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Ameliorative Effects of *Citrus limetta* raw juice on Cyclophosphamide-Induced Organ Toxicity in Rats

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Abstract

The administration of cyclophosphamide (CYP) as a chemotherapeutic drug has been associated with organ toxicities by generative oxidative stress through its reactive metabolites. Thus, current study is aimed to explore the ameliorative effect of Citrus limetta (CL) raw juice treatment against CYP-induced nephrotoxicity and hepatotoxicity in rats. Following one week acclimatization, female Albino rats (N=24), were equally and randomly divided into four groups: control negative (CN) provided with normal feed and water intake, positive control group (CYP) was given intra-peritoneal injection of CYP at 20mg/kg/bw, positive control group (CL) was given oral CL treatment at 25ml/kg/bw without toxicity induction by CYP and treatment group (CYP+CL) received CL juice (25mL/Kg BW orally) after injection of CYP (20mg/kg intra-peritoneal). After 7 days of treatment, blood and tissue samples were collected and processed for biochemical markers and histopathology of liver and renal tissue. After statistical analyses, our results showed that CL juice significantly ($p \leq 0.05$) restored hepatic serum markers (ALT, AST, and total proteins), renal profile (creatinine, BUN, and uric acid), and cell injury markers (LDH and CK). Histopathological findings further supported biochemical findings and confirmed that CL juice preserved the architectural integrity and restored functions of the liver and kidney. Based on our findings, it is implied that CL may offer hepatic and renal protection by ameliorating oxidative stress produced by CYP administration.

Key Words: Cyclophosphamide, *Citrus limetta*, Nephrotoxicity, Hepatotoxicity.

1. Introduction:

The limited effectiveness of chemotherapy is mainly due to its inability to distinguish between healthy and abnormal tissues, causing widespread damage. This not only reduces the patient's quality of life but also results in life-threatening consequences (Mayne et al., 2006, Manavi et al., 2024). Among these immunosuppressant and antitumor drugs is cyclophosphamide (CYP), which is used to treat a wide range of neoplastic disorders and autoimmune diseases (Jalali et al., 2012, Sinanoglu et al., 2012, Zheng et al., 2022). Generally, CYP requires metabolic activation to exert its metabolic and toxic effects. It is activated in the liver by the hepatic cytochrome p450 oxidase system, leading to the formation of its active metabolites, especially 4-hydroxycyclophosphamide, which exists in equilibrium with aldophosphamide. These metabolites diffuse into the cells and are eventually converted into phosphoramide mustard, a potent DNA alkylating agent that interferes with DNA replication and transcription, ultimately leading to cell death (Hall and Tilby, 1992, Krüger-Genge et al., 2023). In addition to its cytotoxic action on rapidly dividing cancerous cells, the metabolic breakdown of CYP also generates several other toxic metabolic byproducts like acrolein and phosphoramide mustard (Al-Amarat et al., 2022). These metabolites are highly reactive, particularly acrolein generate free radical species. Excessive accumulation of these species results in oxidative stress, which causes damage to lipids, proteins, and nucleic acid within cells (Cengiz et al., 2022). This oxidative stress plays a critical role in causing hepatic (Senthilkumar et al., 2006, Rezaei et al., 2023, Tureyen et al., 2025), and renal toxicities (Ayhanci et al., 2010, Tureyen et al., 2025), associated with CYP administration. Since cyclophosphamide cause organ toxicity primarily through oxidative stress, it is essential to explore natural therapies with strong antioxidant properties to help mitigate its effect.

In this context, the Citrus family plants have been valued for their medicinal properties since the Middle Ages, largely due to their limonoids and flavonoids, which possess antitumor and anti-inflammatory properties (Perez et al., 2010). Citrus fruits are cultivated in more than 140 countries worldwide, with China, Brazil, India, and the United States being the major producing countries (Rao et al., 2021). These fruits are enriched with numerous antioxidative biomolecules, including phenolic acids, flavonones, flavanols, and their derivatives (Dadwal and Gupta, 2023), known to prevent, inhibit, or delay the process of oxidation (Hussain et al., 2018). Oxidation is a biochemical process that leads to the generation of free radicals that may directly or indirectly damage the cellular components (DNA, proteins, etc.) (Zandalinas et al., 2017). Citrus produce an ample quantity of endogenous antioxidants such as carotenoids, ascorbic acid (vitamin C), flavonoids, and tocopherols (vitamin E) (Racchi, 2013). These antioxidants detoxify or reduce the negative effects of reactive oxygen species (ROS), thus protecting the cellular components from ROS damage (Khalid et al., 2022). Furthermore, the potential antioxidant application of Citrus plants has been proven to be beneficial in various oxidative stress disorders, including obesity (Huang et

al., 2020), inflammatory diseases (Rong et al., 2021), atherosclerosis (Hu et al., 2021), neurodegenerative diseases (Cirmi et al., 2021), and cancer (Kitagawa et al., 2021).

As CYP administration is associated with organ toxicity, the current study aims at exploring the ameliorative effects of *Citrus limetta* raw juice against CYP-induced hepatotoxicity and nephrotoxicity in rats.

2. Material and Methods:

2.1. Procurement of materials:

Citrus limetta fruits were procured from the local market in Lahore and identified at the Botany Department of Government College University, Lahore (GC.Herb BOT.3472). Fresh juice was manually extracted daily and stored in a glass bottle at 4°C until use. Cyclophosphamide, available under the brand name Cyclomide (500 mg/1000 mg), was purchased from Sehat Clinic, Lahore.

