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Therapeutic Efficacy of Bi-Herbal Extracts of *Aegle Marmelos* and *Annona Squamosa* on Adenine Induced Chronic Kidney Disease in Rats

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Abstract
This study aimed to assess the therapeutic impact of bi-herbal aqueous and alcoholic extracts from *Aegle marmelos* (AM) and *Annona squamosa* (AS) on adenine-induced chronic kidney disease (CKD) in rats. The extracts were combined in a 0.5:1 and 1:0.5 ratio for AM and AS, respectively, as determined by *in-vitro* nucleation assay. Thirty-six male Sprague Dawley rats were divided into six groups, each consisting of six rats. Control group I remained untreated and did not receive adenine or plant extracts. Group II, serving as the adenine control, received oral adenine at 200 mg/kg once a day for 28 days. Chronic kidney disease was induced in groups II to VI by administering a daily dose of 200 mg/kg b.wt. adenine via intra-gastric route for 28 days, establishing an adenine-induced CKD rat model. Following 28 days of CKD induction, groups III and IV received oral administration of aqueous bi-herbal extracts of AM and AS (in a 0.5:1 ratio) at doses of 250 mg/kg and 500 mg/kg b.wt., respectively. Meanwhile, groups V and VI were given alcoholic bi-herbal extracts of AM and AS (in a 1:0.5 ratio) at doses of 250 mg/kg and 500 mg/kg b.wt., respectively, once daily for an additional 42 days. In this study, various parameters including body weight, feed consumption, haemato-biochemical analysis, urine assessment, ultrasonographic examination, and histopathological findings were evaluated after CKD induction. Treatment with aqueous bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* leaves at a dosage of 250 mg/kg b.wt, and alcoholic bi-herbal extracts at 500 mg/kg b.wt showed superior efficacy compared to the lower dosage. Remarkably, the alcoholic extracts exhibited greater efficacy compared to the aqueous extracts. Conclusively, the study suggests that administering bi-herbal aqueous extracts of *Aegle marmelos* and *Annona squamosa* at 250 mg/kg b.wt in a 0.5:1 ratio and bi-herbal alcoholic extracts at 500 mg/kg b.wt in a 1:0.5 ratio orally for 42 days post CKD induction in rats exhibited enhanced effectiveness in treating CKD. Further exploration in larger animals is warranted based on these findings.

Keywords: *Aegle marmelos*, *Annona squamosa*, Bi-herbal extracts, Therapeutic efficacy, CKD rats

Introduction
Chronic kidney disease (CKD) is a gradual and relentless pathological condition characterized by the presence of both structural and functional anomalies within the renal system, stemming from a diverse array of etiological factors. The prevailing contributors to CKD on a global scale are predominantly diabetes and hypertension (Ezekiel *et al*., 2019) [1]. Prolonged disease progression may culminate in end-stage kidney disease characterized by unfavourable clinical outcomes, necessitating the implementation of dialysis or kidney transplantation. Rat models induced through adenine-supplemented diets offer valuable insights into the pathophysiology of CKD, facilitating the evaluation of more effective therapeutic interventions (Yokozawa *et al*., 1982) [2]. Supplementation with 0.75% adenine in the diet rapidly induced kidney damage, including enlargement with granular appearance, apoptotic lesions, 70-80% kidney tissue damage with fibrosis, increased BUN and plasma creatinine, azotemia, abnormal electrolyte metabolism, and oxidative stress within 4 weeks. Currently, there is no single drug for treating kidney function in CKD patients, and long-term allopathic drug therapy has many adverse effects. Developing novel therapies or dietary supplements, especially from natural products or herb-derived sources, is crucial to mitigate the effects of the disease or slow down/reverse the deterioration in kidney function (Shuvy *et al*., 2011; Badreldin *et al*., 2017; Diwan *et al*., 2017) [3, 4, 5].

Aegle marmelos or Bael are belongs to the citrus family Rutaceae and grows in the dry forests of central India and Bangladesh (Sharma *et al*., 2007) [6]. Different parts of the Bael tree possess potential curative properties for various diseases, including reported effects like cardiotonic, hypoglycaemic, antidyslipidemic and anticancer properties in its leaf extract. Additionally, Bael is used to treat conditions like cholera, dropsy, ulcers, ophthalmia and ulcerative colitis (Knight, 1980; Karunanayake *et al*., 1984; Chockalingam *et al*., 2012 and Sankeshi *et al*., 2013) [7, 8, 9, 10]. *Annona squamosa* Linn, a member of the Annonaceae family, is native to South America and the West Indies, widely grown in Thailand and India (Suresh *et al*., 2006) [11]. Previously reported studies showed that the *Annona squamosa* leaves have various health beneficial effects due to their phytochemical compositions including anticancer, hepatoprotective, lipid-lowering, antidiabetic, anti-obesity and antioxidant properties. The plant has historically been used to treat epilepsy, diarrhoea, cardiac issues, worm infections, constipation, bleeding, antibiotic infections, dysuria, fever and ulcers and it also possesses anticancer, antifertility, and abortifacient activities (Biba *et al*., 2014) [12].

2 Materials and Methods

2.1 Experimental Animals

The study was conducted using thirty-six healthy male Sprague Dawley rats, aged 8-10 weeks, were procured from the Zydus Research Centre, Ahmedabad, Gujarat. The rats were kept in the Small Animal House facility at Veterinary College, Kamdhenu University, Anand. The research protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science and Animal Husbandry (IAEC/372/VPT/2022) and all the animal procedures were carried out according to the guidelines of Committee for Control and Supervision of Experiments on Animals (CCSEA).

2.2 Preparation of plant extracts

Leaves of *Aegle marmelos* and *Annona squamosa* were dried, ground into a powder, and stored in air-tight containers. Aqueous extracts were prepared by soaking 100 g of each dried leaf powder in 1 liter of distilled water with thrice-daily shaking. The obtained extracts were concentrated in a rotary evaporator under reduced pressure at 50-60 °C, resulting in a residue that was labeled, stored. To prepare alcoholic extracts, 100 g of coarse powdered *Aegle marmelos* and *Annona squamosa* leaves were extracted in a soxhlet extractor with alcohol as the solvent. The obtained extracts were concentrated in a rotary evaporator at 50-60°C under reduced pressure, resulting in a dark brown residue. The alcoholic extracts were transferred to a petri dish and evaporated over a hot water bath (50°C) until the solvent completely evaporated, after which they were stored at 4°C for further use. Both the aqueous and alcoholic extracts were preserved in a refrigerator at 4°C for future experimental use.

