

*Research Article***Gossypin attenuates methotrexate-induced nephrotoxicity: Role of COX II/MMP-9 and fas ligand.****Heba M. Hafez^{*}, Azza A.K. El-Sheikh^{**} and Nagwa M. Zenhom^{***}**^{*} Department of Pharmacology, Faculty of Medicine, Minia University, Egypt.^{**} Department of Basic Health Sciences, Faculty of Medicine, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.^{***} Department of Biochemistry, Faculty of Medicine, Minia University, Egypt.**Abstract**

Methotrexate (MTX) is one of commonly used chemotherapeutic drugs, with well-known nephrotoxic effect. This study aimed to investigate the protective mechanisms of gossypin; a bioflavonoid present in plants, against MTX-induced nephrotoxicity. Rats were allocated into 4 groups: control, gossypin, MTX, and gossypin/MTX groups. Evaluation of renal function and histological images were done. Renal tissue oxidative markers of lipid peroxidation, total nitrite/nitrate (NO) level, reduced glutathione (GSH) and superoxide dismutase (SOD) were measured. Inflammatory and apoptotic markers as matrix metalloproteinase-9 (MMP-9) and beta-cell lymphoma-2 (Bcl-2) mRNA and protein expressions, respectively, as well as renal immunohistochemical expression of cyclooxygenase-II (COX-II) and fas ligand, were assessed. Renal P-glycoprotein (P-gp) expression was also measured. MTX caused a significant elevation in kidney functions, with noticeable elevation in oxidative stress markers' levels, as well as COX-II and fas ligand expressions. Moreover, MTX decreased renal antioxidant enzymes, P-gp level, and Bcl-2 expression. Gossypin administration significantly improved renal functions and oxidative insults, in addition to significant improvement of inflammatory and apoptotic parameters. Gossypin administration before MTX also ameliorated renal damage, at least in part, via modulation of P-gp level. In conclusion, gossypin may be an effective nephroprotective adjuvant to MTX through antioxidant, anti-apoptotic, and anti-inflammatory mechanisms.

Keywords: Gossypin; Methotrexate; Nephrotoxicity; P-glycoprotein; Matrix metalloproteinase-9**Introduction**

Methotrexate (MTX) is an anticancer drug, broadly used for the treatment of different types of malignancies and autoimmune disorders (Purcell and Ettinger 2003; Behrens et al., 2015; Malaviya 2016). Unfortunately, its cytotoxic effect was not restricted to cancer cells only, but also exerted a harmful effect on other essential organs, prominently the kidneys (El-Sheikh et al., 2016; Hassanein et al., 2019). Several molecular pathways were explored for MTX-induced toxicities, including the direct toxic effect of MTX metabolic product (7-hydroxy-MTX) on renal tubules with a subsequent reduction in glomerular filtration rate due to arteriolar vasoconstriction (Chan and Cronstein 2013).

Variable oxidative and inflammatory cascades, with subsequent injurious cytokine production,

were also supposed as causes of a toxic effect of MTX on renal tissues (Cetiner et al., 2005; Hafez et al., 2015). Also, pro-inflammatory mediators such as cyclooxygenase (COX)-II and matrix metalloproteinase (MMP)-9 could trigger various pro-apoptotic signals resulted in programmed cell death (Rizk et al., 2018), aggravating MTX-induced nephrotoxicity.

Interestingly, previous reports have shown that various apical efflux drug transporters expressed in the kidney, such as P-glycoprotein (P-gp), have a pivotal role in MTX excretion and detoxification (Leslie et al., 2005; Shibayama et al., 2009) and may participate as well in multidrug resistance against cancer chemotherapy. Therefore, P-gp might be a target for drug interaction and its up-regulation might participate in increasing MTX elimination, thus ameliorating renal toxicity. It is

essential to try to overcome such renal toxicity by using safe and effective agents. For this, great efforts were exerted for the amelioration of MTX-induced renal injury by using naturally originated compounds (Erboga et al., 2015; Abdel-Daim et al., 2017).

Flavonoids, widely existing in most plants and fruits, display several pharmacological benefits. One of these interesting agents is gossypin; a pentahydroxy glucosyl flavone obtained from hibiscus vitifolius flowers (Vijayaraghavan et al., 2008). Gossypin was reported to have many biological properties, including antioxidant, anti-inflammatory, and anticancer actions (Mada et al., 2009; Gautam P and Flora 2010). Furthermore, gossypin was previously suggested to possess antiviral and anti-diabetic properties (Vijayan et al., 2004; Venkatesan and Sorimuthu Pillai 2012). Additionally, gossypin protects various organs such as the liver (Anon et al., 1992) and brain (Gautam P and Flora 2010) against injury caused by free radicals, via its reactive oxygen species (ROS) scavenging effect. Gossypin has also shown ameliorating effects against angiogenesis, carcinogenesis, and inflammation by mechanisms related to nuclear factor- κ B (NF- κ B) and COX-II (Babu et al., 2003).

In the kidney, gossypin was shown to protect against gentamicin-induced nephrotoxicity, probably through antioxidant and/or anti-inflammatory properties (Katary and Salahuddin 2017). Nevertheless, cellular targets explaining the protective mechanisms of gossypin have not yet been completely revealed. For this aim, the current study tried to explore the possible protective effect of gossypin on MTX-induced nephrotoxicity focusing on pathways involved in inflammation and apoptosis, as well as exploring possible effects of gossypin on the renal efflux transporter P-gp.

