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Quercetin mediated inhibition of creatinine-induced eryptosis in human erythrocytes: a novel therapeutic approach against eryptosis based anemia

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Quercetin mediated inhibition of creatinine-induced eryptosis in human erythrocytes: a novel therapeutic approach against eryptosis based anemia.

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Abstract

Background: Pleiotropic effects of quercetin include antioxidant activity and inhibits the inflammatory cascade by inhibition of cyclooxygenase-2 with decrease of PGE_2 formation. In erythrocytes, PGE_2 and oxidation activate Ca^{2+}-permeable cation channels. The influx of calcium ions (Ca^{2+}) activates Ca^{2+}-sensitive K^+ channels leading to cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Both these are hallmarks of suicidal cell death (eryptosis). Eryptotic cells adhere to the vascular wall may lead to impairment of microcirculation and these cells are rapidly cleared from circulating blood, leading to anemia. Anemia is a major complication of chronic kidney disease, major global public health problem with a steadily increasing prevalence. **Objectives:** This study aimed to elucidate the mechanisms underlying the dysfunction of erythrocytes induced by uremic toxin creatinine and to investigate the potential protective role of quercetin against creatinine-induced toxicity. **Methods:** Flow cytometry was used to estimate erythrocyte phosphatidylserine (PS) exposure at cell surface from annexin V binding, reactive oxygen species formation from DCFDA (2',7'dichlorodihydrofluorescein diacetate) dependent fluorescence. Spectrophotometer was also used to estimate the antioxidant enzymes (SOD, GPx and CAT) activities and hemolysis %. Determination of role of Ca^{+2} and mean cell volume (MCV) from automated hematology analyser. Moreover, molecular docking analysis was also performed to evaluate the ligand protein binding affinity and interaction patterns by using AutoDock. **Results:** Treatment of uremic-erythrocytes with quercetin (15 µM), shows significantly increased forward scatter, significantly decrease reactive oxygen species (ROS), significantly decrease annexin-V-binding, significantly decrease $[Ca^{2+}]i$, significant decrease in hemolysis %, restoration of MCV, reversed or increase the antioxidant enzymes (SOD, CAT and GPx) activities in uremic-erythrocytes. **Conclusion**: Creatinine is potent stimulator of eryptosis whereas quercetin is potent inhibitor of suicidal erythrocyte death during oxidative stress. Therefore, the antieryptotic effect of quercetin could counteract the development of anemia and impairment of microcirculation with excessive eryptosis.

Key Words: Annexin; Calcium; Cell volume; Anemia; Oxidative Stress, Chronic Kidney Disease.

Introduction

Chronic kidney disease (CKD) can be defined as the presence of renal damage (morphological changes in renal parenchyma, albuminuria, etc.) and an estimated glomerular filtration rate (eGFR) less than 60 ml/min/1.73 m^2 lasting more than 3 months (Romagnani *et al*., 2025). Patients experience with chronic kidney disease show extensive production of uremic toxins (Ribeiro *et al*., 2025), defined as a molecule that accumulates