2.3 Bi-herbal extract ratio determination and procedure of *In Vitro* nucleation assay

In this study, an *in vitro* nucleation assay was conducted to determine the proportion of plant extracts. Stock solutions of *Aegle marmelos* and *Annona squamosa* extracts were prepared, and different proportions of bi-herbal extracts were derived from these stock solutions. A buffer solution with Tris-HCl (0.05 mol/L) and NaCl (0.15 mol/L) at pH 6.5 was used to create a calcium chloride solution (5 mmol/L) and sodium oxalate solution (7.5 mmol/L). Bi-herbal extracts were combined with calcium chloride solution, and after adding sodium oxalate solution, crystallization occurred at a constant temperature of 37 °C. The optical density (OD) of the solution was measured at 620 nm after 30 minutes to calculate the rate of nucleation. The most significant inhibition was observed at a ratio of 0.5:1 in aqueous bi-herbal extracts and a ratio of 1:0.5 in alcoholic bi-herbal extracts. Hence, for evaluating the effectiveness of combined extracts of AM and AS in adenine-induced chronic kidney disease, a 1/2 part of AM and 1 part of AS were selected for aqueous bi-herbal extract, and 1 part of AM and 1/2 part of AS were chosen for alcoholic bi-herbal extract, administered at doses of 250 mg/kg and 500 mg/kg.

2.4 Experimental design

Control group I was kept untreated and do not received adenine or plant extracts. Group II was administered by adenine @ 200 mg/kg daily once orally for 28 days and was considered as adenine control group. Groups III, IV, V and VI were subjected to a 28-day administration of adenine to induce chronic kidney disease (CKD). After 28 days of induction of CKD model in rats, Groups III and IV received bi-herbal aqueous extracts of *Aegle marmelos* and *Annona squamosa* (ratio 0.5:1) orally @ 250 & 500 mg/kg b.wt., respectively daily once for another 42 days. Groups V and VI received bi-herbal alcoholic extracts of *Aegle marmelos* and *Annona squamosa* (ratio 1:0.5) orally @ 250 & 500 mg/kg b.wt., respectively daily once for another 42 days.

2.5 Research protocol

2.5.1 Clinical observation and mortality

Throughout the experimental period, all the rats from groups I to VI were monitored daily for any abnormal physical or behavioural changes as well as mortality.

2.5.2 Estimation of feed consumption

The quantity of feed offered to the group of animals kept in each cage depending on their needs was recorded and the leftover feed was measured every week.

2.5.3 Measurement of body weight

The body weight of all animals in Groups I to VI was measured on the day before starting of the experiment (Day 0), and then further weight was taken on 1st, 2nd, 3rd, 4th,5th,6th,7th,8th,9th,10th week of the experiment.

2.5.4 Blood collection and haemato-biochemical analysis

Blood samples were collected twice during the experiment: on the 28th and 70th day from normal control and therapeutic group of rats. Blood was collected in K_3EDTA vials for the haematological examination whereas in serum clot activator vials for serum-biochemical analysis. The collected blood samples were then subjected to a detailed haematological analysis using an automatic whole blood analyser (Abacus Junior Vet-5, Austria), on the same day to ensuring accurate and precise haematological results. Simultaneously, blood samples were collected from all the rat groups in serum clot activator vials and then subjected to separate serum and stored at -80 °C for further serum-biochemical analysis.

Serum uromodulin concentration was determined using the Enzyme-linked Immunosorbent Assay Kit (MyBioSource, California, USA). Other serum biochemical parameters like serum creatinine, Uric Acid, Blood Urea Nitrogen (BUN), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Albumin, Total Protein, Calcium and Phosphorus were analysed using standard assay kits on the Clinical Chemistry Analyzer (CKK 300, Bangalore, India).

2.5.5 Urine analysis
On day 28th and 70th urine samples from the control and prophylactic groups of rats were collected using metabolic cages. The urine samples were then analyzed for different qualitative parameters like urine pH and specific gravity using automated urine analyzer (Uriscan Optima II) and various biochemical parameters such as total protein, creatinine, calcium and phosphorus using a Mindray BS-120 chemistry analyzer.

2.5.6 Ultrasonography
All the experimental rats were examined by ultrasound technology on day 28th and 70th using Esaote MyLab40 VET (Esaote Europe B.V., Philipsweg 1, 6227 AJ Maastricht, Netherlands). Real-time B-mode imaging of the kidneys was performed using linear transducers with a frequency range of 3.5 to 12 MHz.

2.5.7 Histopathological examination
On 70th day, all the rats were sacrificed and kidney was collected. Tissues were fixed in the formalin. The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections from all the tissues were cut at 5-6 microns' thickness by automatic section cutting machine (Leica, Automatic Microtome Machine, Germany) at the department of veterinary pathology and stained with Haematoxylin and Eosin (H &E) stains (Luna, 1968). The H & E stained slides were observed under microscope and lesions were recorded.

2.5.8 Statistical analysis
Completely randomized design and one-way analysis of variance (ANOVA) was used to compare the means of various parameters by using IBM SPSS software (version 26.0). Significant differences ($P<0.05$) between different experimental groups were analyzed by Duncan's multiple range test (Duncan, 1995). All the data are presented as mean ± SE.

3 Results
The findings of the present study to evaluate the therapeutic efficacy of bi-herbal aqueous and alcoholic extracts of *Aegle marmelos* (AM) and *Annona squamosa* (AS) on adenine induced chronic kidney disease (CKD) in rats were carried out and results of various parameters like feed consumption, body weight, haemato-biochemical estimation, urine assessment, ultrasonographic examination and histopathological findings in rats were presented.

3.1 Clinical observational assessments
A wealth of fundamental and characteristic behavioural cues imparts valuable insights into the timing, zenith and longevity of the test drug's impact. These cues also illuminate the diverse range of pharmacological effects engendered by the drug. Rats belonging to Group I (control group) exhibited consistent and anticipated responses throughout the experimental course. In contrast, adenine-administered rats within Group II exhibited a variety of deviant behaviours, including manifestations of weakness, lethargy, diminished alertness, despondency, instances of diarrhoea, dehydration, coprophagia, salivation, polyuria and polydipsia. The rats in the III, IV, V, and VI groups exhibited some behavioural symptoms, such as polyuria, dehydration, and weakness for up to 28 days. Following day 28, rats in the III, IV, V, and VI groups were noticeably more active till the end of experiment on day 70.

3.2 Estimation of feed consumption
In this study, the average feed consumption in the adenine control group (Group II) was significantly lower than the control group in the first four weeks, as indicated in Table 1. However, feed consumption significantly improved in rats from therapeutic groups (III, IV, V, VI) after oral daily administration of aqueous and alcoholic extracts of *Aegle marmelos* and *Annona squamosa* at doses of 250 and 500 mg/kg body weight for 42 days following the induction of CKD and feed consumption restored to its normal physiological state.

