mechanism involved in renal tissue damage.[23]

Many ethnomedicinal plants from traditional system of medicine have nephroprotective properties and are commonly used to treat the various renal disorder. Studies reveal that synthetic nephroprotective agents have adverse effect besides reduce nephrotoxicity. Phytochemical studies assure that medicinal plants are a source of wide variety of natural antioxidants. They can be important source of managing many renal diseases and their complications, helping millions of patients struggling to reverse or at least slowdown the progression of their disease.[24]

NEFPRO liquid formulation contain a combination of some of the known nephroprotective, antioxidant agents which include Phyllanthus embelica, Withania somnifera, Terminalia belerica, Phyllanthus niruri, Ricinus communis, Tribulus terrestris, Balsamodendron mukul, Terminalia chebula, Naedostachys jatamansi, Centella asiatica, Cperus rotundus, Eclipta alba,

Piper nigrum, Piper longum, Boerhaavia diffusa, Pluchea lanceolata, Zingiber officinale, Adhatoda vasica respectively. Embelicanins A, Embelicanins B, Gallic acid, Ellagic acid, Withaniloids, Withaferi A, Withasomniferin A, β-sitosterol, Chebulic acid, Phyllanthin, Ricinine, Methyl ricinoleate, Harmane, Tribulusterine, Jatamansin, Piperine, Piperlongumine, Stigmasterol are some of the major chemical constituents in Nefpro formulation. Some of the herbs are reported for suppressing the reactive oxygen species mediated signaling pathways, inhibiting synthesis of inflammatory cytokines and mediators. Some have diurectic, lipid lowering activity, antihypertensive activity, hepatoprotective activity. [25, 26]

Some of the marketed polyherbal formulation proven to be renoprotective consist some of the herbs present in composition of nefpro liquid formulation. For eg.

RIKABA: India's 1st Natural Herbal Antioxidant product of Centaur Pharmaceuticals proven its potential as renoprotective is composed of Terminalia chebula, Withania somnifera, Embelica officinalis, tribulus terrestris, etc.[27]

Varunadi Vati - Herbal Remedy for Renal Disorders and Rencure Formula -Natural Kidney Remedy products of Krishna Herbal Company consist of Boerhaavia diffusa, Tribulus terrestris, Commiphora mukul, Tribulus terrestris proven to be potentially renoprotective.[28]

The herbal formulation 'NEFPRO' has been widely prescribed by Ayurvedic practitioners for renal injury treatment. Despite their use, there is no scientific evidence for its potential activity, safety and efficacy.

Hence, the present study is designed to evaluate the activity of 'NEFPRO' herbal formulation in diet and drug induced renal damage in rats.

2.0 OBJECTIVE

To evaluate the effect of **NEFPRO** herbal liquid formulation, an Ayurvedic proprietary medication on diet and drug induced renal damage in Sprague Dawley rats.

Following are the parameters to be evaluated:

1) Evaluation of the changes in the body weight.
2) Estimation of serum Urea level.
3) Estimation of serum and urine Creatinine levels.
4) Estimation of Blood Urea Nitrogen (BUN).
5) Urine analysis.
6) Estimation of anti-oxidant enzymes in kidney tissue
    a. Superoxide-dismutase (SOD).
    b. Malonaldehyde (MDA).
    c. Reduced Glutathione (GSH).
    d. Catalase (CAT).
7) Kidney histopathological study.

3.0 REVIEW OF LITERATURE:

3.1 KIDNEY:

Kidney is one of the vital organs for excretion in our body. Most of the drugs administered get eliminated through kidney. The paired kidneys are reddish, kidney-bean shaped organs located just above the waist between the peritoneum and the posterior wall of the abdomen. They are said to be retroperitoneal as their position is posterior to the peritoneum of the abdominal cavity. The right kidney is slightly lower than the left because the liver occupies considerable space on the left side superior to the kidney. The kidney forms about 0.5% of the total mass. Through renal artery kidney receives 20-25% of the total arterial blood. Each kidney consists of about ten lakh nephrons. It is the unit of the kidney. Nephron forms urine by filtering the blood. The small ions, water molecules and other small molecules get reabsorbed back into the peritubular capillaries by reabsorption. The waste molecules, ions remain in the urine and they get eliminated through urine.

3.1.1 ANATOMY:

A typical adult kidney is 10-12 cm long, 5-7 cm wide and 3cm thick. It has a mass of 135-150gm. Near the center of the concave border is a deep vertical fissure called the renal hilum through which the ureter emerges from the kidney along with blood vessels, lymphatic vessels and nerves. Each kidney is surrounded by three layers of tissue. The deep layer, the renal capsule, is a smooth transparent sheet of dense irregular connective tissue that is continuous with the outer coat of ureter. It serves as a barrier against trauma and helps maintain the shape of the kidney. The middle layer, the adipose capsule, is a mass of fatty tissue surrounding the renal capsule. It also protects the kidney from trauma and holds it firmly in place within the abdominal cavity. The superficial layer, the renal fascia, is another thin layer of the dense irregular connective tissue that anchors the

kidney to the surrounding structures and to the abdominal wall. On the anterior surface of the kidneys, the renal fascia is deep to the peritoneum. A frontal section through the kidney reveals two distinct regions:

1) Superficial, smooth-textured reddish area called the renal cortex.

2) Deep reddish-brown inner region called the renal medulla.

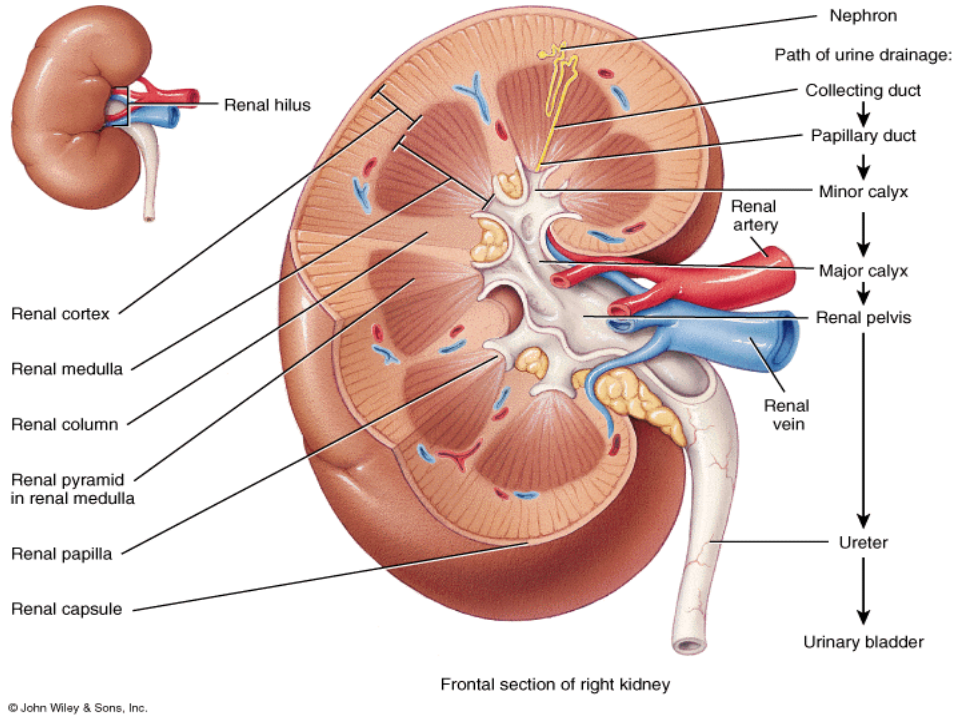


Fig.1: Anatomy of Kidney

The renal cortex is the smooth-textured area extending from renal capsule to the base of the renal pyramids and into the spaces between them. It is divided into an outer cortical zone and an inner juxta medullary zone. Those portions of the renal cortex that extend between renal pyramids are renal columns. The renal medulla consists of several cone-shaped renal pyramids. The base of each pyramid faces the renal cortex and its apex, called the renal papillae, point towards the renal hilum. The renal cortex and renal

pyramids of the renal medulla constitute the parenchyma (functional portion) of the kidney. Within the parenchyma are the functional units of the kidney called Nephrons.

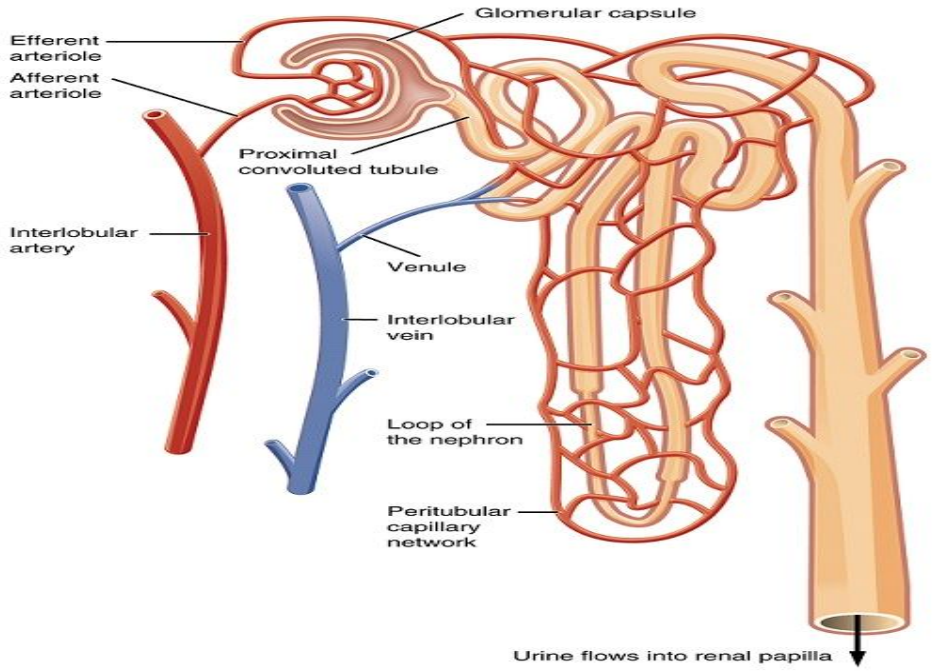


Fig. 2: Structure of Nephron

Urine formed by the nephrons drains into large papillary ducts, which extend through the renal papillae of the pyramids. The papillary ducts drains into cup-like structures called minor and major calyces; each kidney has 8-18 minor calyces and 2-3 major calyces. A minor calyx receives urine from the papillary ducts of one renal papillary and delivers it to a major calyx. From the major calyx, urine drains into a single large cavity called the renal pelvis and then out through the urinary bladder.

The hilum expands into a cavity within the kidney called the renal sinus, which contains part of the renal pelvis, the calyces, and the branches of the renal blood vessels and nerves. Adipose tissue helps stabilize the position of these structures in the renal sinus.[29,30]

3.1.2 FUNCTIONS:

The kidney participates in whole-body homeostasis, regulating acid-base balance, electrolyte concentrations, extra cellular fluid volume and regulation of fluid blood pressure.

- **Excretion of wastes:**

The kidneys excrete a variety of waste products produced by metabolism. These include the nitrogenous wastes called "urea", from protein catabolism, as well as uric acid, from nucleic acid metabolism. Formation of urine is also the function of the kidney.

- **Acid-base homeostasis**

The kidneys have two important roles in maintaining the acid-base balance: to reabsorb bicarbonate from urine, and to excrete hydrogen ions into urine. Two organ systems, the kidneys and lungs, maintain acid-base homeostasis, which is the maintenance of pH around a relatively stable value.

- **Osmolarity regulation**

Any significant rise in plasma osmolarity is detected by the hypothalamus, which communicates directly with the posterior pituitary gland. An increase in osmolarity causes the gland to secrete antidiuretic hormone (ADH), resulting in water reabsorption by the kidney and an increase in urine concentration. ADH binds to principal cells in the collecting duct that translocate aquaporins to the membrane, allowing water to leave the normally impermeable membrane and reabsorbed into the body by the vasa recta, thus increasing the plasma volume of the body.

There are two systems that create a hyperosmotic medulla and thus increase the body plasma volume: Urea recycling and the 'single effect.'

Urea is usually excreted as a waste product from the kidneys. However, when plasma blood volume is low and ADH is released the aquaporins that are opened are also

permeable to urea. This allows urea to leave the collecting duct into the medulla creating a hyperosmotic solution that 'attracts' water. Urea can then re-enter the nephron and be excreted or recycled again depending on whether ADH is present or not.

- **Blood pressure regulation**

Long-term regulation of blood pressure depends upon the kidney. This primarily occurs through maintenance of the extracellular fluid compartment, the size of which depends on the plasma sodium concentration. Although the kidney cannot directly sense blood pressure, changes in the delivery of sodium and chloride to the distal part of the nephron alter the kidney's secretion of the enzyme renin. When the extracellular fluid compartment is expanded and blood pressure is high, the delivery of these ions is increased and renin secretion is decreased. Similarly, when the extracellular fluid compartment is contracted and blood pressure is low, sodium and chloride delivery is decreased and renin secretion is increased in response.

Renin is the first in a series of important chemical messengers that comprise the renin-angiotensin system. Changes in renin ultimately alter the output of this system, principally the hormones angiotensin II and aldosterone. Each hormone acts via multiple mechanisms, but both increase the kidney's absorption of sodium chloride, thereby expanding the extracellular fluid compartment and raising blood pressure. When rennin levels are elevated, the concentrations of angiotensin II and aldosterone increase, leading to increased sodium chloride reabsorption, expansion of the extracellular fluid compartment, and an increase in blood pressure. Conversely, when renin levels are low, angiotensin II and aldosterone levels decrease, contracting the extracellular fluid compartment, and decreasing blood pressure.[31]

3.2 ACUTE KIDNEY INJURY (AKI):

Acute kidney injury (AKI) is the abrupt loss of kidney function defined by a rapid (over hours to days) decline in the GFR resulting in the retention of metabolic nitrogenous waste products and dysregulation of fluid, electrolyte, and acid-base homeostasis. It is the broad term for the clinical condition that occurs when the renal excretory function is critically and acutely decreased to a point at which the body accumulates "uremic" waste products and becomes unable to maintain electrolyte, acid-base, and water balance.[32, 33]

In 2002, the **Acute Dialysis Quality Initiative (ADQI)** Group proposed the first consensus definition of AKI. They proposed classification scheme with three grades based on the magnitude of the increase in serum creatinine level and/or decrease in urine output.

RIFLE defines three grades of increasing severity of ARF (risk, injury, and failure, respectively, R, I, and F) and two outcome variables (loss and end-stage kidney disease, respectively, L and E). A unique feature of the RIFLE classification is that it offers for three grades of severity of renal dysfunction on the basis of a change in serum creatinine, reflecting changes in GFR or duration and severity of decline in urine output from the.[34]

Recently, the **Acute Kidney Injury Network (AKIN)** proposed a modification of the RIFLE classification that includes the Risk, Injury, and Failure criteria with the addition of a 0.3mg/dL or higher increase in the serum creatinine level with 48 hours to the criterion that define risk.

Finally, **Kidney Disease: improving global outcomes (KDIGO)** proposed a consensus definition utilizing 48hours time frame from AKIN for an absolute increase in serum creatinine of 0.3mg/dl and 7 days time frame for relative 50% increase in serum creatinine above baseline.[35]

3.3 ETIOLOGY:

Causes of renal injuries can be classified into three broad groups: (1) pre-renal or hemodynamic (i.e., hypoperfusion to the kidney), (2) intrinsic (i.e.structural damage to the kidney), and (3) post-renal (i.e., obstruction of urinary outflow).[36]

PRE-RENAL: Pre-renal AKI is the leading cause of kidney injury. Decreased renal perfusion of the kidney can cause AKI with or without systemic arterial hypotension. Inadequate fluid intake, excessive vomiting, diarrhea, and fever can lead to dehydration. Trauma resulting in massive hemorrhage decreases circulating volume, resulting in hypoperfusion to the kidney.[37]

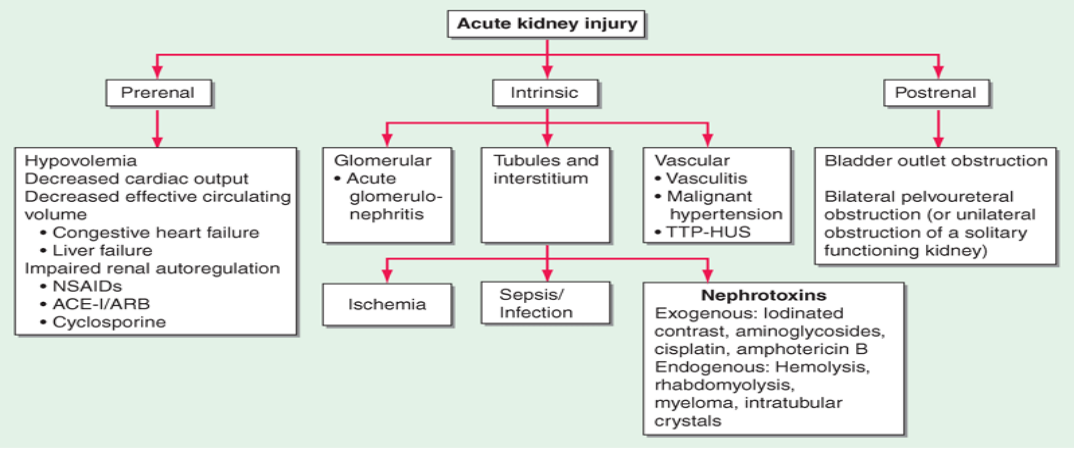
INTRINSIC: Intrinsic kidney injury includes damage to the glomerulus, tubules, interstitium, and vasculature. Drugs causing intrinsic injury may be direct nephrotoxins, or they may stimulate an immune response. In some cases, drugs can cause injury through more than one mechanism (i.e., tubular injury and interstitial injury).

GLOMERULAR: Glomerular injury may occur from immune mediated diseases or conditions such as lupus nephritis, immunoglobulin A nephropathy, Wegner syndrome, post-streptococcal infection. Oncology drugs are the most commonly implicated agents in glomerular injury. Medications causing glomerular injury include interferon, pamidronate, gemcitabine, and vascular endothelial growth factor inhibitors. Renal injury is accompanied by hypertension caused by decreased endothelial nitric oxide production.

TUBULAR: Tubular injury is commonly caused by antimicrobials and nephrotoxic drugs. ATN is a common etiology of AKI in critically ill patients and is the most common type of AKI caused by ischemia or exposure to nephrotoxins.

- **Acute interstitial nephritis (AIN)** may be caused by infections, medications, or immune disorders. The most common infection includes pyelonephritis, but AIN can also be associated with renal tuberculosis and fungal nephritis.
- **Vascular/Thrombotic**: Renal vascular disorders, which may cause AKI, include vasculitis, malignant hypertension, scleroderma, thrombotic thrombocytopenic purpura/hemolytic-uremic syndrome, thrombotic microangiopathies, disseminated intravascular coagulation, mechanical renal artery occlusion (surgery, emboli, thrombotic occlusion), and renal venous thrombosis.

POST-RENAL: Post-renal AKI is the result of kidney obstruction. The most common causes of post-renal AKI include nephrolithiasis, benign prostatic hypertrophy, and surgical causes. The four main chemical types of renal calculi are calcium, uric acid, struvite, and cysteine, with calcium stones being the most common type. Certain drugs have relatively low solubility in the urine and may crystallize, obstructing the collecting system.[36,37, 38, 39]



Source: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine, 18th Edition*: www.accessmedicine.com

Fig. 3: Classification of Etiology of Renal Injury

3.4 RISK FACTOR FOR DRUG INDUCED RENAL DAMAGE:

3.4.1 Kidney and drug specific factors:

Toxicity of therapeutic and diagnostic compounds may be inherent to pharmacological compounds itself and toxicity potential may be high in renal environment. Kidney receive relatively higher amount of cardiac output and it is regularly exposed to drug and drug metabolites through robust blood flow and some of them would achieve entry into the renal epithelial parenchyma cells through pinocytosis and phagocytosis. Other drugs are transported by organic cation and organic anion transporters and then enter into tubular lumens. Kidney uses cytochrome p45 and other enzymes system to oxidize the drugs into active metabolites and it suggest that reactive oxygen species and drug metabolites have a mechanistic link in the intrarenal toxicity.[40]

In addition, certain drugs may attenuate the toxicity in a particular kidney environment e.g. Methotrexate causes kidney toxicity by the generation of crystallization by the parent drug and its metabolites and it is found to be highly favorable in acidic urine. This points out that the renal environment and drug specific characters play an important role in the nephrotoxicity. Tubular cells in the collecting duct and loop of Henle are at greater risk for nephrotoxicity because they are highly metabolically active and, as a result, reside in a relatively hypoxic microenvironment.[41]

3.4.2 Patient specific risk factors:

Patient specific factors show some important causes of drug induced renal damage. Certain patient characteristics predispose to drug-induced nephrotoxicity.