in the circulating blood and organs of patients with CKD, where it exerts deleterious effects on various biological functions (Lu *et al*., 2025). Creatinine is a nitrogenous waste product produced and excreted by the human body. Normal blood creatinine values are observed 0.6-1.3 mg/dl in healthy individuals. Red blood cells lack mitochondria and nuclei and therefore cannot undergo the release of cytochrome c from mitochondria and DNA fragmentation, both hallmarks of apoptosis (Tkachenko and Havranek, 2025). However, erythrocytes can undergo programmed cell death (eryptosis) characterized by cell shrinkage and cell membrane scrambling (Alghareeb *et al*., 2023). Eryptosis may be induced by an increase in intracellular calcium concentration ($[Ca^{2+}]i$) following calcium influx through calcium-permeable cation channels. These channels are activated by oxidative stress (Kempe *et al*., 2023). Elevated intracellular calcium ion concentrations activate calcium-sensitive potassium channels, causing cell shrinkage due to potassium ion (K^+) exit, hyperpolarization and chloride ion (Cl^-) exit, resulting in loss of potassium chloride and water from the cell (Hamill, 1983). Eryptosis is triggered by a wide range of xenobiotics, including many food contaminants (Alghareeb *et al*., 2023; Lang *et al*., 2012). Furthermore, eryptosis is observed in numerous clinical disorders including renal insufficiency, diabetes mellitus, sepsis, hemolytic uremic syndrome, sickle cell anemia, iron deficiency, malaria, hypophosphatemia, wilson's disease and possibly metabolic syndrome (Lang *et al*., 2012). Quercetin (polyhydroxyflavonoid) effect exhibits immune modulating, anti-inflammatory, antiviral and antioxidant and reactive oxygen species scavenging properties (Jiang *et al*., 2025). It is shown that exposure of uremic-erythrocytes to quercetin counteracts the eryptotic effect of oxidative stress, energy depletion and isoosmotic cell shrinkage. The present study might provide novel perspectives for eryptosis based anemia treatment.

Methods

1. *In-Vitro* cytotoxicity of phytochemical

In this study, we exposed the healthy erythrocytes to quercetin concentrations (5 µM, 10 µM and 15 µM) in order to assess their cytotoxicity.

2. Treatment of phytochemical with uremic-erythrocytes

The present experiment aimed to evaluate whether quercetin can inhibit the eryptosis in uremic-erythrocytes**.** Therefore, for this purpose, uremic-erythrocytes were exposed to the various quercetin concentrations (5 µM, 10 µM and 15 µM).

3. Incubation of erythrocytes

Erythrocytes were incubated *in vitro* at 0.4% hematocrit in ringer solution (isotonic solution, contains different salts dissolved in water) maintained at pH 7.4. All incubated treatments (samples) were performed for 48 h at 37°C (Jilani *et al*., 2011).

4. Evaluation of oxidative stress

4.1. Superoxide dismutase (SOD)

For measurement, pipetting was done at room temperature into a series of test tubes; a tube containing mixture reagents produced maximum inhibition and total reaction volume was 1000 µL. Finally tube sample approximately 20 µL was transfer on ELISA plate and read at A_{560} and were determined the amount of enzymes which produced the one-half of maximum inhibition (Rana *et al.*, 2019).

4.2. Catalase (CAT)

For measurement, solution contained 100 µL hydrogen peroxide, 100 µL PB (pH 7.0), 50 µL CAT and 100 µL sample in an Eppendorf tube. 200 µL mixture reaction solutions pour into ELISA plate read at A_{240} for determination of enzyme catalase activity (Sattar *et al.* 2022).

4.3. Glutathione peroxidase (GPx)

For measurement, pipetting was done in a series of a tube: 800 µL potassium phosphate buffer, 100 µL sample, 100 µL guaicol and finally to initiate the reaction added hydrogen peroxide or cumene hydroperoxide. Total 200 µL mixture pour in ELISA plate and taken absorbance at 490nm (Rana *et al.*, 2019).

5. Measurement of hemolysis

To measure hemolysis, hemoglobin concentration of supernatant was photo-metrically determined at 450 nm. The degree of absorbance was used to determine the completeness of hemolysis which is called 100%. Then calculate according to the following formula: Hemolysis (%) = absorbance (control or test samples) × 100 (1) absorbance (distilled water) (Rana *et al*., 2019; Naveed *et al*., 2020).

6. Cell size measurements

Automated hematology analyser was used to measure the mean cell volume or cell size. However, mean cell volume of normal and treated erythrocytes were calculated to compare the similarities and differences in cells (Shabir *et al*., 2019; Ilyas *et al*., 2020).

7. Confirmation of the role of Ca^{+2}

Amlodipine (calcium channel blocker) was used to determine the participation of Ca^{2+} in the stimulation of eryptosis. For this purpose, healthy erythrocytes were exposed to uremic toxin creatinine, followed by treatment with quercetin with and without amlodipine (10 µM). (Rana *et al.,* 2019; Bigdelou and Farnoud, 2020).