Table 1: Comparison of mean (±SE) total feed consumption (g/day) of rats in different CKD treatment and control groups

Group	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC10
I	24.60±0.40^{b}	28.98±0.98^{b}	27.86±0.24^{b}	31.12±1.17^{b}	30.76±0.81^{b}	31.60±1.27^{c}	29.88±1.26^{c}	33.14±1.72^{d}	29.40±0.60^{c}	33.75±1.04^{e}
II	10.13±0.09^{a}	9.21±0.07^{a}	8.16±0.25^{a}	7.89±0.21^{a}	8.43±0.14^{a}	9.54±0.27^{a}	11.03±0.23^{a}	10.77±0.32^{a}	11.54±0.21^{a}	11.85±0.23^{a}
III	12.33±1.67^{a}	10.26±0.98^{a}	8.16±0.22^{a}	5.15±1.37^{a}	9.60±0.84^{a}	16.29±0.57^{b}	18.17±0.98^{b}	24.29±0.29^{c}	28.86±0.29^{c}	30.71±0.14^{cd}
IV	9.98±0.98^{a}	8.14±1.10^{a}	7.88±0.12^{a}	5.01±0.35^{a}	11.24±1.19^{a}	16.05±0.95^{b}	15.43±0.29^{b}	18.57±0.57^{b}	26.55±0.30^{b}	26.21±0.36^{b}
V	9.74±1.26^{a}	10.07±1.36^{a}	7.86±0.43^{a}	6.32±1.46^{a}	11.46±0.73^{a}	14.31±0.69^{b}	18.93±0.36^{b}	25.43±0.86^{c}	29.71±0.29^{c}	30.29±0.57^{c}
VI	12.12±1.98^{a}	9.76±2.00^{a}	7.83±0.40^{a}	6.20±3.58^{a}	10.71±1.71^{a}	14.80±1.70^{b}	21.29±1.15^{b}	26.00±0.57^{c}	31.43±0.57^{d}	32.71±0.71^{de}

Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c,….) within a same column differ significantly from each other ($P<0.05$). Group-I: Normal control, Group-II: Adenine control, Group-III: Adenine contain solution up to 28 days. After 28 days bi-herbal Aq. extract of AM and AS @ 250 mg/ kg b.wt. orally for another 42 days, Group-IV: Adenine contain solution up to 28 days. After 28 days bi-herbal Aq. extract of AM and AS @ 500 mg/ kg b.wt. orally for another 42 days, Group-V: Adenine contain solution up to 28 days. After 28 days bi-herbal Al. extract of AM and AS @ 250 mg/ kg b.wt. orally for another 42 days Group-VI: Adenine contain solution up to 28 days. After 28 days bi-herbal Al. extract of AM and AS @ 500 mg/ kg b.wt. orally for another 42 days

3.3 Measurement of body weight
The present study shows that mean body weight of adenine treated rats (group II) during week 1 to 10 were significantly lower to that of control group (I) shown in table 2. In therapeutic study, bi-herbal aqueous extracts in group III, IV

produced significant improvement in mean body weight as compare to adenine control group. However, bi-herbal alcoholic extracts in group V and VI produced dose-dependent significant improvement in mean body weight as compare to adenine control groups after induction of CKD.

Table 2: Comparison of mean (±SE) Body weight (g) in rats under different CKD treatment and control groups

Group	BW0	BW1	BW2	BW3	BW4	BW5	BW6	BW7	BW8	BW9	BW10
I	419.67±19.61	451.50±13.68^{c}	488.00±14.22^{c}	523.17±13.55^{b}	522.83±15.98^{b}	564.33±15.39^{c}	582.67±20.79^{c}	585.33±19.42^{c}	604.67±18.47^{d}	595.17±17.04^{e}	616.67±15.93^{d}
II	384.67±12.97	325.50±16.66^{a}	299.00±17.94^{a}	295.67±18.92^{a}	264.33±17.82^{a}	274.33±17.15^{a}	280.50±11.10^{a}	288.17±13.38^{a}	305.17±15.85^{a}	325.00±13.50^{a}	342.67±10.57^{a}
III	417.83±8.34	345.83±10.51^{ab}	325.00±10.66^{ab}	311.83±10.65^{a}	300.67±6.66^{a}	313.83±4.23^{b}	336.33±3.13^{b}	358.83±3.26^{b}	404.33±3.56^{b} ^{c}	424.00±1.88^{c} ^{d}	449.83±2.52^{c}
IV	404.83±11.28	358.67±11.00^{ab}	337.00±10.09^{ab}	307.17±13.17^{a}	293.50±16.51^{a}	317.83±7.53^{b}	345.83±6.07^{b}	367.67±6.56^{b}	382.33±4.39^{b}	395.17±1.54^{b}	402.33±1.54^{b}
V	414.83±11.28	368.67±11.00^{b}	347.00±10.09^{b}	317.17±13.17^{a}	303.50±16.51^{a}	327.83±7.53^{b}	355.83±6.07^{b}	377.67±6.56^{b}	392.33±4.39^{b} ^{c}	405.17±1.54^{b} ^{c}	412.33±1.54^{b}
VI	405.83±8.01	367.50±8.18^{b}	352.00±10.09^{b}	322.17±13.17^{a}	307.00±16.93^{a}	326.17±6.33^{b}	350.00±4.64^{b}	373.67±4.15^{b}	420.33±4.05^{c}	433.83±1.92^{d}	442.33±1.54^{c}

Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c, d, e) within a same column differ significantly from each other (P<0.05).

3.4 Haematological analysis

Haematological analysis was performed on 4th week (day 28) after induction of CKD and 10th week (day 70) of experiment have been presented in Table 3. On the 28th day of the experiment, the adenine control group-II (CKD) and therapeutic groups (III, IV, V, VI) showed a significantly decrease in hemoglobin (Hb), total erythrocyte count (TEC) and lymphocyte counts. Meanwhile, there was a significant increase in total leukocyte count (TLC) and granulocyte counts compared to the normal control group due to induction of CKD. After inducing CKD, administering aqueous and alcoholic bi-herbal extracts of AM and AS leaves for 42 days notably enhanced hemoglobin levels, TEC and lymphocytes, while significantly lowering TLC and granulocytes compared to CKD-induced rats (group II). Notably, the most pronounced positive impact on hematological parameters was observed in rats treated with 500 mg/kg body weight of bi-herbal alcoholic extracts.

Table 3: Comparison of mean (±SE) heamatological parameters in rats under different CKD treatment and control groups

Group	Haemoglobin (g/dl)		TEC (10^6/µl)		TLC (10^3/µl)		Granulocytes (%)		Lymphocytes (%)		Monocytes (%)	
Days	28th day	70th day	28th day	28th day	70th day	28th day	28th day	70th day	28th day	70th day	28th day	70th day
I	14.02±0.34^{b}	15.12±0.60^{e}	7.28±0.42^{b}	24.05±1.70^{a}	22.16±1.35^{a}	73.83±1.65^{b}	24.05±1.70^{a}	22.16±1.35^{a}	73.83±1.65^{b}	74.66±1.50^{d}	2.12±0.25	3.18±0.29
II	10.51±0.25^{a}	7.82±0.55^{a}	4.18±0.44^{a}	43.85±1.06^{b} ^{c}	42.42±1.42^{d}	53.60±1.43^{a}	43.85±1.06^{b} ^{c}	42.42±1.42^{d}	53.60±1.43^{a}	54.71±1.64^{a}	2.55±0.47	2.87±0.32
III	10.71±0.40^{a}	13.50±0.32^{c}	4.11±0.18^{a}	41.16±1.70^{b} ^{c}	27.55±0.16^{b}	56.18±1.57^{a}	41.16±1.70^{b} ^{c}	27.55±0.16^{b}	56.18±1.57^{a}	70.09±0.49^{c}	2.67±0.49	2.37±0.27
IV	10.83±0.37^{a}	11.98±0.37^{b}	4.63±0.44^{a}	40.17±1.24^{b}	33.24±0.89^{c}	57.39±1.29^{a}	40.17±1.24^{b}	33.24±0.89^{c}	57.39±1.29^{a}	64.09±0.63^{b}	2.45±0.19	2.67±0.49
V	10.98±0.22^{a}	13.85±0.18^{c} ^{d}	4.46±0.30^{a}	44.98±1.09^{c}	26.78±0.69^{b}	53.36±0.97^{a}	44.98±1.09^{c}	26.78±0.69^{b}	53.36±0.97^{a}	70.49±0.51^{c}	1.67±0.26	2.73±0.82
VI	11.02±0.25^{a}	14.88±0.22^{d} ^{e}	4.94±0.30^{a}	43.49±1.51^{b} ^{c}	24.45±1.21^{a} ^{b}	54.53±1.47^{a}	43.49±1.51^{b} ^{c}	24.45±1.21^{a} ^{b}	54.53±1.47^{a}	73.16±0.99^{c} ^{d}	1.98±0.07	2.40±0.31

Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c,…) within a same column differ significantly from each other (P<0.05).

3.5 Serum biochemistry analysis

The serum biochemical analysis outcomes for experimental therapeutic and control groups on days 28 and 70 of the study have been presented in Table 4. In adenine control group-II and therapeutic groups (III, IV, V, VI) the serum biochemical profile exhibited marked elevations in serum creatinine, BUN, ALT, uric acid, and phosphorus levels, alongside noteworthy reductions in serum uromodulin, total protein, albumin, and calcium, in comparison to the normal control group. These observed changes substantiate that the administration of daily doses of 200 mg/kg b.wt. adenine over a 28-day period led to compromised kidney function and induced CKD in rats. Following the induction of CKD, the administration of aqueous and alcoholic bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* leaves over a period of 42 days in therapeutic groups (III, IV, V, VI) yielded notable results. These results included a significant reduction in serum creatinine, uric acid, BUN, ALT and phosphorus levels, as well as a significant increase in serum uromodulin, serum albumin, total protein and calcium levels as compared to CKD-induced adenine control rats (group II). Remarkably, group III demonstrated remarkable restoration of the serum biochemical imbalances. Furthermore, groups V and VI exhibited dose-dependent effectiveness in ameliorating the severe changes induced by adenine. These findings underscore the considerable therapeutic potential of the bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* leaves in mitigating the detrimental effects of adenine-induced biochemical disruptions.

Table 4: Comparison of mean (±SE) serum parameters in rats under different CKD treatment and control groups

Group	Serum Uromodulin (ng/ml)		Creatinine (mg/dl)		Uric acid (mg/dl)		BUN (mg/dl)		ALT (U/L)		AST (U/L)	
Days	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day
I	19.60±0.58^{b}	19.03±0.63^{d}	0.56±0.05^{a}	0.61±0.05^{a}	2.38±0.16^{a}	2.31±0.15^{a}	23.23±0.54^{a}	25.60±0.74^{a}	26.23±0.22^{a}	27.30±0.25^{a}	110.29±2.08	116.07±2.12
II	10.61±0.34^{a}	10.24±0.56^{a}	3.40±0.10^{b}	3.05±0.09^{e}	5.01±0.14^{b}	4.90±0.10^{d}	154.42±2.53^{b}	121.70±2.63^{d}	51.88±0.44^{b}	50.78±0.34^{c}	117.84±2.96	116.35±2.51
III	10.53±0.41^{a}	16.54±0.70^{bc}	3.57±0.09^{b}	1.46±0.06^{cd}	5.26±0.10^{b}	2.87±0.08^{bc}	149.58±2.27^{b}	58.16±0.85^{c}	51.26±0.34^{b}	33.77±0.72^{b}	114.80±2.55	116.86±2.26
IV	10.28±0.47^{a}	14.83±0.67^{c}	3.59±0.03^{b}	1.56±0.08^{d}	4.93±0.09^{b}	3.15±0.07^{c}	152.15±2.03^{b}	59.63±0.41^{c}	51.44±0.49^{b}	33.93±0.68^{b}	116.31±2.65	117.48±2.15
V	10.12±0.47^{a}	16.99±0.55^{c}	3.44±0.08^{b}	1.36±0.04^{c}	5.03±0.06^{b}	2.59±0.09^{ab}	151.29±1.69^{b}	56.08±0.22^{c}	52.21±0.26^{b}	33.03±0.37^{b}	112.20±2.31	113.89±1.48
VI	11.22±0.66^{a}	18.94±0.64^{d}	3.52±0.08^{b}	0.65±0.08^{a}	5.12±0.07^{b}	2.40±0.10^{a}	150.07±1.11^{b}	49.27±0.59^{b}	51.33±0.37^{b}	32.27±0.65^{b}	111.01±1.58	112.21±1.36

Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c,…) within a same column differ significantly from each other (P<0.05).

Group	Albumin (g/dl)		Total protein (g/dl)		Calcium (mg/dl)		Phosphorus (mg/dl)	
Days	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day
I	3.68±0.07^{b}	3.76±0.06^{d}	6.62±0.16^{b}	6.49±0.19^{b}	10.36±0.27^{b}	10.21±0.23^{c}	6.83±0.15^{a}	7.04±0.17^{a}
II	2.73±0.12^{a}	2.74±0.11^{a}	5.58±0.14^{a}	5.75±0.08^{a}	7.42±0.19^{a}	8.27±0.09^{a}	9.92±0.26^{b}	9.41±0.15^{c}
III	2.60±0.10^{a}	3.44±0.13^{bc}	5.55±0.10^{a}	6.36±0.16^{b}	7.72±0.21^{a}	9.44±0.14^{b}	10.06±0.17^{b}	7.54±0.15^{b}
IV	2.63±0.13^{a}	3.35±0.09^{b}	5.36±0.08^{a}	6.26±0.12^{b}	7.66±0.23^{a}	9.17±0.26^{b}	10.17±0.15^{b}	7.75±0.19^{b}
V	2.67±0.14^{a}	3.39±0.09^{bc}	5.67±0.12^{a}	6.40±0.10^{b}	7.40±0.25^{a}	9.52±0.09^{b}	10.09±0.14^{b}	7.48±0.17^{ab}
VI	2.69±0.06^{a}	3.67±0.09^{cd}	5.39±0.14^{a}	6.52±0.13^{b}	7.69±0.19^{a}	10.17±0.30^{c}	10.48±0.26^{b}	7.26±0.14^{ab}

Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c,…) within a same column differ significantly from each other (P<0.05).