- Aged people and females have decreased muscle mass and total water content which result in lower serum creatinine level leading to an appropriate higher dosing of drug and leading to nephrotoxicity.
- In addition, lower water content in the body of older ages and female causes the accumulation of drug in serum.
- Hypoalbuminemia also carries the risk of inducing toxic drug levels by increasing the unbound drug fraction in the serum.
- Hepatic failure is a particular risk factor for drug-induced renal impairment because cirrhotic patients tend to have reduced muscle mass and hypoalbuminemia
- In neonates, particularly those with premature delivery, drug nephrotoxicity bears a significant burden for AKI compared to adult patients, with some data suggesting that drug-induced renal impairment leads to 16% of AKI cases in newborns.
- Elderly patients have high incidence of non-communicable diseases as they consume multiple medications together may causes toxic insult to kidney.
- People with increased volume depletion is more prone to drug toxicity e.g. diarrhea and vomiting. Moreover, congestive heart failure and hepatic patients with loss in volume may experience AKI and causes drug induced nephrotoxicity.

- Renal toxicity by drugs and potential toxins may increase due to metabolic disturbances. Disturbances in the metabolism may causes kidney to highly vulnerable to drug toxins.[42, 43]

3.5 DRUG INDUCED RENAL DAMAGE:

In the era of modern medicine, patients are exposed to an expanding variety of drugs for diagnostic and therapeutic purposes. Nephrotoxic agents have been implicated as etiologic factors in 17%–26% of in-hospital AKI. Studies of AKI have documented the frequency of drug-induced nephrotoxicity to be approximately 14-26% in adult populations. Nephrotoxicity is a significant in pediatrics with 16% of hospitalized AKI events being attributable primarily to a drug.[44]

Drug-induced nephrotoxicity is a common problem in clinical medicine and the incidence of drug-related AKI may be as high as 60%. It involves many classes of drugs and includes prescription agents as well as commonly encountered over-the-counter drugs. The use of potentially nephrotoxic medications is often unavoidable; however, the contribution of treatment induced renal injury is frequently overlooked as a preventable cause of AKI.

Some of the classes of drugs inducing renal injuries:

3.5.1 ANTIMICROBIAL AGENTS:

Aminoglycosides (AGs): AGs are well known to cause nephrotoxicity and ototoxicity. Neomycin is the most toxic drug in this group, followed by gentamicin, tobramycin, amikacin, and streptomycin. AGs are non-protein bound and freely filtered at the glomeruli. Due to their cationic structure, AGs can undergo proximal tubule reabsorption by megalin-mediated endocytosis. This leads to a preferential accumulation of the drug in the cortical tubular cells, which results in tubular cytotoxicity.[45]

Sulfamethoxazole–trimethoprim and sulfa-based antibiotics: Sulfamethoxazole is probably the most widely used sulfa-based antibiotic. It is generally prescribed along with synergistically acting trimethoprim (TMP) as a combination antimicrobial agent. TMP inhibits proximal tubular secretion of creatinine and can result in elevation of measured serum creatinine. TMP can also result in hyperkalemia by inhibiting the epithelial sodium channel at the distal convoluted tubule, which provides the driving force for potassium excretion. Overall incidence of renal injury may be as high as 11.2%[46]

Vancomycin: Vancomycin (VCM), a glycopeptide antibiotic, is commonly used in the critical care setting since it is a first-line agent in the treatment of severe methicillin-resistant *Staphylococcus aureus* infections. VCM-related nephrotoxicity is generally due to ATN. VCM-induced oxidative stress results in the tubular damage.[47]

Other antibiotics: Ciprofloxacin, a commonly prescribed fluoroquinolone antibiotic, has been reported to cause crystalluria. Ciprofloxacin crystallizes in alkaline urine. Penicillins, cephalosporins and Polymyxins cause AKI by toxic tubular injury.[48]

3.5.2 ANTIVIRAL AGENTS

Acyclovir: High-dose intravenous use of acyclovir can induce AKI secondary to crystal precipitation in the renal tubules.[49]

Foscarnet: Foscarnet is nephrotoxic by inciting ATN, although a case report also suggested the possibilty of a crystal-related injury. In addition to AKI, foscarnet can also cause significant electrolyte abnormalities.[50]

3.5.3 ANTIRETROVIRAL DRUGS:

Tenofovir: The most prominent of the nephrotoxic antiretroviral agents is tenofovir, a nucleoside reverse transcriptase inhibitor that can cause AKI with or without proximal tubulopathy. AKI results from direct toxicity to tubular cells, mediated by mitochondrial injury, resulting in ATN.[51]

Indinavir: A once-prominent protease inhibitor, can crystallize in renal tubules, resulting in crystal-related kidney injury and nephrolithiasis.[52]

3.5.4 ANTIFUNGAL AGENTS

Amphotericin B: Amphotericin B (AmB) is frequently used in the treatment of serious, life-threatening fungal infections. AmB can bind to cholesterol molecules in cellular membranes, thereby altering membrane permeability. This effect can be toxic to renal tubular cells and result in ATN and tubular dysfunction.[53]

3.5.5 CHEMOTHERAPEUTIC AGENTS [54,55]

Chemotherapeutic drugs play a central role in the treatment of various neoplasms. Renal damage is common with many chemotherapeutic agents and can result in a wide spectrum of renal complications.

Cisplatin: The main mechanism for causing AKI is thought to be a direct cellular toxic injury primarily to the proximal tubule. The selective injury to proximal tubules is due to preferential accumulation of the drug in proximal tubular cells. This cellular uptake happens via the copper transporter-1 and organic cation transporter 2 (OCT2).

Ifosfamide: Ifosfamide, a structural isomer of cyclophosphamide, is an alkylating agent undergoes cellular uptake at the proximal tubule via OCT2. Once inside the cell, the drug is then metabolized into chloroacetaldehyde, which is chiefly responsible for cellular toxicity. Fanconi-like syndrome is a common manifestation of ifosfamide nephrotoxicity.

Methotrexate(MTX): MTX is an anti-folate agent widely used as chemotherapy against several malignancies. 90% of MTX is cleared by kidneys. MTX-induced AKI occurs from crystallization in the renal tubules as well as direct tubular toxicity resulting in ATN. The crystallization is enhanced by high urinary MTX concentration, low urine volume, and acidic urine pH.

3.5.6 IMMUNOSUPPRESSIVE AGENTS

Calcineurin inhibitors: Tacrolimus and cyclosporine are widely used calcineurin inhibitors (CNIs). CNIs possess a very narrow therapeutic window and are susceptible to multiple drug–drug interactions, which often lead to toxic serum drug levels. Acute toxicity is usually related to altered renal hemodynamics secondary to afferent arteriolar vasoconstriction. Long-term CNI exposure can cause interstitial fibrosis and tubular atrophy.[56]

3.5.7 MISCELLANEOUS:

Lithium: Lithium has been the mainstay of treatment for patients with bipolar disorder for many years. The most common renal disorder associated with chronic lithium use is NDI. The mechanism for lithium-induced NDI is possibly by downregulation of acquaporin-2 channels in the collecting duct.

Acetaminophen: Acetaminophen is probably the most common over-the-counter analgesic and antipyretic medication. The incidence of AKI in acetaminophen overdose is around 2%. Mechanism of nephrotoxicity is acute tubular injury leading to ATN

Osmotic agents: AKI from osmotic agents, frequently referred to as osmotic nephrosis or osmotic nephropathy, occurs when renal tubules are exposed to hyperosmolar substances causing severe swelling of tubular cells and apparent obliteration of tubular lumens.[57]

3.6 AMINOGLYCOSIDE INDUCED RENAL DAMAGE:

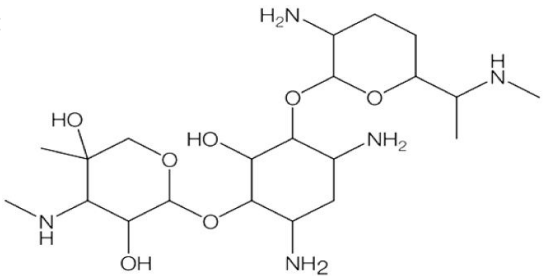
The first AGs, streptomycin, were introduced into clinical practice in 1944, and has since been followed by many drugs of this class. Clinically, AGs may be used to provide targeted therapy, such as for the treatment of pulmonary exacerbations in children with cystic fibrosis colonised with Pseudomonas aeruginosa. They are also used for the empirical treatment of suspected systemic sepsis, where they are given in combination with other antibiotics (such as gylcopeptide or betalactam antibiotics) to provide broad spectrum coverage of Gram-positive and Gram-negative bacteria species.[58,59]

GENTAMICIN (GM) a typical AGS antibiotic is widely used in clinical practices for treatment of life threatening gram-negative infections. This antibiotic generally cause drug induced dose-dependent nephrotoxicity in 10-20% of therapeutic courses. GM induced nephrotoxicity is characterized by direct tubular necrosis, without morphological changes in glomerular structures.

GM is not systemically active when given orally. This is because it is not absorbed to any appreciable extent from the small intestine. It is administered intravenously, intramuscularly or topically to treat infections. It appears to be completely eliminated unchanged in the urine. Urine must be collected for many days to recover all of a given dose because the drug binds avidly to certain tissues.[60,61]

3.6.1 GENTAMICIN :

Chemical Structure:



Pharmacology of Gentamicin

- Half life - 2 – 3 Hours
- Peak Plasma Time – IM 30 to 90 minutes

  IV 30minutes (after 30 minutes of infusion)

Varying dosage–The concentration of gentamicin is increased by edema, ascites, fluid overload. The concentration of gentamicin is decreased with dehydration.

Neonates	–0.4 to 0.6 L/kg
Children	–0.3 to 0.35L/kg
Adults	–0.2 - 0.3L/kg
Excretion	–Urine (as unchanged drug)
Clearance	–Directly related to renal function

Mechanism of Action – Synergistic with beta-lactamase against enterococci. Interferes with bacterial protein synthesis by binding to 30S and 50S ribosomal subunits.

Adverse effects – Neurotoxicity (vertigo, ataxia), Gait instability, Ototoxicity (auditory, vestibular), Nephrotoxicity, Edema, Drowsiness, Headache, Pseudomotor cerebri, Photosensitivity, Allergic reaction, Erythema, Anorexia, Nausea / vomiting, Weight loss, Increased salivation, Enterocolitis, Burning, Increased thirst, Stinging, Tremors, Muscle cramps, Weakness and Dyspnea.

Contraindications –

- Prior aminoglycosides toxicity or hypersensitivity.
- In patients with impaired renal function
- Higher dosage or prolonged therapy

Pregnancy – D Category. Use in LIFE-THREATENING emergencies when no safer drug available. Positive evidence of human fetal risk.

Lactation – Enters breast milk; use with caution (AAP Committee states "compatible with nursing")

Cautions – In premature infants and neonates because of renal immaturity. There is risk of ototoxicity, neurotoxicity & nephrotoxicity soon after anaesthesia or after giving muscle relaxants. Avoid concurrent or sequential use of neurotoxic or nephrotoxic drugs. Avoid potent diuretics because of increased risk of ototoxicity.

Guidelines – Narrow therapeutic index (not intended for long-term therapy); caution in renal failure (not on dialysis), myasthenia gravis, hypocalcemia, and conditions that depress neuromuscular transmission; adjust dose in renal impairment.

3.6.2 MECHANISM OF RENAL DAMAGE:

GM is causing tubular damage through:

1) Necrosis of tubular epithelial cells, predominantly in proximal segment and

2) Alteration of function of main cellular components involved in transport of water and solutes.

The central aspect of GM induced renal damage is tubular cytotoxicity, usually epithelial cells of proximal tubules. While cells of distal tubules and collecting ducts are significantly less affected by cytotoxic effects.

The increased accumulation of GM in proximal tubules is related to expression of transport molecule for proteins and cations (megalin and cubilin complex) in proximal tubules. It is known that this complex is responsible for transport of GM by endocytosis. The drug is then transported to lysosomes, Goldzi apparatus and endoplasmic reticulum. GM binds to membrane phospholipids, alters its function and lead to a condition known as phospholipidosis.[62]

3.6.3 TUBULAR EFFECTS

The tubular toxicity of GM presents two aspects:

(i) The death of tubular epithelial cells, mainly within the proximal segment, with a very important inflammatory component associated and

(ii) The nonlethal, functional alteration of key cellular components involved in water and solute transport.

MECHANISMS OF TUBULAR CELL DEATH:

ACCUMULATION OF GENTAMICIN IN TUBULAR CELLS

GM cytotoxicity occurs in those cell types in which the drug accumulates. In the kidneys, these cells constitute the epithelial cells in the cortex, mainly in the proximal tubule and also in the distal and collecting ducts. A higher accumulation of GM in these cells is consistent with the expression of a transporter of proteins and cations, namely, the giant endocytic complex formed by megalin and cubilin, which is restricted to the proximal tubule. This complex is known to transport gentamicin and, in general, AGs, by endocytosis. These drugs then travel through the endosomal compartment and accumulate mostly in lysosomes, the Golgi, and endoplasmic reticulum. GM binds to membrane phospholipids, alters their turnover and metabolism, and, as a consequence, causes a condition known as phospholipidosis. Lysosomal phospholipidosis results from

(i) The reduction in the available negative charge necessary for the correct function of phospholipases.

(ii) Inhibition of A1, A2, and C1 phospholipases.[63, 64, 65]

CYTOSOLIC REDISTRIBUTION AND MITOCHONDRIAL TARGETING

When the concentration of AGs in endosomal structures exceeds an undetermined threshold, their membrane is disrupted and their content, along with the drug, is poured into the cytosol. Cytosolic GM then acts on mitochondria directly and indirectly, and thus activates the intrinsic pathway of apoptosis, interrupts the respiratory chain, impairs adenosine triphosphate (ATP) production, and produces oxidative stress by increasing superoxide anions and hydroxyl radicals, which further contributes to cell death.

In addition, the lysosomal content bears highly active proteases named cathepsins, which are capable of producing cell death. Cathepsins catalyze the proteolytic activation of

executor caspases 3 and 7 and activate the mitochondrial pathway of apoptosis through the activation of Bid. In the absence of ATP, cathepsins in the cytosol produce a massive proteolysis that leads to necrotic cell death. The effect of GM on mitochondria is produced in a direct and also in an indirect fashion. GM induces the release of proapoptotic proteins from the intermembrane space for the activation of the intrinsic pathway of apoptosis. The indirect action is mediated by Bax, and it is inhibited by overexpression of Bcl-2.[66,67]

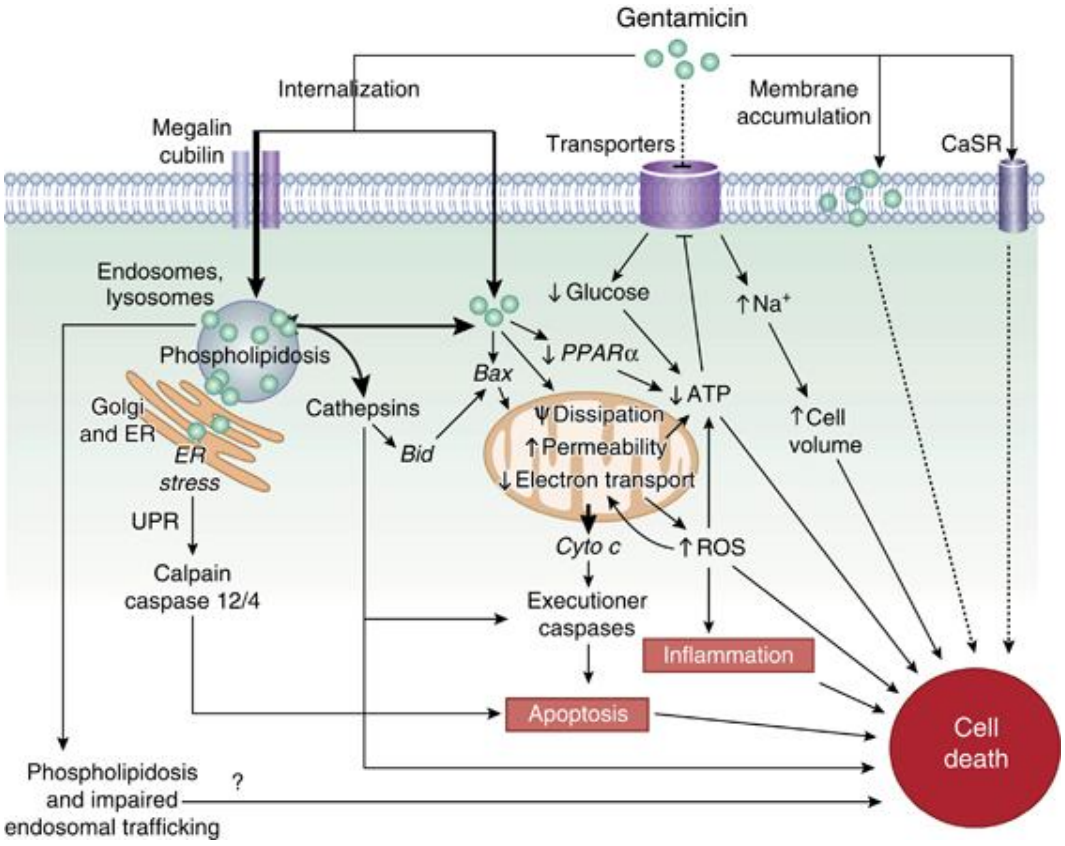


Fig. 4: Accumulation of Gentamicin in tubular cells and mechanism underlying the cytotoxic effect.

ENDOPLASMIC RETICULUM(ER) STRESS AND UNFOLDED PROTEIN RESPONSE (UPR)

In the ER, gentamicin inhibits protein synthesis, impairs translational accuracy, and might interfere with the correct posttranslational protein folding. This generates endoplasmic reticulum stress and activates the unfolded protein response that, on continuous stimulation, activates apoptosis through calpains and caspase.

Under circumstances of UPR overload, the cell undergoes apoptosis, which is mediated by the classical route of calpains and caspase 12 activated by the release of calcium (Ca) from the ER. UPR-activated apoptosis also involves Jun kinase and C/EBP homologous protein transcription factor.[68, 69]

CALCIUM-SENSING RECEPTOR (CaSR) STIMULATION

Activation of the extracellular calcium-sensing receptor (CaSR) with GM and other AGs has also been shown to induce a mild degree of apoptosis in CaSR-expressing tubule cells and not in those lacking it.[70]

3.6.4 GLOMERULAR EFFECTS

The glomerulus is the first part of the nephron to come into contact with chemical agents. GM has glomerular effects that alter filtration.

(i) GM produces mesangial contraction and results in Kf (ultrafiltration coefficient) and GFR reduction;

(ii) GM also stimulates mesangial proliferation paralleled by an increase in apoptosis of these cells, which basically compensate each other;

(iii)Despite the fact that GM does not generate significant morphological changes in the glomerulus, in high-dose treatments, a slight increase in size, alteration of their round shape and density, and a diffuse swelling of the filtration barrier associated with neutrophil infiltration have been detected, although their pathophysiological significance is uncertain.

(iv)Loss of glomerular filtration barrier selectivity, due to the neutralization of its negative charges, contributes to proteinuria, especially under circumstances in which tubular reabsorption is impaired such as in tubular necrosis.[71]

Several factors induced by gentamicin increase intracellular calcium concentration and cause mesangial cell contraction. They include

(i) Platelet-activating factor (PAF) secretion and autocrine action;

(ii) Activation of the renal renin–angiotensin system;

(iii)Production and action of vasoconstrictors such as endothelin-1 and thromboxane A2 arising from endothelial dysfunction or imbalance;

(iv) CaSR stimulation; and

(v) Increase in reactive oxygen species (ROS) production and oxidative stress.

Mesangial proliferation is mediated by calcium-dependent AP-1 activation. Mesangial cell apoptosis is mediated by increased ROS and probably by nitric oxide (NO) overproduction. GM stimulates inducible nitric oxide synthase (iNOS) expression and NO production in isolated glomeruli and mesangial cells. Excessive NO production due to expression of iNOS, especially under oxidative stress circumstances, interacts with superoxide anion to forms peroxynitrite, which causes nitrosative stress and cytotoxic effects.[72,73]

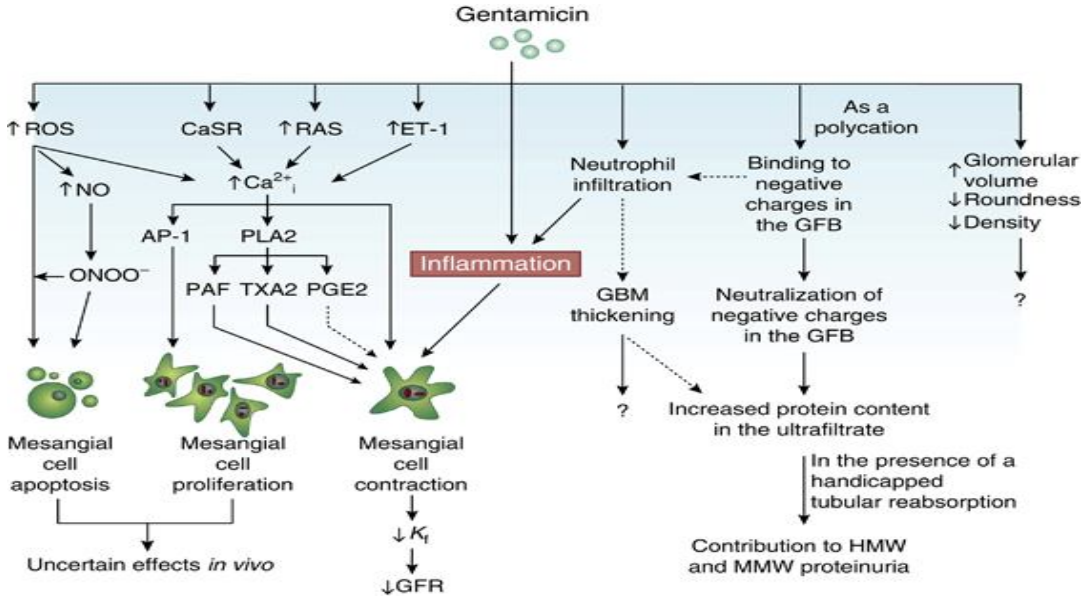


Fig. 5: Glomerular effects of Gentamicin.

