INVESTIGATING THE EFFECTS OF BETULINIC ACID ON ACUTE KIDNEY INJURY TO CHRONIC KIDNEY DISEASE TRANSITION IN MICE

Thesis

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Dr. Bency Elsa Johnson
Roll No. M-6183

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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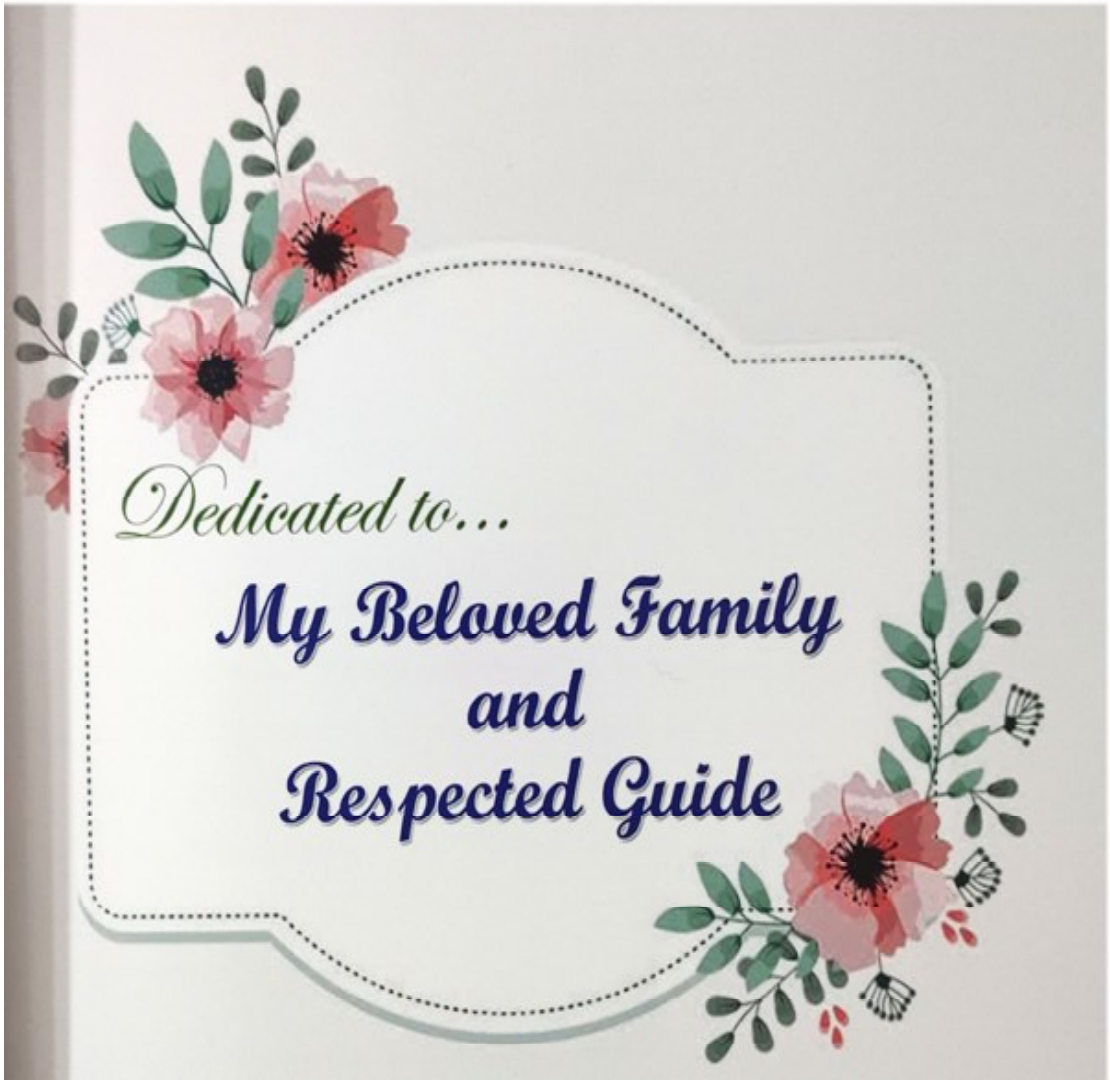
Dedicated to...
My Beloved Family
and
Respected Guide

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
इज्जतनगर –243122, (उ.प्र.), भारत

DIVISION OF PHARMACOLOGY AND TOXICOLOGY
ICAR-INDIAN VETERINARY RESEARCH INSTITUTE
(Deemed University)
IZATNAGAR - 243 122, U.P., INDIA

Dr. Madhu C.L.
M.V.Sc., Ph.D.
Scientist (SS)

Dated: 22. 12. **2022**

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Investigating the effects of betulinic acid on acute kidney injury to chronic kidney disease transition in mice"** *submitted by* **Dr. Bency Elsa Johnson, Roll No. M-6183,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Pharmacology** *at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

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We the undersigned members of Advisory Committee of **Dr. Bency Elsa Johnson**, *Roll No.* **M-6183**, *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Pharmacology**, *agree that the thesis entitled* "**Investigating the effects of betulinic acid on acute kidney injury to chronic kidney disease transition in mice**" *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

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Signature S.K. Tandan
Name S.K. TANDAN
External Examiner

Date : 31.01.2023

(Madhu C.L.)
Chairman
Advisory Committee

Date : 19.12.2022

MEMBERS OF STUDENT'S ADVISORY COMMITTEE

Dr. T.U. Singh, Senior Scientist
Division of Pharmacology and Toxicology, ICAR-IVRI, Izatnagar

Dr. Subhashree Parida, Senior Scientist
Division of Pharmacology and Toxicology, ICAR-IVRI, Izatnagar

Dr. Manish Mahawar, Principal Scientist
Division of Biochemistry, ICAR-IVRI, Izatnagar

Dr. M. Karikalan, Scientist (SS)
Centre for Wildlife, ICAR-IVRI, Izatnagar

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Place: *ICAR-IVRI, Izatnagar*

ABBREVIATIONS

%	:	Percentage
µg	:	Microgram
µl	:	Microlitre
AAN	:	Aristolochic acid nephropathy
AKI	:	Acute Kidney Injury
AMP	:	Adenosine Mono Phosphate
APS	:	Ammonium persulphate
ATP	:	Adenosine Tri Phosphate
BA	:	Betulinic acid
BSA	:	Bovine serum albumin
BUN	:	Blood urea nitrogen
BW	:	Body weight
$CaCl_2$	:	Calcium chloride
CAT	:	Catalase
CBB	:	Coomassie Brilliant Blue
CKD	:	Chromic Kidney Disease
CLP	:	Cecal ligation puncture
CTGF	:	Connective tissue growth factor
DAB	:	3, 3'- diaminobenzidine
DMSO	:	Dimethyl sulphoxide
DPX	:	Dibutylphthalate polystyrene xylene
ECM	:	Extracellular matrix
ESKD	:	End stage kidney disease
ESRD	:	End Stage Renal Disease
FA	:	Folic acid
GFR	:	Glomerular Filtration Rate
GSH	:	Glutathione
GSH-Px	:	Glutathione peroxidase
h	:	Hour
HCl	:	Hydrochloric acid
HMW	:	High molecular weight
HO-1	:	Haeme oxygenase
HRP	:	Horseradish peroxidase
IL-13	:	Interleukin-13
IL-4	:	Interleukin-4

iNOS	:	Inducible nitric oxide synthase
IRI	:	Ischemia reperfusion injury
LPO	:	Lipid peroxidation
MDA	:	Malondialdehyde
MMP	:	Matrix metalloproteinase
NaCl	:	Sodium chloride
NDMA	:	N- nitrosodimethylamine
NEDD	:	N-naphthylethylenediamine
NF-κB	:	Nuclear factor kappa B
NO	:	Nitric oxide
Nrf2	:	Nuclear factor erythroid-2- related factor 2
PVDF	:	Polyvinylidene fluoride
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
RRT	:	Renal replacement therapy
SDS-PAGE	:	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SOD	:	Superoxide dismutase
TBA	:	Thiobarbituric acid
TBST	:	Tris bufferd saline with tween
TCA	:	Trichloroacetic acid
TEC	:	Tubular epithelial cell
TEMED	:	Tetramethylethylenediamine
TGFβ	:	Transforming growth factor beta
TMB	:	3,3'5,5'- Tetramethylbenzidine
TNF-α	:	Tissue necrosis factor alpha
$ZnCl_2$	:	Zinc chloride
α-SMA	:	Alpha smooth muscle actin

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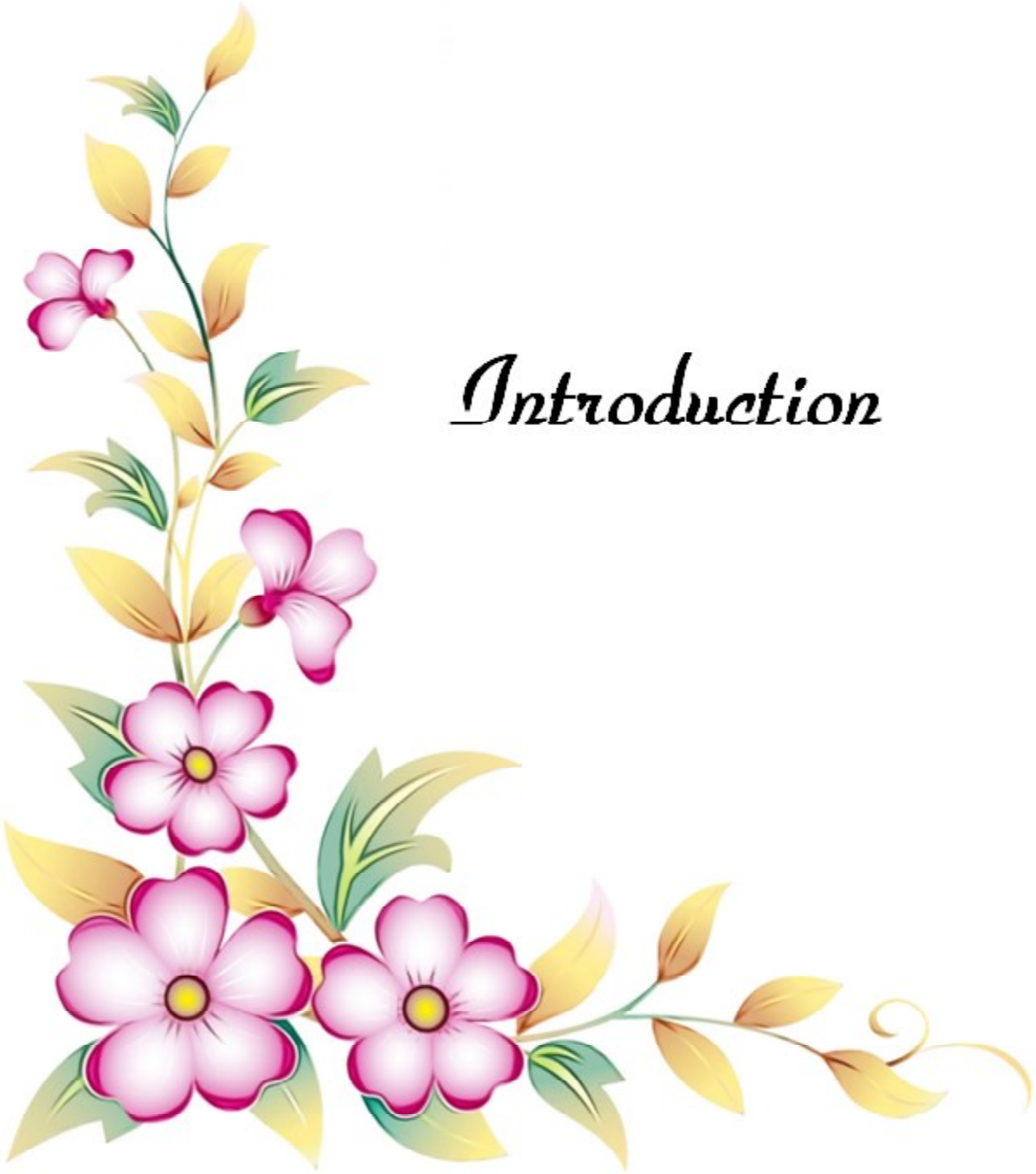


Introduction

1 INTRODUCTION

Acute kidney injury (AKI) is a complex clinical syndrome, associated with high short- and long-term morbidity and mortality in critically ill patients (Singbartl and Kellum, 2012) and it continues to impose significant healthcare and economic burdens (Leung, Tonelli, James, 2013). Recent data demonstrated that AKI occurs in about 3.2-9.6% of human admissions with overall mortality around 20% (Fang *et al*., 2010, Lafrance *et al*., 2010). The underlying mechanism of AKI is complex and encompasses ischemia, sepsis, drug toxicity, trauma, and a precipitous fall in glomerular filtration rates (GFR) (Huang *et al*., 2021). In veterinary medicine, infection is reported as the second most common etiology of AKI (Eatroff *et al*., 2012), and the incidence of AKI secondary to sepsis is 12% (Kenney *et al*., 2010). A steady decline in GFR which leads to imperceptible deterioration of renal function is the defining characteristic of chronic kidney disease (CKD). CKD is caused by a multitude of disease pathways that permanently impair the function and structure of the kidney over months or years. CKD may develop as a result of renal damage or as a complication of conditions like diabetes or hypertension (Sun *et al*., 2016; Yang *et al*., 2020). Animals are also susceptible to CKD, in addition to humans. According to reports, the likelihood of CKD rises correspondingly with age, reaching 80% in elderly cats, and the ubiquity ranges from 0.5-1% for dogs and 1-3% for cats (IRIS, 2019). AKI-CKD transition refers to the progression of CKD after AKI (Coca *et al*., 2012; Hsu, 2012). Because of the long-held assumption that the kidney's incredible regenerating power was sufficient to reverse acute renal failure, acute and chronic renal failure were formerly thought to be two separate illnesses. However, complete recovery from AKI is less common than previously thought, and the field now understands that AKI and CKD are

interrelated. AKI appears to play a crucial role in the development and progression of CKD, according to recent epidemiological and experimental research. (Ferenbach and Bonventre, 2015; Basile *et al*., 2016). AKI outcomes can range from complete resolution to partial or incomplete recovery of renal function, all of which can lead to an elevated risk of death, prolonged hospitalization, and risk for chronic comorbidities, like cardiovascular disease, CKD and subsequent progression to end-stage renal disease (ESRD) (Coca *et al*., 2012; Goldstein *et al*., 2014). The possibility of transition from AKI-CKD is upto 25.8%, and the likelihood of acquiring end-stage renal failure from AKI is 8.6% (Coca *et al*., 2012). As a result, limiting the shift from AKI to CKD is critical for lowering the social and economic cost of CKD. AKI episodes accelerate the progression of CKD whereas patients with pre-existing CKD are more likely to develop AKI. (Chawla *et al*., 2014; He *et al*., 2017). Moreover, AKI and CKD both increase the risk of cardiovascular adverse events (Chawla *et al*., 2014; Odutayo *et al*., 2017). Several studies have demonstrated that AKI is linked to the development of CKD and the severity, frequency, and duration of AKI are important factors in this process (Chawla and Kimmel, 2012; Heung *et al*., 2016).

The precise mechanism of AKI-to-CKD transition is complex and little understood, especially in humans, and numerous pathways have been put forward. Different animal studies have used ischemia/ reperfusion and nephrotoxic injuries to investigate the pathophysiologic events involved in AKI to CKD progression (Basile *et al*., 2016). Cellular damage due to any reason cause decrease in intrarenal blood flow (RBF) and this leads to renal ischemia which in turn causes a rapid degradation of intracellular adenosine triphosphate (ATP) to adenosine diphosphate and adenosine monophosphate (AMP). AMP may be further degraded to other adenine nucleotides that diffuse out of cells, preventing ATP re-synthesis. Decreased intracellular ATP leads to several metabolic and structural changes within renal tubular cells. It raises intracellular calcium levels, which can activate proteases and phospholipases, resulting in cellular damage. Hydrogen peroxide and superoxide may be produced as a result of the decomposition of other substances. In renal tubular cells, ischemia activates nitric oxide synthase. Peroxynitrite, which may directly oxidise compounds like lipids and sulfhydryls, can be formed when nitric oxide reacts with superoxide. Peroxynitrite can also prevent renal tubular cell-matrix attachment,

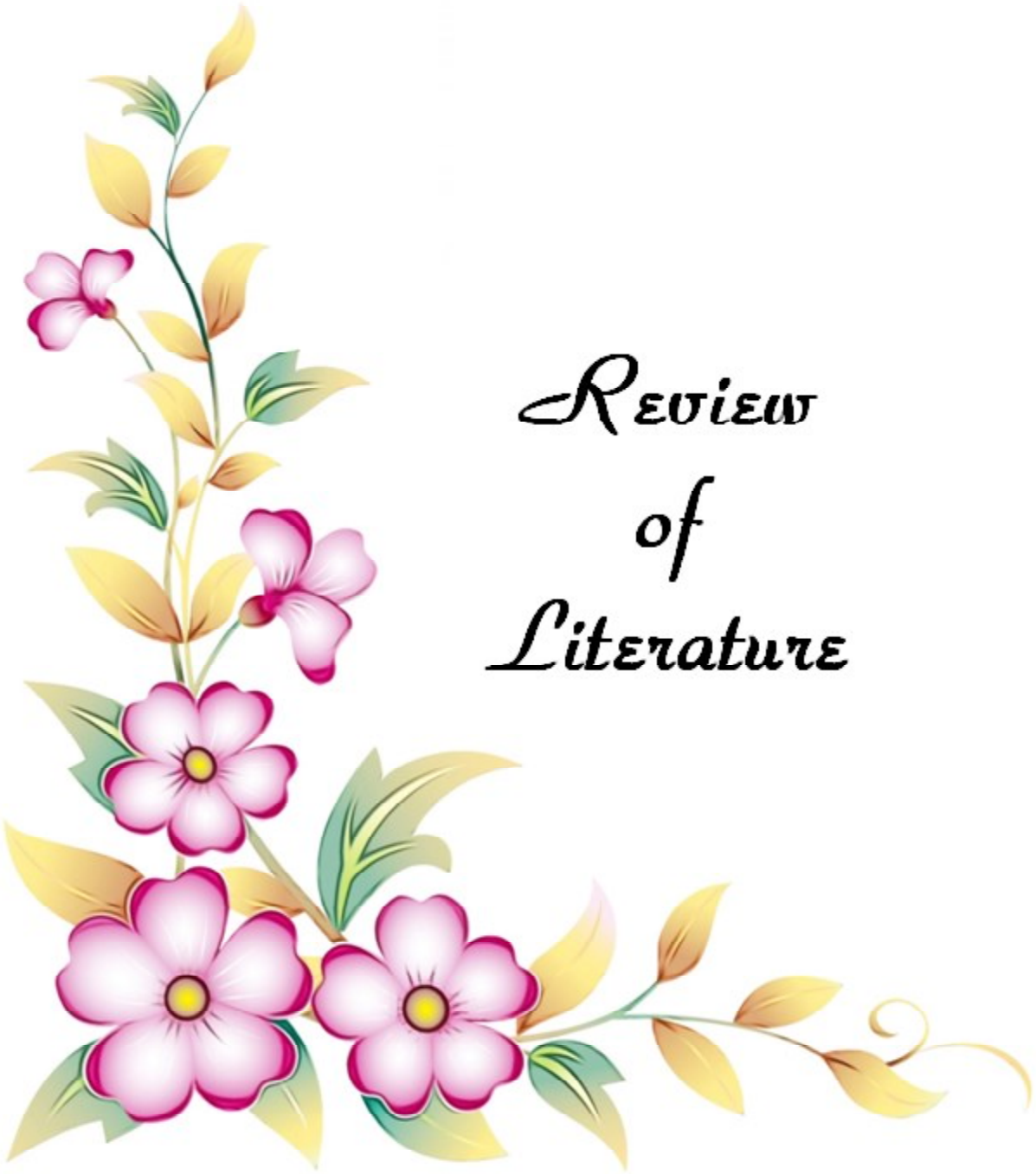
causing tubular epithelial regeneration to be delayed (Goligorsky *et al*., 1999; Devarajan, 2006). This maladaptive repair process is the characteristic feature of AKI-to-CKD transition. Dedifferentiation and activation of damaged tubular epithelial cells leads to renal fibrosis. In injured renal tubular cells, the Nrf2 antioxidative pathway is inadequately and transiently activated. Inadequate Nrf2 activation may underpin the AKI-to-CKD transition, and activating Nrf2 has the potential to stop the transition (Strausser *et al*., 2018). Despite substantial effort put forth, there has been minimal progress in lowering the severity of CKD. To find novel strategies for the treatment of this disease, extensive research is necessitated.

Natural products have been utilised for thousands of years to treat human diseases because they offer a wide range of biological features that can be harnessed for medical purposes (Newman *et al*., 2003). Naturally occurring compounds are becoming increasingly important in drug discovery and development. Triterpenoids are a broad class of secondary metabolites found in plants, which have been demonstrated to exhibit a variety of biological activities. Triterpenes have the chemical formula $C_{30}H_{48}$ and are terpenes made up of six isoprene units. Lupine, oleanane and ursane are the three classes of pentacyclic triterpenes (Laszczyk, 2009). Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid) (BA) is a naturally occurring pentacyclic lupine type triterpenoid but can also be synthesized chemically from betulin, a compound abundant in the outer bark of white birch trees (*Betula alba*) (Alakurtti *et al*., 2006). It is present in the bark of various plant species, the most common of which is the white birch (*Betula pubescens*) (Tan *et al*., 2003). BA is a minor bio-compound yet having a high efficacy in treating different diseases. The biological effects of BA and its derivatives are diverse, including anti-inflammatory (Lingaraju *et al*., 2015a), anti-oxidant properties (Lingaraju *et al*., 2015b). Numerous studies have revealed the beneficial effects of BA in experimentally-induced kidney injury models (Eksioglu-Demiralp *et al*., 2010; Wang *et al*., 2016; Sutariya *et al*., 2017; Xie *et al*., 2017; Fan *et al*., 2018; Adeleke and Adaramoye, 2021; Huang *et al*., 2021). Further, in our previous studies BA was found to reduce both AKI and CKD-associated renal fibrosis by reducing the oxidative stress, inflammation and profibrotic markers in the rodent models (Lingaraju *et al*., 2015b; Sharma *et al*., 2017). However, none of these studies report the effects of BA on AKI-CKD transition and is yet to be investigated.

