

Exploring the Effect of Phytochemical Extract (*Benincasa sp.*) on the Inflammatory Mediators and Regulatory Pathways of Acute Cisplatin Nephrotoxicity in Pre-clinical Mouse Model

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ABSTRACT

Introduction: Cisplatin-induced acute nephrotoxicity is typically manifested by a decrease in kidney function, electrolyte imbalances, and acute kidney injury (AKI). Most therapies for AKI are costly and have serious side effects. Utilizing phytotherapy for the prevention of kidney disease has the potential to reduce both treatment expenses and the occurrence of associated side effects. *Benincasa cerifera* (BC) offers extensive advantages owing to its anti-inflammatory, antioxidant, and anti-diabetic characteristics. This study aims to demonstrate the protective effects of BC on the inflammatory mediators and regulatory pathways in a cisplatin-induced acute kidney injury (AKI) model. **Methods:** Fifteen 6–8-week-old female BALB/c mice (weighing 20–25 g) were divided into three groups as follows: (I) Control, (II) Cisplatin, (III) Cisplatin + BC. The disease was induced with a single dose (15 mg/kg) of cisplatin on day 0, followed by treatment with an ethanolic extract of BC (500 mg/kg). On day 7, the sacrificed mice were used to study the histopathological, biochemical, immunological, and inflammatory parameters. **Results:** Treatment with BC successfully restored the clonogenic potential of the kidney, blood, spleen, and bone marrow cells. Flow cytometry showed that cisplatin-induced abnormalities in cellular circulation and the migration of CD45⁺B220⁺ B cells, CD4⁺, CD8⁺ T lymphocytes, and F4/80⁺ cells in the blood were significantly recovered with the treatment of BC. This study also found that BC inhibited the upregulation of different inflammatory cytokines, which were involved in the Th₁, Th₂, and Th₁₇ response. **Conclusion:** Phytochemical-based novel treatment strategies developed from this work will provide safe options for patients and assist in reducing the disease burden of AKI.

Key words: Mechanism of acute kidney injury, Cisplatin, *Benincasa cerifera*, Immunological Markers, Nephrotoxicity, Inflammatory Cytokines

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INTRODUCTION

Acute Kidney Injury (AKI), characterized by a sudden decline in kidney function, arises from a variety of causes including urinary tract blockages, glomerulonephritis, sepsis, and reduced blood flow due to organ failure or significant blood loss. It is an urgent health concern, particularly in hospitalized children and critically ill pediatric patients, where it affects around 5–10% and exhibits higher incidence rates respectively, leading to increased morbidity and mortality^{1,2}. A notable cause of AKI, particularly relevant in cancer treatment, is nephrotoxicity induced by cisplatin—a widespread chemotherapeutic agent. While cisplatin is efficacious in treating various cancers, its associated nephrotoxic effects, which manifest in 21–31.5% of treated patients, severely limit its clinical application, necessitating dose adjustments or discontinuation to mitigate renal damage^{3–5}. Cisplatin-induced AKI, marked by tubular

cell death, inflammation, and oxidative stress, underscores the need for innovative therapeutic approaches and deeper understanding of its pathology⁶. However, the progression from acute cisplatin nephrotoxicity to chronic kidney disease in mouse models remains underexplored due to the absence of standard protocols⁷.

In West Bengal, the surge in kidney diseases has been attributed to increased prevalence of hypertension⁸. Standard AKI treatments involve managing hypovolemia—a critical reduction in blood plasma volume—using isotonic saline, albumin, and tailored fluid therapy to prevent rapid heartbeat, weakness, confusion, or unconsciousness. Vasopressors such as Noradrenaline, Vasopressin, and Terlipressin offer further support; however, these treatments can be expensive and yield severe side effects⁹. Ultimately, for both acute and chronic kidney diseases, dialysis or kidney transplantation are the primary interventions, each carrying substantial impacts on patient quality of

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life and potential risks, including immunological rejection and ethical concerns such as organ trafficking. Given these challenges, there's growing interest in alternative treatments like those offered by Ayurveda, including phytochemical extracts known for their regenerative properties and minimal side effects. Notably, extracts from *Benincasa* sp., commonly known as winter melon, are gaining attention for their antioxidant properties and potential in treating kidney diseases¹⁰⁻¹⁵. This study aims to explore the effects of *Benincasa* sp. extract on the inflammation and regulatory pathways involved in cisplatin-induced AKI, using a mouse model treated with cisplatin followed by oral administration of the extract. Through this research, we hope to uncover novel therapeutic pathways and validate the efficacy of traditional medicines in the context of modern-day ailments.

METHODS

Ethical approval

Every experiment was conducted in compliance with the guidelines established by the animal ethics committee of the University of Calcutta (No- ERB/ZOO/2023/III, Dated 02.08.2023). The mice were housed in the University of Calcutta's Department of Zoology animal facility under specific pathogen-free conditions.

Mice

Female *BALB/c* mice aged 6-8 weeks (weighing 20-25 g) were divided into three groups, each comprising n = 5: (I) Control, (II) CIS, and (III) CIS+BEN.

Collection and Preparation of *Benincasa cerifera* (BC)

BC was collected from the Indian Market in Kolkata during the summer. The rind was peeled, and the fruit was cut into small pieces, dried in a freeze-dryer, and then ground using an electric blender. The powder was then dissolved in 70% v/v ethanol and shaken for an entire day. A gas dryer was used to evaporate and dry the mixture. To remove water and yield a solid powder, the evaporated solution was placed in a freeze-dryer again. The resultant powder was extracted in water and administered orally (Figure 1).

Induction of Acute Kidney Injury (AKI) and treatment with *Benincasa cerifera*

Cisplatin (Cisplatin injection, Cizcan) was administered intraperitoneally once. For the diseased and treatment groups, an intraperitoneal injection of cisplatin was given on day 0 (15 mg/kg body weight).

The control group received normal saline. For the treatment group, *Benincasa* sp. extract (500 mg/kg body weight) dissolved in water was administered orally on day 0 (after 6 hours of cisplatin induction), and then on days 2, 4, 6. Animals were euthanized on day 7 (Figure 2).

Sacrifice and collection of tissues

On day 7, mice were euthanized by cervical dislocation, and the following tissues were collected. 1 ml of peripheral blood (PB) was collected via cardiac puncture into tubes containing EDTA as an anticoagulant. A smear was made on a microscope slide for a differential cell count.

For assays

The kidney, spleen, and liver were placed in a Petri dish and finely chopped. The kidney was then treated with a 1X cocktail of collagenase/hyaluronidase (Stem Cell Technology) overnight at 37°C. Once a single-cell suspension was achieved, it was filtered through a no. 60 sieve (Sigma Aldrich).

For gene expression

One kidney was taken whole, washed with PBS, and stored in RNeasy lysis solution (Qiagen, Inc) at -80°C.

For protein expression

One kidney was taken whole, washed with PBS, and stored in PBS at -80°C.

For histology

One kidney was collected whole in 10% buffered formalin.

Total cell (TC) count

The TC count of PB, kidney, bone marrow, spleen, and liver cells was performed according to Mitra *et al.*, 2022¹⁶.

Differential cell (DC) count

The DC count of PB was conducted according to Mitra *et al.*, 2022¹⁶.

BUN and Serum Creatinine Level Test

PB from each group of mice was collected in uncoated tubes and allowed to stand for at least 30 minutes. The samples were then centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was collected in fresh 1.5 ml tubes. The serum was stored at -20°C for later analysis. The BUN and serum creatinine levels were

Table 1: List of primers used for determination of gene expression by RT-PCR

Gene	Primer sequence (5'-3')		Tm
GAPDH	F	GAGGGGCCATCCACAGTCTTC	62.8°C
	R	CATCACCATCTTCCAGGAGCG	
IFN γ	F	AGCGGCTGACTGAACTCAGATTGTAG	62.9°C
	R	GTCACAGTTTTCAGCTGTATAGGG	
TNF α	F	GGCAGGTCTACTTTGGAGTCATTGC	64.6°C
	R	ACATTGAGGCTCCAGTGAATTCGG	
IL1 β	F	TCATGGGATGATGATGATAACCTGCT	61.5°C
	R	CCCATACTTTAGGAAGACACGGATT	
IL-4	F	ACCTTGCTGTCACCCTGTTC	58.3°C
	R	TTGTGAGCGTGGACTCATTC	
IL-13	F	CGGCAGCATGGTATGGAGTG	60.4°C
	R	ATTGCAATTGGAGATGTTGGTCAG	
IL-10	F	ATTTGAATTCCCTGGGTGAGAAG	54.1°C
	R	CACAGGGGAGAAATCGATGACA	

