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**Original Paper**

# Camel Milk Ameliorates 5-Fluorouracil-Induced Renal Injury in Rats: Targeting MAPKs, NF-κB and PI3K/Akt/eNOS Pathways

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**Key Words**
Camel milk • 5-fluorouracil • MAPKs • NF-κB • PI3K • Akt

**Abstract**
***Background/Aims:*** The clinical utility of 5-fluorouracil (5-FU) is limited by its nephrotoxicity. Camel milk (CM) has previously displayed beneficial effects in toxicant-induced nephropathies. The current study aimed to investigate the potential of CM to attenuate 5-FU-induced nephrotoxicity in rats. ***Methods:*** Renal tissues were studied in terms of oxidative stress, inflammation and apoptosis. The levels of renal injury markers, inflammatory cytokines along with NOX-1, Nrf-2 and HO-1 were assessed by ELISA. The expression of MMP-2, MMP-9, NF-κBp65, p53, Bax and PCNA were detected by Immunohistochemistry. To gain an insight into the molecular signaling mechanisms, we determined the effect of CM on MAPKs, NF-κB and PI3K/Akt/eNOS pathways by Western blotting. ***Results:*** CM lowered 5-FU-triggered increase of creatinine, BUN, Kim-1 and NGAL renal injury biomarkers and attenuated the histopathological aberrations. It suppressed oxidative stress and augmented renal antioxidant armory (GSH, SOD, GPx, TAC) with restoration of NOX-1, Nrf-2 and HO-1 levels. CM also suppressed renal inflammation as indicated by inhibition of MPO, TNF-α, IL-1β, IL-18 and MCP-1 proinflammatory mediators and downregulation of MMP-2 and MMP-9 expression with boosting of IL-10. Regarding MAPKs signaling, CM suppressed the phosphorylation of p38 MAPK, JNK1/2 and ERK1/2 and inhibited NF-κB activation. For apoptosis, CM downregulated p53, Bax, CytC and caspase-3 proapoptotic signals with enhancement of Bcl-2 and PCNA. It also enhanced PI3K p110α, phospho-Akt and phospho-eNOS levels with augmentation of renal NO, favoring cell survival. Equally important, CM preconditioning enhanced 5-FU cytotoxicity in MCF-7, HepG-2, HCT-116 and PC-3 cells, thus, justifying their concomitant use. ***Conclusion:*** The current findings pinpoint, for the first time, the marked renoprotective effects of CM that were mediated via ROS scavenging, suppression of MAPKs and NF-κB along with activation of PI3K/Akt/eNOS pathway.



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## Introduction

5-fluorouracil (5-FU), a pyrimidine antimetabolite, has displayed remarkable anti-cancer actions against diverse malignancies including breast, stomach, colorectal, head and neck along with skin cancer [1]. Following metabolic transformation to 5-fluoro-2-deoxyuridine monophosphate, it irreversibly inhibits thymidylate synthase resulting in suppression of thymine nucleotide synthesis driving apoptosis in malignant cells and even in normally replicating cells [2]. Thus, 5-FU exhibits significant toxicity and adverse actions that curbs its clinical use including leucopenia, mucositis, cardio-, hepato- and nephrotoxicities. In particular, the renal injury is a major drawback of 5-FU that is thought to be mediated by its metabolic cleavage into α-fluoro-β-alanine, ammonia and urea [1].

Evidence has indicated that oxidative stress plays a central role in mediating the 5-FU-induced renal injury [1, 3]. The generation of reactive oxygen species (ROS) incurs membrane lipid peroxidation and oxidative cellular damage. Several studies have suggested that ROS and proinflammatory cytokines are engaged in the activation of mitogen-activated protein kinases (MAPKs) including p38 MAPK, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Stimulation of p38 MAPK and JNK contributes to the upregulation of cytokines and renal apoptotic cell death in several renal pathologies [4, 5]. In addition, stimulation of ERK pathway drives the activation of transcription factors such as nuclear factor kappa B (NF- κB) which controls the expression of diverse proinflammatory cytokines and proteins [6, 7].

A previous report has indicated that 5-FU-induced renal injury is associated with enhanced renal apoptosis marked with increased caspase-3 and increased Bax/Bcl-2 ratio [1]. An intimate pathway linked to the apoptotic cell death is the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway which can be modulated by several renal toxicants. PI3K/Akt has been reported to control cellular survival signals via interaction with the Bcl-2 family [8, 9]. Akt has also been linked to renal NO production through activation of the endothelial nitric oxide synthase (eNOS) [10].

Recently, there has been a revival of interest for the search for potential protective agents against chemotherapy-associated adverse effects. Previous studies have revealed the protective effects of natural antioxidants such as propolis and chrysin in 5-FU-induced renal damage [1, 11]. Hence, agents that possess antioxidant features with minimal adverse effects may represent a potential cost-effective intervention against 5-FU nephrotoxicity. In this regard, Camel milk (CM) has displayed remarkable antioxidant/anti-inflammatory actions that encouraged us to investigate its potential alleviating effects against 5-FU-induced renal injury. CM possesses unique antioxidant features that are ascribed to its high content of vitamins C and E besides its high lactoferrin content, a bioactive protein with marked anti-oxidant and anti-inflammatory actions [12]. Several clinical and experimental studies have revealed the mitigating actions of CM against rheumatoid arthritis [13], inflammatory bowel disease [14], Diabetes mellitus [15], alcohol-induced hepatic injury [16], steatohepatitis [17] and wound healing [18]. More important, reports have indicated the efficacious role of CM in diabetes- [19], gentamicin- [20] and cisplatin-induced nephropathies [21]. Yet, whether CM can protect against 5-FU-evoked renal injury remains to be investigated. Hence, the present study focuses on gaining an insight into the molecular mechanisms underlying 5-FU renal damage, in particular, the role of MAPKs, NF-κB and PI3K/Akt/eNOS signaling pathways. More important, the potential protective role of CM was investigated in 5-FU renal injury.

## Materials and Methods

*Ethical statement*

Animal care and experimental work were approved by the Research Ethical Committee of Taif University, Saudi Arabia and strictly followed the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996).

*Reagents and drugs*

5-Fluorouracil and Quercetin were obtained from Sigma-Aldrich, St. Louis, MD, USA. All other chemicals were of the highest purity and analytical grade. Camel milk (CM) was obtained from Al-Turath Al-Saudia Company, KSA (3% fat and 6.3 % milk non-fat solids). Notably, the marked anti-inflammatory and antioxidant features of CM are considerably attributed to lactoferrin (220 mg/L) which represents the highest lactoferrin content among the milks of several animal species e.g., buffalo, cow, goat, sheep and mare [22, 23]. Compared to the bovine milk, CM contains 3-5 folds of vitamin C content (24-52 mg/kg) and similar vitamin E levels [23].