8. Estimation of ROS formation

ROS levels were measured by utilizing 2', 7'dichlorodihydrofluorescein diacetate (DCFDA). Following incubation, 50 µL of erythrocytes stained with 10 µM DCFDA, kept in dark at 37 °C for 30 minutes and washed with ringer solution. Following DCFDA loading, erythrocytes were re-suspended in 200 µL of Ringer solution and fluorescence intensity corresponding to ROS production was analyzed in the FL-1 channel by flow cytometry (Jilani *et al*., 2013).

9. FACS analysis of forward scatter and annexin-V-binding

In present study, FACS analysis was applied to determine multiple parameters of eryptosis. Cell volume was determined by forward scatter (FSC) without the need for staining. FSC was recorded simultaneously with annexin V-binding. Following incubation under indicated experimental condition, 50 µL of the cell suspension was washed in Ringer solution and stained with Annexin-V FITC (1:200 dilution). In parallel, forward scatter was determined and annexin-V fluorescence intensity was analysis in FL-1 chennel using by FACS Calibur (Jilani *et al*., 2013).

10. Molecular docking

Quercetin was created by utilization of ChemDraw Pro 12.0 and target enzyme (protein) scramblase and NADP (H) oxidase were obtained from protein data bank. Molecular docking was done using AutoDock Vina to determine their binding affinity and interaction patterns. Discovery Studio and PyMOL were opted to determine the bonding between proteins and ligands (Bastin *et al*., 2023; Shah *et al*., 2022).

11. Statistics

Data was presented as arithmetic means ± SEM. Statistical analysis was performed using ANOVA and t-tests, as applicable. *N* represents total number of distinct erythrocyte samples analysed. (Aslam *et al*., 2022; Naveed *et al*., 2020).

Result and Discussion

This study explored whether quercetin inhibit the eryptosis. To this end a first set of experiments were evaluated the potential effect of quercetin on enzymatic antioxidant activity, cell volume and percentage of hemolysis in uremic-erythrocytes.

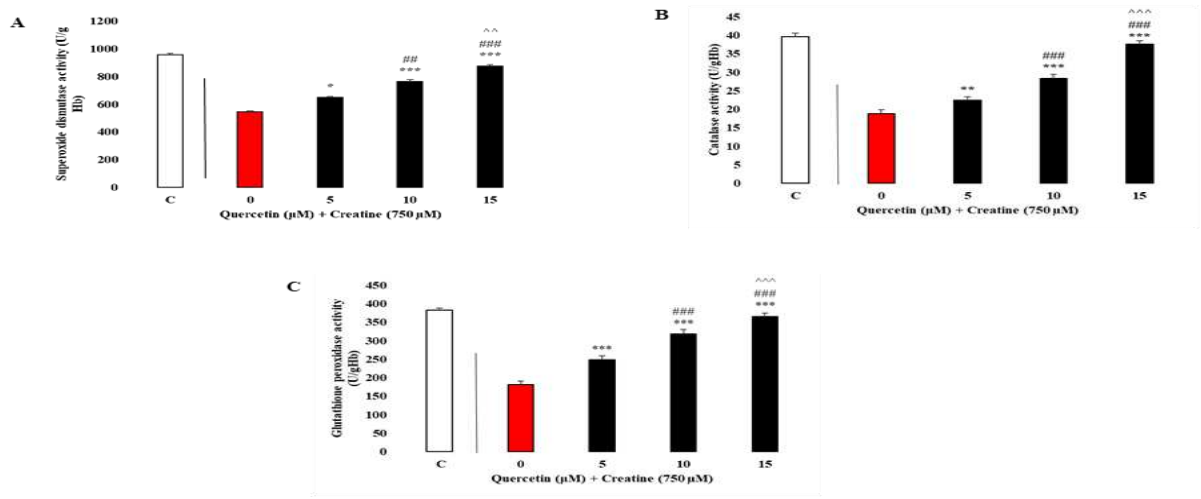


Figure 1. Effect of quercetin on activities of antioxidant enzymes in uremic-erythrocytes.