BA is a potent bioactive compound with the ability to improve compromised biological functioning. In CKD patients, the AKI-CKD transition is a significant event that causes renal fibrosis and eventually resulting in ESRD. Hence, investigating the effect of BA on the AKI-CKD transition is a subject with enormous potential that is currently unexplored.

Objective

- **To evaluate the effects of BA on AKI-CKD transition in the mouse model.**



Review of Literature

2 REVIEW OF LITERATURE

2.1 Acute kidney injury

AKI is a severe and common disease directly related to patient short and long term morbidity and mortality. It is a precipitous drop in kidney function caused by both injury (structural damage) and impairment (loss of function) and is associated with expedited CKD (Siew and Davenport, 2015). To determine a relative change in serum creatinine level and stage the severity of AKI, the RIFLE, AKIN and KDIGO classification schemes use a reference (baseline) serum creatinine estimation (Bagshaw *et al.*, 2005). Latest consensus diagnostic criteria include an elevation in serum creatinine of more than 0.3 mg/dL (≥26.5 µmol/L) in the preceding 48 h; an increase in serum creatinine to more than 1.5 times baseline which is known

Grade	Glomerular filtration rate (GFR) criteria	Urine output (UO) criteria
R, Risk	Serum creatinine increase: 1.5-fold; GFR decrease > 25%	UO < 0.5 ml/kg/h for 6 h
I, Injury	Serum creatinine increase: 2-fold; GFR decrease > 50%	UO < 0.5 ml/kg/h for 12 h
F, Failure	Serum creatinine increase: 3-fold; GFR decrease > 75%; serum creatinine > 350 µmol/liter (4 mg/dl) with acute increase 44 µmol/liter (0.5 mg/dl)	UO < 0.3 ml/kg/h for 24 h Or anuria for 12 h
L, Loss	Persistent ARF = complete loss of renal function for> 4 weeks	
E, Endstage	End stage renal disease (ESRD) = complete loss of renal function for> 3 months	

Fig. 1: RIFLE Classification

or supposed to have occurred well within the past 7 days; or urine volume of less than 0.5ml/kg/h for 6 h (Khwaja, 2012). The proposed criteria for AKD encompass the definition for AKI, but may also be defined by a GFR <60 ml/min/1.73 m^2 for <3 months, a decrease in GFR by ≥35% for <3 months, or an increase in SCr by >50% for <3 months.

Ischemia, sepsis, drug toxicity, and trauma are all implicated in the pathogenesis of AKI. Prerenal, renal, and postrenal etiologies are the three main causes of AKI in clinical terms (Thadhani *et al*., 1996). Reduced renal perfusion and GFR are linked to prerenal acute kidney damage, which is produced by intravascular volume depletion due to hypovolemia, peripheral vasodilation, reduced arterial pressures, and altered cardiac function, resulting in lower cardiac output (Badr *et al*., 1988). Extrarenal blockage of urine flow causes postrenal AKI. Neurogenic bladder, retroperitoneal fibrosis, and the tumour load of bladder, prostate, or cervical cancer are all possible causes. In elderly men, the most prevalent reason is prostatic hypertrophy (Khalil *et al*., 2016). The interstitial or vascular sections of the kidney are included in intrinsic renal causes of AKI, that are classified by the location of the injury, which is most typically the glomerulus or tubule. Damage to the tubular cells of the kidney from ischemic or nephrotoxic etiologies cause acute tubular necrosis, the most frequent intrinsic kidney injury. Long durations of severe hypotension, hypovolemia, or hypoperfusion of the kidneys (e.g., from haemorrhage, shock, sepsis, cirrhosis, peritonitis, or infarcts) that do not improve with rehydration are examples of ischemic causes (Khalil *et al*., 2016). Endogenous and exogenous toxins are both nephrotoxic causes.

A systematic review of 312 cohort studies including 49 million patients revealed that one in every five adults and one in every three children in the world had AKI during a hospital episode of care (Susantitaphong *et al*., 2013). The combined AKI-related death rates in adults were 23.9% (95% CI 22.1–25.7) and 13.8% in children (95% CI 8.8–21.0). A definite inverse link exists between AKI-related mortality and the percentage of GDP spent on total healthcare expenditure. A multinational cross-sectional study including 289 centres from 72 countries revealed that 7-day mortality of patients with AKI ranged from 10% in high-income countries to 12% in low-income countries (Mehta *et al*., 2016). AKI is particularly common in the Intensive Care Unit (ICU) where it affects >50% of patients during their first week of

admission (Hoste *et al*., 2015). In the acute setting, the mortality rate of AKI is 24% in adults and 14% in children (Ostermann *et al*., 2018). Consequences of AKI include the requirement for renal replacement therapy (RRT) and the progression to CKD in about 20% of patients and a decreased quality of life (Josef *et al*., 2018). From 1996 and 2003, the incidences of non-RRT-requiring and RRT-requiring AKI in this population increased significantly from 322.7 to 522.4 per 100,000 person-years (38%) and from 19.5 to 29.5 per 100,000 person-years (33%), respectively. The importance of AKI is demonstrated by the recent aggressive International Society of Nephrology initiative to avert all deaths due to AKI throughout the world that could have been avoided by 2025 (Mehta *et al*., 2015). In one 2014 study in the United Kingdom, the annual AKI hospitalization cost was $1.3 billion, which was slightly over 1% of their National Health Service budget (Kerr *et al*., 2014). Furthermore, the lifetime estimated costs of AKI survivors in the United Kingdom in 2010 to 2011 was US$ 233 million. In the United States, one source stated that AKI was associated with increasing hospitalization costs ranging from US$ 5.4 to 24.0 billion annually (Silver *et al*., 2017). The lifetime economic burden in the United States was also reported to be enormous, with the estimated total cost as $ 88 195 per patient.

2.2 Chronic kidney disease

CKD is described as the appearance of kidney damage (albuminuria) or reduced renal function (ie, GFR < 60 mL/min per 1·73 m^2) for three months or more, regardless of clinical diagnosis (NKF/KDOQI., 2002; Vassalotti *et al*., 2007; Stevens *et al*., 2009). Since GFR plays such an important part in the pathophysiology of complications, the disease is divided into five stages based on GFR: more than 90 mL/min per 1·73 m^2 (stage 1), 60–89 mL/min per 1·73 m^2 (stage 2), 30–59 mL/min per 1·73 m^2 (stage 3), 15–29 mL/min per 1·73 m^2 (stage 4), and less than 15 mL/min per 1·73 m^2 (stage 5). Proteinuria appears to play a crucial role in the pathophysiology of disease development, according to evidence from experimental and clinical studies (Remuzzi *et al*., 2006). In addition to, and independent of, low GFR and risk elements for cardiovascular events, epidemiological studies have indicated graded relationships between increasing albuminuria and mortality and kidney outcomes in various study populations (De Jong *et al*., 2006; Hemmelgarn *et al*., 2010).

Although other factors like HIV (Ekrikpo *et al*., 2018) and exposure to heavy metals and pollutants have an auxillary impact on CKD in underdeveloped nations, hypertension and diabetes are the most prominent and leading causes of CKD (Couser *et al*., 2011). The etiology of greater incidence of CKD cases in different regions of the world remains to be undetermined (Jha *et al*., 2013). CKD exacerbates the clinical condition of people with diabetes and hypertension and elevates their risk of death from cardiovascular disease (Chronic Kidney Disease Prognosis Consortium, 2010; Couser *et al*., 2011; Matsushita *et al*., 2015). According to the USRDS (2012), type II diabetes and hypertension account for more than 65% of instances of CKD and ESRD, and 40% of diabetic individuals are said to have nephropathy that progresses to CKD. Between 1990 and 2012, CKD-related mortality among diabetic patients increased to 94% of all deaths (Jha *et al*., 2013). When it comes to the adverse consequences of CKD patients, hypertension is a crucial factor because it has been linked to the course of the disease on a cause-and-effect basis. Predictably, individuals with hypertension have been found to have reduced GFR along with proteinuria (Townsend and

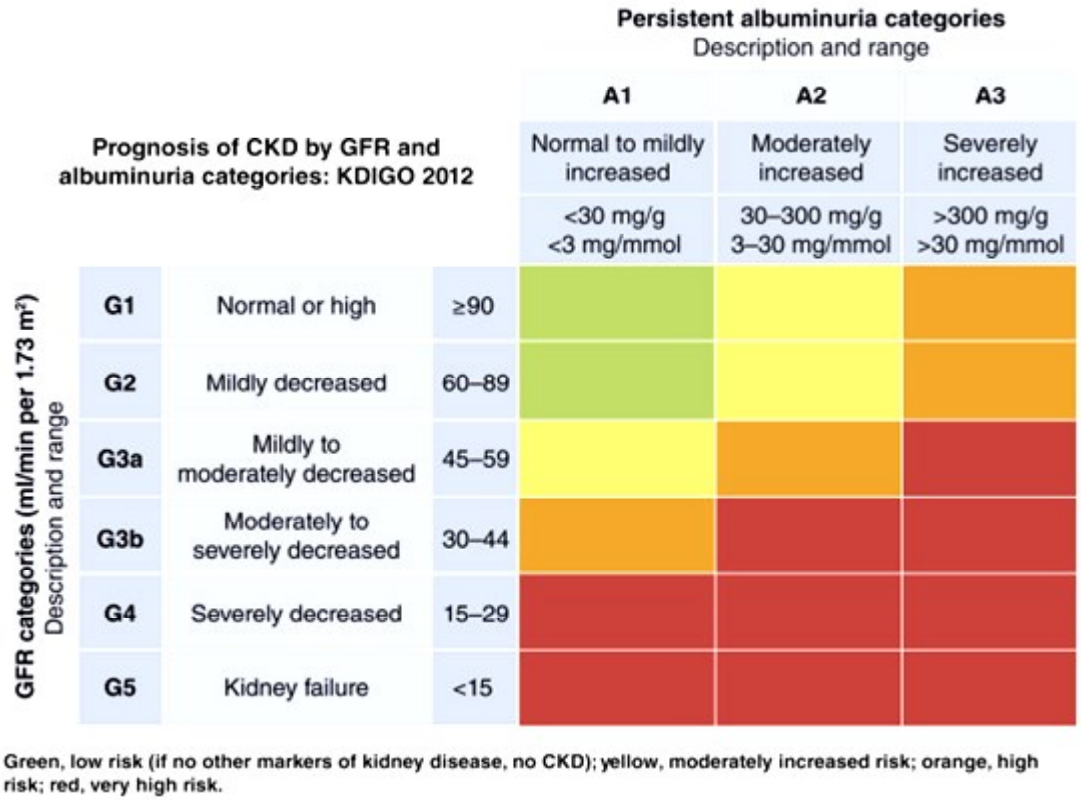


Fig. 2: Risk of CKD given by KDIGO based on GFR and albuminuria

Taler, 2015). In many parts of the world, the impact of CKD and related risk factors is still poorly understood. Moreover, in nations where data are accessible, neither the general public nor the health care workers are aware of the disease (Tuot *et al*., 2016), because of this, many nations do not have highly established nephrology workforces and focus more on addressing ESRD than the initial stages of CKD.

CKD is a significant cause for noncommunicable disease morbidity and mortality. Care expenses for CKD increased from the 1960s, when renal replacement procedures became available, allowing patients with end-stage kidney disease (ESKD) to receive life-saving but expensive treatment on a long-term basis (Himmelfarb *et al*., 2010). The number of persons on RRT has surpassed 2.5 million, with the figure expected to double to 5.4 million by 2030 (Liyanage *et al*., 2015). However, renal replacement services are limited in many countries and due to the lack of access to this treatment, an estimated 2.3–7.1 million adults have expired prematurely (Liyanage *et al*., 2015). In 2017, there were 697·5 million (95% UI 649·2 to 752·1) cases of CKD worldwide. Nearly a third of the CKD patients resided in China (132·3 million [95% UI 121·8 to 143·7] cases) and India (115·1 million [106·8 to 124·1] cases) respectively. The global occurence of CKD was approximated to be 9·1% (95% UI 8·5 to 9·8) in 2017, with CKD stages 1–2 accounting for 5·0% (4·5 to 5·5) of the population, stage 3 for 3·9% (3·5 to 4·3) of the population, stage 4 for 0·16% (0·13 to 0·19), stage 5 for 0·07% (0·06 to 0·08), dialysis for 0·041% (0·037 to 0·044), and kidney transplantation for 0·011% (0·010 to 0·012). Females (9·5% [8·8 to 10·2]) had a 1·29 (95% UI 1·28 to 1·30) times higher age-standardised incidence of CKD than in males (7·3% [6·8 to 7·9]). Out of 133 conditions, CKD is the 12th major cause of death according to GBD (Carney, 2020). In 2017, 1.2 million people died as a result of CKD (95% UI 1·2 to 1·3) (GBD, 2017). According to studies, 10% of dogs and 30% of cats over the age of 15 are identified with CKD, with males being more afflicted than females (Polzin, 2011; Oburai *et al*., 2015).

Fig. 3: World-wide prevalence of kidney diseases (Jager *et al*., 2019; Mills *et al*., 2015)

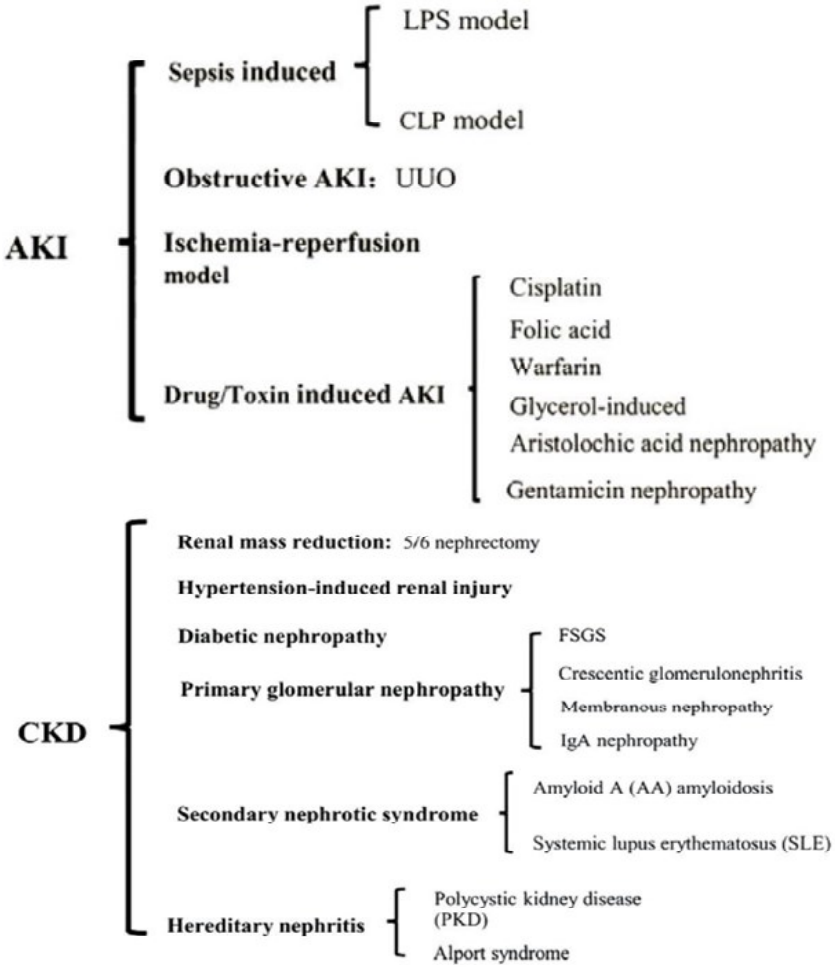


Fig 4: Summary of major acute kidney injury (AKI) and chronic kidney disease (CKD) models

2.3 AKI-CKD Transition

The progression from AKI-CKD has recently been one of the popular topics in renal disease research (Chawla *et al*., 2012). AKI has long been considered to be a self-limiting, minor illness that resolves quickly and spontaneously. In fact, after AKI, most of the patients regain their baseline kidney function, and only a few need hemodialysis after being discharged from the hospital (Levey *et al*., 2017; Vanmassenhove *et al*., 2017). However, a rising body of findings suggests that the number of individuals with incomplete renal recovery may have been undercounted in the past (Lameire *et al*., 2103; Lewington *et al*., 2013; Susantitaphong *et al*., 2013; Mehta *et al*., 2015).

AKI is a risk factor for CKD that is unrelated to the disease. The intensity, prevalence, and duration of AKI are linked to the ensuing development of CKD (Singer *et al*., 2011; Thakar *et al*., 2011). AKI and CKD are now regarded as a single clinical syndrome (Chawla *et al*., 2014). Transition from AKI to CKD or ESRD suggests the prolonged renal disease as a result of either continuing or recurrent cellular injury and/or abnormal repair processes.

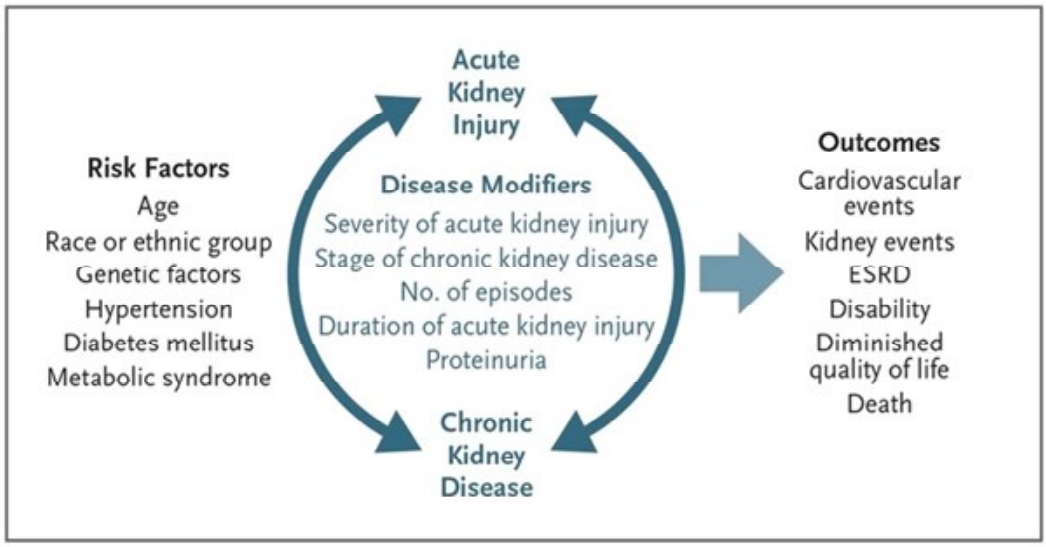


Fig 5: Nexus of AKI and CKD/ESRD with risk factors and disease modifiers

Dedifferentiation, stress adaptation, metabolic alteration, inflammatory cell infiltration, extracellular matrix synthesis, and remnant nephron hypertrophy are all repair processes that occur after a kidney injury. These mechanisms are mutually supporting and require numerous cell types in the damaged kidneys to work together. Injured kidneys restore their structure and

develop new equilibrium if the procedure is effective. The reparative process becomes misdirected or maladaptive when there is severe and repeated assaults, that results in persistent inflammation, matrix deposition, and renal atrophy (Schnaper *et al*., 2017).

After AKI, the cell cycle G2/M was halted in renal tubular epithelial cells, which can activate the pro-fibrotic signalling pathway and lead to the generation of profibrotic cytokines (Liu *et al*., 2018). The study by Lee and co-workers showed that macrophages infiltrating at early time points after ischemia-reperfusion AKI exhibited primarily a pro-inflammtory M1 phenotype, but switched to a reparative M2 phenotype at later time points. Macrophage depletion at early time points led to less severe kidney injury, but macrophage deletion at later time points led to impaired recovery, underscoring the importance of polarization of macrophages in recovery from ischemia-reperfusion AKI.

Tubular atrophy, excessive and chronic inflammation, extracellular matrix accumulation, vascular rarefaction, and reduced nephron mass are pathologic characteristics of maladaptive repair, which result in progressive deterioration of renal function (Chawla *et al*., 2011; Takaori *et al*., 2016). After an insult, tubular epithelial cells are thought to be a key trigger of CKD development (Venkatachalam *et al*., 2015). For tissue repair, injured epithelial cells dedifferentiate and initiate several gene programmes. Inflammation and fibrosis continue as a result of an unresolved and excessive/misdirected reparative effort.

Hypoxic and oxidative stress have long been thought to be major exacerbating factors in renal disorders such as AKI and CKD (Shelton *et al*., 2013; Tanaka *et al*., 2014). Cells under these stressors activate adaptive gene programs driven by master transcription factors including hypoxia-inducible transcription factor (HIF) for hypoxia and Nrf2 for oxidative stress (Strausser *et al*., 2018). HIF is a master regulator switch for the adaptive response to cellular and tissue hypoxia. Both ischemic and non ischemic AKI leads to hypoxia of kidney which in turn activates HIF. Modification of HIF is protective in multiple animal models of AKI but has a controversial role in the pathogenesis of CKD, needing further investigative efforts (Andriga *et al*., 2014). Nrf2 activation, which governs cellular antioxidative adaptation, is insufficient and transient in AKI and CKD mouse models whereas the oxidative stress is a major pathogenic

driver of disease progression (Shelton *et al*., 2013; Ruiz *et al*., 2013). Nrf2 is a fundamental regulatory transcription factor of the cellular antioxidative response and maintains cellular redox equilibrium. This is performed via transcriptional activation of its target genes, which comprise components of redox regulatory mechanisms like glutathione synthesis and recycling, as well as NAD(P)H synthesis, an essential reducing equivalent for cells (Itoh *et al*., 2010; Telorack *et al*., 2016). In preclinical and clinical investigations, Nrf2 has been intensively explored as a promising disease-modifying target against a variety of disorders, including renal ailments (Shelton *et al*., 2013; Ruiz *et al*., 2013). Hence, harnessing cellular stress responses, such as the Nrf2 antioxidative stress pathway, will be a potential therapeutic method to prevent or even reverse the detrimental AKI-to-CKD transition process (Strausser *et al*., 2018).