*Animals*

Adult 12-week-old Wistar rats (180 g ±20 g) were provided by King Fahd medical research center, Jeddah, Saudi Arabia and were maintained in our Animal Laboratory facility under standard conditions of temperature 24-26°C, humidity 50-70% and 12L/12D lighting regimen. Rats were randomly distributed to clean polypropylene cages (4 per cage) and were fed standard rodent chow (15% protein, 3.5 % fat, 6.5% crude fibers plus vitamins/minerals mixture) and water *ad libitum*. Animals were kept for one week for the purpose of acclimatization before any experimental treatment.

*Experimental design and treatment protocol*

A total of 56 rats were used in the present study; they were randomized into 7 experimental groups (8 rats per group) as follows: *Group I (Control gp)*: Normal rats that received five i.p. saline injections (one injection per day) on the 17$^{th}$ – 21$^{st}$ days. *Group II (Control + CM 10 gp)*: Normal rats that received oral Camel milk (10 ml/kg p.o.) for 21 days and five i.p. saline injections as in gp I. *Group III (5-FU gp):* Rats that received five i.p. injections of 5-FU (50 mg/kg/day) on the 17$^{th}$ – 21$^{st}$ days. *Group IV (5-FU + BM gp):* Rats that received isocaloric doses of Bovine milk daily for 21 days and five i.p. injections of 5-FU (50 mg/kg) on the 17$^{th}$ – 21$^{st}$ days. *Group V (5-FU + CM 5 gp):* Rats that received oral Camel milk (5ml/kg) for 21 days and five i.p. injections of 5-FU on the 17$^{th}$ – 21$^{st}$ days. *Group VI (5-FU + CM 10 gp):* Rats that received oral Camel milk (10ml/kg) for 21 days and five i.p. injections of 5-FU on the 17th – 21st days. *Group VII (5-FU + QRC gp):* Rats that received oral Quercetin (QRC; 50 mg//kg) for 21 days and five i.p. injection of 5-FU on the 17$^{th}$ – 21$^{st}$ days.

The selected regimen of 5-fluorouracil renal injury is based on previous reports [24-26] and our preliminary results. The selected doses for Camel milk are consistent with previous studies [13, 14, 16] and the dose of the reference antioxidant Quercetin is in line with previous literature [6].

*Blood and kidney collection*

At the end of study (day 22), rats were anesthetized with thiopental (50 mg/kg; ip) and blood samples were obtained from the retro-orbital vein for serum separation. Animals were euthanized and kidneys were dissected out, washed with ice-cold saline and immediately frozen at -80°C. One part of frozen kidney was homogenized in protease inhibitor-complemented lysis buffer for estimation of renal injury markers, inflammatory markers, oxidative stress markers and apoptotic markers. Another part of the kidney was homogenized in ice-cold saline for determination of MDA, NO, GSH and TAC. A third part was lysed for Western blot analysis. In addition, a sample part was used for the histopathological and immunohistochemical examination.

*Renal injury markers*

Blood urea nitrogen (BUN) and creatinine were determined in serum using the corresponding commercial kits (Stanbio, Texas, USA). Renal kidney injury molecule-1 (Kim-1) and neutrophil gelatinase associated lipocalin (NGAL) levels were assayed with ELISA kits (Cusabio Biotech) as described by the manufacturer.

*Histopathology and immunohistochemistry*

The kidney tissues were fixed in neutral buffred 10% formalin, embedded in paraffin and 5 μm renal sections were stained with hematoxylin-eosin (H&E) [27]. For immunohistochemistry, the sections were blocked with 5% bovine serum albumin (BSA) for 2 h, then, incubated in humidified chamber overnight

at 4 °C with primary antibody against MMP-2, MMP-9, NF-κBp65, p53, Bax and PCNA (Thermo Scientific, IL, USA). Slides were washed, incubated with HRP-linked secondary antibody. The immunoreactions were developed with DAB (Sigma-Aldrich) and counterstained with hematoxylin as described [28]. All images were acquired by light microscopy (Leica Microsystems, Germany) by an observer blinded to the treatment group.

*MPO and Cytokine assays*

Commercial ELISA kits were utilized for the determination of renal myeloperoxidase (MPO; Hycult Biotech, Uden, Netherlands), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-10 (RayBiotech, USA), MCP-1 (Cusabio Biotech) and IL-18 (LifeSpan Biosciences, USA), as instructed by the manufacturer.

*Oxidative stress markers*

Renal lipid peroxides, expressed as malondialdehyde (MDA) were assayed as previously described [29] while the reduced glutathione (GSH) levels were measured using Ellman's reagent [30]. The enzymatic activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined as described [31] and [32], respectively. ELISA kits were used for the estimation of NADPH oxidase-1 (NOX-1; USCN Life Science, China), total antioxidant capacity (TAC; Cayman, USA), and Heme oxygenase-1 (OH-1;Enzo life Sciences, USA) as instructed by the manufacturer's protocol. The transcription factor Nrf-2 was quantified using an ELISA kit purchased from Cayman, USA.

*Nitric oxide (NO)*

Total nitric oxide levels were converted to the stable nitrite/nitrate and determined using Greiss reagent according to Miranda, et al [33]. with the modification of using zinc sulfate for protein precipitation [34].

*Caspase-3, Cyt C and Bcl-2*

A colorimetric assay kit (R&D systems, USA) was used for measuring caspase-3 activity whereas ELISA kits were used for the determination of Cytochrome c (Cyt c; EIab ELISA kit) and B cell lymphoma-2 (Bcl-2;Calbiochem, Germany) protein levels as described by the manufacturer.

*Immunoblotting*

Protein samples were prepared and separated using SDS-PAGE as described [35, 36]. Proteins were electrophoretically transferred into PVDF membrane. After blocking, membranes were probed overnight at 4 °C with specific primary antibodies: rabbit anti-phospho-NF-κB (Ser536), rabbit phospho-IκBα (Ser32), rabbit anti-NF-κBp65, rabbit anti-phospho-p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK, rabbit anti-phospho-ERK1/2 (phospho-p44/42; Thr202/Tyr204)), rabbit anti-ERK1/2 (p44/42), rabbit anti-phospho-JNK1/2 (Thr 183/185), rabbit polyclonal anti-JNK1/2, rabbit PI3K p110α, rabbit phospho-Akt (Ser473), rabbit anti-Akt, rabbit phospho-eNOS (Ser1177), rabbit anti-eNOS, rabbit cleaved caspase-3 (Asp175), rabbit anti-PARP, and rabbit monoclonal anti-Lamin B (Cell Cell Signaling Technology, USA). The blots were developed using Clarity Western ECL substrate (Biorad, CA, USA). Densitometric analysis was performed by normalizing to total corresponding target protein and signals were quantified via Image J software (Bethesda, MD, USA).