Vertical bars represent values of antioxidant enzymes (n=20). *, ##, ^^, ^^^, ###, *** (P<0.05, P< 0.01, P< 0.01, P< 0.0001, P< 0.0001 P< 0.0001) demonstrate effect reaching statistical significance at 15 µM quercetin concentration in uremic-erythrocytes as determined by ANOVA.

As illustrated in Fig. 1, the effect of increasing quercetin concentrations (5-15 µM) on the antioxidant enzymes (SOD, GPx and CAT) activities in uremic-erythrocytes. In control groups (white bars), erythrocytes exhibits higher and ideal antioxidant enzymes activities reflecting neutralization of superoxide radicals and maintaining cellular redox homeostasis as healthy erythrocytes. In uremic groups (red bars), erythrocytes exhibits marked reduction antioxidant enzymes (SOD, GPx and CAT) activities in uremic-erythrocytes and reflecting significant oxidative stress, impaired enzymatic antioxidant capacity, subsequent induced oxidative damaged and lead to eryptosis. In treatment groups (black bars), erythrocytes demonstrate a significant restoration of enzymatic antioxidant capacity with closer to healthy erythrocytes. This improvement in enzymatic antioxidant capacity demonstrate that quercetin provides comprehensive protection against oxidative damage to erythrocyte membranes and stabilization of the erythrocyte redox balance.

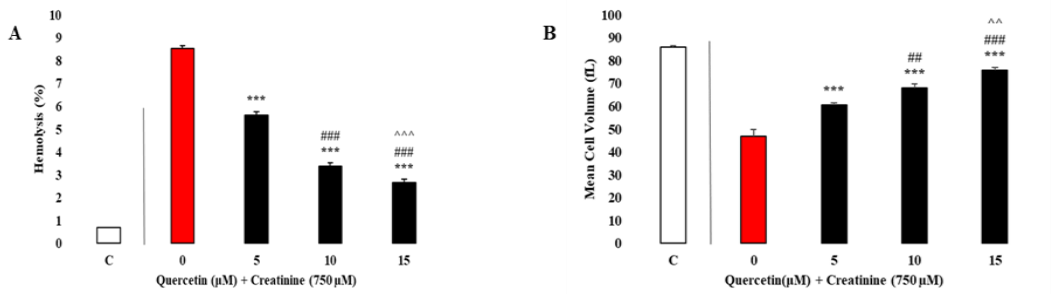


Figure 2. Effect of quercetin on hemolysis (%) and mean cell volume (fL) in uremic-erythrocytes.

Vertical bars represent values of hemolysis (n=20). ***, ##, ###, ^^, ^^^ (P< 0.0001, P< 0.01, P< 0.0001, P< 0.01, P< 0.0001) demonstrate effect reaching statistical significance at 15 µM quercetin concentration in uremic-erythrocytes as determined by ANOVA.

This experiments explored whether quercetin exert protective effects in uremic-erythrocytes. In control group (white bars), erythrocytes exhibits baseline hemolysis % and average mean cell volume (MCV), reflecting erythrocytes membrane integrity under physiological conditions. In uremic group (red bars), exhibits decrease mean cell volume (erythrocyte shrinkage) and a marked increase in hemolysis % and with significant oxidative stress and disruption of cytoskeletal of erythrocytes. In treatment group (black bars), reflecting a gradually decline in hemolysis % and restoration of mean cell volume towards control levels, reflecting quercetin counteracts cell shrinkage as induced by creatinine.

