Research Article

Protective activity of phalaris canariensis against methotrexate-induced nephrotoxicity via BCL2 pathway.

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Abstract

This study aimed to evaluate the protective effects of the ethanolic extract obtained from the seeds of phalaris canariensis, against nephro-toxicity induced by methotrexate (MTX). Toxicity was induced by a single dose of MTX (20 mg/kg) and ethanolic extract administered in three different doses (100, 200 and 400 mg/kg) daily for 15 days. Urea and creatinine were measured in serum. In addition, malondialdehyde (MDA) and total nitrites, were measured in kidney tissues. At the same time, expression of kidney injury molecule-1 (KIM-1) and expression of BCL2 in kidney were measured. The ethanolic extract showed dose-dependent reduction in urea, creatinine, renal MDA, renal total nitrites and expression of KIM-1, which were increased with MTX administration, however, the extract dose-dependently, increased BCL2 expression that was reduced with MTX administration. in conclusion, this study suggests that the seed extracts from *P.* canariensis exhibit protective potentials against MTX-induced nephro-toxicity and can be considered a potential therapeutic agent in prevention of MTX-induced toxicity.

Key words: Methotrexate, Phalaris canariensis, BCL2, nephrotoxicity.

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1. Introduction

Plant derived active principles represent an opportunity for treatment of different types of diseases. According to the WHO, medicinal herbal drugs can treat 80% of the world population's health problems[1,2].

Free radicals as well as oxidative stress-activated signaling, play an important role in the pathogenesis of human disease conditions such as aging, cancer, immune dysfunction as well as renal impairment. Therefore, the antioxidants are expected to improve the therapeutic process of these diseases' conditions, at least in terms of their free radical scavenging effects and by the process of induction of anti-oxidative pathways.

Natural anti-oxidants represent a safer and sometimes more effective alternatives to synthetic ones. Various plant active ingredients, especially flavonoids and other phenolic compounds scored important achievement in the oxidative stress research field[3,4].

It was suggested the use of *Phalaris canariensis* L. as human cereal grain which also reported to show a hepatoprotective effect[5]. The canary seed or alpiste, *P. canariensis*. is a member of a family of grasses. In certain countries, the seeds are used as a food for human and birds. The seeds have been used traditionally in folk medicine in the form of tea as a co-adjuvant in the treatment of hypertension, diabetes mellitus, renal diseases and hypercholesterolemia[6,7,8,9].

Methotrexate (MTX), a folic acid antagonist, is used in the treatment of cancer and as immunosuppressive agent in diverse inflammatory diseases. It is on the WHO's list of essential medicines[10]. Unfortunately, its toxicity and severe adverse effects including nephrotoxicity restrict efficacy of MTX[11]. Free radical production and oxidative stress

induction play a critical role in MTX toxicity[12].

The hypothesis of the study was that the ethanolic extract of *P. canariensis* seeds will protect against nephrotoxicity induced by MTX in rats.

2. Material and Methods

2.1. Plant material

Mature seeds of *P. canariensis* are collected in December 2017 from plants grown in the Food Technology Research Institute, Faculty of Agriculture, Cairo University, Giza. Seeds were identified by prof. Dr. Osama El Kopasy, professor of Botany, Faculty of Agriculture, Cairo University. Voucher specimen (PC-PH-17) was kept at the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Deraya University, Minia, Egypt.

2.1.1. General

Silica gel H (Merk, Darmstadt, Germany) was used for vacuum liquid chromatography (VLC), silica gel 60 (70-230 mesh ASTM, Fluka, Stein-helm, Germany) and Sphadex LH-20 (Pharmacia, Stockholm, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} pre-coated plates (Fluka) using the following solvent system: S_1, Chloroform-Methanol (9:1v/v); S_2, Petroleum ether - Ethyl acetate - Formic acid (45:16:3.6 v/v/v); S_3, Chloroform - Acetone - Formic acid (65:15:15 v/v/v); S_4, Chloroform - Acetone - Formic acid (75:16.5:8.5 v/v/v); S_5, Ethyl acetate - Methanol - Water (100:16.5:13.5 v/v/v); S_7, Chloroform-Methanol (8:2v/v).

