Journal of Traditional and Complementary Medicine 10 (2020) 478–486

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Ethyl acetate extract of *Ceiba pentandra* (L.) Gaertn. reduces methotrexate-induced renal damage in rats via antioxidant, anti-inflammatory, and antiapoptotic actions

Mohamed E. Abouelela [a], Mohamed A.A. Orabi [a, b], Reda A. Abdelhamid [a], Mohamed S. Abdelkader [c], Hafez R. Madkor [d], Faten M.M. Darwish [e], Tsutomu Hatano [f], Bakheet E.M. Elsadek [d, *]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, P.O. Box 71524, Assiut, Egypt
[b] Department of Pharmacognosy, College of Pharmacy, Najran University, P.O. Box 1988, Najran, Saudi Arabia
[c] Department of Pharmacognosy, Faculty of Pharmacy, Sohag University, Nasr City, Eastern Avenue, P.O. Box 11432, Sohag, Egypt
[d] Department of Biochemistry, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, P.O. Box 71524, Assiut, Egypt
[e] Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, P.O. Box 71526, Assiut, Egypt
[f] Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Tsushima, Kita-ku, Okayama, 700-8530, Japan

ARTICLE INFO

Article history:
Received 15 January 2019
Received in revised form
13 August 2019
Accepted 26 August 2019
Available online 28 August 2019

Keywords:
Ceiba pentandra (L.) Gaertn.
Methotrexate
Nephrotoxicity
Antioxidant

ABSTRACT

Methotrexate (MTX) is a chemotherapeutic agent and an immunosuppressant used to treat cancer and autoimmune diseases. However, its use is limited by its multi-organ toxicity, including nephrotoxicity, which is related to MTX-driven oxidative stress. Silencing oxidative stressors is therefore an important strategy in minimizing MTX adverse effects.

Medicinal plants rich in phenolic compounds are probable candidates to overcome these oxidants. Herein, *C. pentandra* ethyl acetate extract showed powerful *in vitro* radical-scavenging potential ($IC_{50} = 0.0716$) comparable to those of the standard natural (ascorbic acid, $IC_{50} = 0.045$) and synthetic (BHA, $IC_{50} = 0.056$) antioxidants. The effect of *C. pentandra* ethyl acetate extract against MTX-induced nephrotoxicity in rats was evaluated by administering the extract (400 mg/kg/day) or the standard antioxidant silymarin (100 mg/kg/day) orally for 5 days before and 5 days after a single MTX injection (20 mg/kg, i.p.).

C. pentandra showed slight superiorities over silymarin in restoring the MTX-impaired renal functions, with approximately twofold decreases in overall kidney function tests. *C. pentandra* also improved renal antioxidant capacity and reduced the MTX-induced oxidative stress. Moreover, *C. pentandra* inhibited MTX-initiated apoptotic and inflammatory cascades, and attenuated MTX-induced histopathological changes in renal tissue architecture.

Phytochemical investigation of the extract led to the purification of the phenolics quercitrin (**1**), cinchonains 1a (**2**) and 1b (**3**), *cis*-clovamide (**4**), *trans*-clovamide (**5**), and glochidioboside (**6**); a structurally similar with many of the reported antioxidant and nephroprotective agents. In conclusion, these data demonstrate that *C. pentandra* exhibits nephroprotective effect against MTX-induced kidney damage via its antioxidant, antiapoptotic and anti-inflammatory mechanisms.

Taxonomy: Functional Disorder, Traditional Medicine, Herbal Medicine.

1. Introduction

The kidney is a crucial organ that is responsible for filtering the blood to remove wastes, maintains water and electrolytes homeostasis, and regulates blood pressure.[1] The use of the antimetabolite Methotrexate (MTX), at high doses in cancer therapy or at

* Corresponding author. Al–Azhar University, Assiut Branch, P. O. Box 71524, Assiut, Egypt.
E-mail address: bakheet.elkot@azhar.edu.eg (B.E.M. Elsadek).
Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2019.08.006

List of abbreviations

BHA	Butylated hydroxyanisole
Bcl-2	B-cell lymphoma 2
BUN	Blood urea nitrogen
CAT	Catalase
CMC	Carboxy methyl cellulose
C. pentandra	*Ceiba pentandra* (L.) Gaertn.
CRP	C-reactive protein
DCM	Dichloromethane
DPPH	2,2-Diphenyl-1-picryl hydrazyl
EtOAc	Ethyl acetate
GSH	Reduced glutathione
IL-18	Interleukin-18
KIM-1	kidney injury molecule-1
MDA	Malondialdehyde
MeOH	Methanol
MTX	Methotrexate
NO	Nitric oxide
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor alpha

lower doses for long periods in treating non-malignant autoimmune disorders, is associated with acute toxicity in multiple organs including kidneys.[2] Because ~90% of the administered dose is excreted unchanged through the kidney, MTX is more likely to damage the kidneys than other organs by crystal nephropathy and/or tubular toxicity as a consequence of the overwhelming production of reactive oxygen species within the kidney.[2] Abnormal generations of inflammatory mediators and neutrophil infiltration as well as apoptotic renal cell death are also implicated in MTX-induced renal damage.[3] These severe adverse effects restrict, to a large extent, the clinical use of MTX. Therefore, attenuation of these devastating events of MTX, especially towards the kidney, is considered a fundamental goal to improve its therapeutic index.