Materials and Methods

Materials

Gossypin (pentahydroxyflavone glucoside) powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). MTX (50 mg/2 ml ampules) was bought from Ebewe Co. (Unterach, Austria). COX-II and fas ligand anti-rat/rabbit

polyclonal antibodies were procured from Thermo Fisher Inc./Lab Vision (Fremont, CA, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers for beta-cell lymphoma-2 (Bcl-2), MMP-9, and glyceraldehyde 3-phosphate dehydrogenase (GABDH) genes were obtained from (Kapa, Biosystems, USA). Enzyme-linked immunosorbent assay (ELISA) P-gp kit was purchased from Biological Life Sciences (Salem, MA, USA). ELISA kits for MMP-9 and Bcl-2 were purchased from Elabscience Biotechnology Inc (Texas, USA). Other used chemicals and kits were obtained from Sigma and Biochem companies, respectively.

Laboratory animals and study design

Twenty-four Wister rats of 195 ± 15 g weight were obtained from the animal house center, El-Giza, Egypt, and were left to acclimatize for one week before the start of the experiments. A regular diet of rat chow and water ad libitum were permitted to the rats. The experiments were done in accordance with the ethical guidelines of Minia University, Egypt, for the care and use of laboratory animals. Rats were distributed into 4 groups, 6 rats per group. The first group served as control. The second group received gossypin (10 mg/kg/day, p.o) freshly suspended in 0.5% carboxymethyl cellulose daily for 7 days (Katary and Salahuddin 2017). The third group was administered MTX as a single i.p dose of 20 mg/kg at day 4 of the experiment (El-Sheikh et al., 2016). The fourth group received a combination of both gossypin and MTX as indicated in the second and third groups. The control rats received the drugs' vehicles. At the end of the study duration, rats fasted from food for 12 h, and then they were anesthetized with urethane (1.5 g/kg). Blood samples were collected and centrifuged at 5.000 rpm for 15 min for collection of serum. The animals were then decapitated, and the 2 kidneys were quickly detached and washed with cold saline. Segments of left kidneys were sectioned and fixed in neutral buffered 10% formalin solution for further histopathological and immunohistochemical examination. The rest of the kidneys were well kept frozen at -80°C till used.

Kidney function and oxidative stress parameters

Kidney functions were assessed by evaluating serum creatinine and urea levels via colorimetric kits according to kit's manufacturers' instructions. Determination of renal oxidative stress markers was performed in kidney tissue. For this, the kidneys were homogenized via Glas-Col homogenizer in a ratio of 1 to 5 w/v in ice-cold phosphate buffer (0.01M, pH 7.4), followed by centrifugation at 3000g for 15 min at 4°C. The supernatant was divided into different containers to avoid thawing and refreezing, and the samples were kept at -20°C until used. For evaluation of the renal reduced glutathione (GSH) level; Ellman's reagent was reduced to yield 2-nitro-5-mercaptobenzoic acid and a yellow chromogen was observed, whose absorption was colorimetrically measured at 412 nm (Moron et al., 1979). The activity of superoxide dismutase (SOD) was determined based on the principle of pyrogallol autoxidation inhibition by SOD as previously described colorimetrically at 420 nm (Marklund and Marklund 1974). Level of lipid peroxidation was based on malondialdehyde (MDA) reaction with thiobarbituric acid and the absorbance was determined colorimetrically at 535 nm (Tsikas 2017). Renal nitrites (NO) were measured using Griess reagent (Sastry et al., 2002) and the absorbance of each sample was measured at 540 nm.

Renal histopathological examination and immunohistochemical expression of COX-II and fas ligand

Kidney slices fixed in neutral buffered 10% formalin solution were dehydrated in ascending alcoholic degrees, cleared in xylene and embedded in paraffin. Five µm-thick segments were cut using a microtome and stained with hematoxylin and eosin. Under a light microscope, a pathologist performed a blinded examination of the slides. The pathological injuries were graded as previously described (Houghton et al., 1978), where normal renal histology was given score of 0, focal tubular cell degeneration with >1% of the total tubular epithelial cell desquamation was given score of 1, cortical tubules desquamation and necrosis in a range of 1-25% was given score of 2, proximal tubules desquamation and necrosis in a range of 26-75% was given a score of 3, and score of 4 was given when more than

75% of proximal tubular were necrotic. Three different fields were examined for each animal specimen, and the average is taken. This was performed for all animals tested (n=6). Expression of renal tissue COX-II and fas ligand followed the instructions of the kits' manufacturers. Briefly, slides were deparaffinized, and then the antigen was retrieved, followed by a block of endogenous peroxidase activity. After that, renal sections were incubated overnight at 4°C with anti-fas ligand and COX-II primary antibodies (1:500). Afterward, the secondary antibody and diaminobenzidine were applied to yield a brown colored reagent. Semi-quantitative immunostaining assessment was done and positively stained cells for both antibodies were counted. The mean values of positive cells were calculated (El-Sheikh et al., 2014; Hafez et al., 2015).

Detection of renal mRNA expression of MMP-9 and Bcl-2 genes by qRT-PCR

Total RNA was extracted from renal tissue homogenate using RiboZol reagent (Amresco, Solon, USA) following the manufacturer's instructions. qRT-PCR was performed with 50 ng RNA template per reaction using Thermo Scientific one-step kits in 25 µl reaction volume containing 70 nM of specific primers in qRT-PCR detection system (Kapa, Biosystems, USA). The SYBR green data were analyzed with a relative quantification to 18s RNA as the reference gene. The sets of primers used in renal tissue for MMP-9 is; Sense:

5'-TCGAAGGCGACCTCAAGTG-3';

Antisense:

5'-TTCGGTGTAGCTTTGGATCCA-3' and GAPDH; sense:

5'- GTCGGTGTGAACGGATTTG -3'

antisense:

5'- CTTGCCGTGGGTAGAGTCAT -3'

and for Bcl-2 are; sense:

5'-GTATGATAACCGGAGATCG-3';

antisense:

5'-AGCCAGGAGAAATCAAACAG-3'.