*Effect of Camel milk on 5-FU cytotoxicity*

We assessed the effect of Camel milk on 5-FU-induced cytotoxicity using MTT assay [37] in MCF-7, HepG-2, HCT-116 and PC-3cells. CM was first defatted and then lyophilized and the obtained dry powder was solubilized in DMSO. Cells were preconditioned with the calculated $IC_{10}$ of Camel milk for 24h, then treated with 5-FU for 72h and the MTT was added to the cultures for 2 hours. Concentration-response curves were generated and $IC_{50}$ for each curve was calculated (Graph Pad, Prism software, version 5).

*Statistical analysis*

All values are expressed as arithmetic mean ± SEM. Data were evaluated with SPSS, version 17 and statistical comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test. P values of $<0.05$ were regarded as statistically significant.

## Results

*Camel milk alleviates 5-FU-induced renal dysfunction*

5-FU triggered a marked renal damage as indicated by elevated levels of BUN (3.4 fold), serum creatinine (3.6 fold) and the sensitive renal injury indices [38]; Kim-1 (3.6 fold) and NGAL (4.5 fold) as compared to control rats (Fig. 1). Interestingly, CM, dose-dependently, lowered these injury biomarkers, indicating attenuation of 5-FU renal damage. Notably, the higher dose of CM (10 ml/kg) afforded a marked lowering of BUN (55%), creatinine (49.9%), renal kim-1 (47.4%) and NGAL levels (57.9%) as compared to the 5-FU group. These favorable effects were similar to those provoked by QRC, a reference antioxidant agent with marked reno-protective actions [6, 9]. To a lesser extent, the lower dose of CM (5 ml/kg) displayed marginal alleviation of these nephrotoxicity markers. Thus, the 10 ml/kg dose of CM was selected for further experiments. Worthy to mention that treatment of rats with isocaloric doses of bovine milk did not mitigate the 5-FU renal dysfunction, thus, ruling out that the caloric content of CM is responsible for the observed renal protection. Instead, the unique composition of CM is the crucial factor for the observed renal protection.

*Camel milk attenuates 5-FU-induced histopathological changes*

We next investigated whether CM can mitigate the 5-FU-evoked renal histopathological alterations. Sections from control and control + CM groups showed normal architecture of renal parenchyma (Fig. 2A,B and Table 1). In contrast, 5-FU treatment afforded several morphological changes and tissue damage including congestion of glomerular tufts, focal tubular necrosis associated with inflammatory cell infiltration, vacuolization of tubular epithelium and congestion of the intertubular blood capillaries (Fig. 2C, D). Interestingly, CM protected against these pathological alterations and preserved the renal architecture (Fig. 2E). Likewise, QRC attenuated the pathological changes with some areas displaying congestion of renal blood vessels

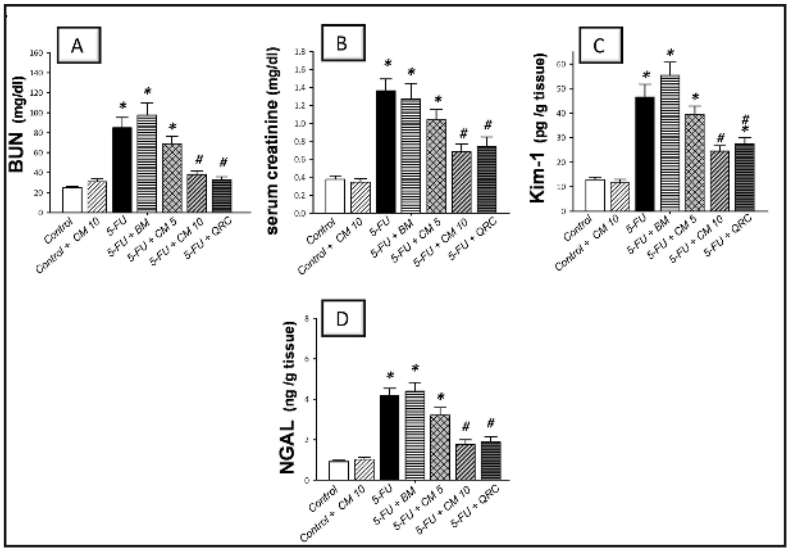


**Fig. 1.** Camel milk diminishes nephrotoxicity markers in rats with 5-FU-induced renal injury. (A) Blood urea nitrogen (BUN). (B) Serum creatinine. (C) Renal kidney injury molecule-1 (Kim-1). (D) Renal neutrophil gelatinase-associated lipocalin (NGAL). Data are expressed as mean ± SEM.* Significant difference from control gp at $p<0.05$, # Significant difference from 5-FU gp at $p<0.05$. CM 5, Camel milk (5 ml/kg); CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; BM, Bovine milk; QRC, Quercetin (50 mg/kg).

**Table 1.** Histopathological alterations in renal tissues of 5-fluorouracil-treated rats. +++ severe, ++ moderate, + mild, – Nil. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg)

| Histopathological alteration | Control | Control + CM 10 | 5-FU | 5-FU + CM 10 | 5-FU + QRC |
|---|---|---|---|---|---|
| Congestion and hypertrophy of glomerular tuft | – | – | +++ | – | – |
| Vacuolization of tubular epithelium | – | – | + | – | – |
| Focal tubular necrosis | – | – | ++ | – | – |
| Congestion of intertubular blood capillaries | – | – | +++ | – | + |

(Fig. 2F, G). These data indicate that CM and QRC mitigated the 5-FU renal histopathological damage.

*Camel milk diminishes renal inflammation in 5-FU-treated rats*

5-FU elicited a 4.1-fold increase of MPO, a reliable index for neutrophil recruitment [39], as compared to the control group (Fig. 3). In the same context, 5-FU triggered elevation of renal pro-inflammatory cytokines e.g., IL-1β (4.8 fold), TNF-α (4.3 fold), MCP-1 (5.3 fold) and IL-18 (6.9 fold) as compared to control rats. This was accompanied with a decline in the renal anti-inflammatory IL-10 levels (0.46 fold). In addition, 5-FU increased the protein expression of the proteolytic signals [40] MMP-2 (2.15 fold) and MMP-9 (2.2 fold; Fig. 4). Interestingly, CM reversed these changes and suppressed the inflammatory response, thereby, contributing to the attenuation of 5-FU renal injury.

