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Short-Communication

Crocin mitigates colistin-induced toxicity in HEK-293 cells through antioxidant and antiapoptotic mechanisms

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Abstract

Objective: The glycopeptide antibiotic colistin (polymyxin E) causes dose-dependent nephrotoxicity. The pathophysiology of colistin-induced nephrotoxicity involves processes such as ischemia, inflammation, and apoptosis. By alleviating oxidative stress, inflammation, and apoptosis, crocin, a key component of *Crocus sativus* protects the kidneys from toxicity. This study designed to explore the protective effects of crocin against colistin-induced cytotoxicity in HEK-293 cells.

Materials and Methods: HEK-293 cells were treated with varying concentrations of crocin (1.25 - 40 µM) for 24 hr before exposure to 160 µM colistin (the IC50 value). Cell viability was measured using an MTT assay. Intracellular reactive oxygen species (ROS) levels were measured using DCFH, and Western blotting was performed to analyze the expression of Bcl-2, Bax, and caspase-3 proteins.

Results: Pre-treatment with crocin at concentrations of 1.25 ($p<0.001$), 2.5, and 5 ($p<0.01$) µM effectively enhanced cell viability, which had been diminished by colistin. Furthermore, crocin mitigated the elevated levels of ROS induced by colistin ($p<0.001$). The findings reveal that crocin prevented the upregulation of pro-apoptotic proteins Bax ($p<0.05$) and caspase-3 ($p<0.01$), and countered the downregulation of the anti-apoptotic protein Bcl-2 ($p<0.05$) triggered by colistin in HEK-293 cells.

Conclusion: Crocin serves as a natural compound that alleviates colistin-induced cytotoxicity through inhibiting oxidative stress and preventing apoptosis induction. As a result, crocin stands out as a promising antioxidant for preventing colistin-induced cytotoxicity, especially in renal cells.

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Introduction

Colistin (polymyxin E), is a peptide antibiotic which belongs to the polymyxin class with poor gastrointestinal absorption (Biswas et al. 2012). It is available in two forms: 1) Colistimethate sodium, and 2) Colistin sulfate (Pastor et al. 2019). Due to its role as a last-line antibiotic against infections from several multidrug-resistant Gram-negative bacteria, colistin is being used more frequently. However, nephrotoxicity stands out as a common side effect of colistin (Andrade et al. 2020). *In vivo* studies have indicated that the toxicity of colistin is dose- and duration-dependent, and typically reversible (Aggarwal and Dewan 2018; Ghlissi et al. 2014; Heidari et al. 2019). Colistin can bind with the tubular cell membranes, thereby increasing membrane permeabilization, and leading to cell swelling and lysis (Ordooei Javan et al. 2015). Colistin induces the demise of proximal tubule cells through mechanisms such as the elevation of reactive oxygen species (ROS) and the initiation of apoptosis, ultimately resulting in nephrotoxicity (Lee et al. 2015; Yousef et al. 2012). Colistin administration leads to a notable deterioration in kidney function through various mechanisms, including the impact on nuclear factor erythroid 2-related factor 2 (Nrf2), oxidative stress parameters, and signal transducer and activator of transcription 3 (STAT3) (Ozkan et al. 2013). Despite its widespread use as an antibiotic, colistin's administration is limited due to the development of nephrotoxicity and antibiotic resistance.