Then the samples were placed in a thermal cycler (Applied Biosyst 7500 fast, Techne (Cambridge) LTD, UK). The relative gene expression was calculated using the formula 2^(-ΔΔCt) as previously described (VanGuilder et

al., 2008). They were scaled relative to controls where control samples were set at a value of one.

Assessment of P-gp, MMP-9, and Bcl-2 protein expressions in renal tissue by ELISA

A sandwich enzyme immunoassay for quantitative measurement of P-gp, MMP-9 and Bcl-2 protein levels in rat tissue by ELISA technique was used according to the instructions of the manufacturer.

The color changed spectrophotometrically at a wavelength of 450 ± 10 nm. Concentrations of P-gp, MMP-9, and Bcl-2 in the renal samples were then determined by comparing the optic density of the sample to the standard curve.

Statistical analysis

Values were presented as means \pm SEM. The results were statistically analyzed using one-way analysis of variance (ANOVA) test followed by Tukey's post-test, employing Graph Pad Prism 5.01 software (San Diego, CA, USA). Differences were considered significant if the p-value was < 0.05 .

Table (1): Effect of gossypin on kidney function tests in methotrexate (MTX)-induced nephrotoxicity in rats.

	Creatinine (mg/dl)	Urea (mg/dl)
Control	1.50 ± 0.02	44 ± 3
Gossypin	1.55 ± 0.02	49 ± 2
MTX	2.48 ± 0.05^a	146 ± 7^a
Gossypin/MTX	$1.94 \pm 0.06^{a,b}$	$75 \pm 3^{a,b}$

Data were expressed as means \pm S.E.M (n = 6). Significantly different results are reported when $p < 0.05$. ^a Significant compared to control, ^b significant compared to MTX group.

Effect of gossypin on renal oxidative stress markers in MTX-treated rats

To investigate the effect of gossypin on markers of oxidative stress, lipid peroxidation product; MDA, total nitrite; NO, the antioxidant enzyme; SOD and the powerful antioxidant; GSH were assessed (Table 2). Group treated with MTX, showed renal oxidative stress, manifested by a significant increase of both MDA and NO

Results

Effect of gossypin on kidney function and histopathological picture in MTX-treated rats

To determine kidney function, serum creatinine and urea were evaluated (Table 1). Serum levels of urea and creatinine were not significantly different from control in the group treated with gossypin 10 mg/kg/day for 7 days. A single i.p dose of 20 mg/kg of MTX, on the other hand, significantly elevated both creatinine and urea compared to control. Gossypin/MTX group showed significantly decreased creatinine and urea, indicate the reverse of nephrotoxicity. The functional findings were confirmed by the histopathology, where both control and gossypin groups showed normal kidney architecture (Fig 1A and 1B, respectively). MTX treatment, however, caused necrosis of renal glomeruli, with vacuolation of tubules (Fig 1C). Pre-treatment with gossypin caused improvement in renal histology (Fig 1D). The histopathological results were semi-quantitatively scored, and the significance of the reported results was confirmed (Fig 1E).

levels, with a significant decrease of SOD enzymatic activity and GSH levels compared to control groups. Pre-treatment with gossypin before MTX challenge reversed MTX-induced renal oxidative stress, as manifested by a significant decrease of MDA and NO levels, with a significant increase of SOD enzymatic activity and GSH levels compared to the group treated with MTX alone.

Table (2): Effect of gossypin on renal tissue oxidative stress markers in methotrexate (MTX)-induced nephrotoxicity in rats.

	MDA (nmol/g tissue)	NO (nmol/g tissue)	SOD (U/g tissue)	GSH (mg/g tissue)
Control	76.37 ± 1.8	114.3 ± 4.6	2376 ± 18.3	850.6 ± 1.3
Gossypin	76.14 ± 2.5	120.2 ± 6.3	1505 ± 16.4	864.9 ± 15.3
MTX	154.6 ± 5.5 ^a	480.8 ± 2.8 ^a	1226 ± 16.6 ^a	685.8 ± 26.7 ^a
Gossypin/MTX	99.88 ± 7.4 ^{a,b}	240.7 ± 11.7 ^{a,b}	1719 ± 0.6 ^{a,b}	795.1 ± 31.9 ^b

Data were expressed as means ± S.E.M (n = 6). Significantly different results are reported when p < 0.05. ^a Significant compared to control, ^b significant compared to the MTX group.

Effect of gossypin on renal COX-II and fas ligand expression in MTX-treated rats

The effect of gossypin on inflammation and apoptosis was assessed by determining the expression level of the pro-inflammatory enzyme COX-II and the apoptotic marker fas ligand using immunohistochemical staining method (Fig 2) and the results were semi-quantitatively scored (Fig 3). Treatment with gossypin alone did not affect the level of expression of either COX-II or fas ligand (Fig 2B and 2F, respectively) compared to the respective controls (Fig 2A and 2E, respectively). Treatment with MTX alone caused significant up-regulation of the expression levels of both COX-II and fas ligand (Fig 2C and 2G, respectively), whereas treatment with both gossypin and MTX showed significantly less expression compared to MTX alone (Fig 2D and 2H, respectively).

Effect of gossypin on renal MMP-9 and Bcl-2 mRNA expression in MTX-treated rats

To confirm the process of inflammation and apoptosis, using qRT-PCR, renal mRNA expression of the inflammatory marker; MMP-9, and the anti-apoptotic marker; Bcl-2 were assessed. The results showed that treatment with gossypin alone didn't affect either

parameter (Fig 4). Treatment with MTX, on the other hand, produced a significant elevation in the renal mRNA level of MMP-9 with a significant decrease in the Bcl-2 mRNA levels in comparison with the respective controls. Gossypin administration before MTX resulted in a significant decrease in MMP-9 and an increase in Bcl-2 as compared with the group treated with MTX alone.