The chromatograms were visualized under UV light (at 254 and 366 nm), exposed to ammonia vapor, sprayed with $AlCl_3$ and sprayed with anisaldehyde/sulphuric acid reagent. Melting points (uncorrected) were determined on a D. Electrothermal 9100 instrument (Labe-quip, Markham, Ontario, Canada). UV spectra were recorded using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). A Varian Mercury -VX-300 NMR instrument (Palo Alto, CA, USA) was used for ^1H NMR (300 MHz) and ^{13}C NMR (75 MHz). The NMR spectra were recorded in DMSO-d_6 and chemical shifts are given in δ (ppm) relative to TMS as an internal standard.

2.1.2. Extraction, fractionation, and isolation.

The air-dried powdered seeds of *P. canariensis* (1Kg), was percolated with ethanol 70% until exhaustion to yield 115g of the alcoholic extract. The residue was suspended in distilled water and partitioned successively using n-hexane, chloroform, ethyl acetate, and n-butanol saturated with water to yield the following sub-fractions: Hx Fr., Cl Fr., Et Fr., and Bu Fr. Fractions were separately concentrated under reduced pressure to yield 58.5, 7.25, 6.4 and 4.8g, respectively. Cl Fr. (5g) was chromatographed over a VLC column (5x20 cm, silica gel H, 250). Gradient elution was carried out using n-hexane/methylene chloride mixtures, methylene chloride - ethyl acetate mixtures, and ethyl acetate-methanol mixtures.

Fractions of 200 ml each were collected and monitored by TLC to yield nineteen fractions (Ac-Sc). Fraction Gc (60% methylene chloride in ethyl acetate) was re-chromatographed on a silica gel 60 column, using n-hexane - ethyl acetate (8.5:1.5 v/v) as an eluent to give compound F_1 (20mg, 0.002%w/w seeds, 0.017%, yellow powder, R_f=0.72 in S_2). Fraction Hc (70% methylene chloride in ethyl acetate) was re-chromatographed on a silica gel 60 column, using n-hexane-ethyl acetate (9:1. v/v) as an eluent to give compound F_2 (15mg, 0.0015%w/w seeds, 0.013%, yellow powder, R_f=0.70 in S_2). Fraction Kc (70% ethyl acetate in methanol) was purified using, several sephadex LH-20 column to yield compound F_3 (18mg, 0.0018%w/w seeds, 0.016%, yellow powder, R_f=0.59 in S_2).

Et.Fr. (5g) was chromatographed over polyamide column (250g, 5x120 cm). Gradient elution was carried out with water, followed increasing amount of methanol starting with 5% up to 90% methanol. Fractions of 250ml each were collected and monitored by TLC to yield seven main fractions (A_{et}-G_{et}).

Fraction C_{et} (40% methanol in water) was re-chromatographed over cellulose column (50g, 3.5x120 cm) by using 15% methanol in water as an eluent to give compound F_4 (15mg, 0.0015%w/w seeds, 0.013%, yellowish brown powder, R_f=0.18 in S_4). Fraction E_{et} (60% methanol in water) was purified several times over cellulose columns (50g, 3.5x120 cm) by using 15% methanol in water as an eluent to

give compound F_5 (22mg, 0.0022%w/w seeds, 0.019%, yellowish powder, R_f=0.44 in S_4). Fraction F_{et} (80% methanol in water) was purified by using several sephadex LH-20 columns to yield compound F_6 (17mg, 0.0017%w/w seeds, 0.015%, yellowish white powder, R_f=0.68 in S_4) and F_7 (15mg, 0.0015%w/w seeds, 0.013%, yellowish white powder, R_f=0.73 in S_4).

2.2. Drugs and chemicals

Methotrexate was purchased from SM pharma (Egypt, Cairo). All other chemicals were of analytical grade and obtained from commercial sources.

2.3. Animals

The present study was conducted on adult male wistar ~~albino~~ rats weighing 205-280 g. Rats were obtained from the animal house, El-Giza, Egypt. Rats were fed a standard diet of commercial rat chow and tap water and left to acclimatize to the environment for one week prior to inclusion in the experiments. All experiments were conducted according to the ethical standards approved by the Faculty Board Committee of the Faculty of Medicine, Minia University, Egypt.

2.4. Experimental design

2.4.1. Induction of nephrotoxicity

Nephrotoxicity induced by single intraperitoneal MTX at dose of 20 mg/kg. The dose of MTX as well as the duration of the study was selected on the light of our pilot experiment and with previous studies [13,14,15].

