ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 3, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ESTABLISHMENT OF NOVEL URINARY KIDNEY DISEASE NEW BIOMARKERS AND THERAPEUTIC EFFECT OF METHANOL FRACTION OF *TERMINALIA ARJUNA* ON ACETAMINOPHEN INDUCED KIDNEY DISEASE IN RATS

DEBLINA GIRI[1], KOUSHIK DAS[1]*, DILIP KUMAR NANDI[2]

[1]Department of Nutrition and Research Unit, [2]Department of Post Graduate Physiology of Raja Narendra Lal Khan Women's College, Midnapore, Paschim Medinipur, West Bengal, India. Email: koushikphysiology@yahoo.com

Received: 04 August 2018, Revised and Accepted: 17 January 2019

ABSTRACT

Objective: There were main two objectives, first was the identification of best biomarkers for early screening of kidney diseases whether plasma urea and creatinine or novel urinary low molecular weight protein biomarkers Interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), and cystatin-C. Second was the therapeutic efficacy of methanol fraction of *Terminalia arjuna* (MFTA) on urinary novel biomarkers.

Methods: A total of 35 adult male rats were divided into three Groups (n=5), Group 1 was fed normal food, Group 2, normal food with administration of acetaminophen (APAP) for 5 days, 10 days, and 15 days, and Group 3, normal food with administration of APAP and coadministration of MFTA for 5 days, 10 days, and 15 days. All rats were sacrificed at 15th day of the experiment.

Results: Results showed 5 days, 10 days, and 15 days administrations of APAP increased novel urinary biomarkers as IL-18, KIM-1 near two-folds and cystatin-C near six-folds increased than old biomarkers plasma urea and plasma creatinine. Administration of APAP with coadministration of MFTA represented the protective effect by decreasing old and new novel biomarkers with superoxide dismutase and catalase but malondialdehyde level increased. Sodium dodecyle sulphate-polyacrylamide gel electrophoresis showed new low molecular weight urinary protein bands in APAP administration rats, the protective effect of MFTA presents no band at this molecular level as normal rats.

Conclusion: MFTA is the most potent nephroprotective agent, and urinary low molecular proteins are the best thing diagnostic tools for early detection of kidney disease over common plasma urea and creatinine.

Keywords: Kidney Disease, Acetaminophen, Nephrotoxicity, Biomarkers, *Terminalia arjuna*, Nephroprotective, Rat.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i3.28485

INTRODUCTION

Chronic kidney disease (CKD) is an irreversible progressive disorder of structural and functional changes in kidney parenchyma into five stages on the presence of decreased kidney function and measured by the concentration of serum creatinine and estimated glomerular filtration rate [1]. However, the use of serum creatinine to detect kidney disease which is poor in assay specificity and sensitivity to delayed diagnosis and treatment timing. Kidney disease becomes more fatal and severe with comorbid disorders, reactive oxygen species, diabetes mellitus, hypertension, etc. [2]. From the earlier background of our research, different anti-uremic and renoprotective plant extract such as phytocompounds from *Terminalia arjuna* (TA) [3], *Asparagus racemosus* [4], and *Withania somnifera* [5] which efficiently played as a remedy on experimentally induced uremic and oxidative stress rats. Alternative therapy such as alpha lipoic acid [6] and probiotic [7] shown brilliance nephroprotective action against the administration of acetaminophen (APAP)-induced renal failure male rats in our laboratory [8]. In early research anti-uremic and anti-oxidative activity of TA (Family - Combretaceae) is already established [9]. Therefore, the aim of the study was to evaluate early urinary biomarkers such as kidney injury molecule-1 (KIM-1), Interleukin-18 (IL-18), and cystatin-C at an early stage of kidney diseases. KIM-1 appears in urine by specific mild renal tubular disorder [10], IL-18 for proximal tubular necrosis [11], and cystatin-C for kidney tissue macrophages cell in collecting duct [12] after duration dependent induction of kidney disease by peritoneal administration of acetaminophen in rats and also evaluate the urinary protein band by protein gel electrophoresis that was best result of protein marker would be sequence and identified in future.

METHODS

Reagents

Major biochemical parameters such as urea and creatinine were measured by diagnostic kits supplied by Spinreact, S.A.U. - Ctra, Santa Coloma, and Spain. We used Enzyme-linked immunosorbent assay (ELISA) kit like IL-18; KIM-1 supplied by Thermofisher Scientific, *in vitro* gen Bioservices India Pvt. Ltd., Bengaluru, India. Other chemicals were purchased from SRL, India and MERCK, India, SD FINE-CHEM Limited, India, HiMedia Laboratories Pvt. Ltd. Mumbai, India, and Crest Biosystems Goa, India, and Gland Pharma Limited, India.

Instruments

Biochemical parameters such as urea and creatinine were monitored by semi autoanalyzer (Diasil-100). Superoxide dismutase (SOD), catalase, and malondialdehyde (MDA) were monitored by Ultraviolet-visible spectrophotometer (UV-VIS spectrophotometer) (Systronics, India). IL-18, KIM-1, and Cystatin-C were measured by ELISA Plate Reader (Thermofisher Scientific, India). Plant material fraction extract was done using an electrical blender (Japan, India), Microcentrifuges (Remi, India), Digital weight balance (Accuracy-0.1 mg Adhair Dutta and Sons), and pH meter (Mettler, India). Gel electrophoresis was done by a full set up of gel apparatus (Tarsons, India).

Preparation of the methanol fraction of TA (MFTA) bark

Bark of TA was collected from Raja Narendra Lal Khan Women's College campus of Midnapore, Paschim Medinipur district of West Bengal, India. The material was designated by the taxonomist of the botany department at our college. Voucher specimens were deposited

in the department of botany. Preparation of MFTA was done as earlier modified work in our laboratory [2].

Animal model and administration of MFTA

The study was performed on 35 healthy adult male albino rats of *Wister strain* (Supplied from Saha Enterprise, 386/2, Nilachal, Birati, Kolkata, West Bengal 700051, Regd. No.:1828/PO/BT/S/15/Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA]) having a body weight of 200±15 g. They were acclimatized to laboratory condition for 2 weeks before experimentation. Animals were housed five rats/cage in a temperature-controlled room (22±2°C) with 12–12 h dark–light cycles (8.00–20.00 h light, 20.00–8.00 h dark) at a humidity of 50±10%. They were provided with standard food and water *ad libitum* throughout the experimental period. Animal care was provided according to the guiding principle for the care and use of animals (Olfert *et al.* 1993)[13]. This experiment was approved by our Institutional Animal Ethics Committee (Regd. No.: 1905/PO/Re/S/2016/CPCSEA), guidelines followed by CPCSEA.