Effect of gossypin on renal protein levels of P-gp, MMP-9, and Bcl-2 proteins in MTX-treated rats

Since the renal efflux transporter P-gp contributes to MTX renal elimination, testing the effect of gossypin on the P-gp level was performed using the ELISA technique. We also used the same technique to confirm the effect on MMP-9 and Bcl-2. Gossypin alone had no significant effect on renal expression of P-gp, MMP-9 and Bcl-2 proteins compared to the control (Fig 5). The group treated with MTX alone significantly decreased renal P-gp and Bcl-2 and increased MMP-9 protein levels compared to the control group. Pre-treatment with gossypin before MTX succeeded in significantly increasing renal P-gp and decreasing MMP-9 protein levels compared to MTX alone. However, the renal Bcl-2 protein level showed no significant improvement.

Fig 1

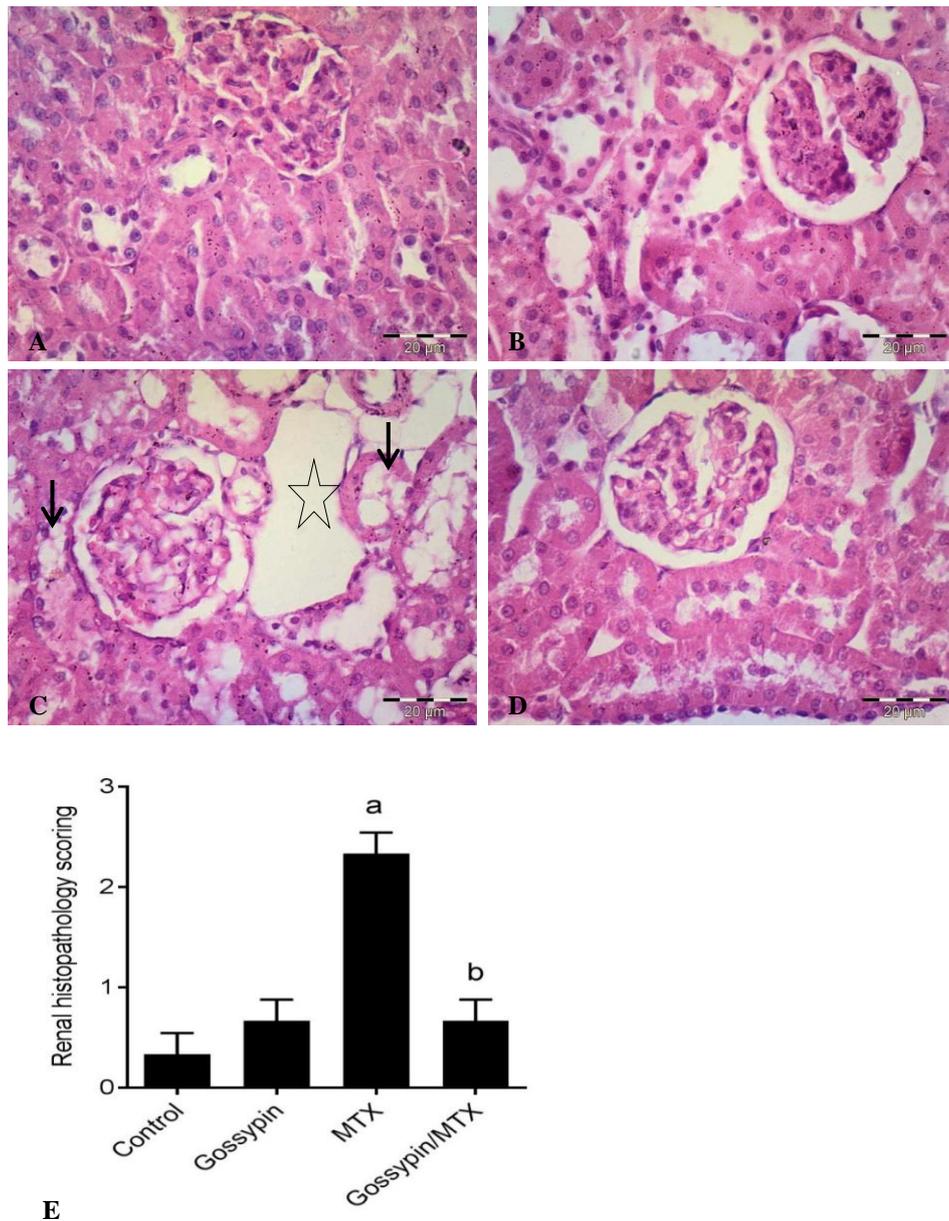


Fig 1: Renal histopathological changes in methotrexate (MTX)-treated rats. Photomicrographs of rat kidney (200×) of (A) control, (B) gossypin-treated, (C) MTX-treated and (D) gossypin/MTX-treated groups. Star: degenerative necrotic area with absent glomerulus; black arrows: vacuolation inside renal tubules. Histopathological scoring of different groups is summarized in figure 1E. Sample tissue from each animal was tested (n=6). Significantly different results are reported when $p < 0.05$. ^a Significant compared to control, ^b significant compared to the MTX group.