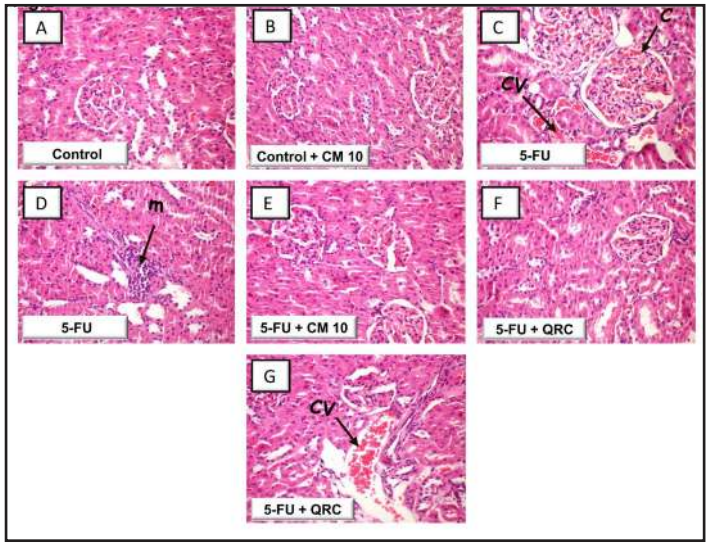


**Fig. 2.** Camel milk alleviates 5-FU-induced renal histopathological damage. Representative photomicrographs of sections from renal samples. Hematoxylin and eosin staining (× 200 magnification). C; Congestion of glomerular tufts, CV; Congestion of the intertubular blood capillaries, m; Inflammatory cell infiltration. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

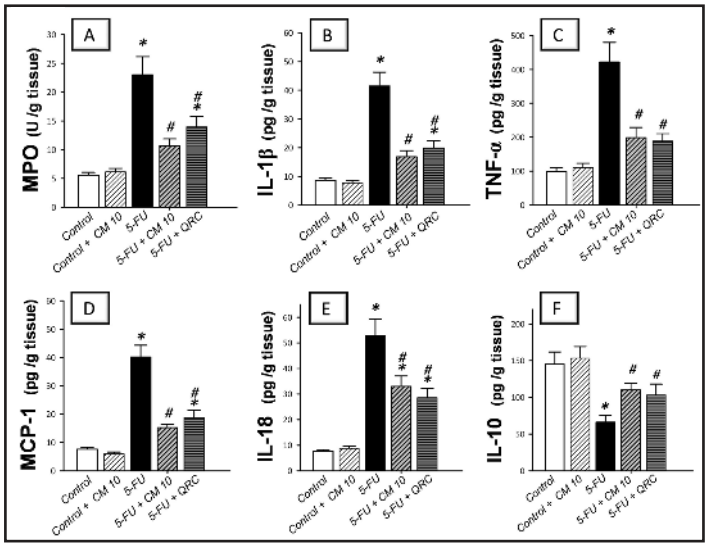


**Fig. 3.** Camel milk attenuates proinflammatory cytokines and augments IL-10 in 5-FU-induced renal injury. (A) Myeloperoxidase (MPO). (B) Interleukin-1β (IL-1β). (C) Tumor necrosis factor-α (TNF- α). (D) Monocyte chemoattractant protein-1 (MCP-1). (E) Interleukin-18 (IL-18). (F) Interleukin-10 (interleukin-10). Data are expressed as mean ± SEM. * Significant difference from control gp at $p<0.05$, # Significant difference from 5-FU gp at $p<0.05$. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

*Camel milk suppresses NF-κB and MAPKs activation in 5-FU-induced renal injury*

To further explore the molecular mechanisms associated with 5-FU renal injury, the role of NF-κB and MAPKs (p38 MAPK, JNK1/2 and ERK1/2) signaling pathways was determined. 5-FU activated NF-κB as evidenced by the increased immunohistochemical detection of activated NF-κB p65 (3.18 fold; Fig. 4C) and the increased phosphorylation of NF-κB p65 (2.88 fold) and IκBα proteins (3.68 fold; Fig. 5A-C). For MAPKs pathway, 5-FU triggered its activation as demonstrated by increased phosphorylation of p38 MAPK (3.97 fold), ERK1/2 (2.75 fold) and JNK1/2 (2.36

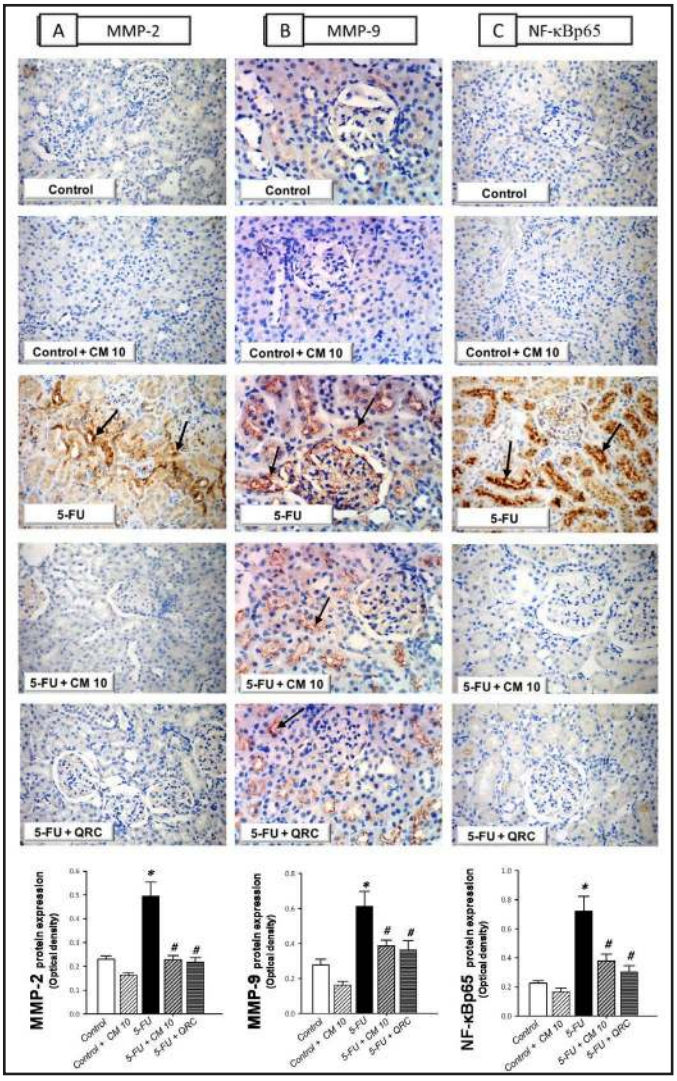


**Fig. 4.** Camel milk downregulates the protein expression of MMP-2, MMP-9, and NF-κBp65 in 5-FU-induced renal injury. Representative images for the immunohistochemical detection of target protein expression in renal tissues. (A) Matrix metalloproteinase 2 (MMP-2). (B) Matrix metalloproteinase 9 (MMP-9). (C) Nuclear factor kappa B (NF-κBp65). The optical density of the corresponding protein expression is shown in the lower panel. * Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