A total of 35 rats were divided into three Groups (n=5). Group 1; five animals were normal animals without inducing kidney injury and fed a normal diet with *ad libitum* water. Group 2; 15 animals were divided into three subgroups (A–5, A–10, and A–15); each subgroup was with five rats, APAP at the concentration of 500 mg 5 mL de-ionized water/kg of body weight/day was injected with peritoneal route at zero "0" day, at 5th day, at 10th day for 14 days (A-15, at "0" day denotes 15 days treated; A-10, at 5th day for 10 days treated and A-5, at 10th day for 5 days treated by APAP) to achieve uremia and feed normal diet with *ad libitum* water. Group 3; 15 animals were subjected same as Group 2 with coadministered MFTA (TA-15, at zero "0" day denotes 15 days treated; TA-10, at 5th day for 10 days treated and TA-5, at 10th days for 5 days treated by APAP), at the dose of 40 mg/kg body weight by orally and feed normal diet with *ad libitum* water for 14 days daily at 11.00 A.M. before giving the food. APAP-induced uremia was established in our earlier research [8].

Animal scarification to collect plasma, organs, and urine

All rats were sacrificed on the 15th day. Blood was collected from the aorta and was centrifuged with heparin anticoagulant to collect the plasma. Plasma and kidneys were stored in −20°C for different biochemical analysis. Urine samples were collected directly from the bladder using a needle through the syringe. Collected sample was stored at −20°C. Urine samples were centrifuged using a micro centrifuge operating 10,000 rpm for 10 min, supernatants were separated [14]. A small part of the kidney was fixed in Bouin's fixative for histology.

Biochemical analysis by semi autoanalyzer, UV-VIS Spectrophotometer, and ELISA Plate Reader

Biochemical estimation of urea and creatinine level of plasma and urine were measured by commercially available standard kit with standard protocol for photometric determination by semi autoanalyzer [6,8]. SOD, catalase, MDA level of kidney tissue measured in UV-VIS Spectrophotometer by standard protocol [9,15,16]. IL-18 [17,18], KIM-1 [18,19], and cystatin-C [20] were measured by commercially available standard kit with standard protocol for kit literature by an ELISA plate reader.

Polyacrylamide gel electrophoresis (PAGE)

Denaturing sodium dodecyl sulfate (SDS)-PAGE was made after over 14 day's period of either APAP administration or coadministration of MFTA in rats with normal feeding rats. Urine sample was centrifuged at 10,000 rpm for 10 min, and the supernatant was discarded. Pellet was mixed with an equal volume of ×2 electrophoresis sample buffer (0.125 m Tris-Hydrochloride, 4% SDS, 20% v/v glycerol, 0.2 M Dithiothreitol, and 0.02% bromophenol blue, pH 6.8), 5 µg protease cocktail and heated at 95°C for 5 min, was analyzed by SDS-PAGE according to the method of Laemmli using 15% acrylamide resolving gels and 10% stacking gel. Electrophoresis was performed on Tarson electrophoresis apparatus. After washing in water for 5 min, the gels were stained with Coomassie Brilliant Blue R250 (0.1% in 25% methanol, and 10% acetic acid). The distaining step was done with Coomassie Brilliant Blue by incubation in 30% acetic acid distinguished with blue stain for protein bands [21,22].

Histological examination

Kidney fixed in Bouin's fixative for 24 h. Then kidney tissues were dehydrated with an elevated degree of ethanol and embedded in paraffin for preparing paraffin blocks. Sections from kidney tissue (4–5 mm) were stained with hematoxylin-eosin. The sections were examined and photographed by a Magnus MLX PLUS microscope (Nagano, Japan) [23].

Statistical Analysis

Data were analyzed using the origin software package (version 9.1). Analysis of variance (ANOVA) was followed by a multiple two-tail "t"-test at the significance level ($p<0.05$) and expressed as SEM (n=5) [24].

RESULTS

Changes of old kidney disease biomarkers versus new kidney disease biomarkers of progression of kidney disease by APAP administration

APAP administration for 5 days in Group 2 (A-5) rats rises plasma urea 38.39% and plasma creatinine 51.61% significantly ($p<0.05$) than untreated Group 1 rat, while IL-18, KIM-1 and cystatin-C level in urine raised in 75.41%, 93.50%, and 307.69%, respectively, then Group 1. All biomarkers in plasma and urine significantly increased ($p<0.05$) in Group 2 (A-5) than Group 1 (Table 1, Fig. 1 G-2a and G-2b).

APAP administration for 10 days in Group 2 (A-10) elevates plasma urea 112.11% and plasma creatinine 96.77% significantly ($p<0.05$) than untreated Group 1 rats, while IL-18, KIM-1, and cystatin-C level of

Table 1: Changes of new biomarkers either APAP administration or co-administration of MFTA with APAP on plasma, urine, and kidney tissue. Data are expressed as SEM (n=5). ANOVA followed by multiple two-tail t-test and data with different superscripts letters (a, b, c, d, e, f, and g) revealed significant difference from each other (P<0.05). Group 1, normal rats without inducing kidney injury. Group 2, APAP administration rats groups (A-5, A-10, and A-15, respectively, APAP administration for 5 days, 10 days, and 15 days), Group 3: APAP with MFTA coadministration rats (TA-5, TA-10, and TA-15, respectively, APAP with MFTA coadministration for 5 days, 10 days, and 15 days)