2.2. Experimental design and animal grouping:

Twenty-four female wistar albino rats (6–8 weeks old, 230–250 g) were acclimatized for one week in the experimental animal facility at the Department of Physiology, University of Veterinary and Animal Sciences (UVAS). The rats were maintained under standard housing conditions, including a 12-hour light/dark cycle, a temperature of 22°C ± 3°C, and 60–70% humidity. They were provided standard chow feed and water *ad libitum.* The Institutional Ethical Committee of UVAS, Lahore approved all experimental procedures (DR #/1326). The rats were randomly and equally divided into 4 groups (n=6) as (a) control negative (CN) rats received basal diet, (b) CYP positive control group received intraperitoneal injection of CYP at 20mg/kg/bw (Kurdi and Alamri, 2016), (c) CL positive control group received orally CL juice at 25 ml/kg/bw (Ezeigwe et al., 2022), and (d) treatment group that was treated with CL juice at 25ml/kg/bw orally following CP 20mg/kg intra-peritoneal injection. Treatment was administered for seven days after CYP induction.

2.3. Body measurements and blood sample collection:

Body weight (g) was measured at the start and end of the experiment. Daily feed consumption (g/day) was measured throughout the trial by using equation 1 (Laaksonen et al., 2013). Body mass index (g/cm^2) was measured by using equation 2 (Bastías-Pérez et al., 2020).

$$\text{Daily feed intake} = Feed\ intake\ (g) - Feed\ leftover\ (g) \qquad Eq.1$$

$$\text{BMI} = \frac{\text{Body Weight(g)}}{\text{Nasoanal Length (cm2)}} \qquad Eq.2$$

At the end of 7-day, the rats in all the groups were sacrificed by cervical dislocation and blood samples from rats was processed into two different vials i.e. clot activator and anticoagulants. Anticoagulant vials were processed for complete hematology analysis by using Abacus Junior Vet hematology analyzer (Diamond Diagnostics Inc. Holliston, US). Procoagulant vials were processed for serum separation. After centrifugation 2000× g for 10 mins, serum was separated and stored at -20°C.

2.4. Biochemical parameters: Determination of hepatic profile

Serum liver parameters included total proteins (TP; Ref#10570) , albumin (A; Ref#10560), globulin (G), albumin-to-globulin ratio A/G ratio, and serum enzymes including aspartate transaminase (AST; Ref#12017), alanine transaminase (ALT; 12117), alkaline phosphatase (ALP; Ref#12117), and gamma-glutamyl transferase (GGT; Ref#12033) were measured using commercially available kits Human Diagnostics, Wiesbaden, Germany were used to assess all the parameters. The readings of these were taken on the Epoch™ microplate reader (Biotek Instruments Inc. Winooski, US).

Globulin levels were measured by using following formula:

$$Globulin\ \left(\frac{g}{dL}\right) = Total\ protein\ \left(\frac{g}{dl}\right) - Albumin\ \left(\frac{g}{dl}\right) \qquad Eq\ 3$$

2.5. Assessment of renal profile:

Renal profile parameters were determined by using commercially available kits by Human Diagnostics, Germany (Creatinine LOT#10053), blood urea nitrogen (BUN; LOT# 16010), and uric acid levels were determined by using (LOT#16005).

2.6. Assessment of cell injury markers:

Serum lactate dehydrogenase (LDH), and creatine kinase (CK) were evaluated by using Commercial kits from Human Diagnostics, Germany, (LOT#16001 for LDH) (LOT# A170245/6 FOR CK).

2.7. Assessment of Lipid profile:

Lipid profile parameters were measured by using human diagnostics, Germany (Total cholesterol LOT# 16008, Triglycerides LOT#16005, and HDL LOT#16002). LDL was measured by using the formula (Nawaz et al., 2024):

$$LDL\_c = Total\ cholesterol - HDL_c - \left(\frac{Triglycerides}{5}\right) \qquad Eq\ 4$$

2.8. Histopathology and histomorphometry:

For histopathology, liver and kidney tissue samples were retrieved from 10% formalin and embedded in paraffin to construct blocks. For analysis, 4-5µm thin sections were mounted on albumin-coated slides and stained with hematoxylin and eosin (H&E). Sections were observed under light microscope at 40x magnification. Histomorphometry was performed by using ImageJ software (1.54d, Java 1.8.0_345).

2.9. Statistical analysis:

Data is expressed as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test to assess the difference between groups. A value of $p \leq 0.05$ were considered statistically significant.

3. Results:

3.1. Anthropometric measurement and analysis:

Anthropometric parameters revealed (Figure 1A-C) that there was a significant (P≤0.05) reduction in body weight (225±12), feed intake (66±2.3), and body mass index (BMI, 0.45±0.04) in the CYP group as compared to control negative group (body weight: 255±11, feed intake: 79±3.2, and BMI: 0.56±0.04), indicating significant anthropometric effects of cyclophosphamide. Whereas, supplementation with Citrus limetta resulted in significant (P≤0.05) restoration of these levels, exhibited in the CYP+CL group (body weight: 240±13, feed intake: 71±2.3, and BMI: 0.51±0.03) showing its protective and restorative effects, potentially mitigating cyclophosphamide-induced metabolic and nutritional disturbance.