3.6 Urine analysis: The presented data in Table 5 encompasses the average urine parameters among rats across distinct therapeutic and control groups on both the 28th and 70th days of the experiment. The analysis of urine in adenine control group (Group II) and the therapeutic groups revealed a statistically significant reduction in urine pH, urine creatinine and phosphorus levels. In contrast, there was a substantial increase observed in urine total protein and urine calcium concentrations. These discernible alterations within the urine profile substantiate the contention that the daily administration of adenine at a dosage of 200 mg/kg body weight for a period of 28 days' precipitates impaired renal function and the consequent induction of CKD in rats. Subsequent to CKD induction, administration of aqueous and alcoholic bi-herbal extracts from *Aegle marmelos* and *Annona squamosa* leaves resulted in noteworthy enhancements of urine pH, urine creatinine, and urine phosphorus levels in group III. In contrast, in group V and VI, a dose-dependent response was observed, exhibiting significant improvements in these parameters along with substantial reductions in urine total protein and urine calcium, when compared to CKD-induced rats.

Table 5: Comparison of mean (±SE) urine parameters in rats under different CKD treatment and control groups

Group	Urine pH		Urine Specific Gravity		Urine Total Protein (g/dl)		Urine Creatinine (mg/dl)		Urine Calcium (mg/dl)		Urine Phosphorus (mg/dl)	
Days	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day	28th day	70th day
I	8.25±0.11^{b}	8.50±0.18^{d}	1.014±0.003	1.018±0.002	7.32±0.29^{a}	7.42±0.24^{a}	11.14±0.23^{b}	11.69±0.18^{d}	2.28±0.09^{a}	2.54±0.12^{a}	2.47±0.12^{b}	2.59±0.14^{c}
II	6.17±0.11^{a}	5.98±0.18^{a}	1.008±0.002	1.025±0.006	25.52±0.67^{b}	26.54±0.62^{d}	4.80±0.21^{a}	5.06±0.09^{a}	4.75±0.29^{b}	5.34±0.32^{c}	1.75±0.06^{a}	1.37±0.16^{a}
III	6.08±0.15^{a}	7.92±0.24^{c}	1.010±0.001	1.015±0.003	26.18±0.64^{b}	12.18±0.27^{b}	4.77±0.24^{a}	8.09±0.19^{c}	4.61±0.07^{b}	3.09±0.21^{a}	1.74±0.05^{a}	2.33±0.04^{c}
IV	6.00±0.22^{a}	6.83±0.21^{b}	1.009±0.002	1.010±0.004	26.01±0.70^{b}	14.18±0.39^{c}	4.48±0.19^{a}	7.20±0.39^{b}	4.73±0.16^{b}	4.24±0.18^{b}	1.81±0.06^{a}	1.99±0.03^{b}
V	6.08±0.15^{a}	7.08±0.15^{b}	1.011±0.002	1.014±0.004	26.69±0.59^{b}	13.56±0.17^{c}	4.53±0.32^{a}	7.34±0.19^{b}	4.54±0.22^{b}	3.91±0.22^{b}	1.76±0.05^{a}	2.01±0.06^{b}
VI	6.08±0.15^{a}	8.18±0.12^{d}	1.009±0.002	1.015±0.004	24.71±0.83^{b}	11.88±0.18^{b}	4.29±0.24^{a}	8.49±0.29^{c}	4.50±0.17^{b}	2.82±0.20^{a}	1.83±0.05^{a}	2.41±0.05^{c}

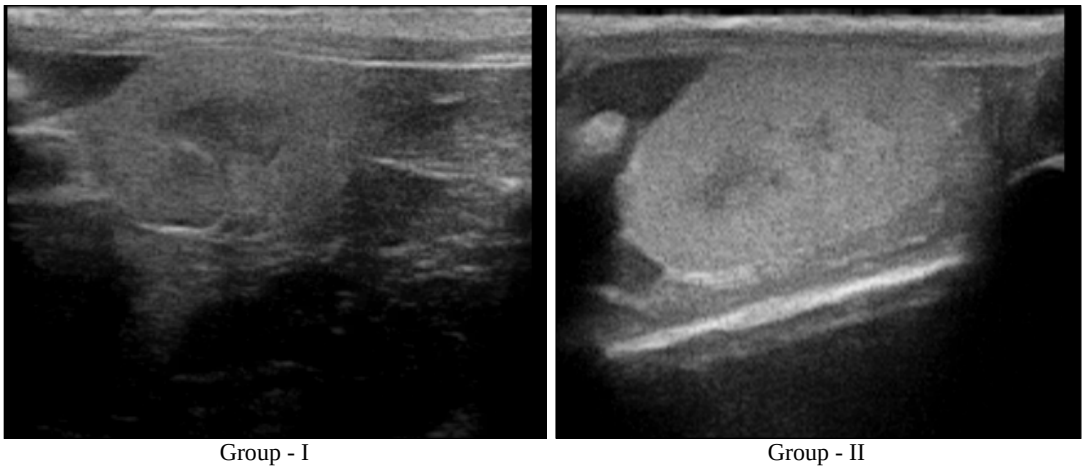
Values (Mean ± S.E., n=6) bearing different superscripts (a, b, c,…) within a same column differ significantly from each other (P<0.05).

3.7 Renal ultrasound examination

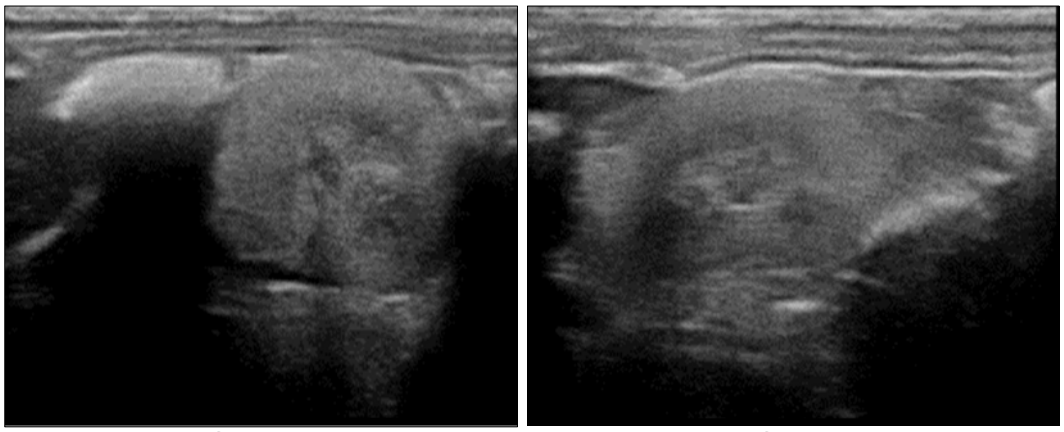
Renal ultrasound assessments were conducted on the 28th and 70th days of the experiment to validate the impact on kidney structure. Ultrasonography of kidneys in rats induced with chronic kidney disease on the 28th day across groups II, III, IV, V, and VI revealed the presence of multiple hyperechoic foci situated at the cortico-medullary junction, along with an indistinct cortico-medullary junction.

Subsequently, a distinct parenchymal swelling (manifesting as an increase in kidney width) was observed, leading to the development of a spherical kidney morphology with hypoechoic renal tissue. In contrast, the cortico-medullary junction remained clearly discernible in the normal control group. Post-therapeutic intervention (on the 70th day) involving the administration of aqueous and alcoholic bi-herbal extracts sourced from Aegle marmelos and Annona squamosa, a reduction in hyperechoic foci was evident. Furthermore, the cortico-medullary junction regained clarity in groups III, IV, V and VI. These findings underscore the therapeutic potential of these plant extracts, as illustrated in Figure 1.