Parameters	Groups						
	Group 1	Group 2			Group 3		
		A-5	A-10	A-15	TA-5	TA-10	TA-15
Plasma urea (mg/dl)	25.42±0.18^{a}	35.18±0.17^{b}	53.92±0.39^{c}	76.26±0.20^{d}	31.46±0.46^{e}	34.56±0.27^{f}	35.44±0.35^{g}
Urine urea (mg/dl)	88.30±0.26^{a}	39.68±0.52^{b}	38.04±0.30^{c}	30.36±0.39^{d}	40.96±0.27^{e}	47.72±0.38^{f}	56.32±0.82^{g}
Plasma creatinine (mg/dl)	0.31±0.01^{a}	0.47±0.01^{b}	0.61±0.01^{c}	0.76±0.01^{d}	0.38±0.01^{e}	0.41±0.01^{f}	0.39±0.01^{g}
Urine creatinine (mg/dl)	3.54±0.05^{a}	1.34±0.05^{b}	0.74±0.04^{c}	0.51±0.03^{d}	0.79±0.03^{e}	1.03±0.03^{f}	1.84±0.14^{g}
Urine KIM-1 (pg/ml)	168.30±1.88^{a}	295.22±1.73^{b}	395.11±1.58^{c}	495.04±1.98^{d}	174.90±1.17^{e}	194.20±3.40^{f}	182.21±2.13^{g}
Urine IL-18 (pg/ml)	14.94±0.17^{a}	28.91±0.34^{b}	39.07±0.16^{c}	49.38±0.33^{d}	18.26±0.17^{e}	22.46±0.66^{f}	27.22±0.96^{g}
Urine cystatin C (µg/ml)	0.13±0.01^{a}	1.13±0.01^{b}	1.30±0.01^{c}	1.44±0.01^{d}	0.44±0.01^{e}	0.34±0.01^{f}	0.33±0.01^{g}

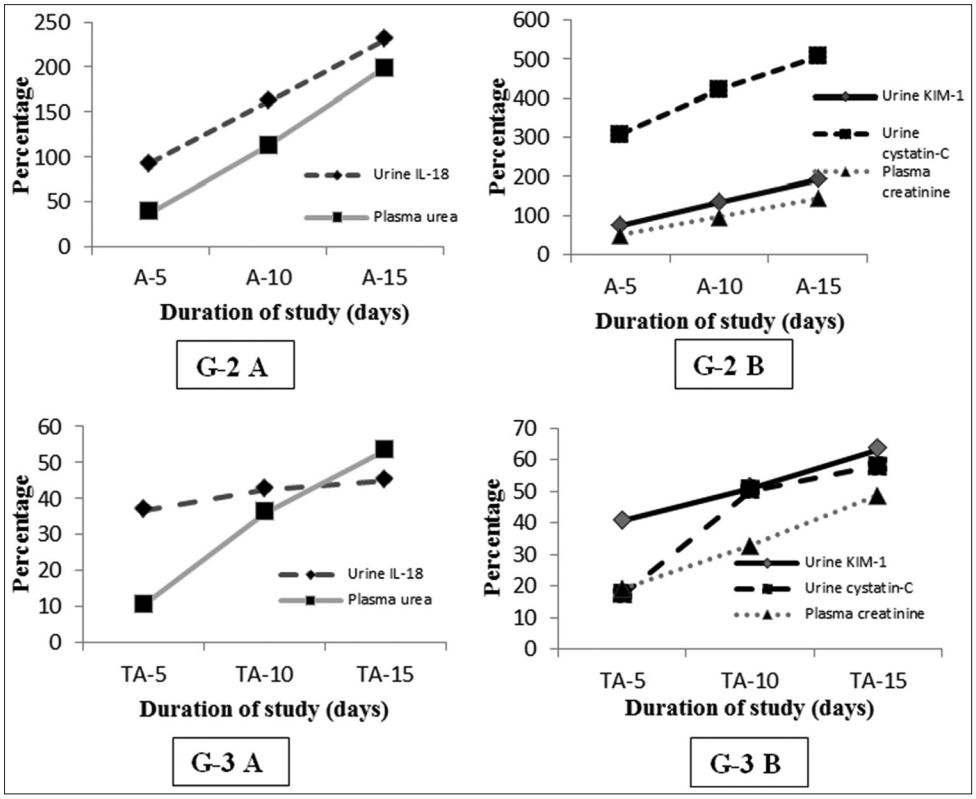

Fig. 1: Line diagram represents the percentage against duration of study (days). Urine IL-18 and plasma urea shows of group 2 (G-2a) and group 3 (G-3a) and urine KIM-1, cystatin-c, plasma creatinine shows of group 2 (G-2b) and group 3 (G-3 b). Fig.1: Line diagram represents the percentage against duration of study (days). Urine IL-18 and plasma urea shows of group 2 (G-2a) and group 3 (G-3a) and urine KIM-1, cystatin-c, plasma creatinine shows of group 2 (G-2b) and group 3 (G-3b)

urine elevated in 161.51%, 134.76%, and 423.07%, respectively, than Group 1. All biomarkers in plasma and urine significantly increased (P<0.05) in Group 2 (A-10) than Group 1 (Table 1, Fig. 1 G-2a and G-2b).

APAP administration for 15 days in group 2 (A-15) increases plasma urea 200.00% and plasma creatinine 145.16% significantly (p<0.05) than untreated Group 1 rats, while IL-18, KIM-1, and cystatin-C level of urine increased in 230.52%, 194.14%, and 507.69%, respectively, then Group 1. All biomarkers in plasma and urine significantly increased (p< 0.05) in Group 2 (A-15) than Group 1 (Table 1, Figs. 1 G-2a and G-2b).

Effect of APAP administration for 5 days, 10 days, and 15 days created a vast difference between plasma old biomarkers and urinary proteo-immuno new biomarkers. KIM-1 and cystatin-C are low molecular weight signaling proteins molecules. IL-18 is synthesized from tissue macrophages in epithelial cells of the proximal tubule.

Effect of MFTA on changes of old and new kidney disease biomarkers

Peritoneal administration of APAP with coadministration of MFTA orally for 5 days decreases plasma urea 23.76% and plasma creatinine 19.14% in Group 3 (TA-5) then Group 2 (A-5) rats, while IL-18, KIM-1, and cystatin-C level in urine decreased in 22.22%, 3.9%, and 16.98%, respectively, in TA-5 then A-5. All biomarkers in plasma and urine significantly decreased (p<0.05) in TA-5 than A-5 (Table 1, Figs. 1 G-3a and G-3b).

Peritoneal administration of APAP with coadministration of MFTA orally for 10 days in Group 3 (TA-10) reduces plasma urea 35.95% and plasma creatinine 32.78% then Group 2 (A-10) rats, while IL-18, KIM-1, and cystatin-C level in urine reduced in 50.33%, 15.38%, and 50.00%, respectively, in TA-10 than A-10. All biomarkers in plasma and urine significantly reduced (p<0.05) in TA-10 than A-10 (Table 1, Figs. 1 G-3a and G-3b).