Saffron (*Crocus sativus* L.) is recognized as the most precious spice in the world, with its primary production occurring in the Khorasan Province of northeastern Iran. Iran is the leading producer of saffron globally, responsible for over 90% of saffron exports and covering 60% of the world's saffron cultivation area (Duan et al., 2021; Hosseinzadeh et al. 2013). For centuries, saffron extracts and tinctures have a long tradition of use in medicine where they have been employed to treat various conditions and disorders, exhibiting properties from sedative and eupeptic to antispasmodic, expectorant, abortifacient, and emmenagogue effects. Historically, saffron has been recognized for its benefits in treating genital disorders and assisting with menstrual regulation and discomfort. Additionally, during the Middle Ages, midwives often utilized saffron for its sedative and antispasmodic effects during childbirth (Alonso et al. 2012; Mollazadeh et al. 2015). Saffron contains important compounds such as crocin, crocetin, safranal, and picrocrocin with different properties. Among these, anti-depressive (Vahdati Hassani et al. 2014), anti-arteriosclerosis (He et al. 2005), and anti-cancer (Escribano et al. 1996; Garc-Olmo et al. 1999; Rastgoo et al. 2013), as well as vascular and cardiotoxicity protective (Razavi et al. 2014; Razavi et al. 2013) effects of crocin have been well known. Crocin exhibits these effects through its anti-apoptotic (Razavi et al. 2016), anti-inflammatory (Akbari et al. 2018), and antioxidant (Rameshrad et al. 2018; Singla and Bhat 2011) properties, commonly. Crocin has been shown to mitigate the elevation of creatinine and urea-nitrogen, as well as kidney tissue malondialdehyde (MDA) levels. It also restores the decreased levels of ferric reducing/antioxidant power (FRAP) in renal tissue induced by gentamicin. Moreover, crocin ameliorated various histopathological manifestations of gentamicin-induced renal damage (Yarijani et al. 2016). Studies in rats suggest that crocin can ameliorate renal injury caused by ischemia/reperfusion, an effect associated with its antioxidant and anti-inflammatory actions (Hosseinzadeh et al. 2005; Yarijani et al. 2017). Evidence suggests that saffron, particularly its active component crocin, can lessen oxidative stress associated with renal ischemia-reperfusion (IR) injury in rats (Boozari and Hosseinzadeh 2017; Hosseinzadeh et al. 2005). Additionally, research suggests that crocin prevents α- and β-zearalenol-induced endoplasmic reticulum stress and

apoptosis in HEK-293 cells (Ben Salem et al. 2016).

Because colistin, as an important antibiotic, is known to cause kidney damage, and crocin has demonstrated nephroprotective properties, this study designed to examine whether crocin could protect HEK-293 cells from colistin-induced cytotoxicity.

Materials and Methods

Materials

This study utilized the HEK-293 cell line, sourced from the Pasteur Institute in Tehran, Iran. Acrylamide, DCF (2′, 7′-Dichlorofluorescin diacetate), NaF (Sodium fluoride), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Merck (Germany). PVDF membranes were purchased from Bio-Rad (USA), and all antibodies used in this research were obtained from Abcam (USA).

Cell preparation and treatment

HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed every two days. Once the cells reached a confluence of over 80%, they were treated with colistin and crocin, either individually or in combination. For combined treatment, cells were pretreated with crocin for 24 hr prior to the addition of colistin, to assess toxicity and perform subsequent experiments.

Examining the toxicity of colistin and determining its IC50

To assess the mitochondrial function and survival rate of HEK-293 cells, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted. Reduced cell viability is reflected by a decrease in the optical absorption of the resulting purple MTT solution (Firdaus et al. 2019; Mosmann 1983).

Briefly, HEK-293 cells in logarithmic growth were seeded into 96-well plates. After 24 hr of incubation, the cells were treated with varying concentrations of colistin (20 to 320 µM). Following a 24-hr treatment, cell viability was evaluated using the MTT assay, measuring absorbance at 545 and 630 nm. The resulting data was analyzed using GraphPad Prism software to determine the IC50 value of colistin, which was then used in further experiments.

Investigating the protective effect of crocin against colistin toxicity

To investigate the protective effect of crocin against colistin toxicity, 24 hr after seeding HEK-293 cells in 96-well plates, first cells were treated with crocin at 1.25, 2.5, and 5 µM concentrations, and 24 hr later, colistin at 160 µM (IC50) was added to cells. Finally, the viability of cells (%) was determined using the MTT (Mosmann 1983) test 24 hr later. Given the lack of a significant change in cell viability in comparison with the control in cells exposed to crocin over a wide range of concentrations (1.25, 2.5, 5, 10, 20, and 40 µM), the lowest concentrations were used to investigate the dose-dependent effects of crocin on colistin cytotoxicity.