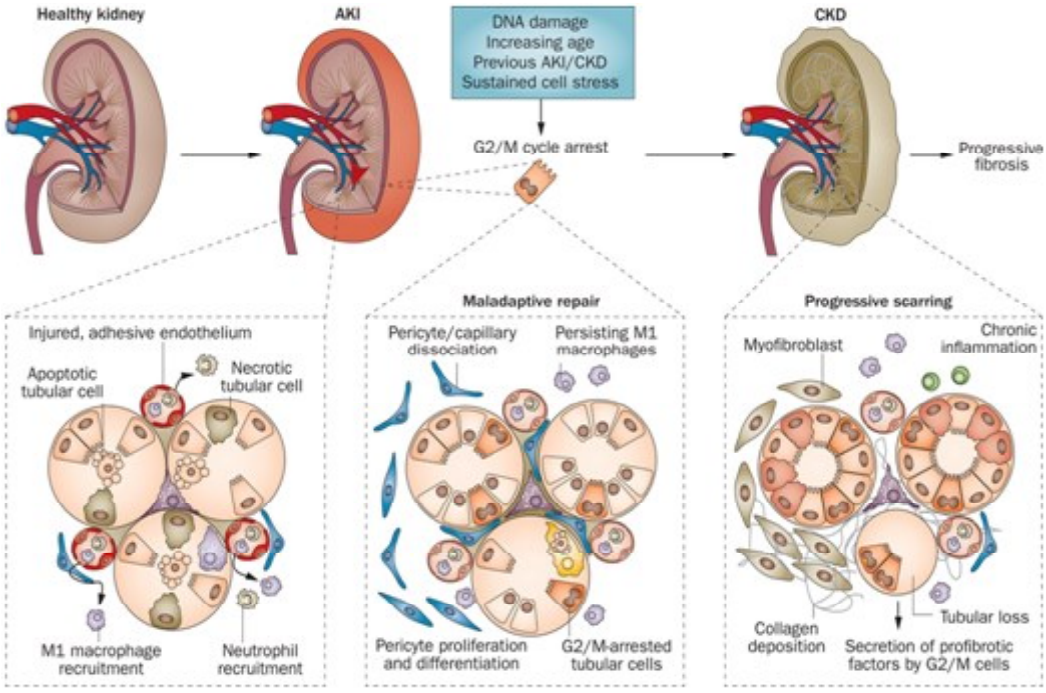


Fig 6: A description of some of the mechanisms involved in initial tissue damage and subsequent kidney repair in acute kidney injury. Fibrosis and, eventually, chronic kidney disease occur as a result of maladaptive and inadequate recovery

2.4 Animal models of AKI-CKD transition

AKI is an influencing element in the development and progression of CKD. Despite quick advancements, the mechanism underlying the AKI-CKD switch is still unknown. There

are currently no efficient therapies for either AKI or CKD, highlighting the ongoing need to interpret the underlying pathogenic mechanisms of AKI and CKD (Yan, 2021). In this regard, animal models have been utilised widely to elucidate the pathophysiology and underlying processes of renal disease. Mice and rats are the most often used models for studying nephropathy events and prospective treatment targets, as well as identifying specific disease biomarkers. Mice and rats are easy to breed and keep, as well as being quite affordable to shelter and care for (Wei and Dong, 2012). Surgery or introduction of medications or toxins can be used to induce classic acute kidney disease in a wide range of murine models (Singh *et al*., 2012; Ortiz *et al*., 2015). IR (pre-renal acute kidney failure), intra-renal injections of medicines, toxins, or endogenous toxins, and ureteral obstruction (post-renal acute kidney failure) are all current models of AKI (Singh *et al*., 2012; Sanz *et al*., 2013).

Renal ischemia-reperfusion injury (IRI) is among the most likely reasons of AKI and it can occur in a variety of clinical settings such as major surgery vascular blockage, postoperative hypoperfusion, haemorrhage, dehydration, shock, and sepsis. Renal IRI occurs when oxygen and nutrient transport to kidney cells is temporarily disrupted and then restored, triggering a series of harmful cellular reactions that result in tubular cell damage and death, inflammation, and vascular dysfunction (Linkermann *et al*., 2014; Agarwal *et al*., 2016). In addition to these acute changes, in rodents, post-IRI kidneys may acquire chronic renal diseases over the course of a few weeks to many months, offering clinically useful research paradigms of AKI-CKD transition. Bilateral IRI (bIRI), unilateral IRI (uIRI), and unilateral IRI with contralateral nephrectomy (uIRIx) are now the most extensively utilised rodent IRI models for AKI-CKD transition research.

2.4.1 bIRI model of AKI-CKD transition

Blockage of blood flow to both kidneys causes bIRI, with the influence on renal hemodynamics being more significant to human pathology. Basile and co-workers created a rat model of bIRI in 2001 to investigate the long-term effects of AKI. Male rats were given 60 minutes of bilateral renal ischemia followed by 4, 8, or 40 weeks of reperfusion in their study (Basile *et al*., 2001). Renal function studies revealed that serum creatinine elevated early in the mild IRI model, then reverted to normal levels by day 7 following the initial injury, and these

mice showed no fibrotic alterations by day 42, implying that renal integrity had been completely restored. (Yang *et al*., 2010). The mice in the severe IRI model, on the other hand, had persistently high serum creatinine levels that did not recover to baseline at day 42 following injury, and these mice exhibited interstitial fibrosis, as seen by histological investigation and immunostaining of fibrosis markers. Overall, these findings suggest that severe bilateral IRI causes chronic kidney alterations, including tubulointerstitial fibrosis, which is a characteristic of the AKI-CKD transition. The bIRI model's major flaw is its instability or fluctuation. As previously stated, if AKI is too severe, death of mice may occur during the acute damage phase, whereas if too low, the kidneys may fully recover and do not proceed to chronic conditions or CKD (Skrypnyk *et al*., 2013; Wei and Dong, 2012).

2.4.2 uIRI model of AKI-CKD transition

An obstruction of blood flow to one kidney causes uIRI. The contralateral kidney is unaffected and functional in this paradigm, enabling for long-term animal survival in studies of AKI to CKD transition. In 2011, Zager and co-workers exposed CD-1 mice to 30 minutes of uIRI at 37°C and measured renal abnormalities at 1 day, 1 week, and 3 weeks. The uIRI model has a significant benefit in terms of long-term observation. In general, bIRI in mice is observed for a few days to two weeks, whereas uIRI permits for substantially longer time investigations without considerable animal loss (Zager *et al*., 2011; Le Clef *et al*., 2016). Furthermore, because the animals in uIRI have a functional contralateral kidney, they can endure severe renal ischemia caused by a comparatively extended clipping period, culminating in more constant AKI and more repeatable chronic pathologies, such as interstitial fibrosis. Furthermore, uIRI generates greater renal fibrosis than bIRI or uIRI with contralateral nephrectomy for the same period of ischemia (Le Clef *et al*., 2016). Because of these characteristics, uIRI is a considerably more dependable model to study the post-ischemic AKI-CKD transition. However, tracking alterations in renal function over time in the same animal is complicated and necessitates more advanced approaches (Roberts *et al*., 2007).

2.4.3 uIRIx model of AKI-CKD transition

On the ground of uIRI, the contralateral kidney is removed in the uIRIx model, facilitating functional assessment of the IRI-injured kidney. Finn and co-workers used 60 minutes of total

unilateral renal artery blockage to generate IRI in rats. They discovered that removing the contralateral kidney before ischemia improved blood flow to the postischemic kidney, preserving renal tubular structure and function which is favourable for recovery. However, they did not apply this model to AKI-CKD transition studies (Finn, 1980; Finn *et al*., 1984). With respect to this, Yang and co-workers performed a right nephrectomy three days after the left kidney underwent uIRI. They discovered that the group with nephrectomy had considerably less fibrosis than the uIRI-only animals 42 days following uIRI. These results suggested that the uIRIx model could not only advance into chronic renal pathologies, but also endure higher levels of ischemia therapy, indicating that the long-term model was more sustainable. However, if the initial damage is severe, this paradigm is linked to considerable animal death. After 30 minutes of left uIRI with right nephrectomy in our hands, 30% of mice died within 2 weeks. It was also discovered that there are more variances in this model than in uIRI alone.

2.4.4 Repeated IRI model of AKI-CKD transition

Similar results should be expected following repeated IRI insults based on the formation of fibrotic damage by several incidents of tubular damages (Grgic *et al*., 2012; Kim *et al*., 2015). A study of repeated bIRI in female Sprague-Dawley rats was published in 1984 by Zager and co-workers when compared to a single damage, repeated bIRI did not result in substantial decrease in GFR and had no considerable renal histopathological degradation. Rather than raising the risk of further ischemic injury, they discovered that repeated IRI had a beneficial effect (Zager *et al*., 1984). Preconditioning refers to the phenomena of protection provided by a previous occurrence of IRI, which has been confirmed by numerous other investigations. Given the preventive effect of preconditioning, it appears unlikely that recurrent IRI will cause renal tissue degeneration and thereby development of CKD. Furthermore, repeated sessions of IRI (more than two times) have not been thoroughly evaluated in rodent models. Also, the duration of ischemia as well as the period between damage events must be carefully considered.

2.4.5 Nephrotoxic models of AKI-CKD transition

The nephrotoxic effect of medications is a frequent way to research acute and chronic kidney injury because it is a major reason of kidney damage.

2.4.5.1 Repeated low-dose cisplatin model of AKI-CKD transition

Cisplatin is a chemotherapeutic drug that is widely used. On the other hand, it is known for its side effects in healthy tissues, particularly nephrotoxicity in the kidneys, which restrict its application and usefulness in cancer treatment. Cisplatin is frequently administered to cancer patients in clinical trials for numerous rounds or cycles. While acute nephrotoxicity has been extensively noted within 1–2 weeks of cisplatin chemotherapy, repeated cisplatin therapy can have long-term repercussions. In FVB/n mice, Sharp *et al*. investigated the effects of a single high dosage of cisplatin (25 mg/kg) vs repeated low doses of cisplatin (7 or 9 mg/kg once a week for 4 weeks). They found that inflammatory chemokines and cytokines were strongly increased in the repeated low-dose paradigm, although cell death was decreased. Furthermore, after 4 weeks, the repeated low-dose model had higher values of fibrotic markers (fibronectin, TGF-β, and α-smooth muscle actin) as well as interstitial fibrosis (Sharp *et al*., 2016). As a result, repeated low-dose cisplatin treatment can cause some of the essential characteristics of CKD in both the tubulointerstitium and the glomerulus, and if surveillance period is adequately long, it can lead to true CKD. The documented research are only for reference purposes due to the recurrent injection of low-dose cisplatin, the long period of investigation, and changes in laboratory settings. Cisplatin dosage and treatment in each study plan must be precisely adjusted. Cisplatin ought to be freshly made and diluted for injection to ensure its stability and efficacy. Various animal strains and sub-strains may require different environments that should also be adjusted. C57/BL6 mice, for example, are more resistant to fibrosis than FVB/n mice (Katagiri *et al*., 2016).

2.4.5.2 Aristolochic acid (AA) model of AKI-CKD transition

In clinic, aristolochic acid nephropathy (AAN) is a gradual renal tubulointerstitial nephritis with escalating proximal tubule atrophy and interstitial fibrosis (Depierreux *et al*., 1994; Debelle *et al*., 2008). AA may be utilised to construct a model for the transition of AKI to CKD (Yang *et al*., 2018) because of its feature of an early phase of AKI culminating in CKD (Jadot *et al*., 2017). Jadot and co-workers fed 8-week-old C57Bl/6J male mice 3.5 mg/kg AA daily for 4 days to analyse them at 5, 10, and 20 days later. By morphometric analysis in sirius red staining and quantitative RT-PCR analyses, these mice demonstrated profound renal damage

and characteristic histopathological signs of AAN, including interstitial cell infiltration and tubulointerstitial fibrosis. The findings show that repeated AA treatment causes AKI, which can develop to CKD (Jadot *et al*., 2017).

2.4.5.3 Folic acid model

Despite the nutritional benefits of a small amount of FA, a large dose can damage the kidneys and lead to renal fibrosis (Fan *et al*., 2017). The use of high-dose folinic acid in conjunction with other anticancer medicines for the treatment of metastatic gastrointestinal cancer has increased the rate of FA-induced kidney injury clinically (Metz-Kurschel *et al*., 1990). The direct toxicity to the epithelium and the production of luminal crystals are the principal causes of kidney injury produced by high-dose FA injection. FA also has direct nephrotoxic impacts that are not dependent on intraluminal FA accumulation (Fink *et al*., 1987). FA injection frequently causes the formation of pointed, needle-like FA crystals in renal tubules, causing physical/obstructive tissue damage, which was once assumed to be the cause of renal toxicity. Nevertheless, Fink and co-workers found that intravenous injection of FA lowered crystallisation in rats pretreated with sodium bicarbonate to generate alkalosis (raising FA solubility), but the harm to the renal collecting ducts continued, suggesting a direct nephrotoxic impact of FA on these cells. Moreover, unlike experiments using the rat model, studies employing mice use intraperitoneal injection of FA (Baserga *et al*., 1968; Doi *et al*., 2006), and although FA crystal formation in renal tubules has never been observed, acute kidney injury and fibrosis still happened solely in the collecting ducts. Among all the animal models of renal disease produced using various techniques, the FA-induced model offers some benefits that other models do not. Regular laboratory handling of FA presents no hazards as it is a vitamin that is not harmful to the environment. FA is given as a simple injection that demands no surgery and is non-invasive and animal friendly in contrast to ischemic surgery for kidney injury. The FA model only damages the kidney and has no negative effects on other organs, unlike the toxicity models for cadmium and cisplatin that cause multiple organ injury (Rattanasinganchan *et al*., 2016). A single injection of FA can be used to explore AKI, CKD, or the AKI–CKD transition, according on the experimental demands (Aparicio-Trejo *et al*., 2020). In mice, a large dose of FA can also cause AKI (Wen *et al*., 2012).

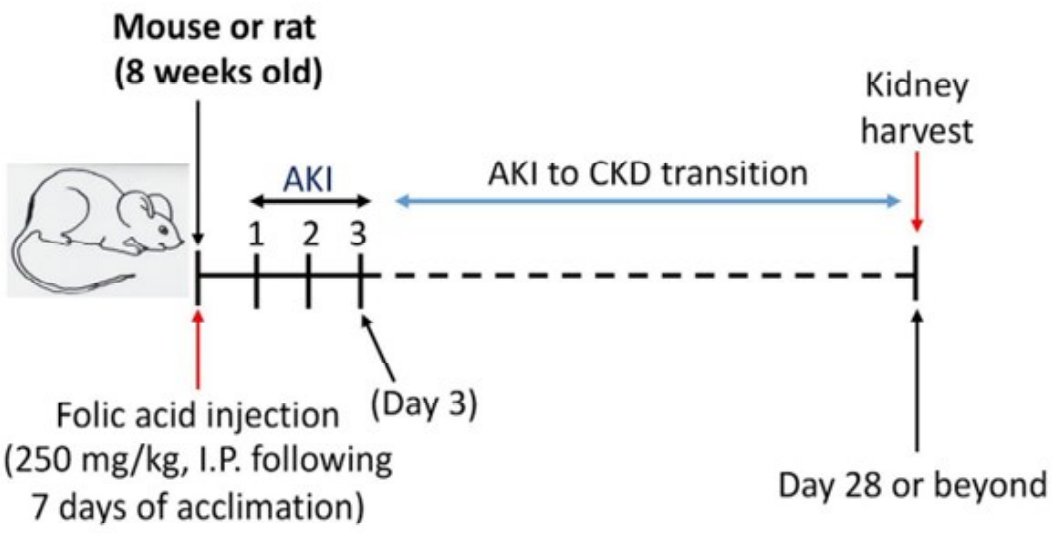


Fig. 7: General experimental scheme of folic acid (FA)-induced acute kidney injury (AKI) and chronic kidney disease (CKD) (Yan, 2021).

In mice, a 250 mg/kg intraperitoneal injection of FA (dissolved in 0.3 mmol/L $NaHCO_3$) can produce severe renal toxicity and damage (Wen *et al*., 2012; Soofi *et al*., 2013). Mice acquired AKI after folic acid injury, which progressed to CKD with varying grades of interstitial fibrosis, tubular cell atrophy, and growth arrest. This persisting renal tubule impairment was linked to chronic oxidative stress and a weakened Nrf2 antioxidant defence, as evidenced by reduced Nrf2 nuclear aggregation and reduced stimulation of its specific antioxidant enzymes (Ruiz *et al*., 2013). FA nephropathy is thought to be caused by FA crystal accumulation in the tubular lumen, which causes blockage and necrosis (Szczypka *et al*., 2005; Kumar *et al*., 2015). AKI can be induced by a single intraperitoneal injection of FA at a dose of 250 mg/kg body weight (Kumar *et al*., 2015; Nikolic *et al*., 2020), resulting in proteinuria and elevated blood urea nitrogen (BUN) and creatinine levels (Jiang *et al*., 2019). Within 72 h of FA administration, AKI can be examined. CKD will develop if left untreated, and it can be examined for up to 4 weeks following FA injection (Aparicio-Trejo *et al*., 2020).

The signs of renal disease may also be triggered by repeated injections of a smaller dose of FA (125–150 mg/kg body weight) (Newbury *et al*., 2019) or a single injection of FA (less than 200 mg/kg body weight) that can be employed to explore the pathological processes of AKI or CKD. FA-induced kidney disease can therefore encompass AKI, CKD, and the AKI–CKD transition (Perales-Quintana *et al*., 2019). Furthermore, the development process

of kidney disease is simple and straightforward, requiring no surgery, since FA is water-soluble and the injection is intraperitoneal. FA-induced kidney disease is a highly repeatable model that can replicate the clinical signs of human kidney disease. Oxidative stress, mitochondrial abnormalities, redox imbalance, and disordered fatty acid oxidation are all included in the FA-induced AKI–CKD transition paradigm (Jiang *et al*., 2020).

Tubular blockage and oxidative stress, which cause tubular epithelial cell (TEC) necrosis and cytokine release, are the key pathophysiological characteristics underpinning FA-induced AKI (Aparicio-Trejo *et al*., 2019). The contribution of oxidative stress in renal injury development has been identified as a crucial player in the pathophysiologic processes of a broad range of developing and experimental renal disorders (Haugen *et al*., 1999). Because of its transport function, the kidney has a very active oxidative metabolism, which leads to the generation of reactive oxygen species (ROS), which if left unattended can injure all main cellular structures and results in oxidative stress (Maser *et al*., 2002). A study found a link between elevated prooxidant levels and acute kidney damage, which was then treated with folic acid. Renal hypertrophy and serious deterioration of renal function were observed in FA-treated mice. After FA treatment, glutathione levels (GSH) and antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were dramatically reduced, but LPO levels were elevated (Gupta *et al*., 2012).

2.5 Betulinic acid and its biological activities

Despite a lack of understanding of their mechanisms of action, herbal medicines generated from plant extracts are increasingly being used to treat a wide range of illnesses. Terpenes, commonly referred to as terpenoids or isoprenoids, are the most abundant class of chemical molecules found in plants. They play a range of crucial physiological roles due to their diversity and widespread distribution (Breitmaier, 2006). Terpenoids are classified according to number of isoprene units they possess. Monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40), and polyterpenes are the various kinds (Withers and Keasling, 2007; Rabi and Bishayee, 2009). Triterpenoids are found practically everywhere in plants and are shown to have a broad range

of biological activities. This resulted in a constantly growing eagerness to elucidate their potential. In the plant kingdom, BA, 3b-hydroxy-lup-20(29)-en-28-oic acid (Fig. 8), is a common and widespread pentacyclic lupane-type triterpene present in the bark of various kinds of plants, most notably the white birch (*Betula pubescens*), from which the chemical gets its name (Tan *et al*., 2003).