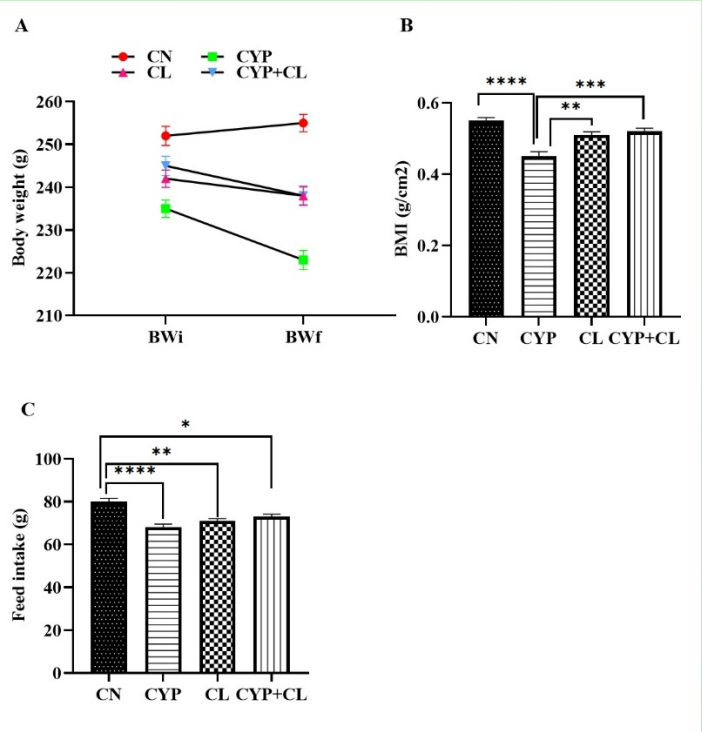

Figure 1. *Citrus limetta* supplementation mitigated cyclophosphamide-induced organ toxicity by improving key anthropometric parameters.

These parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). These parameters include (A) Body weight, (B) Body mass index (BMI), and (C) Feed intake. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

3.2. Hematological analysis and results:

Analyses of hematological parameters revealed significant (P≤0.05) suppression in white blood cells (WBC: 3.2±0.8 10^3/µl), lymphocytes (2.2±0.9 10^3/µl), and platelets (350±21 10^3/µl) in CYP group as compared to the control negative group (WBC: 7.2±2.8, lymphocytes 2.2±0.9, and platelets: 980±23), indicating cyclophosphamide-induced hematotoxicity (Fig 2A-C). However, *Citrus limetta* supplementation significantly (P≤0.05) restored these levels showing its immunomodulatory and hematoprotective properties and mitigating the myelosuppressive effects of cyclophosphamide. Moreover, no significant difference was observed in hematocrit levels (HCT), RBCs, and hemoglobin (Hb) among all groups (Fig 2 D-F).

3.3. Biochemical assessment of liver function:

Liver enzymes and protein levels were analyzed to assess liver function (Fig 3A-F). Cyclophosphamide administration significantly (P≤0.05) elevated ALT (175±13.2), and GGT (1.25±0.34) levels, along with the increase in total proteins levels (7.2±1.01) and decreased albumin to globulin ratio (A/G; 0.67±0.07) as compared to control negative (ALT: 118±12.4, GGT: 0.45±0.21, total proteins: 5.3±1.05, and A/G ratio:1.52±0.03), indicating hepatic toxicity and dysfunction. However, *Citrus limetta* supplementation significantly (P≤0.05) restored these biomarkers showing its potent hepatoprotective potential against cyclophosphamide-induced hepatotoxicity.