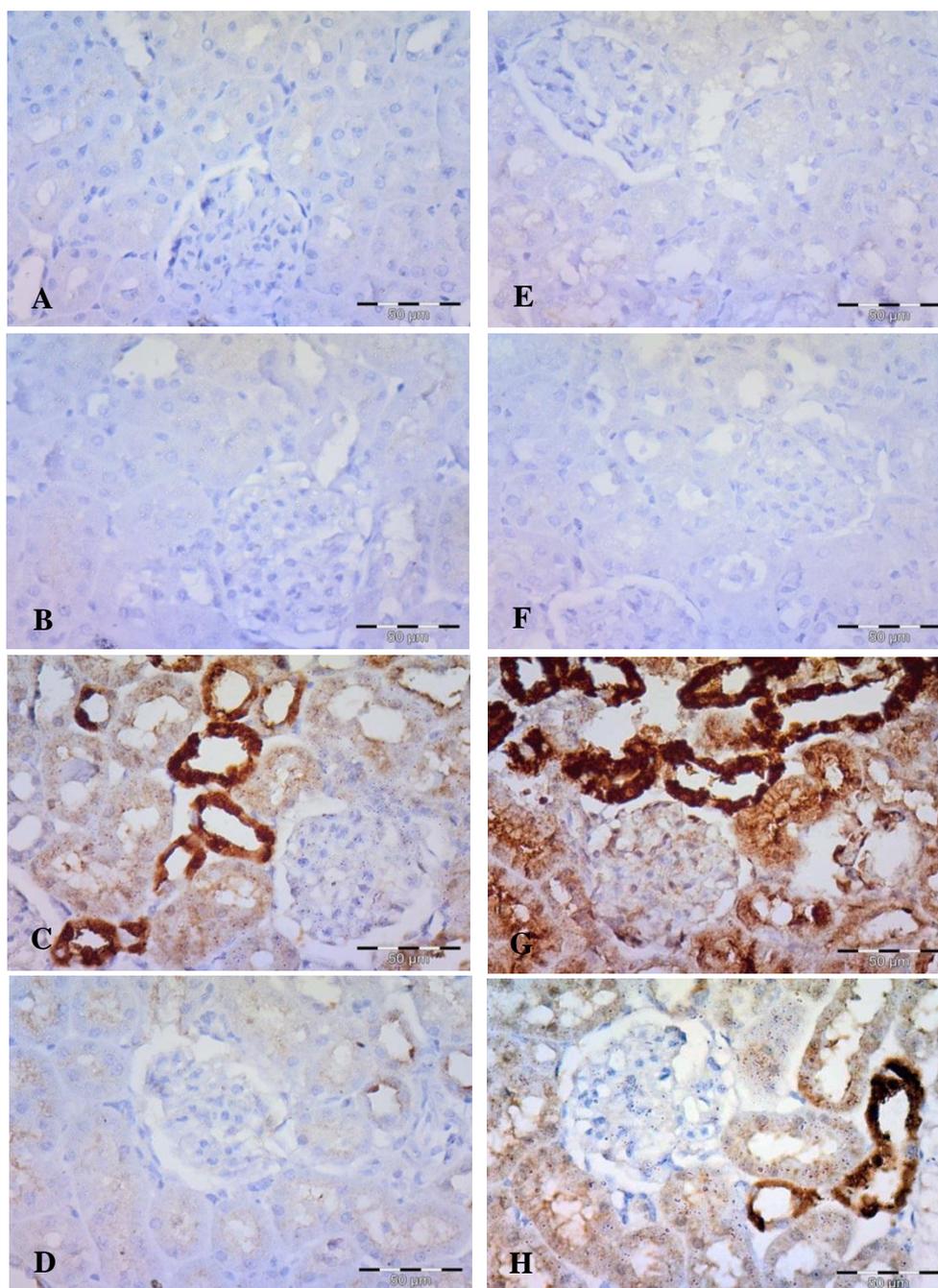
Fig 2

Fig 2: Renal expression of COX-II and fas ligand in methotrexate (MTX)-treated rats. Immunohistochemical localization ($\times 200$) of COX-II (left panel) and fas ligand (right panel) of (A and E) control, (B and F) gossypin-treated, (C and G) MTX-treated and (D and H) gossypin/MTX-treated groups, respectively