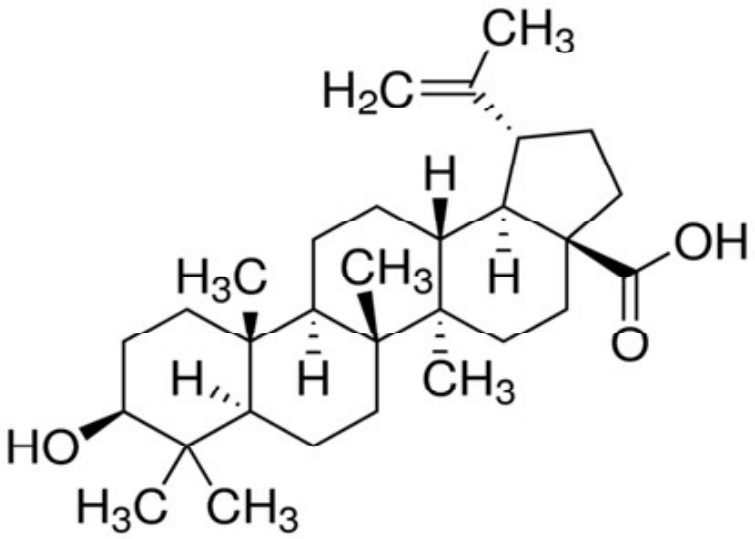


Fig. 8: Chemical structure of betulinic acid

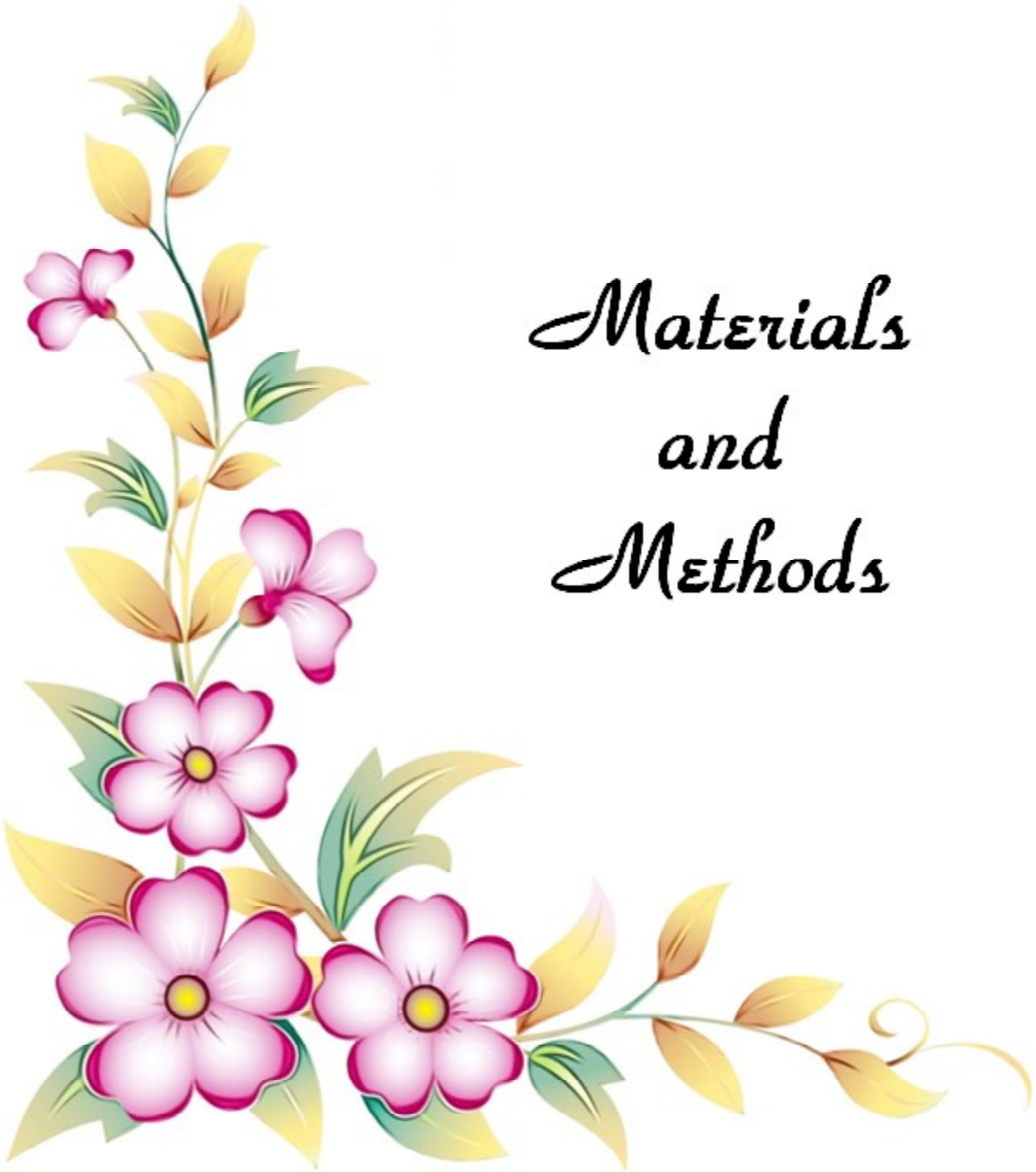
Numerous research in recent years have revealed many beneficial effects of both betulin and betulinic acid, including antiviral (anti-influenza, anti-HIV), antiinflammatory, antiallergenic, antihypoxic, liver protectant, and antituberculosis characteristics (Alakurtti *et al*., 2006). The most major part, nonetheless was its all-encompassing anti-cancer action. BA has been shown to be safe and non-toxic in mice at dosages up to 500 mg/kg body weight (Alakurtti *et al*., 2006), and its pharmacokinetics and tissue distribution in mice have also been explored (Udeani *et al*., 1999). BA and its derivatives could be useful therapeutically since they have strong action against a variety of tumors and malignancies while having little effect on normal cells, suggesting selectivity (Zuco *et al*., 2002).

Betulin and betulinic acid have been shown as potent phospholipase A2 inhibitors (Bernard *et al*., 2001). Because of their resemblance to steroidal chemicals, they have been given a mechanism comparable with that of these anti-inflammatory drugs. Many experimental investigations have shown BA to have anti-inflammatory and anti-oxidant properties, involving sepsis, with no antibacterial action (Takada and Aggarwal, 2003; Fontanay *et al*., 2008;

Eksioglu-Demiralp *et al*., 2010; Viji *et al*., 2011; Nader and Baraka, 2012; Lingaraju *et al*., 2015a,b; Kalra *et al.,* 2018).

2.5.1 Betulinic acid and kidney diseases

In the adenine-induced CKD rat model, BA treatment at 30 mg/kg body weight for 28 days diminished renal fibrosis by preventing levels of pro-fibrotic proteins like TGF, connective tissue growth factor (CTGF), fibronectin, collagen type I, and hydroxyproline, along with improving renal structure and function (Sharma *et al*., 2017). Furthermore, in STZ-induced diabetic rat kidneys, BA therapy (20 mg/kg, orally, daily) for 8 weeks attenuated diabetic renal fibrosis (Wang *et al*., 2016). After 7 days of STZ injection, BA (40 mg/kg) once daily for 30 days had a preventive impact on diabetic nephropathy, presumably via the AMPK/ NF-B/Nrf2 pathway (Xie *et al*., 2017). NDMA-induced liver and kidney damage of rats can be reduced by treatment with BA at 25 mg/kg for 14 days interval due to its antioxidative, anti-inflammatory, and lipid-lowering properties (Adeleke and Adaramoye, 2021). Cadmium-induced apoptosis in the kidney is prevented by BA treatment for 10 days (Fan *et al*., 2018). By controlling the apoptotic function of leukocytes and reducing neutrophil infiltration, BA (250 mg/kg, i.p.) at the 6^{th} h attenuates I/R-induced oxidative effects, restores microscopic damage, and renal function (Eksioglu-Demiralp *et al*., 2010). BA treated at doses of 10 and 30 mg/kg have shown to reduce the CLP-induced acute kidney injury by correcting the aforementioned inflammatory mediators, oxidant and anti-oxidant imbalances (Lingaraju *et al*., 2015a,b). BA (25 and 50 mg/kg) given orally for 4 weeks improved NF-κB, iNOS, TNF-α, Nrf2, HO-1 and NQO1 mRNA and protein expression in the kidney of a passive Heymann nephritis rat model. BA also improved malondialdehyde levels and antioxidant enzyme activity in the kidney in the same model (Sutariya *et al*., 2017). By stimulating the Nrf2 signalling pathway, BA reduced oxidative stress and inflammation in T-2 toxin-induced kidney injury (Huang *et al*., 2021). By lowering oxidative stress, BA (100 mg/kg) therapy significantly reduced in the 30 days arsenic-induced renal tissue damage (Prakash *et al*., 2018). In an 8-day research, betulinic acid demonstrated considerable nephroprotective effect in animal models of gentamicin-induced kidney injury (Noushida *et al*., 2020).



Materials and Methods

3 MATERIALS AND METHODS

3.1 Experimental Animals

In the present study Swiss albino male mice weighing around 18-25 g were used. Healthy animals were procured from Laboratory Animal Resource Section of IVRI, Izatnagar. Prior to the experiment, the animals were given a one-week acclimatisation period. These animals were kept in polypropylene cages in ambient environment of room temperature 24 ± 2°C; relative humidity 60-70%; 12-h light-dark cycle. Throughout the study, all animals were maintained on a balanced ration produced by the Feed Technology Unit of the Institute, and fresh drinking water was provided to them *ad libitum* on a daily basis. All the experiments on animals were carried in accordance with the ethical norms set forth by Institutional Animal Ethics Committee Guidelines, IVRI.

Table 1: Feed composition

Wheat bran	30%
Maize mash	40%
Soya meal	20%
Mineral mixture	8%
Common salt	2%

3.2 Drugs and Chemicals

Table 2: List of chemicals, kits and antibodies used

Sl. No.	Item	Catalogue no	Company
1	Betulinic acid	855057	Sigma Aldrich
2	Creatinine kit	1101060035	Coral Clinical Systems
3	Folic acid (FA)	03860	Loba Chemie
4	Mouse Interleukin 4 ELISA KIT	CSB-E04634m	CUSABIO
5	Mouse Interleukin 13 ELISA KIT	CSB-E04602m	CUSABIO
6	DAB Substrate Kit	34002	ThermoFisher SCIENTIFIC
7	Immobilon -E membrane	IEVH00005	Merck Millipore
8	Prestained protein ladder	MBT092-100LN	HiMedia
9	Nitrotyrosine	N0409	Sigma
10	β-actin	ADI-905-733	Enzo
11	α-smooth muscle actin	MAB1420	R&D systems
12	Mouse anti-rabbit IgG-HRP	SC-2357	Santa Cruz
13	Goat anti-mouse IgG-HRP	SC-2005	Santa Cruz

3.3 Experimental Design

Experiment was conducted in two phases. Phase I experiment was done for 3 days for investigating AKI. Phase II experiment was done for 28 days for investigating AKI-CKD transition. In the phase I study mice were initially divided into four groups containing 5 animals in each. Group C served as control, group T served as treatment control, group I served as AKI group, group IT served as AKI and treatment. Similarly, mice were divided into 4 groups with 5 animals each in phase II study where group C served as control, group I served as CKD group, groups IT3 and IT7 served as CKD and treatment groups.

In phase I study, vehicle at a dose of 1 ml/kg was administered intraperitoneally to the animals in the C group. Single dose of FA at the dose of 250 mg/kg was administered intraperitoneally to induce AKI in I group on day 1 (Perales-Quintana *et al*., 2019, Aparicio-Trejo *et al*., 2020). BA in vehicle (5% DMSO in pea nut oil) was given to the animals in T group for 3 days intraperitoneally at the dose of 30 mg/kg. Animals in IT group received FA at 250 mg/kg intraperitoneally on day 1 and BA at the dose of 30 mg/kg intraperitoneally for 3

days. All the animals were sacrificed on 3rd day of the experiment and blood and kidney samples were collected for further studies.

In phase II study, the animals in C group received vehicle at a dose of 1ml/kg intraperitoneally. AKI-CKD transition was induced in I group by intraperitoneal administration of FA at dose of 250 mg/kg on day 1 (Perales-Quintana *et al*., 2019, Aparicio-Trejo *et al*., 2020). The mice in IT3 group were administered with FA (250 mg/kg i/p) on day 1 and BA (30 mg/kg i/p) on days 1,2,3 whereas those in IT7 were given FA (250 mg/kg, i/p) on day 1 and BA (30 mg/kg i/p) on days 1,2,3,7,14,21 and 28. All the animals were sacrificed on 28th day and blood and kidney samples were collected for further analysis.

Table 3: Animal grouping and drug administration protocol

Group	Treatment
Phase I study	
C	1 ml/kg vehicle (5 % DMSO in pea nut oil; 0.3 M $NaHCO_3$), i/p
T	BA (30 mg/kg BW) for 3 days, i/p in vehicle (5 % DMSO in pea nut oil).
I	Folic acid (250 mg/kg BW) (vehicle: 0.3 M $NaHCO_3$) single dose, i/p
IT	FA (250 mg/kg BW) (vehicle: 0.3 M $NaHCO_3$) single dose, i/p; BA (30 mg/kg BW) for 3 days, i/p (5 % DMSO in pea nut oil).
PHASE II study	
C	1 ml/kg vehicle (5 % DMSO in pea nut oil; 0.3 M $NaHCO_3$), i/p
I	Folic acid (250 mg/kg BW) (vehicle: 0.3 M $NaHCO_3$) single dose, i/p
IT3	FA (250 mg/kg BW) (0.3 M $NaHCO_3$) single dose i/p; BA (30 mg/kg BW) for first 3 days, i/p (5 % DMSO in pea nut oil).
IT7	FA (250 mg/kg BW) (0.3 M $NaHCO_3$) single dose, i/p; BA (30 mg/kg BW) on days 1,2,3,7,14,21,28 i/p (5 % DMSO in pea nut oil).

3.4 Serum and urine collection

Mice were anaesthetized with ketamine (50 mg/kg BW) and xylazine (5 mg/kg BW). The blood samples were collected from the retro-orbital plexus of anesthetized animals with

minimum stress on day 3 from phase I study and on day 28 from phase II study in vacutainers without anticoagulant. Serum was then separated by keeping those tubes in slanting position at room temperature for 1-2 h and centrifuging the tubes at 2000 rpm for 10 minutes. The collected serum was stored at -20 °C for later biochemical analysis. The animals were kept in metabolic cage prior to the day of sacrifice for 24 h for collection of urine. Collected urine samples were centrifuged at 2000 rpm for 10 minutes and were then stored for creatinine, total protein and urine nitrite estimation.

3.5 Animal sacrifice and preparation of homogenate

Following blood collection, mice were sacrificed by cervical dislocation at the end of experimental period (i.e, on Day 3 for phase I study and on Day 28 for phase II study). The kidneys were removed from all the animals, rinsed with isotonic saline, dried with wipes and weighed. The relative kidney weight (mean kidney weight (g)/ body weight (g)) of all animals in phase II study were recorded by taking mean weight of right and left kidneys and dividing them with body weight of respective mouse. Each kidney was excised into two equal halves and one half was fixed for one week by immersion in 10% neutral buffered formalin at room temperature for histopathological examination. Other part of the kidney tissue was weighed and homogenized in NP-40 lysis buffer (150 mM sodium chloride, 1 % NP-40 (Triton X-100 can be substituted for NP-40), 50 mM Tris, pH 8.0) (pH 7.4) at 4 °C to make 5 or 10 % tissue concentration. The samples were then subjected for ultrasonication at 20 kHz for four cycles of 10 seconds each. The homogenate was then centrifuged at 2000 rpm for 10 min. The resulting supernatant was collected and stored at -80 °C until assayed for further biochemical parameters and protein analysis.

3.6 Serum and urine creatinine

Serum and urine creatinine were estimated by alkaline picrate method using commercially available kit on day 3 in phase I study and on day 28 in phase II study according to manufacturer's protocols. Absorbance of the standard and test samples against blank was measured at 520 nm.

Creatinine (mg/dl) = OD of Test / OD of Standard x 2

Urine creatinine (g/l) = OD of Test / OD of Standard x 1

3.7 Total protein estimation by Bradford method

Reagents required

1. BSA (Bovine Serum Albumin) stock solution: 1 mg/ml
2. Bradford reagent solution: dilute 2 mg of CBB with an equal volume of methanol and phosphoric acid and make up the volume to 20 ml with distilled water. Filter the solution with 0.45 µm syringe filter.

Procedure

3. Different dilutions of BSA solution ranging from 10-100 µg/ml were prepared by mixing specific volume of BSA stock solution of 100 µg/ml (by adding 1mg BSA in 10 ml of 0.9 % NaCl).
4. From these different dilutions, pipetted out 5 µl of different dilutions of BSA standards and the lysate and phase II urine samples to different test tubes and added 245 µl Bradford reagent.
5. The reaction mixture was incubated for 3 min at room temperature.
6. The colorimeter absorbance was made zero with blank and optical density of the reaction mixture was measured in a UV visible spectrophotometer at 595 nm.
7. The absorbance was plotted against protein concentration to obtain standard calibration curve using Graph pad Prism software.

The unknown protein concentrations of tissue and urine samples were extrapolated from the standard curve.

3.8 Estimation of nitrite by Griess reaction (Green *et al.*, 2004)

Reagents required

1. Sulphanilamide: 10 mg/ml in 5 % phosphoric acid
2. N-naphthylethylenediamine (NEDD): 1 mg/ml in distilled water
3. Acetonitrile

Procedure

1. Griess reagent was prepared by mixing 25 µl of 1 % sulfanilamide (prepared in 5 % phosphoric acid) and 25 µl of 0.1 % N-naphthylethylenediamine (NEDD) (prepared in distilled water).
2. 75 µl of acetonitrile was added to equal volume of urine samples for deproteinization and kept for 10 min.
3. Centrifuged at 1000 rpm for 5 min. Supernatant was collected for measuring nitrite.
4. 10 µl of Griess reagent and 65 µl of distilled water were added to 75 µl of deproteinized samples and incubated at room temperature for 20 min.
5. After incubation the absorbance was measured against reference blank containing all the reagents except lysate sample at 540 nm.
6. The results were expressed in nmol urine nitrite/day.

Calculation

Nitrite (M) = Optical Density/Molar extinction coefficient × Dilution factor

Molar extinction coefficient, E= 36900

Dilution factor= 4

3.9 Estimation of lipid peroxidation in terms of malondialdehyde (MDA) by Shafiq-ur-Rehman (1984) method with few modifications

Reagents required

1. Thiobarbituric acid: 3.7 mg/ml in distilled water
2. Trichloroacetic acid: 150 mg/ml in distilled water
3. 0.25 N Hydrochloric acid

Procedure

1. TBA-TCA-HCl reagent -0.37 % TBA, 0.25 N HCl, 15 % TCA was prepared.
2. 50 µl of tissue homogenates were treated with 200 µl of TBA-TCA-HCl reagent and placed in boiling water bath for 15 min and then cooled.
3. Reaction mixtures were then centrifuged at 2000 rpm for 10 min.

4. The absorbance of clear supernatant was measured against reference blank containing all the reagents except lysate sample at 535 nm.
5. The results were expressed in nmol MDA/mg tissue.

Calculation

MDA (M) = OD/E × D.F

E= Molar extinction coefficient of MDA, 1.56×10^5

D.F= dilution factor

3.10 Estimation of IL- 4 and 13 levels by ELISA kit method

Cytokines IL-4 and 13 levels were measured by ELISA kit as per manufacturer's instructions to assess the inflammatory response.

Materials required

1. Biotin- antibody
2. HRP-avidin
3. Biotin-antibody diluent
4. HRP-avidin diluent
5. Sample diluent
6. TMB Substrate
7. Stop solution
8. Wash buffer

Procedure

1. Prepare reagents, samples and standards as instructed.
2. Add 100 µl standard or sample to each well. Incubate for 2 h at 37 °C.
3. Remove the liquid of each well. Don't wash.
4. Add 100 µl Biotin- antibody (1x) to each well. Incubate for 1 h at 37 °C.
5. Aspirate and wash three times with wash buffer.
6. Add 100 µl HRP-avidin (1x) to each well. Incubate for 1 h at 37 °C.
7. Aspirate and wash five times with wash buffer.

8. Add 90 µl TMB substrate to each well. Incubate for 15-30 min at 37 °C. Protect from light.
9. Add 50 µl stop solution to each well. Read at 450 nm within 5 min.

3.11 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

3.11.1 SDS-PAGE

About 100 µg of protein lysate and urine samples from phase II study were mixed with appropriate volume of 5X sample buffer. They were heated in boiling water for 5-10 min and then cooled to 4 °C. These samples were subjected to SDS-PAGE and Western blotting analysis.

3.11.1.1 Gel casting

1 % agarose solution was prepared by boiling and poured in between two glass plates in gel casting assembly separated by two spacers at both ends each of 1.5 mm thickness to seal the bottom. Then 10 % resolving gel (pH 8.8) was prepared and poured over the agar. It was kept for polymerization for 30 minutes. After polymerization of separating gel, stacking gel (4 % in tris buffer of pH 6.8) was poured above it and gel comb was placed and left for 30 min for polymerization.

3.11.1.2 Electrophoresis

Following polymerization, the gel was transferred from casting apparatus to an electrophoretic apparatus, the gel comb was gently removed and the buffer chambers were filled with running buffer (25 mM Tris base, 190 mM glycine, 0.1 % SDS). 5 µl of prestained molecular weight standard for proteins was used as marker and 100 µg of protein samples or urine samples were loaded into different wells of gel and the gel was then subjected to electrophoresis at 90 V until the tracking dye reached the base of the separating gel.

Gel loaded with urine samples was stained with Coomassie Brilliant Blue stain for 45 min and then destained the gel in aqueous 4 % methanol-8 % acetic acid (v/ v) to visualise blue stained bands against transparent background.

Composition for 10 % resolving gel (5 ml) is as follows:

Components	**10 % gel**
Distilled water	2.425 ml
30 % Acrylamide mix	1.25 ml
1.5 M Tris (pH 8.8)	1.25 ml
SDS (10 %)	50 µl
TEMED	2.5 µl
APS (10 %)	25 µl

Composition for 4 % stacking gel (1 ml) is as follows:

Components	**4 % gel**
Distilled water	636 µl
30 % Acrylamide mix	100 µl
0.5 M Tris (pH 6.8)	252 µl
SDS (10 %)	10 µl
TEMED	1 µl
APS (10 %)	5 µl

3.11.2 Western Blot Analysis

3.11.2.1 Transfer/blotting

The gel was removed from plates and washed with transfer buffer (10 % methanol, 25 mM Tris (pH 8.3), 192 mM glycine, 0.03 % SDS) after gel electrophoresis and protein were transferred onto a polyvinylidene fluoride (PVDF) (0.45 µm) membrane using a semidry transfer apparatus. The foam pad, whatman filter paper and PVDF membrane soaked in transfer buffer were placed on anode plate (+) of transfer apparatus above which gel was placed carefully and whatman filter paper and foam pad soaked in transfer buffer were arranged over the gel that formed a sequence of layers from anode (+) to cathode (-). A glass rod was used as roller to remove air bubbles in between the layers in each step and utmost care was taken to prevent drying of membrane and damage to gel. After attaching the electrodes to specific slots of transfer apparatus, transfer was done at 90 V, 230 mA for 45 min in cold chamber.

3.11.2.2 Blocking the membrane

After transfer process, the PVDF membrane was removed and immersed in blocking buffer (5 % Bovine Serum Albumin in TBST) (TBST-50 mM Tris, 150 mM NaCl, 0.1 % Tween 20) to block the non-specific sites on the membrane for 1 h at room temperature on a shaker.

3.11.2.3 Incubation with the antibodies

Following blocking, membrane was kept for overnight incubation with primary antibody (Table 4) appropriate for the protein of interest. The next day, membrane was washed thrice for 5 min each with TBST wash buffer. The membrane was then incubated with appropriate HRP-conjugated secondary antibody (Table 4) for 2 h at room temperature under darkness. Again, washed the membrane with TBST wash buffer for 3 times for developing.

Table 4: List of antibodies and dilutions used

Sl. No.	Antibody	Host and antibody type	Dilution
1	Anti-nitrotyrosine	Rabbit polyclonal	1:500
2	Anti-β-actin	Rabbit polyclonal	1:3000
3	Anti-α-smooth muscle actin	Mouse monoclonal	1:2000
4	Anti-rabbit HRP	Mouse	1:2000
5	Anti-mouse HRP	Goat	1:5000

3.11.2.4 Developing

After washing with TBST wash buffer, the membrane was incubated with 3, 3' diaminobenzidine tetrahydrochloride (DAB kit) for 5 min to develop the blot. The bands were photographed and images were subjected for band density analysis by image J software.

3.12 Gelatin zymography

Gelatin zymography was done for analysing gelatinase activity of MMP-2 enzyme.