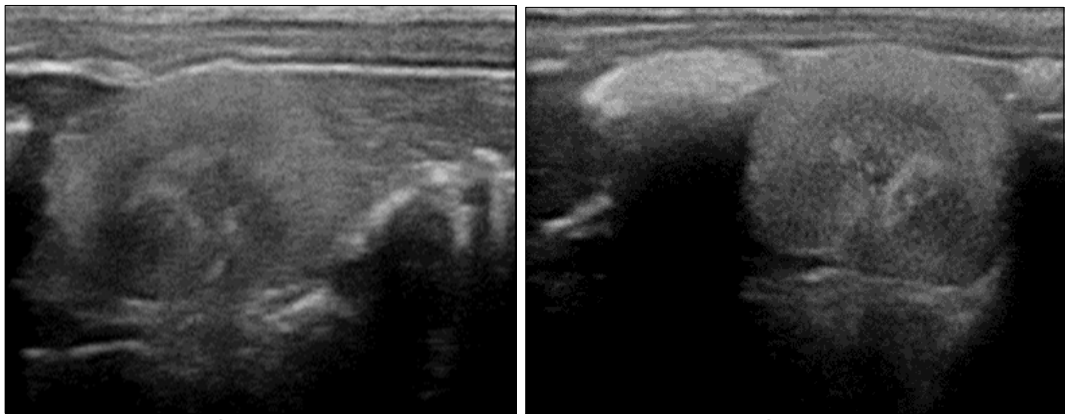
3.8 Histopathological examination of kidney: The histopathological assessment of kidney revealed a range of pathological modifications. In the adenine-induced groups, discernible pathological variations were observed, including pronounced tubular atrophy, cystic dilatation, severe fibrosis, congestion, inflammatory exudates and the presence of tubular casts as evident in the H&E stained sections. However, subsequent to the administration of aqueous and alcoholic bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* leaves following the induction of CKD in groups III, IV, V, and VI, the histopathological examination exhibited comparatively diminished pathological alterations. This reduction in pathological changes is particularly noteworthy when contrasted with the adenine control group and the observed differences are visually documented in Figures 2 to 7.



Group - I Group - II



Group - III Group - IV



Group - V Group - VI

Fig 1: Renal ultrasound of normal and treatment groups

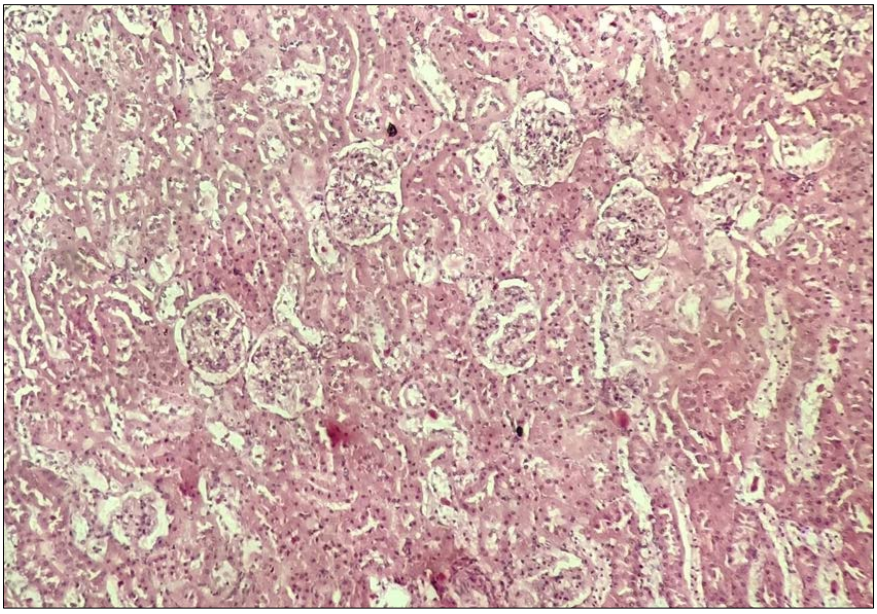


Fig 2: Microphotographs (H & E, 120X) of kidney showed normal histo-architexture details of normal control rats (Group-I)

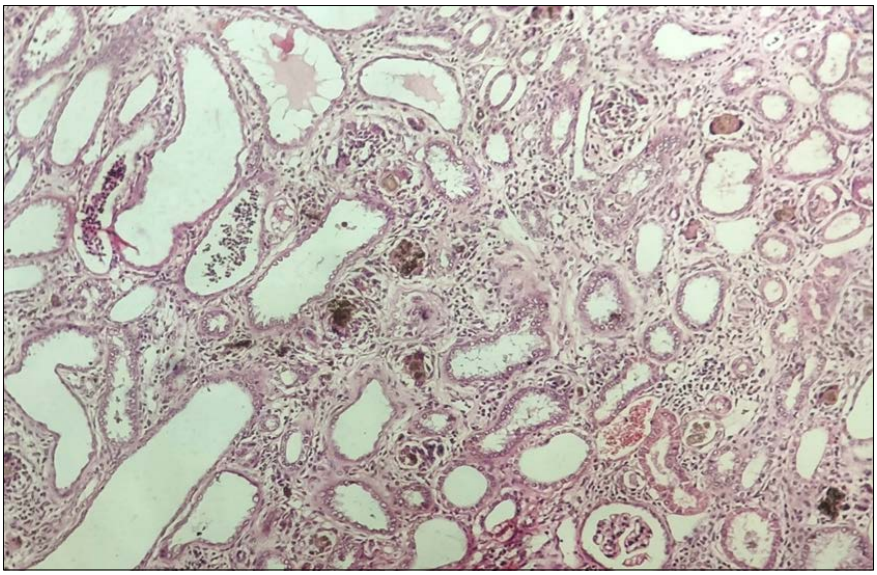


Fig 3: Microphotographs (H & E, 120X) of kidney showed marked tubular atrophy, cystic dilatation, mild fibrosis, congestion, inflammatory exudates and tubular cast in adenine control rats (Group-II)