3.6.5 VASCULAR EFFECTS

GM induces a reduction in renal blood flow (RBF), which is the consequence of an increased resistance of the renal vascular bed rather than that of a lower perfusion pressure. A lower RBF causes GFR to fall, and sensitizes tubule cells to cell death by reduction of oxygen and ATP availability.

RBF reduction arises initially (i) from the activation of TGF by the handicapped tubular reabsorption, in order to prevent massive fluid and electrolyte loss and (ii) progressively, superseding TGF adaptation, by production of vasoconstrictors within the renal vascular tree and mesangial compartment; and by direct effects of GM on vascular cells. In addition to stimulating the production of vasoconstrictors, GM also blocks the synthesis of vasodilator prostaglandins. Endothelial NO synthase derived NO, at low levels, mediates physiological vasodilatation, whereas excessive NO production due to the over expression of iNOS can cause cytotoxic effects in surrounding cells.

NO interacts with superoxide anion to form peroxynitrite, which induces protein and cell damage and uncouples endothelial NO synthase to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress. GM also impairs vascular smooth muscle-relaxing capacity. Leukocyte migration, leading to vascular plugging, congestion, and infarction, is induced by gentamicin in retinal vessels after 48–72 h of treatment.[74,75]

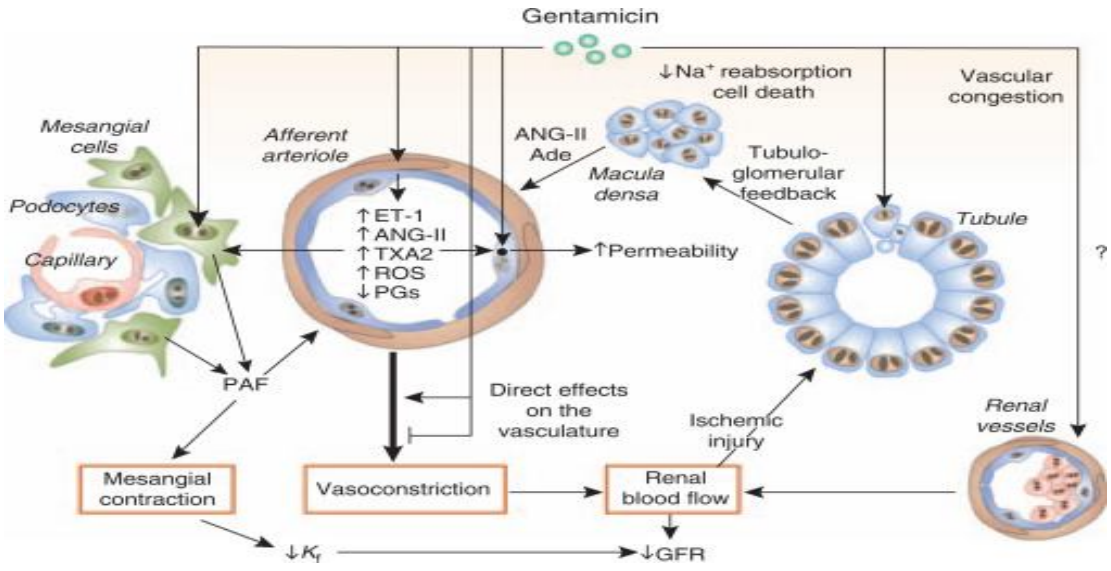


Fig. 6: Vascular effects of Gentamicin.

3.6.6 CENTRAL ROLE OF OXIDATIVE STRESS AND INFLAMMATION:

Oxidative stress has been suggested to have a key role in gentamicin induced renal damage. GM directly increases the production of mitochondrial ROS, which

(i) are able of damaging many cellular molecules including proteins, lipids, and nucleic acids, thus impairing cell function and leading to cell death;

(ii) Contribute to mesangial and vascular contraction.

(iii) Participate in inflammation.[76]

ROS, mainly superoxide anions and hydroxyl radicals, cause cellular damage and death through diverse mechanisms, including the following:

(1) Inhibition of the electron transport chain and suppression of cellular respiration and ATP production;

(2) Stimulation of the release of cytochrome c, AIF, etc. from the mitochondrial inter membrane space;

(3) DNA damage, which triggers an increase in poly ADP ribose synthase activity, a decrease in the cell's ATP reserve, and cell cycle arrest;

(4) Lipid peroxidation, destabilization of the cellular membrane, activation of death receptors (Fas, etc.) by alteration of lipid rafts, and generation of proapoptotic lipid metabolites, such as 4-hydroxynonenal and ceramide;

(5) Stress on different organelles and cellular structures, such as the ER

(6) Inhibition of transmembrane sodium flow, by oxidative inhibition of the Naþ/Kþ ATPase pump and of sodium channels, which originates cellular swelling, loss of membrane integrity, and necrosis.[77]

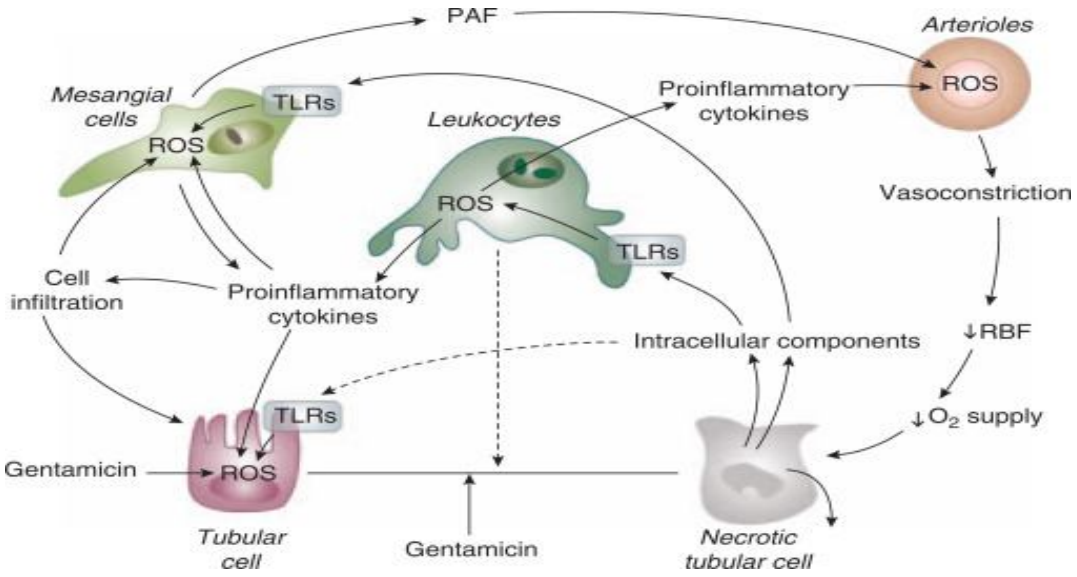


Fig. 7: Role of inflammation in the amplification of tubular, glomerular, and vascular effects of Gentamicin.

An increased or unbalanced ROS production and oxidative stress mediate the inflammatory response unleashed by gentamicin. Superoxide anion and hydrogen peroxide activate nuclear factor kB (NFkB), a key mediator of several inflammatory pathways. NFkB induces the expression of proinflammatory cytokines and iNOS. Over expression of iNOS can cause cytotoxic effects in surrounding cells. Excessive iNOS derived NO can react with superoxide anion and produce peroxinitrite, a highly reactive radical that contributes to cell damage and reduced vascular relaxation. Inflammatory cytokines, such as tumor necrosis factor alpha can activate tubular apoptosis, especially in the pathological environment.[78,79,80,81,82]

3.7 FRUCTOSE:

Fructose is a simple sugar, a monosaccharide that is present primarily in added dietary sugars, honey, and fruit. Fructose is used extensively in our modern diet in carbonated beverages, dairy products, canned fruits and baked goods. The industrialized form of fructose is a major health threat in almost every organ system. Fructose consumption has been linked to obesity, metabolic syndrome, and hypertension in large epidemiologic studies and small randomized, controlled trials of short term dietary interventions. Intake of fructose has increased dramatically over the last century, has accelerated since the introduction of high fructose corn syrup.[83]

3.7.1 FRUCTOSE METABOLISM:

Fructose is distinct from glucose in its initial metabolism. The Glut5 transporter in the intestine absorbs fructose with 60 to 70% being taken up by Glut2 and possibly other transporters in the liver and 30 to 40% by the kidney, adipose tissue, and other organs. Most fructose is metabolized by fructokinase (KHK), which phosphorylates fructose to fructose 1-phosphate. The phosphorylation of fructose results in a decrease in intracellular phosphate and ATP depletion, resulting in transient inhibition of protein synthesis.[84]

Literature depicts that excessive consumption of a high-fructose diet may lead to the epidemic of chronic renal disease and metabolic syndrome characterized by visceral adiposity, dyslipidemia and insulin resistance. Preliminary evidence demonstrates that high fructose consumption induces kidney damages in both rats and mice. Increase catabolism of fructose is associated with the cellular energy depletion that can increase the susceptibility of cells to lipid peroxidation. It has been postulated that increased catabolism of fructose can accelerate free radical production similar to glucose and

impairs the free radical defense system leading to oxidative stress. Fructose has been demonstrated to induce the production of macrophage associated MCP-1 in kidney proximal tubular cells that promotes monocyte and macrophage migration and activation. The activated macrophages produce numerous proinflammatory cytokines such as TNF-α, which have been shown to mediate inflammation in several models of renal injury, including tubulointerstitial injury.[85]

Nakayama T et.al. studied the effects of a high-fructose diet on normal rat kidneys. Fructose diet, but not glucose diet, significantly increased kidney weight by 6 wk. The primary finding was tubular hyperplasia and proliferation involving all segments of the proximal tubules while glomerular changes were not observed. Fructose undergoes a unique metabolism in which it is taken up by specific transporters and then phosphorylated by ketohexokinase. KHK is most heavily expressed in the intestinal epithelium, the liver, and the kidney where it is expressed only in the proximal tubule, but not other segments of the nephron.[84]

3.7.2 FRUCTOSE METABOLISM IN PROXIMAL TUBULAR CELLS:

Most fructose is metabolized in the liver, especially when small amounts of fructose are ingested. However, in the setting that a large amount of fructose is taken, some fructose escapes from the liver, enters into the systemic circulation, and is filtered into urine in the kidney. One of the 206 major sites of fructose metabolism is by the proximal tubules in the kidney. The proximal tubular epithelial cell expresses KHK, which is a primary enzyme for fructose, and that fructose transporters (GLUT5) are also located in the brush border. It is likely that GLUT2 is a main transporter of fructose in the S1 and S2 segments of the proximal tubule, whereas GLUT5 is expressed only in the S3 segment of the normal kidney. Thus tubular cell proliferation and hyperplasia found could be a consequence of fructose metabolism.

Various studies carried out portrays that chronic ingestion of fructose induces mild tubulointerstitial disease, and this might be because of a direct proliferative effect of fructose on the proximal tubular cell, induces renal microvascular disease that alters renal autoregulation and results in glomerular hypertension, increase kidney weight in association with hyperplasia and proliferation.[85,86,87]

3.8 KIDNEY BIOMARKERS FOR DRUG INDUCED RENAL DAMAGES:

Early detection of drug-induced kidney injury is important in drug development. Generally accepted biomarkers such as creatinine and blood urea nitrogen (BUN) lack sensitivity and early injury responses are missed. Many new biomarkers to detect nephrotoxicity have been utilized. However, guidance on appropriate biomarkers is minimal.

Glomerular filtration rate (GFR) remains the ideal marker of kidney function. Other biomarkers such serum creatinine (SCr) and cystatin C (CysC), albuminuria may precede kidney function decline.

New potential biomarkers have arisen with the promise of detecting kidney damage.

- CysC is a small molecule cysteine proteinase inhibitor synthesized by all nucleated cells and filtered freely by the glomerulus. After filtration it is not secreted nor reabsorbed by the tubules, but catabolized completely and thus reflects true GFR when measured in blood.
- Neutrophil gelatinase-associated lipocalin (NGAL) is an acute phase protein secreted as a response to acute injury of proximal and distal tubular epithelial cells. It is freely filtered by the glomerulus after which rapid clearance occurs via receptor binding and endocytosis. NGAL has been reported to be the most sensitive marker for proximal tubular damage.

- N-acetyl-beta-glucosaminidase (NAG) is a lysosomal enzyme which is contained abundantly in the renal tubular epithelia. Its size precludes glomerular filtration and elevated urinary concentrations are considered to reflect tubular dysfunction.
- Kidney injury molecule 1, a type-1 transmembrane protein, is extensively expressed in proximal tubule cells in biopsy confirming acute tubular necrosis.[88,89]

3.9 TREATMENT APPROACHES:

- **Preventing AG-induced nephrotoxicity**

  Prevention of renal damage is an unmet therapeutic objective that will improve the pharmacotoxicological profile and the clinical utility of many drugs significantly, including AGs.

- **Choice of AGs**:

  The following rank order of nephrotoxicity from most toxic to least toxic: neomycin >gentamicin ≥ tobramycin ≥ amikacin ≥ netilmicin > streptomycin.

- **Extended interval dosing:**

  Higher single dose may result in saturation of megalin-mediated uptake of AG in the proximal tubule, resulting in a greater percentage of the AG being excreted in the urine. Multiple daily dosing is beneficial.

- **Therapeutic drug monitoring:**

  It is helpful for monitoring both efficacy and toxicity. Elevated trough levels suggest reduced renal clearance of AG. Competing with or decreasing drug binding to the brush-border membrane.

- **Decreasing or preventing AG accumulation by kidneys:**

  AG accumulation can be reduced either by impairing their uptake or by enhancing their release. Uptake can be reduced by complexing the aminoglycosides extracellularly. Statins (simvastatin, pravastatin and rosuvastatin) inhibited GM accumulation and cytotoxicity in a dose-dependent manner in an in vitro proximal tubule model by inhibiting megalin-mediated endocytosis.

- **Preventing or decreasing the lysosomal phospholipidosis induced by the cell-associated AGs.**

  A reduction in lysosomal phospholipidosis can be achieved either by use of an aminoglycoside modified to bind less tightly to phospholipids at an acidic pH or by the administration of an agent that would prevent the binding of the antibiotic to phospholipids. Polyaspartic acid has emerged as a very successful protectant against AG-induced nephrotoxicity from the screening of various polymers that are likely to impair the binding of aminoglycosides to kidney membrane vesicles.

- **Clinical drugs**:

  Some clinical drugs have been shown to be protective. disease-modifying antirheumatic drugs (DMARD), cholesterol-cutting statins, neuroprotective agents for cerebral infarction, selective vitamin D receptor agonist (VDRA), tetracycline antibiotics, phosphodiesterase-5 (PDE5) inhibitors, angiotensin II receptor antagonist, mammalian target of rapamycin (mTOR) inhibitor, immunosuppressant drug, and steroid hormones.[90,91]

There are no or very few tailored preventive strategies for individual nephrotoxic drugs, based on specific mechanisms of action.

Herbal medicine is still the mainstay of about 75-80% of the whole population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with human body and fewer side effects. However, the last few years have seen a major increase in their use in the developed world.[92]

Medicinal plants have been used for centuries as remedy for diseases because they provide an important source of new chemical substances with potential therapeutic effects. These medicinal plants have been used in traditional medicine for the treatment of several diseases. Many plants synthesize active constituents that are useful for the maintenance of health in humans and animals. These include flavonoids, glycosides, saponins, triterpenoids, oxepins, phytosterols and phenols or their oxygen-substituted derivatives such as tannins.

The World Health Organization (WHO) defines herbal medicine as "finished, labelled, medicinal product that contain active ingredients, aerial or underground parts of the plants or other plant material or combinations thereof, whether in the crude state or as a plant preparation" The WHO estimates that 4 million people, 80% of the world population presently use herbal medicine for some aspects of primary health care.[93]

NEFPRO liquid formulation contain a combination of some of the known nephroprotective, antioxidant agents which include *Phyllanthus embelica, Withania somnifera, Terminalia belerica, Phyllanthus niruri, Ricinus communis, Tribulus terrestris, Balsamodendron mukul, Terminalia chebula, Naedostachys jatamansi, Centella asiatica, Cperus rotundus, Eclipta alba, Piper nigrum, Piper longum,*

Boerhaavia diffusa, Pluchea lanceolata, Zingiber officinale, Adhatoda vasica respectively.

Relevant data obtained from various investigations regarding the active ingredients present in nefpro liquid formulation is summarized below:

Phyllanthus embelica: [94, 95]

Chemical Constituents*:*

Phyllanthus embellica also known as Amla or Indian Gooseberry is an atural fruit that serves as a rich source of vitamin. It also contains flavonoids, tannins, terpenoids and alkaloids.

It is used as a 'rejuvenating herb' in traditional system of Indian medicine. It has been shown to possess antioxidant, anti-inflammatory and anti-apoptotic effects.

Research Investigations:

Theactive extracts of *Phyllanthus embellica* have been demonstrated to exert a wide range of activities such as antioxidant, anti-apoptotic, anti-inflammatory. Several studies have also been conducted to investigate its beneficial effect in kidney injury including cyclophosphamide induced renal injury and mycotoxin induced renal toxicity. study demonstrated that EO attenuated cisplatin-induced nephrotoxicity in rats through suppression of MAPK induced inflammation and apoptosis. Dose of (600 mg/kg) inhibited MAPK phosphorylation which was instrumental in preserving renal function and morphology.

Withania somnifera: [96]

Chemical constituents*: Withania somnifera*, commonly known as ashwagandha or winter cherry, is used for therapeutic purpose in Indian traditional medicine for more than 3000 years for its ability to strengthen the immune system. Among the Indian medicinal plants, thirteen positive alkaloids and to date around 138 withanolides have been reported from *W. somnifera*.

Research Investigations:

Prominent medicinal properties, such as immunomodulatory, antiinflammatory, endocrine, anti-stress, anti-cancer, adaptogenic, anti-tumor, central nervous system, cardiovascular, and neuroprotective. Study concluded that *W. somnifera* (750 mg) exhibits the nephroprotective effect probably by promoting enhanced antioxidant activity with natural antioxidants and by scavenging the free radicals.

Terminalia belerica: [97]

Chemical Constituents*: Terminalia belerica* Roxb. is one of the oldest medicinal herb of India, is an ingredient of Indian Ayurvedic drug 'triphala' used for the treatment of digestion and liver disorders. Fruit of *Terminalia belerica* have also been reported for the presence of sitosterol, gallic acid ellagic acid, ethyl gallate, chebulagic acid, mannitol, glucose, galactose, fructose and rhamnose. Active principle such as gallic acid (3,4,5 trihydroxybenzoic acid) has also been identified. It shows marked bile stimulating activity and has strong antioxidant properties.

Research Investigations:

The fruit is reported to have purgative, cardiac depressant, hypotensive and choleretic effects. Study depicts that 200 mg/kg dose of gallic acid to be most effective against carbon tetrachloride induced kidney damage due to presence of some phenolic components that have membrane stabilizing effects. Gallic acid may directly combine

with free radicals and lead to inactivate them which may suppress the intracellular concentration of free radicals.

Phyllanthus niruri: [98]

Chemical Constituents:

P. niruri is a small erect annual herb of the family Euphorbiaceae having active phytochemicals, flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins.

Research Investigations:

Plant extracts have been evaluated in human trials for the treatment of hypertension, jaundice, diabetes, hypercalciuria and urolithiasis. Flavonoids from *P. niruri* showed anti-oxidant activity.

Ricinus communis: [99]

Chemical Constituents:

Ricinus communis; Family: Euphorbiaceae popularly known as 'castor plant'. The preliminary phytochemical study of *R. communis* presence of steroids, saponins, alkaloids, flavonoids, and glycosides. The dried leaves of R. communis showed the presence of alkaloids, ricinine(0.55%) and N-demethylricinine and six flavones glycoside. The monoterpenoids (1, 8-cineole, camphor and pinen) and sesquiterpenoid (β-caryophyllene), gallic acid, quercetin, gentisic acid, rutin, epicatechin and ellagic acid are the major phenolic compounds isolated from leaves.