Following non-reducing SDS-PAGE in which the gel was co-polymerized with 0.1 % w/v gelatin, proteins were separated from tissue homogenate. Methods and conditions for electrophoresis were comparable to that of SDS-PAGE except that mercaptoethanol was absent in 5X buffer composition and boiling step was avoided. After electrophoresis, gel was washed with 2.5 % Triton X-100 twice for 15 min at room temperature to remove SDS. Washed again with developing buffer for 15 min followed by incubation with developing buffer for 16-24 h. (50 mM Tris HCl, 5 mM $CaCl_2$, 200 mM NaCl,1 μm $ZnCl_2$, 1 % Triton X-100). After incubation, gel was stained with 0.05 % Coomassie Brilliant Blue-G-250 in a mixture of methanol-acetic acid-water (2.5:1:6.5 v/v) for 45 min destained in aqueous 4 % methanol-8 % acetic acid (v/ v). Gelatinolytic activity was detected as clear transparent bands against the dark blue background. The bands were photographed and images were subjected for band density analysis by image J software.

3.13 Histopathology

3.13.1 Haematoxylin-Eosin (H&E) Staining

As described in sacrifice and tissue processing section the kidney specimens were fixed in in 10 % neutral buffered formalin for one week at room temperature and then embedded in paraffin wax for sectioning. Serial paraffin sections of 5 μm thickness were stained with H&E routinely and observed under a light microscope for conventional morphological evaluation.

3.13.2 Masson's trichrome staining

1. Processed tissue sections were stained with Weigerts's Iron Haematoxylin working solution (Sigma, USA) for 20 mins.
2. Followed by rinsing in running tap water for 5 min (2-3 times).
3. The tissue sections were then stained with Biebrich scarlet-acid fuchsin solution (Sigma, USA) for 15 min.
4. Rinsed in tap water for next 5 min.
5. The sections were differentiated in 1:1 phosphomolybdic-phosphotungstic acid solution for 15 min.
6. Without rinsing transferred them directly to warm aniline blue solution for 15 min.

7. Then rinsed briefly in distilled water and excess stain was removed by one dip in 1 % acetic acid solution.
8. Rewashed the slides with distilled water.
9. Dehydrated quickly in absolute alcohol and then cleared in xylene.
10. Mounted with DPX.

3.13.3 Picro Sirius Red staining

The tissue sections were stained with Picro sirius red for one hour followed by washing twice in acidified water. After removing most of the water from slides by vigorous shaking, they were dehydrated in three changes of absolute alcohol and then cleared in xylene and at last mounted in a resinous medium. Then sections were observed under polarized microscope & photographed.

3.14 Statistical analysis

Data were analyzed by one way ANOVA followed by Bonferroni post hoc test for comparision between groups. In phase I study data of group I and T were compared with group C and data of group IT was compared with group I. In phase II study data of group I was compared with group C and data of groups IT3 and IT7 were compared with group I. p value less than 0.05 was assumed as statistically significant.

Results

4 RESULTS

4.1 Phase I

4.1.1 Effect of BA on serum creatinine in AKI mice

The results of serum creatinine levels (mg/dl) are shown in Fig. 9 and Table 5. Serum creatinine levels inversely correlate with glomerular filtration rate (GFR) and are an indicator of renal function. Serum samples of AKI group (I) had significantly higher levels of creatinine as compared to control (C) mice. BA treated AKI mice (IT) showed significantly lower levels of serum creatinine when compared to AKI (I) group. BA treatment control group (TC) didn't show any significant change in serum creatinine levels when compared to control group (C).

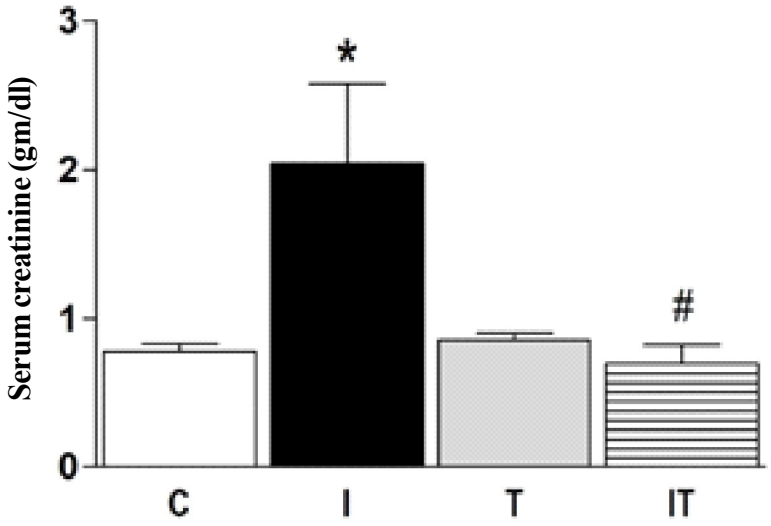


Fig. 9: Serum creatinine levels in mice from different groups (mg/dl). Column shapes represent mean and lines on the shapes represent S.E.M. * p<0.05 vs. C; # p<0.05 vs I.

4.1.2 Effect of BA on tissue MDA in AKI mice

MDA is the product of lipid peroxidation which is considered as an important factor of tissue damage. At the end of the experiment tissue MDA levels remained statistically insignificant among the groups. The results of tissue MDA levels (nanomoles/mg protein) of different groups in this study are shown in Fig. 10 and Table 5.

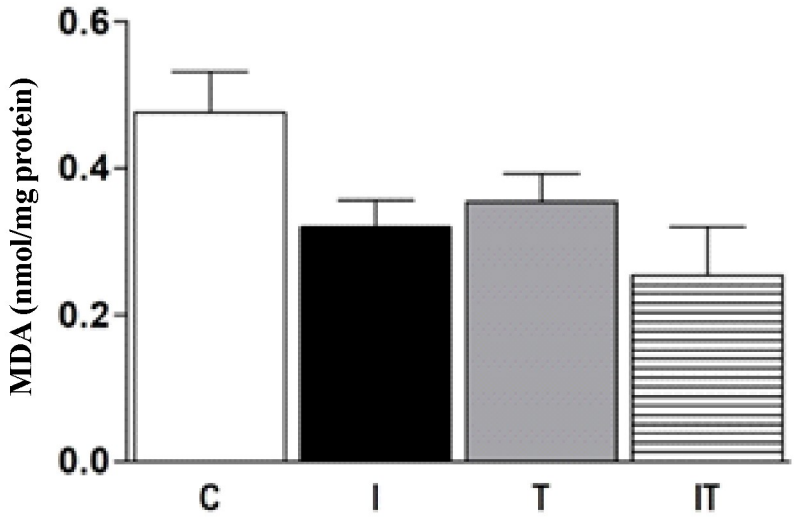


Fig. 10: Kidney MDA levels in mice from different groups (nmol/mg protein). Column shapes represent mean and lines on the shapes represent S.E.M. (MDA: malondialdehyde).

4.1.3 Effect of BA on urine nitrite in AKI mice

Nitrite recognized as nitric oxide metabolite is an indicator of nitrosative stress. The results of urine nitrite levels (nanomoles/day) are shown in Fig. 11 and Table 5. No significant change was observed in urine nitrite levels in the AKI (I) group when compared to the control group (C). Even though BA treatment significantly attenuated the levels of urine nitrite in AKI treatment group (IT), the results were not conclusive.

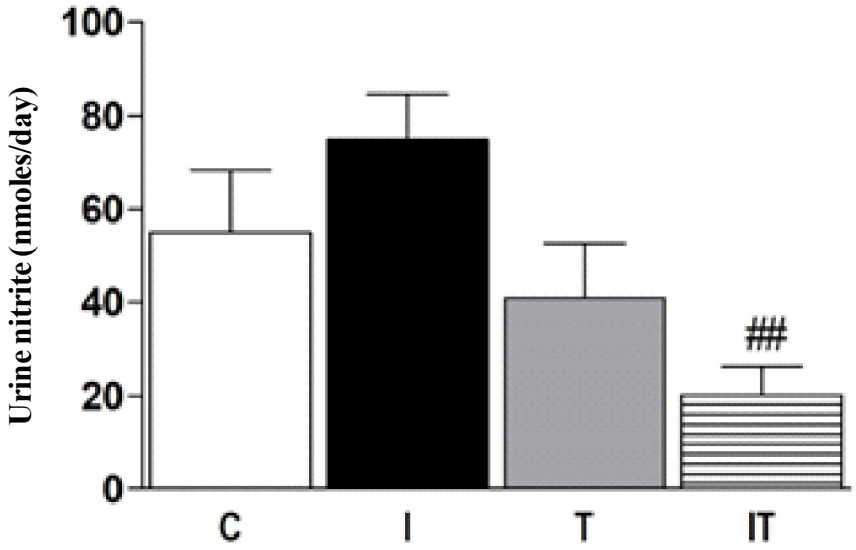


Fig. 11: Urine nitrite levels in mice from different groups (nmoles/day). Column shapes represent mean and lines on the shapes represent S.E.M. ## p<0.01 vs I.

Table 5: Effect of BA on serum creatinine, urine nitrite and kidney MDA in AKI mice

Group	C	I	T	IT
Serum creatinine (mg/dl)	0.78±0.05	2.04*±0.53	0.85±0.05	$0.69^{\#}$±0.13
Urine nitrite (nmoles/day)	54.87±13.49	74.78±9.75	40.85±11.84	$20.16^{\#\#}$±6.02
Kidney MDA (nmol/mg protein)	0.48±0.05	0.32±0.04	0.35±0.04	0.25±0.06

Values are presented as mean ± S.E.M. *p<0.05 vs C. #p<0.05, ##p<0.01 vs I. (MDA: malondialdehyde).

4.1.4 Effect of BA on histopathological changes in AKI mice

Histopathological findings are shown in Fig. 12. H&E stained kidney sections from the mice of control (C) and treatment control (TC) groups showed a normal intact tubular arrangement and glomerular architecture and no microscopic changes were noticed. However, mice from AKI (I) group showed degenerative changes such as tubular epithelial swelling leading to luminal occlusion (yellow), pyknotic nuclei (brown), flattened nuclei (blue) and tubular necrosis (red). However, treatment of AKI mice with BA (IT) produced partial amelioration of these degenerative changes.

4.2 Phase II

4.2.1 Effect of BA on relative kidney weight in CKD mice

No significant difference in relative kidney weight was observed among the groups. The results of relative kidney weight is shown in Fig. 13 and Table 6.

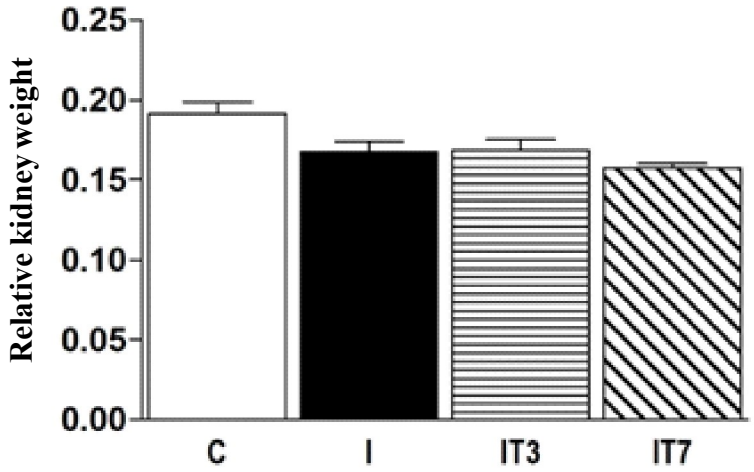


Fig. 13: Relative kidney weight of mice from different groups. Column shapes represent mean and lines on the shapes represent S.E.M.

4.2.2 Effect of BA on serum creatinine in CKD mice

Serum samples of CKD group (I) had significantly higher levels of creatinine as compared to control group (C). BA treatment significantly reduced the serum creatinine levels in both IT3 and IT7 groups in comparison to CKD (I) group (Fig. 14; Table 6).

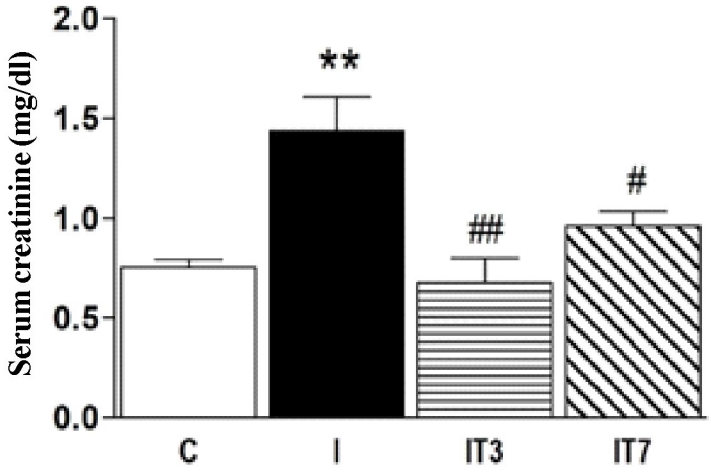


Fig. 14: Serum creatinine levels in mice from different groups (mg/dl). Column shapes represent mean and lines on the shapes represent S.E.M. ** p< 0.01 vs. C; # p <0.05 vs. I; ## p <0.01 vs. I.

4.2.3 Effect of BA on urine creatinine in CKD mice

Fig. . 15 and Table 6 show the urine creatinine levels (mg/day) estimated from different groups in the study. CKD (I) group had significantly lower level of urine creatinine compared to the control group (C). BA treatment significantly attenuated the urine creatinine levels in IT7 group. No significant change was observed in IT3 group when compared to CKD (I) group.

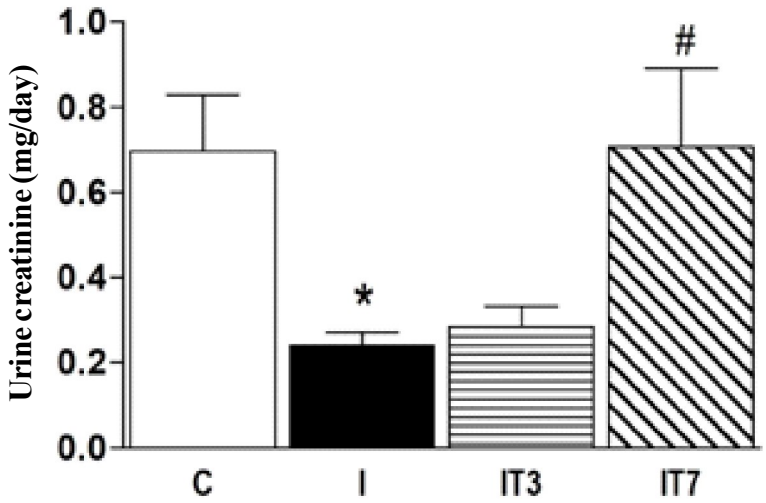


Fig. 15: Urine creatinine levels in mice from different groups (mg/day). Column shapes represent mean and lines on the shapes represent S.E.M. * p< 0.05 vs. C; # p <0.05 vs. I.

4.2.4 Effect of BA on the urine protein excretion in CKD mice

Fig. 16 and Table 6 show the total protein levels (mg/ml) in urine estimated from various groups in this study. The values of urine protein remained statistically non-significant among groups. No high molecular weight proteins (HMW) were detected in injury group when the samples were subjected to SDS-PAGE and staining indicating the absence of HMW proteinuria in folic acid model of kidney injury. However, small molecular weight proteins (~10-30 kDa) were found more in control & IT3 group.

Table 6: Effect of BA on relative kidney weight, serum creatinine, urine creatinine and urine protein in CKD mice

Group	C	I	IT3	IT7
Relative kidney weight	0.19±0.01	0.17±0.01	0.17±0.01	0.16±0.003
Serum creatinine (mg/dl)	0.75±0.04	1.44**±0.17	0.68$^{\#\#}$±0.12	0.96$^{\#}$±0.07
Urine creatinine (mg/day)	0.69±0.13	0.24*±0.03	0.28±0.05	0.71$^{\#}$±0.18
Urine protein (mg/ml)	7.23±0.11	5.47±2.23	5.85±0.80	5.18±1.21

Values are presented as mean ± S.E.M. *p<0.05, ** p< 0.01 vs C. #p<0.05, ##p<0.01 vs I.

4.2.5 Effect of BA on kidney MDA in CKD mice

Increase in MDA levels is an important marker of oxidative stress and kidney injury. Results of MDA levels (nmoles/mg of protein) in tissue are shown in Fig.17 and Table 7. MDA levels were significantly higher in CKD group (I) as compared to control group mice (C). BA treatment for 3 days (IT3) did not show any significant changes in MDA levels when compared to CKD group (I). However, BA treated IT7 group showed significantly lower levels of MDA when compared to CKD (I) as well as IT3 groups.

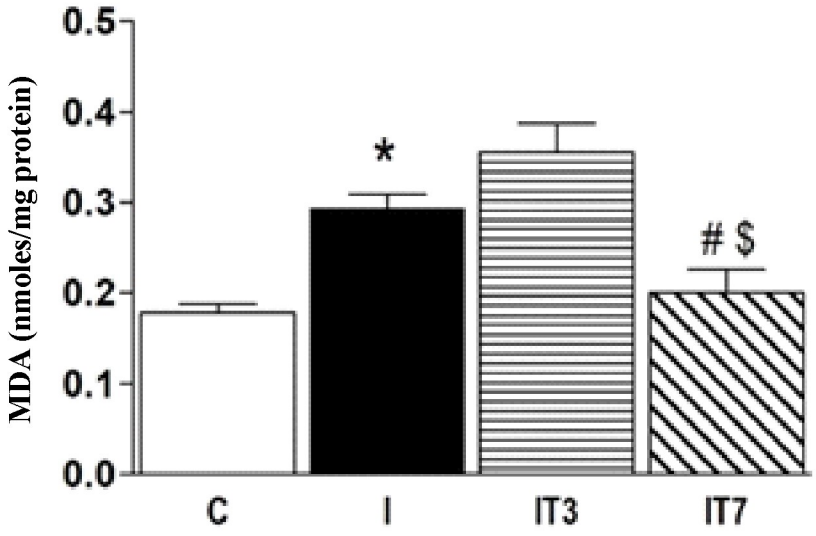


Fig. 17: Kidney MDA in mice from different groups (nmol/mg protein). Column shapes represent mean and lines on the shapes represent S.E.M. * p< 0.05 vs. C; # p <0.05 vs. I; $ p <0.05 vs. IT3. (MDA: malondialdehyde).

4.2.6 Effect of BA on urine nitrite in CKD mice

The urine nitrite levels (Fig. 18 and Table 7) were determined by Griess reaction. A significant increase in urine nitrite levels was observed in CKD (I) group when compared to control mice (C). BA treatment for 3 days (IT3) as well as 7 days (IT7) significantly reversed the nitrite excretion in urine when compared to the CKD group (I).

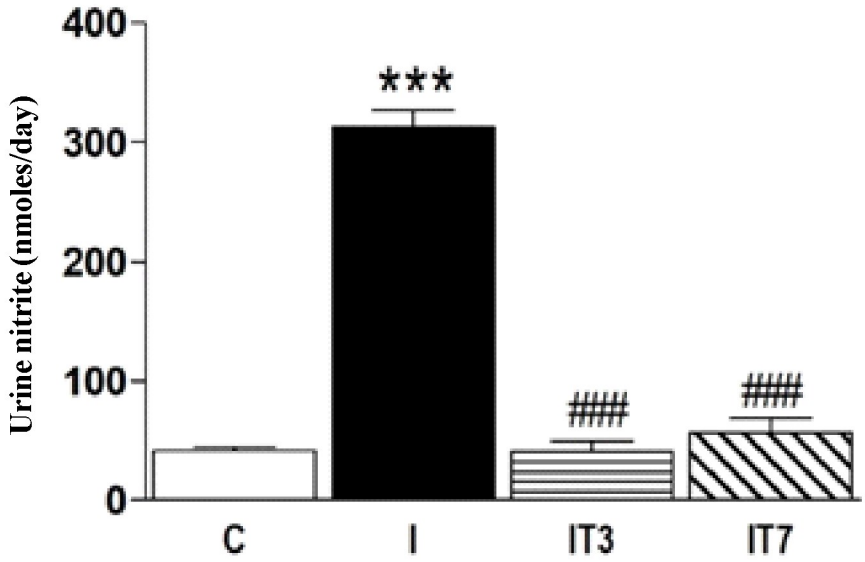


Fig. 18: Urine nitrite levels in mice of different groups (nmoles/day). Column shapes represent mean and lines on the shapes represent S.E.M. *** p< 0.001 vs. C; ### p <0.001 vs. I.

4.2.7 Effect of BA on the expression of nitrotyrosine in the kidney of CKD mice

The results of nitrotyrosine expression by Western blot are shown in Fig. 21A & 21B and Table 7. The expression of nitrotyrosine was significantly higher in CKD group (I) in comparison to the control group (C). BA treatment for 3 days (IT3) didn't affect the expression of nitrotyrosine significantly in mice kidneys compared to CKD group (I). However, IT7 group had significantly less expression of nitrotyrosine when compared to CKD group (I).

Table 7: Effect of BA on kidney MDA, urine nitrite and kidney nitrotyrosine expression in CKD mice

Group	C	I	IT3	IT7
Kidney MDA (nmol/mg protein)	0.18±0.01	0.29*±0.02	0.35±0.03	0.2#$±0.03
Urine nitrite (nmoles/day)	41.92±2.53	312.6***±14.03	40.82###±8.87	56.79###±12.33
Kidney nitrotyrosine/β-actin	0.52±0.04	1.17*±0.18	0.59±0.17	0.41#±0.13

Values are presented as mean ± S.E.M. *p<0.05, *** p< 0.001 vs C. #p<0.05, ###p<0.001 vs I. $ p <0.05 vs. IT3 (MDA: malondialdehyde).

4.2.8 Effect of BA on the level of renal IL-4 in CKD mice

IL-4 is a tissue reparative cytokine which plays an important role in recovery from kidney injury. IL-4 level was significantly decreased in CKD group (I) when compared to the control group. No significant difference was observed in IL-4 level in IT3 group when compared to CKD group (I). But IL-4 level was restored significantly in IT7 group as compared to CKD group (I) (Fig. 19 and Table 8).