Peritoneal administration of APAP with coadministration of MFTA orally for 15 days in Group 3 (TA-15) declines plasma urea 39.41% and plasma creatinine 48.68% then Group 2 (A-15) rats, while IL-18, KIM-1, and cystatin-C level in urine declined by 82.19%, 8.26%, and 58.22%, respectively, in TA-15 when compared to A-15. All biomarkers in plasma and urine significantly decreased (p<0.05) in TA-15 then A-15 (Table 1, Figs. 1 G-3a and G-3b).

Effect of MFTA on SOD, Catalase, and MDA (oxidative biomarkers)

Effect of APAP in co administration with MFTA on kidney tissue levels of SOD, catalase and MDA were shown in Table 2. Quantification level of SOD and catalase significantly low (P<0.05) in kidney tissue of A-5, A-10, and A-15 rats was compared to other two groups, but MDA level is significantly high (P<0.05) in compare to Group 1, TA-5, TA-10, and TA-15. MFTA coadministration with APAP causes improvement of oxidative stress in kidney tissues.

Correlation between old biomarkers and new biomarkers

Correlation analysis was also represented graphically by a scatter diagram. Urine IL-18 correlates with plasma urea and urine KIM-1 correlates with plasma creatinine. Urine IL-18 and urine KIM-1 values were taken on vertical axis versus plasma urea and plasma creatinine values on horizontal axis (Figs. 2 and 3). Group 1 represented a medium uphill (positive, r=0.434) relationship between urine IL-18 and plasma urea (Fig. 3 G-1a) and a weak downhill (negative, r=0.257) relationship between urine KIM-1 and plasma creatinine (Fig. 3 G-1b). Group 2 showed APAP administration for 5 days (Group A-5), 10 days (Group A-10), and 15 days (Group A-15) a downhill (negative, r=0.302, 0.066, and 0.754, respectively) relationship between urine IL-18 and plasma urea (Figs. 2 A-5A, 2 A-10A, A-15A). KIM-1 and plasma creatinine represent a strong uphill (positive, r=0.772) relationship (Fig. 3 A-5B), a medium downhill (negative, r=0.541) relationship (Fig. 3; A-10B) and a weak downhill (negative, r=0.294) relationship (Fig. 3 A-15B) in

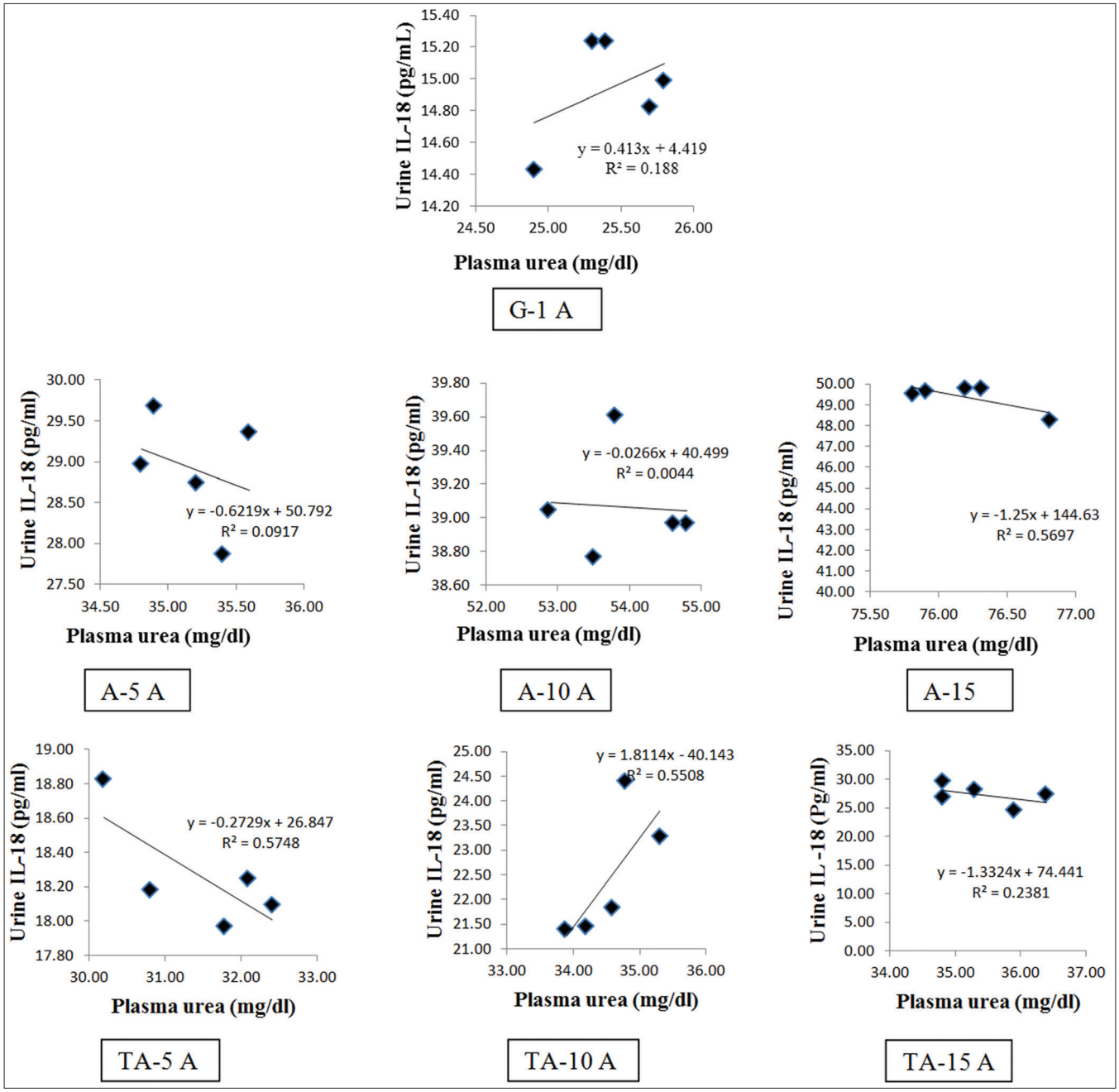

Fig. 2: Scatter diagram represents the correlation in between Urine interleukin-18 (IL-18) and Plasma urea, G-1 A: normal rats group. A-5 A: A-5 group rats (acetaminophen administration for 5 days), A-10 A: A-10 group rats (acetaminophen administration for 10 days), A-15 A: A-15 group rats (acetaminophen administration for 15 days). TA-5 A: TA-5 group rats (methanol fraction of Terminalia arjuna [MFTA] co-administration with acetaminophen administration for 5 days), TA-10 A: TA-10 group rats (MFTA co-administration with acetaminophen administration for 10 days), TA-15 A: TA-15 group rats (MFTA co-administration with acetaminophen administration for 15 days)