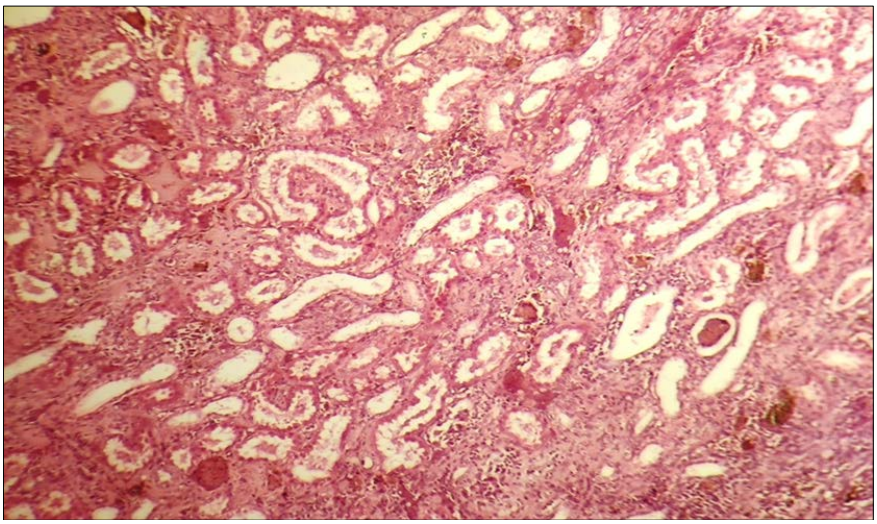


Fig 4: Microphotographs (H & E, 120X) of kidney showed tubular atrophy, degeneration and moderate fibrosis of interstitium in rats treated with Aq. bi-herbal extract of AM & AS @ 250 mg/kg b.wt after induction of CKD for 42 days(Group-III)

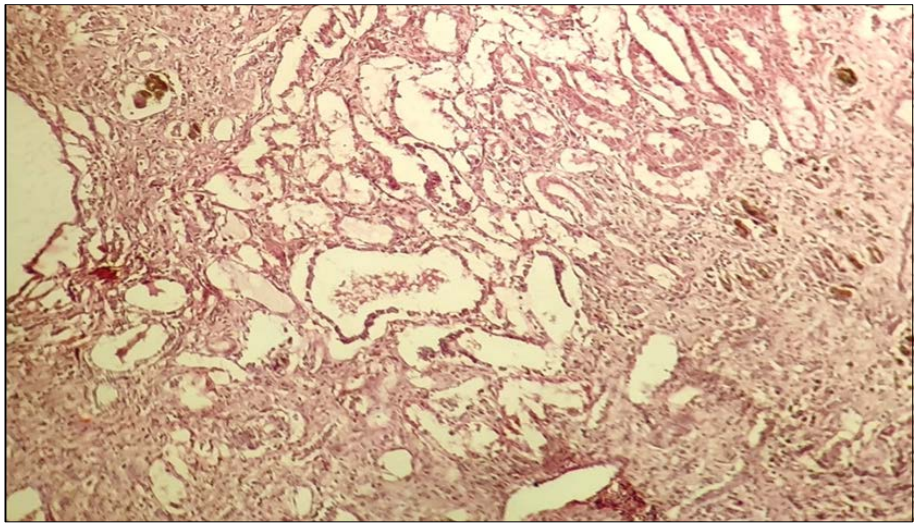


Fig 5: Microphotographs (H & E, 120X) of kidney showed severe interstitial fibrosis and marked tubular dilatation along with presence of cast in rats treated with Aq. bi-herbal extract of AM & AS @ 500 mg/kg b.wt after induction of CKD for 42 days (Group-IV)

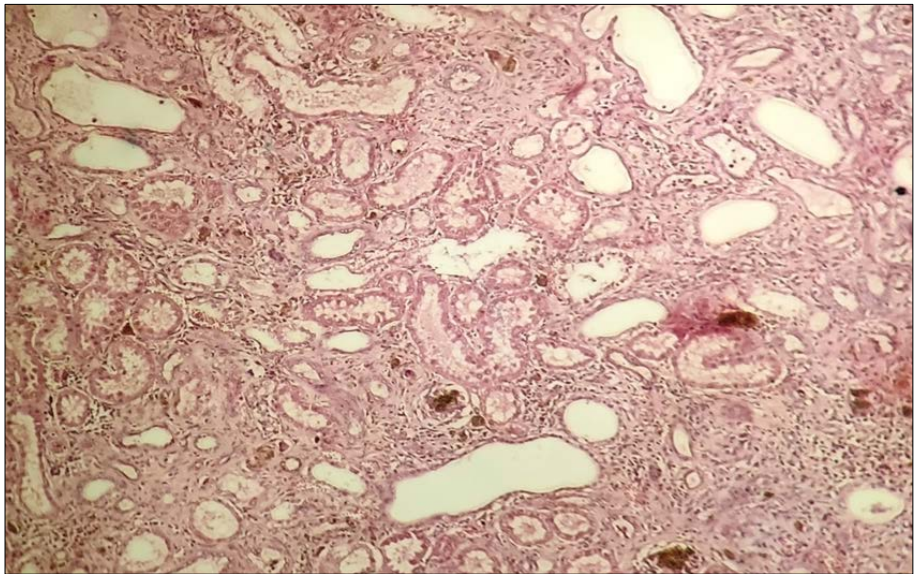


Fig 6: Microphotographs (H & E, 120X) of kidney showed moderate tubular atrophy with cystic dilatation and fibrosis of interstitium in rats treated with Al. bi-herbal extract of AM & AS @ 250 mg/kg b.wt after induction of CKD for 42 days (Group-V)

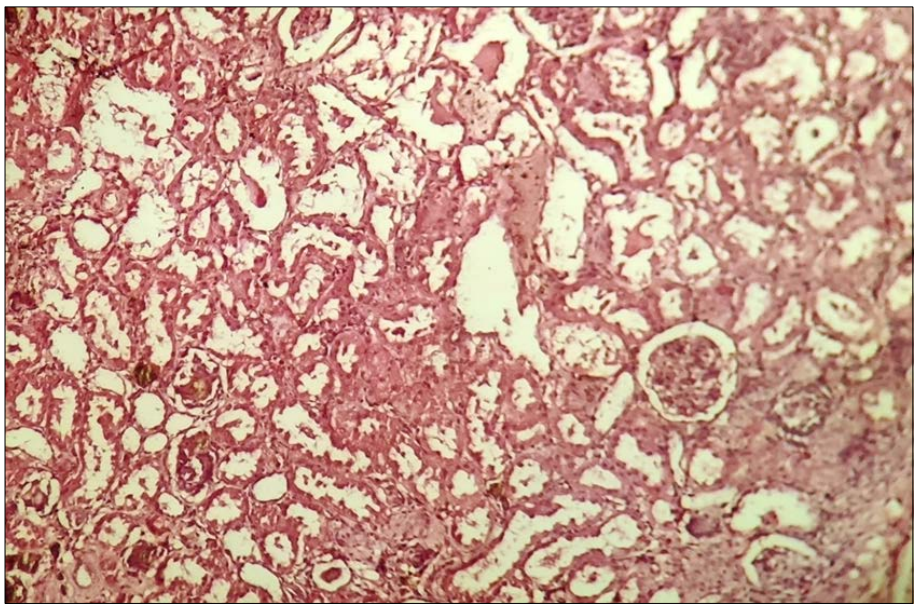


Fig 7: Microphotographs (H & E, 120X) of kidney showed mild degenerative changes in tubular epithelial cells in rats treated with Al. bi-herbal extract of AM & AS @ 500 mg/kg b.wt after induction of CKD for 42 days (Group-VI)

4. Discussion

The present results on feed consumption align with earlier studies. Diwan *et al*. (2017) [5] noted that rats receiving dietary adenine exhibited a gradual decrease in feed intake compared to normal control rats. Similarly, studies by Li *et al*. (2018) [13] and Rahman *et al*. (2018) [14] reported diminished food consumption in rats with adenine-induced chronic renal failure. In another context, Kaleem *et al*. (2006) [15] demonstrated that administering *Annona squamosa* to diabetic rats led to a significant increase in body weight gain, nearly restoring levels seen in control rats with streptozotocin-induced diabetes.