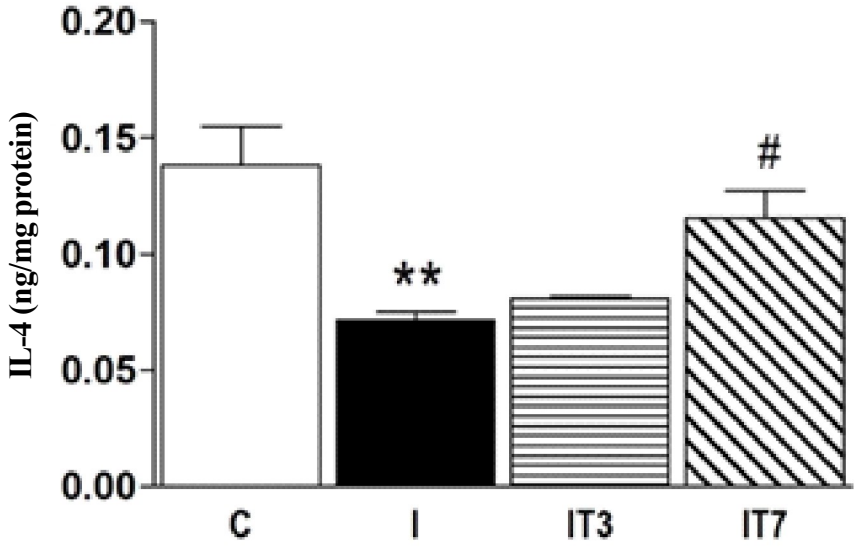


Fig. 19: Kidney IL-4 (ng/mg protein) levels in mice from different groups. Column shapes represent mean and lines on the shapes represent S.E.M. ** p< 0.01 vs. C; # p <0.05 vs. I. (IL-4: Interleukin-4).

4.2.9 Effect of BA on the level of renal IL-13 in CKD mice

IL-13 performs similar function as that of IL-4. Levels of kidney IL-13 (Fig. 20 and Table 8) are shown below. Protein levels of IL-13 was significantly attenuated in kidney of CKD group (I) mice when compared to control group (C). IL-13 level was restored significantly in both IT3 and IT7 groups as compared to the CKD group (I).

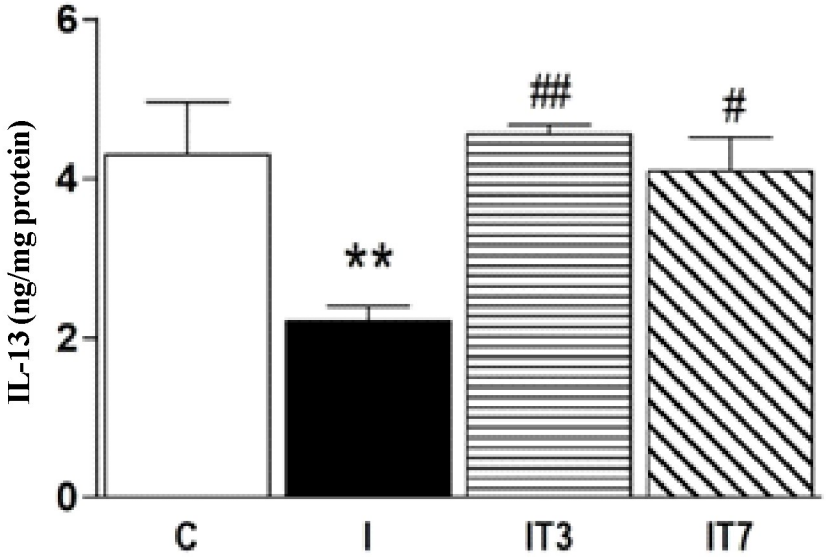


Fig. 20: Kidney IL-13 levels in mice from different groups (ng/mg protein). Column shapes represent mean and lines on the shapes represent S.E.M. ** p< 0.01 vs. C; ## p <0.01 vs. I; # p <0.05 vs. I. (IL-13: Interleukin-13).

Table 8: Effect of BA on kidney IL-4 and IL-13 in CKD mice

Group	C	I	IT3	IT7
IL-4 (ng/mg protein)	0.14±0.02	0.07^{**}±0.003	0.08±0.001	$0.11^{\#}$±0.01
IL-13 (ng/mg protein)	4.3±0.67	2.21^{**}±0.19	$4.56^{\#\#}$±0.11	$4.09^{\#}$±0.42

Values are presented as mean ± S.E.M. ** p< 0.01 vs C. #p<0.05, ##p<0.01 vs I. (IL- interleukin)

4.2.10 Effect of BA on the expression of α-SMA in the kidney of CKD mice

α-SMA is the marker of myofibroblasts which is main source of extracellular matrix. The expression of α-SMA was estimated by Western blotting and shown in the Fig. 21A & 21C and Table 9 with β actin as internal control. A significantly higher expression of α-SMA was observed in CKD group (I) when compared to the control group (C). IT3 group showed

no significant difference in α-SMA expression compared to the CKD group (I). But IT7 group had significantly lower expression of α-SMA as compared to CKD group (I).

4.2.11 Effect of BA on the MMP-2 activity in the kidney of CKD mice

MMP-2 is an enzyme that degrade extracellular matrix. MMP-2 levels in kidney of different groups were determined by gelatin zymography. As shown in the Fig. 22 and Table 9, significantly higher MMP-2 activity in CKD group (I) was observed as compared to control group (C). BA treatment for 7 days (IT7) significantly reduced MMP-2 activity when compared to both CKD (I) and IT3 groups.

Table 9: Effect of BA on kidney α-SMA/ β-actin and MMP-2 activity in CKD mice

Group	C	I	IT3	IT7
α-SMA/ β-actin	0.42±0.12	1.38^{*}±0.17	0.61±0.34	$0.26^{\#}$±0.14
MMP-2	1±0	2.38^{*}±0.08	1.82±0.04	$0.89^{\#\#\$}$±0.28

Values are presented as mean ± S.E.M. *p<0.05. #p<0.05, ##p<0.01 vs I. $p<0.05 vs IT3 (α-SMA: Alpha smooth muscle actin, MMP2: Matrix metalloproteinase 2).

4.2.12 Effect of BA on histopathological changes in the kidney of CKD mice

As shown in Fig. 23, H&E stained kidney sections from control group (C) mice showed a normal intact tubular arrangement and glomerular architecture and no microscopic changes. However, mice from CKD group (I) showed parenchymatous tissue replaced with non-parenchymatous tissue including fibroblasts (yellow) and matrix (encircled pink stained areas), compressed tubules due to compromised space (matrix deposition) (blue). There was no significant difference in these degenerative changes in IT3 group. BA treatment for 7 days (IT7) significantly attenuated the histopathological changes, as evident from reduced fibroblasts and matrix material.

4.2.13 Effect of BA on Masson's trichrome staining findings in the kidney of CKD mice

Masson's trichrome staining revealed normal amounts of dispersed ECM around Bowman's capsule and renal tubules in control group (C) mice with no abnormal ECM deposits

in the interstitium. CKD group (I) exhibited increased deposition of ECM in the interstitium (yellow) and ECM deposits in periglomerular area (periglomerular fibrosis) (green). Comparatively same histological picture was apparent in IT3 group. However, IT7 group showed minimal ECM deposition when compared to the CKD group (I) (Fig. 24).

4.2.14 Effect of BA on Picro sirius red staining findings in the kidney tissue section of mice from different groups

Picro sirius red staining method is simple, sensitive and specific for collagen staining which is the main component of extra cellular matrix. The results of picro sirius red staining are shown in Fig. 25. Intense staining indicating higher collagen deposition was observed in CKD group (I) when compared to the control group (C). Among the treatment groups, when IT3 group showed similar findings to that of CKD group (I), the density of staining was less in IT7 group revealing the decreased collagen deposition.

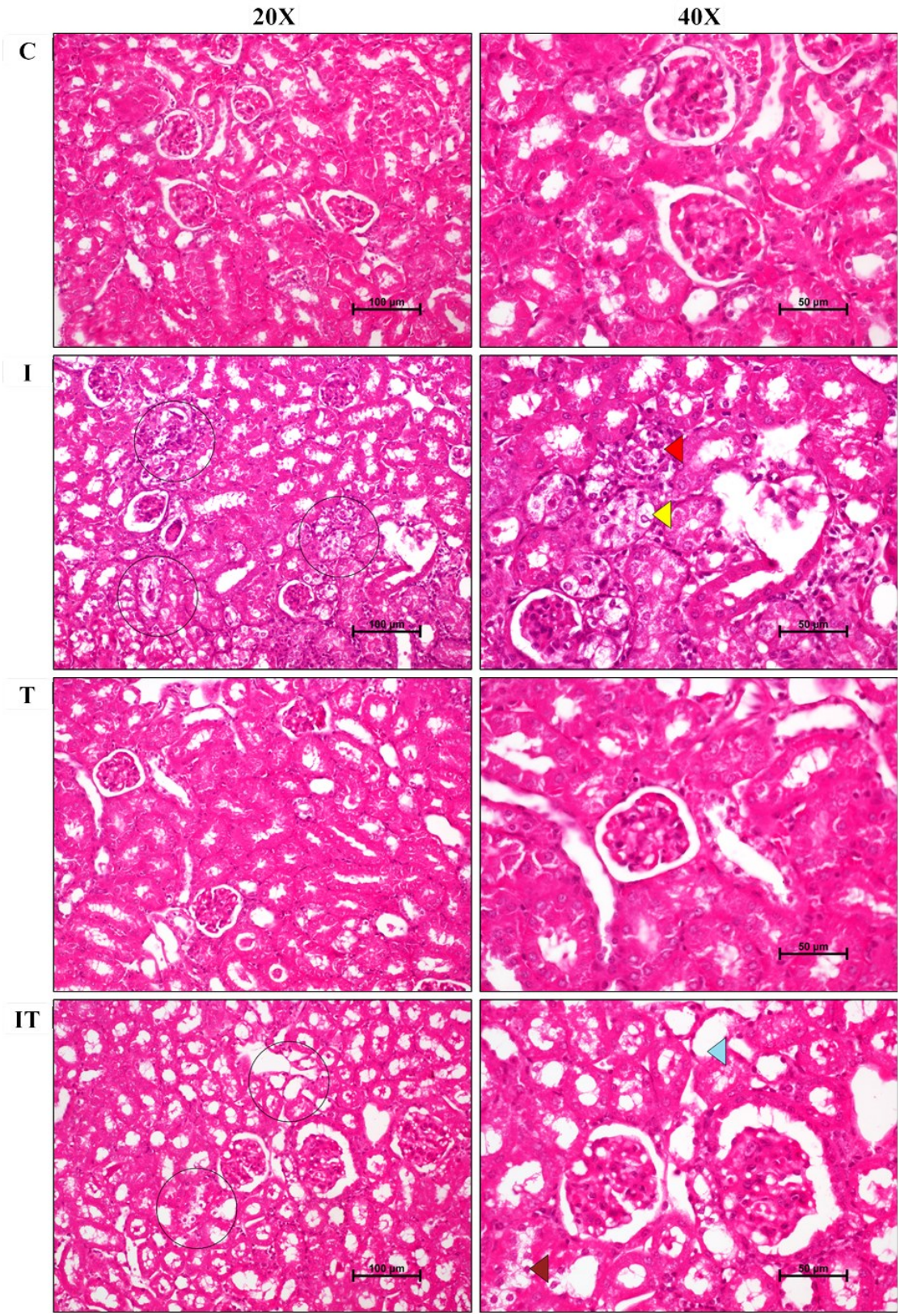
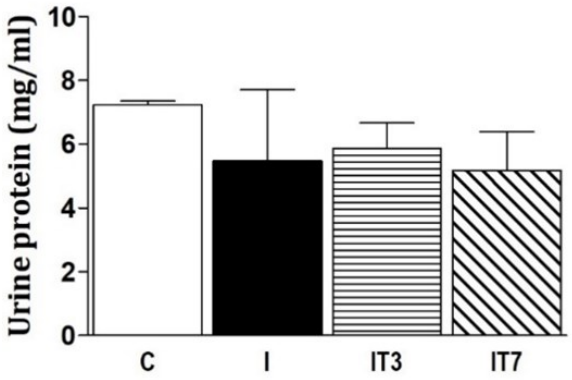
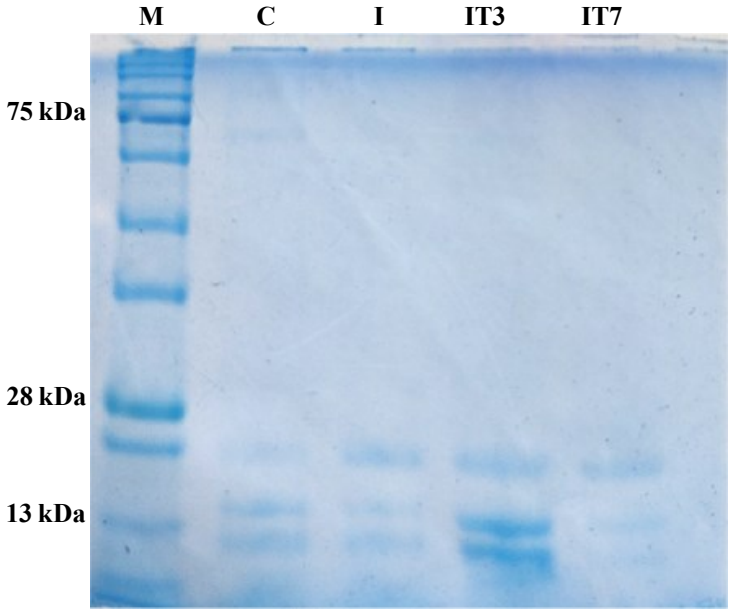


Fig. 12: Photomicrographs of representative kidney tissue sections from different groups (Objective piece magnification: 20X; 40X)

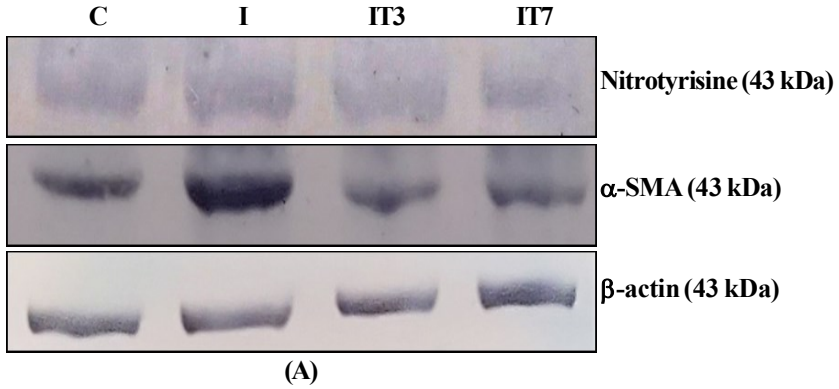


(A)

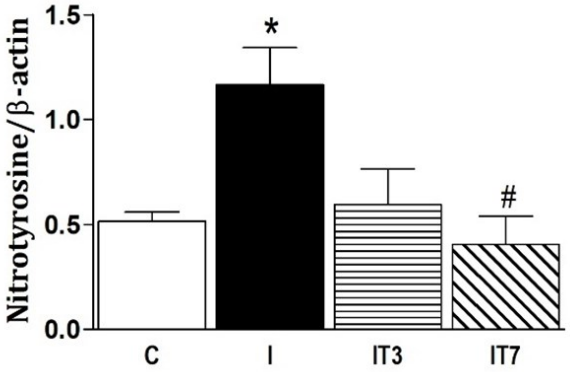


(B)

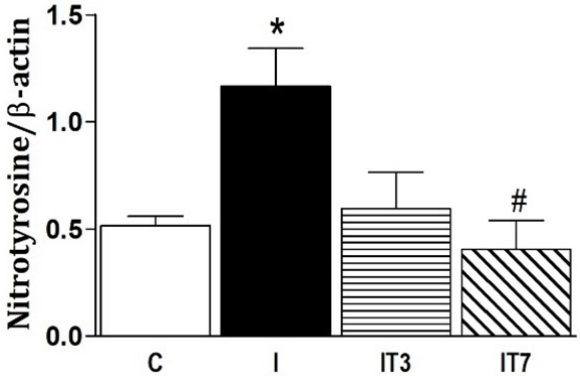
Fig. 16: A. Urine protein levels in mice from different groups (mg/ml). Column shapes represent mean and lines on the shapes represent S.E.M. B. Representative photograph of SDS-PAGE of urine samples from different groups.



(A)

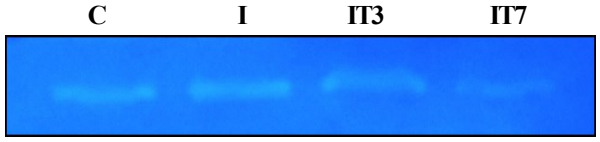


(B)

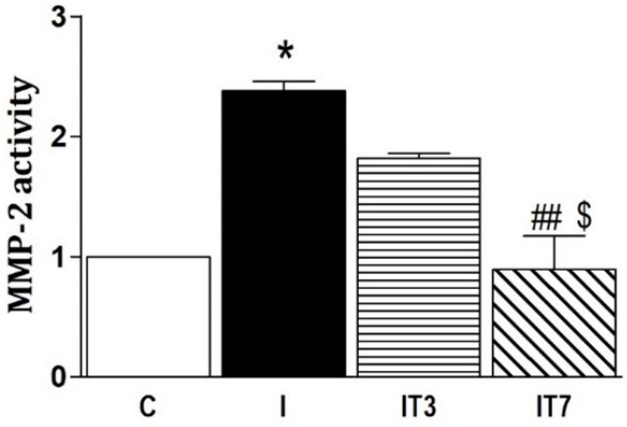


(C)

Fig. 21: A. Representative photograph of Western blot analysis of nitrotyrosine and renal ±-SMA in different groups with β-actin as internal control. B. Relative expression of nitrotyrosine (nitrotyrosine/ β-actin ratio). C. Relative expression of kidney α- SMA (α-SMA/ β-actin ratio). Column shapes represent mean and lines on the shapes represent S.E.M. * p<0.05 vs. C and # p<0.05 vs. I. (α-SMA: Alpha smooth muscle actin).



(A)



(B)

Fig. 22: A. Representative photograph of zymography analysis of renal MMP-2 activity in different groups. B. Kidney MMP-2 activity. Column shapes represent mean and lines on the shapes represent S.E.M. * p< 0.05 vs. C; # # p <0.01 vs. I; $ p <0.05 vs. IT3. (MMP2: Matrix metalloproteinase 2).

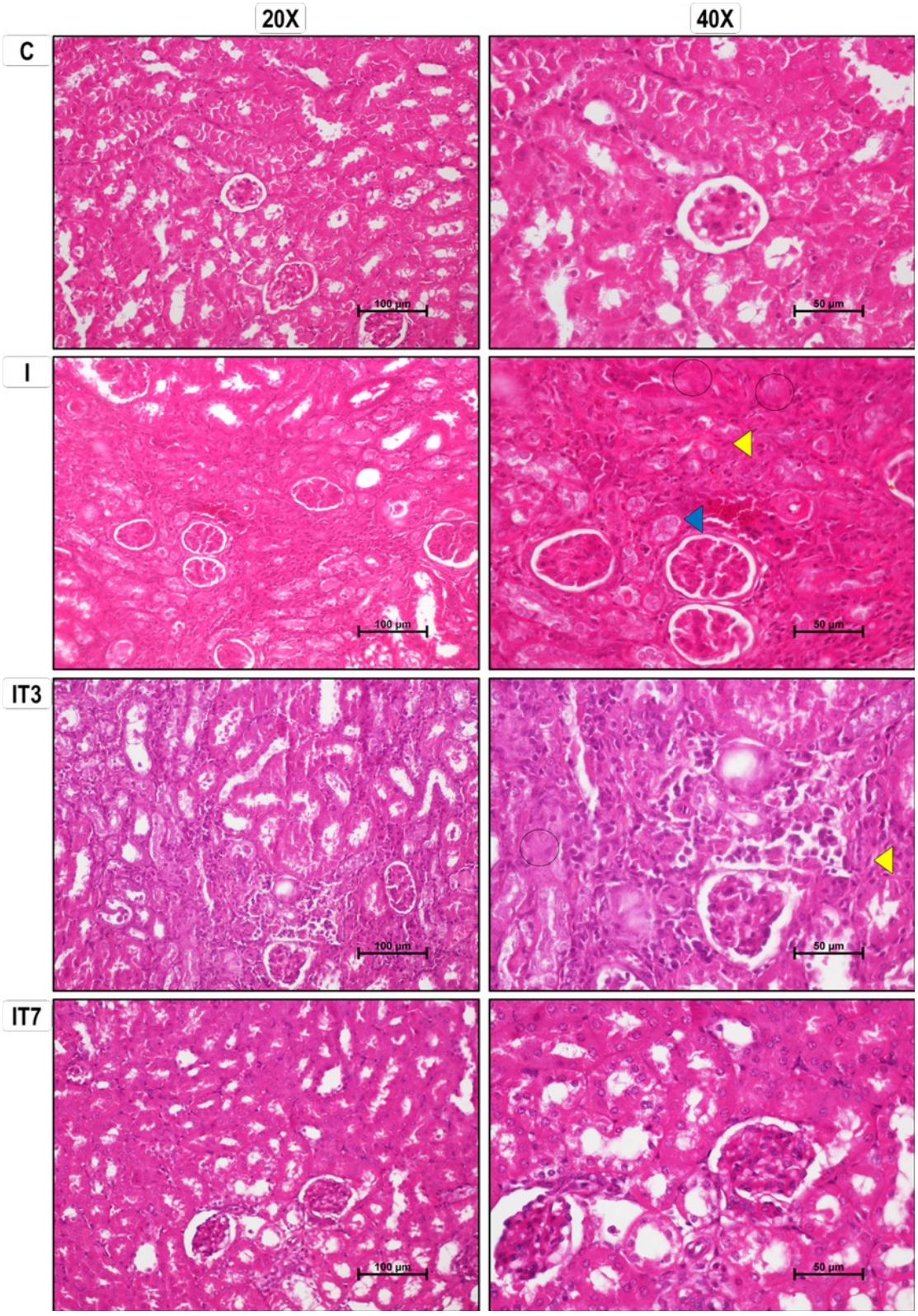


Fig. 23: Photomicrographs of representative H&E stained kidney tissue sections of mice from different groups. (Objective piece magnification: 20X; 40X).