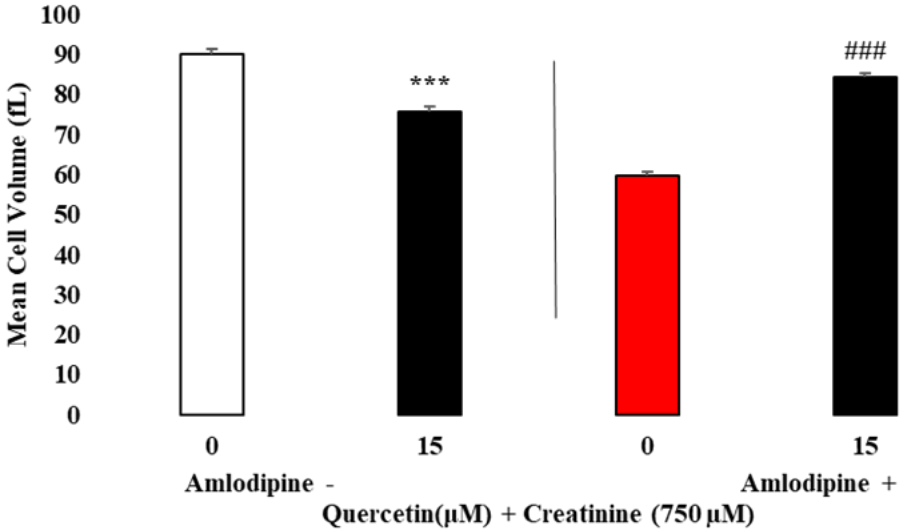


Figure 3. Effect of quercetin on cell size with and without Ca^{2+} blocker in uremic-erythrocytes.

Vertical bars represent values of MCV (n=20). ***, ### (P< 0.0001, P< 0.0001) demonstrate significance at 15 µM quercetin concentration with amlodipine (Ca^{2+} blocker) in uremic-erythrocytes as determined by ANOVA.

As illustrated in Fig. 3, the effect of creatinine on annexin-V-binding was significantly reduced in the nominal absence of extracellular Ca^{2+}. Thus creatinine did increase cytosolic Ca^{2+} concentration and trigger eryptosis with presence of Ca^{2+}. Treatment shows normal mean cell volume, reflecting minimum cell shrinkage and membrane scramblase. Thus, quercetin inhibit the eryptosis induced by uremic toxin creatinine.

In second set of experiments, cell shrinkage and cell membrane scrambling was assessed by measurement of phosphatidylserine abundance at cell surface. Phosphatidylserine (PS) exposing erythrocytes were determined by annexin-V-binding in FACS analysis.

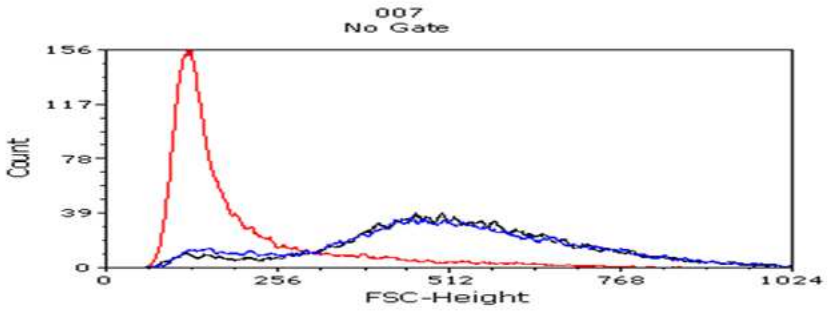


Figure 4. Effect of quercetin on forward scatter in uremic-erythrocytes.

Original forward scatter histograms of erythrocytes following 48 h incubation in Ringer solution with and without quercetin concentration (15 μM) in uremic-erythrocytes.

Forward scatter (FSC) was positively correlated with cell volume and size. As illustrated in Fig. 4, control group (black curve) indicates the erythrocytes were normal size with intact membranes and regular morphology. Uremic group (red curve) shows decrease in mean cell volume or size while treatment group (blue curve) shows significant increase in forward scatter and reflecting erythrocytes size was preserved and heterogeneity was significantly reduced.