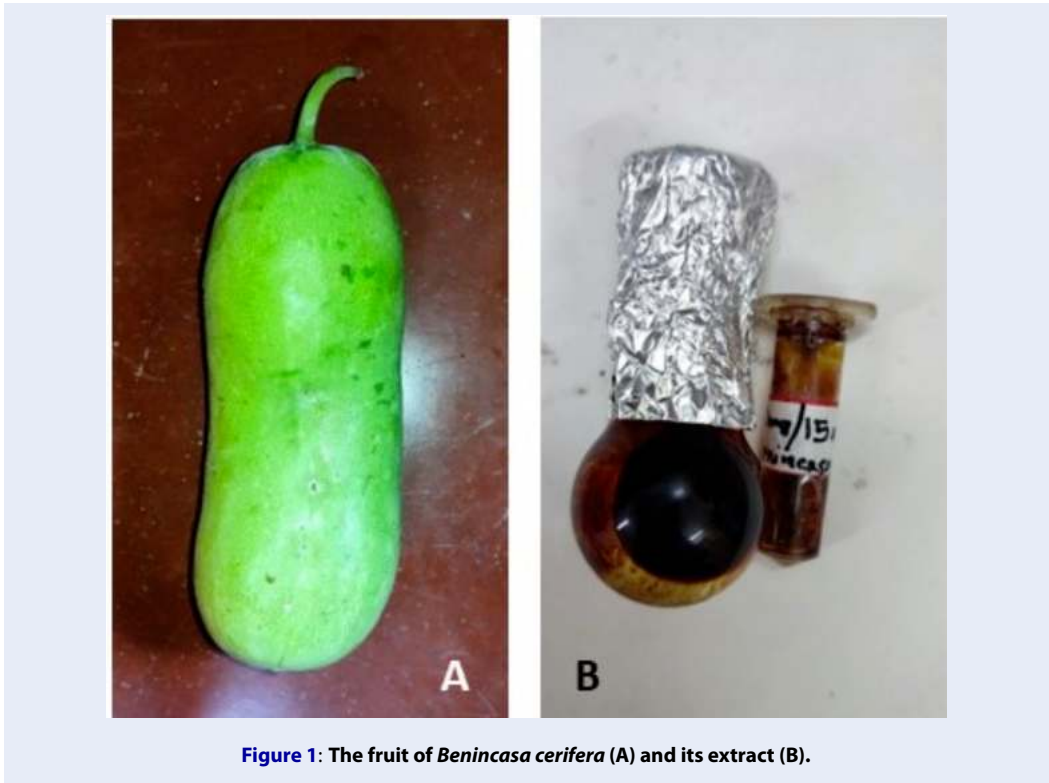


Figure 1: The fruit of *Benincasa cerifera* (A) and its extract (B).

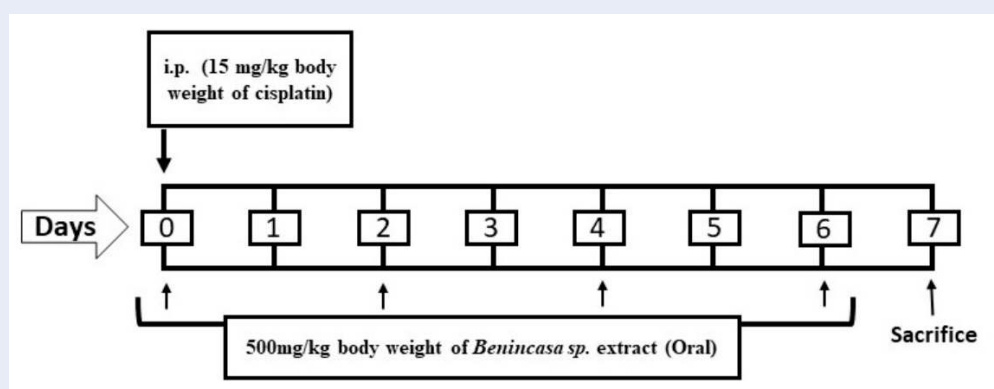


Figure 2: Diagrammatic representation of induction of disease (AKI) and treatment with *Benincasa cerifera*.

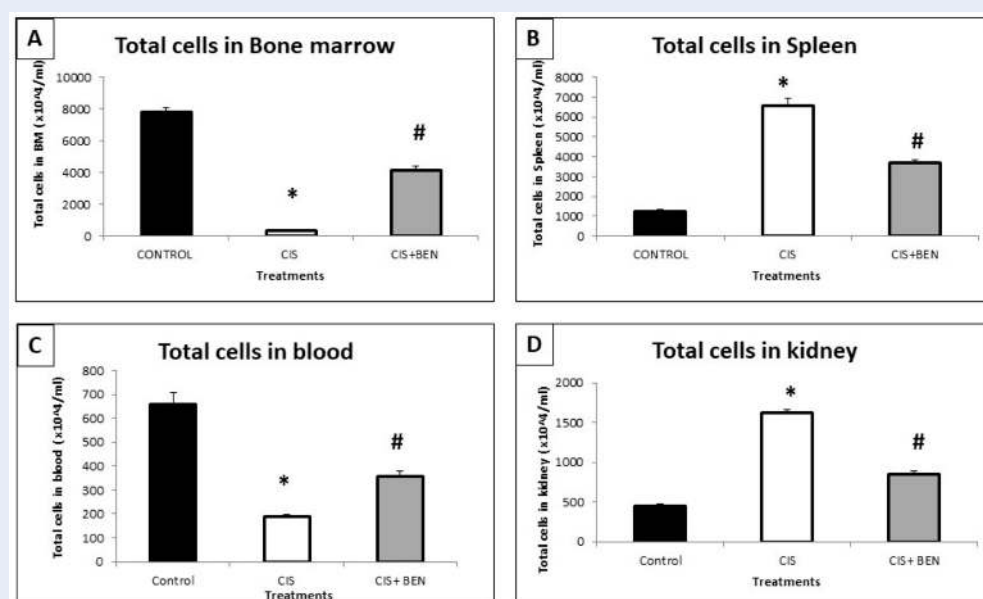


Figure 3: Effect of *Benincasa sp.* on total cell count in case of peripheral blood, kidney, spleen and bone marrow in cisplatin induced AKI (* $p < 0.05$, compared to control; # $p < 0.05$, compared to cisplatin; significance value: ≤ 0.05).

evaluated using the Autospin Urea Test kit and Autospin Liquid Gold Creatinine, both from ARKRAY Healthcare Pvt. Ltd, respectively.

CFU-c assay

To assess the clonogenic capability of the tissue cells, a colony-forming unit-cell assay was performed using methylcellulose semisolid media (Himedia, India), following a protocol standardized in the lab of the University of Calcutta.

Histology

Kidney tissues were fixed in 10% formalin, dehydrated, and then embedded in molten paraffin to form blocks. The tissues were then sectioned into 5 μm slices. Hematoxylin and eosin-stained kidney sections showed changes in the glomeruli and renal tubules. A light microscope (Dewinter Fluorex LED, camera- DIGIEYE-510CCD) was used to capture images of the H&E-stained kidney sections. A slightly modified version of the previously disclosed semi-quantitative pathological scoring system was applied. A grading scale (0, 1, 2, 3) was used to determine the

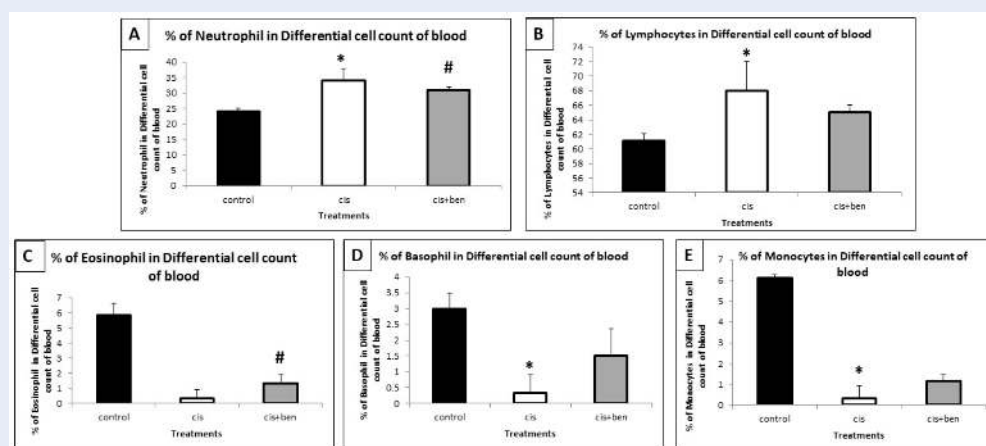


Figure 4: Effect of *Benincasa sp.* on Differential cell count of peripheral blood in cisplatin induced AKI (*p < 0.05, compared to control; #p < 0.05, compared to cisplatin; significance value ≤ 0.05).