Fig 3

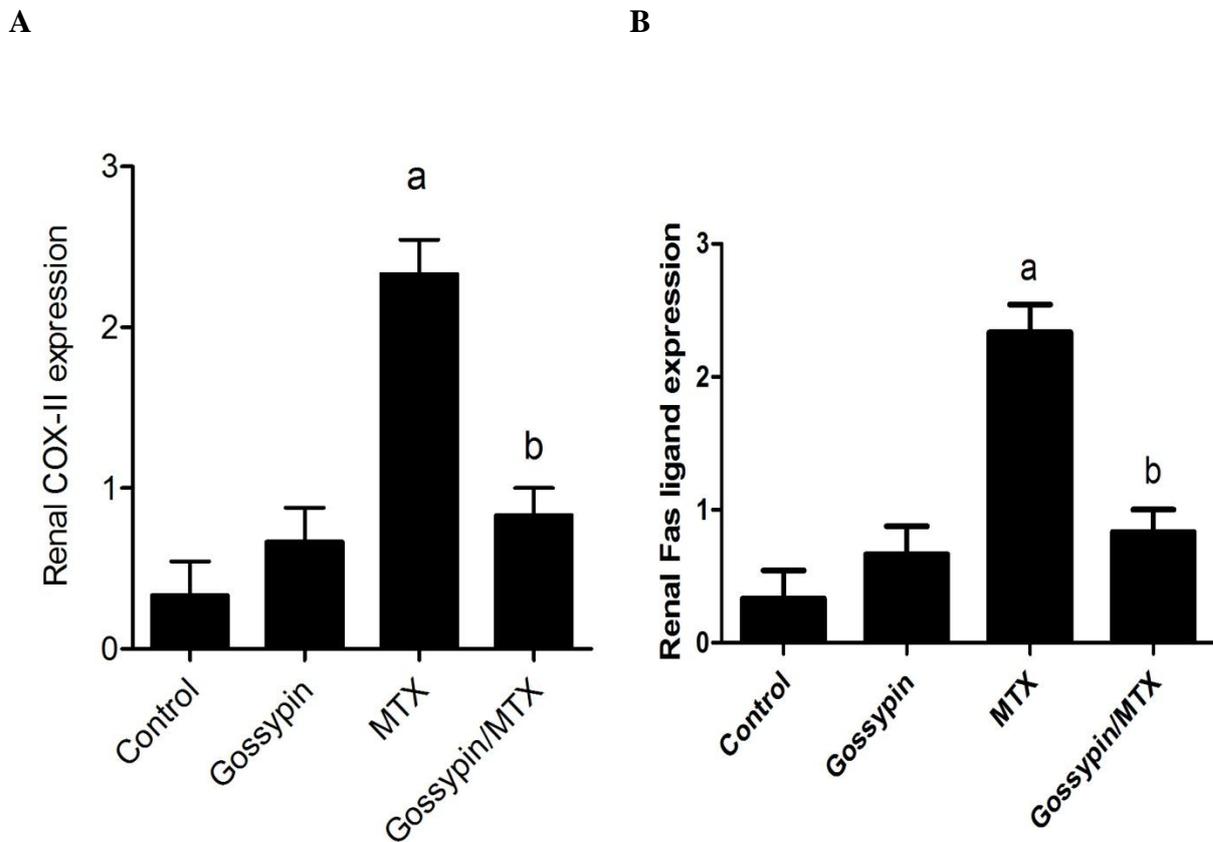


Fig 3: Semi-quantitative analysis of renal expression of COX-II and fas ligand in methotrexate (MTX)-treated rats. Analysis of the results for (A) COX-II and (B) fas ligand were evaluated semi-quantitatively, where values represent means \pm SEM. Significantly different results are reported when $p < 0.05$. ^a Significant compared to control, ^b significant compared to the MTX group.

Fig. 4

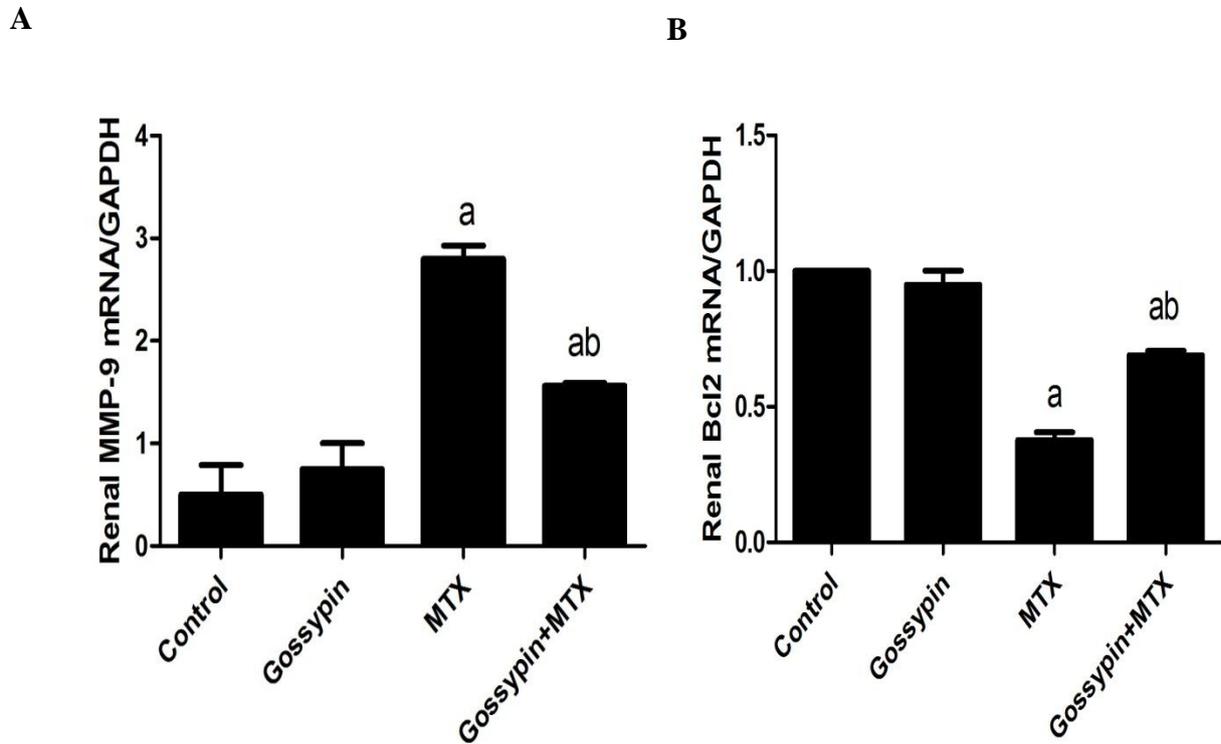


Fig 4: RT-PCR of renal mRNA expression of MMP-9 and Bcl-2 in methotrexate (MTX)-treated rats. Relative gene expressions of renal mRNA of (A) MMP-9 and (B) Bcl-2. Data were expressed as means \pm SEM (n = 6). MMP-9, matrix metalloproteinase-9; Bcl-2, B cell lymphoma-2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. Significantly different results are reported when $p < 0.05$. ^a Significant compared to control, ^b significant compared to the MTX group.