Research Investigations:

Phytopharmacological screening revealed the high antioxidant activity of the seed of ***communis*** at low concentration shows that it could be very useful for the treatment of disease resulting from oxidative stress.

Tribulus terrestris: [100]

Chemical Constituents:

Tribulus terrestris (family Zygophyllaceae), commonly known as *Gokshur or Gokharu* or puncture vine, has been used for a long time in both the Indian and Chinese systems of medicine for treatment of various kinds of diseases. It is a popular leafy prostrate branching herb used in folk medicine as a diuretic and urinary antiseptic. The preliminary phytochemical study reported that furostanol and spirostanol saponins of tigogenin, neotigogenin, gitogenin, neogitogenin, hecogenin, neohecogenin, diosgenin, chlorogenin, ruscogenin, and sarsasapogenin types are frequently found in this plant.

Research Investigations:

The dry fruits extract of T. terrestris exhibited free radical scavenging activity. The methanol extract of the fruits provided protection against the mercuric chloride induced nephrotoxicity in the mice. Study by Maged S. Abdel-Kader et.al. confirmed the positive effect of the plant on the kidney tissues and function.

Terminalia chebula: [101]

Chemical Constituents:

Terminalia chebula Retz. commonly known as 'Black myrobalan' in English. The fruits of T. chebula is rich in tannins (about 32%-34%). researchers found 14 components of hydrolysable tannins (gallic acid, chebulagic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulinic acid, casuarinin, terchebulin)

Research Investigations:

It has been reported that it possesses antioxidant activity, anti-inflammatory and anti-arthritic activities It has been shown to be effective in cadmium and GM-induced nephrotoxicity model. Study discovered that T. chebula ameliorated oxidative and histological damage caused by cisplatin.

Naedostachys jatamansi: [102,103]

Chemical Constituents*:*

Nardostachys jatamansi [Family *Valerianaceae*] is a perennial herb found in Alpine Himalayas. *Nardostachys jatamansi* consist of following constituents but the main active constituents in the plant material are sesquiterpenes and coumarins. Jatamansone or valeranone is the principal sesquiterpene. Antioxidant property of jatamansi is been reported.

Eclipta alba: [104]

Chemical Constituents*:*

Eclipta alba (*Asteraceae*) is an annual herbaceous plant, commonly known as false daisy. It is also known as Bhringaraj and Karisilakanni. It contains wide range of active principles which includes coumestans, alkaloids, flavonoids, glycosides, polyacetylenes, triterpenoids. The leaves contain stigmasterol, β-terthienylmethanol, wedelolactone, demethylwedelolactone and demethylwedelolactone-7-glucoside43. The roots give hentriacontanol and heptacosanol. The roots contain polyacetylene substituted thiophenes.

Research Investigations:

Literature study proves eclipta alba to be very good antioxidant with a dose of 500ugm/ml. also its other activities like anti-inflammatory, antidiabetic, antiproliferative are proven which supports its potential of nephroprotective considering these mechanisms.

Piper nigrum: [105]

Chemical Constituents*:*

Piper nigrum L. is widely distributed in the Asian continent, especially in Hainan and Yunnan, China. It contains major pungent alkaloid Piperine (1-peperoyl piperidine). Other compounds are Brachyamide B, Dihydro-pipericide, (2E,4E)-N-Eicosadienoyl-pereridine, N-trans-Feruloyltryamine, N-Formylpiperidine, Guineensine, pentadienoyl as piperidine, (2E,4E)- Nisobuty- ldecadienamid, isobutyl-eicosadienamide, Tricholein, Trichostachine, isobutyl-eicosatrienamide, Isobutyl-octadienamide, Piperamide, Piperamine, Piperettine, Pipericide, Piperine, Piperolein B, Sarmentine, Sarmentosine, Retrofractamide A.

Research Investigations:

Study demonstrated that Ethanol extract of piper nigrum at a dose of has a significant antioxidant activity on lipid peroxidation compared with Vitamins E and C.

Literature depicts Piperine inhibited free radicals and reactive oxygen species, therefore known to possess protective effects against oxidative damage. Piperine also found to reduce the synthesis of prostaglandin E2 in dose dependant comportment at the concentrations of 10-100 μg/mL which confirms its anti-inflammatory activity.

Piper longum: [106,107]

Chemical constituents:

Piper longum Linn. is a native of the Indo-Malaya region, belongs to family Piperaceae. Major chemical constituents are alkaloids piperine, piperlongumine, piperlonguminine and also methyl-3, 4, 5 - trimehoxycinnamate.

Research Investigations: Study reveals that administration of P. longum provide a significant protection to liver and kidney from the oxidative stress at a dose of 300mg/kg. Also it has promising hepatoprotective and anti-inflammatory activity proven in ccl4 induced hepatotoxicity.

Boerhaavia diffusa: [108,109]

Chemical Constituents:

Boerhaavia diffusa L. (Nyctaginaceae), commonly known as 'Punarnava' in the Indian system of medicine. The plant contains a large number of such compounds as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins. Punarnavine, boeravinone A , hypoxanthine 9-Larabinofuranoside, ursolic acid, punarnavoside, lirodendrin. Punarnava also contains arachidic acid, Sitosterol, B-sitosterol, palmitic acid, ester of B-sitosterol, tetracosanoic, hexacosonoic, stearic, urosilic acid, Hentriacontane, Ecdysone, triacontanol etc.

Research Investigations:

Its various pharmacological and biological activities such as immunomodulatory effects, immunosuppressive activity, antimetastatic activity, antioxidant activity, antidiabetic activity antiproliferative and antiestrogenic activity, analgesic and anti-inflammatory activity, antibacterial activity, antistress and adoptogenic activity, antilymphoproliferative activity, nitric oxide scavenging activity, hepatoprotective activity are reported.

Pluchea lanceolata: [110]

Chemical Constituents:

Pluchea lanceolata (Family: Asteraceae) is a rapidly spreading perennial herb, considered valuable for the management of anti-inflammatory disease. Boehmerol acetate, sorghumol acetate, monoterpenic ester, chromenone, steroidal latone were isolated from

roots of *pluchea lanceolata*. Pleuchiol, a stigmastanol derivative and pleuchioside, a ursane derivative were isolated from leaves. Pluchein and plucheinol (sequiterpenes) and cuauhtemone have isolated from stems.

Research Investigations:

Activities of P. lanceolata, including anti-inflammatory, anti-arthritis, anticancer, muscle relaxant, CNS stimulant, anti-implantation, as well as immunosuppressant, contraceptive, and toxicological effects and their use in traditional system are critically evaluated. A methanolic fraction of a chloroform extract of defatted Pluchea indica roots was investigated for its anti-inflammatory potential against several models of inflammation, showing significant inhibitory activity.

Zingiber officinale: [111]

Chemical Constituents:

Zingiber officinale roscoe, commonly known as ginger, is one of the most commonly used spices in India and around the world. Belongs to Zingiberaceae family. Over 50 components of the oil have been characterized and these are mainly monoterpenoids [b-phellandrene, (+)-camphene, cineole, geraniol, curcumene, citral, terpineol, borneol] and sesquiterpenoids [a-zingiberene (30–70%), b-sesquiphellandrene (15–20%), b-bisabolene (10–15%), (E-E)-a-farnesene, arcurcumene, zingiberol], amongst phenols most abundant is most abundant is [6]-gingerol. Rhizome of ginger has been recommended for use as carminative, diaphoretic, antispasmodic, expectorant, peripheral circulatory stimulant, astringent, appetite stimulant, anti-inflammatory agent, diuretic and digestive aid.

Research Investigations:

Study reports 200mg/kg dose of Z. officinale significantly protected the nephrotoxicity either by enhancing the DXN-induced declined renal antioxidant status or by its direct antioxidant activity.

Adhatoda vasica: [112]

Chemical Constituents:

Adhatoda vasica Nees. (Acanthaceae), with the common name vasaka. Main chemical constituents of this plant are vasicine (derived from leaves), 2'-hydroxy-4-glucosyloxychalcone, vasicol (from leaves), vasicinone (from leaves, stem and roots), vasicinol (contained in stem and roots), and deoxyvasicinone (from leaves).

Research investigations:

Plant is reported for the activities like antioxidant, genoprotective, muscle stimulant activity, abortifacient activity, anti-diabetic activity, anticestodal activity, antileishmanial activity, anti-helminthic activity, anti-bacterial activity, anti ulcer activity.

Combinations of these polyherbal formulations is not been scientifically evaluated. Hence the present study was effectively designed to attain the scientific evidence and nephroprotective efficacy and safety of NEFPRO herbal formulation.

4.0 MATERIALS AND METHODS

4.1 Source of Herbal Formulation

Herbal liquid formulation NEFPRO required for the study was been supplied by Progen research laboratory, Belagavi.

4.2 Selection of dose: According to the LD 50 studies of various herbal extracts in the formulation, human dose was converted to animal dose and animals were administered with 5 and 10ml/kg NHF.

4.3 Phytochemical Investigation

The liquid herbal formulation Nefpro was subjected to different qualitative Phytochemical tests to determine the phytochemical constituents present in the extract.

4.4 Drugs and Chemicals

The various chemicals, drugs and kits used for the study are listed below.

Table. 1: List of drugs, chemicals and kits used in present study.

Sr.No	Materials used	Source of the Material
01	Nefpro liquid formulation	Progen Research lab, Belagavi.
02	Simvastatin	Micro Labs Ltd, Bangalore.
03	Gentamicin	Gentamicin injection IP 80mg/2ml ampoules, Abbott.
04	Ellmans reagent	Sigma-Aldrich, USA.
05	Thiobarbituric acid	Himedia Pvt. Ltd, Mumbai.
06	Creatinine kit	ERBA Diagnostics Manheim GmbH, Germany.
07	Urea(BUN) kit	ERBA Diagnostics Manheim GmbH, Germany.
08	Uric acid kit	ERBA Diagnostics Manheim GmbH, Germany.
09	Total protein kit	ERBA Diagnostics Manheim GmbH, Germany.

10	Albumin kit	ERBA Diagnostics Manheim GmbH, Germany.
11	Fructose powder	NIHAL Marketing, Bangalore.
12	Glucometer	On call plus, India.
13	Di-sodium hydrogen phosphate	Himedia Pvt. ltd, Mumbai
14	Potassium di-hydrogen phosphate	Merck Pvt. ltd, Mumbai.
15	Glacial acetic acid	Himedia Pvt. ltd, Mumbai
16	Hydrochloric acid	Himedia Pvt. ltd, Mumbai
17	Ellman's reagent	Himedia Pvt. ltd, Mumbai
18	Sodium azide	Himedia Pvt. ltd, Mumbai
19	Hydrogen peroxide	Himedia Pvt. ltd, Mumbai
20	Trichloro acetic acid (TCA)	Himedia Pvt. ltd, Mumbai
21	Thiobarbutaric acid (TBA)	Himedia Pvt. ltd, Mumbai
22	Potassium di-chromate	Himedia Pvt. ltd, Mumbai
23	Sodium hydroxide	Himedia Pvt. ltd, Mumbai
24	Tris hydrochloric acid	Himedia Pvt. ltd, Mumbai
25	Glutathione	Himedia Pvt. ltd, Mumbai
26	EDTA	Himedia Pvt. ltd, Mumbai
27	Pyrogollol	Himedia Pvt. ltd, Mumbai
28	Potassium chloride	Himedia Pvt. ltd, Mumbai
29	Sodium phosphate monobasic	Himedia Pvt. ltd, Mumbai
30	Anhydrous sodium phosphate dibasic	Himedia Pvt. ltd, Mumbai
31	Formalin	Himedia Pvt. ltd, Mumbai
32	Chloroform	Himedia Pvt. ltd, Mumbai

All reagents used were of analytical grade.

Table. 2: List of instruments used in present study.

Sr.No.	Instuments	Description
01	Electronic balance	Adventurer, OHAUS, USA.
02	U.V. Spectrophotometer	1800 Shimadzu Corporation, Japan.
03	Auto analyzer	Star 21 plus.
04	Homogenizer	Remi Motors Pvt. Ltd, India.
05	Centrifuge	Remi Motors Pvt. Ltd, India.

4.5. Experimental Animals

Sprague Dawley rats of either sex weighing 130-150g, were procured from Invivo Biosciences, Bangalore. The animals were housed in solid bottom polypropylene cages with a stainless steel grill on top and a bedding of clean paddy husk, at an ambient temperature and humidity, with 12 – 12 h light and dark cycle. The rats has been provided with normal feed and water *ad libitum* for acclimatization to laboratory conditions for a period of 10 days and later they were provided with a high fructose diet.

The experimental protocol was been approved by the Institutional Animal Ethics Committee (IAEC Reg.No.:221/PO/Re/S/2000/CPCSEA Res.25-09/09/2017) Belagavi. All the protocols and the experiments conducted were in strict compliance with the ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

4.6. Experimental model

For the induction of renal damage methodology proposed by Zaid O. Ibrahem et al. has been slightly modified and followed.[115] Sprague Dawley rats, induced with renal damage by administration of 30% fructose in diet and 10% in drinking water for 42 days along with Gentamicin (40mg/kg/day) for last 10 days of induction period. This experimental model has been employed in order to attain drug as well as diet induced renal damage in rats. 30% of food grade fructose powder was mixed with powdered normal rat feed and were moulded into laddoos and fed to experimental rat groups (groups II - VI). Drinking water was substituted with 10% of fructose water solution. Gentamicin 40mg/kg was administered to rats through intraperitoneal route for last 10 days of induction period. Nefpro herbal liquid formulation(5ml/kg) was administered orally to rats of group VI from day 1st and continued throughout the induction period to evaluate the renoprotective activity of the formulation.

After 6 weeks of induction of renal damage in rats, urine creatinine levels were estimated and the animals with creatinine levels below the normal baseline were selected for the study. Simvastatin (10mg/kg) solution administered orally was prepared by inclusion complexation method using beta cyclodextrin. Drug simvastatin and beta cyclodextrin in 1:1 ratio of their molecular weight were weighed and kneaded with little quantity of water. Later this paste was then dried in hot air oven at 45°C and the dry powder obtained was suspended in distilled water. The Nefpro formulation (NF) was administered in different doses to the rats per orally.

Body weight, food and water intake was monitored daily throughout the experiment. At the end of the 30 days treatment period, blood samples were collected from overnight fasted, ether anesthetized rats via retro orbital plexus puncture. Blood serum has been used for biochemical estimations. Animals were further sacrificed by cervical dislocation and the

kidney homogenate supernatant has been employed for antioxidant enzymes estimation. Kidneys were isolated for histological examination

Table. 3: Experimental Design.

Thirty six Sprague Dawley rats were divided into following 6 groups for the present study. (6 animals per group).

Groups	**Treatment**
Group I (normal control)	Receives normal rat feed and drinking water.
Group II (Disease control)	Receives 30% fructose diet and 10% fructose drinking water for 6 weeks along with Gentamicin (40mg/kg/day) last 10 days of induction period
Group III (Treated animals)	Treatment with simvastatin 10mg/kg p.o for 30days in renal damage induced rats.
Group IV (Treated animals)	Treatment with 5ml/kg Nefpro formulation twice daily for 30 days.
Group V (Treated animals)	Treatment with 10ml/kg Nefpro formulation twice daily for 30 days.
Group VI (Treated animals)	Recieves 30% fructose diet and 10% fructose drinking water for 6 weeks, and Gentamicin (40mg/kg/day) last 10 days of induction period, along with 5ml/kg Nefpro formulation twice daily for renoprotective acitivity assessment.

4.7 Parameters Assessed

4.7.1 Body Weight

The body weight of all the animals was recorded initially, at the start of the study and further weekly during the entire study period. The changes in body weights were monitored and tabulated.

4.7.2 Food Intake: 100gm of modified diet with 30% fructose feed was given to the animals per cage each day, and feed intake was recorded.

4.7.3 Kidney Weight: After sacrificing the animals, the kidneys was dissected out, rinsed in normal saline and blotted on filter paper. The kidneys were then weighed and ratio of kidney weight to body weight was calculated.

4.7.4 Urine volume:

Urine of the each group animals was collected one day before the last day of experiment. Animals were kept in metabolic cages with proper food and water supply for 24h. The volume of urine collected was measured and it was utilized to analyze urine Creatinine and Microalbumin.

4.7.5 Serum Analysis

At the end of the experimental period, rats were anaesthetized with ether. Blood samples were collected via retro orbital puncture, stored undisturbed for 2 hours at ambient temperature and centrifuged at 2000 rpm for 5 minutes. The serum was separated and used for the estimation of biochemical parameters viz; urea, creatinine, blood urea nitrogen, uric acid, total protein, albumin, electrolyte balance.

4.8 Procedure for Estimation of Biochemical Parameters:

A) Estimation of Serum and Urine Creatinine (CRE)

Methodology: Modified Jaffe's reaction. [117]

Principle: Creatinine reacts with alkaline picrate to produce an orange yellow colour (the Jaffe's reaction) specificity of the assay has been improved by the introduction of an initial rate method. The absorbance of the orange-yellow color formed is directly proportional to creatinine concentration and is measured at 500-520nm.

Specimen collection and handling:

Serum, plasma (heparin, EDTA) or urine.

For determination in urine use 24 hours specimen. Dilute the urine samples in 1+ 19 ratio with distilled water and multiply results by 20.

Procedure: The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. The samples were prepared as per the protocol The samples were mixed well and absorbance read at 505nm, 20 seconds after mixing (A1) and finally at 80 seconds (A2).

Pipette	Standard	Test
Working Reagent	1000ul	1000ul
Standard	100ul	-
Test	-	100ul

Calculation: $\Delta A = A1 - A2$

$$\text{CRE (mg/dl)} = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{concentration of standard (mg/dl)}$$

B) Estimation of Blood Urea Nitrogen (BUN)

Methodology: Talke and Schubert, Tiffany et al.[118]

Principle: The estimation of Urea in serum involves the following enzyme catalyzed reactions:

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2\,NH_3 + CO_2$$

$$NH_3 + \alpha\text{-KG} + \text{NADH} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}$$

α -KG: α- Ketoglutarate

GLDH: Glutamate dehydrogenase

The rate of decrease in absorbance is monitored at 340nm and is directly proportional to urea concentration in the sample.

Specimen collection and handling:

Use unheamolytic Serum, Plasma (heparin, EDTA) or Urine.

For determination in urine use 24 hours specimen. Dilute the urine samples in 1+ 100 ratio with distilled water and multiply results by 101.

Procedure: The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. The samples were prepared as per the protocol. The samples were mixed well and absorbance read at 340nm, 20 seconds after mixing (A1) and finally at 80 seconds (A2).

Pipette into tubes marked	Standard	Test
Working reagent	1000ul	1000ul
Standard	20ul	-
Test	-	20ul

Calculation: $\Delta A = A1 - A2$

$$\text{Urea (mg/dl)} = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{Concentration of Standard (mg/dl)}$$

C) Estimation of Uric Acid:

Methodology: Trivedi and Kabasakalian with a modified trinder peroxidase method using TBHB.[119]

Principle:

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{TBHB} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + H_2O_2$$

4-AAP: 4-Aminoantipyrine

TBHB: 2,4,6-Tribromo-3-hydroxy benzoic acid

The intensity of chromogen (Quinoeimine) formed is proportional to the uric acid concentration in the sample when measured at 505nm (500-540nm)

Procedure: The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. The samples were prepared as per the protocol. The samples were mixed well and incubated for 5 minutes at 37˚C. Absorbance of standard and each test was read at 505nm.

Pipette into tubes marked	Blank	Standard	Test
Working Reagent	1000ul	1000ul	1000ul
Distilled water	20ul	-	-
Standard	-	-	-
Test	-	-	20ul

Calculations:

$$\text{Uric Acid (mg/dl)} = \frac{\text{Abs of Test}}{\text{Abs of Standard}} \times \text{Concentration of standard (mg/dl)}$$

D) Total protein[120]

Principle:

The peptide bonds of protein react with copper II ions in alkaline solution to form blue-violet complex, (biuret reaction). Each copper ion complexing with 5 or 6 peptide bonds. Tartarate is added as a stabilizer whist iodide is used to prevent auto-reduction of the alkaline copper complex. The color formed is proportional to the protein concentration and is measured at 546nm (520-560nm).

Specimen Collection and Handling: Use unheamolytic serum, plasma(heparin, EDTA), urine.

Procedure:

This was done using kit of Erba as per Biuret Methods, End point method. The samples were prepared as per the protocol. The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. Test tubes were incubated for 10 mins at 37oC. Reading was obtained by measuring the absorbance at 546 nm (520-560nm) in the autoanalyser of standard and each test against reagent blank.