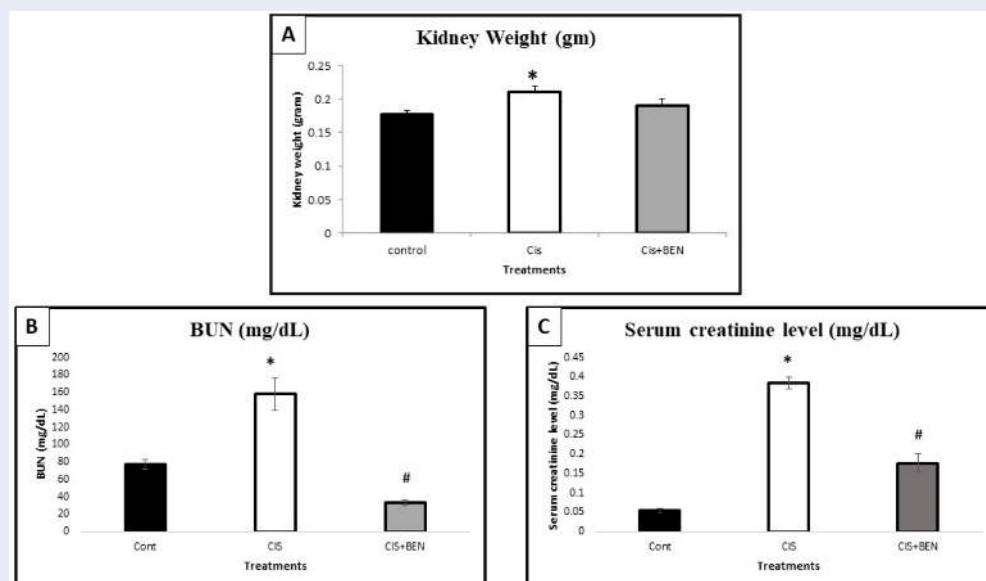


Figure 5: Effect of *Benincasa sp.* on kidney weight, serum creatinine level and blood urea nitrogen (BUN) level in cisplatin induced AKI (*p < 0.05, compared to control; #p < 0.05, compared to cisplatin; significance value ≤ 0.05).

degree of kidney damage, with detailed criteria for each grade. For the cortex and outer stripe of the outer medulla in each kidney section, ten fields (40x) were counted. The sum of all scores from 100 fields, with a maximum score of 300, was used to determine the overall score for each kidney. The percentage of cast formation was calculated from 20 fields for the cortex and medulla in each kidney section.

Flow cytometry

Cells from the blood, kidney, bone marrow, and spleen were stained with suitable antibodies and analyzed using flow cytometry on a BD FACSVerse (BD Biosciences, USA), with results processed using the FACSSuite (BD Biosciences, USA) software. The antibodies used for cell surface staining included: CD45-PerCPCy5.5 (BioLegend), CD4-V450 (BD Biosciences, USA), CD3e-PE (BD Biosciences, USA), B220-FITC (BD Biosciences, USA), CD8a-

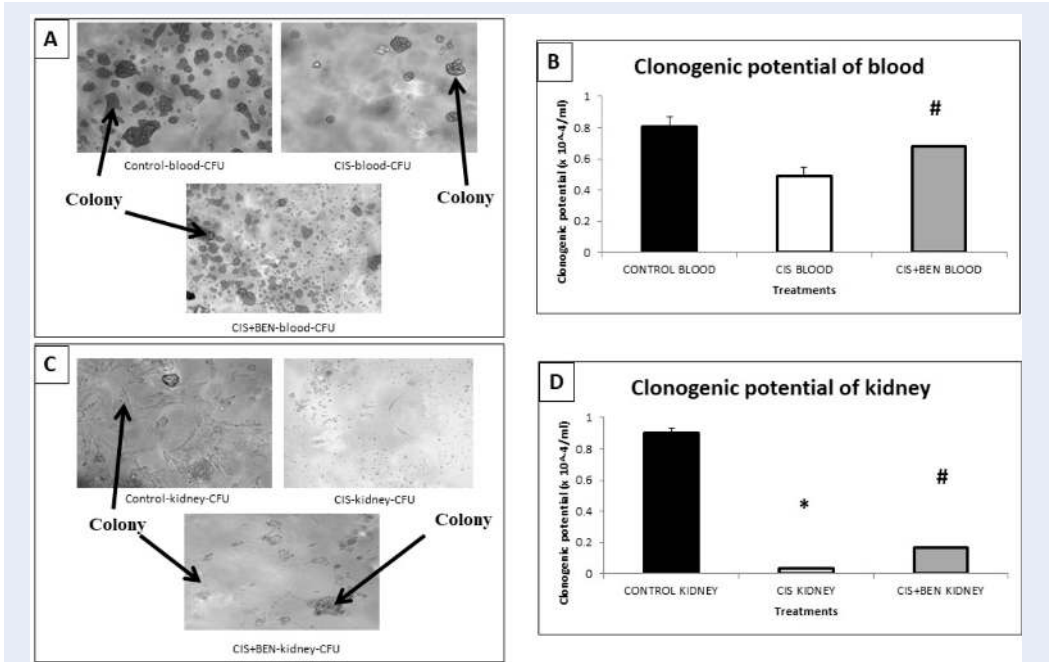


Figure 6: Results of the CFU-c assay. (*p < 0.05, compared to control; #p < 0.05, compared to cisplatin; significance value ≤ 0.05).

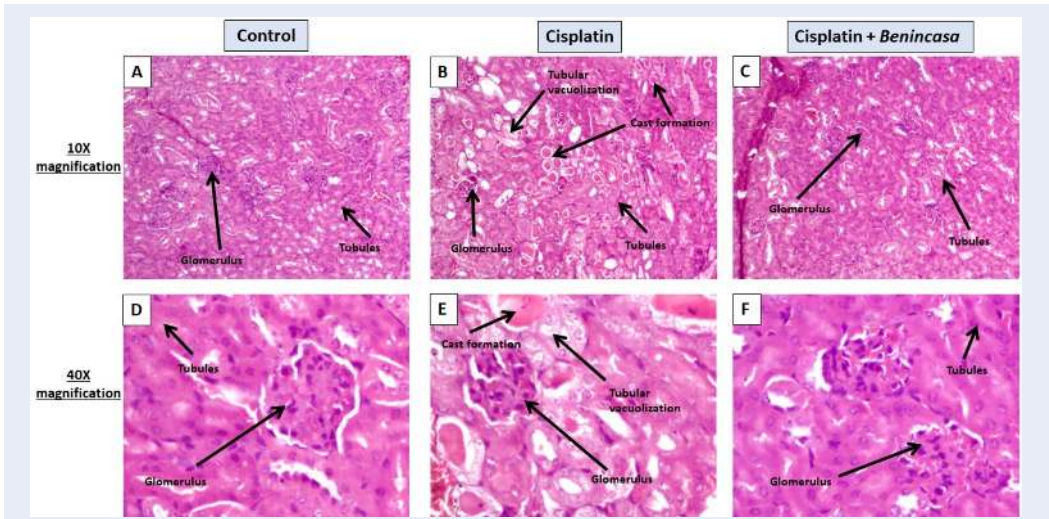


Figure 7: Study the microanatomy of kidney using Haematoxylin-eosin staining.

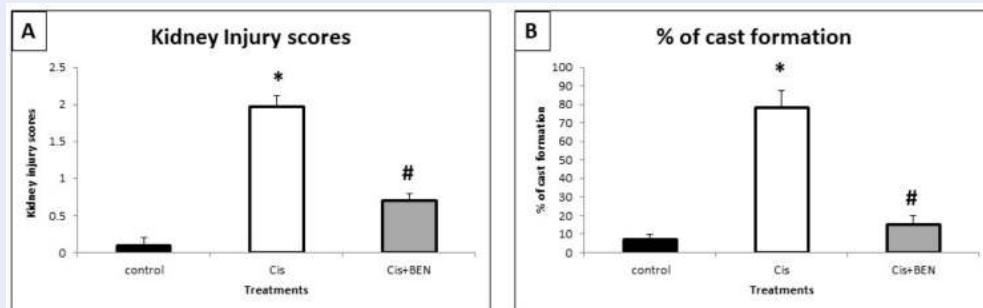


Figure 8: Graphical representation of the effects of *Benincasa sp.* on kidney's microanatomy (as seen in the figure 7) in cisplatin-induced Acute kidney injury (* $p < 0.05$, compared to control; # $p < 0.05$, compared to cisplatin; significance value ≤ 0.05).

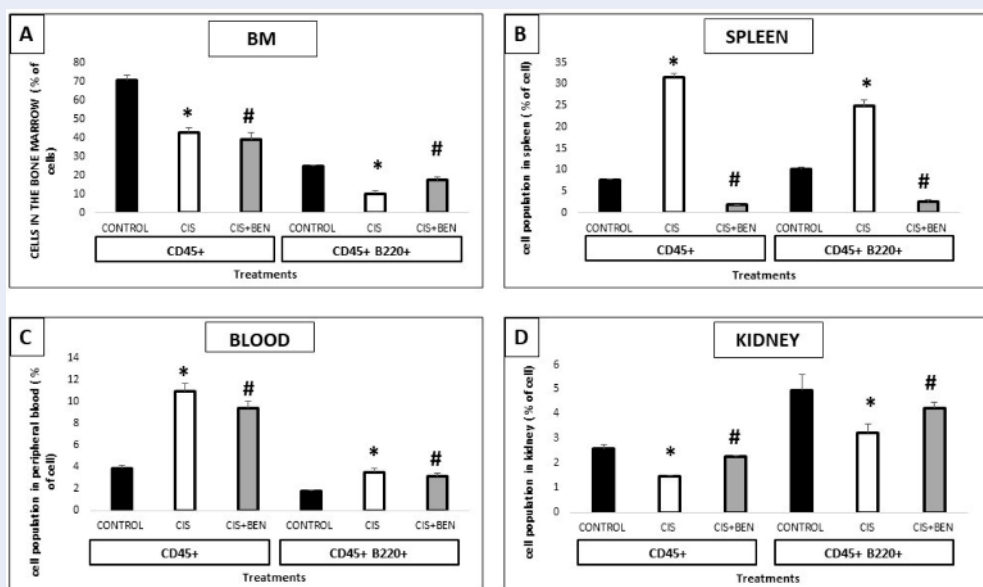


Figure 9: Study the effect of *Benincasa sp.* on the cellular status and migration pattern of $CD45^+ B220^+$ B cells in bone marrow (BM), spleen, blood and kidney in cisplatin induced AKI (* $p < 0.05$, compared to control; # $p < 0.05$, compared to cisplatin; Significance value ≤ 0.05).