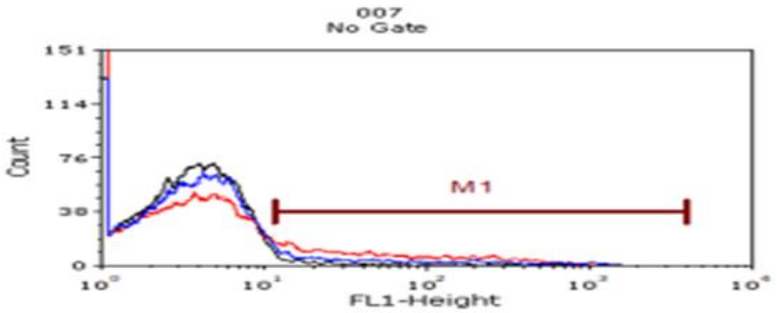


Figure 5. Effect of quercetin on phosphatidylserine exposure in uremic-erythrocytes.

Original annexin-V-binding histograms of erythrocytes following 48 h incubation in Ringer solution with and without quercetin concentration (15 μM) in uremic-erythrocytes.

As illustrated in Fig. 5, a 48 h exposure to creatinine increased the percentage of annexin-V-binding erythrocytes, thus creatinine exposure was followed by membrane scrambling with phosphatidylserine exposure at cell surface. Treatment with quercetin could restore to normal levels.

Molecular docking studies

Molecular docking of the ligands with each of target enzyme was done using AutoDock Vina to determine their binding affinity and interaction patterns.

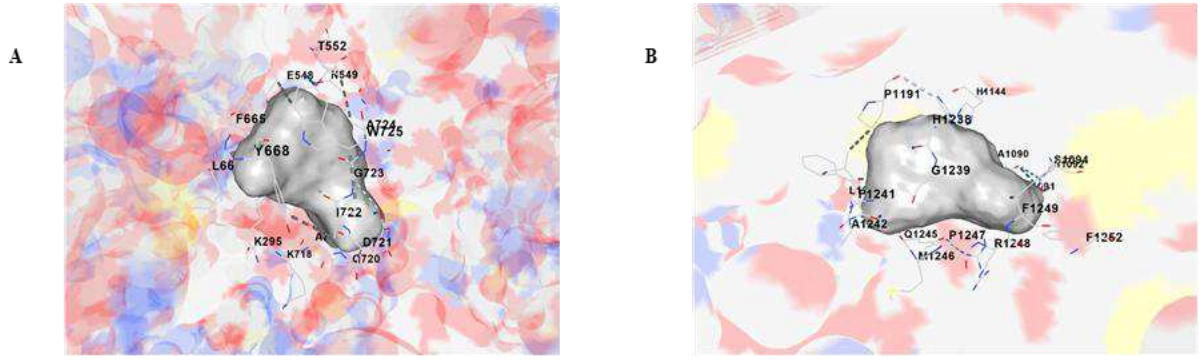


Figure 6. Inhibitory effect of quercetin on enzyme NADP (H) oxidase and enzyme scramblase.

As illustrated in Fig. 6A, the spatial complementarity of quercetin within region (pocket) with hydrophobic attractions and hydrogen bonding facilitate molecular basis for underlying inhibitory mechanism. This binding configuration suggests quercetin potentially inhibiting the enzyme scramblase activity by occupy the active site and regulate the scramblase function in erythrocytes. As illustrated in Fig. 6B, shows strong binding stability interaction and block the structural rearrangement in which NADPH cannot reduce FAD. Thus, electron flow was blocked, which can reduced the production of reactive oxygen species.