Calculations:

Calculate the results as follows:

$$\text{Total protein (g/dl)} = \frac{\text{Abs of Test}}{\text{Abs of Standard}} \times \text{Concentration of standard (g/dl)}$$

E) Albumin (ALB)

Methodology: Doumas et al[121]

Principle:

Albumin binds with bromocresol green (BCG) at pH 4.2 causing a shift in absorbance of yellow BCG dye. The blue-green colour formed is proportional to the concentration of albumin present, when measured photometrically between 580-630nm with maximum absorbance at 625 nm.

Specimen Collection and Handling:

Use Unheamolytic serum or plasma (EDTA, heparin)

Procedure:

This was done using kit of Erba as per BCG Dye Method, End Point. The samples were prepared as per the protocol. The autoanalyser instrument was set to the specifications mentioned in the protocol supplied with the kit. Mix well the test tubes with samples, read the

absorbance of standard and each test at 630nm (580-630nm) against reagent blank, after one minute incubation at 37˚C.

Pipette into tubes marked	Blank	Standard	Test
Albumin Reagent	1000ul	1000ul	1000ul
Distilled Water	10ul	-	-
Standard	-	10ul	-
Test	-	-	10ul

Calculations:

$$\text{Albumin (g/dl)} = \frac{\text{Abs of Test}}{\text{Abs of Standard}} \times \text{Concentration of standard (g/dl)}$$

4.9 Estimation of kidney Antioxidant Enzymes:

Preparation of kidney Homogenate: Animals were sacrificed by cervical dislocation. On dissection, the kidneys were isolated and washed immediately with cold saline to render them free from blood clots. Kidney homogenates (10% w/v) were been prepared in cold phosphate buffer in the ratio 1:4 using homogenizer. The unwanted cell debris was separated by centrifugation at 3000 rpm for 15 minutes (4º C), using a cold centrifuge. Furthermore, the supernatant obtained was been utilized for the estimation of superoxide dismutase (SOD), reduced glutathione (GSH),malondialdehyde (MDA) and catalase (CAT)concentration.

A. Estimation of Superoxide dismutase (SOD)[123]

Principle: SOD is a metalloprotein and the first enzyme involved in the antioxidant defense against ROS by lowering the steady state level oxygen. SOD scavenges the superoxide ions produced as cellular byproducts. SOD is a major defense for aerobic cells, combating the toxic effect of superoxide radicals. The SOD activity is been determined by the ability of the enzyme to inhibit auto oxidation of pyrogallol.

Reagent preparation:

1. Tris-HCl buffer mixture: 3.0275 g of tris hydrochloride and 0.186 g EDTA was been added to 300 ml of distilled water. The resultant solution's pH was adjusted to 8.5 with 50 mM hydrogen chloride and its volume was made up to 500 ml with distilled water.

2. Pyrogallol solution: 25 mg of pyrogallol was been dissolved in 10 ml of distilled water.

Sr. No	Reagent	Blank(B)	Control(C)	Test(T)
1	Tris-HCl buffer (0.05M	2.9ml	2.9ml	2.9ml
2	Pyrogallol solution	-	0.1ml	0.1ml
3	Heart homoginate	-	-	10µl
3	Heart homoginate	-	-	10µl

The reagents were been combined with the homogenate according to the assay procedure and absorbance of the resultant solution was taken at 420 nm. Absorbance was measured at two intervals, viz; 90 and 120 seconds respectively using UV spectrophotometer.

Formula for calculation:

$$\textbf{SOD (unit /ml)} = \frac{\textbf{(C-T)} \times \textbf{100}}{\textbf{(C}\times\textbf{50)} \times \textbf{h}}$$

Where, h= volume of homogenate taken.

C (Control) =(absorbance at 210 seconds-absorbance at 60 seconds)÷ 2.

T (Test)= (absorbance at 210 seconds-absorbance at 60 seconds) ÷ 2.

Note: 1unit of SOD = Amount of enzyme required for 50% inhibition of Pyrogallol autooxidation.

B) Estimation of Catalase (CAT)[124]

Principle:

CAT is a heme-protein, localized in the micro-peroxisome. It reduces the hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals, thereby protecting the cellular constituents from oxidative damage in the peroxisome. The enzyme catalysis the decomposition of hydrogen peroxide to water and oxygen. Thus protecting the cell from oxidative damage caused by hydrogen peroxide. The assay method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H2O2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced is been measured calorimetrically at 570-610 nm.

Requirements:

1. Dichromate/acetic acid reagent: The reagent was prepared by mixing 5% solution of K2Cr2O7withglacial acetic acid in the ratio 1:3.
2. Hydrogen peroxide (0.2 M).
3. Phosphate buffer (0.01 M-pH7).

Preparation of standard graph: Different amounts of H_2O_2 ranging from 10-160 µ moles were taken in small test tubes (6ml). 2ml of 5% solution of $K_2Cr_2O_7$ and glacial acetic acid (1:3 ratio) was added to each of them. The resulting solution turned blue due to the unstable blue precipitate of perchromic acid. On heating all the tubes for 10mins in boiling water bath, the solution changed to a stable green color due to the formation of chromic acetate. The tubes were cooled at room temperature and then optical density was measured at 570 nm. Further, the graph of linear relationship was obtained.

Preparation of test: 4 ml of H_2O_2 solution (800 µmoles) and 5 ml phosphate buffer were mixed together. To this1ml of properly diluted enzyme preparation was added and the reaction was allowed to stand at room temperature. Further,1 ml of the resulting solution was taken and blown into 2 ml of dichromate/acetic acid reagent at 60 sec intervals.

$$K = 1/t \log_{10} \frac{so}{s}$$

K = Pseudo monomolecular reaction velocity constant at time t

S0 = Initial concentration of H_2O_2 (800µM).

S = Concentration of H_2O_2 at t time

K values are plotted against time in min and points were extrapolated to the Y-axis to get K(0) or K at.f

K(0)= Pseudo monomolecular reaction velocity constant at time 0.

K at.f = k at alasefaahigkeit (catalase contents of the enzyme preparation according to von Euler and Josephson)

$$Kat.f = \frac{K_0}{G \text{ of protein/ml}}$$

Calculation:

Graph between optical density and µm of H_2O_2 (standard) was plotted. At 60 sec interval different readings in the experiment were noted. Those values were plotted in standard graph. The resultant values were put in equation ($K=1/t \log 10\ S_0/S$). Different values of K were obtained. K versus time graph was plotted. K was extrapolated to 0 to obtain K(0) as per the formula to obtain the result.

C) Estimation of Reduced glutathione (GSH)[125]

Principle:

GSH is a major non-protein thiol, endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell's structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reaction**.**

Reagent preparation: 1. Reaction buffer:0.1 M Sodium phosphate, pH8 containing 1mM EDTA.

Stock 1-2.78 g Sodium phosphate monobasic was dissolved in 100 ml distilled water.

Stock 2- 2.84 g anhydrous Sodium phosphate dibasic was dissolved in 100 ml distilled water.

2.65 ml of Stock 1 and 47.35 ml of Stock 2 were mixed together and the volume was made up to 100 ml with distilled water. Further, 37.2 mg EDTA was added to the above solution.

Ellmans reagent solution: 4 mg of Ellmans reagent was dissolved in 1 ml of reaction buffer.

Assay procedure:

Sr. No	Test	Blank
1	50 µl of Ellaman's reagent solution	50 µl of Ellaman's reagent solution
2	2.5 ml reaction buffer	2.5 ml reaction buffer
3	250 µl heart homogenate	250 µl reaction buffer

The reagents were mixed well with the liver homogenate in the above-mentioned proportion and incubated at room temperature for 15 minutes. Absorbance of the resulting reaction was read at 412 nm using UV spectrophotometer.

Formula for calculation:

$$\text{GSH (Mole)} = \frac{\text{Absorbance}}{1.4150\times10^{4}} \times 11.2$$

For the conversion of Mole to micro Mole, the whole equation is multiplied by 10^6.

D) Estimation of Malondialdehyde (MDA)[126]

Principle: Oxidative stress is associated with peroxidation of cellular lipids, which is been determined by measuring Thiobarbituric acid reacting substance (TBARS). The concentration of MDA products may reflect the degree of oxidative stress. The increased level of TBARS results in increase of oxygen free radicals, which attack the polyunsaturated fatty acids present in the cell membranes and cause lipid peroxidation. The Malondialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS.

Requirements:

1) 1.5% KCl solution.

2) 1% phosphoric acid solution.

3) 0.6% Thio-barbituric acid solution.

4) n–butanol.

Procedure:

Tissue malondialdehyde (MDA) levels as a marker of lipid peroxidation were been analysed using thio-barbituric acid reactive substances.

- 10% liver homogenate prepared with 1.5% cold KCl was been used for MDA analysis.
- 3 ml of 1% phosphoric acid 1 ml of 0.6% thio-barbituric acid solution was added to 0.5 ml of 10% liver homogenate contained in each of the test tubes.
- The above mixture was heated for 45 minutes and on cooling, 4 ml of n-butanol was been added to it and mixed thoroughly. Absorbance of the clear solution obtained on standing was measured at 535 and 520 nm respectively.
- The difference between the two measurements defined the level of MDA (μM/g of tissue).

Histopathology: At the end of the treatment period, all animals were sacrificed, their kidneys were dissected out and fixed overnight in 10% formalin. Sections of the tissues fixed in paraffin were prepared, stained with hematoxylin and eosin and observed for pathological changes.

Statistical Analysis:

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test (Graph Pad Prism software, version 5.01). $p < 0.05$ was considered statistically significant.

5.0 RESULTS

5.1 Phytochemical Investigation

The herbal formulation NEFPRO was subjected to various qualitative analysis to determine the Phyto constituents present in the extract. (Table 04)

Table. 04: Results of Qualitative Phytochemical tests

S.No.	Chemical Test		Result
1.	**Test for Carbohydrates** • Molisch's test (general test)		+
	A.	**Test for Reducing Sugars**	
		• Fehling's test	-
		• Benedicts test	-
	B.	**Test for Monosaccharides**	
		• Barfoed's test	-
	C.	**Test for Hexose sugars**	
		• Cobalt chloride test	-
	D.	**Test for Non-reducing sugars**	-
	E.	**Test for Non-reducing polysaccharides (starch)**	
		• **Iodine test**	-
		• **Tannic acid test tor starch**	-
2.	**Test for Proteins**		
	• Biuret test (General test)		-
	• Millon's test		-
	• Xanthoprotein test		-
	• Test for protein containing sulphur		-

3.	**Test for Steroids**	
	• Salkowski reaction	+
	• Liembermann-Burchard reaction	+
	• Liebermann reaction	+
4.	**Test for Flavonoids**	
	• Shinoda test	+
	• Ferric chloride test	+
	• Alkaline reagent test	+
	• Lead Acetate test	+
5.	**Test for Alkaloids**	
	• Dragendroff's test	+
	• Mayer's test	+
	• Hager's test	+
	• Wagner's test	+
6.	**Test for Tannins**	
	• Ferric chloride test	+
	• Lead acetate test	+
	• Bromine water test	+
	• Dilute iodine test	+
7.	**Test for Organic Acids**	
	• Oxalic acid	+
	• Tartaric acid	+
	• Citric acid	+
8.	**Test for Vitamin C**	+

5.2 Pharmacological Investigation

Nefpro herbal herbal formulation was been administered at doses of 5ml/kg and 10ml/kg twice a day to evaluate its pharmacodynamic effects on diet and drug induced renal damage in rats.

5.2.1 Effect of Nefpro herbal formulation (NHF) on Body weight (BW):

The change in body weight of animals in different groups has been compared at the end of study period. There was an initial statistical non-significant increase in BW in the drug and diet induced renal damage rats 149.66±10.03gms, followed by a gradual increase in weight 208.16 ±17.70gms by week 6 when compared to that of normal control group having mean BW of about 169.16 ±7.49gms. Treatment of the renal damaged rats with NHF at 5 and 10ml/kg showed a significant ($p<0.01$ & $p<0.001$) dose dependant decrease in BW of about 190.33 ±10.211gms and 170.83±8.90gms respectively when compared to the disease control group. However, treatment with standard drug Simvastatin (10 mg/kg) in renal damaged rats showed a significant ($p<0.001$) dose dependant decrease in BW to 181.66 ±9.62gms when compared to diseased group. The results are tabulated in table.05 fig: 08

5.2.2 Changes in food intake in renal damaged rats:

The change in the food intake of modified diet in rats has been compared at the end of induction period. Diet with 30% of fructose was fed to rats and the food intake was recorded. Initially in week 1 there was non-significant change observed in food intake of about 46.26±0.40g/day when compared to the normal diet fed group i.e.52.70±0.26g/day. Gradual significant ($p<0.001$) increase in food intake i.e. 84.90±g/day was observed by week 4 which further significantly decreased ($p<0.01$) by 62.50±0.20g/day was found till the end of week 6 of induction period. The results are tabulated in table.06 fig: 09

5.2.3 Effect of Nefpro herbal formulation (NHF) on Kidney weight and kidney to body weight ratio.

The change in kidney weight of animals and kidney weight to body weight ratio in different groups has been compared at the end of study period. Kidney weight was significantly ($P<0.001$) increased 2.045±0.40g in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) i.e 1.008±0.36g. Similarly kidney weight to body ratio in percent also showed significant ($P<0.001$) increase 0.914% in renal damaged rats (Group II) compared to normal control (Group I) i.e 0.535%. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent reduction in kidney weight with mean value of 1.417±0.25g and 1.000±0.09g respectively, also reduction in ratio by 0.742% and 0.585% respectively. Similarly kidney weight non-significantly elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 1.295(±0.15)g and ratio by 0.665%. Simvastatin treated group (group III) showed significant ($P<0.001$) decrease in kidney weight when compared to group II. The results are tabulated in table.07

5.2.4 Effect of Nefpro herbal formulation (NHF) on Urine parameters:

A. Urine volume:

Urinary output of the normal, control and formulation treated rats on last day of experiment are shown in Table.04 The urine volume of the normal rats (Group I) was 10.95 ±2.30ml/24 h, while it was significantly ($P<0.001$) increased in diet and drug induced renal damaged rats (Group II) by 17.07 ±1.56 ml/24h. However the urinary output of NHF 5 and 10ml/kg treated rats (Groups IV& V) significantly ($p<0.05$ & $p<0.001$) reduced to 15.70 ±1.43 and 12.11 ±0.86ml/24h respectively when compared to disease induced group. Similarly urine output of preventive group (Groups VI) showed non-significant ($p<0.001$) increase to 10.41

±1.28ml/24h. Std drug treated group (group III) showed significant ($p<0.001$) decrease in Urine output by 11.84 ±1.33ml/24h when compared with disease induced group (Group- II). The results are tabulated in table.08 fig.10

B. Urine Creatinine (UCr):

The change in Urine Creatinine (UCr) of animals in different groups has been compared weekly throughout induction period till week 6 and at the end of treatment period. There was an initial non-significant decrease in UCr in the drug and diet induced renal damage rats 52.85±12.13mg/dl, followed by a gradual significant ($p<0.001$) decrease in UCr 12.53±2.69mg/dl by week 6 when compared to that of normal control group having mean UCr of about 55.16±10.42mg/dl. The results are tabulated in table.09 fig. 11

Furthermore after treatment period there was significant ($p<0.001$) decrease in UCr 18.50±4.231mg/dl in the renal damage induced group (Group II) when compared to normal control group(Group I) 55.00±8.367 mg/dl. However the treatment with NHF 5 and 10ml/kg treated rats (Groups IV& V) significantly ($p<0.05$ & $p<0.001$) increased Creatinine to 28.67±4.179 and 49.67±6.022 mg/dl respectively when compared to disease induced group. Similarly preventive group (Groups VI) showed non-significant ($p<0.001$) decrease to 51.50±5.431mg/dl compared to normal control group. The results are tabulated in table.10 fig. 12

C. Urine Microalbumin:

Urine Microalbumin was significantly ($P<0.001$) increased 185.0±101.5 mg/Lin drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 27.67 ±10.60mg/L. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent reduction in elevated microalbumin level with mean value of 107.8 ±28.35 and 21.67 ±6.563 mg/L respectively.

Similarly microalbumin level non-significantly elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 25.17±5.115 mg/dl. Simvastatin treated group (group III) showed significant p<0.001 decrease when compared to group II. The results are tabulated in table.10 fig. 13

5.2.5 Effect of Nefpro herbal formulation (NHF) on Serum parameters

A. Serum Creatinine (SCr)

Serum Creatinine was significantly (P<0.001) increased 2.008±0.313mg/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 2.008±0.313mg/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant (p<0.01 & p<0.001 respectively) dose dependent reduction in elevated SCr level with mean value of 1.047±0.330 mg/dl and 0.627±0.243mg/dl respectively. Similarly SCr level non-significantly elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 0.850±0.277mg/dl. Simvastatin treated group (group III) showed significant p<0.001 decrease in SCr when compared to group II. The results are tabulated in table.11 fig: 14

B. Serum Albumin (Alb)

Serum Albumin was significantly (p<0.05) decreased 3.042±0.519g/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 3.920±0.584g/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant (p<0.01 & p<0.001 respectively) dose dependent elevation in Alb level with a mean value of 3.665±0.297g/dl and 4.268±0.350g/dl respectively. Similarly Alb level non-significantly decreased in preventive groups (Groups VI) when compared to disease induced (group- II) by 3.788±0.356g/dl. Simvastatin treated group (group III) showed significant p<0.01 elevation 3.843±0.568g/dl in Alb when compared to group II. The results are tabulated in table.11 fig.15

C. Serum Total Protein (TP)

Serum Total protein was significantly ($p<0.05$) decreased 5.887±0.400g/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 8.472±0.610g/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent elevation in TP level with a mean value of 6.242±1.052g/dl and 7.482±0.357g/dl respectively. Similarly TP level non-significantly decreased in preventive groups (Groups VI) when compared to disease induced (group- II) by 7.550±0.400g/dl. Simvastatin treated group (group III) showed significant $p<0.001$ elevation 7.467±0.385g/dl in TP when compared to group II. The results are tabulated in table.11 fig:16

D. Serum Uric Acid (UA):

Serum Uric Acid was significantly ($P<0.001$) increased by 4.797±0.649mg/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 1.950 (±0.435) mg/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent reduction in elevated UA level with mean value of 2.420±0.460mg/dl and 2.008±0.227 mg/dl respectively. Similarly UA level non- significantly elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 1.928±0.496mg/dl. Simvastatin treated group (group III) showed significant ($P<0.001$) decrease in UA when compared to group II. The results are tabulated in table.11 fig.17

D. Serum Urea:

Serum Urea was significantly ($P<0.001$) increased 93.33±13.75mg/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 47.12±18.03mg/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.05$ & $p<0.001$ respectively) dose dependent reduction in elevated urea level

with mean value of 75.43±3.602 mg/dl and 55.71±8.639mg/dl respectively. Similarly urea level significantly ($p<0.01$) elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 64.46±19.12 mg/dl. Simvastatin treated group (group III) showed significant decrease 63.87±5.244 mg/dl in urea when compared to group II. The results are tabulated in table. 12 fig. 18

E. Blood Urea Nitrogen (BUN):

BUN was significantly ($P<0.001$) increased 43.68±6.435 mg/dl in drug and diet induced renal damaged group (group II) when compared to normal control (Group I) 19.89±7.850 mg/dl. Treatment with NHF at 5 and 10 ml/kg in the renal damaged rats showed significant ($p<0.05$ & $p<0.001$ respectively) dose dependent reduction in elevated BUN level with mean value of 35.77±2.021 mg/dl and 29.22±6.207 mg/dl respectively. Similarly BUN level significantly ($p<0.01$) elevated in preventive groups (Groups VI) when compared to disease induced (group- II) by 30.17±8.950mg/dl. Simvastatin treated group (group III) showed significant ($P<0.001$) decrease 29.89±2.453mg/dl in BUN level when compared to group II. The results are tabulated in table. 12 fig. 19

5.2.6 Effect of Nefpro herbal formulation on kidney Antioxidant Enzymes (SOD, CAT, GSH and MDA).

Kidney homogenate's of experimental rats to whom High fructose diet and gentamicin 40mg/kg has been administered exhibited a highly significant reduction in SOD, CAT and GSH ($P<0.001$) while, a highly significant elevation in MDA ($P<0.001$) levels. Treatment of renal damaged rats with NHF at 5 ml/kg showed a significant increase in SOD & CAT levels ($P<0.001$) and a significant decrease in MDA level ($P<0.05$). However, treatment of renal damage rats with NF at 10 ml/kg exhibited a significant increase in the diminished SOD, CAT ($P<0.001$) as well as GSH levels ($P<0.01$) while a significant decrease in elevated MDA levels ($P<0.001$). In addition, treatment with standard drug Simvastatin (10 mg/kg) significantly increased the level of GSH ($p<0.01$), SOD & CAT ($p<0.001$) respectively while it also exhibited a highly significant ($p<0.001$) decrease in MDA levels. The results are tabulated in table no.13 fig. 20, 21, 22, 23

Table 5: Effect of various treatments on Body weight:

Body Weight / GROUPS	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 10
NORMAL CONTROL	165.83(±7.27)	169.16(±7.49)	173.16(±7.70)	175.66(±8.59)	181.00(±7.95)	185.33(±7.06)	188.33(±5.98)
DISEASE CONTROL	142.83(±9.19)	149.66(±10.03)	168.50(±11.22)	183.33(±13.45)	196.50(±17.55)	208.16(±17.70)	223.16(±10.68)#
TREATMENT WITH STD DRUG	151.83(±14.59)	158.83(±13.27)	172.50(±13.64)	188.66(±15.61)	200.00(±19.60)	209.50(±18.23)	181.66(±9.62)***
TREATMENT WITH NHF (5ml/kg)	157.66(±15.68)	163.16(±19.48)	173.66(±15.99)	179.00(±16.29)	187.66(±15.26)	196.50(±14.44)	190.33(±10.21)**
TREATMENT WITH NHF (10ml/kg)	157.66(±18.43)	167.83(±16.48)	177.50(±14.93)	181.83(±12.85)	188.00(±12.97)	193.66(±14.01)	170.83(±8.90)***
PRETREATED WITH NHF (5ml/kg)	144.50(±12.81)	152.50(±11.84)	159.33(±11.50)	162.83(±9.283)	169.50(±8.45)	171.66(±7.06)	174.50(±7.31)

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using two-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 6: Effect of various treatments on Food Intake:

Duration / Groups	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
NORMAL CONTROL	55.46(±0.32)	52.70(±0.26)	62.46(±0.20)	68.50(±0.19)	65.43(±0.41)	62.63(±0.20)	67.43(±0.41)
DISEASE CONTROL	45.03(±0.15)	46.26(±0.40)	66.93(±0.15)	79.10(±0.20)	84.90(±0.09)	77.46(±0.30)	62.50(±0.20)**
TREATMENT WITH STD DRUG	35.33(±0.41)	54.63(±0.25)	71.46(±0.30)	74.46(±0.15)	92.13(±0.25)	72.40(±0.36)	56.86(±0.15)**
TREATMENT WITH NHF (5ml/kg)	32.50(±0.20)	58.26(±0.30)	77.80(±0.10)	82.33(±0.20)	88.50(±0.26)	81.40(±0.26)	59.13(±0.15)**
TREATMENT WITH NHF (10ml/kg)	41.43(±0.25)	49.53(±0.37)	72.63(±0.20)	80.53(±0.47)	91.10(±0.20)	83.36(±0.25)	52.73(±0.20)**
PRETREATED WITH NHF (5ml/kg)	38.86(±0.15)	56.10(±0.26)	69.33(±0.11)	71.66(±0.15)	67.63(±0.20)	69.23(±0.20)	62.76(±0.32)

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using two-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 07: Effect of various treatments on Kidney weight and kidney to body weight ratio.