Group 2. Group 3 reveals MFTA coadministration with APAP a downhill (negative, r=0.758, 0.488) association (Figs. 2 TA-5A, TA-15A) and a strong uphill (positive, r=0.742) association (Fig. 2; TA-10A) in between urine IL-18 and plasma urea. KIM-1 and plasma creatinine correlation reveals a weak downhill (negative, r=0.024) relationship (Fig. 3 TA-15B) and a weak uphill (positive, r=0.035) relationship (Fig. 3 TA-5B) but a strong uphill (positive, r=0.972) relationship (Fig. 3 TA-10B) in Group 3.

Denaturing SDS-PAGE analysis

SDS-PAGE showed different protein aggregated bands (Fig. 4) corresponding to the molecular weight marker (MWM). MWM represented eight separate protein bands showing 75 Kilodalton (kDa) to 11 kDa in descending orders. C1 and C2 lane represented the urinary protein bands at 63, 20, and 11 kDa of Group 1 rats and there was no other band at 48, 38, 25, and 17 kDa. Urinary protein bands in Group 2 observed new bands at 35 kDa in A1 and A2 lane when compared to C1 and C2. It was also observed that band intensity at 11 kDa in A1 and A2 lane was less than C1 and C2. The MFTA coadministration represented the urinary protein bands in Group 3 – 63, 20, and 11 kDa and less intensity bands in 11 kDa of TA1 and TA2. It also has shown no band in 35 kDa that indicates improvement toward the normal condition.

Histological examination

The histological architecture of kidney indicated (Fig. 5) necrosis and atrophy in proximal tubule (black arrow in Fig. 5b) and glomerulus (black circle in Fig. 5b) in sub-group of Group 2 (APAP treated) compares

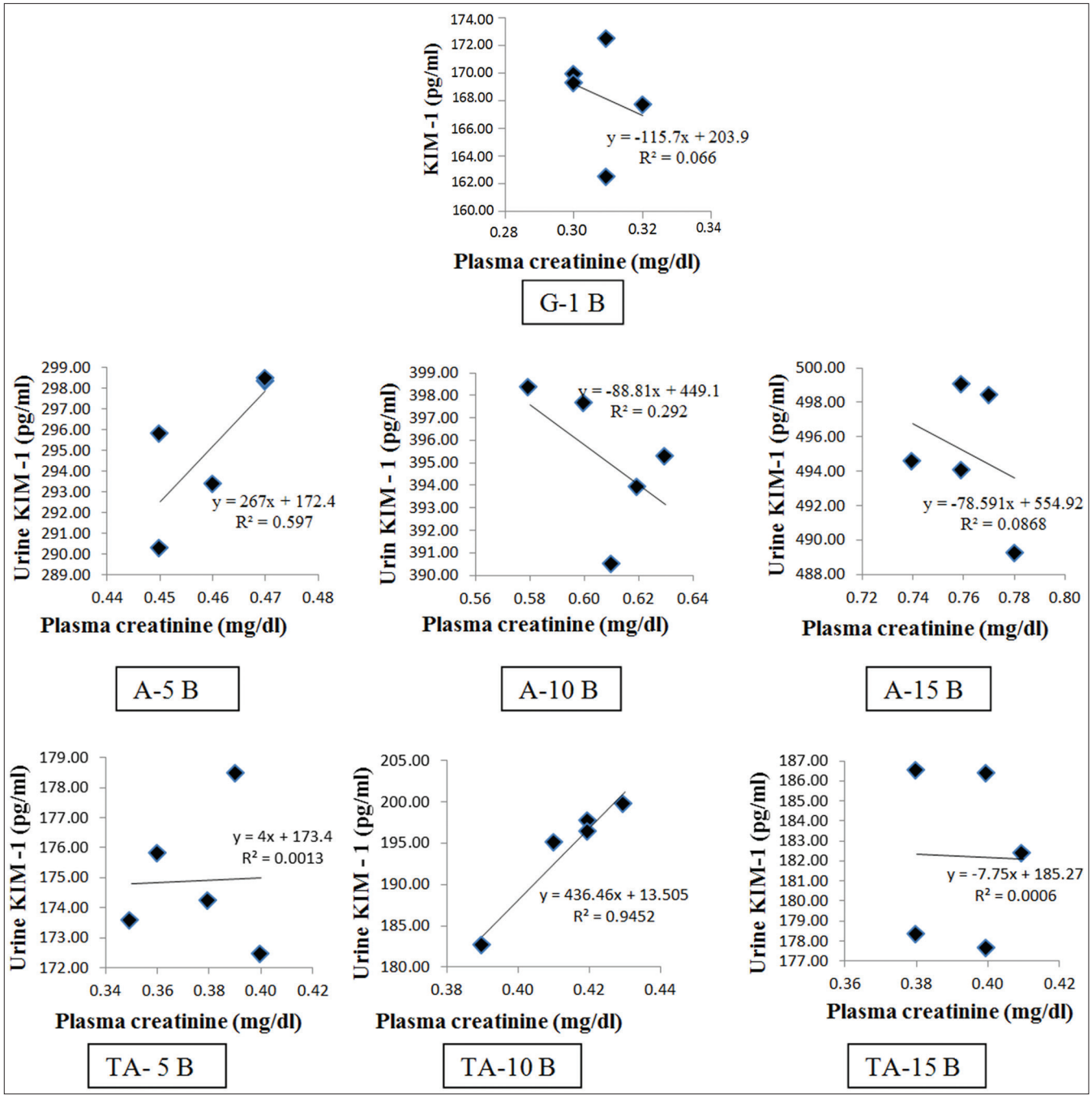

Fig. 3: Scatter diagram represents the correlation in between Urine KIM-1 and Plasma creatinine, G-1 B: normal rats group. A-5 B: A-5 group rats (acetaminophen administration for 5 days), A-10 B: A-10 group rats (acetaminophen administration for 10 days), A-15 B: A-15 group rats (acetaminophen administration for 15 days). TA-5 B: TA-5 group rats (methanol fraction of *Terminalia arjuna* [MFTA] co-administration with acetaminophen administration for 5 days), TA-10 B: TA-10 group rats (MFTA co-administration with acetaminophen administration for 10 days), TA-15 B: TA-15 group rats (MFTA co-administration with acetaminophen administration for 15 days)