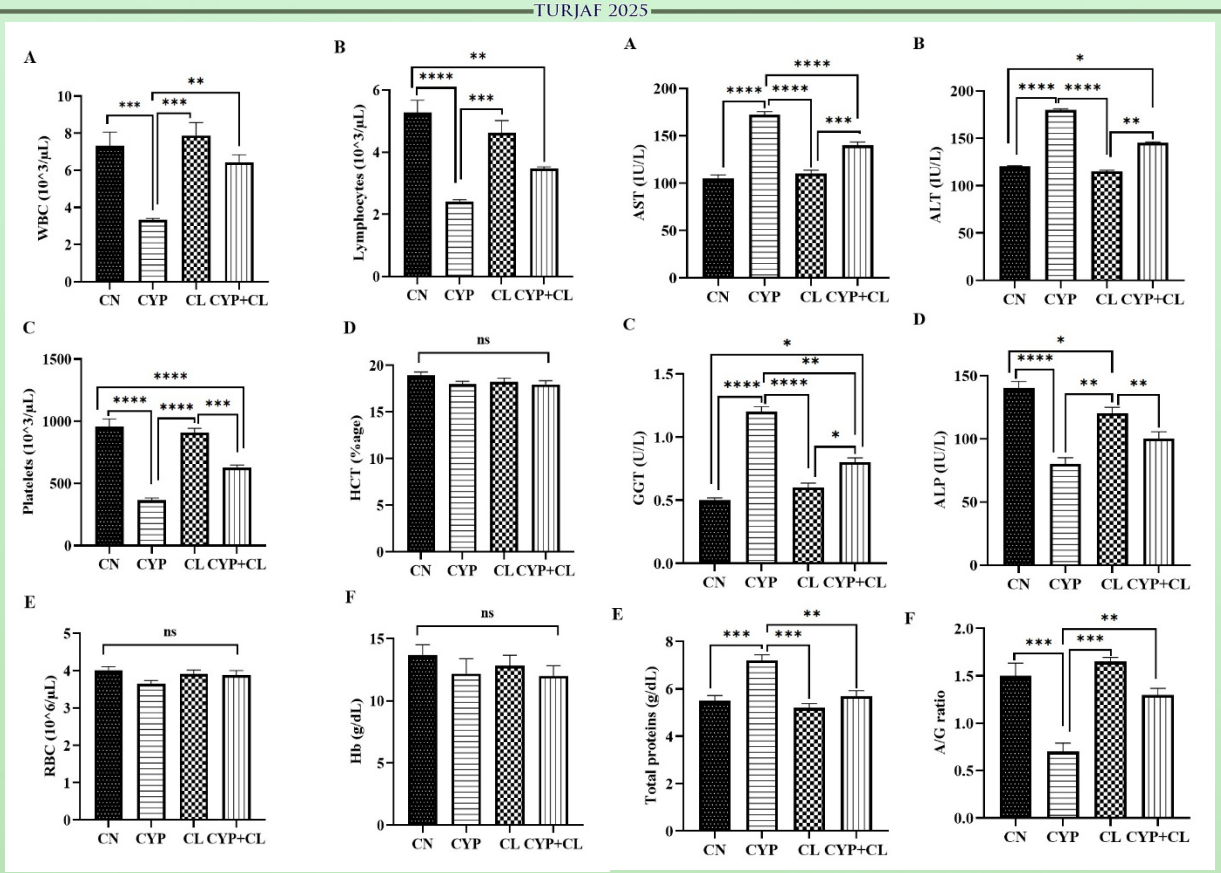

Figure 2. *Citrus limetta* supplementation facilitated hematological restoration against cyclophosphamide-induced organ toxicity.

The following parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). These parameters include (A) White blood cells (WBC), (B) Lymphocytes, (C) Platelets, (D) Hematocrit (HCT), (E) Red blood cells (RBCs), and (F) Hemoglobin (Hb). Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. ns=non-significant.

Figure 3. *Citrus limetta* supplementation alleviated cyclophosphamide-induced hepatotoxicity by improving liver enzyme profiles and protein homeostasis.

The following parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). These parameters include (A) AST, (B) ALT, (C) GGT, (D) ALP), (E) Total proteins, and (F) A/G ratio. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

3.4. Biochemical assessment of renal function:

Renal function markers including creatinine, blood urea nitrogen (BUN), and uric acid levels were monitored to evaluate renal health (Fig 4A-C). This study revealed a significant (P≤0.05) increase in the levels of creatinine (1.39±0.23), BUN (23.5±3.2), and uric acid (8.5±2.7) in the CYP group as compared to control negative (creatinine: 0.68±0.11, BUN: 13.5±2.3, and uric acid: 5.2±1.9), indicating renal damage. However, *Citrus limetta* effectively mitigated these elevations, reducing creatinine (0.81±0.13), BUN (17.5±2.4), and uric acid (6.1±1.9), indicating its nephroprotective potential against cyclophosphamide-induced nephrotoxicity.

3.5. Biochemical assessment of cell injury markers:

Citrus limetta raw juice consumption alongside cyclophosphamide administration indicated a significant effect (P≤0.05) on lactate dehydrogenase (LDH), and creatine kinase (CK), key markers of cell injury (Fig 5A-B). This study revealed a substantial elevation in LDH (810±18) and CK (126±15) levels in the CYP group compared to normal control (LDH: 570±17, CK: 72±12), indicating increased cellular damage. However, *Citrus limetta* supplementation significantly (P≤0.05) reduced these levels (LDH: 650±12, CK: 83±17), highlighting its cytoprotective potential against cyclophosphamide-induced toxicity.

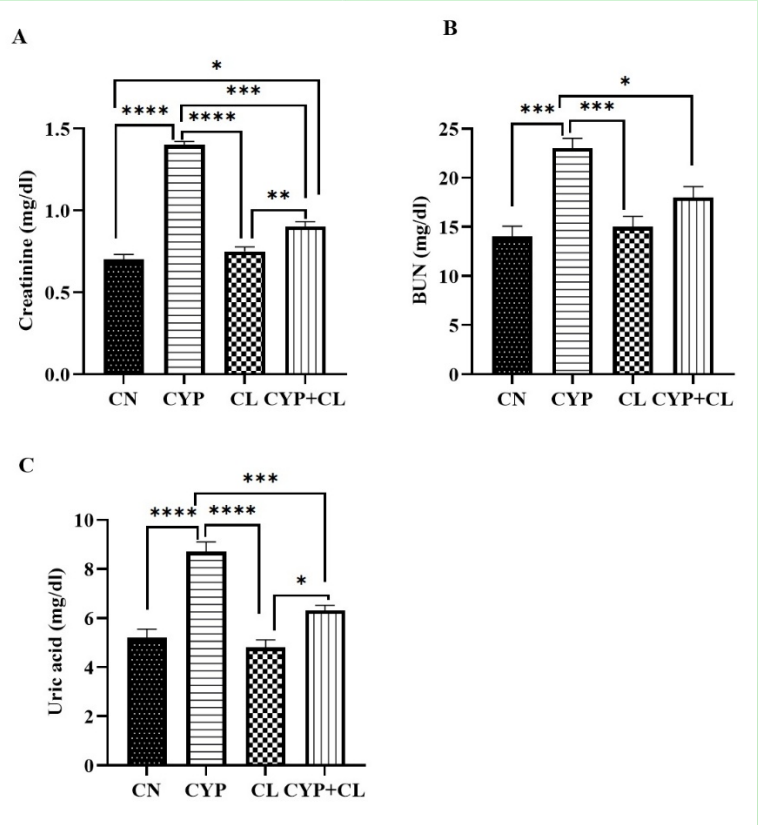

Figure 4. *Citrus limetta* supplementation mitigated cyclophosphamide-induced nephrotoxicity by restoring creatinine, BUN, and uric acid concentrations.