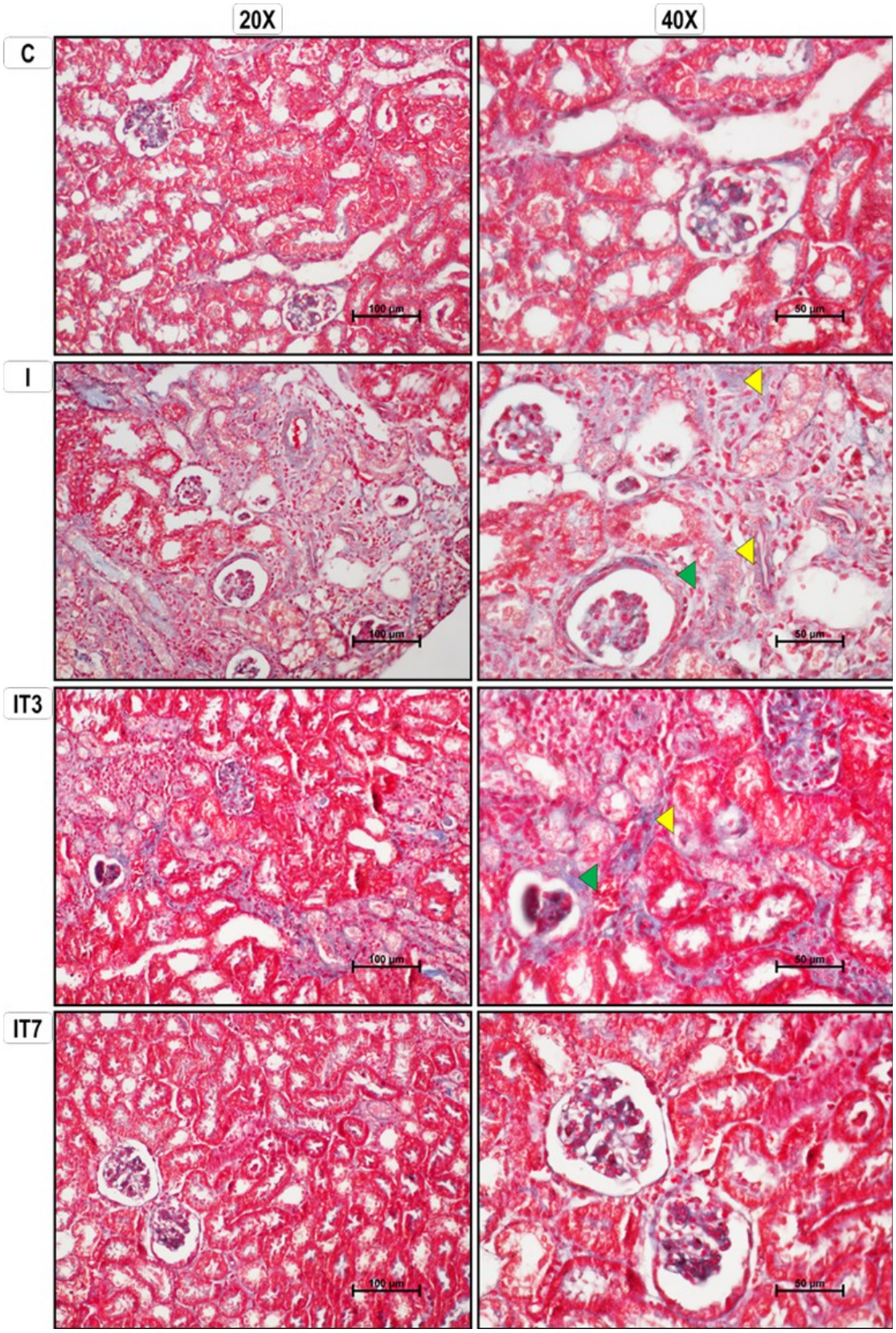


Fig. 24: Photomicrographs of representative Masson's trichrome stained kidney tissue sections depicting ECM deposition in the kidney tissue of different groups. (ECM: extracellular matrix) (Objective piece magnification: 20X; 40X).

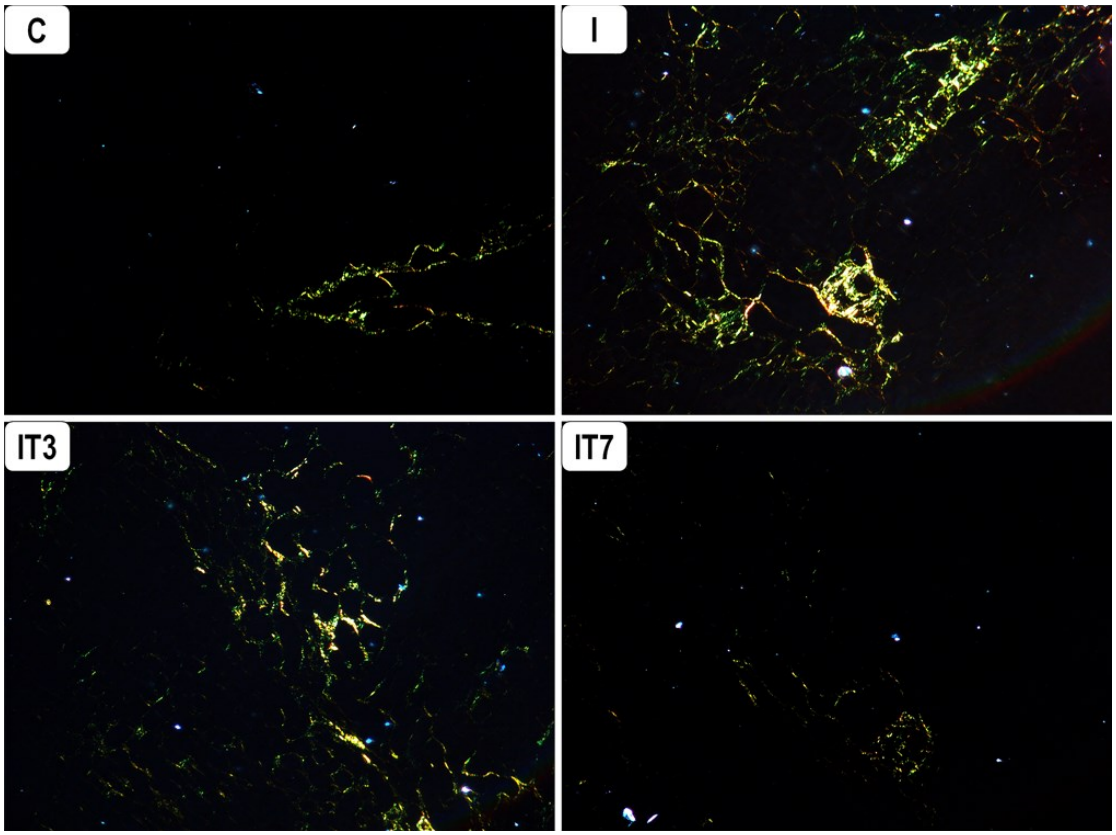


Fig. 25: Photomicrographs of representative kidney tissue sections stained with picrosirius red stain depicting collagen deposition in the kidney tissue sections of mice from different groups. (Objective piece magnification: 10X).

Discussion

5 DISCUSSION

Development of CKD condition from the existing AKI is popularly termed as AKI-CKD transition. There is a strict correlation between AKI and CKD, a public health problem with a high level of morbidity and mortality (Liyanage *et al*., 2015; Negi *et al*., 2018). AKI severity, duration and frequency are associated with the development of CKD, but even mild episodes are associated with an increased risk of disease progression. The lack of recovery of the tubular structure's integrity after AKI promotes the progression of interstitial injury. Thus, the incomplete repair of tubular injury during the course of AKI could lead to the development of CKD (Venkatachalam *et al*., 2015; Strausser *et al*., 2018). Naturally occurring compounds are becoming increasingly important in drug discovery and development. Various components of the natural medicines can simultaneously hit different targets involved in the pathogenesis of the diseases. Terpenoids are the largest group of phytochemicals and are comprised of both primary and secondary metabolites (Taiz *et al*., 2010). Triterpenoids are usually classified into three groups: acyclic, tetracyclic and pentacyclic (Price *et al*., 1987; Hostettmann *et al*., 1995). Pentacyclic triterpenoids are the most common and widely distributed triterpenoids and are biosynthesized in plants by the cyclization of squalene, a triterpene hydrocarbon and precursor of all steroids. The pentacyclic triterpenes can be divided into three main classes: oleanane, ursane and lupine. Triterpenoids are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and above all, for their cytostatic effects (Dzubak *et al*., 2006).

BA is a naturally occurring pentacyclic lupine type triterpenoid that is derived from betulin, a compound abundant in the outer bark of white birch trees (*Betula alba*) (Alakurtti *et al*., 2006). BA exhibits a variety of biological and medicinal properties such as inhibition of human immunodeficiency virus (HIV) (Kashiwada *et al*., 1994; Fujioka *et al*., 1994), anti-bacterial (Fujioka *et al*., 1994; Chandramu *et al*., 2003), anti-malarial (Bringmann *et al*., 1997), anti-inflammatory (Huguet *et al*., 2000; Bernard *et al*., 2001; Alakurtti *et al*., 2006), anthelmintic (Enwerem *et al*., 2001), anti-nociceptive (Kinoshita *et al*., 1998) anti-HSV-1 (Ryu *et al*., 1993), and anti-cancer activities (Fulda and Debatin, 2000; Zuco *et al*., 2002). BA has also been found to inhibit human melanoma cells selectively while leaving healthy cells alive and also several BA derivatives are potent and highly selective inhibitor of HIV-1 (Aiken and chen, 2005). BA exhibits specific cytotoxic effect on several tumor cell lines by inducing apoptosis in cells (Ehrhardt *et al*., 2004) suggesting its anti-tumor activity. BA has been found to protect against ischemia/reperfusion-induced renal damage suggesting its nephroprotective action (Demiralp *et al*., 2010). BA also appears to be a key regulator of experimentally induced AKI and CKD and improves kidney function thereby becoming an interesting modality agent for AKI and CKD (Lingaraju *et al*., 2015b; Sharma *et al*., 2017).

This study aimed at investigating the effects of BA on AKI-CKD transition in mice which is yet unknown. This study was conducted in two phases. In phase I we established AKI animal model by intraperitoneal administration of single dose of FA (250 mg/kg BW). BA was administered at a dose of 30 mg/kg BW for 3 days. Animals were sacrificed on day 3 to assess the AKI and its modulation by BA. In phase II study, progression of AKI to CKD was produced by single dose (250 mg/kg BW) intraperitoneal administration of FA in mice on first day and left for 28 days period. Excess mice of this group were again divided into two separate groups and BA was administered at dose rate of 30 mg/kg BW for 3 days (on days 1, 2, 3) in one group (IT3) and for 7 days (on days 1, 2, 3, 7, 14, 21, 28) in another group (IT7) to assess the effect of BA on AKI-CKD progression under two different therapeutic regimen.

In phase I study we found that AKI (I) was associated with increased serum creatinine (Fig. 9). Reduced creatinine clearance in acute renal failure may result from either impaired creatinine filtration as a result of decreased GFR (Perrone *et al*., 1992). After FA injection,

urinary volume shows a decrease, as does GFR and the filtration fraction. This is followed by an elevation in the concentration of BUN and creatinine (Rattanasinganchan *et al*., 2016; Li *et al*., 2021). The rate of increase and the final concentration of serum creatinine depend on many factors, including the severity and time course of resolution of renal injury, rate of generation of creatinine, volume of distribution of creatinine. In principle, if the renal function stabilizes, the serum creatinine concentration will reach a plateau at a value where renal creatinine excretion equals the difference between production and extrarenal elimination of creatinine (Kassirer, 1971). The administration of BA improved the renal function and glomerular filtration thereby significantly reducing the serum creatinine levels. Confirming this, BA was also found to improve GFR as reflected by serum creatinine levels in a different model of AKI (Lingaraju *et al*., 2015b).

Oxidative stress is an imbalance among the rate of production and removal of oxidant and common causative factor of various diseases such as ischemia, atherosclerosis, neurodegenerative diseases and aging (Sorg, 2004; Salmon *et al*., 2010). Increased ROS/ RNS production was linked to kidney injury in oxidative stress-related AKI, which results in the oxidation of numerous macromolecules (e.g. protein, DNA and lipid). Production of lipid peroxidation (LPO) in oxidative stress-related AKI results in large production of secondary products such as MDA and 4-hydroxynonenal (Cristol *et al*., 1996). Prolonged oxidative stress eventually shift the balance of nitric oxide that undergo a sequential oxidation or nitrosation of water to form the more stable NO metabolites, nitrite and nitrate (Bryan *et al*., 2007). In a study conducted by Mian and co-workers in 2011 it was found that lower concentration of urinary nitrate, but not nitrite, was significantly associated with AKI. Renal MDA levels were considerably higher in the IR model, showing the presence of enhanced lipid peroxidation due to renal injury (Baud and Ardaillou, 1993; Ergin *et al*., 2015). However, in our study kidney MDA and urine nitrite levels didn't show any significance among the groups (Fig. 10 & 11). Histopathological examination of kidney sections from control (C) and treatment control (T) animals showed no signs of damage. However, kidney from AKI group (I) revealed the widespread damage of tubules as shown by degenerative changes such as tubular swelling leading to occlusion of lumen (yellow) and tubular epithelial necrosis (red). Only partial

amelioration of these changes was observed in BA treated group (IT) (Fig. 12). So, AKI induced by single dose of FA was partially ameliorated by BA treatment as revealed by serum creatinine levels and histopathological findings.

In phase II study which was conducted for 28 days to assess CKD condition and its modulation by two different treatment regimen of BA, it was found that experimental groups did not have significant changes with respect to the relative kidney weight (Fig. 13). In CKD animals, reduced body weight and additional structural pathology will lead to increase in the kidney weight to body weight ratio (relative kidney weight) (Diwan *et al*., 2017; Thakur *et al*., 2018). However, it was not evident in our study (Fig. 13) therefore the result of BA on relative kidney weight in CKD mice could not be drawn. Body weight is a multifactorial determinant and is controlled by many factors (Kurniawan *et al*., 2019; Toda *et al*., 2022). This can be one of the reasons that the results of relative kidney weight are not conclusive in this study. Anyhow, in apparently healthy mice, BA did not show any negative impact on relative kidney weight (Fig. 13).

Creatinine is formed as a result of the nonenzymatic dehydration of muscle creatine which is the precursor of creatinine (Hahn and Meyer, 1928; Borsook and Dubnoff, 1947). The serum creatinine concentration, which is utilised as a crucial screening test for assessing renal function, is frequently interpreted as a marker of the GFR (Duncan *et al*., 2001). In this study renal function was examined by assessing serum and urine creatinine levels. Serum creatinine levels were increased in CKD group (I) after administration of FA, which is comparable to the study conducted by Liang-Jun Yan in 2021. Several studies have shown that urinary creatinine excretion is reduced in patients with elevated serum creatinine (Tynkevich *et al*., 2014; Hessels *et al*., 2018). In chronic renal disease, a decreased GFR results in reduced creatinine filtration (Perrone *et al*., 1992). BA administered animals in IT7 group in this study had significant reduction in serum creatinine levels. This group also showed significant improvement in urine creatinine levels when compared to the CKD group (I) indicating the improved renal function (Fig. 14 & 15).

Urine protein levels, another marker of renal damage was done by total protein quantification by Bradford method. In our study, urine protein were detected but values

remained statistically non-significant among the groups. Androgen-dependent proteins (lipocalins), which are tiny (~ 18 kDa) and freely pass into glomerular filtrate, are present in the blood of male rats and mice. Some are reabsorbed in the proximal nephrons, but some leak out in the urine (Vettorazzi *et al*., 2013). Albuminuria is commonly used as an early marker of renal injury because it often precedes a decline in renal function. No HMW protein (albumin) were detected in CKD (I) and other groups when the samples were subjected to SDS-PAGE which indicates the absence of HMW proteinuria (Fig. 16). Albumin excretion (HMW protein in urine) cannot distinguish different types of proteinuric kidney disease and has a limited ability to predict disease progression and determine therapeutic efficacy (Tesch, 2010).

By damaging the cellular integrity through a mechanism whereby ROS react with the unsaturated fatty acids of cellular and subcellular membranes, oxidative stress plays a significant role in kidney damage (Crimi *et al*., 2006). This mechanism is thought to be one of the basic mechanisms of tissue damage caused by free radicals (Wheeler, 2011). The most common oxidative stress biomarker employed in many diseases like cancer, chronic obstructive pulmonary disease, or cardiovascular illnesses is MDA. One of the ultimate products of polyunsaturated fatty acids peroxidation in the cells is MDA. When ROS and PUFA interact, they generate peroxides and lipid hydroperoxides, which break down to produce MDA and other cytotoxic byproducts. So, an increase in free radicals causes overproduction of MDA. These reactive aldehydes, can easily react with proteins to form advanced lipoxidation end products (Ayala *et al*., 2014). In our study a significantly higher level of MDA in kidney was observed in the CKD group (I) compared to the control mice (C). MDA correlated negatively with the glomerular filtration rate and was significantly different among CKD patients with stages 2, 3, 4, and 5 (Kirkman *et al*., 1999). Higher levels of serum MDA were also found in haemodialysis patients (Kirkman *et al*., 1999; Kaya *et al*., 2012). High serum MDA levels were found in patients with advanced chronic renal failure (Zwolinska *et al*., 2004; Rutkowski *et al*., 2006) and in subjects on peritoneal dialysis (Martin-Mateo *et al*., 1998; Samouilidou *et al*., 2003). MDA was found to be significantly elevated showing a pro-oxidant status in CRF patients (Sreenivasulu *et al*., 2020). BA treatment significantly reduced MDA levels proposing its antioxidant activity (Fig. 17).

Oxidative /nitrosative stress as a result of the increase in the content of reactive oxygen/ nitrogen forms is recognized in turn as a prominent feature of many acute and chronic diseases (Dalle Donne *et al*., 2006). Nitrite is a product of nitric oxide oxidation. In acidic environments or under oxidative stress conditions it can be converted to a range of reactive nitrogen species (RNS) (D'Ischia *et al*., 2011). Levels of nitrosative stress are primarily influenced by RNS concentration, exposure time, and cellular antioxidant capacity to remove them (Calcerrada *et al*., 2011). Results in our study indicated significantly higher levels of urine nitrite in CKD group (I). Nitrite occurrence at relatively high levels indicate higher levels of NO which occur under nitrosative stress conditions. However, its levels were significantly reduced on treatment with BA for both 3 days (IT3) and 7 days (IT7) (Fig. 18 and Table 6). ROS generated avidly react with and inactivate NO and, in the process, produce highly reactive and cytotoxic products, such as peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH). Peroxynitrite in turn reacts with and modifies various molecules, such as lipids, DNA, and proteins. When peroxynitrite reacts with the tyrosine residues in protein molecules, nitrotyrosine, which are considered to be footprints of NO-ROS-protein interaction is produced (Beckman *et al*., 1996; Halliwell *et al*., 1997). In this study, expression of nitrotyrosine was significantly enhanced in CKD group (I) compared to the control group (C). In IT7 group BA treatment significantly reduced the expression of nitrotyrosine suggesting the decreased level of oxidative stress in the tissue (Fig. 21A and 21B).

In the initial repair phase after a kidney injury, cells may enter the G2/M phase and become stalled, releasing cytokines and growth factors that encourage continuing inflammation by retaining inflammatory cells inside the kidney. A possible reason for the higher incidence of CKD progression following AKI in the elderly is that ageing makes tubular cells more susceptible to G2/M arrest in response to cell stress and DNA damage (Ferenbach and Bonventre, 2015). IL-4 has been shown to be an important cytokine in modulating the recovery of tubular injury in renal disease (Liang *et al*., 2017; Zhang *et al*., 2017). Zhang *et al*. (2017) demonstrated using an AKI animal model that the polarisation of the M2 macrophage phenotype caused by IL-4 and IL-13 was associated with the recovery of tubular damage. The study by Lee and co-workers showed that macrophages infiltrating at early time points after ischemia-reperfusion

AKI exhibited primarily a proinflammatory M1 phenotype, but switched to a reparative M2 phenotype at later time points. The importance of macrophage polarisation in the recovery from ischemia-reperfusion AKI was highlighted by the fact that macrophage depletion at early time points resulted in less severe kidney injury, but macrophage deletion at later time points resulted in decreased recovery (Lee *et al*., 2011). In our study both IL-4 and 13 levels were significantly decreased in the CKD group (I) which indicates the predominating proinflammatory stage creating a dampened environment for tubular recovery. IL-13 may also influence directly the function of infiltrating or circulating inflammatory cells. IL-13 reduces production of proinflammatory cytokines, such as IL-1, TNF-α, IL-6, IL-8, and MIP-1α, and induces expression of IL-1 decoy receptor by neutrophils and macrophages (Minty *et al*., 1993; Berkman *et al*., 1996). Furthermore, IL-13 decreases respiratory burst in macrophages (Girard *et al*., 1996; Reglier-Poupet *et al*., 1998). The levels of IL-4 and 13 were significantly improved in the BA treated CKD group (IT7) (Fig. 19 and 20) suggesting the favourable condition for recovery from renal damage.

CKD is brought on by a wide range of primary renal illnesses, but no matter the initial insult, the kidney always responds in a predictable way, with expansion of myofibroblasts and deposition of extracellular fibrotic matrix (Kramann *et al*., 2013). Collagens, elastin, and a number of glycoproteins and proteoglycans create the complex network that makes up the interstitial space and basal membranes of the renal ECM (Bülow and Boor, 2019). Myofibroblasts are the cells that appear to participate actively in tissue repair and in production of ECM that reduces the filtration surface area in the kidney. In addition, these contractile cells are characterized by the presence of cytoplasmic microfilament bundles expressing α SMA, the actin isoform characteristic of vascular smooth muscle cells (Skalli *et al*., 1986; Darby *et al*., 1990). Numerous glomerular disorders have interstitial myofibroblast infiltration, which has been demonstrated to substantially correlate with its prognosis (Badid *et al*., 2002). In this study, α-SMA (myofibroblast marker) expression was significantly higher in CKD group (I) compared to the control mice (C). In the normal kidney, immunohistochemical expression of contractile protein α-SMA is limited to the vascular smooth muscle cells. In pathological conditions, the expression of α-SMA is found in the glomerular mesangial cells and the interstitial

myofibroblasts. α-SMA expression in interstitium is also connected with serum creatinine and creatinine clearance and consecutively with decrease of renal function (Novakovic *et al*., 2012; Haritha *et al*., 2022) which was evident in this study as well (Fig. 14). α-SMA expression was significantly reduced in IT7 group after treatment with BA revealing the inhibitory potential of BA on myofibroblast proliferation which is a characteristic feature of CKD (Fig. 21A and 21C). These findings give preliminary clue that BA has potential to retard the progression of AKI to CKD.