Parameters / Groups	Body weight(g)	Kidney weight (g)	Kidney weight/ Body weight ratio%
NORMAL CONTROL	188.33(±5.98)	1.008(±0.36)	0.535%
DISEASE CONTROL	223.16(±10.68)	2.045(±0.40)	0.914%
TREATMENT WITH STD DRUG	181.66(±9.62)	0.838(±0.26)	0.465%
TREATMENT WITH NHF (5ml/kg)	190.33(±10.211)	1.417(±0.25)	0.742%
TREATMENT WITH NHF (10ml/kg)	170.83(±8.90)	1.000(±0.09)	0.585%
PRETREATED WITH NHF (5ml/kg)	174.50(±7.31)	1.295(±0.15)	0.665%

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 08: Effect of various treatments on Urine Volume:

PARAMETER / GROUPS	URINE OUTPUT(ml)
NORMAL CONTROL	10.95(±2.30)
DISEASE CONTROL	17.07(±1.56)$^{###}$
TREATMENT WITH STD DRUG	11.84(±1.33)^{ns} ***
TREATMENT WITH NHF (5ml/kg)	15.70(±1.43)$^{### ns}$
TREATMENT WITH NHF (10ml/kg)	12.11(±0.86)^{ns} ***
PRETREATED WITH NHF (5ml/kg)	10.41(±1.28)^{ns} ***

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.
Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 09: Effect of various treatments on Urine Creatinine:

Urine Creatinine (mg/dl) / Groups	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
NORMAL CONTROL	58.25(±13.42)	58.85(±13.26)	59.85(±12.72)	59.96(±11.58)	55.23(±12.08)	55.16(±10.42)
DISEASE CONTROL	52.76(±10.62)	52.85(±12.13)	48.07(±10.37)	44.25(±8.68)	35.60(±6.98)	12.53(±2.69)
TREATMENT WITH STD DRUG	55.73(±14.11)	55.60(±15.62)	49.55(±12.41)	46.75(±13.05)	36.71(±9.94)	14.03(±3.55)
TREATMENT WITH NHF (5ml/kg)	63.38(±13.14)	63.33(±14.72)	56.71(±12.67)	50.26(±10.81)	37.03(±6.83)	13.44(±4.73)
TREATMENT WITH NHF (10ml/kg)	58.53(±12.73)	60.16(±12.34)	52.11(±12.73)	46.16(±12.30)	31.18(±4.04)	12.99(±4.49)
PRETREATED WITH NHF (5ml/kg)	65.43(±9.55)	64.33(±11.64)	62.11(±8.54)	62.58(±8.59)	57.80(±7.79)	56.25(±7.72)

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 10 : Effect of various treatments on Urine Parameters:

PARAMETERS / GROUPS	Urine Microalbumin (mg/L)	Urine Creatinine (mg/dl)
NORMAL CONTROL	27.67 (±10.60)	55.00(±8.367)
DISEASE CONTROL	185.0 (±101.5)	18.50(±4.231)
TREATMENT WITH STD DRUG	84.83 (±19.02)	41.00(±4.940)
TREATMENT WITH NHF (5ml/kg)	107.8 (±28.35)	28.67(±4.179)
TREATMENT WITH NHF (10ml/kg)	21.67 (±6.563)	49.67(±6.022)
PRETREATED WITH NHF (5ml/kg)	25.17 (±5.115)	51.50(±5.431)

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 11: Effect of Nefpro various treatments on Serum Parameters:

Parameters / Groups	SERUM CREATININE (mg/dl)	SERUM ALBUMIN (g/dl)	SERUM TOTAL PROTEIN (g/dl)	SERUM URIC ACID (mg/dl)
NORMAL CONTROL	0.228 (±0.056)	3.920(±0.584)	8.472(±0.610)	1.950 (±0.435)
DISEASE CONTROL	2.008(±0.313)$^{\#\#\#}$	3.042(±0.519)$^{\#}$	5.887(±0.400)$^{\#\#\#}$	4.797(±0.649)$^{\#\#\#}$
TREATMENT WITH STD DRUG	1.095 (±0.247)$^{***\ @@@}$	3.843(±0.568)*	7.467(±0.385)***	2.275(±0.798)***
TREATMENT WITH NHF (5ml/kg)	1.047 (±0.330)***	3.665(±0.297)	6.242(±1.052)$^{@@}$	2.420(±0.460)***
TREATMENT WITH NHF (10ml/kg)	0.627 (±0.243)$^{***\ @}$	4.268(±0.350)***	7.482(±0.357)***	2.008(±0.227)***
PRETREATED WITH NHF (5ml/kg)	0.850 (±0.277)$^{\#\#\ ***}$	3.788(±0.356)*	7.550(±0.400)***	1.928(±0.496)***

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.
$^{@}$p<0.05, $^{@@}$p<0.01, $^{@@@}$p<0.001 when compared with Std drug treatment group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table. 12: Effect of various treatments on Serum Parameters:

PARAMETERS / GROUPS	SERUM UREA (mg/dl)	BLOOD UREA NITROGEN (BUN)
NORMAL CONTROL	47.12(±18.03)	19.89(±7.850)
DISEASE CONTROL	93.33(±13.75)$^{\#\#\#}$	43.68(±6.435)$^{\#\#\#}$
TREATMENT WITH STD DRUG	63.87(±5.244)**	29.89(±2.453)$^{\# **}$
TREATMENT WITH NHF (5ml/kg)	75.43(±3.602)*	35.77(±2.021)$^{\#\#}$
TREATMENT WITH NHF (10ml/kg)	55.71(±8.639)***	29.22(±6.207)**
PRETREATED WITH NHF (5ml/kg)	64.46(±19.12)**	30.17(±8.950)$^{\# **}$

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

Table 13: Effect of various treatments on Kidney Antioxidant Enzymes (SOD, CAT, GSH and MDA).

Parameters / Groups	SOD (U/ml)	CATALASE (U/mg protein)	GSH (µg/g of tissue)	MDA (µM/g of tissue)
NORMAL CONTROL	21.91(±2.32)	19.65(±1.941)	18.67(±3.28)	20.14(±3.40)
DISEASE CONTROL	10.69(±0.86)$^{###}$	7.948(±2.247)$^{###}$	6.094(±3.40)$^{###}$	42.64(±3.43)$^{###}$
TREATMENT WITH STD DRUG	18.84(±1.80)***	17.65(±1.392)***	14.78(±1.71)***	29.63(±1.66)***
TREATMENT WITH NHF (5ml/kg)	15.72(±3.69)**	12.00(±1.086)**	12.45(±3.55)$^{## **}$	38.21(±1.60)*
TREATMENT WITH NHF (10ml/kg)	19.71(±0.98)***	15.62(±0.9595)***	16.69(±2.85)***	27.60(±1.73)***
PRETREATED WITH NHF (5ml/kg)	20.42(±1.28)***	17.15(±0.7971)$^{#}$	16.83(±2.60)***	25.19(±3.04)$^{##}$

# $p<0.05$, # # $p<0.01$, # # # $p<0.001$ when compared with normal control group.
*$p<0.05$, * * $p<0.01$, * * * $p<0.001$ when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

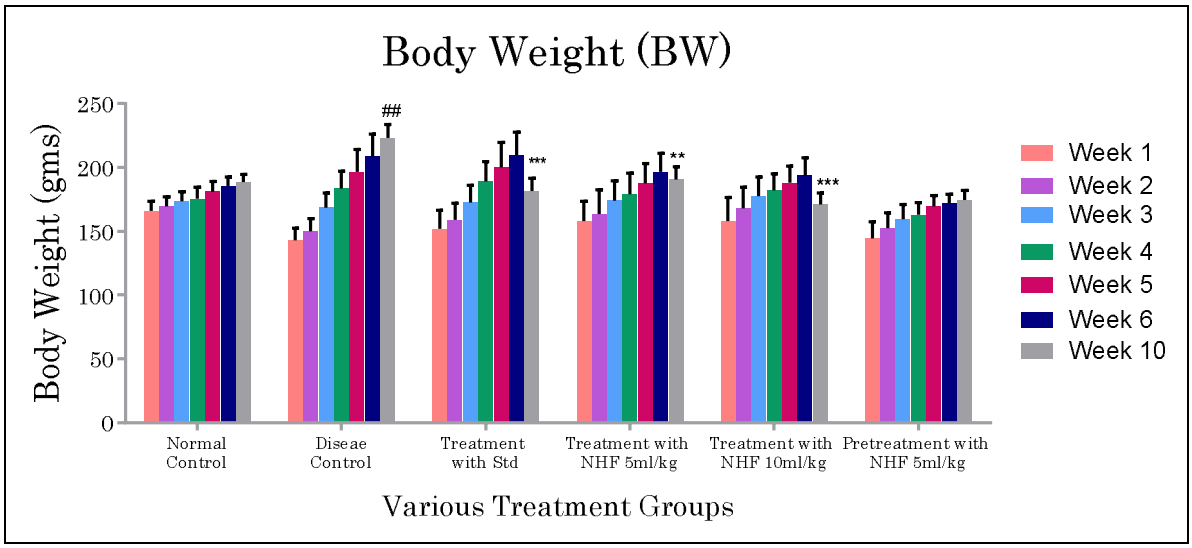


Fig. 08: Effect of various treatments on body weight (BW)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * *p<0.01, * * *p<0.001 when compared with disease control group.

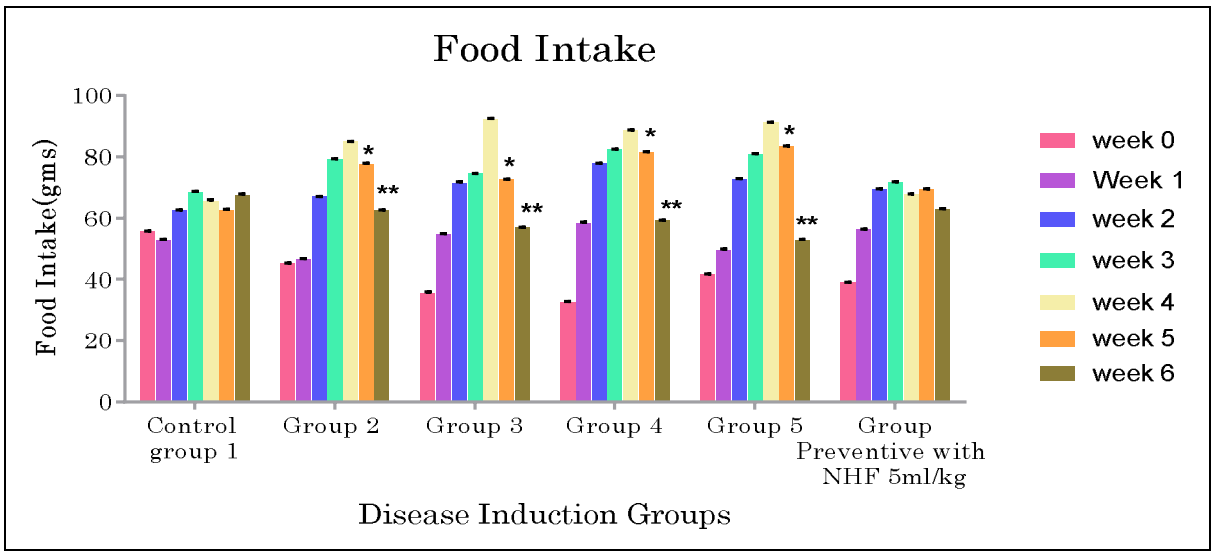


Fig 09: Changes in food intake during induction period in renal damage induced rats.

*p<0.05, * * p<0.01, * * * p<0.0001 when compared with normal control group.
Results are been expressed as mean ± SD. Differences among data were determined using two-way ANOVA followed by Dunnett Multiple Comparison Test.

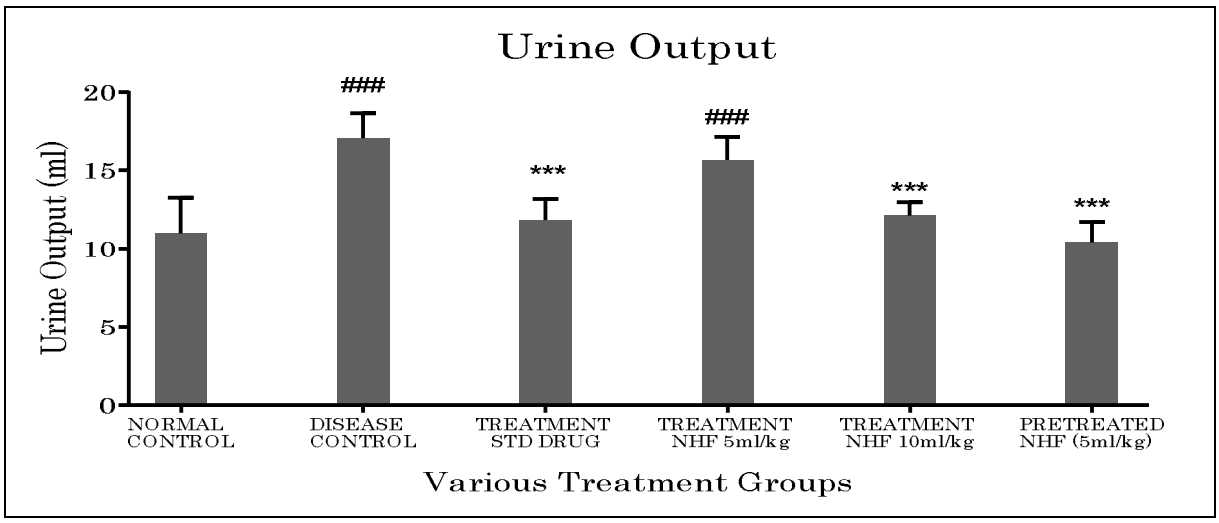


Fig 10: Effect of various treatments on urine output in rats

# p<0.05, # # p<0.01, # # #p<0.001 when compared with normal control group.
*p<0.05, * *p<0.01, * * *p<0.001 when compared with disease control group.

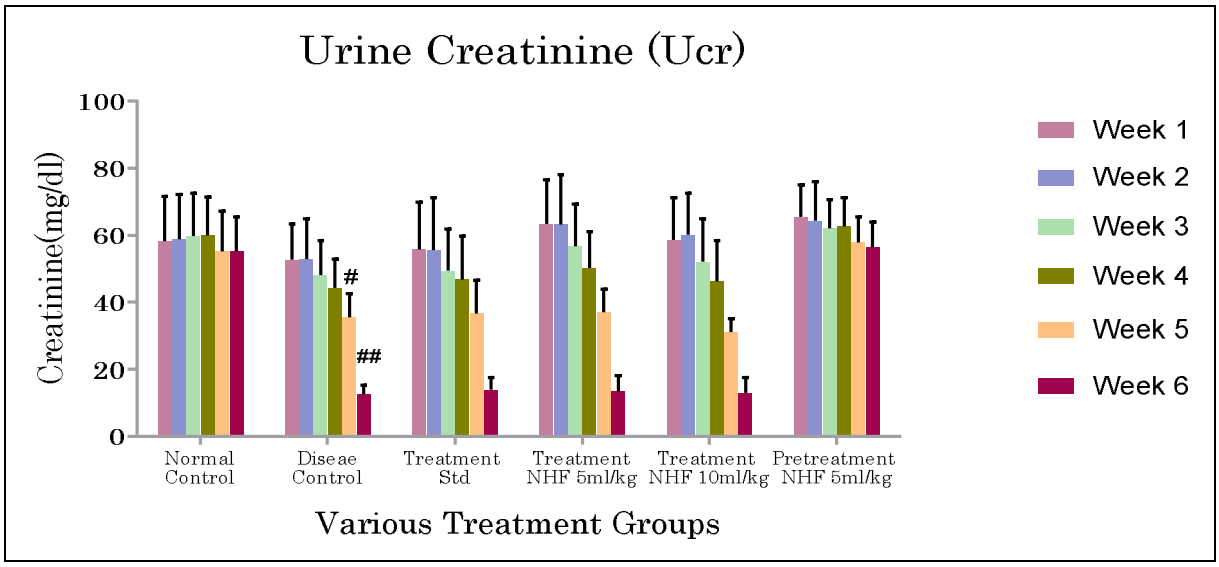


Fig 11: Effect of various treatments on Urine Creatinine.

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group

Results are been expressed as mean ± SD. Differences among data were determined using two-way ANOVA followed by Dunnett Multiple Comparison Test.

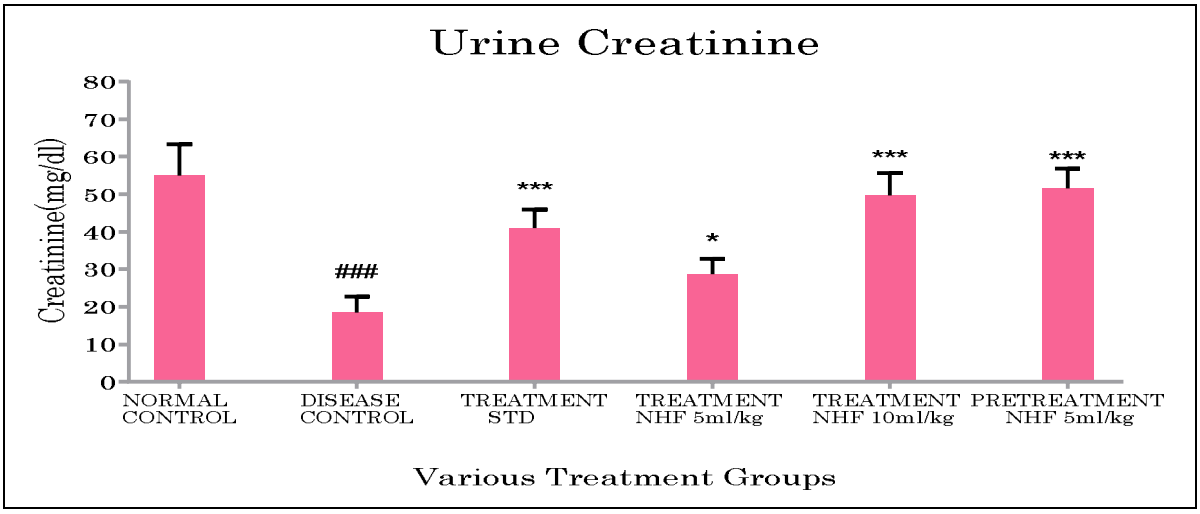


Fig 12: Effect of various treatments on Urine Creatinine.