The following parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), Citrus limetta control (CL), and the treatment group receiving Citrus limetta following cyclophosphamide administration (CYP+CL). These parameters include (A) Creatinine, (B) BUN, and (C) Uric acid. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

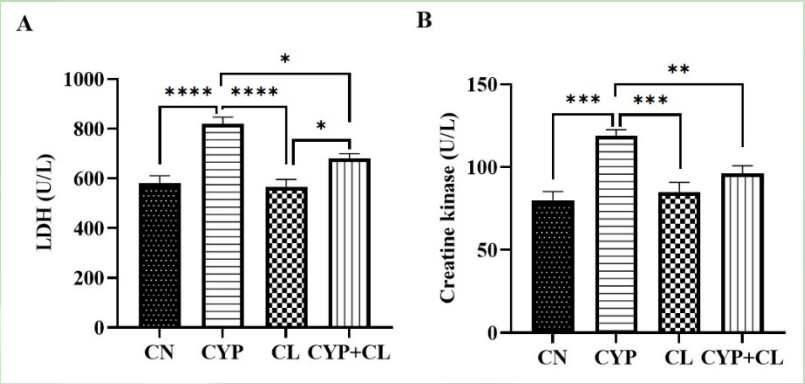

Figure 5. *Citrus limetta* supplementation attenuated cyclophosphamide-induced organ toxicity by reducing cell injury markers.

The following parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). These parameters include (A) LDH, and (B) Creatine kinase. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

3.6. Biochemical assessment of lipid profile:

Lipid profile analyses revealed significant (P≤0.05) dyslipidemia as evidenced by elevation in total cholesterol (149±5.4), and triglycerides (175±4.5) accompanied by a marked reduction in HDL-c (14±1.3) levels in the CYP group (Fig 6A-D), as compared to the control negative group (total cholesterol: 78±4.3, triglycerides: 123±5.7, and HDL-c 25±2.1) showing potential disruption of lipid homeostasis associated with cyclophosphamide. However, supplementation with *Citrus limetta* resulted in the restoration of lipid levels, exhibited in the CYP+CL group, indicative of vital role in mitigating cyclophosphamide-induced dyslipidemia and contributing to lipid profile normalization.

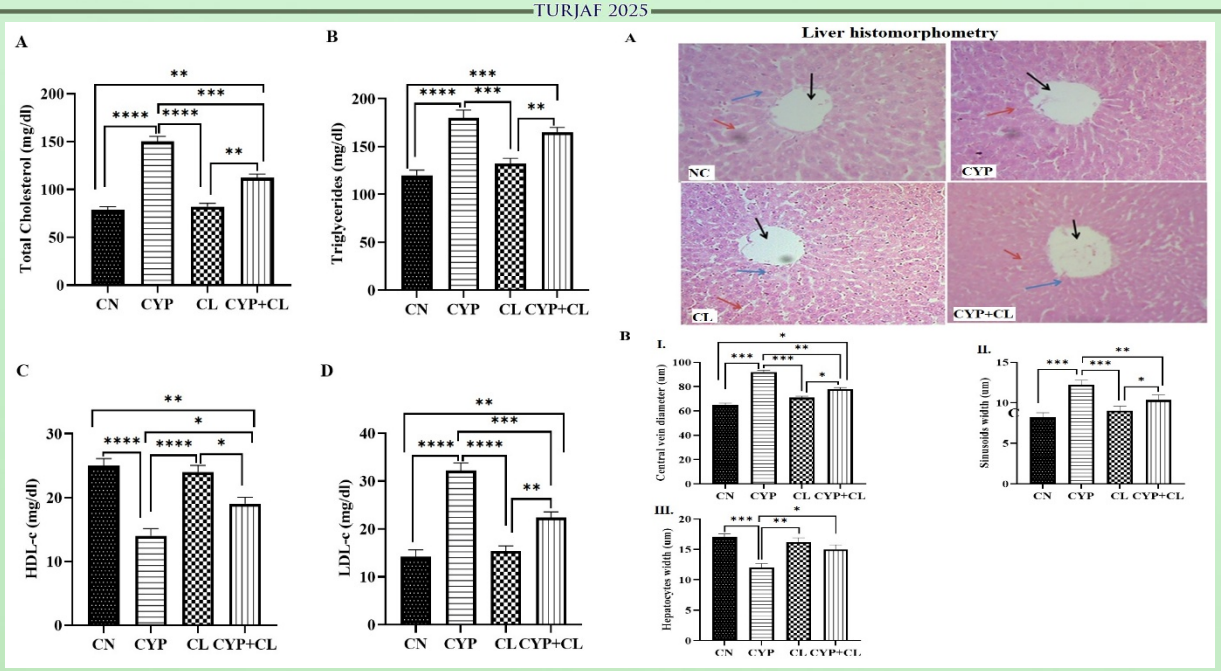

Figure 6. *Citrus limetta* supplementation mitigated cyclophosphamide-induced dyslipidemia by optimizing lipid profile parameters.