MMPs participate in fundamental processes such as cell proliferation, differentiation, adhesion, migration, angiogenesis, apoptosis and inflammation (Le *et al*., 2007). MMP-2 also known as gelatinase A (Nagase *et al*., 1992) is primarily involved in degradation of the ECM proteins. It proteolytically digests gelatin (denatured collagen), and types IV, V, VII, IX and X collagen (Kundu and Patil, 2005). The gelatinases MMP-2 and MMP-9 are produced by both intrinsic glomerular and tubular cells (Chung *et al*., 2008; Peiskerová *et al*., 2009). In our study MMP-2 activity was estimated by gelatin zymography in which higher activity was observed in CKD group (I) than the control group (C) (Fig. 22). It has been shown that the increased activity of MMP-2 and MMP-9 across the kidney tubules may lead to structural alterations in the tubular basement membrane, which starts epithelial-mesenchymal transition (EMT). EMT consists of the process in which the components of epithelial cell adhesion are mislaid and they acquire a mesenchymal phenotype (myofibroblast) by expressing α-SMA and produce matrix protein. In addition to higher MMP-2 activity, CKD group also had higher α-SMA expression in this study All these mechanisms subsequently trigger tubular atrophy and fibrosis (Cheng *et al*., 2006; Ravarotto *et al*., 2018). Interestingly, BA reduced the MMP-2 activity significantly in IT7 group. This indicates the potential of BA to induce less possible circumstances for extracellular matrix deposition in the kidney in CKD condition.

Histological analysis is the gold standard test for both qualitative and quantitative measure of pathological changes in tissue, whether in research or diagnosis. It is employed to evaluate the degree of inflammation or the healing process as well as to keep track of the presence and distribution of degradation products that have dissolved into the surrounding tissue. Different staining is used to identify certain structures, cells or tissues (Paramitha *et al*., 2017). In this

study, three kinds of staining were employed (H&E, Masson's trichrome and picro sirius red) to assess the findings of different experimental groups to support and affirm the biochemical and molecular results. H&E staining results revealed no signs of damage in kidney sections from control mice (C). However, degenerative renal changes such as replacement of parenchymatous tissue with non-parenchymatous tissue including fibroblast and matrix were observed in CKD group (I). IT3 group also revealed similar degenerative changes whereas this severity was significantly reduced in BA treated IT7 group indicating the ameliorated CKD picture (Fig. 23). Same kind of pathological changes were more clearly revealed in masson's trichrome staining which stains the ECM. Almost negligible area of ECM deposition was observed in normal control animals (C). Matrix deposition was higher in CKD group (I) whereas BA treatment for 7 days significantly attenuated these changes in IT7 group as revealed by less density of ECM accumulation (Fig. 24). BA treatment for 3 days (IT3) did not reduce ECM deposition as compared to the CKD group (I). The ECM is composed of two main classes of macromolecules: proteoglycans and fibrous proteins (Jarvelainen *et al*., 2009; Schaefer and Schaefer, 2010). Collagens, elastins, fibronectins, and laminins are the primary fibrous ECM proteins (Alberts *et al*., 2007). Collagen constitutes up to 30% of the total protein content of a multicellular animal and is the most prevalent fibrous protein inside the interstitial ECM (Frantz *et al*., 2010). Collagens, the primary structural component of the ECM, regulate cell adhesion, assist chemotaxis and migration, and guide tissue formation in addition to providing tensile strength (Rozario and DeSimone, 2010). Fibroblasts that either live in the stroma or drawn to it from nearby tissues transcribe and secrete the majority of interstitial collagen (De Wever *et al*., 2008). The inherent birefringence of collagen I and III fibrils is increased when the sirius red molecule binds to it in the tertiary groove. Collagen appears bright when viewed with polarisation contrast against a dark background (Junqueira *et al*., 1978). Yellow-red strong birefringence would be assigned to collagen type I whereas collagen type III would display a weak birefringence associated with a greenish colour (Junqueira *et al*., 1979; Montes and Junqueira, 1991). In our study an increased birefringence was observed in the CKD group (I) as compared to the control group (C) pointing increased collagen deposition. The results were similar in IT3 group whereas the staining intensity was

significantly lesser in the BA treated IT7 group (Fig. 25) which reaffirms the findings of masson's trichrome staining. Altogether microscopic analysis convey that FA injected mice (I) developed histomorphological features of CKD. When these animals were given BA treatment under two regimen (IT3 & IT7), CKD features were found improved in IT7 group. These findings are in alignment with the results of biochemical and biomolecular studies proving the mitigative potential of BA on development and progression of CKD in mice.

Conclusively the results demonstrate that single i/p dose of FA in phase I study induced AKI in 3 days as reflected by higher serum creatinine and picture of renal histological damage in mice. This was partially blunted by 3 days BA administration. AKI progressed to CKD at the end of experiment in 28 days study (phase II) where CKD mice experienced higher serum creatinine, lower urine creatinine and higher urinary nitrite excretion. Tissue oxidative stress markers, reparative factors (IL-4, 13), maladaptive changes were inclined towards noxious change. However, BA administration mostly the 7 days treatment regimen (IT7) improved serum and urine parameters accompanied by reduced oxidative stress, improved reparative cytokines and inhibited maladaptive matrix deposition. However, BA treatment for first 3 days (IT3) failed to produce effect on AKI-CKD progression.

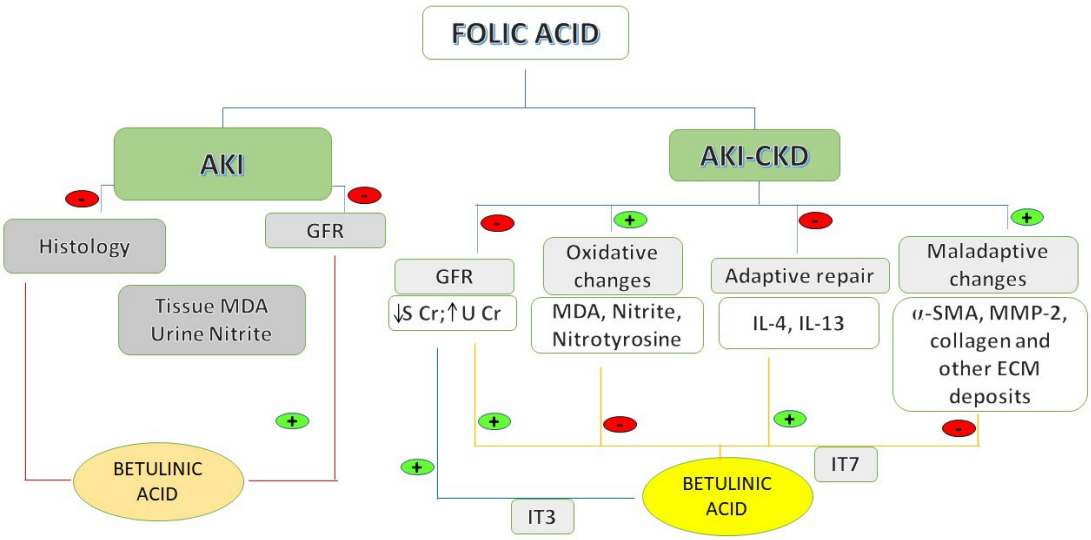
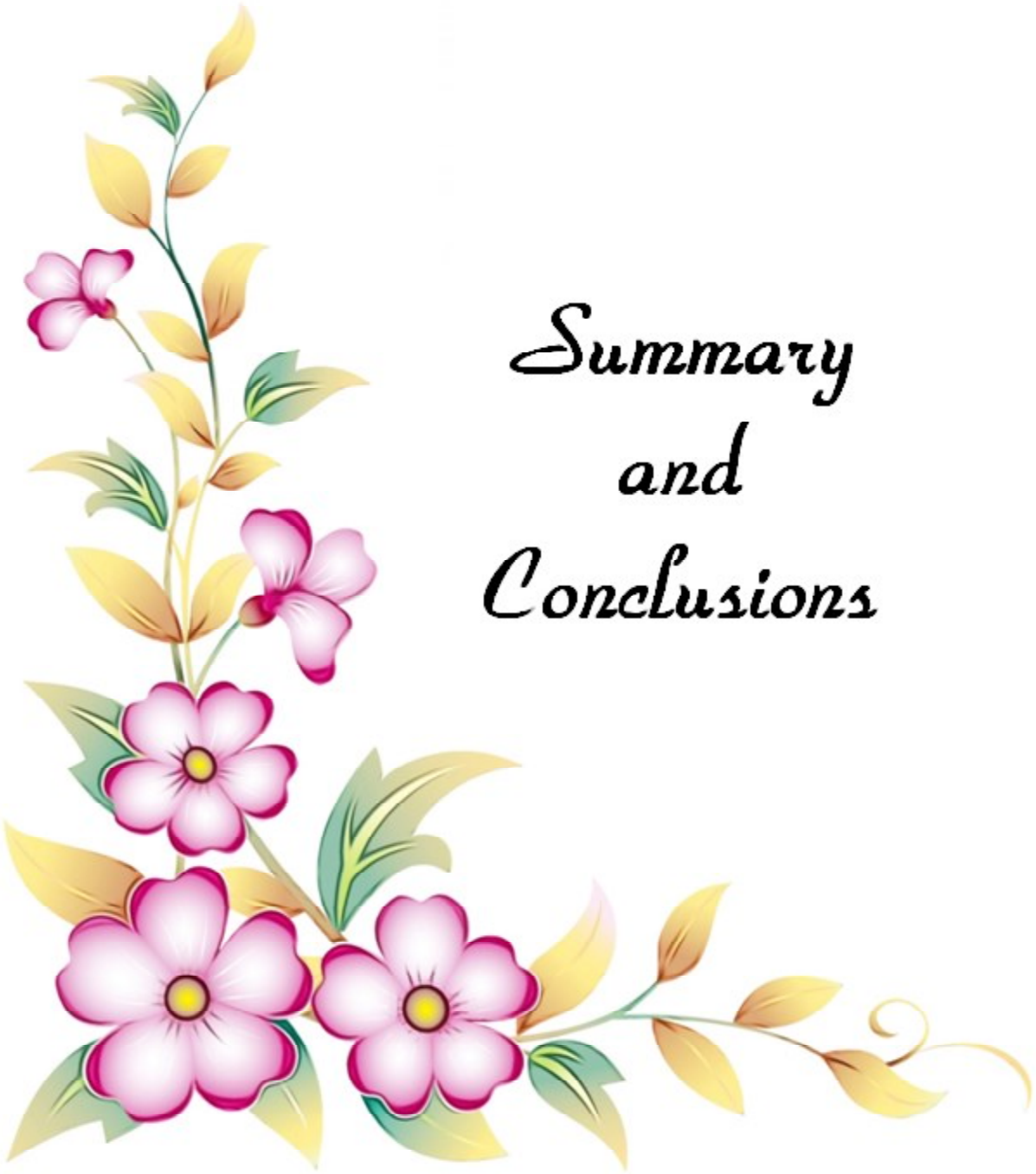


Fig. 26: Schematic illustration of possible mechanism by BA on renoprotection in FA -induced AKI and AKI-CKD transition in mice.



Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

The present study on triterpenoid betulinic acid (BA) was designed with the objective to evaluate its effect on folic acid (FA)-induced acute kidney injury (AKI) to chronic kidney disease (CKD) transition in mice. Single dose of FA (250 mg/kg BW, i/p) was used to induce AKI in mice and mice were sacrificed on day 3 in phase I study. Similar experiment was performed in phase II study but mice were sacrificed on day 28 as they developed CKD condition from AKI.

BA (30 mg/kg BW, i/p) treatment was given for first 3 days in phase I study and days 1, 2, 3, 7, 14, 21, 28 in phase II study using 5 % DMSO in pea nut oil as vehicle. The relative kidney weight, kidney injury markers, oxidants, inflammatory mediators, protein expressions and histopathological findings were determined in kidneys to address our objective.

In phase I study,

- Single high dose of FA-induced AKI and renal dysfunction. Serum creatinine concentrations were increased in AKI mice (I) and this was reduced by BA treatment (IT).
- High dose of FA did not significantly change urine nitrite and tissue MDA levels.
- FA induced injury showed a widespread microscopic damage of kidney in AKI group (I). However, BA partially ameliorated these histopathological changes (IT).

In phase II study,

- One high dose of FA (250 mg/kg BW, i/p) induced CKD from AKI in mice in 28 days.

- Relative kidney weight remained statistically non-significant among the groups.
- FA-induced renal dysfunction and serum creatinine concentrations were increased in CKD group (I). This was reduced after BA treatment (IT3 & IT7).
- FA-induced CKD mice (I) experienced lesser creatinine excretion in urine than control group (C). BA treated CKD animals in IT7 group exhibited similar urine creatinine excretion as that of control animals.
- No significant change in urine protein levels were observed among the groups. SDS-PAGE also revealed absence of any high molecular weight proteins in urine in CKD group (I) compared to the control group.
- BA treatment in IT7 group significantly reduced the MDA levels as compared to the CKD group (I) as well as the IT3 group.
- BA treatment (IT3 and IT7) was successful in decreasing FA-induced elevation in urine nitrite levels (CKD).
- BA-treated mice in IT7 group showed significantly lower relative expression of nitrotyrosine as compared to the CKD group (I).
- Protein levels of IL-4 was markedly attenuated in kidneys of CKD group (I) and they were significantly elevated in IT7 group after treatment with BA. Similarly attenuated levels of IL-13 in CKD animals were significantly restored in BA treatment groups, IT3 and IT7.
- BA-treated IT7 group had significantly less expression of myofibroblast marker, α-SMA protein as revealed by band density when compared to the CKD group (I).
- FA-induced kidney damage produced higher activity of MMP-2 in CKD group (I) which was significantly reduced in IT7 group after treatment with BA.
- CKD group (I) showed widespread damage of kidney. However, BA treatment in IT7 group significantly attenuated these histopathological changes of kidney as revealed by H&E, Masson's trichrome and Picrosirius red staining.

In conclusion, administration of single dose of FA induced AKI in 3 days which progressed to CKD in 28 days. BA partially ameliorated AKI and 7 days BA treatment regimen in CKD reduced the levels of kidney MDA, urinary nitrite excretion and tissue

nitrotyrosine to reduce oxidative & nitrosative stress. Further, reparative cytokines like IL-13 and 4 were improved by BA with concomitant reduction in chronic pathological changes such as MMP-2 activity, α-SMA expression and deposition of extracellular matrix in the FA-injured tissue. These findings indicate the potential of BA to prevent development of CKD after AKI. However, BA treatment for first 3 days failed to produce effect on AKI-CKD progression.


Mini Abstract

7 MINI ABSTRACT

Acute kidney injury to chronic kidney disease (AKI-CKD) transition refers to the progression of CKD after AKI. According to recent epidemiological and experimental research, AKI seems to be essential for the onset and progression of CKD. Transition from AKI to CKD or ESRD suggests the prolonged renal disease as a result of either continuing or recurrent cellular injury and/or abnormal repair processes, that results in persistent inflammation, matrix deposition, and renal atrophy. Betulinic acid (BA) has been shown to exhibit various pharmacological activities under *in-vitro* and *in-vivo* conditions. Studies have shown the protective effect of BA on AKI and CKD, however no reports are available on its effect on AKI-CKD transition. In the present work, the effects of BA in folic acid (FA)-induced AKI-CKD transition was explored. We established AKI model by single intraperitoneal (i/p) administration of FA in male mice in phase I study and BA was administered at 30 mg/kg BW for 3 days and the animals were sacrificed on day 3. In phase II study, mice acquired AKI after single FA (i/p) dose, which progressed to CKD when left untreated for 28 days. BA was administered at dose rate of 30 mg/kg for 3 days (on days 1,2,3) (IT3) in one group and for 7 days (on days 1,2,3,7,14,21,28) in another group (IT7) to assess its effect on AKI-CKD transition under two treatment regimen and animals were sacrificed on day 28. In phase I study, elevated level of serum creatinine and widespread microscopic damage of kidney was observed in AKI group (I). In phase II study, CKD mice (I) showed elevated levels of serum creatinine along with oxidative stress markers such as tissue MDA, urine nitrite, expression of nitrotyrosine and myofibroblast marker α-SMA and MMP-2 activity and attenuated levels of urine creatinine, tissue reparative cytokines such as IL-4 and IL-13. Histological observations that show kidney injury and ECM deposition visible as the replacement of parenchymatous tissue with non-parenchymatous tissue, such as fibroblasts and matrix (deposition of ECM in interstitium and periglomerular area), further support these findings. However, in phase I study, BA partially ameliorated AKI with better serum creatinine status and incomplete microscopical recovery. 3 days BA treatment regimen in phase II study failed to reverse most of the parameters except few. However, 7 days BA treatment regimen significantly improved serum and urine parameters accompanied by reduced oxidative stress, improved reparative cytokines and inhibited maladaptive matrix deposition thus revealing its potential to prevent development of CKD after AKI.

लघु सारांश

8 लघु सारांश

क्रोनिक किडनी डिजीज (एकेआई–सीकेडी) संक्रमण के लिए एक्यूट किडनी इंजरी एकेआई के बाद सीकेडी की प्रगति को संदर्भित करता है। हालिया महामारी विज्ञान और प्रायोगिक अनुसंधान के अनुसार, AKI CKID के विकास और प्रगति में महत्वपूर्ण भूमिका निभाता प्रतीत होता है। एकेआई से सीकेडी या ईएसआरडी में संक्रमण या तो निरंतर या आवर्ती सेलुलर चोट और/या असामान्य मरम्मत प्रक्रियाओं के परिणामस्वरूप लंबे समय तक गुर्दे की बीमारी का सुझाव देता है, जिसके परिणामस्वरूप लागतार सूजन, मैट्रिक्स जमाव और गुर्दे की शोष होती है। बेटुलिनिक एसिड (बीए) इन–विट्रो और इन–विवो स्थितियों के तहत विभिन्न औषधीय गतिविधियों को प्रदर्शित करने के लिए दिखाया गया है। अध्ययनों ने AKI और CKD पर BA का सुरक्षात्मक प्रभाव दिखाया है, हालांकि AKI-CKD संक्रमण पर इइसके प्रभाव की कोई रिपोर्ट उपलब्ध नहीं है। वर्तमान कार्य में, फोलिक एसिड (एफए) प्रेरित एकेआई–सीकेडी संक्रमण में बीए के प्रभावों का पता लगाया गया हमने चरण I अध्ययन में पुरूष चूहों में एफए के एकल इंट्रापेरिटोनियल (आई/पी) प्रशासन द्वारा एकेआई मॉडल की स्थापना की और बीए को 3 दिनों के लिए 30 मिलीग्राम/किग्रा बीडब्लयू पर प्रशासित किया गया और जानवरों को दिन 3 पर बलिदान किया गया चरण II अध्ययन में, चूहों एकल एफए (आई/पी) खुराक के बाद एकेआई प्राप्त किया, जो 28 दिनों तक अनुपचारित रहने पर सीकेडी में आगे बढ़ गया। बीए को एक समूह में 3 दिनों (1, 2, 3 दिनों पर) (आईटी3) और 7 दिनों के लिए (1, 2, 3, 7, 14, 21, 28 दिनों पर) 30 मिलीग्राम/किग्रा की खुराक दर पर प्रशासित किया गया था। एक अन्य समूह (IT7) में AKI-CKD संक्रमण पर इसके प्रभाव का आकलन करने के लिए दो उपचार आहार के तहत और जानवरों की 28 तारीख को बलि दी गई। चरण I के अध्ययन में, AKI समूह (I) में सीरम क्रिएटिनिन का ऊंचा स्तर और गुर्दे की व्लापक सूक्ष्म क्षति देखी गई। दूसरे चरण के अध्ययन में CKD चूहों (I) ने सीरम क्रिएटिनिन के ऊंचे स्तर के साथ–साथ ऑक्सीडेटिव तनाव मार्कर जैसे ऊतक एमडीए, मूत्र नाइट्राइट, नाइट्रोसिन की अभिव्यक्ति और मायोफिब्रोब्लास्ट मार्कर α-SMA और MMP-2 गतिविधि और मूत्र क्रिएटिनिन के क्षीण स्तर को दिखाया। ऊतक पुनरावर्ती साइटोकिन्स जैसे IL-4 और IL-13 हिस्टोलॉजिकल अवलोकन जो गुर्दे की चोट और ईसीएम जमाव को गैर–पैरेन्काइमेटस ऊतक के प्रतिस्थापन के रूप में दिखाई देते हैं, जैसे कि फाइब्रोब्लास्ट्स और मैट्रिक्स (टरस्टिटियम और पेरिग्लोमेरूलर क्षेत्र में ईसीएम का जमाव), इन निष्कर्षों का और समर्थन करते हैं। हालांकि, चरण I के अध्ययन में, बीए आंशिक रूप से बेहतर सीरम क्रिएटिनिन स्थिति और अपूर्ण माइक्रोस्कोपिक रिकवरी के साथ एकेआई में सुधार करता है। दूसरे चरण के अध्ययन में 3 दिनों का बीए उपचार कुछ को छोड़कर अधिकांश मापदंडज्ञें को उलटने में विफल रहा। हालांकि, 7 दिनों के बीए उपचार ने कम ऑक्सीडेटिव तनाव के साथ सीरम और मूत्र मापदंडों में काफी सुधार किया, रिपेरेटिव साइटोकिन्स में सुधार किया और मैलाडेप्टिव मैट्रिक्स जमाव को बाधित किया, जिससे एकेआई के बाद सीकेडी के विकास को रोकने की इसकी क्षमता का पता चला।

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VITAE

Name	:	**Dr. Bency Elsa Johnson**
Father's Name	:	Shri A Y Johnson
Mother's Name	:	Smt. Shailaja Johnson
Date of Birth	:	11-11-1994
Address	:	Adakkaravayalil House, Meenangadi P.O, Wayanad district, Kerala, 673591
Nationality	:	Indian
Email	:	bencyelsajohnson@gmail.com
Phone no	:	8138962028

Educational Qualification

Degree	Board/university	Year of passing	OGPA
B.V.Sc & A.H.	Kerala Veterinary and Animal Sciences University, Pookode	2020	8.018
M.V.Sc	ICAR-Indian Veterinary Research Institute,Izatnagar, Bareilly	2022	8.748

Awards/Membership

- ICAR-JRF (2020-2022)
- Member of Kerala State Veterinary Council
- Member of Indian Veterinary Association (IVA), Kerala Unit.