Discussion

The complications of chronic kidney disease are crucial significance, as determine the patient survival and prognosis (Bello *et al*., 2017; Petho *et al*., 2024). Patients with CKD suffering a markedly higher risk of mortality, due to early onset of common diseases infection, cardiovascular disease and cancer, while these complications develop as a result of renal dysfunction (Kumar *et al*., 2014; Caturano *et al*., 2024). However, the mechanisms triggering of eryptosis in chronic kidney disease remain currently limited (Gok *et al*., 2022). Theoretical, various uremic toxins may induce eryptosis. Eryptosis known to be stimulated by methylglyoxal, acrolein, vanadate and indoxyl sulfate increased in uremic plasma (Hussain, 2013; Pieniazek *et al*., 2021). In our study, uremic toxin creatinine, which known for adverse effects in chronic kidney disease patients, may cause eryptosis (Volpe *et al*., 2014; Chmielewski *et al*., 2013). Erythrocytes exhibit highly susceptible to oxidative injury due to their oxygen rich environment and haemoglobin which together act potentially powerful driver for the oxidative processes (Robaszkiewicz *et al*., 2007). Erythrocytes membrane damage (protein and lipid peroxidation) disturb the cell integrity, implicated in hemolysis subjected with accumulation of uremic toxin (creatinine) with reduction of erythrocyte antioxidant systems (superoxide dismutase (SOD), catalase (CAT) and glutathione

peroxidase (GPx)), increase annexin V positive cells, increased reactive oxygen species (ROS) reduced forward scatter, and lead to anemia (Manikanta *et al*., 2025).

Our study examined the quercetin to identify a novel effect as the inhibition of eryptosis. Quercetin statistically inhibited eryptosis were comparable to those reported for apoptosis (Pan *et al*., 2020; Nassiri-Asl *et al*., 2013). According to our study, no effect of quercetin were observed on healthy erythrocytes hemolysis, antioxidants enzyme, reactive oxygen species production and on mean cell volume (Pawlikowska *et al*., 2003). Our results show that quercetin can protect erythrocytes from oxidative stress under *in-vitro* conditions (Song *et al*., 2020; Pasdar *et al*., 2020; Manful *et al*., 2025). Thus, reactive oxygen species production strongly inhibited by quercetin and promote the increase antioxidant level in plasma, reduced oxidative damaged by reduced lipid peroxidation (Kook *et al*., 2008; Keskin *et al*., 2016; Jomova *et al*., 2025). Inhibit cell membrane scrambling, reduced level of ROS production and maintained erythrocytes membrane integrity, improved survival of erythrocytes when treated with stress erythrocytes (Jain *et al*., 2025). Quercetin minimized the oxidative stress by activating the Nrf2 pathway (Nrf2 highly active in cell) cyto-protective adaptive responses to uremic toxins, downstream effector gene HEC-1A, HO-1 in Hela, A549 and HEK293cells (Franco *et al.*, 2024).

Uremic toxin creatinine significantly increased expression of HO-1 and Nrf2 expression in the kidney tissues (Dash *et al*., 2025; Corte-Iglesias *et al*., 2025) Quercetin can stimulate transcriptional activity of Nrf2 and directly interact with Keap1 binding site of Nrf2 (Feng *et al*., 2023; Baby *et al*., 2016). However, nephron-protective effect of quercetin can increased induction of Nrf2/HO-1 signaling pathway (Erden Inal *et al*., 2001; Bai *et al*., 2025). Our study result showed creatinine exposure to erythrocytes exhibits significantly activity iNOS, NO levels and increased activity of NF-κB inhibited by quercetin. These condition improved antioxidant gene expression, promoting to cellular redox homeostasis (Costa *et al*., 2016; Boo, *et al*., 2025). In accordance to these observations, quercetin inhibit the eryptosis (Arjumand and Sultana, 2011; Akbari *et al*., 2022). However, plant based phytochemical (quercetin) with antioxidant properties have been proposed as an alternative therapeutic agents for management of anemia.

Author Contributions: K. j and M.I: Designed the study; M. I : Investigated, analyzed, wrote, edited the data and organized the final draft; K. J, Z. M and M.S: Reviewed the data. All authors gave their final approval for this draft.

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Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest
There is no financial interest or personal relationship that could influence the work reported in this paper.

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