The following parameters were evaluated across four groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). These parameters include (A) Total cholesterol, (B) Triglycerides, (C) HDL-c, and (D) LDL-c. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

Figure 7. *Citrus limetta* supplementation mitigated cyclophosphamide-induced hepatic damage, preserving liver structure.

(A) Representative photomicrographs of liver sections stained with Hematoxylin and Eosin (H&E) at 40× magnification, illustrating histological variations in different experimental groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). Cyclophosphamide administration led to significant hepatic alterations, including an increased central vein diameter and sinusoidal width, and reduction in hepatocyte size. These structural disruptions were effectively normalized in the Citrus limetta-treated group. Histological analysis (H&E, 40x) highlights central vein dilation (black arrow), sinusoidal congestion (red arrow), and restored hepatocyte morphology (blue arrow) following Citrus limetta supplementation. (B) Quantitative histomorphometric data depicting (I) Central vein diameter, (II) Sinusoid width, and (C) Hepatocyte width. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

3.7. Liver histomorphometry:

Liver histopathological analyses (Fig 7A-B) revealed that cyclophosphamide administration induced significant (P≤0.05) hepatic alterations as evidenced by an increased central vein diameter (91±4.2) and sinusoidal width (13.2±0.78), and with a notable reduction in hepatocyte size 12.5±1.07) in CYP group as compared to control negative group (central vein diameter: 63±3.2, sinusoidal width: 7.5±0.89, and hepatocyte diameter: 16.5±1.03). These alterations indicate extensive hepatocellular damage and vascular remodeling, suggesting hepatotoxicity. However, these structural disruptions were effectively normalized in the *Citrus limetta* supplemented group, depicted by CYP+CL, affirming its hepatoprotective potential in maintaining structural integrity of liver against cyclophosphamide-induced hepatotoxicity.

3.8. Renal histopathology:

Histopathological analyses of renal tissue (Fig 8) revealed that cyclophosphamide administration induced structural disruptions as evidenced by increased capsule and glomerular thickness, along with significant dilation of PCT and DCT diameter in the CYP group as compared to the control negative group. Furthermore, CL supplementation resulted in the restoration of glomerular and tubular structural integrity by mitigating cyclophosphamide-induced nephrotoxicity, thereby promoting renal health and functional recovery.

3.9. Renal histomorphometry:

Quantitative histomorphometric analyses of renal tissue revealed significantly (P≤0.05) increased renal corpuscle thickness, increased PCT and DCT diameter, increased PCT wall-to-lumen (W/L) ratio, and significantly decreased DCT W/L ratio in the CYP group (capsule: 85±2.3, PCT W/L: 0.52±0.04, and DCT W/L 0.14±0.03), indicative of nephrotoxicity, as compared to control negative (capsule: 79±1.9, PCT W/L: 0.41±0.03, and DCT W/L 0.19±0.02), and *Citrus limetta* supplemented group (CYP+CL; capsule: 80±1.8, PCT W/L: 0.45±0.05, and DCT W/L 0.21±0.04). These findings imply that *Citrus limetta* possesses strong nephroprotective properties by mitigating cyclophosphamide-induced structural alterations.

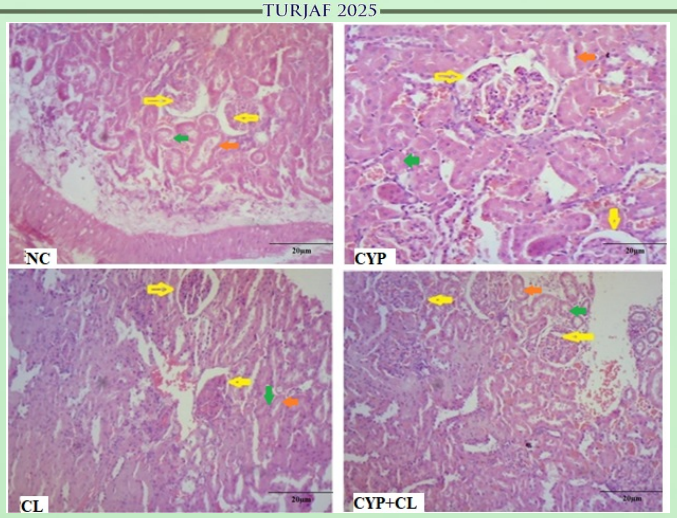

Figure 8. *Citrus limetta* supplementation safeguarded renal tissue integrity by counteracting cyclophosphamide-induced nephrotoxicity.

Representative photomicrographs of renal sections stained with Hematoxylin and Eosin (H&E) at 40× magnification, illustrating histological variations in different experimental groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). Cyclophosphamide exposure resulted in pronounced renal structure disruption including increased capsule and glomerulus thickness, dilation of both proximal (PCT) and distal convoluted tubules (DCT). These pathological alterations were effectively mitigated by *Citrus limetta* supplementation which helped restore renal architecture. Histological features are indicated as follows: renal corpuscle (yellow arrows), proximal convoluted tubules (PCT, orange arrows), and distal convoluted tubules (DCT, green arrows. Scale bar = 20 µm.