to Group 1. Group 1 shows a normal proximal tubule (black arrow in Fig. 5a) and glomerulus (black circle in Fig 5a). Peritubular congestion and epithelial desquamation (black star in Fig. 5b) increased in APAP treated group in compared to MFTA coadministration group (Group 3) and improved or prevented injuries by regenerating normal glomerulus with light Bowmans degeneration and renal tubules (black circle in Fig. 5c, black arrow in Fig. 5c).

Old biomarkers versus new biomarkers on duration dependent study

Our study shown by line diagrams, increased percentage of old biomarkers (urea and creatinine) is less than the increased level of new biomarkers depends on the duration of the study. IL-18 and plasma urea level of Group 2 when compared with Group 1, the result proves the enriched level of IL-18 increased by 93.50%, 161.51%, and 230.52%, respectively, in 5th, 10th, and 15th days but while plasma urea level increased in only 38.39%, 112.11%, and 200.00%, respectively, in 5th, 10th, and 15th days for low sensitivity and protectivity (Fig. 1 G-2a). Furthermore, KIM-1, cystatin-C, and plasma creatinine when compared with Group 1, there the result shows KIM-1 level in 75.41%, 134.76%, and 194.14% and cystatin-C level in 307.69%, 423.07%, and 507.69% increased, respectively, in 5th, 10th, and 15th days but plasma creatinine shows 51.61%, 96.77%, and 145.16%, respectively, in 5th, 10th, and 15th days for poor sensitivity and protectivity in kidney injury

Table 2: Changes of old biomarkers either APAP administration or coadministration of MFTA with APAP on plasma, urine, and kidney tissue. Data are expressed as SEM (n=5). ANOVA followed by multiple two-tail t-test and data with different superscripts letters (a, b, c, d, e, f, and g) revealed significant difference from each other (P<0.05). Group 1, normal rats without inducing kidney injury. Group 2, APAP administration rats groups (A-5, A-10, and A-15, respectively, APAP administration for 5 days, 10 days, and 15 days), and Group 3: APAP with MFTA coadministration rats (TA-5, TA-10, and TA-15, respectively, APAP with MFTA coadministration for 5 days, 10 days, and 15 days).

Parameters	Groups						
	Group 1	Group 2			Group 3		
		A-5	A-10	A-15	TA-5	TA-10	TA-15
MDA (nmol/mg of tissue)	40.05±0.15^{a}	79.07±0.31^{b}	94.73±0.30^{c}	98.92±0.25^{d}	56.05±0.30^{e}	59.47±0.25^{f}	61.13±0.2^{g}
SOD (mmolof H_2O_2 consumption/mg of tissue/min)	0.87±0.01^{a}	0.11±0.01^{b}	0.16±0.01^{c}	0.25±0.01^{d}	0.42±0.01^{e}	0.50±0.0^{f}	0.56±0.01^{g}
Catalase (mmol of H_2O_2 consumption/mg of tissue/min)	0.57±0.01^{a}	0.13±0.01^{b}	0.19±0.01^{c}	0.21±0.01^{d}	0.37±0.01^{e}	0.40±0.01^{f}	0.41±0.01^{g}

MDA: Malondialdehyde

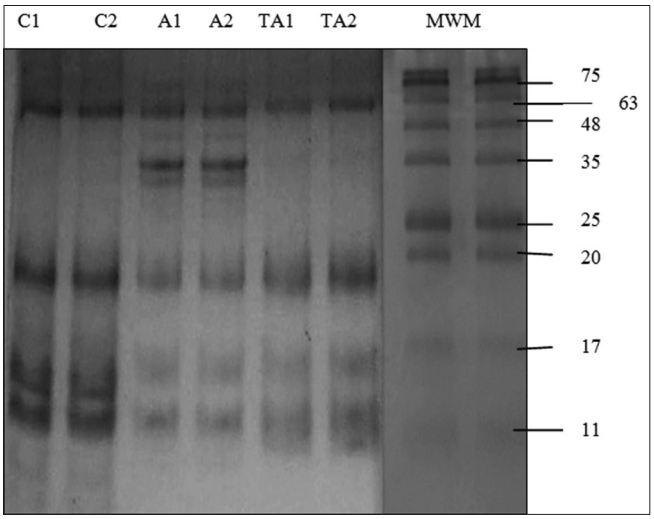

Fig. 4: Electrophoresis of urine samples from normal (C1 and C2), administration of acetaminophen (APAP) administration (A1 and A2), APAP with co-administration of methanol fraction of *Terminalia arjuna* (MFTA) (TA1 and TA2) performed under SDS-PAGE were run along with standard molecular weight markers (MWM). Patterns represent three different urinary fraction of rat's urine. (lane C1 and C2) Group 1 (normal rat urine sample), (lane A1 and A2) A-15 group (APAP treated for 15 days urine sample), (lane TA1 and TA2) TA-15 group (APAP treated for 15 days urine sample with co-administration of MFTA)

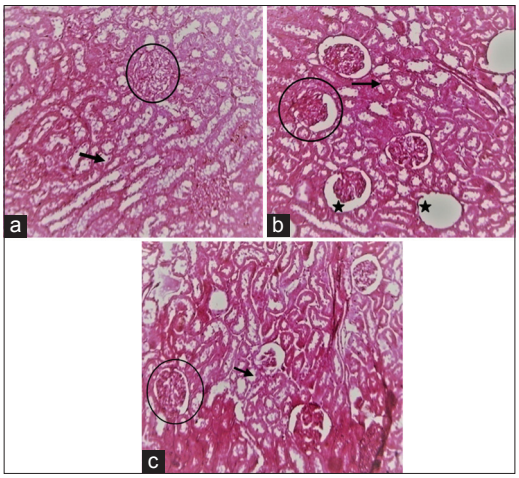

Fig. 5: Histological changes in kidney of rats. (a) Group 1 (normal rats, without inducing kidney injury rat kidney), (b) Group 2 (acetaminophen treated kidney of A-15 sub-group), (c) Group 3 (acetaminophen treated kidney with co-administration of methanol fraction of *Terminalia arjuna* of TA-15 sub-group)