ROS measurement

Intracellular reactive oxygen species (ROS) levels were evaluated using 2',7'-dichlorofluorescein diacetate (DCFH2-DA). HEK-293 cells were seeded in 96-well plates at a density of 5 x 10^3 cells/ well and pretreated for 24 hr with crocin (1.25, 2.5, and 5 µM). Following pretreatment, colistin (160 µM) was added, and the cells were incubated for an additional 24 hr. After removing the supernatant and washing with phosphate-buffered saline (PBS), cells were incubated with 100 µl of 10 µM DCFH2-DA for 30 min. The supernatant was then completely removed, 200 µl of PBS was added to each well, and fluorescence was measured using a fluorimeter at excitation/emission wavelengths of 485/520 nm.

Results were determined as a percentage of the control (Liu et al. 2017).

The effect of crocin and colistin on protein levels of Bax, Bcl-2, and Caspase-3 using the western blotting

Due to the non-significant effects of crocin at various concentrations on colistin-induced cytotoxicity and ROS formation in HEK-293 cells, as well as the high cost of performing western blot, the lowest concentration was selected to evaluate the levels of the target proteins in the present study. Initially, cells were washed with PBS (Aminifard et al., 2024). Then, 200 µl of homogenization buffer was added to the cell pellet. This buffer consisted of 50 mM Tris, 2 mM EDTA, 2 mM EGTA, 10 mM sodium azide, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.2% (w/v) sodium deoxycholate, and 10 mM 2-mercaptoethanol (2-ME). The buffer was further supplemented with 1 µl of a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor (Aminifard et al., 2024). The obtained suspension was vortexed and kept at 4°C for 30 min and vortexed several times during this interval. To disrupt the cells, the samples were sonicated on ice for 40 sec (4 times, 10 sec each time). Following homogenization, the samples were centrifuged at 10,000 g at 4°C for 15-20 min. The supernatant, containing the extracted proteins, was then carefully collected. Finally, protein concentration was quantified using the Bradford assay (Kruger 2009). The extracted proteins were separated by performing SDS-PAGE. In this step, the proteins move along the gel containing acrylamide and separate from each other according to their weight and electric charge. Next, the isolated proteins were transferred from the acrylamide gel to the polyvinylidene fluoride (PVDF) membrane under electrical conditions (350 mA for 45 min). To block non-specific binding, the membrane was incubated for 2 hr in Tris-buffered saline with Tween 20 (TBST) containing 5% nonfat dry milk. The membrane was then washed three times for 5 min each with TBST to remove residual blocking solution. Primary antibodies rabbit anti-Bcl-2 (1:1000, Cell Signaling #2870), rabbit anti-Bax (1:1000, Cell Signaling #2772), rabbit anti-caspase-3 (1:1000, Cell Signaling #9665), and the loading control mouse anti-β-actin (1:1000, Cell Signaling #3700) were applied and incubated overnight. After removing the primary antibody solution, the membrane was washed again three times for 5 min each with TBST. Subsequently, the membrane was incubated for 2 hr with horseradish peroxidase (HRP)-conjugated secondary antibodies: anti-mouse (1:3000, Cell Signaling #7076) and anti-rabbit (1:3000, Cell Signaling #7074). Following a final series of three 5-min washes with TBST, the protein bands were visualized by enhanced chemiluminescence (ECL) upon addition of hydrogen peroxide (H_2O_2). Chemiluminescent detection was performed using a Gel Doc UV Alliance system (Alliance 4.7, England). The intensity of the resulting bands was used to quantify protein expression levels for each sample.

Statistical Analyses

Data from this study were analyzed using GraphPad Prism version 8 software. Colistin IC50 values were determined using the same software. Statistical comparisons among the groups were done using a one-way ANOVA followed by a Tukey-Kramer post hoc test. All data are presented as mean ± standard deviation (SD), and statistical significance was defined as $p < 0.05$.

Results

Determination of IC 50 of colistin

As shown in Figure 1a, the colistin IC50 was estimated at 160±3.6 µM.

Crocin protects HEK-293 cells against colistin-induced cell death

MTT assay data (Figure 1c) showed that 160 µM colistin significantly decreased HEK-293 cell survival compared to the control ($p<0.001$). Crocin pretreatment significantly increased cell survival compared

to the colistin-only treated group ($p < 0.001$ for 1.25 µM, and $p < 0.01$ for 2.5 µM and 5 µM). Crocin alone had no significant effect on cell viability (Figure 1b).