2.4.2. Grouping

The animals were randomly divided into 5 experimental groups of 8-10 animals each. Duration of the study was 15 days. Group 1; received vehicle, group 2; rats were administered MTX on 1th day, group 3; rats administered ethanolic extract daily (100 mg/kg, p.o.) with MTX on 11th day, group 4; rats were administered ethanolic extract daily (200 mg/kg, p.o.) with MTX on 11th day and group 5; rats were administered ethanolic extract daily (400 mg/kg, p.o.) with MTX on 11th day. These doses of the extract that used in the experiment were selected according to a pilot study in which we used the following doses 10, 20, 40, 80, 100 and 200 mg/kg.

2.5. Sample collection and storage

Five days starting from the day of MTX injection, all animals were sacrificed. For biochemical measurements, blood samples were collected from all the rats. Left at room temperature for 15 minutes, serum was collected after centrifugation at 4000 g for 10 minutes was done. Tissue samples were prepared by homogenization in phosphate-buffered saline at a pH of 7.4 which followed by centrifugation at 4000 g for 20 minutes. The supernatant was stored at -80°C until further analysis. For histopathological studies, parts of kidney tissues fixed in 10% formalin for light microscopy.

2.6. Biochemical analysis

2.6.1. Evaluation of kidney functions

Creatinine and urea levels determined by commercial colorimetric kits (Spectrum Diagnostic, Cairo, Egypt).

2.6.2. Renal oxidative stress parameters

Measurement of malondialdehyde (MDA) was evaluated by a method that depends on the reaction between MDA with thiobarbituric acid. At the end of the reaction, the color developed was measured spectrophotometrically at 535 nm against a blank. Using 1,1,3,3-tetramethoxypropane, a standard curve was established and the MDA concentration was determined and expressed as nmol/g tissue [16].

2.6.3. Determination of renal total nitrites

Reduction of nitrate to nitrite by copperized cadmium granules was done and quantitation of nitrites was based on the Griess reaction and the absorbance of developed color was measured at 545 nm against a blank. Standard curve was done by using NaNO3 (0–100 nmol/ml)[17].

2.7. rt-PCR analysis of renal tissues

Total RNA was isolated from frozen kidney tissues according to RNA isolation kit (RiboZol™ RNA Extraction Reagents) AMRESCO, LLC Solon, Ohio USA. The concentration and purity of the purified RNA were determined by spectrophotometry (Nanodrop1000, Thermo Scientific) apparatus. High quality RNAs (A260/280 $\geq$ 1.6), were selected and were used for Quantitative real-time PCR synthesis. In the present study, Kim-1, BCL-2 and β-actin genes were selected as targets and internal reference gene, respectively.

The sequences of interest genes were obtained from NCBI database and primer sets were designed via Thermo Fisher Scientefic.

The sequences of the primer used in this study have been summarized in Table 1. Quantitative real-time PCR was performed using the One-Step TaqProbe qRT-PCR Kit abm. Reaction volume was 50 µL and components were used including 1 µg of total RNA as template (10 µL), TaqProbe qRT-PCR MasterMix1X (25µL), qRT-PCR Enzyme Mix (50X) (1 µL), Forward and reverse Primer (10 µM) (1.5 µL), TaqMan Probe (5 µL), respectively. Then, each sample was completed with 6 µL nuclease free water. Thermal cycling was performed on the Bioer (Fully automated Real Time PCR). By using the following cycling conditions: 50°C for 1 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min.

Table (1): The primer sequences for interest and reference genes.

Gene title	Accession no.	Forward primer 5`-3`	Reverse primer 5`-3`
Kim-1	AF-035963	5′- AAC GCA GCG ATT GTG CAT CC-3′	5′- GTA CAC TCA CCA TGG TAA CC-3′
Bcl-2	S-74122	5′- GCG AAG TGC TAT TGG TAC CTG-3	5′- ATA TTT GTT TGG GGC AGG TCT -3′
B-actin	AF-541940	5′- CCC GCG AGT ACA ACC TTC T-3′	5′- CGT CAT CCA TGG CGA ACT -3′

2.8. Histopathological examination

The specimens of the kidney were fixed in a 10% formalin solution. Then they were embedded in paraffin. Cross-sections 5µm thick, were cut using a microtome. The sections were subjected to hematoxylin-eosin staining and observed under an optical microscope and histopathologically evaluated (10x magnification).

The results were expressed as the sum of individual score grades (0: no findings, 1: mild, 2: moderate, or 3: severe) for each of the following parameters: degeneration, cellular swelling, necrosis, congestion and hemorrhage[18].