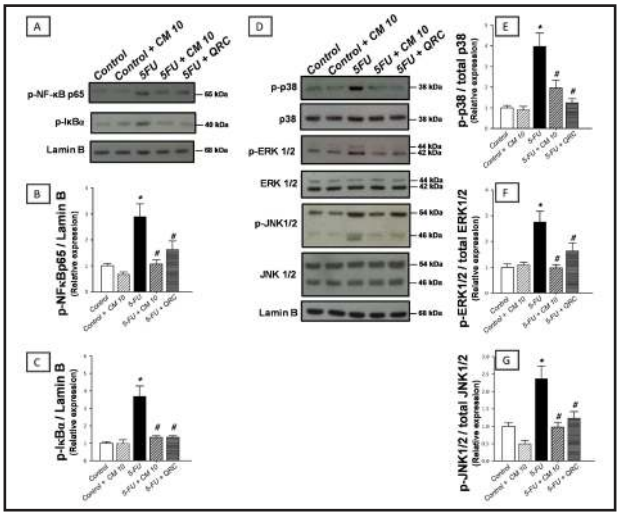


**Fig. 5.** Camel milk inhibits NF-κB activation and MAPKs transduction in 5-FU-induced renal injury. (A) Western blot analysis showing the expression of p-NF-κBp65 and p-IκBα. Lamin B was utilized to prove equal loading of total protein lysate. (B) Relative expression of p-NF-κBp65. (C) Relative expression of p-IκBα. (D) Western blot analysis for the expression of phosphorylated and total protein forms of p38 MAPK, ERK1/2 and JNK1/2. (E) Relative expression of p-p38. (F) Relative expression of p-ERK1/2. (G) Relative expression of p-JNK1/2. The control value was set as 1.0. Data were extracted from at least 3 independent experiments and values were expressed as mean ± SEM. * Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

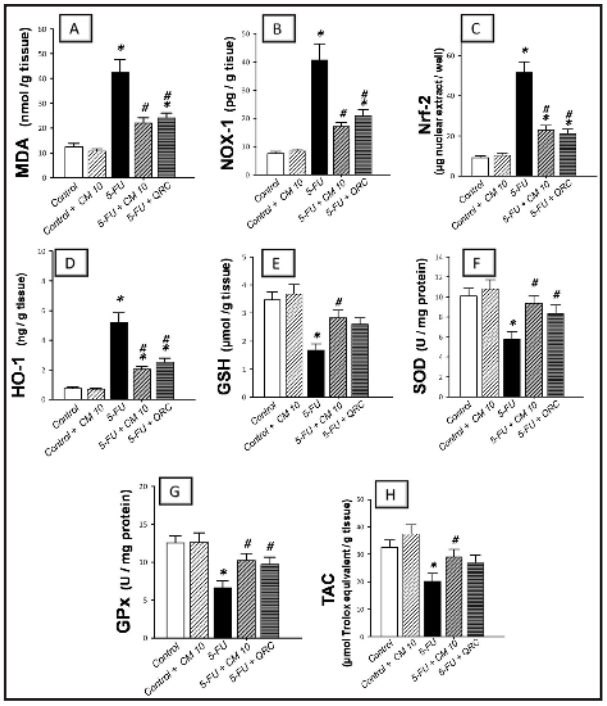


**Fig. 6.** Camel milk inhibits renal oxidative stress and restores Nrf-2 and HO-1 protein expression in 5-FU-induced renal injury. (A) Malondialdehyde (MDA). (B) NADPH oxidase-1 (NOX-1). (C) Nuclear factor erythroid 2-related factor 2 (Nrf-2). (D) Heme oxygenase-1 (HO-1). (E) Reduced glutathione (GSH). (F) Superoxide dismutase (SOD). (G) Glutathione peroxidase (GPx). (H) Total antioxidant capacity (TAC). Data are expressed as mean ± SEM. * Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

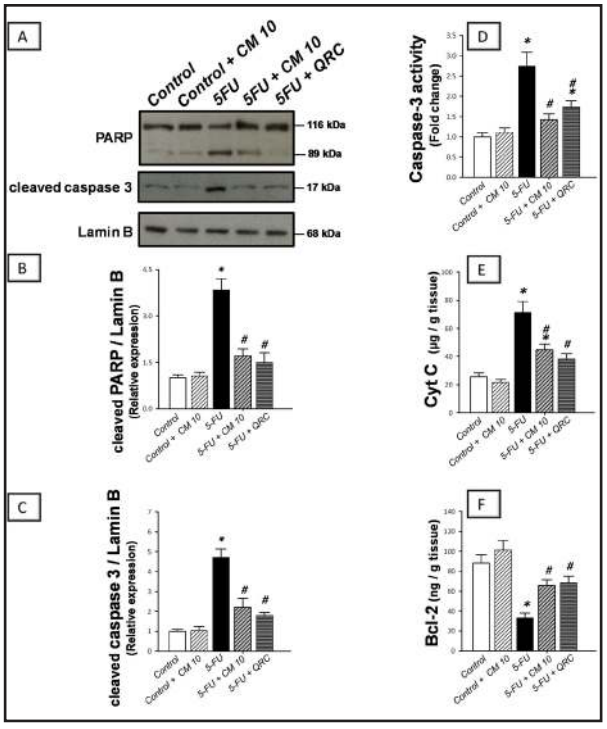


**Fig. 7.** Camel milk downregulates apoptosis in 5-FU-induced renal injury. (A) Western blot analysis for the expression of cleaved forms of PARP and caspase 3 proteins. Lamin B was utilized to prove equal loading of total protein lysate. (B) Relative expression of cleaved PARP. (D) Relative expression of cleaved caspase 3. The control value was set as 1.0. Data were extracted from at least 3 independent experiments. (D) Activity of caspase-3. (F) Expression of Cytochrome C (Cyt C). (F) Expression of B cell lymphoma (Bcl-2). Data are expressed as mean ± SEM.* Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

fold) proteins without affecting the corresponding total protein levels as compared to the control rats (Fig. 5D-G). Administration of CM and QRC curbed these changes as compared to 5-FU-treated rats. Together, these data reveal that the intervention with NF-κB and MAPKs transduction is implicated in CM attenuation of 5-FU nephrotoxicity.

*Camel milk inhibits renal oxidative stress and restores Nrf-2 and HO-1 protein expression in 5-FU treated rats*

Administration of 5-FU triggered a sharp increase (3.4 fold) of lipid peroxides (expressed as malondialdehyde; MDA) in renal tissues of rats as compared to control rats (Fig.