# p<0.05, # # p<0.01, # # #p<0.001 when compared with normal control group.
*p<0.05, * *p<0.01, * * *p<0.001 when compared with disease control group.

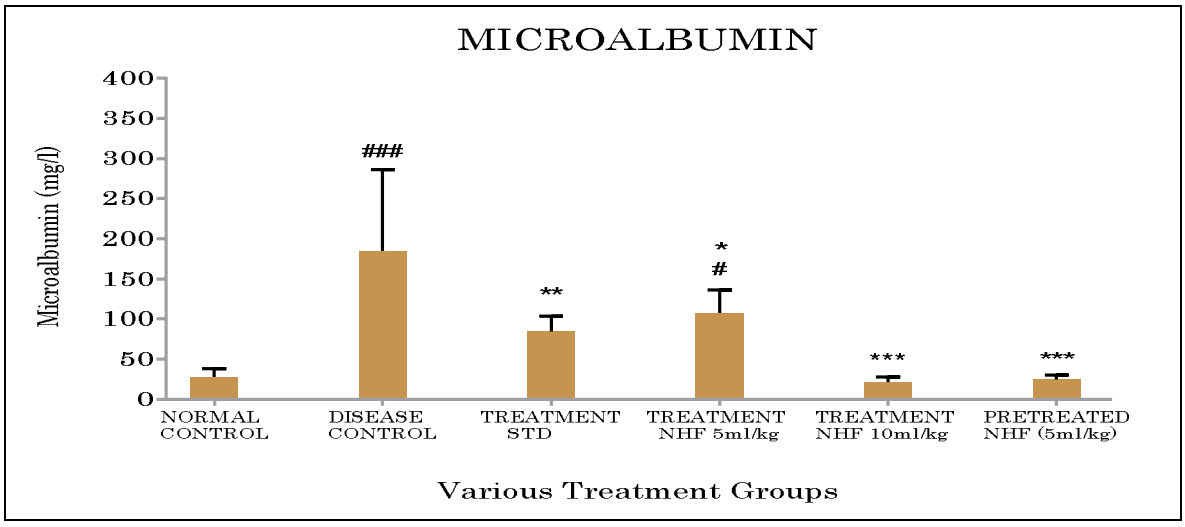


Fig 13: Effect of various treatments on Urine Microalbumin:

# p<0.05, # # p<0.01, # # #p<0.001 when compared with normal control group.
*p<0.05, * *p<0.01, * * *p<0.001 when compared with disease control group.

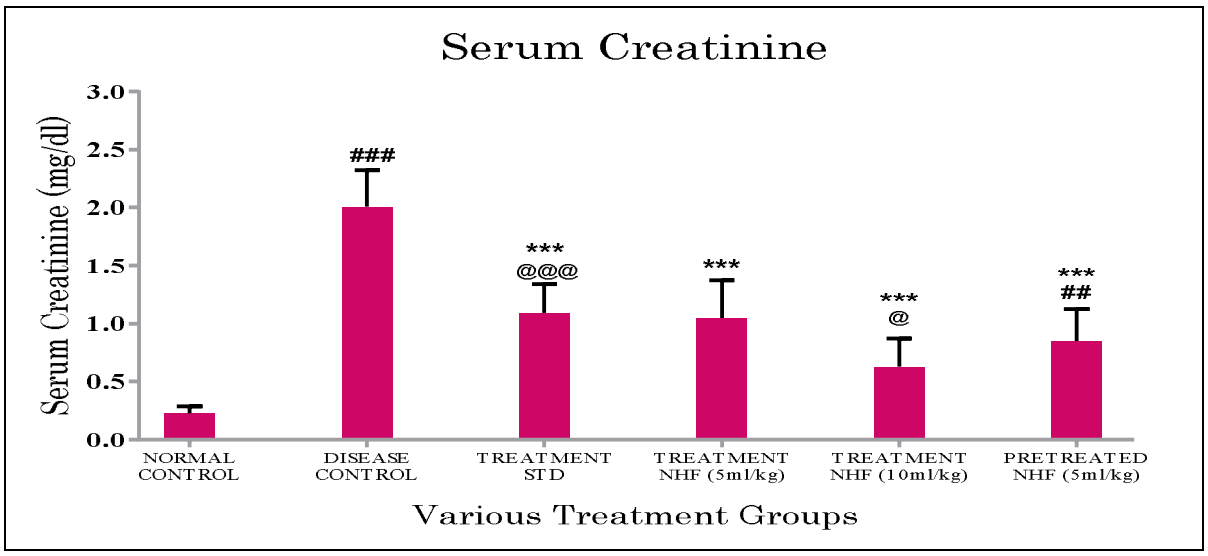


Fig 14: Effect of various treatments on Serum Creatinine (SCr).

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.
$^{@}$p<0.05, $^{@@}$p<0.01, $^{@@@}$p<0.001 when compared with Std drug treatment group.

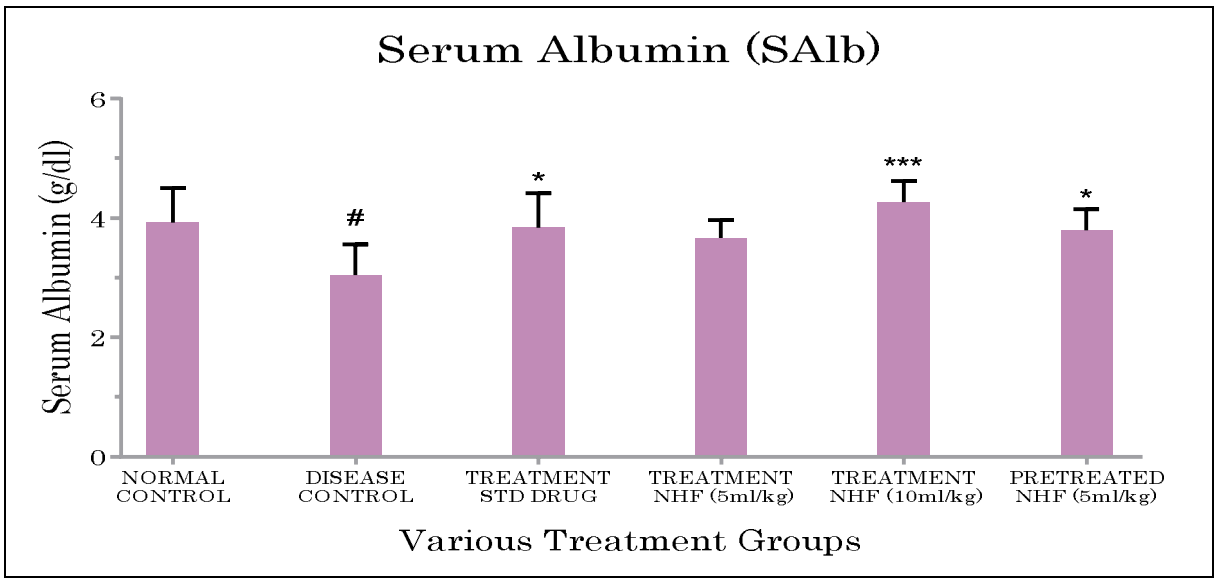


Fig 15: Effect of various treatments on Serum Albumin (Alb)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

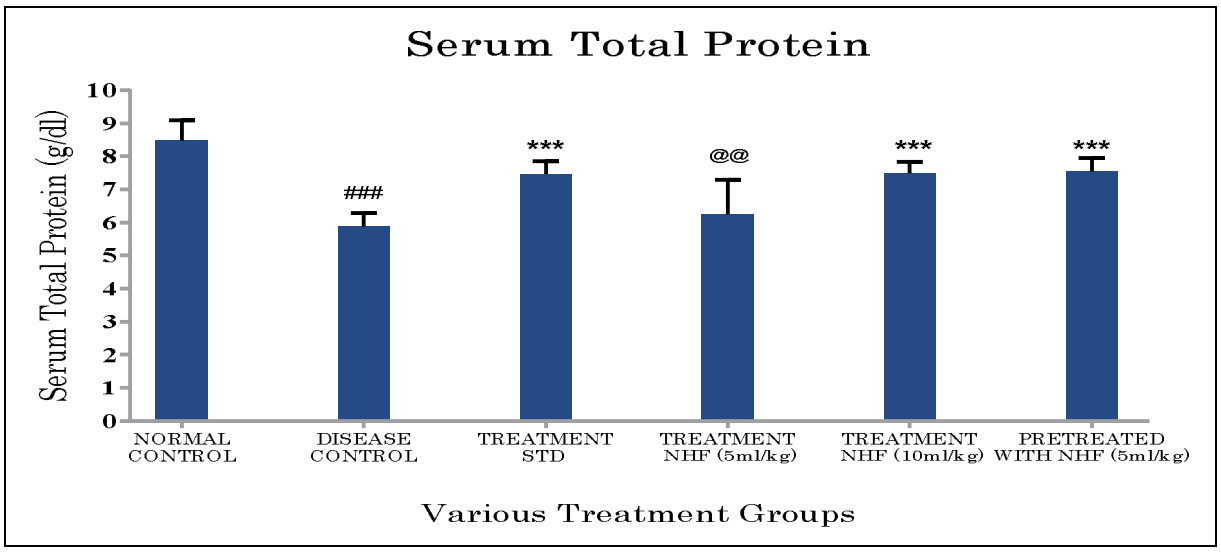


Fig 16: Effect of various treatments on Serum Total Protein (TP)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * *p<0.01, * * *p<0.001 when compared with disease control group.
$^{@}$p<0.05, $^{@@}$p<0.01, $^{@@@}$p<0.001 when compared with Std drug treatment group.

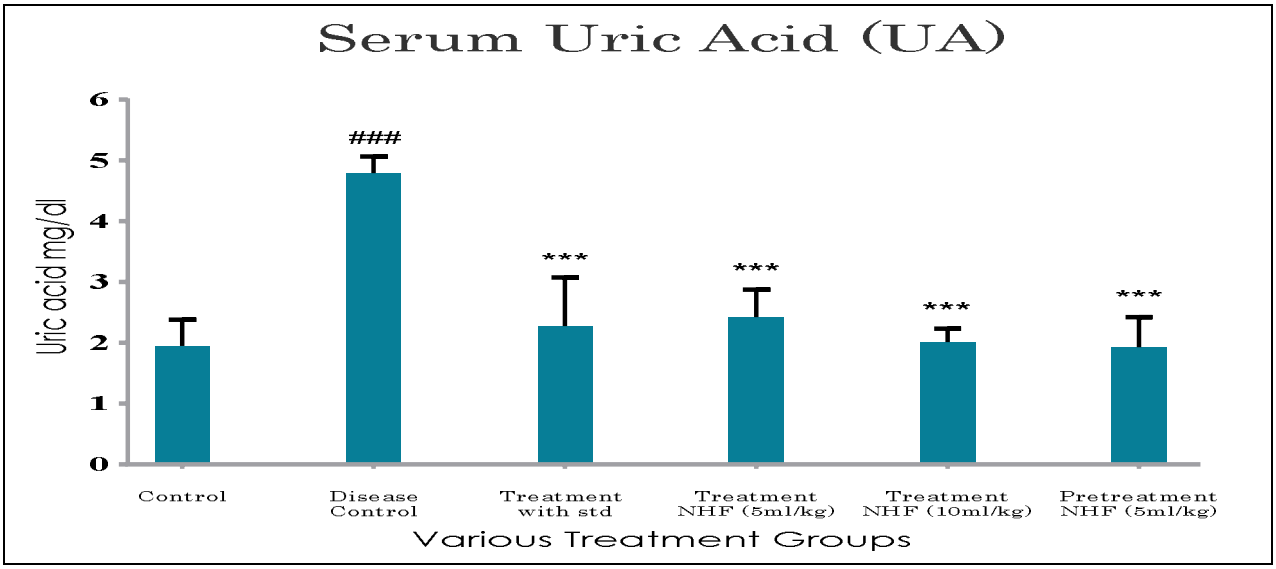


Fig 17. Effect of various treatments on Serum Uric Acid (UA)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

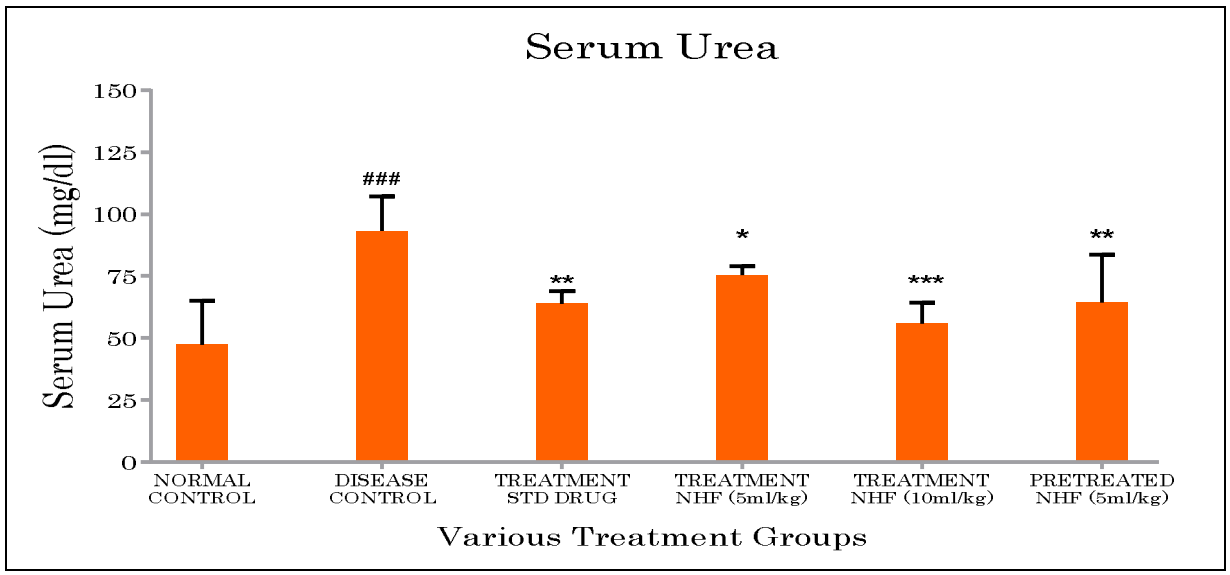


Fig 18: Effect of various treatments on Serum Urea

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

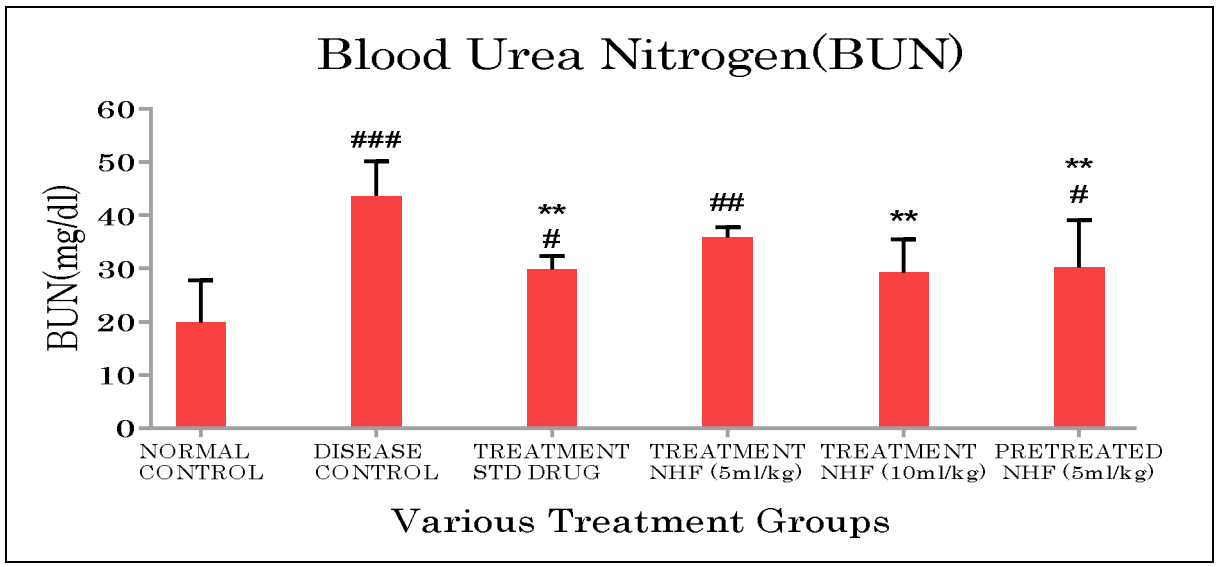


Fig 19: Effect of various treatments on Blood Urea Nitrogen (BUN)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

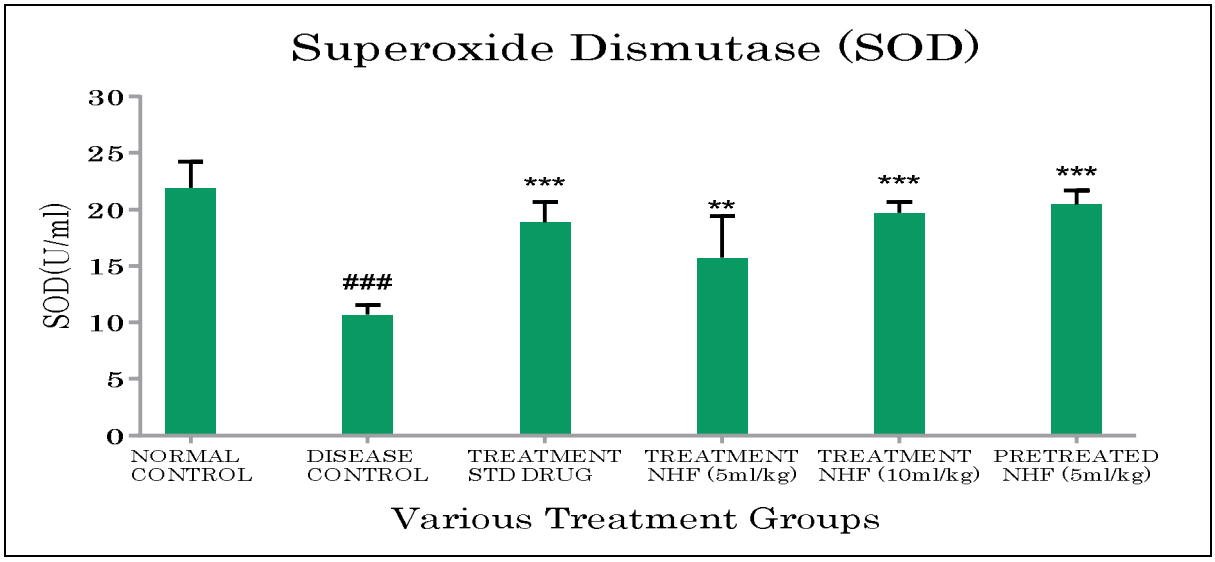


Fig 20: Effect of various treatments Superoxide Dismutase (SOD).