2.9. Statistical analysis

Results were expressed as mean ± standard error (SE). Differences among groups were statistically analyzed by one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test for multiple comparisons. Significance was considered if p value < 0.05. All data analyses were performed using GraphPad Prism7 program.

3. Results

3.1. Phytochemical investigation

Phytochemical study of ethanolic extract of *P. canariensis* led to isolation of seven known compounds, including five flavonoids; 5,7-dihydroxy-3',4'',5'-trimethoxy flavone (**F_1**), 5,7-dihydroxy-4'',5'-dimethoxy flavone (Tricin) (**F_2**), quercetin (**F_3**), rutin (**F_4**) and quercetin-3-O-glucoside (**F_5**), and two phenolic compounds; gallic acid (**F_6**) and P-coumaric acid (**F_7**). All the isolated compounds were first reported in *P. canariensis* seeds. Their structures were established by chemical evidence spectroscopic techniques (UV, ^{1}H NMR, ^{13}C NMR, HMQC and COSY) and by comparison with already existing spectroscopic data (figure1).

3.2. Renal tissue parameters

3.2.1. Effect of ethanolic extract of P. canariensis on serum urea and serum creatinine

In MTX group, serum urea and serum creatinine was increased, as compared to control. On the other hand, treatment with *P. canariensis* produced dose-dependent reduction in serum urea and creatinine, as compared to MTX group (figure 2A, 2B).

3.2.2. Effect of ethanolic extract of P. canariensis on renal MDA and renal total nitrites

In MTX group, renal MDA and renal total nitrites was increased, as compared to control. On the other hand, treatment with *P. canariensis* dose-dependently reduced MDA and total nitrites, as compared to MTX group (figure 2C, 2D).

3.2.3. Effect of ethanolic extract of P. canariensis on expression of BCL2 and KIM-1 in renal tissue

In MTX group, BCL2 expression was reduced, meanwhile, KIM-1 expression was increased, as compared to control. In contrast, *P. canariensis* dose-dependently showed increase in BCL2 expression and decrease in KIM-1

expression, as compared to MTX group (figure 2E).

3.3. Histopathological study

In MTX and *P. canariensis* (100 mg/kg/d)-treated groups, necrosed, collapsed, hypercellular glomeruli and renal tubules degenerations were noticed. In *P. canariensis* (200 mg/kg/d)-treated group, renal glomeruli with narrowed space and increased cellularity were noticed. At the same time, mild degeneration and vacuolations of renal tubule lining epithelium were noticed. However, group treated with *P. canariensis* (400 mg/kg/d) showed normal glomeruli and renal tubules (figure 3). A dose-dependent reduction in semiquantitaive scoring of renal histopathological changes was noticed in groups treated with *P. canariensis*, as compared to MTX group [table 2].