Crocin reduces colistin-induced ROS production in the HEK-293 cells

Figure 2 demonstrates that colistin treatment significantly elevated ROS formation in HEK-293 cells compared to the control group ($p<0.001$). However, pretreatment with crocin at all tested concentrations significantly attenuated the colistin-induced ROS generation, as indicated by a statistically significant decline in ROS levels compared to cells treated with colistin alone ($p<0.001$ for 1.25 µM, and $p<0.01$ for 2.5 and 5 µM).

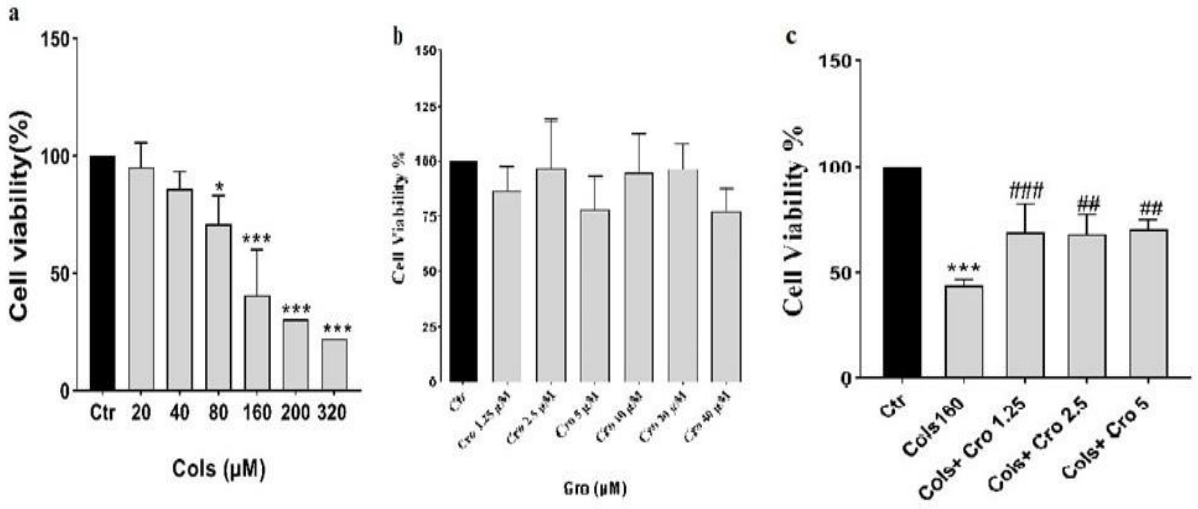

Figure 1. Effect of colistin (Cols) and crocon (Cro) on cell viability in HEK-293 cells; a) Cols (0-320 µM), b) Cro (0-40 µM), c) protective effects of Cro (1.25, 2.5 and 5 µM) against Cols (160 µM)-induced toxicity on HEK-293 cells. Data were analyzed by one-way ANOVA following the Tukey-Kramer post-test and values are expressed as means ± SD. **p<0.01, and ***p<0.001 compared to the control group, and #p<0.05, and ##p<0.01 compared to Clos.

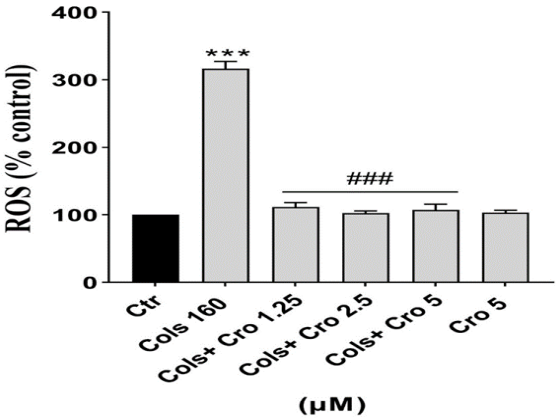

Figure 2. Effect of crocin (Cro) on colistin (Cols)-induced reactive oxygen species (ROS) production in HEK-293 cells. Data were analyzed by one-way ANOVA following the Tukey-Kramer post-test and values are expressed as means±SD. ***p<0.001 compared to the control group, and ###p<0.001 compared to the Cols group.