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

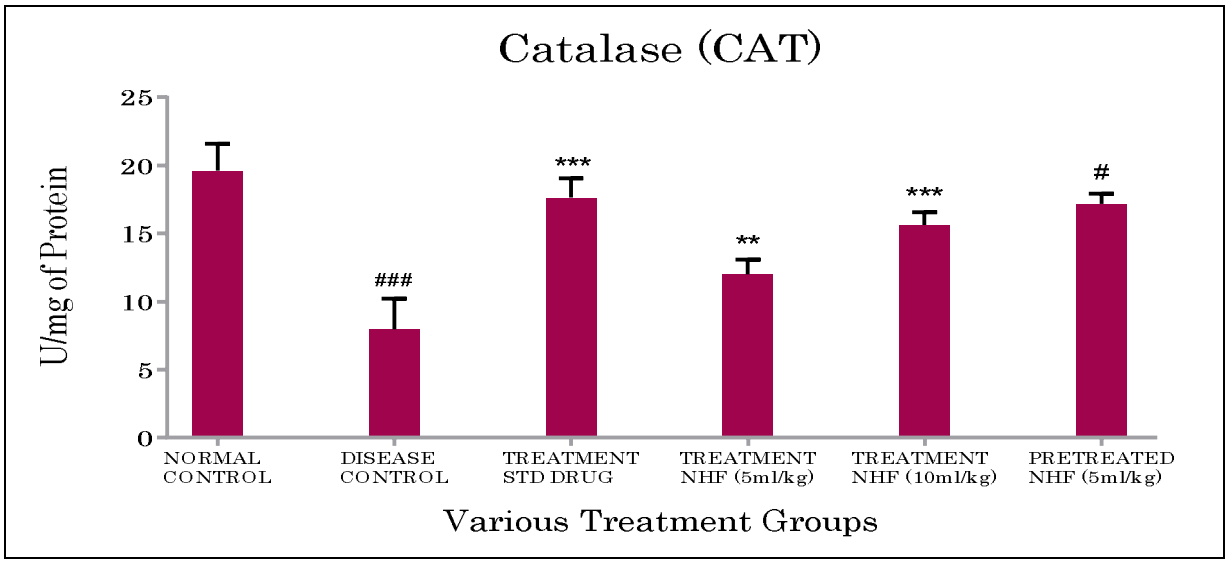


Fig 21: Effect of various treatments on Catalase (CAT)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

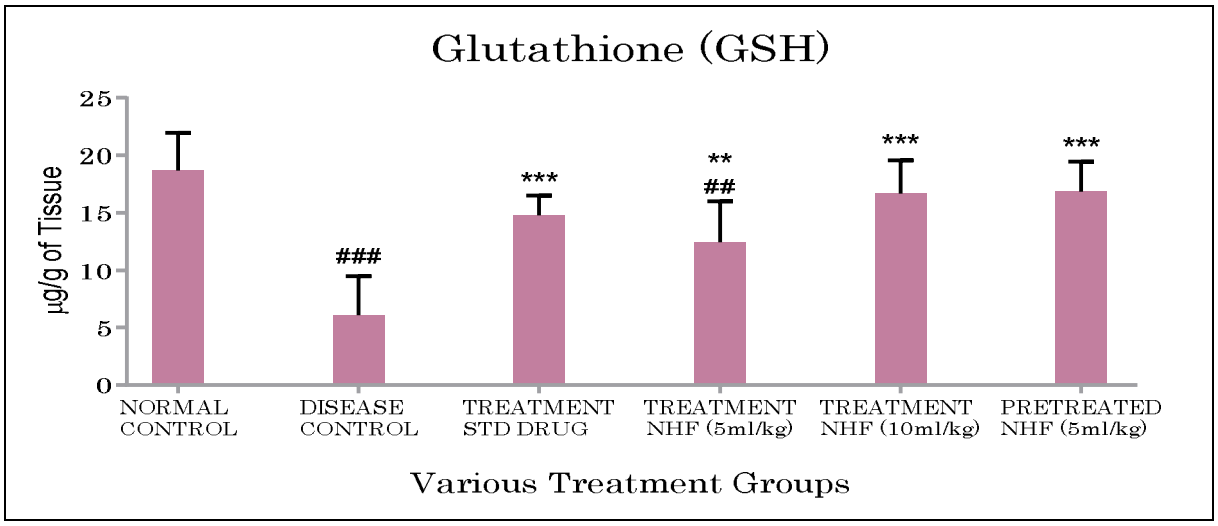


Fig 22: Effect of various treatments on Glutathione (GSH)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

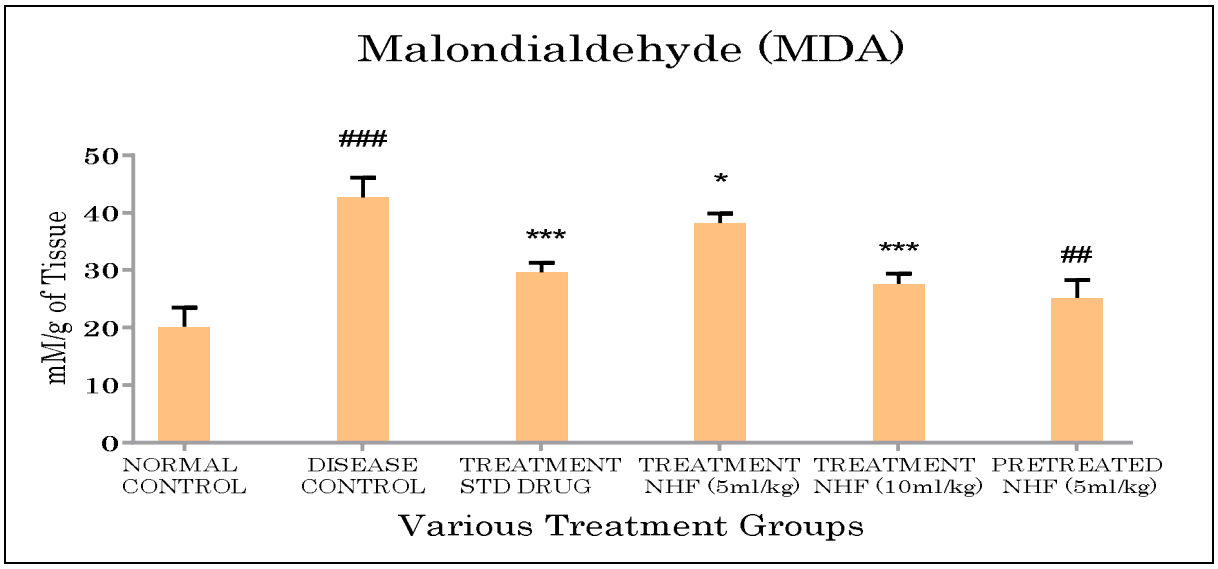


Fig 23: Effect of various treatments on Malondialdehyde (MDA)

# p<0.05, # # p<0.01, # # # p<0.001 when compared with normal control group.
*p<0.05, * * p<0.01, * * *p<0.001 when compared with disease control group.

Results are been expressed as mean ± SD. Differences among data were determined using one-way ANOVA followed by Dunnett Multiple Comparison Test.

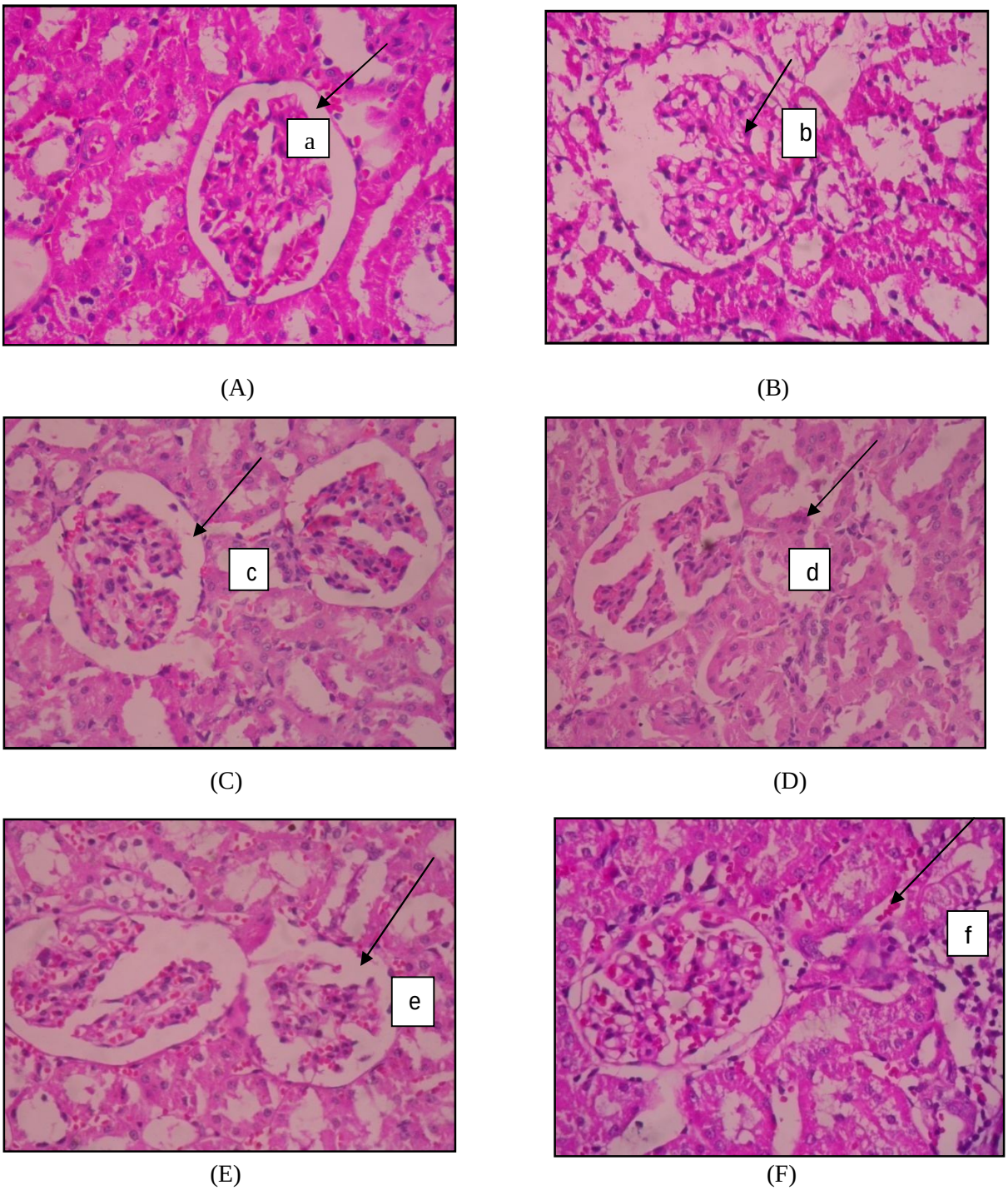


Fig. 24: Effect of various treatments on Histopathology of kidneys.

(A) Normal group: Section of kidneys showing normal intact glomerular architecture. (a)

(B) Disease control group(renal damage rats): Section of kidneys showing presence of tubular congestion, cytoplasmic vacuoles, perivascular inflammation, peritubular inflammation, glomerular congestion. (b)

(C) Treatment of Std drug : Section of kidneys showing tubular congestion but absence of perivascular and peritubular inflammation,cytoplasmic vacuoles. (c)

(D) Treatment with Nefpro Herbal Formulation 5ml/kg: Section of kidneys showing mild decrease in tubular and glomerular congestion.(d)

(E) Treatment with Nefpro Herbal Formulation 10ml/kg: Section of kidneys showing decreased glomerular and tubular congestion and absence of peritubular and perivascular inflammation.(e)

(F) Pretreatment with Nefpro Herbal Formulation 5ml/kg: Section of kidneys showing tubular and vascular congestion, peritubular and perivascular inflammation, tubular degeneration.(f)

6.0 DISCUSSION

The present study has been designed to evaluate the effect of Nefpro herbal liquid formulation, an ayurvedic proprietary medication in experimentally induced renal damage rats. The doses selected for the present study were 5 and 10 ml/kg rat body weight. The results obtained from the present study, suggest that Nefpro herbal liquid formulation improves the renal damage state in a dose dependent manner. In addition, phytochemical investigation of the liquid formulation of Nefpro has revealed the presence of sterols, flavonoids, alkaloids, tannins, vitamin-C and saponins.

Renal damage has been induced in the experimental Sprague dawley rats via the administration of fructose enriched diet ie. 30% of fructose diet and 10% of fructose in drinking water. Along with that gentamicin 40mg/kg through intraperitoneal route for last 10 days of induction period. The clinical usefulness of gentamicin is limited due to its nephrotoxicity, manifested to acute tubular necrosis and impairment in renal function. In the case of gentamicin, renal damage is produced in a two step process. In the early step, the drug is transported and gets accumulated in proximal tubular cells of nephrons. In the later step, it leads to adverse interaction between these polycationic drugs and cellular moieties, also along with oxidative stress it causes cellular damage to nephrons. Accumulation in epithelial tubular cells cause a range of effects starting with loss of the brush border in epithelial cells and ending in tubular necrosis, activation of apoptosis and massive proteolysis. Gentamicin also causes cell death by generation of free radicals, phospholipidosis, extracellular calcium-sensing receptor stimulation and energetic catastrophe, reduced renal blood flow and inflammation.[19, 45]

Literature evidently depicts high doses (40 mg/kg or more for gentamicin) in animals rapidly induce extended cortical necrosis and causes renal dysfunction. At this stage, a large number of structural, metabolic, and functional alterations are observed in tubular cells, and several of

these alterations have been claimed to be responsible for cell death or dysfunction. Various literature studies proved gentamicin induced renal damage as a promising animal model for studying nephroprotective activity.[64]

Furthermore, numerous studies have put forth that a high percentage of fructose in diet initiates the development of metabolic syndrome that may also contribute to the development of chronic kidney disease. Particularly, fructose has now become a major constituent of our modern diet and is used extensively in carbonated beverages, dairy products, canned fruits and baked goods.[83, 87]

Study depicts 30 to 40% of fructose is taken by the kidney, metabolized by fructokinase (ketohexokinase), which phosphorylates fructose to fructose 1-phosphate. Phosphorylation of fructose results in a decrease in intracellular phosphate and ATP depletion, resulting in transient inhibition of protein synthesis.[84]

Experimental studies support fructose intake as a mechanism for kidney injury. Administration of fructose (60% diet) to rats induces renal hypertrophy with tubular cell proliferation and low grade tubulointerstitial injury, generating chemotactic factors such as monocyte chemoattractant protein-1 by tubular cells. These abnormalities are specifically not observed in rats fed an equivalent glucose-based diet.[22]

Results obtained from the present study, confirms that administration of 30% fructose in diet and 10% in drinking water, along with GM 40mg/kg lead to induction of renal damage in experimental rats. Successful induction of renal damage is confirmed by increase in body weight, urine volume, serum Creatinine, Urea, BUN, Uric Acid, MDA. In addition, there was a significant ($p<0.001$) decrease in urine Creatinine, serum Albumin and serum Total protein as well as antioxidant biomarker's (SOD, GSH and CAT). Induction of renal damage was also supported by histopathological changes (Glomerular congestion, tubular necrosis, peritubular inflammation, perivascular inflammation, tubular congestion).

In the present study, the significant ($p<0.001$) increase in urine output was observed in renal damage rats when compared with normal control group. Literature studies indicate that GM causes impairment of urinary concentrating ability in relation to the renal expression of aquaporin 2 water channel. In rats due to the suppression of tubular water reabsorption, osmotic diuresis, expression levels of aquaporin 2 in the inner and outer medulla and cortex are significantly decreased. In this study, the 24-h collection of urine volume in the gentamicin group was significantly higher than in the control group, indicating the presence of gentamicin-induced polyuria. Treatment of renal damage rats with NHF at 5 and 10ml/kg showed significant ($p<0.05$) and ($p<0.001$) dose dependent decrease in elevated urine output compared to disease control group. Also the pretreated group with NHF 5ml/kg showed non-significant increase in urine output. The results clearly indicate that treatment with Nefpro herbal liquid formulation improves increased urine output, with a most prominent effect observed at the dose of 10 ml/kg BW.

An important index of renal function is creatinine determination. Creatinine, is a by-product of muscle metabolism and it is freely filtered but is not reabsorbed or metabolized. Its level rises in the blood if there is deficiency in kidney filtration capacity, suggesting remarkable damage to the nephron. The significant increase ($p<0.001$) in serum creatinine and gradual decrease in urine creatinine was observed in renal damage rats when compared with normal control group. According to the present study, treatment of renal damage induced rats with Nefpro herbal liquid formulation at 5 and 10 ml/kg showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent reduction in elevated Serum Creatinine level. The observed effect in the renal damage rats can be attributed to the phytochemical constituents viz; flavonoids, saponins, alkaloids, triterpinoids, phenolics and tannins, which have been reported for their renoprotective potential in animal experimental studies.

The disease induced group exhibited the significant ($p<0.001$) increase in serum urea levels compared to normal control group. Urea, produced by the liver in the urea cycle and is a sensitive biomarker used in the assessment of renal tissue damage. Therefore, in renal tissue injury, there is retention of urea. It has been established that gentamicin causes inhibition of protein synthesis in renal cells with consequent abundance of amino acid in the kidney resulting in increased urea levels. Treatment of renal damage induced rats with Nefpro herbal liquid formulation at 5 and 10 ml/kg showed significant ($p<0.05$ & $p<0.001$ respectively) dose dependent reduction in elevated Serum Urea levels. Also pretreated group with NF 5ml/kg showed significant ($p<0.05$) elevation in serum urea.

The gentamicin-induced renal damage group significantly ($p<0.001$ & $p<0.05$) showed decreased serum protein and albumin levels compared to normal group. According to literature study this resulted from damage to the filtration mechanism of the glomerular basement membrane as well as reabsorptive failure of the proximal convoluted tubules with consequent leaking of plasma protein into the urine. Treatment of renal damage induced rats with Nefpro herbal liquid formulation at 5 and 10 ml/kg showed significant ($p<0.01$ & $p<0.001$ respectively) dose dependent elevation in decreased Serum albumin and total protein levels. Pretreated group showed non-significant decrease in serum protein and albumin.

Oxidative stress plays a central role in pathological mechanism of GM renal damage. AGs cause stimulation of mitochondrial production of ROS by oxidizing many cellular molecules leading to cell dysfunction, cell death, mesengial and vascular contraction along with inflammation. Reactive oxygen species like superoxide anion and hydrogen peroxide activate nuclear factor kB. Inducible nitric oxide reacts with superoxide anion to produce preoxynitrile - highly reactive radical causing cell damage and vascular contraction. Attributing to this mechanism, herbal extracts present in the Nefpro herbal formulation are reported to be renoprotective according to literature survey.[75]

Literature study shows that ***Boerhaavia diffusa*** contains a large number of compounds such as flavonoids, alkaloids (punarnavine), steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins[109] which can be attributed to the significantly($p<0.001$) reduced the MDA levels and increased the GSH, CAT, SOD levels.

Zingiber officinale extracts reduces inflammation, improves kidney function, and induces healthy state of renal cells, suggesting its role as renal protective agent. This can be attributed to their abundance of natural antioxidants: flavonoids, sterols, and alkaloids like emblicanins A and B, gallic acid present. In-silico studies revealed that ellagic acid might be responsible for its nephroprotective activity, along with high level of polyphenol compounds (6-gingerol and its derivatives), which have a high antioxidant activity. Mehrdad et al. stated that ***Zingiber officinale*** has a beneficial effect for removal of urea and creatinine.[111]

Terminalia chebula possessed high antioxidant activity and phenolics were found to be responsible for this activity.[101]

Therapeutic activity of ***Withania somnifera*** was also supported by its numerous bioactive components, including withaferin A, sitoindosides VII–X, 5-dehydroxy withanolide-R, withasomniferin-A, etc which might reverse the damage caused in renal cells. ***Withania somnifera*** was more prominent in protecting kidney against gentamicin induced nephrotoxicity.[96]

Results obtained from previous studies indicated the extract of ***Tribulus terrestris*** has significant nephroprotective activity have reported the identification of tribulusamides A and B, tigogenin, neotigogenin, terrestrosid F, and gitonin in *Tribulus terrestris* extracts to be responsible for nephroprotection.[100]

Treatment of renal damage rats with Nefpro herbal formulation significantly regulated abnormalities observed in histopathological studies. It showed decreased glomerular and tubular congestion and absence of peritubular and perivascular inflammation. Also Pretreated group with Nefpro formulation maintained the normal cellular integrity.

The results obtained from the present study indicate that Nefpro herbal liquid formulation shows significant dose dependent improvement in drug and diet induced renal damage in Sprague dawley rats. Also pretreatment group showed a prominent renoprotective activity. Based on the present study, it is difficult to establish the exact mechanism of action for improvement in the renal damage. However, the effect of Nefpro herbal liquid formulation can be assumed to be a synergistic effect of mainly phyto-constituents like flavonoids, saponins, triterpinoids, alkaloids, sterols, tannins and vitamin-C present in its contents which act via a plethora of mechanisms to improve renal functions.

7.0 Conclusion

In conclusion, NEFPRO herbal formulation demonstrated a significant dose dependent improvement in renal damage. Moreover, treatment of renal damaged rats with Nefpro herbal formulation exhibited an improvement in serum creatinine, urea, albumin, total protein, uric acid levels. Furthermore, it also demonstrated antioxidant potential by significantly reducing oxidative stress induced in animal experimental model.

Pretreatment with Nefpro herbal formulation significantly declined the renal damage and was found to be effective in maintaining the normal renal function. The beneficial renoprotective effect of Nefpro herbal formulation can be attributed to the synergistic effects of phyto-constituents viz; flavonoids, saponins, triterpinoids, alkaloids, sterols, tannins and vitamin-C present in the contents of the formulation. Hence, Nefpro herbal formulation could be a promising future herbal treatment for drug, and diet induced renal damage.

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9.0 ANNEXURES

- Consent letter from Progen Research Lab.
- Institutional Animal Ethics Committee approval letter.
- Conferences/ Seminars/Workshops attended
    - Attended **16th National Seminar on 'Non- Invasive Diagnostic Procedures in Cardiovascular Physiology – Recent Advances'** organized by Association of Physiologist and Pharmacologist of India, Belgaum Branch, at JNMC, Belgaum.
    - Participated in **Pharmacology CME 2017 "Diabetes Mellitus – A Holistic Approach"** held at JNMC, Belagavi.
    - Participated in Workshop on "**Recent Advances in Preclinical Screening models in Drug Discovery**" (conducted by Dept of Pharmacology KLE COP Belagavi).
    - Attended **17th National Seminar on "Environmental Physiology – High Altitude and Space"** organized by APPI, Belgaum Branch, at JNMC, Belagavi.