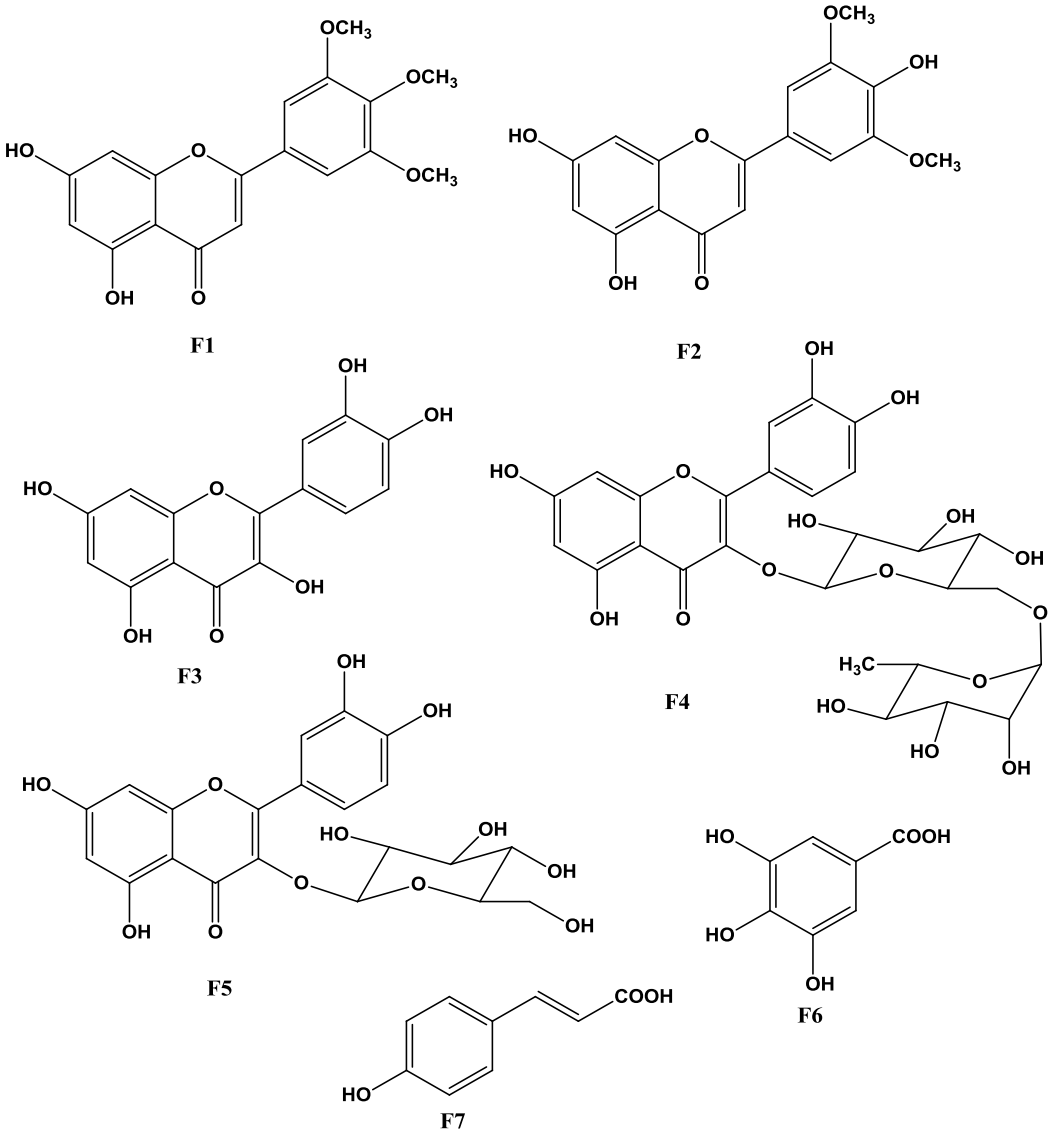

Figure 1. Chemical structures of compound isolated from the chloroform and ethyl acetate fractions.