6). It also increased the levels of NOX-1 (5.4 fold) and the upstream targets Nrf-2 (5.6 fold), HO-1 (6.9 fold). Conversely, 5-FU depleted the antioxidant defenses as evidenced by the diminished levels of GSH (0.48 fold), SOD (0.57 fold), GPx (0.53) and TAC (0.62 fold) as compared to control rats. CM and QRC markedly counteracted renal oxidative stress markers and restored the antioxidant defenses. These effects indicate that attenuation of oxidative injury and boosting of the cellular antioxidants play a role in the alleviation of 5-FU renal damage.

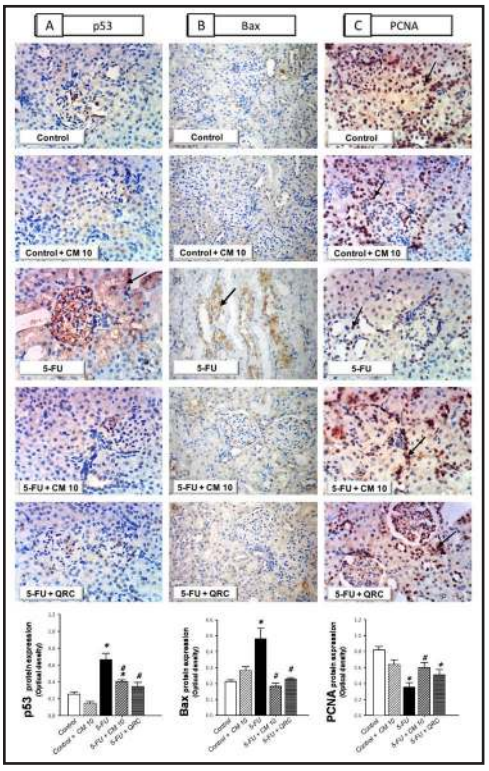


**Fig. 8.** Camel milk downregulates the protein expression of p53 (A) and Bax (B) and increases the expression of PCNA (C) in 5-FU-induced renal injury. Representative images for the immunohistochemical detection of target protein expression in renal tissues. (A) protein 53 (p53). (B) Bcl-2 associated x protein (Bax). (C) Proliferating cell nuclear antigen (PCNA). The optical density of the corresponding protein expression is shown in the lower panel. * Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

*Camel milk inhibits 5-FU-induced renal apoptosis*

5-FU instigated significant renal apoptosis as demonstrated by the marked increase of the cleaved forms of PARP (3.83 fold) and caspase-3 (4.69 fold) together with increased caspase-3 activity (2.7 fold) and Cyt C levels (2.8 fold; Fig. 7). 5-FU also triggered intense expression of p53 (2.59 fold) and Bax (2.3 fold) proapoptotic signals (Figs. 8A, B) with downregulation of the anti-apoptotic Bcl-2 (0.38 fold; Fig. 7F). In the same context, the renal expression of the proliferation signal, PCNA, was downregulated in 5-FU treated rats (0.44 fold; Fig. 8C).

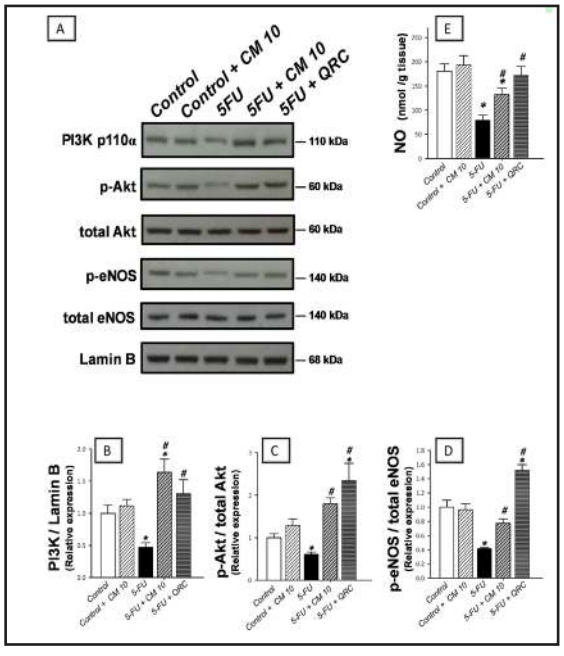


**Fig. 9.** Camel milk activates PI3K/Akt /eNOS pathway in 5-FU-induced renal injury. (A) Western blot analysis for the expression of PI3Kp110α along with phosphorylated and total Akt and eNOS. (B) Relative expression of phosphoinositide 3 kinase (PI3K). (C) Relative expression of phospho- protein kinase B (p-Akt). (D) Relative expression of phospho-endothelial nitric oxide synthase (p-eNOS). Lamin B was utilized to prove equal loading of total protein lysate. The control value was set as 1.0. Data were extracted from at least 3 independent experiments and values. (E) Renal nitric oxide (NO) levels. Data are expressed as mean ± SEM. * Significant difference from control gp at p<0.05, # Significant difference from 5-FU gp at p<0.05. CM 10, Camel milk (10 ml/kg); 5-FU, 5-fluorouracil; QRC, Quercetin (50 mg/kg).

CM and QRC counteracted these changes in favor of cell survival.

*Camel milk mitigation of 5-FU renal injury involves PI3K/Akt /eNOS activation*

The PI3K/Akt/eNOS pathway plays a central role in promoting cell survival and suppression of apoptosis [10]. 5-FU lowered the protein levels of PI3K p110 (0.47 fold) together with the phosphorylation of Akt ($Ser^{473}$; 0.61 fold) and eNOS ($Ser^{1177}$; 0.42 fold) as compared to control rats without affecting their corresponding total protein levels (Fig. 9A-D). In the same context, 5-FU diminished the renal NO levels (0.44 fold; Fig. 9E). CM and QRC counteracted these changes implicating the involvement of PI3K/Akt /eNOS pathway activation for the mitigation of 5-FU nephrotoxicity.