Crocin protects HEK-293 cells against colistin-induced apoptosis

Western blot analysis (Figure 3a) showed that exposure to colistin significantly down regulated the protein level of the Bcl-2 compared to the control group ($p<0.05$). In contrast, colistin significantly up regulated the protein level of Bax and caspase-3 in HEK-293 cells compared to the control group ($p<0.05$ for caspase-3, and $p<0.01$ for Bax) (Figure 3b, c). However, combined treatment with crocin and colistin significantly prevented the colistin-induced up regulation of Bax ($p<0.05$) and caspase-3 ($p<0.01$), maintaining their expression levels closer to baseline compared to cells treated with colistin alone (Figure 3b and c). Additionally, Bax/Bcl-2 ratio was significantly elevated ($p<0.01$) in the

colistin-treated group compared to the control group and significantly reduced in the group treated with crocin in combination with colistin compared to the colistin-treated group ($p<0.01$) (Figure 3e).

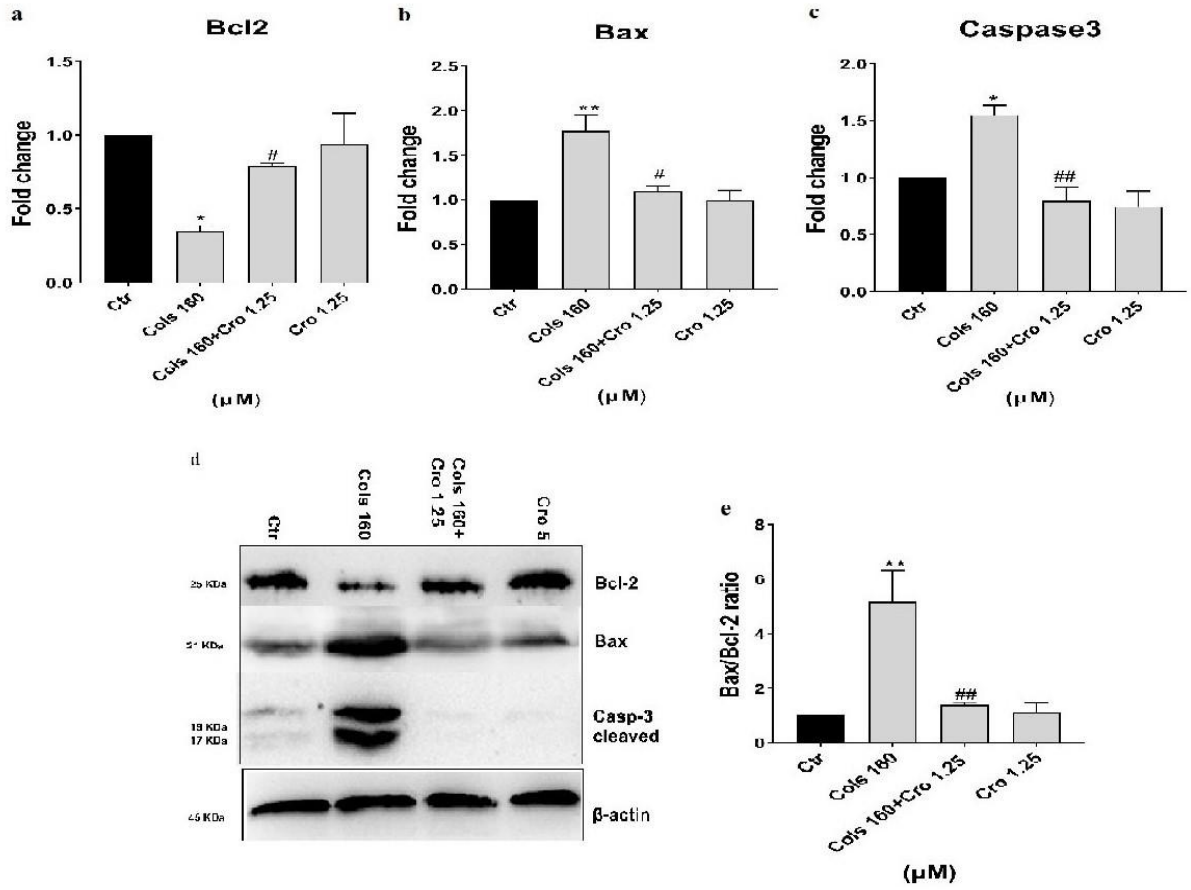

Figure 3. Effect of colistin (Cols) and crocin (Cro) on the levels of proteins involved in apoptosis pathway in HEK-293 cells by Western blotting; a) graph shows analysis results for B-cell leukemia/lymphoma 2 protein (Bcl-2), b) graph shows analysis results for Bcl-2–associated X protein (Bax), c) graph shows analysis results for Caspase 3, d) different bands (25, 21, 17-19 and 45 KDa) belonging to Bcl-2, Bax, Caspase 3 and β-actin and **e**) graph shows analysis results for Bax/Bcl-2 ratio. Data were analyzed by one-way ANOVA following the Tukey-Kramer post-test and values are expressed as means ± SD. *$p<0.05$, and **$p<0.01$ compared to control, #$p<0.05$, and ##$p<0.01$ compared to Cols.

Discussion

The present study was designed to evaluate the protective effects of crocin against colistin-induced cytotoxicity in HEK-293 cells. The HEK-293 cell line is obtained from human embryonic kidney cells, making it suitable for industrial biotechnology and toxicology research (Dumont et al. 2016; Lin et al. 2014; Thomas and Smart 2005).

The findings revealed that crocin enhances cell survival when exposed to colistin by significantly reducing apoptosis.

Colistin, as an antibiotic belonging to the polymyxins family, exerts its action on the bacterial cell membrane (Nation and Li 2009) and causes rapid death by changing the permeability of the bacterial membrane (Yahav et al. 2012). The most common adverse effect reported for colistin therapy is nephrotoxicity, which mostly appears as acute kidney injury (AKI). Colistin mainly accumulates in the kidney cortex regions and targets the cells' mitochondria (Nilsson et al. 2015)