Fig. 5

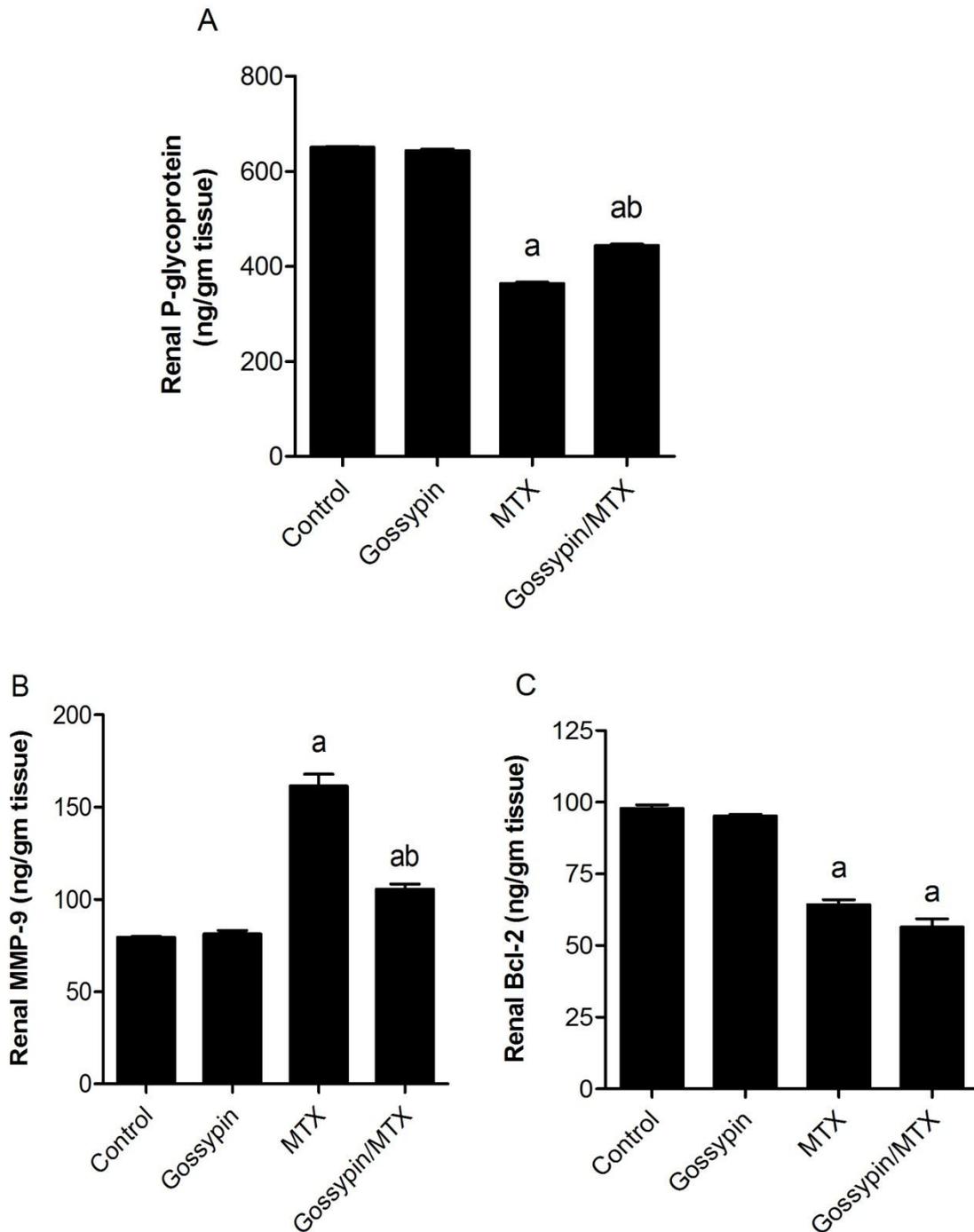


Fig 5: Renal P-gp, MMP-9, and Bcl-2 protein levels in methotrexate (MTX)-treated rats. By using the ELISA technique, data were expressed as means \pm SEM (n = 6). Significantly different results are reported when $p < 0.05$. ^a Significant compared to control, ^b significant compared to the MTX group.

Discussion

Although being one of the broadly applied anticancer drugs and successful therapy of a wide array of autoimmune diseases, MTX's clinical use has been challenged by its dangerous effect on vital organs like the liver (Fayez et al., 2018), kidneys (Arab et al., 2018), and lung (Arpag et al., 2018). The renal system is considered one of the susceptible targets for such toxicities and one of the common causes of therapy discontinuation (Kitai et al., 2015). Until now, there is no clearly defined safe and effective adjuvant treatment for MTX to ameliorate its nephrotoxicity; therefore, the current study was designed to examine the role of gossypin on MTX-induced renal damage.

In the present study, MTX resulted in a significant deterioration of renal functions measured by serum urea and creatinine levels that was confirmed by the histopathological picture in the form of renal tubular degeneration and necrosis. This was in line with previous reports (Heidari et al., 2018). MTX-induced renal toxicity was related to the generation of ROS, which deteriorates the renal system by oxidative stress assaults due to in balance between pro-oxidants and antioxidants (Rjiba-Touati et al., 2018). Subsequently, the elevation of lipid peroxidation products MDA and NO, alongside with a reduction in the defense mechanisms provoked by SOD and GSH were discussed in various studies, which supported our presented data (Gad et al., 2017; Hassanein et al., 2019).