The body weight measurements obtained in this study align with prior research. Jia *et al*. (2013) [16], Ali *et al*. (2014) [17], Tani *et al*. (2017) [18], Ali *et al*. (2017) [19] and Abellan *et al*. (2019) [20] all reported a notable decline in body weight as a consequence of the adenine-induced CKD model in rats. Additionally, Kaleem *et al*. (2006) [15] demonstrated that administering *Annona squamosa* to diabetic rats led to a significant increase in body weight, effectively counteracting the weight loss observed in streptozotocin-induced diabetic rats.

The hematological analysis findings of the present study harmonize with those from previously reported research by Sun *et al*. (2013) [21], Ali *et al*. (2014) [17] and Patel *et al*. (2020) [22]. Furthermore, Rahman *et al*. (2018) [14] demonstrated that there was no significant alteration in haematocrit values following adenine treatment at a dose of 200 mg/kg in rats. However, when administered at 600 mg/kg, adenine led to a noteworthy reduction in haematocrit levels by day 24, compared to vehicle control rats. They proposed that the diminished total erythrocyte count (TEC) observed in adenine-treated rats could be linked to an inadequate production of erythropoietin hormone, resulting in uremic anemia and impaired oxygen metabolism. Paul *et al*. (2018) [23] reported that experimental fish infected with *Aeromonas hydrophila* exposed to 100% concentrations of *Annona squamosa* aqueous leaf extract exhibited an increase in Hb. Likewise, Patel *et al*. (2020) [22] documented that administering bi-herbal extracts of *Boerhavia diffusa* and *Tribulus terrestris*, along with adenine, led to elevated mean values of hemoglobin (Hb), total erythrocyte count (TEC), and lymphocytes in prophylactic groups, when contrasted with the adenine control group. These findings, along with the present results, collectively underscore the potential nephron-protective and anti-anemic roles attributed to the bi-herbal aqueous and alcoholic leaf extracts of *Aegle marmelos* and *Annona squamosa* in CKD rats.

The results obtained from the serum biochemical analysis of adenine-induced CKD are consistent with the outcomes documented in research by Sun *et al*. (2013) [21], Seo *et al*. (2022) [24], Ali *et al*. (2014) [17], Ali *et al*. (2016) [18], Tani *et al*. (2017) [18], Rahman *et al*. (2018) [14], and Patel *et al*. (2020) [22] for a range of serum biochemical parameters. This alignment with prior studies serves to reinforce the credibility and significance of our current findings, underscoring their resonance with established research in the field. Neelima *et al*. (2020) [25] discovered that the ethanolic leaf extract of *Aegle marmelos* notably reduced serum creatinine, BUN and uric acid levels in a paracetamol-induced nephrotoxicity model in rats. In parallel, Kaleem *et al*. (2008) [26] reported that *Annona squamosa* aqueous extract exhibited significant decreases in serum creatinine and uric acid levels, while concurrently elevating albumin levels in streptozotocin (STZ)-induced diabetic mellitus. Furthermore, Bhalerao and Shendre (2018) [27] observed considerable reductions in serum creatinine, BUN, uric acid and ALT levels with the ethanolic leaf extract of *Aegle marmelos* in a gentamicin-induced nephrotoxicity rat model. In concurrence Singh *et al*. (2022) [28] documented that administering aqueous and alcoholic extracts of *Boerhavia diffusa* and *Bryophyllum calycinum* to rats subsequent to CKD induction led to a noteworthy restoration in mean values of creatinine, uric acid, BUN, ALT and phosphorus within the therapeutic groups, while simultaneously eliciting an elevation in total protein levels in comparison to the adenine control group. The serum biochemistry analysis revealed that the administration of bi-herbal extracts derived from *Aegle marmelos* and *Annona squamosa* effectively mitigated the renal and hepatic damage induced by adenine. Notably, these extracts exhibited a promising potential as therapeutic agents with nephron-protective attributes in the context of chronic kidney disease in rats.

The observations stemming from the urine analysis in adenine-induced CKD rats harmonize with the conclusions outlined by Singh *et al*. (2022) concerning various urine parameters. Studies by Yokozawa *et al*. (1986) [29] and Ali *et al*. (2016) [30] highlighted that rats subjected to adenine treatment displayed heightened urine concentrations of total protein in contrast to the control group. Further investigations conducted by Ali *et al*. (2014) [17] and Ali *et al*. (2017) [18] confirmed a noteworthy decline in creatinine clearance as a consequence of adenine treatment. Abellan *et al*. (2019) [20] have reported a notable upsurge in the mean urine calcium values within rats subjected to adenine administration. Korah *et al*. (2020) [31] documented a significant elevation in urine pH within rats administered *Annona squamosa* ethanolic leaf extracts. Likewise, Singh *et al*. (2022 [28] demonstrated that the administration of aqueous and alcoholic extracts of *Boerhavia diffusa* and *Bryophyllum calycinum* subsequent to CKD induction led to an augmentation in urine pH and creatinine levels, coupled with a reduction in urine total protein and calcium concentrations within therapeutic groups when compared to the adenine control group.

The histopathological examination findings of the kidneys in the present study resonate with previous research. Ali *et al*. (2016) [30] examined the histopathology of adenine-induced CKD in rat kidneys, revealing the presence of fibrosis, tubular lumen dilatation, and inflammatory changes. Dwivedi *et al*. (2017) [32] observed that treatment with hydro-alcoholic and ethyl acetate extracts of *Aegle marmelos* leaves exhibited pronounced protective effects, preserving kidney cellular structure without any signs of inflammation. Similarly, the observations align with Neelima *et al*. (2020) [25] reported that treatment with ethanolic extracts of *Annona squamosa* leaves manifested a substantial protective effect, effectively maintaining kidney structure with minimal swelling, cellular necrosis, and cellular desquamation evident upon histopathological examination in rats.

In conclusion, the integration of body weight and feed consumption evaluations, alongside heamato-biochemical analysis, urine assessments, ultrasonographic examinations and histopathological findings collectively indicate that the administration of aqueous bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* leaves at a dosage of 250

mg/kg body weight after CKD induction showcases promising therapeutic efficacy. In contrast, the alcoholic bi-herbal extracts at a dosage of 500 mg/kg body weight exhibit notably superior therapeutic effectiveness compared to the 250 mg/kg dosage. Importantly, the alcoholic bi-herbal extracts show better results compared to the aqueous bi-herbal extracts. Ultimately, these outcomes underscore the heightened therapeutic potential of alcoholic bi-herbal extracts of *Aegle marmelos* and *Annona squamosa* at a dosage of 500 mg/kg body weight in ameliorating the impacts of CKD induction in rats.

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