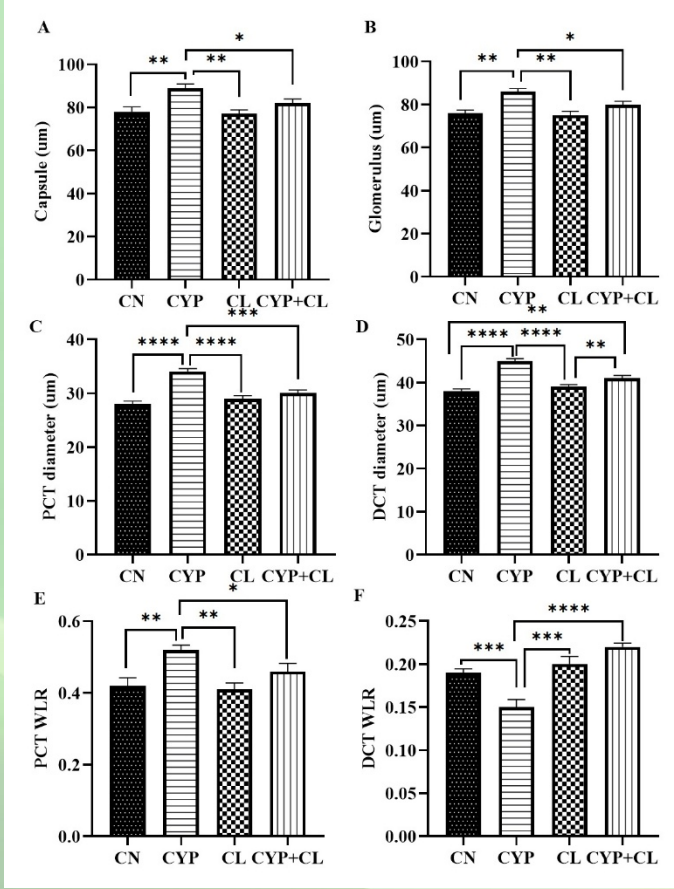

Figure 9. *Citrus limetta* effectively preserved the structural integrity of renal tissues, mitigating cyclophosphamide-induced nephrotoxicity and preventing structural alterations.

Quantitative histomorphometric analysis of renal structural parameters across different experimental groups: the control negative (CN), cyclophosphamide-induced positive control (CYP), *Citrus limetta* control (CL), and the treatment group receiving *Citrus limetta* following cyclophosphamide administration (CYP+CL). Quantitative histomorphometric data depicting: (A) capsule thickness (µm), (B) glomerulus diameter (µm), (C) proximal convoluted tubule (PCT) diameter (µm), (D) distal convoluted tubule (DCT) diameter (µm), (E) PCT wall-to-lumen ratio, and (F) DCT wall-to-lumen ratio. Mean±SEM values are displayed on bars, N=6. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.

4. Discussion:

Currently, there is significant research interest in medicinal plants, which confer therapeutic effects, both in modern medicine and allopathy, due to their pronounced antioxidant and antihyperglycemic properties, reduced side effects, and low cost (Nawaz et al., 2024). Among these, the *Citrus* species stands out due to its potent antioxidant and anti-inflammatory properties, which effectively reduce oxidative stress and inflammation __ key contributors to cellular injury and organ damage. Cyclophosphamide, a widely used chemotherapeutic agent induces organ toxicity primarily through oxidative stress. Thus current study is aimed at exploring the protective and restorative effects of *Citrus limetta* raw juice against cyclophosphamide-induced organ toxicity.

Anthropometric data indicated a reduction in body weight, feed intake, and BMI in the CYP group, likely due to the ability of CYP to impair the integrity of the intestinal mucosa, disrupt the morphology of the intestinal villi, increase the permeability of the intestinal mucosal barrier, and resultant nutrient malabsorption leading to weight loss (Zhao et al., 2024). Whereas, supplementation with *Citrus limetta* restored these parameters (**Fig 1A-C**) which could be attributed to its rich flavonoid content. *Citrus* flavonoids, such as naringenin and hesperidin, have been demonstrated to upregulate the expression of tight junction proteins, thereby strengthening the intestinal barrier function and reducing permeability (Stevens et al., 2019, Noda et al., 2013). Thus it is implied that *Citrus limetta* may exert protective and restorative effects on gut health by mitigating CYP-induced intestinal disturbances.

Hematological analysis revealed that CYP remarkably reduced WBC, lymphocytes, and platelets levels in the CYP group likely due to its myelosuppressive effects. CYP disrupts the bone marrow niche by disrupting the interactions between hematopoietic stem cells and their supportive microenvironment, leading to impaired hematopoiesis (Lévesque et al., 2003). Whereas these alterations were restored by *Citrus limetta* (**Fig 2A-F**)which could be due to its strong flavonoid content. As flavonoids have been shown to positively modulate gut microbiota enhancing the production of short-chain fatty acids (SCFAs), which play a crucial role in immune modulation and hematopoiesis (Zhao et al., 2023). Furthermore, no significant effects were seen on hematocrit, hemoglobin, and RBC levels due to selective toxicity of CYP towards rapidly dividing cells. As an alkylating agent, CYP targets rapidly dividing cells by interfering with DNA replication, leading to cell death and immunosuppression (De Jonge et al., 2005). On contrary, mature RBC are non-dividing cells and lack nuclei, rendering them less susceptible to the direct cytotoxic effects of CYP (Sheng et al., 2020). Additionally, RBCs have a longer lifespan, and the study's short duration was insufficient to impact their levels unless severe hemolysis occurred. These findings suggest *Citrus limetta* may exert immunomodulatory effects due to its flavonoid-rich composition.