Alexa Fluor 488 (BD Biosciences, USA), GR-1-FITC (MACS), and F4/80-PE (eBiosciences). Among the $CD45^+$ hematopoietic cells, $CD45^+ B220^+$ are B cells, and $CD45^+ CD3^+$ are T cells, with subsets identified as helper T (TH) cells ($CD3^+ CD4^+$) and cytotoxic T (TC) cells ($CD3^+ CD8^+$), while neutrophils and macrophages are identified as $CD45^+ Gr1^+$ and $CD45^+ F4/80^+$, respectively.

Gene expression Study

Total RNA was extracted from kidney tissues using TRIzol solution according to the manufacturer's instructions (Life Technologies, USA). The expression

of $TNF\alpha$, $IFN\gamma$, $IL1\beta$, $IL13$, $IL4$, and $IL10$ was evaluated using GAPDH as the housekeeping gene. To visualize the expression of inflammatory genes, PCR products were run on 1% agarose gels, and band intensities were quantified using ImageJ software following visualization under UV light in a gel-doc system (BioRad).

Protein expression by Western blot

Kidneys were homogenized, and total cellular protein was extracted in RIPA buffer solution. Western blotting was performed using antibodies against GAPDH, $TGF\beta$, AQP1, and AQP5, followed by de-

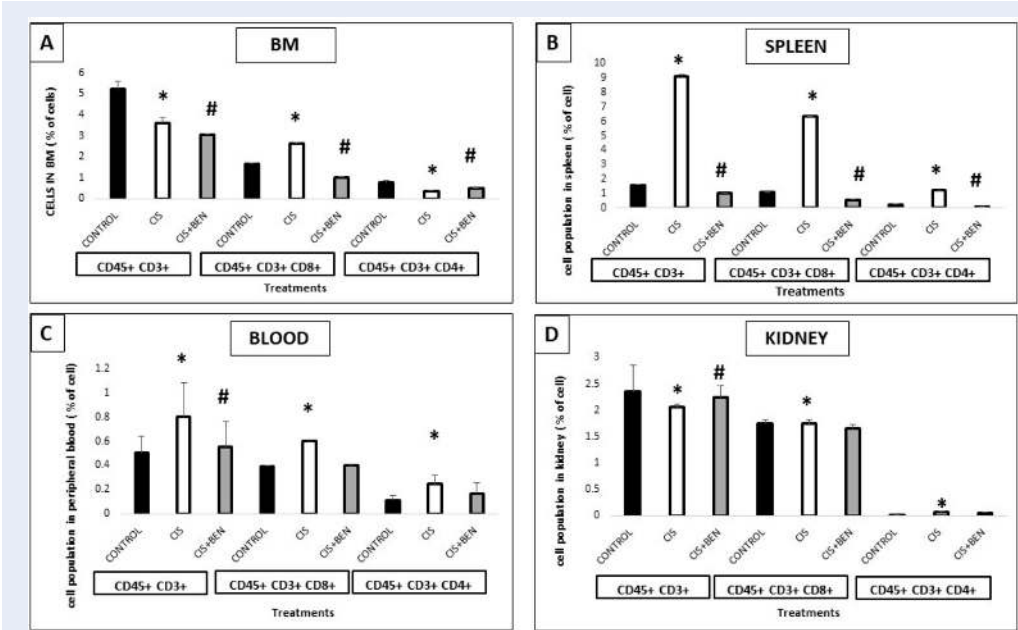


Figure 10: Study the effect of *Benincasa sp.* on the cellular status and migration pattern of CD45⁺ CD3⁺ CD8⁺ and CD45⁺ CD3⁺ CD4⁺ T cells in bone marrow (BM), spleen, blood and kidney in cisplatin induced AKI (* p < 0.05, compared to control; # p < 0.05, compared to cisplatin; Significance value ≤ 0.05).

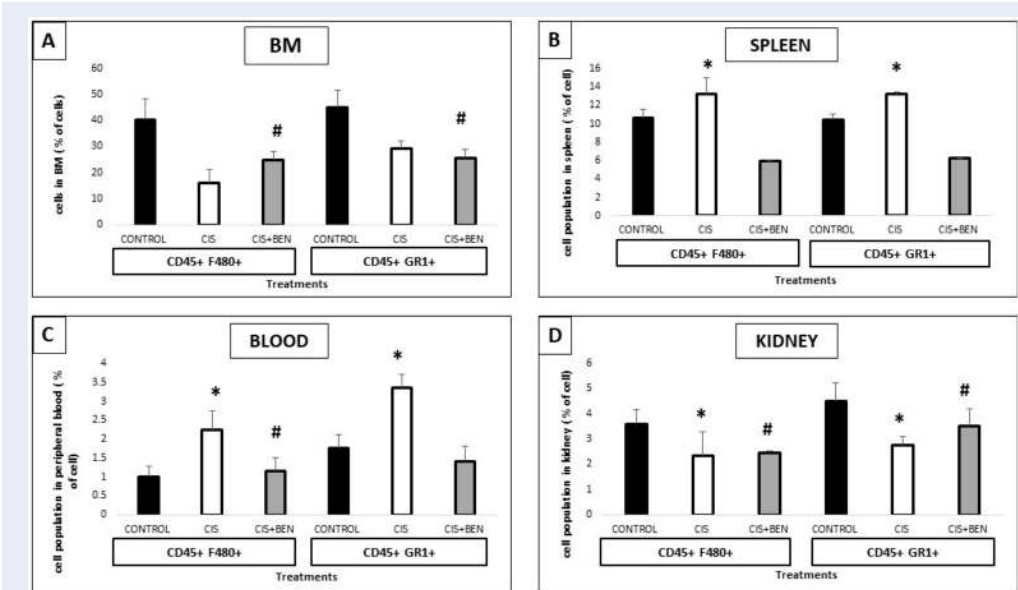


Figure 11: Study the effect of *Benincasa sp.* on the cellular status and migration pattern of CD45⁺ F480⁺ Macrophages and CD45⁺ GR1⁺ Neutrophils in bone marrow (BM), spleen, blood and kidney in cisplatin induced AKI (* p < 0.05, compared to control; # p < 0.05, compared to cisplatin; Significance value ≤ 0.05).

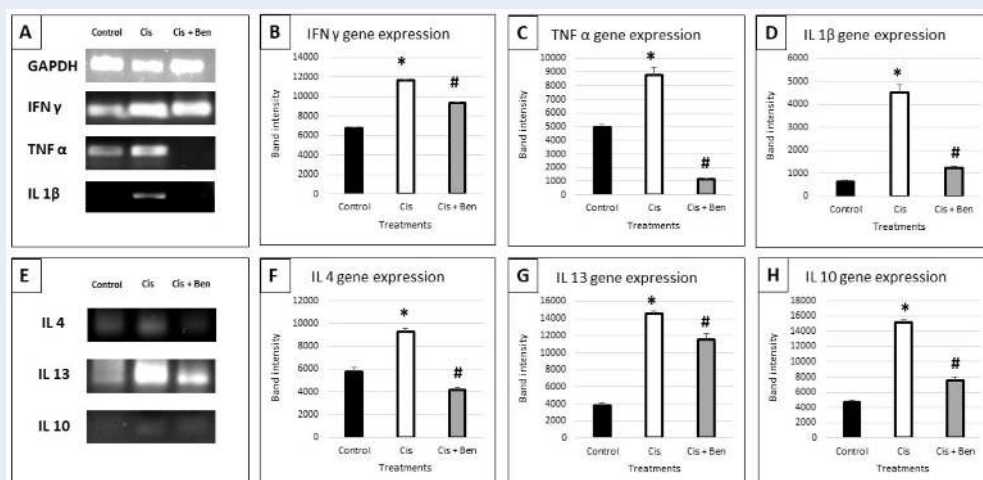


Figure 12: *Benincasa* extract reduced the expression of inflammatory cytokines and signalling molecules at the gene level in cisplatin induced AKI (* $p < 0.05$, compared to control; # $p < 0.05$, compared to cisplatin; Significance value ≤ 0.05).

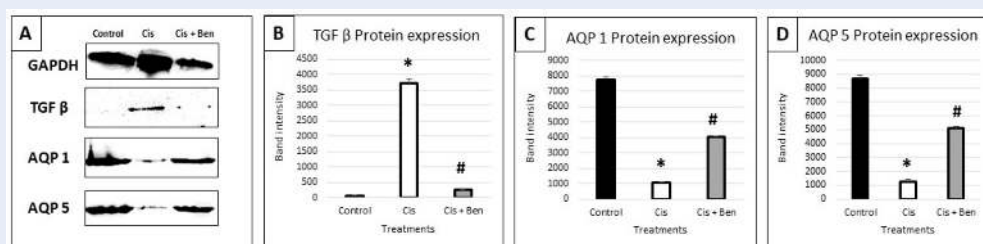


Figure 13: *Benincasa* restored the expression of signalling molecules and transmembrane channels at the protein level in cisplatin induced AKI (* $p < 0.05$, compared to control; # $p < 0.05$, compared to cisplatin; Significance value ≤ 0.05).

tection with HRP-conjugated secondary antibodies. The blots were developed using BioRad Western ECL substrate and visualized in a chemidoc system (BioRad). The band intensities were calculated using ImageJ software.