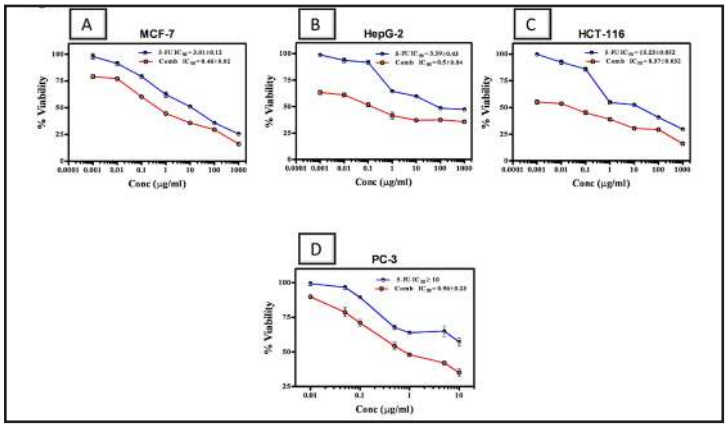


**Fig. 10.** Camel milk enhances the cytotoxicity of 5-FU in MCF-7 (A), HepG-2 (B), HCT-116 (C) and PC-3 (D) cells. Comb, Combination of 5-FU and CM; 5-FU, 5-fluorouracil; CM, Camel milk.

*Preconditioning with Camel milk enhances 5-FU-induced cytotoxicity in MCF-7, HepG-2, HCT-116 and PC-3 cancer cells*

To exclude the possibility that CM may interfere with 5-FU cytotoxicity, we examined the anti-proliferative effects of 5-FU alone and in combination with CM in MCF-7, HepG-2, HCT-116 and PC-3 cancer cell lines. The cancer cells were preconditioned with the $IC_{10}$ of Camel milk (1 mg/ml) for 24h followed by different concentrations of 5-FU for 72h. Treatment with 5-FU alone showed evident cytototoxicity with $IC_{50}$ values of 3.01±0.12 µg/ml (MCF-7), 3.39±0.43 µg/ml (HepG-2), 1.8±0.2 µg/ml (HCT-116) and > 10 µg/ml (PC-3; Fig. 10). Preconditioning with CM prior to 5-FU augmented the cytotoxicity of 5-FU in all tested cancer cell lines. This was evident by the markedly lowered $IC_{50}$ values that reached 0.461±0.026 µg/ml in MCF-7, 0.15±0.04 µg/ml in HepG-2, 0.13±0.052 µg/ml in HCT-116 and 0.96±0.23 µg/ml in PC-3.

## Discussion

Chemotherapy with 5-FU, a pyrimidine antimetabolite has been associated with renal toxicity as a serious adverse reaction [1]. Interestingly, the current findings pinpoint, for the first time, the marked renoprotective effects of CM that were mediated via ROS scavenging, suppression of MAPKs and NF-κB along with activation of PI3K/Akt/eNOS pathway.

The current data revealed that 5-FU instigated marked renal injury that was associated with increased BUN and serum creatinine and renal Kim-1 and NGAL; findings that coincide with previous literature [1]. Renal Kim-1 is upregulated in proximal tubular injury whereas increased NGAL confirms the damage to renal nephrons [38]. These events can be regarded as compensatory mechanisms against renal damage since Kim-1 serves to suppress apoptosis and aids in tubular re-epithelization while NGAL behaves as an adhesion molecule that abrogates epithelial shedding [41]. Interestingly, current findings demonstrated that CM exerted renoprotective actions as confirmed by leveling-off these injury biomarkers; events that are in agreement with the reported CM attenuation of cisplatin- [21], Diabetes mellitus- [19] and gentamicin-induced nephrotoxicities [20].

Emerging evidence has indicated the involvement of oxidative stress in the pathogenesis of 5-FU-induced renal injury via excessive release of free radicals and ROS [1, 3]. The current

findings revealed that 5-FU instigated lipid peroxidation and associated damage to the cellular membranes in addition to depletion of cellular GSH, SOD, GPx and TAC antioxidants; findings that coincide with previous reports [1, 42, 43]. A plausible explanation for ROS generation is the observed infiltration of inflammatory cells and associated increase of NOX-1 activity which generates a surplus of superoxide anions [44]. Additionally, the increased MPO levels trigger overshooting of hypochlorous acid, a robust cytotoxic oxidant [39, 45]. The current findings also revealed an upregulation of Nrf-2 and HO-1 upstream signals, probably as compensatory mechanisms to counteract ROS generation. Upon oxidative stimulation, the Nrf-2 is liberated from the inhibitory Keap1 protein and translocates to the nucleus for binding antioxidant response element essential for transcription of HO-1 and antioxidant enzymes [46].

A unique finding in the current study is the attenuation of renal dysfunction together with oxidative stress markers. These observations support the premise that CM antioxidant features are implicated in the alleviation of 5-FU renal injury, likely via scavenging free radicals. These data are in accordance with the marked antioxidant features of CM in toxicant-induced renal damage [20, 21]. These beneficial effects have been attributed to CM significant content of the vitamins C and E along with the high levels of zinc, an essential element for SOD, and selenium, a component of selenoproteins and GPx enzyme [22, 23]. A major contributor for CM antioxidant features is the lactoferrin, a bioactive protein which scavenges free iron with consequent suppression of hydroxyl radical generation [12]. A Plausible explanation for CM attenuation of oxidative stress is the observed inhibition of leukocyte infiltration and NOX-1 [44] along with the inactivation of the redox-sensitive MAPKs and NF-κB pathways which play essential roles in driving oxidative stress [4, 44]. The observed CM restoration of Nrf-2 and HO-1 probably reflects the attenuation of ROS generation, since the activation of Nrf-2 and HO-1 is instigated by overshooting of pro-oxidant signals [46].

The current study revealed robust inflammatory events in response to 5-FU administration. This was evidenced by increased renal MPO, TNF-α, IL-1β, IL-18 and MCP-1 proinflammatory signals; findings which are consistent with previous literature [41]. Evidence indicates that the activation of monocytes and macrophages is linked to the release of an array of proinflammatory cytokines which trigger and perpetuate the inflammatory response in diverse renal pathologies [6]. This was accompanied with increased renal expression of MMP-2 and MMP-9 in 5-FU-treated animals. Several AKI models have described increased expression of MMP-2 and MMP-9 in response to oxidative stress [40]. MMPs play a central role in the cellular inflammatory response particularly, the activation, chemotaxis and proliferation of immune cells. For example, MMP-9 generates collagen fragments that act as robust chemostactic signals for neutrophils.

An upstream landmark in the inflammatory/apoptotic pathways is the MAPKs super-family which involves ERK, JNK and p38 MAP kinases. Activation of p38 MAPK and JNK occurs in response to proinflammatory cytokines e.g., TNF-α and other cellular stresses such as ROS [4, 5]. The current study reports, for the first time, the involvement of MAPKs activation in mediating the injurious events of 5-FU chemotherapy on rat kidney. Stimulation of JNK and p38 MAPK signaling drives pro-inflammatory cytokine production and renal apoptosis [4, 5] which correlates well with the renal damage in human and experimental models [5]. In addition, ERK stimulation has been reported to activate downstream transcription factors including NF-κB [6, 7]. Previous reports have characterized the activation of NF-κB in 5-FU-induced nephrotoxicity [11]. In addition to MAPKs activation, NF-κB can be driven by excessive ROS generation and diverse cytokines [6]. These signals trigger the dissociation of the inhibitory IκBα from NF-κB with consequent liberation of the active heterodimer (p50 and p65 subunits) that translocates to the nucleus for transcription of target inflammatory genes including TNF-α, IL-1, IL-18 and MCP-1. Thus, the protein expression of NF-κB p65 has been commonly recognized as an activation index of NF-κB. [6].