The results of the present study showed that colistin causes cell toxicity (IC50: 160

μM) and consequently significantly reduces the viability of HEK-293 cells. Several studies showed that colistin can cause a toxic effect on various cell lines (Gallardo-Godoy et al. 2016; Naghmouchi et al. 2013). According to Lee et al., colistin's toxicity in human proximal tubular cells is dependent on both the dose and the time of exposure (Lee et al. 2015). Studies reported that crocin and trans sodium crocetinate derived from saffron extract have anti-cytotoxic effects (Ben Salem et al. 2016; Rajabian et al. 2023). The results of our study indicated that crocin prevents colistin cytotoxicity and preserves cell survival. Previous studies, in line with the current research, have shown that crocin with a concentration of 250 μM can increase the viability of HEK-293 cells against alpha and beta zearalenol and reduce cell stress, and it has also been able to improve the reduced cell viability of cells exposed to carbon tetrachloride (Ben Salem et al. 2016; Erdemli et al. 2018).

Colistin dramatically increased ROS production in HEK-293 cells and interestingly, crocin suppressed this effect. In this regard, Jiang et al. published that colistin-induced ROS formation results in cytotoxicity and induction of apoptosis (Jiang et al. 2014). It has been indicated that 24-hr exposure of PC12 cells to colistin resulted in a significant enhancement in ROS formation (Liu et al. 2013). In another study, increased intracellular ROS levels were reported in neuroblastoma 2-a cells treated with 50, 100, and 200 μM concentrations of colistin (Dai et al. 2016). More studies have also shown that crocin can act as a strong radical quencher and antioxidant (Ali et al. 2022).

According to the present study, pretreatment with crocin has preserved cell viability against colistin, which indicates the strong antioxidant role of this compound. Studies demonstrated that saffron extract containing crocin protects renal against ischemia reperfusion (IR)-induced oxidative stress in rats (Hosseinzadeh et al. 2005). Crocin has strong antioxidant activity (Bastani et al. 2022) and directly prevents the release of ROS from mitochondria to the cytoplasm by scavenging superoxide radicals or by stabilizing mitochondrial membrane potential (inhibition of mitochondrial permeability) (Ben Salem et al. 2016). In this regard, the study of Ochiai et al showed that crocin can act as a strong antioxidant and prevent cytotoxicity in PC-12 cells (Ochiai et al. 2004).