RESULTS

Total cell counts of blood, kidney, spleen, and bone marrow

Reduction of total cell (TC) count in blood and bone marrow is an indication of cisplatin-induced toxicity. The TC of bone marrow and PB decreased by 23.11-fold ($p < 0.05$) and 3.48-fold ($p < 0.05$) after treatment with cisplatin, compared to control, and increased by 12.29-fold ($p < 0.05$) and 1.89-fold ($p < 0.05$), respectively, with *Benincasa* treatment (Figure 3A, C). The TC of spleen and kidney increased by 5.42-fold ($p < 0.05$) and 3.57-fold ($p < 0.05$), respectively, with cis-

platin treatment, and reduced by 1.79-fold ($p < 0.05$) and 1.91-fold ($p < 0.05$), respectively, with *Benincasa* extract (Figure 3B, D).

Differential count of peripheral blood

Cisplatin treatment led to an increase in the count of both neutrophils and lymphocytes in the blood by 1.41-fold ($p < 0.05$) and 1.11-fold ($p < 0.05$), respectively. *Benincasa* therapy successfully reduced both counts by 1.09-fold ($p < 0.05$) and 1.04-fold, respectively (Figure 4A, B). The count of eosinophils, basophils, and monocytes decreased by 17.5-fold, 9-fold ($p < 0.05$), and 18.3-fold ($p < 0.05$), respectively, with cisplatin treatment and increased by 4-fold ($p < 0.05$), 4.5-fold, and 3.5-fold, respectively, with *Benincasa* extract (Figure 4C, D, E).

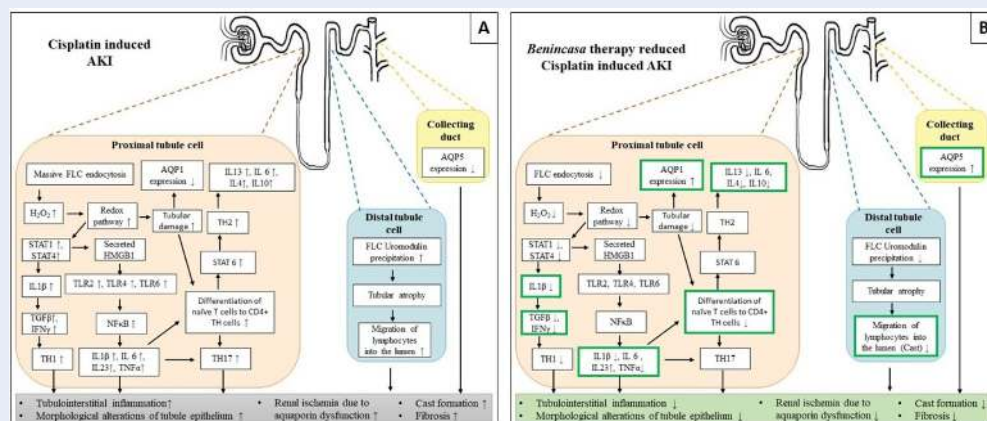


Figure 14: Mechanism of cisplatin induced acute kidney injury- Free monoclonal light chains (FLCs) were endocytosed in the proximal tubule following the injection of cisplatin by the tandem receptors megalin and cubilin expressed on proximal epithelial cells. (A) Massive FLC reabsorption within proximal cells led to the production of hydrogen peroxide (H_2O_2), which in turn activates the nuclear factor- κ B (NF- κ B) and signal transducer and activator of transcription 1, 4, (STAT1, STAT4) pathways, promoting the production of inflammatory cytokines and transforming growth factor β (TGF β), and other molecules. The complementarity determining region 3 (CDR3) of FLCs interacts with a nine-amino-acid domain on uromodulin in the lumen of the distal tubule. In cisplatin-induced AKI, these binds cause casts to clog distal tubules. Inflammatory giant cells can also arise as a result of casts, especially if the tubular basement membrane is damaged. Cisplatin also decreased the proximal tubule cells' expression of aquaporin 1 (AQP1) and decreased the expression of aquaporin 5 (AQP5) in collecting ducts. Collectively, these lesions cause morphological changes in the tubular epithelium, fibrosis, and severe tubulointerstitial inflammation. **(B)** Possible mechanism (green boxes) of action of *Benincasa* extract in reducing the symptoms of cisplatin induced acute kidney injury. In addition to reducing the production of inflammatory cytokines and transforming growth factor β (TGF β), *Benincasa* also lowered the formation of hydrogen peroxide (H_2O_2) and tube damage. The *Benincasa* extract also raised the expression of aquaporin 5 (AQP5) in collecting ducts and aquaporin 1 (AQP1) in proximal tubule cells while decreasing cast formation in distal tubule cells. Overall, this *Benincasa* treatment reduced fibrosis, structural changes in the tubular epithelium, and significant tubulointerstitial inflammation.

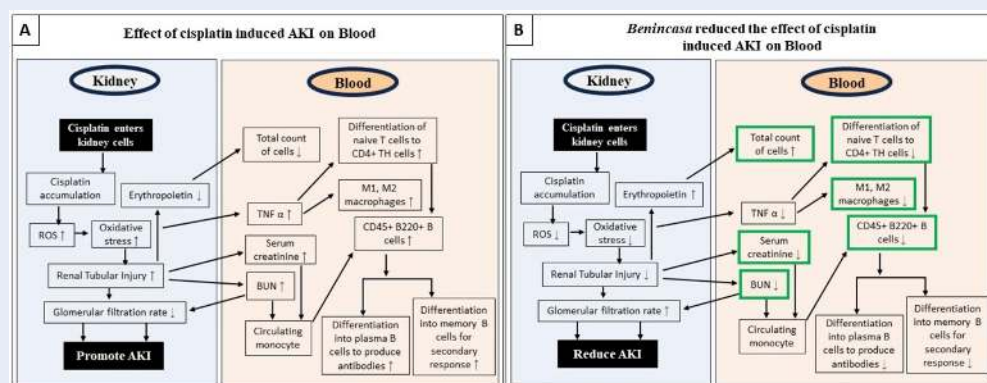


Figure 15: The effect of cisplatin induced AKI on blood. (A) After cisplatin administration, tubular damage and inflammations were started in kidney. Damaged tubules can't produce erythropoietin which reduce red blood cell production in bone marrow. Simultaneously, in blood, total count of cells decreased and serum creatinine and BUN (blood urea nitrogen) increased. These lesions activate CD4 $^{+}$ T cells, CD45 $^{+}$ B220 $^{+}$ B cells and macrophages. **(B)** Possible mechanism (green boxes) of action of *Benincasa* extract in reducing the symptoms of cisplatin induced acute kidney injury in blood as *Benincasa* reduced the inflammation in kidney, it increased the total count of blood cells and reduced serum creatinine and BUN and inflammation in blood.

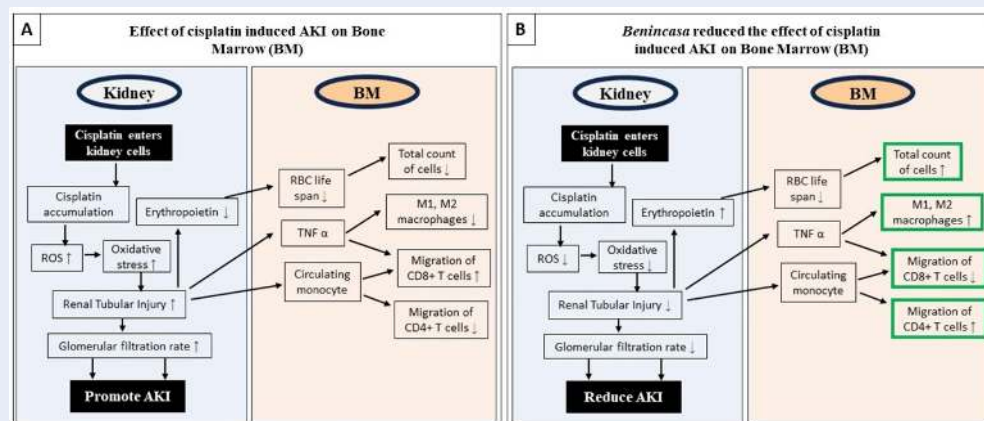


Figure 16: The effect of cisplatin induced AKI on bone marrow (BM). (A) After cisplatin administration, tubular damage and inflammations were started in kidney. Damaged tubules can't produce erythropoietin which reduce red blood cell production in bone marrow. Simultaneously, total count of cells decreased. These lesions activate CD4⁺ T cells, CD45⁺ B220⁺ B cells and macrophages. (B) Possible mechanism (green boxes) of action of Benincasa extract in reducing the symptoms of cisplatin induced acute kidney injury in bone marrow. As *Benincasa* reduced the inflammation in kidney, it increased the total count of bone marrow and reduced the inflammation in bone marrow.