The current findings demonstrated that CM effectively suppressed the proinflammatory MAPKs and NF-κB pathways along with their downstream cytokines, signifying the multipronged anti-inflammatory actions of CM. These findings are in harmony with the

reported CM suppression of MAPKs pathway in Adjuvant arthritis [13] and LPS-induced respiratory distress [47] along with abrogation of NF-κB signaling in wounds of diabetic rats [18]. Interestingly, blockade of MAPKs signaling, in particular, p38 and JNK pathways, has been shown to protect against acute renal failure, tubular cell apoptosis and histopathological damage in multiple renal injury models [5, 48]. The observed CM inhibition of MAPKs pathway together with downstream effectors including NF-κB and inflammatory cytokines has been regarded as an effective strategy for the management of diverse renal injuries [4, 6]. In the same context, lactoferrin, a major anti-inflammatory component of CM, has been acknowledged for curbing the production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6 in mononuclear cells [12].

The current findings further demonstrated that 5-FU instigated renal apoptosis as confirmed by the cleavage of PARP and caspase-3, reliable indices of apoptosis [49] and enhanced the expression of the tumor suppressor p53, Bax and Cyt C pro-apoptotic signals with decline of the anti-apoptotic Bcl-2 and the proliferation signal PCNA. These data are consistent with previous literature [1, 50]. Apoptotic cell death correlates well with the level of renal dysfunction in murine models of kidney injury [10]. In kidney injury models, the surge of ROS and proinflammatory signals has been recognized to activate renal apoptosis at multiple steps of the cell death pathway. In this regard, ROS drive conformational changes of Bax which then translocates to the mitochondria resulting in Cyt C release to the cytosol with consequent activation of caspase-9 and eventually the executioner caspase-3 [9, 10, 49].

In the context of renal apoptosis, PI3K/Akt/eNOS is an upstream signaling pathway that, upon activation, favors cell survival and suppresses apoptosis. Akt activation phosphorylates and inactivates several apoptosis-inducing factors, including Bax [10]. The phosphorylation of Akt ($Ser^{473}$) and eNOS ($Ser^{1177}$) upregulates the anti-apoptotic Bcl-2 family proteins and switches-off caspase-3 activity via S-nitrosylation of its active center p17 [8, 9]. Meanwhile, activation of PI3K/Akt pathway plays a critical role in modulating Nrf2-dependent attenuation of oxidative stress [8]. In the current study, 5-FU suppressed the PI3K/Akt/eNOS pathway with consequent decline of NO levels. The observed decline of renal NO level is in line with data from previous AKI models and is probably due to ROS-triggered inactivation and sequestration of NO [9]. In addition to driving apoptotic cascade via inhibition of PI3K/Akt pathway, the current study revealed that 5-FU activated p38 MAPK and JNK; known triggers of apoptotic cell death [4, 51].

The present data indicated that CM suppressed renal apoptosis in 5-FU-induced renal injury as evidenced by counteracting the apoptotic events which is consistent with previous literature [14, 16, 52]. One possible explanation for these findings is the reported and observed scavenging of ROS, major trigger for apoptosis [9, 10]. The inhibition of proinflammatory cytokines e.g., TNF-α and their upstream NF-κB pathway can also contribute to the dampening of the apoptotic pathway [6]. In addition, activation of PI3K/Akt/eNOS pathway and inhibition of MAPKs pathway can contribute to the abrogation of renal tubular apoptosis [10]. Virtually, the current finding of PI3K/Akt/eNOS pathway activation is consistent with previous studies that described enhanced phosphorylation of Akt by CM whey protein supplementation in wounds of diabetic mice and in B- and T-lymphocytes isolated from type 1 diabetic mice [53].

## Conclusion

In conclusion, the present work demonstrates, for the first time, the renoprotective actions of CM against 5-FU-induced renal injury. These events were mediated, at least partly, via suppression of oxidative, inflammatory and apoptotic aberrations. Mechanistically, the inhibition of MAPKs and NF-κB along with the activation of PI3K/Akt/eNOS signaling cascade played crucial roles in the mitigation of 5-FU-induced renal injury. Notably, CM preconditioning augmented 5-FU cytotoxicity in MCF-7, HepG-2, HCT-116 and PC-3 cells, excluding the doubt about their concomitant use and confirming the "dual benefit" for the use of CM with 5-FU. Together, the current study findings advocate the use of CM as a safe

adjunct approach for minimizing nephrotoxicity associated with 5-FU. Additional studies may be warranted to explore the correlation between the plasma/renal levels of lactoferrin and other CM bioactive ingredients and the extent of renal injury. Additionally, the potential efficacy of CM in the clinical setting as a complementary strategy for minimizing 5-FU nephrotoxicity needs to be explored.

## Abbreviations

5-FU (5-fluorouracil); Akt (protein kinase B); Bax (Bcl-2 associated x protein); Bcl-2 (B cell lymphoma-2); CM (Camel milk); CytC (cytochrome C); eNOS (endothelial nitric oxide synthase); ERK1/2 (extracellular signal-regulated kinase 1/2); GSH (reduced glutathione); GPx (glutathione peroxidase); HO-1 (heme oxygenase-1); IL-1β (interleukin-1β); IL-10 (interleukin-10); IL-18 (interleukin-18); JNK1/2 (c-Jun N-terminal kinase 1/2); Kim-1 (kidney injury molecule-1); MAPKs (mitogen activated protein kinases); MMP-2 (matrix metalloproteinase 2); MMP-9 (9 matrix metalloproteinase 9); MCP-1 (monocyte chemattractant protein-1); MDA (malondialdehyde); MPO (myeloperoxidase); NGAL (neutrophil gelatinase associated lipocalin); NF-κB (nuclear factor kappa B); NO (nitric oxide); NOX-1 (NADPH oxidase-1); Nrf-2 (nuclear factor erythroid 2-related factor 2); PARP (poly (ADP-ribose) polymerase); PCNA (proliferating cell nuclear antigen); PI3K (phosphoinositide 3 kinase); ROS (reactive oxygen species); SOD (superoxide dismutase); TAC (Total anti-oxidant capacity); TNF-α (tumor necrosis factor-α).

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## Disclosure Statement

The authors declare that there are no conflicts of interest.

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