(Fig. 1 G-2b). The percentage levels in Group 3 compared to Group 2 of IL-18, and plasma urea was decreased at an equal level at the presence of MFTA, 36.83%, 42.51%, and 44.87% and 10.57%, 35.90%, and 53.52%, respectively, in 5th, 10th, and 15th days (Fig. 1 G-3a). Identical same result was also found with KIM-1(40.75%, 50.84%, and 63.19%, respectively, in 5th, 10th, and 15th days), cystatin-C (16.98%, 50.00%, and 58.22%, respectively, in 5th, 10th, and 15th days), and plasma creatinine (19.14%, 32.78%, and 48.68%, respectively, in 5th, 10th, and 15th days) (Fig. 1 G-3b).

DISCUSSION

Our earlier investigation, acetaminophen-induced nephrotoxicity [8], MFTA having three major polyphenols details published earlier by corresponding author [2] showed nephroprotective activity on old biomarkers those were urea and creatinine [6] and also MFTA established as a powerful antioxidant evidenced on SOD, catalase, and MDA on kidney tissue [5]. Plasma urea is synthesized in liver, and plasma creatinine is a breakdown product of muscle protein, both were expected in late stages of kidney disease [29]. Plasma urea is screened high at late stages of kidney diseases. Used of plasma urea old biomarker at late stage patients suffer irreversible destruction and damage of kidney interstitium [25]. The study demonstrated that APAP administration in rats raises plasma urea 38.39% for 5 days, 112.11% for 10 days, and 200.00% for 15 days while urinary IL-18 raise 75.41% for 5 days, 161.51% for 10 days, and 230.52% for 15 days. Urinary biomarker is IL-18 (24 kDa), a proinflammatory and immunomodulatory cytokine in distal convoluted tubule and collecting tubule in kidney [26]. A mild injury or stress in distal and collecting tubule, elevation of IL-18 occurs invasive amount [27]. Plasma creatinine, end product of muscle protein breakdown, showed high in the late destructive stage of kidney [28]. We elaborate APAP administration in rats elevates plasma creatinine 51.61% for 5 days, 96.77% for 10 days, and 145.16% for 15 days even as urinary KIM-1 lifts up 75.41% for 5 days, 134.76% for 10 days, and 194.14% for 15 days. KIM-1, which found in proximal tubular epithelium transmembrane glycoprotein (90 kDa) absent in normal kidney tissue or urine. KIM-1 increased and excreted in urine a huge amount by mild tubular stress [29]. A low molecular weight protein (13 kDa) cystatin-C is prepared in all organs in our body while oxidative damage based necrosis of cells and tissues. It is completely assured by normal nephrons. It is contained in urine when any part of nephrons gets damaged [30]. First 5 days APAP administration increases urinary cystatin-C level 307.69%, thereafter, 423.07% for 10 days, 507.69% for 15 days. However, it is distinctly clear urinary IL-18, KIM-1, and cystatin-C showing a higher level in duration dependent APAP administration than plasma urea and plasma creatinine. In our study, coadministrated MFTA with APAP to another groups of rats which was effective in reducing old kidney biomarkers plasma urea, creatinine, and new kidney biomarkers urinary IL-18, KIM-1, and cystatin-C. The authors speculate that antioxidant enzymes, SOD, and catalase

in kidney tissues significantly enhanced and significantly diminished MDA by MFTA feeding orally [6]. It is difficult to conclude with the exact mechanism by which MFTA controls the kidney biomarkers. It should be hypothesized that three major compounds (earlier published, [2]) have a significant main effect on renal tubules.

Evidence from correlation study in between plasma urea and urine IL-18 in untreated rats, APAP administration for 5 days and 10 days, there was no significant relation of urea and IL-18 while significantly related to 15 days APAP administration. Plasma creatinine was not significantly correlated with urinary KIM-1 where there were strongly significant correlated with APAP administration for 5 days. MFTA causes negative correlation with plasma creatinine and urinary KIM-1.

Urinary IL-18, KIM-1, and cystatin-C are low molecular protein measured by ELISA kit method which was strong evidenced with the presence of extra band around 25–30 kDa of APAP administration by SDS-PAGE. Histological observations showed according to Abhirama *et al.*, tubular degeneration, desquamation, necrosis, intertubular hemorrhage, and hyaline in tubules with congestion and swelling in glomerulus evaluated due to nephrotoxicity in animals [31]. However, coadministration of MFTA caused marked histological protection on tissue injuries including tubular necrosis, capillary congestion, and epithelial desquamation against APAP administration in Group 3. Cirevalli *et al.* suggested that CKD patient with low-income also against the provision of various drugs to reduce the financial burden on pharmaceutical costs not only for the patient but also to caregivers to their family [32].

CONCLUSION

It can be possible to conclude that the use of these urinary low molecular weight proteins biomarkers could be used to monitor at risk in kidney disease patients. These biomarkers will allow to diagnose kidney disease at a much earlier stage. MFTA possess anti-uremic and urinary biomarkers suppressive capacity. The kidney protective effect in this study provides a mechanistic evidence for why tribal people in our Jangalmahal area of Paschim Medinipur and Jhargram, state of West Bengal, India found it useful for treating kidney ailments as well as being used to cure other health disorders.

ACKNOWLEDGEMENT

Authors are grateful to acknowledge the Department of Science and Technology, Government of West Bengal for financial support as a major project (Memo no.-804(Sanc.)/ST/P/S&T/1G-32/2016, Dated-06/12/2016).

AUTHOR'S CONTRIBUTIONS

1. Deblina Giri had contributed in definition of intellectual content, literature search, data acquisition, data analysis, manuscript preparation, and manuscript editing.
2. Koushik Das had contributed in concept, design, manuscript preparation, and manuscript editing.
3. Dilip Kumar Nandi had contributed in concept, statistical analysis, and data acquisition.