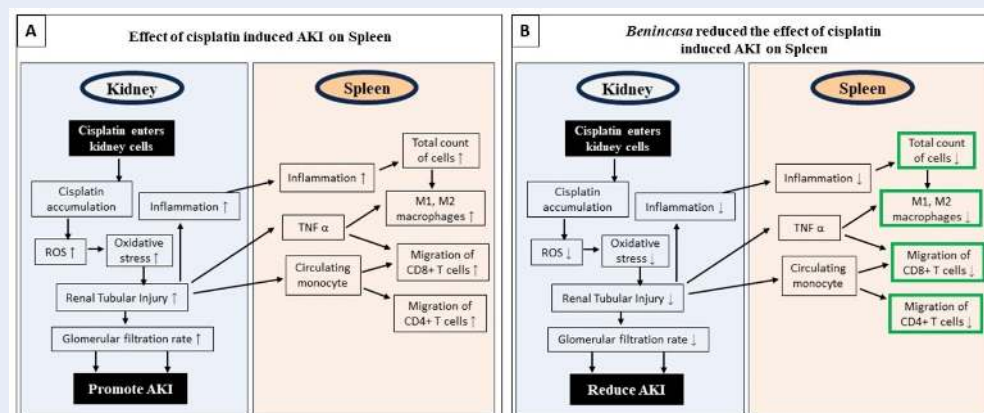


Figure 17: The effect of cisplatin induced AKI on spleen. (A) After cisplatin administration, tubular damage and inflammations were started in kidney. These lesions activate CD4⁺ T cells, CD8⁺ T cells, and macrophages in spleen. (B) Possible mechanism (green boxes) of action of Benincasa extract in reducing the symptoms of cisplatin induced acute kidney injury in spleen. As *Benincasa* reduced the inflammation in kidney, it decreased the total count of bone marrow and reduced the inflammation in spleen.

Estimation of kidney weight, serum creatinine level, and blood urea nitrogen (BUN) level

An increase in kidney weight, serum creatinine level, and BUN are indications of cisplatin-induced toxicity. The kidney weight increased by 1.18-fold ($p < 0.05$) after treatment with cisplatin, compared to control, and decreased by 1.10-fold with Benincasa treatment (Figure 5 A). Levels of serum creatinine and BUN increased by 7.09-fold ($p < 0.05$) and 2.04-fold ($p <$

0.05), respectively, with cisplatin treatment and were successfully reduced by 2.16-fold ($p < 0.05$) and 4.92-fold ($p < 0.05$), respectively, with Benincasa extract (Figure 5 B, C).

Estimation of clonogenic potential (assessed by the CFU-c assay) of cells

Loss of the ability to proliferate to their full potential of cells is an indication of inflammation or degeneration. Cisplatin treatment led to a decrease in

the clonogenic potential of blood (Figure 6 A, B) and kidney (Figure 6 C, D) by 1.65-fold and 25.5-fold ($p < 0.05$), respectively. Benincasa extract successfully restored the clonogenic potential by 1.38-fold ($p < 0.05$) and 4.80-fold ($p < 0.05$), respectively.

Hematoxylin-eosin staining of kidney sections

Representative images (Figure 7) of the renal pathology (H&E staining, magnification 10x, 40x) on day 7 after a single administration of cisplatin showed renal damages. Control groups showed normal renal structure. In the case of cisplatin-treated kidney sections, kidney injury scores, and the percentage of cast formation increased by 19.66-fold ($p < 0.05$) and 11.14-fold ($p < 0.05$), respectively, with cisplatin treatment. Benincasa extract significantly reduced the damage by 2.80-fold ($p < 0.05$) and 5.2-fold ($p < 0.05$), respectively (Figure 8 A, B).

Immunophenotyping and migration pattern of immune cells in the bone marrow, spleen, blood, and kidney

Cisplatin treatment increased the CD45⁺B220⁺ B cell populations by 2.42-fold ($p < 0.05$) and 1.94-fold ($p < 0.05$) in the spleen and blood, respectively, and decreased by 2.38-fold ($p < 0.05$) and 1.52-fold ($p < 0.05$) in bone marrow and kidney, respectively. Benincasa therapy successfully reduced the B cell population by 9.24-fold ($p < 0.05$) and 1.09-fold ($p < 0.05$) in spleen and blood, respectively (Figure 9 B, C), and increased by 1.69-fold ($p < 0.05$) and 0.6-fold ($p < 0.05$) in bone marrow and kidney, respectively (Figure 9 A, D).

Cisplatin treatment increased the CD45⁺CD3⁺CD8⁺ T_C cell populations by 1.57-fold ($p < 0.05$), 6-fold ($p < 0.05$) and 1.51-fold ($p < 0.05$) in bone marrow, spleen and blood respectively. Benincasa therapy successfully reduced the population by 2.5-fold ($p < 0.05$), 11.5-fold ($p < 0.05$) and 1.5-fold ($p < 0.05$) in bone marrow, spleen and blood respectively (Figure 10 A, B, C). In cisplatin induced mice, the CD45⁺CD3⁺CD4⁺ T_H cell populations were increased by 3.75-fold ($p < 0.05$), 5.17-fold ($p < 0.05$) and 2.27-fold ($p < 0.05$) in kidney, spleen and blood respectively and decreased by 2.22-fold ($p < 0.05$) in bone marrow. Benincasa therapy successfully reduced the T_H cell population by 1.36-fold ($p < 0.05$), 16.2-fold ($p < 0.05$) and 1.51 fold ($p < 0.05$) in kidney, spleen and blood respectively (Figure 10 B, C, D) and increased by 1.42 fold ($p < 0.05$) in bone marrow (Figure 10 A).

Cisplatin treatment increased the CD45⁺F480⁺ macrophage population by 1.23-fold ($p < 0.05$) and

2.25-fold ($p < 0.05$) in spleen and blood respectively and decreased by 2.48-fold ($p < 0.05$) and 1.53-fold ($p < 0.05$) in bone marrow and kidney respectively. Benincasa therapy successfully reduced the macrophages by 2.2-fold ($p < 0.05$) and 1.95-fold ($p < 0.05$) in spleen and blood respectively (Figure 11 B, C) and increased by 1.52-fold ($p < 0.05$) and 1.04-fold ($p < 0.05$) in bone marrow and kidney respectively (Figure 11 A, D). Cisplatin treatment increased the CD45⁺GR1⁺ neutrophil cell populations by 1.26-fold ($p < 0.05$) and 1.91-fold ($p < 0.05$) in spleen and blood respectively and decreased by 1.55-fold ($p < 0.05$) and 1.63-fold ($p < 0.05$) in bone marrow and kidney respectively. Benincasa therapy successfully reduced the neutrophil populations by 2.12-fold ($p < 0.05$) and 2.39-fold ($p < 0.05$) in spleen and blood respectively (Figure 11 B, C) and increased by 1.27-fold ($p < 0.05$) in kidney (Figure 11 A, D).

Estimation of the expression of proinflammatory cytokines and signaling molecules at the gene level

Using RT-PCR, this study evaluated the gene expression of signaling molecules and pro-inflammatory cytokines such as TNF α , IFN γ , IL1 β , IL13, IL4, and IL10. The treatment in the study had no effect on the expression of GAPDH, which served as a house-keeping gene (Figure 12 A and E). However, the intensity of the bands on the gels showed that the expression of the genes of interest significantly rose after cisplatin treatment compared to control. With cisplatin treatment (lanes marked "Cis" in Figure 12 A, E), the expression of IFN γ , TNF α , IL1 β , IL4, IL13, and IL10 increased by 1.72-fold ($p < 0.05$) (Figure 12 B), 1.77-fold ($p < 0.05$) (Figure 12 C), 7.18-fold ($p < 0.05$) (Figure 12 D), 1.60-fold ($p < 0.05$) (Figure 12 F), 3.88-fold ($p < 0.05$) (Figure 12 G), and 3.19-fold ($p < 0.05$) (Figure 12 H), respectively, compared to control (lanes marked "Control" in Figure 12 A, E). With Benincasa extract treatment (lanes marked "Cis+Ben" in Figure 12 A, E), the expression of IFN γ , TNF α , IL1 β , IL4, IL13, and IL10 decreased by 1.23-fold ($p < 0.05$) (Figure 12 B), 7.76-fold ($p < 0.05$) (Figure 12 C), 3.64-fold ($p < 0.05$) (Figure 12 D), 2.22-fold ($p < 0.05$) (Figure 12 F), 1.26-fold ($p < 0.05$) (Figure 12 G), and 2.01-fold ($p < 0.05$) (Figure 12 H), respectively, compared to the diseased group. These results imply that the expression of inflammatory molecules has been downregulated.