Elevated levels of ROS can provoke cytochrome c release and induce DNA damage, subsequently activating the tumor suppressor protein p53. The p53 activation leads to downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic Bax proteins. This change in the Bax/Bcl-2 ratio promotes further cytochrome c release, thereby activating caspase-9. Activation of caspase-9 subsequently triggers the activation of caspase-3, culminating in the initiation of the cell death cascade. Therefore, colistin can indeed activate apoptotic pathways and induce cytotoxicity (Jiang et al. 2014). The elevated protein levels of Bax and caspase-3 caused by the exposure of HEK-293 cells to colistin can be due to the increase in ROS formation. In the present study, colistin also increased the level of caspase-3 protein expression. While pretreatment with crocin reversed the increasing effect of colistin on the protein level of caspase-3 and inhibited the induction of apoptosis. Caspases are a group of cysteinyl proteases belonging to the C14 family which are necessary for inducing apoptosis (Kumar 2007). Previously, Lee et al. demonstrated that caspase 3/7 activity increased in a dose-dependent manner when exposed to colistin different concentrations of 25, 50, and 100 μg/ml at 6 hr (Lee et al. 2015).

Detergent-like properties of colistin and other polymyxins enable them to interact with and disrupt the integrity of bacterial cell membranes (Falagas and Kasiakou 2006). High levels of colistin accumulated intracellularly (Yun et al. 2015) may cause disintegration of the bilayer membrane of kidney cells. Damage to the membrane of

proximal tubular cells of the kidney causes the excessive passage of ions through the damaged membrane, changes in osmotic homeostasis, and eventually can trigger a signaling cascade leading to apoptosis (Kunin 1970; Mohamed et al. 2016).

Our current study results, which mostly focused on the mechanism of crocin protection effects against colistin-induced cytotoxicity in HEK-293 cells, are in line with the study of Rajabalizadeh et al. who showed that crocin ameliorates kidney damage caused by colistin through its antioxidant effects (Rajabalizadeh et al. 2023). Several studies have reported the anti-apoptotic effects of crocin (Mohammadzadeh et al. 2022; Wang et al. 2019) and other compounds derived from saffron extract, such as trans sodium crocetinate (Aminifard et al. 2023; Aminifard et al. 2024). Accordingly, preventing excessive ROS production is the possible mechanism of crocin in inhibiting colistin-induced cytotoxicity and apoptosis.

The underlying mechanism of action of colistin leading to cell death is the disruption of bacterial cell membranes. Conversely, crocin, with its antioxidant and anti-apoptotic effects, counters these cytotoxic effects, suggesting a potential protective role against colistin-induced toxicity in renal cells. These results highlight the possible therapeutic value of crocin as a protective agent against the adverse effects of colistin. Considering the increasing use of colistin in treating multidrug-resistant bacterial infections and its associated nephrotoxicity, incorporating crocin-rich sources such as saffron into the diet could offer a nutritional approach to mitigate these side effects.

The study's limitations include the failure to assess crocin impact on the antimicrobial properties of colistin, and the lack of exploration into different molecular mechanisms and pathways involved. To enhance future research, it is recommended to explore the crocin effects on the antimicrobial properties of colistin and to conduct more detailed mechanistic studies.

Additionally, exploring the roles of other pathways involved in colistin-induced cytotoxicity in kidney cells is essential.

Future studies should also examine combination therapies for potential synergistic effects, investigate various renal cell lines, and assess specific molecular pathways, including autophagy. Furthermore, evaluating the implications of chronic exposure is crucial. Addressing these aspects could significantly improve our understanding of crocin protective properties and its potential for clinical applications.

In conclusion, this study highlights the protective effects of crocin against colistin-induced cytotoxicity in HEK-293 cells. Crocin significantly improved cell survival, decreased ROS formation and modulated the expression of key apoptotic proteins. Notably, crocin pretreatment restored the balance between pro- and anti-apoptotic factors, evidenced by reduced Bax and caspase-3 protein levels and increased Bcl-2 protein expression (Figure 4). While these findings are promising, future research should emphasis on elucidating the detailed molecular mechanisms by which crocin exerts its protective effects and exploring its potential therapeutic applications in preventing or treating colistin-induced nephrotoxicity.