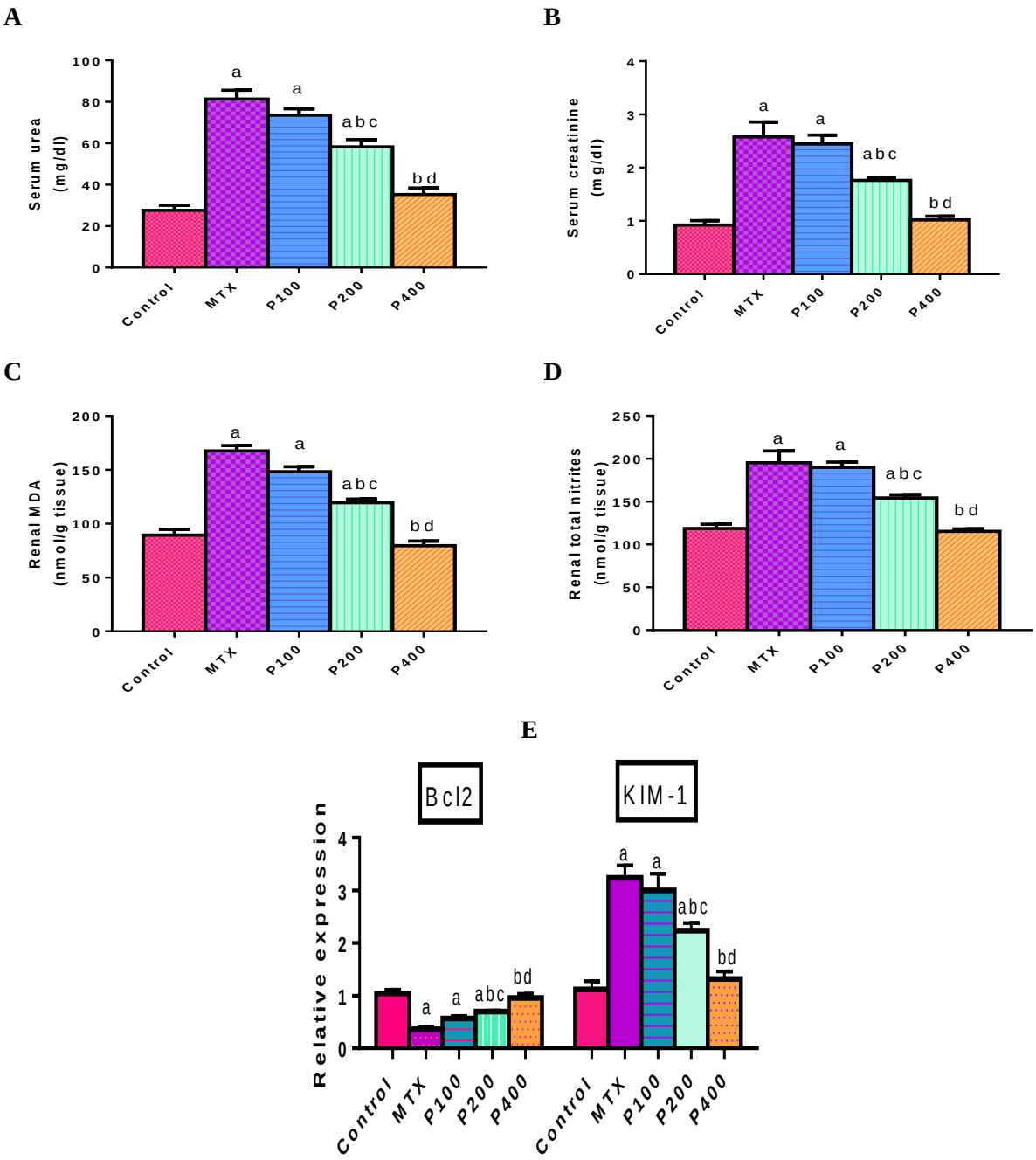

Figure 2: Effect of ethanolic extract of *P. canariensis* on serum urea (A), serum creatinine (B), renal malondialdehyde (C), renal total nitrites (D) and expression of BCL2 and KIM-1 in renal tissue (E).

Results represent the mean ± SE (n = 8-10).

[a] Significant difference from control group, [b] significant difference from MTX group, [c] significant difference from P100 group, [d] significant difference from P200 group. Significance is at ($p < 0.05$). MTX; methotrexate, P100 group; *P. canariensis* 100 mg/kg-treated group, P200 group; *P. canariensis* 200 mg/kg-treated group and P400 group; *P. canariensis* 400 mg/kg-treated group.