CONFLICTS OF INTEREST

All the authors have an equal contribution in the manuscript and declare no conflicts of interest.

REFERENCES

1. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 2004;351:1296-305.
2. Das K, Roy S, Mandal S, Pradhan S, Debnath S, Ghosh D, *et al.* Renal protection by isolated phytocompounds from TA bark fraction on dehydration induced uremia, oxidative stress and kidney disease rats. Int J Rec Sci Res 2016;7:13787-95.
3. Das K, Ghosh D, Nandi DK. Reno-protective effect of bark extract of TA on dehydration induced uremia of rat. Int J Physiol Allied Sci 2011;65:43-58.
4. Roy S, Das K, Mandal S, Pradhan S, Patra A, Nandi DK. Crude root extract of *Asparagus racemosus* ameliorates acetaminophen induced uremic rats. Int J Pharm Sci Res 2013;4:3004-12.
5. Das K, Samanta TT, Samanta P, Nandi DK. Effect of extract of *Withania somnifera* on dehydration-induced oxidative stress-related uremia in male rats. Saudi J Kidney Dis Transpl 2010;21:75-80.
6. Pradhan S, Mandal S, Roy S, Mandal A, Das K, Nandi DK. Attenuation of uremia by orally feedingalpha – Lipoic acid on acetaminophen induced uremic male rats. Saudi Pharm J 2013;21:187-92.
7. Mandal A, Nandi DK, Roy S, Das K, Mondal KC. *In vivo* assessment of bacteriotherapy on acetaminophen induced uremic rats. J Nephrol 2013a;26:228-36.
8. Roy S, Pradhan S, Das K, Mandal A, Mandal S, Patra A, *et al*. Acetaminophen induced kidney failure in rats: A dose response study. J Biol Sci 2015;15:187-93.
9. Das K, Chakraborty PP, Ghosh D, Nandi DK. Protective effect of aqueous extract of TA against dehydrating induced oxidative stress and uremia in male rat. Iran J Pharm Res 2010a;9:153-61.
10. Bonventre JV. Kidney injury molecule-1 (KIM-1): A urinary biomarker and much more. Nephrol Dial Transplant 2009;24:3265-8.
11. Liang XL, Liu SX, Chen YH, Yan LJ, Li H, Xuan HJ, *et al*. Combination of urinary kidney injury molecule-1 and interleukin-18 as early biomarker for the diagnosis and progressive assessment of acute kidney injury following cardiopulmonary bypass surgery: A prospective nested case-control study. Biomarkers 2010;15:332-9.
12. Herget-Rosenthal S, Marggraf G, Hüsing J, Göring F, Pietruck F, Janssen O. Early detection of acute renal failure by serum cystatin C. Kidney Int 2004;66:1115-22.
13. Olfert ED, Cross BM, McWilliam AA, editors. Guide to the Care and Use of Experimental Animals. Ottawa, Canada: Canadian Council on Animal Care; 1993. p. 82-93.
14. Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, *et al.* Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 2006;69:1471-6.
15. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
16. Marklund S, Marklund G. Involvement of superoxide anion radical in auto oxidation of pyrogallol and a convenient assay of superoxide dismutase. Eur J Biochem 1974;47:469-74.
17. Dinarello CA, Novick D, Puren AJ, Fantuzzi G, Shapiro L, Mühl H, *et al*. Overview of interleukin-18: More than an interferon-gamma inducing factor. J Leukoc Biol 1998;63:658-64.
18. Ren H, Zhou X, Dai D, Liu X, Wang L, Zhou Y, *et al*. Assessment of urinary kidney injury molecule-1 and interleukin-18 in the early post-burn period to predict acute kidney injury for various degrees of burn injury. BMC Nephrol 2015;16:142.
19. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney injury molecule-1 (KIM-1): A novel biomarker for human renal proximal tubule injury. Kidney Int 2002;62:237-44.
20. Jiang R, Xu C, Zhou X, Wang T, Yao G. Detection of cystatin C biomarker for clinical measurement of renal disease by developed ELISA diagnostic kits. J Transl Med 2014;12:205.
21. Dunn MJ. Gel Electrophoresis PROTEIN. Oxford: BIOS Scientific Publishers Ltd.;1993.
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
23. Palani S, Raja S, Kumar RP, Jayakumar S, Kumar BS. Therapeutic efficacy of *Pimpinella tirupatiensis* (*Apiaceae*) on acetaminophen induced nephrotoxicity and oxidative stress in male albino rats. Int J Pharm Tech Res 2009;1:925-34.
24. Sokal RR, Rohle FJ. Introduction to analysis of variance. In: Biometry. New York; W H Freeman and Company: 1997. p. 179-206.
25. Sirota JC, Klawitter J, Edelstein CL. Biomarkers of acute kidney injury. J Bras Nefrol 2013;35:229-36.
26. Parikh CR, Jani A, Melnikov VY, Faubel S, Edelstein CL. Urinary interleukin-18 is a marker of human acute tubular necrosis. Am J Kidney Dis 2004;43:405-14.
27. Lin X, Yuan J, Zhao Y, Zha Y. Urine interleukin-18 in prediction of

acute kidney injury: A systemic review and meta-analysis. J Nephrol 2015;28:7-16.
28. de Geus HR, Betjes MG, Bakker J. Biomarkers for the prediction of acute kidney injury: A narrative review on current status and future challenges. Clin Kidney J 2012;5:102-8.
29. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, *et al.* Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem 1998;273:4135-42.
30. Gong R, Rifai A, Dworkin LD. Anti-inflammatory effect of hepatocyte growth factor in chronic kidney disease: Targeting the inflamed vascular endothelium. J Am Soc Nephrol 2006;17:2464-73.
31. Abhirama BR, Shanmuga Sundaram R, Raju A. Amelioration of gentamicin-induced renal damage in rats by ethanol extract of the whole plant *Biophytum sensitivum* (Linn.) Dc. Int J Pharm Pharm Sci 2018;10:130-5.
32. Cirevalli P, Nimmanapalli HD, Parlapalli LK, Nuthalapati LS, Kuttiappan A, Bhotla SK. Pill burden, drug class distribution and financial burden for buying medicines in different modalities of chronic kidney disease patients: Cross-sectional study. Int J Pharm Pharm Sci 2018;10:165-70.