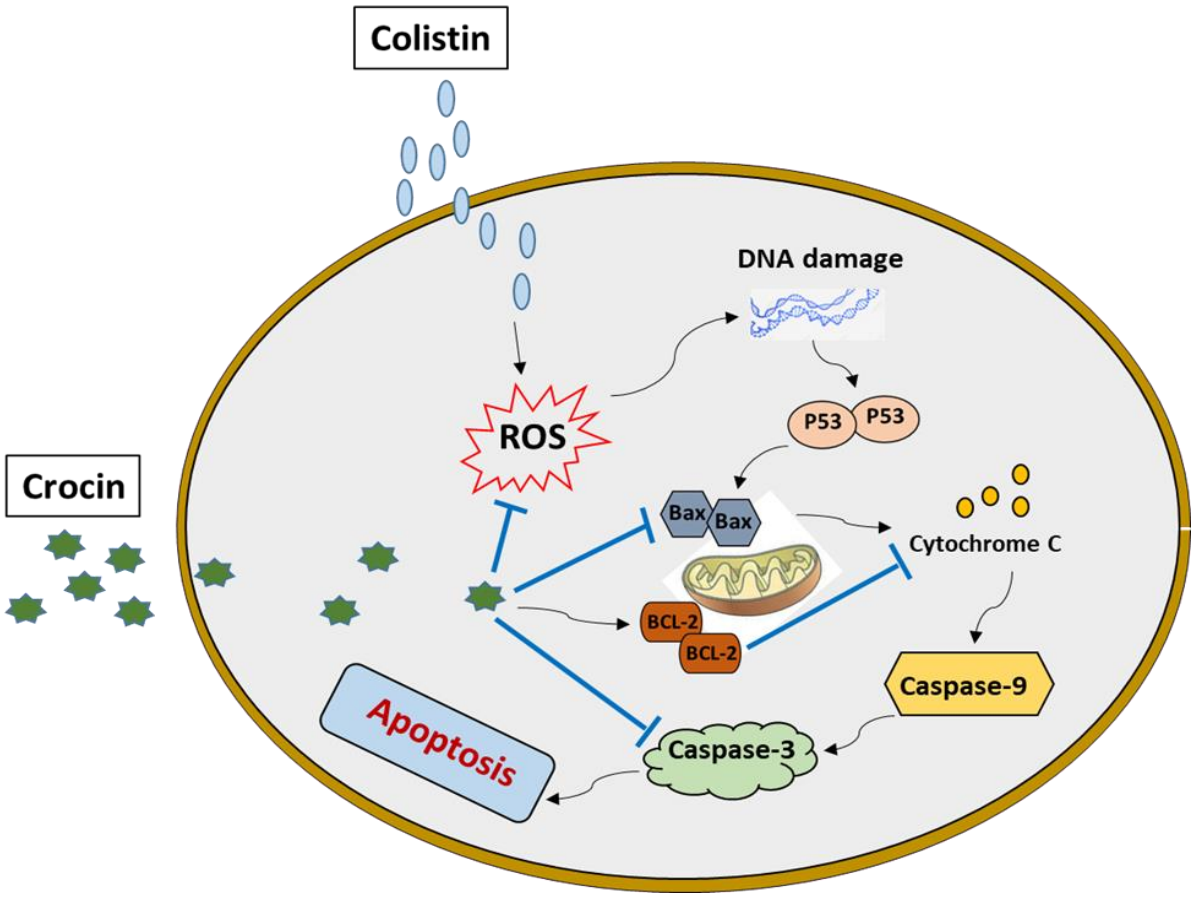

Figure 4. Crocin inhibits colistin-induced cytotoxicity and apoptosis. Exposure to colistin can cause the production of ROS, increase the Bax/Bcl-2 ratio, and activate apoptosis cascade factors such as caspase-3. Crocin prevents the induction of apoptosis caused by colistin by reducing the production of ROS and increasing the Bcl-2 protein which stabilizes the mitochondrial membrane. B-cell leukemia/lymphoma 2 protein (Bcl-2), Bcl-2–associated X protein (Bax), reactive oxygen species (ROS).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

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