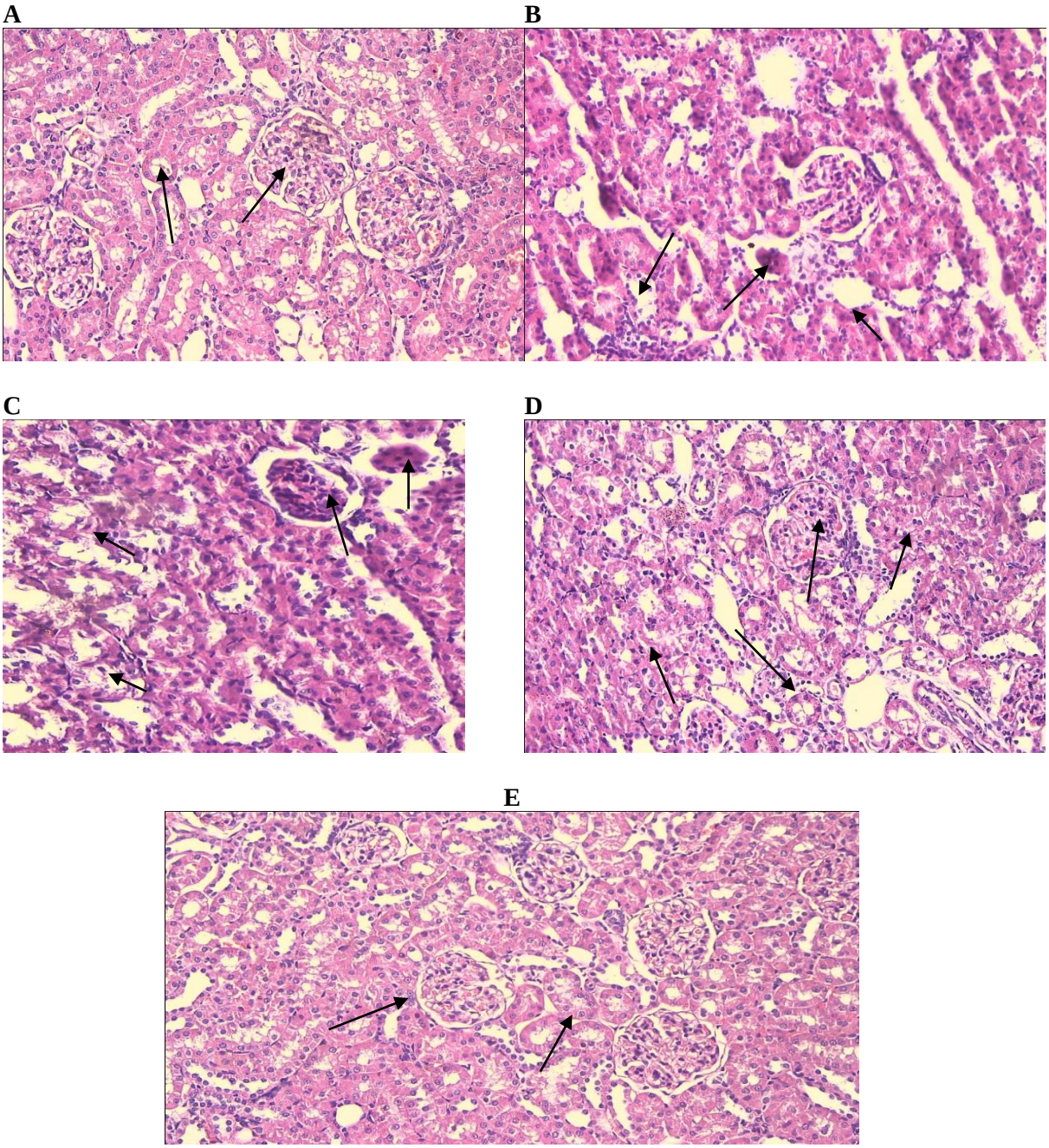

Figure 3: Effect of *P. canariensis* on kidney histopathological picture. A representative photomicrograph of a section in renal tissue. A) control group, B) MTX group,arrows referto necrosed, collapsed and hypercellular glomeruli and degenerated renal tubules, C) P100 group; arrows refer to necrosed glomeruli and degenerated tubules, D) P200 group; arrows refer to renal glomeruli with narrowed space and increased cellularity, renal tubules with mild degeneration and vacuolation of its lining epithelium, E) P400 group; arrows refer to normal glomeruli and renal tubules. MTX; methotrexate, P100, 200, 400 groups; *P. canariensis* 100, 200 and 400 mg/kg-treated groups, respectively.

Table 2: kidney lesion scores in control and different experimental groups.

Lesion	Control	MTX	P100	P200	P400
Vascular congestion	0.00	2.88 ± 0.13^{a}	2.50 ± 0.19	1.25 ± 0.16^{bc}	0.38 ± 0.18^{bd}
Cloudy swelling	0.00	2.75 ± 0.16^{a}	2.38 ± 0.18	1.38 ± 0.18^{bc}	0.50 ± 0.19^{bd}
Interstitial and tubular hemorrhage	0.00	2.63 ± 0.18^{a}	2.50 ± 0.19	1.50 ± 0.19^{bc}	0.63 ± 0.19^{bc}
Focal tubular necrosis	0.00	2.75 ± 0.25^{a}	2.25 ± 0.16	1.25 ± 0.33^{bc}	0.25 ± 0.16^{bd}

Results represent the mean ± SE (n = 6-8).
[a] Significant difference from control group, [b] significant difference from MTX group. [c] significant difference from P100 group, [d]significant difference from P200 group. Significance is at $p < 0.05$. MTX; methotrexate, P100 group; *P. canariensis* 100 mg/kg-treated group, P200 group; *P. canariensis* 200 mg/kg-treated group and P400 group; *P. canariensis* 400 mg/kg-treated group.

4. Discussion

There is currently an unmet need for adjuvant therapy to modulate MTX-induced toxicity. MTX is efficacious and unique in that it can be administered safely in a multitude of dosing strategies. However, the limitation of MTX in clinical use is its side effects associated with oxidative stress, apoptosis and inflammatory responses, and clinicians face the challenge of its adverse effects[19]. Many pharmacological interventions, were done to mitigate MTX toxicities, including leucovorin rescue that have not substantially yielded desired results[20]. Abundant evidence suggests that the antioxidant phytochemicals in natural products may abrogate oxidative toxicity[21,22]. The present study aimed at investigating the protective effect of *P. canariensis* against the development of MTX-induced toxicity in rats. Different doses of the extract (100, 200, and 400 mg/kg/d) when given to normal rats; rats did not receive MTX; no significant change was noticed in renal parameters, as compared to control group (unpublished data).