Estimation of the expression of signaling molecules and transmembrane channels at the protein level

This study also evaluated the protein expression of signaling molecules and transmembrane channels like TGF β (molecular weight 44 kDa), AQP1 (molecular weight 34 kDa), and AQP5 (molecular weight 35 kDa). The treatment in the study had no effect on the expression of GAPDH, which served as a house-keeping protein (Figure 13 A). However, the intensity of the bands on the blots showed that the expression of the study proteins significantly changed following cisplatin treatment when compared to control. With cisplatin treatment (lanes marked "Cis" in Figure 13 A), the expression of TGF β increased by 73.99-fold ($p < 0.05$) (Figure 13 B), compared to control (lanes marked "Control" in Figure 13 A). With Benincasa extract treatment (lanes marked "Cis+Ben" in Figure 13 A), the intensity of TGF β decreased by 13.45-fold ($p < 0.05$) (Figure 13 B). With cisplatin treatment (lanes marked "Cis" in Figure 13 A), the expression of AQP1 and AQP5 decreased by 0.14-fold ($p < 0.05$) and 0.14-fold ($p < 0.05$), respectively (Figure 13 C, D), compared to control (lanes marked "Control" in Figure 13 A). With Benincasa extract treatment (lanes marked "Cis+Ben" in Figure 13 A), the intensities of AQP1 and AQP5 increased by 0.26-fold ($p < 0.05$) and 0.24-fold ($p < 0.05$), respectively (Figure 13 C, D). These results imply that the expression of signaling molecules and transmembrane channels has been restored with the use of Benincasa therapy.

DISCUSSION

Cisplatin-induced acute kidney injury (AKI) is a significant complication of chemotherapy, characterized by tubular cell injury, inflammation, and oxidative stress. Cisplatin, a widely used chemotherapeutic agent, exerts nephrotoxic effects primarily by accumulating in renal tubular cells, leading to cellular apoptosis and necrosis. This nephrotoxicity often limits the clinical utility of cisplatin, necessitating dose reductions or discontinuation of treatment, which can compromise the efficacy of cancer therapy¹⁷⁻²¹. Effective management of cisplatin-induced AKI remains a critical challenge, highlighting the need for novel protective strategies to mitigate renal damage without impeding the anti-cancer efficacy of cisplatin²¹. Few investigations have clarified the roles of oxidative stress injury, apoptosis, and inflammation as causes of nephrotoxicity, and the mechanism by which cisplatin causes kidney injury is still poorly understood. Renal replacement therapy, kidney transplantation, and dialysis are associated with

several risk factors. The synthetic drugs for AKI are expensive and have side effects on other organ functions. Therefore, scientists are interested in natural compounds that have beneficial antioxidant properties. This study used *Benincasa*, a widely used vegetable and fruit in India, which contains beneficial constituents like triterpenoids, vitamins, amino acids, and uronic acids. *Benincasa* sp. has been successfully used for renal failure in the human body, but the mechanism is still unknown. There is no *in vivo* animal AKI model to investigate the immune profile of this therapy. This study aimed to investigate whether the peel extract of *Benincasa* sp. showed a protective role against renal cell injury in the case of cisplatin-induced AKI. *Benincasa* sp. has been used at a dose of 500 mg/kg/day in previous investigations, and therapy with *Benincasa* sp. extract produced good ameliorative effects on renal ischemia and diabetes¹⁵. The identical dosage of 500 mg/kg/day of *Benincasa* sp. in an aqueous solution was utilized in this investigation. Furthermore, *Benincasa* sp. was given orally rather than intraperitoneally, intravenously, or intratracheally as had been done in other investigations. Oral medication operates systemically on the disease and offers a more convenient route of treatment.

Most people with kidney disease develop anemia. In AKI patients, the occurrence of anemia is frequent and rapid²². A crucial hormone called erythropoietin (EPO) is produced by the kidneys. Production of red blood cells is regulated by EPO. In renal disease, the kidneys are unable to produce enough EPO, leading to a drop in the total blood cell count and subsequent anemia. Treatment with cisplatin led to a 3.48-fold decrease in the total cell count of the blood compared to the untreated control ($p < 0.05$), indicating that the kidneys cannot make enough EPO to produce sufficient blood cells. Oral treatment with *Benincasa* extract led to a slight increase in the total cell count of blood, showing its ability to significantly increase blood cell production. The total cell counts at the site of inflammation (*i.e.*, the kidney) increased 3.57-fold after treatment with cisplatin, indicating the presence of inflammation. *Benincasa* extract significantly reduced the cell count ($p < 0.05$), indicating that it is significantly successful in reducing inflammation when applied orally. Cisplatin had a significant toxic effect on both cellular and vascular components of the spleen, increasing the total cell (TC) count in the spleen as an indication of cisplatin-induced toxicity. The TC count of the spleen decreased after treatment with *Benincasa* extract. In the bone marrow, *Benincasa* extract successfully reduced inflammation (Figures 14, 15 and 3). Both the

number of neutrophils and lymphocytes in the blood increased after receiving the chemotherapeutic drug cisplatin. Neutrophil and lymphocyte counts were successfully reduced by *Benincasa* therapy. With cisplatin treatment, the count of eosinophils, basophils, and monocytes decreased. The number of lymphocytes was successfully boosted by *Benincasa* extract (Figures 4, 14 and 15). The amount of nitrogen in the blood that originates from the waste product urea is measured by a BUN test. When protein is broken down by the body, urea is produced. The body excretes urea through urine, which is generated in the liver. A BUN test is performed to assess how effectively the kidneys are functioning. A rise in BUN level indicates the inability of kidneys to regularly eliminate urea from the blood. Kidneys also remove creatinine from the body. Thus, a rise in the level of creatinine in the blood indicates renal malfunction. Increases in serum creatinine, BUN, and kidney weight are signs of cisplatin-induced kidney damage. The toxicity was successfully decreased by the *Benincasa* extract (Figures 5, 14 and 15). Clonogenic potential measures a cell's capacity to grow into colonies. Stressed cells typically lose some of their capacity for clonogenic reproduction. The clonogenic potential of kidney cells and blood cells from mice given cisplatin decreased after 6 days of incubation as compared to controls. All of these cell types had their clonogenic potential successfully recovered after treatment with *Benincasa* extract (Figures 6 and 14). The formation of monoclonal immunoglobulin free light chains (FLCs), which coprecipitate with Tamm-Horsfall glycoprotein (THP) in the lumen of the distal nephron and block tubular fluid flow, is directly associated with renal lesions (casts). Additionally, FLCs that evade tubular reabsorption are delivered to the distal nephron where they, under the right circumstances, create intraluminal casts that block tubular fluid flow. AKI and progressive renal failure are two clinical symptoms of this condition, also known as cast nephropathy²³. Urinary casts are a helpful predictor of AKI in acute heart failure, and AKI is associated with a poor prognosis among patients with acute heart failure (AHF)²⁴. When compared to the control group in this investigation, the cisplatin-injected kidneys displayed glomerular damage, collapsing Bowman's space, cast formation, epithelial necrosis, and vacuolization in the proximal tubules. Treatment with *Benincasa* extract significantly reduced these alterations (Figures 7, 8 and 14).

By making cell suspensions and tissue sections from lymphoid and nonlymphoid organs, it has been possible to evaluate the frequency and distribution of B

lymphocyte subsets in numerous human tissues and fluids. B cells may be a component of a circulating system that helps kidney disease patients' immune systems by acting as antigen-presenting cells and a source of cytokines that encourage T-cell proliferation. Therefore, in this cisplatin-induced AKI, the number of matured B cells is enhanced in the blood and spleen and decreased in the kidney and bone marrow (Figure 9). The dynamics of CD45⁺B220⁺ B cell populations in the bone marrow, spleen, blood, and kidney were successfully restored by *Benincasa* therapy (Figures 9 and 14). The relationships between the various lymphocyte subsets vary in most tissues and fluids. Between B and T lymphocytes, there is more compositional heterogeneity than between CD3⁺CD4⁺ and CD3⁺CD8⁺ T lymphocytes. Peripheral blood and the spleen have fewer CD3⁺ T lymphocytes in a healthy person than they do in renal disease. It has been established that T lymphocytes have a role in ischemia AKI²⁵. Compared to T cell replete mice, the T cell deficient mice experienced less renal dysfunction and tubular damage and fared better in terms of survival²¹. It was found that CD3⁺CD4⁺ T cells and, to a lesser extent, CD3⁺CD8⁺ T cells were the main mediators of the negative effects of T cells. T cells are thought to play a role in the delayed cell-mediated immune response according to conventional immunology theories²⁶. *Benincasa* therapy dramatically decreased the infiltration of T cell populations in the spleen, blood, and kidney in cisplatin-induced mice, whereas the cell populations of CD3⁺CD4⁺ and CD3⁺CD8⁺ T lymphocytes increased in the blood, spleen, and kidney (Figures 10, 14 and 15). After being activated, naive CD3⁺CD4⁺ T cells in cisplatin-induced AKI develop into T-helper type 1 (Th₁) and type 2 (Th₂), which generate cytokines such as interferons like IFN γ and TNF α and interleukins like IL4, IL13, and IL10, respectively. When exposed to infections, CD3⁺CD8⁺ T cells can develop into cytotoxic effector cells that produce tumor necrosis factor (TNF α) and interferon (IFN γ) and migrate to eradicate the infection. Antigen-presenting cells in the lymph nodes and spleen prime CD3⁺CD8⁺ T cells during an infection²⁶⁻²⁹. Regulatory T cells (Tregs), Th₁, Th₂, and Th₁₇ can all be functionally differentiated from CD4⁺ T cells, and they can also activate the TGF signaling pathway. IFN γ and TNF α , two significant pro-inflammatory cytokines produced by Th₁ cells, and IL4, IL10, and IL13, anti-inflammatory cytokines produced by Th₂ cells, are proinflammatory cytokines. Anti-inflammatory cytokines like IL4, IL10, and IL13 showed higher levels of expression