Liver enzymes profile was monitored to evaluate liver function, as liver is the primary site of drig metabolism, primarily mediated by cytochrome p450 system (Allameh et al., 2023). During CYP metabolism by the liver, acrolein- a highly reactive byproduct, induces excessive free radicals production, overwhelming the liver's antioxidant defenses. These free radicals initiate lipid peroxidation by attacking polyunsaturated fatty acids in cell membranes (May-Zhang et al., 2021), compromise membrane integrity, increasing permeability and ultimately induce hepatocyte injury. Consequently, intracellular enzymes like ALT and GGT leak into the bloodstream, serving as biomarkers of liver dysfunction (Afzal et al., 2023). It was consistent with our findings as AST, ALT, ALP, and GGT levels were significantly raised in CP group along with increased total proteins levels. Whereas these perturbations were restored in CL treated group, as shown in **Fig 3A-F**, which could be attributed to its strong antioxidant activity exerted by flavonoids. Flavonoids are reported to neutralize ROS and inhibit lipid peroxidation, reducing membrane damage and preventing enzyme leakage (restoring **ALT** and **GGT** levels) (Shirani et al., 2020). These restorative activities of CL were further confirmed by histopathology revealing structural preservation, restored central vein diameter, sinusoids width, and hepatocytes diameter as shown in **Fig 7 A-B**. These findings suggest that Citrus species, including *Citrus limetta*, may enhance the liver's antioxidant defenses, thereby aiding in hepatic regeneration and functional recovery.

Serum creatinine, BUN, and uric acid are kidney-specific biomarkers used to monitor renal health. Our findings indicated a significant elevation of these biomarkers after CYP administration (**Fig 4A-C**). Although CYP is an effective chemotherapeutic drug, it has certain toxic effects on different organs like kidneys. Acrolein, a highly toxic metabolite, is filtered by the kidneys and accumulates in the bladder and renal tubules (Hałka et al., 2022). This accumulation directly damages renal tubular cells and causes apoptosis and necrosis (Mohamed et al., 2023). These toxic effects were further confirmed by renal histopathology (**Figure 8-9**)which exhibited glomerular congestion, increased PCT and DCT diameter, and significant tubular damage. These functional and structural alterations were however, ameliorated by *Citrus limetta* juice supplementation which could be attributed to its strong antioxidant defense system, and its citrus-derived compounds known to stabilize podocyte functions (Gong et al., 2023), and ultimately maintain filtration barrier integrity. Thus, *Citrus limetta* protects against CYP-induced nephrotoxicity by enhancing antioxidant defenses and preserving renal structure and function.

Cell injury markers CK and LDH were significantly increased in CYP administered group which indicates widespread tissue injury (**Fig 5A-B**). As explained earlier, CYP metabolism leads to hepatic injury which causes the release of intracellular enzyme LDH leakage into the plasma (Bale et al., 2014). However, an increase in CK levels indicates the myotoxic effects of CYP as seen in some cases of rhabdomyolysis (Shima et al., 2002).

Whereas *Citrus limetta* showed cytoprotective effects due to the presence of vitamin C which enhances levels of glutathione, a key cellular antioxidant in the cellular membrane (Heaney et al., 2008, Metwaly et al., 2022).
The metabolism and physiology of lipids and lipoproteins is a dynamic integrated process with the liver playing a central role in lipid homeostasis. Given the hepatotoxic effects of CYP, the current study revealed that the CYP-administered group showed elevated total cholesterol and triglycerides levels (**Fig 6A-D**) which could be due to the acrolein-lysine adducts known to cause hypertriglyceridemia and hypercholesterolemia by increasing cellular cholesterol accumulation, and by reducing cholesterol efflux by generation of free radicals and inhibition of lipoprotein-lipase (Yahya et al., 2022). *Citrus limetta* exhibited significant hypolipidemic effects, primarily due to its rich flavonoid content. Flavonoids function as cellular signaling molecules, modulating sterol signal transduction pathways, which enhance lipoprotein reuptake and thereby lower circulating LDL levels (Oboh et al., 2014). Thus it is implied that *Citrus limetta* mitigates CYP-induced dyslipidemia by enhancing lipid metabolism and reducing circulating LDL levels through its flavonoid-mediated regulatory effects.

5. Conclusion:

Bases on our findings it is concluded that *Citrus limetta* demonstrated significant protective and restorative effects against cyclophosphamide-induced organ toxicity. Its rich flavonoid content contributed to antioxidant defense, immunomodulation, and lipid metabolism regulation. The juice effectively mitigated CYP-induced hepatotoxicity, nephrotoxicity, dyslipidemia, and myelosuppression by reducing oxidative stress, stabilizing cellular structures, and enhancing metabolic pathways. These findings suggest that *Citrus limetta* holds therapeutic potential in counteracting chemotherapy-induced toxicity, supporting its role as a natural cytoprotective agent.

6. Strengths, limitations and future prospective

This study provides valuable insights into the protective effects of *Citrus limetta* against cyclophosphamide-induced organ toxicity, demonstrating its antioxidant, immunomodulatory, and hypolipidemic potential. The integration of biochemical, hematological, and histopathological analyses strengthens the reliability of the findings. Despite its promising findings, the study has some limitations. The sample size was limited, and the study duration may not have been sufficient to observe long-term protective effects. Additionally, molecular mechanisms underlying the observed protective effects were not extensively explored, warranting further investigation into specific signaling pathways and gene expressions involved.

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