However, adjunctive treatment with antioxidant, anti-inflammatory, and antiapoptotic agents during MTX chemotherapy may restore normal kidney functions and protect against MTX-induced toxicity. Screening natural herbs and their products as nephroprotective is now advancing.[4] Recent epidemiologic and experimental studies demonstrate that many herbal plants with well-known high content of phenolic compounds such as *Pomegranate*, Green tea, Grape and *Curcuma longa* exhibit promise in this area, through their antioxidant, anti-inflammatory, and apoptosis-modulating activities.[5,6] Silymarin which is a lipophilic extract of the *Silybum marianum* seeds is also an example of the great natural therapeutic discoveries. Its hepatoprotective, nephroprotective, antiproliferative, antiviral and antifibrotic activities through several mechanisms, including anti-oxidation, inhibition of lipid peroxidation, immunomodulation and anti-inflammatory effects, are well established.[7,8]

Ceiba pentandra (L.) Gaertn. (*C. pentandra*), also known as the silk-cotton tree or dum, is an ornamental tree belonging to the family Bombacaceae that is cultivated in Southeast Asia and Africa.[9] Traditionally, *C. pentandra* is used as a diuretic, aphrodisiac and to treat headache. Pharmacologically, extracts of some morphological parts of *C. pentandra* exhibited hepatoprotective, hypolipidemic, anti-inflammatory, antihypertensive, and hypoglycemic effects in type II diabetes.[9] A wide array of flavonoids, tannins and related phenolics is found in *C. pentandra*.[10]

Here, we report the *in vitro* antioxidant activity of an 80% aqueous methanol (v/v) extract of *C. pentandra* and its fractions. We estimate the oral acute toxicity of the ethyl acetate fraction. Additionally, we assess the *in vivo* protective effects of this ethyl acetate fraction against MTX-induced nephrotoxicity in rats. Finally, to relate these biological effects to phytomolecules of the extract, an isolation experiment was conducted.

2. Material and methods

2.1. Plant material

The fresh aerial parts of *C. pentandra* were collected in September 2016 from the field of ornamental plants, College of Agriculture, Assiut University. The plant was botanically authenticated by Dr. Essam Youssef, Professor of Horticulture, College of Agriculture, Assiut University. A voucher specimen (No. Cpp2) was kept in Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Assuit branch. The plant material were dried under shade and finely powdered.

2.2. Extraction, isolation, and purification of phytomolecules from the ethyl acetate fraction of C. pentandra extract

The air-dried powdered aerial parts of *C. pentandra* (2.5 kg) were extracted three times with 80% methanol (MeOH) at room temperature. The alcoholic extract was concentrated at 45 °C under reduced pressure. The dry extract (250 g) was suspended in 0.5 L distilled water and submitted to a separating funnel where the phytoconstituents were sequentially partitioned between the aqueous layer and dichloromethane (DCM), ethyl acetate (EtOAc), and *n*-butanol, respectively, till exhaustion and the obtained fractions were dried under reduced pressure. The obtained dry DCM (52 g), EtOAc (28.06 g), *n*-butanol (19.23 g) fractions were kept in air tight bottles in refrigerator for chemical and/or biological investigations. Subsequent purification of phytomolecules from the ethyl acetate fraction of *C. pentandra* extract was carried out according to standard procedures as described minutely in the supplementary material.

2.3. In vitro antioxidant assay

The 2,2-Diphenyl-1-picryl hydrazyl (DPPH$^{\bullet}$) radical scavenging assay was used to determine the antioxidant potential of different *C. pentandra* extracts according to standard procedures using ascorbic acid and butylated hydroxyanisole (BHA) as positive natural and synthetic antioxidant controls, respectively.[11] For more details, see the supplementary material.

2.4. Animal experiments

In the current study, all experimental procedures were achieved according to the National Institutes of Health guide (NIH 1985) for the Care and Use of Laboratory Animals, and were approved by the Ethical Committee of the College of Pharmacy, Assiut University, Assiut, Egypt (12/2016). Healthy male Wistar rats (weighing 165 ± 10 g) were obtained from Pre-Clinical Animal House, Pharmacology Department, College of Medicine, Assiut University. Animals were housed (2 per cage) at regular environmental conditions (temperature, 22 ± 2 °C; humidity, 50 ± 5%; night/day cycle, 12 h) with a free access to an ordinary rodent diet (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) and tap water *add libitum*. Body weight of each animal was recorded every day.