in cisplatin-induced animals, which may have activated STAT6-mediated Th₂ differentiation. The expression of the IL4, IL10, and IL13 genes was dramatically decreased by Benincasa therapy (Figures 12 and 14). According to previous research, the pattern of Th₂ inflammatory cells is protective in a kidney disease model, while the pattern of Th₁ inflammatory cells is pathogenic and causes more damage²⁶⁻²⁹. Th1 differentiation is mediated by the signal transducer and activator of transcription (STAT4) gene, whereas Th₂ differentiation requires STAT6. Studies employing STAT4- and STAT6-knockout mice in an ischemia AKI model demonstrated that STAT6 deficiency resulted in decreased renal function and tubular damage due to impaired Th₂ cell development. In STAT6-deficient mice, IFN γ expression was shown to be upregulated, whereas IL4 expression was downregulated. According to studies by scientists²⁶⁻²⁹, ischemia-reperfusion injury (IRI) in STAT4-deficient mice led to a slight improvement in renal function. IFN γ , TNF α , and TGF β gene expression levels rose in cisplatin-induced mice, possibly activating the TGF β signaling pathway or STAT4-mediated Th1 differentiation. IFN γ , TNF α , and TGF β expression at the gene and protein levels were all dramatically reduced by Benincasa therapy (Figures 12 and 13). In the pathogenesis of AKI, Th₁₇ cells have more recently become important participants²⁸. Different studies have shown that the kidney-infiltrating lymphocytes known as Th₁₇ are the most prevalent after AKI in mice. Researchers have found that after developing AKI due to ureteral obstruction, resident dendritic cells and tubular epithelial cells (TEC) release IL-1 (α and β of IL-1), IL-23, and IL-6 to encourage intrarenal IL-17 migration and activation²⁶⁻²⁹. In cisplatin-induced kidneys, increased levels of IL-1 gene expression may trigger Th₁₇ differentiation, which was significantly suppressed by Benincasa therapy (Figures 12, 14, 15 and 16). During the first few days of AKI, this study looked at the dynamics of leukocyte populations in the bone marrow, spleen, blood, and kidney. As previously mentioned, extravascular kidney leukocytes were identified by administering a CD45⁺ antibody just before organ harvest. A dispersed mononuclear phagocyte cellular system (MPS) made up of tissue macrophages helps the body react to physiologic changes and infectious threats. As macrophages are flexible cells, they can quickly change their gene expression patterns and functionalities to match the changing renal microenvironment. The pathogenesis of ischemic AKI has been linked to macrophages. Their contribution to cisplatin nephrotoxicity is unclear, though.

After cisplatin administration, researchers observed a 2-fold increase in CD11⁺ macrophages in the kidney and F480⁺ macrophages in the blood and spleen. Through their functional characteristics, neutrophils, the most prevalent leukocyte population in circulation, are important effectors of the inflammatory cascade. There is debate concerning neutrophils' contribution to acute renal failure. There is no doubt that the absence of neutrophils can result in acute renal failure. According to scientists²⁵, neutrophil retention is influenced by the level of neutrophil activation and the length of renal ischemia. It is possible that this relationship explains why acute renal failure occurs so frequently²⁶⁻²⁹. Neutrophil (CD45⁺GR1⁺) counts decreased in bone marrow after cisplatin-induced AKI (Figure 11 A), and a reciprocal increase was seen in blood and spleen (Figure 11 B, C). According to studies, the quantity of neutrophils in the blood is positively connected with the severity of AKI. The dynamics of leukocyte populations (CD45⁺F480⁺ and CD45⁺GR1⁺ cells) in the bone marrow, spleen, blood, and kidney were dramatically restored by Benincasa therapy (Figures 12, 14 and 16). The elevated incidence of acute renal failure may be explained by this interaction. As a result of cisplatin-induced AKI, neutrophil (CD45⁺GR1⁺ cells) counts decreased in bone marrow (Figure 11 A), and a corresponding rise in blood and spleen (Figure 11 B, C) numbers was observed. According to scientists²⁷⁻²⁹, there is a positive correlation between the blood's neutrophil count and the severity of AKI. In the bone marrow, spleen, blood, and kidney, Benincasa therapy greatly improved the dynamics of leukocyte populations (CD45⁺F480⁺ and CD45⁺GR1⁺ cells) (Figures 12, 14, 16 and 17). A family of highly selective transmembrane channels called aquaporins (AQPs) primarily transports water across cells while also facilitating the passage of some low-molecular-weight solutes. The apical and basolateral plasma membrane of the proximal tubule, the descending thin limbs of Henle, and the descending vasa recta all contain the first water channel, known as AQP1, which mediates water reabsorption. A very selective water-permeable channel is AQP1. Polyuria in AQP1-deficient mice demonstrates this protein's critical function in the development of hypertonicity. AQP5 is said to be present in type B intercalated cells of collecting ducts, according to research published a few years ago³⁰. In this investigation, it was found that cisplatin-induced mice had lower levels of AQP1 and AQP5 protein expression, and Benincasa therapy significantly corrected the deficiency in these two transmembrane channels (Figures 13 and 14). With

the worldwide increase in kidney disease, the high cost of therapy, and the side effects of current therapeutic strategies, our study of *Benincasa* extract in a pre-clinical mouse model may provide a starting point for the development of a safer, more efficient, and more cost-effective treatment for acute kidney disease.

CONCLUSIONS

Recent findings from a preclinical study involving mouse models suggest that oral *Benincasa* extract, delivered in an aqueous solution, offers a cost-effective and safe intervention for the early stages of acute kidney disease, primarily during its acute inflammatory phase. These findings, supported by colony-forming unit (CFU) data, indicate that *Benincasa* extract holds promise as a therapeutic agent for kidney regeneration and the treatment of anemia induced by acute kidney injury (AKI).

The mechanism by which *Benincasa* extract exerts its therapeutic effects appears to involve the inhibition of several pathological processes. Notably, the extract prevents the synthesis and migration of CD3⁺CD4⁺ T helper cells and mitigates increases in blood urea nitrogen (BUN) and serum creatinine levels, which are critical markers of kidney function. Moreover, it inhibits the activation of key inflammatory mediators, including TGFβ, IFNγ, and TNFα, along with Th₂ and Th₁₇ cytokines, during the critical acute phase of kidney disease. Additionally, in cases of acute renal illness triggered by cisplatin, *Benincasa* extract has been shown to counteract the loss of aquaporin transmembrane water channels, as evidenced by Figure 14 through Figure 17.

Overall, this study strongly suggests that *Benincasa* extract could be a valuable therapeutic option for addressing the early stages of acute kidney illness, offering a new avenue for the treatment of this condition and its complications, including AKI-related anemia.

ABBREVIATIONS

AQP1: Aquaporin 1, **AQP5:** Aquaporin 5, **AHF:** Acute Heart Failure, **AKI:** Acute Kidney Injury, **BC:** *Benincasa* cerifera, **BUN:** Blood Urea Nitrogen, **CD:** Cluster of Differentiation, **CFU-c:** Colony-Forming Unit-Cell, **DC:** Differential Cell, **ECL:** Enhanced Chemiluminescence, **EDTA:** Ethylenediaminetetraacetic Acid, **EPO:** Erythropoietin, **FLC:** Free Light Chain, **GAPDH:** Glyceraldehyde 3-Phosphate Dehydrogenase, **H&E:** Hematoxylin and Eosin, **HRP:** Horseradish Peroxidase, **IFNγ:** Interferon

Gamma, **IL10:** Interleukin 10, **IL13:** Interleukin 13, **IL1β:** Interleukin 1 Beta, **IL4:** Interleukin 4, **MPS:** Mononuclear Phagocyte System, **PB:** Peripheral Blood, **PBS:** Phosphate-Buffered Saline, **RT-PCR:** Reverse Transcription Polymerase Chain Reaction, **STAT4:** Signal Transducer and Activator of Transcription 4, **STAT6:** Signal Transducer and Activator of Transcription 6, **TC:** Total Cell, **TGFβ:** Transforming Growth Factor Beta, **Th₁:** T Helper 1, **Th₂:** T Helper 2, **Th₁₇:** T Helper 17, **THP:** Tamm-Horsfall Glycoprotein, **TNFα:** Tumor Necrosis Factor Alpha, **T_{regs}:** Regulatory T Cells

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AUTHOR'S CONTRIBUTIONS

Ms. Payal Pal performed the immunological experiments, assays, analyzed data and drafted manuscript. Prof. Ena Ray Banerjee initiated the project, designed the whole experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

A project proposal no ERB/ZOO/2023/III submitted by Ms. Payal Pal has been approved/recommended by the Institutional Animal Ethics Committee of the Department of Zoology, University of Calcutta on 02.08.2023 using *BALB/c* mouse, to support the work done in this manuscript.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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