In the current study, the administration of gossypin before MTX succeeded to ameliorate nephrotoxicity with a reduction in serum markers, in addition to an improvement in light microscopic pictures of renal tissues. Gossypin exerted its nephroprotective effect via free radical scavenging and antioxidant properties, together with its impending action on nitrosative stress by decreasing renal NO and MDA levels. This was associated with a marked increase in renal SOD and GSH levels as a defense against such toxicity. In accordance with previous reports, gossypin was reported to decrease oxidative stress markers and exerted a protective action in different models of the liver (Gautam A and Vijayaraghavan 2007), kidney

(Katary and Salahuddin 2017), and brain (Gautam P and Flora 2010) injuries.

Since there is a substantial correlation between oxidative stress and inflammatory pathways, several pro-inflammatory mediators could be provoked in response to MTX-induced oxidative injury (Rizk et al., 2018; Wang et al., 2018). MTX-induced renal damage in the present study was evidenced by modulation of expression of different pro-inflammatory markers such as COX-II and MMP-9. COX-II is an inflammatory enzyme that is up-regulated in renal tissues in stressful conditions induced by various chemotherapeutic drugs, including MTX (Natarajan et al., 2018). COX-II exerts its pro-inflammatory effect on body systems by yielding prostaglandins and thromboxane A₂ from arachidonic acid metabolism. Elevated expression of COX-II is considered one of the crucial signals of inflammation (Ali et al., 2017; Li et al., 2018).

The MMPs, an important family of inflammatory enzymes, are sets of structurally associated proteins that are tangled in the extracellular matrix, inducing remodeling of several physiological and pathological circumstances (Cavdar et al., 2017). MMP-9 is one of the MMPs family locally secreted in response to renal pathological settings and may be involved in the initiation and severity of inflammatory kidney disorders (Chen et al., 2017). Furthermore, it was proved that elevated levels of MMP-9 were linked to tubular epithelial/mesenchyme alteration in vitro (Strutz et al., 2002). In the present study, MTX significantly increased MMP-9 mRNA expression in renal tissues confirming its role in renal inflammation, which was supported by previous studies (de Araujo et al., 2014). Data in the present study revealed that gossypin could mitigate MTX-induced nephrotoxicity by decreasing the pro-inflammatory COX-II and MMP-9 enzymes. Gossypin was reported in previous in vitro studies to have COX-II inhibitory action owing to the three-dimensional structure that would possibly facilitate proper binding with COX-II active site, which possibly participated in the anti-inflammatory and anti-nociceptive activities of gossypin (Mada et al., 2009). Accordingly, with such a

high grade of in vitro selectivity, an expected in vivo selectivity could be attained. Confirming the anti-inflammatory effect in the present study, gossypin has been able to decrease the level of MMP-9. This was in accordance with a study demonstrating a gossypin binding affinity to MMP-9 active site, with an inhibitory effect that resulted in improving cartilage destruction and inflammation (Pradiba et al., 2018).

The current study also demonstrated that MTX enhanced renal injury by targeting apoptotic/anti-apoptotic markers as exhibited by the up-regulation of fas ligand and down-regulation of Bcl-2 mRNA, two important consecutive steps in the apoptotic pathway. The pro-inflammatory mediators and oxidative states generated by MTX were reported to trigger specific pro-apoptotic signals, which were fas-dependent, with consequent caspase cascades and cell death (El-Gowilly et al., 2015). MTX down-regulation of the anti-apoptotic marker Bcl-2 was in line with previous findings (Mahmoud et al., 2018). Interestingly, despite that pretreatment with gossypin caused up-regulation of Bcl-2 mRNA, it did not affect the Bcl-2 protein level. This might be due to the acute nature of the study, where Bcl-2 mRNA was up-regulated, but the time was not enough for protein synthesis. Pretreatment with gossypin exerted a protective effect partially through anti-apoptotic mechanisms that increased the expression of Bcl-2 mRNA and decreased the apoptotic fas ligand. Conversely, gossypin exerted a potential programmed cell death in different cancer models targeting Bcl-2/Bax and fas (Kunnumakkara et al., 2007). Thus, gossypin could be expected to be a worthy adjuvant in cancer chemotherapy, with proper cytotoxicity on malignant cells, while defending organ toxicities. It is worth noting that gossypin, as other flavonoid glycosides, has high polarity and is quite sizable, thus, it does not readily cross membranes (Fernandez et al., 2009).

Interestingly, in the present study, MTX resulted in decreasing the expression of renal transporter, P-gp that was reversed by gossypin treatment. Previous studies have demonstrated that the kidney via several drug transporters eliminates MTX (Van Aubel et al., 2000; El-

Sheikh et al., 2016). P-gp is one of multi-drug resistance efflux pumps belonging to the ATP binding cassette family that is responsible for MTX resistance during cancer treatment (de Graaf et al., 1996; Jia et al., 2016). Previous reports explored a reduction in P-gp expression with renal impairment (Naud et al., 2007).

Additionally, MTX administration was also reduced the P-gp expression level in renal tissues and ileum (Shibayama et al., 2009) and this supported the current finding. Therefore, MTX may affect other adjuvant drugs by changing the level of P-gp.

To the best of our knowledge, the current study is the first to report that gossypin reversed the down-regulation resulted from MTX on renal P-gp in vivo and this may confer a further unique nephroprotective pathway against MTX-induced renal damage. Despite these promising results, further researches are needed to explore gossypin/MTX pharmacokinetic interactions at different levels.

Conclusion

Taken together, Gossypin exerted a nephroprotective effect on MTX by different mechanisms, including antioxidant, anti-apoptotic, and anti-inflammatory signaling pathways. In addition, gossypin mitigated MTX toxicity by restoring P-gp efflux transporter in renal tissues that could present gossypin as a novel, safe, and effective adjuvant with MTX chemotherapy.

Acknowledgements

The authors thank Dr. Hanaa Hassanein for her great help in the histopathology and immunopharmacology images.

Declaration of interest

The authors declare no conflict of interest.

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