The BCL2 proteins are central regulators of the mitochondrial apoptotic pathway[23]. BCL2 was initially discovered as the protooncogene involved in follicular lymphoma[24]. There are many intrinsic and extrinsic pathways for apoptosis but the Bcl-2 family is considered as a major signaling regulatory protein in cell apoptosis pathways[25] and is a key regulator of apoptosis[26, 27, 28].

Kidney toxicity can occur with MTX treatment in both low and high doses. High doses of MTX make kidney damage by causing tubular injury with the precipitation of MTX in kidney tubules, and decrease in the glomerular filtration rate[29]. MTX also induces apoptosis that aggravates renal injury[22,30]. In patients receiving MTX treatment, the risk of kidney toxicity was 1.8%[31]. In the current study, MTX elevated serum urea and serum creatinine, which are indicators of the renal function. Induction of oxidative stress in kidney by MTX was noticed by increase in MDA and total nitrites. These results are in agreement with Armagan and coworkers who reported that the same single MTX dose induced similar biochemical findings, which indicates the impairment of renal function induced by MTX[32]. Similarly, previous studies which reported that MTX increased blood urea and creatinine levels together with increase in MDA and nitric oxide[33,34]. In these studies, nephrotoxicity was also induced by single dose of MTX.

KIM-1 is a type I trans-membrane glycoprotein discovered in 1998, which is undetectable in healthy kidney. Several reports mentioned the very high levels of KIM-1 exprssion on the apical membrane of proximal tubule cells after nephrotoxic injury[35,36]. Expression of KIM-1 is associated with renal fibrosis and inflammation that was confirmed in kidney biopsy tissue collected from patients with kidney disease[37]. MTX-induced kidney toxicity was associated with increase in KIM-1 expression indicating the renal damage occurred with MTX. Similarly, Freedman and coworkers reported that kidney express kidney injury molecule-1 after nephrotoxic chemical injury[38].

In the current study, MTX reduced expression of BCL2 in renal tissue. This result is in

agreement with previous studies, which reported the downregulation of BCL2 expression in rats, which was, administered MTX[39]. The toxic effect of MTX was confirmed by the histopathological findings such as renal tubule degeneration and necrosed glomeruli. Similarly, in a previous study where the equivalent dose was used, MTX caused marked degenerative changes, such as tubular degeneration, tubular dilatation, tubular cell swelling, and tubular damage[15].

At doses 200 and 400 mg/kg/d, and not at dose 100 mg/kg/d, *P. canariensis* reduced serum urea, serum creatinine, renal MDA, renal total nitrites and renal expression of Kim-1, and increased expression of BCL2. Histologic improvements in arteriolopathy and tubular injury were found in *P. canariensis* (200, 400 mg/kg/d)-treated rat kidneys and not at dose 100 mg/kg/d. to our knowledge, this is the first time to study the effect of *P. canariensis* on a model of renal injury. The noticed dose dependent effect was accompanied by anti-oxidative stress action that reduced reactive oxygen species production, which results in a cytoprotective effect.

Excessive apoptosis or its dysregulation can lead to nephrotoxicity[22,40]. The results of the present study indicated that MTX-induced renal apoptosis through decreasing the expression of anti-apoptotic protein BCL2. In this study, *P. canariensis* inhibited apoptosis might be dependent on increase in BCL2 protein expression. The results demonstrated that inhibition of renal apoptosis by *P. canariensis* may potentially attenuate renal injury and BCL2 may be the target for the reno-protective strategy of *P. canariensis*.

5. Conclusions

In conclusion, ethanolic extract of *P. canariensis*, at doses 100, 200 and 400 mg/kg/day, dose-dependently showed a protective effect against nephrotoxicity induced by MTX. These recoveries were associated with the reduction of oxidative stress, apoptosis, and tubular damage. We suggest that reduction of oxidative stress and antagonizing apoptosis evidenced by increase in BCL2 may be possible mechanisms.

So that, the administration of *P. canariensis* could be a target for protecting against the progression of renal injury occur with MTX administration, but further studies will be needed to facilitate such a potential therapy.

6. References

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