2.4.1. Acute toxicity study of the ethyl acetate fraction of C. pentandra on rats

In this experiment, twenty healthy rats was used to explore the

acute oral toxicity of the ethyl acetate fraction of *C. pentandra* according to literatures.[12] Four subgroups of rats (n = 5) were treated with 100, 1000, 3000, or 5000 mg/kg body weight p.o. of the ethyl acetate fraction of *C. pentandra*. The rats were monitored for 24 h to report toxicities and/or mortalities. We also observed animals for possible behavioral, physiological, and toxicological changes for further 14 days post treatment.

2.4.2. In vivo nephroprotective activity of C. pentandra

This experiment was performed using forty healthy male Wistar rats that were randomly classified into 4 groups (10 rats each) after one week acclimatization period. Animals in group 1 (healthy control group) received orally the suspending vehicle that consists of 0.5% (w/v) carboxy methyl cellulose (CMC) for 10 consecutive days. Animals in group 2 (MTX group) were injected with a single MTX dose (20 mg/kg, i.p) on day 5 of the experiment.[13] In group 3 (MTX + silymarin), animals were injected with a single MTX dose (20 mg/kg, i.p) in addition to silymarin (100 mg/kg body weight, daily) as positive control by oral gavage suspended in 0.5% (w/v) CMC.[14] Administration of silymarin started 5 days before the MTX injection and continued for another 5 consecutive days. Animals in group 4 (MTX + *C. pentandra*) were injected with a single MTX dose (20 mg/kg, i.p) in addition to the ethyl acetate extract of *C. pentandra* by oral gavage (400 mg/kg body weight, daily) suspended in 0.5% (w/v) CMC.[15] Administration of *C. pentandra* started 5 days before MTX injection and continued for another 5 consecutive days. Our primarily conducted acute toxicity study revealed that the ethyl acetate extract is safe in rats and well tolerated at doses up to 5000 mg/kg for the 14 days ($LD_{50} > 5000$ mg/kg). In accordance with several previous studies, a similar dose of *C. pentandra* extract was used, as a common effective dose investigated in some *in vivo* activities.[16–19]

On the 6th day post the MTX injection, each animal was kept in an individual metabolic cage to obtain urine samples for assessment of urinary bioindices. On the 7th day post the MTX injection, blood samples were withdrawn from retro-orbital plexus and the rats were sacrificed by cervical decapitation under isoflurane anesthesia. Both kidneys of each animal were subsequently removed, washed in ice-cold isotonic saline, and divided into three parts. One part was kept in 10% neutral buffered formalin solution for histopathological examinations. The other two parts were instantly flash frozen in liquid nitrogen and stored separately at −80 °C for biochemical and molecular assays.

2.4.2.1. Estimation of nephrotoxicity markers. Serum creatinine was estimated using a commercially available kinetic assay kit (Human Diagnostic, Wiesbaden, Germany). Blood urea nitrogen (BUN) was also estimated using a commercially available kinetic assay kit (Agappe Diagnostics, Switzerland GmbH). Serum cystatin C was estimated by immunoassay procedures using a kit provided from R&D Systems® Inc. (USA). Urinary kidney injury molecule-1 (KIM-1) was assayed using enzyme-linked immunosorbent assay (ELISA) assay kit according to the manufacturer instructions (Glory Science.co., ltd, Hangzhou, China). Microalbuminuria was measured by colorimetric procedures (BioSystems (Barcelona, Spain).

2.4.2.2. Estimation of oxidative stress markers. Renal lipid peroxidation was assessed spectrophotometrically in the form of thiobarbituric acid reacting substance (TBARS) and is expressed as equivalents of malondialdehyde (MDA), using *1,1,3,3* tetramethoxypropane as a standard.[20] Renal tissue content of the reduced glutathione (GSH) was assayed spectrophotometrically according to Ellman method.[21] Renal superoxide dismutase (SOD) activity was assayed by xanthine oxidase method.[22] Renal catalase (CAT) activity was estimated according to Sinha method.[23] Renal tissue nitric oxide (NO) level was measured spectrophotometrically by measuring its stable metabolites, particularly, nitrite and nitrate.[24] Determination of total protein levels in renal tissue homogenates was achieved using corresponding kinetic kits manufactured by slandered company.

2.4.2.3. Assessment of the apoptotic and inflammatory markers. Quantitative estimation of serum levels of tumor necrosis factor alpha (TNF-α), an important proinflammatory cytokine in the innate immune response, was carried out using a rat specific ELISA assay kits (Biospes Co., Ltd., China) according to the manufacturer instructions. Serum C-reactive protein (CRP), an acute-phase protein that plays an important physiological role in complement system activation, was assayed using an enhanced MISPA *i2* based nephelometry assay kit (Agappe Diagnostics Switzerland GmbH).

Detection of the mRNA expression of caspase-3, a pro-apoptotic factor, B-cell lymphoma 2 (*Bcl-2*), an anti-apoptotic factor and interleukin-18 (IL-18), a pro-inflammatory cytokine in renal tissue homogenates was achieved using RT–PCR according to standard procedures, as fully summarized in the supplementary material.

2.4.3. Histopathological examinations

Assessment of histopathological changes in renal tissue samples that were fixed in 10% neutral buffered formalin solution was carried out after routine processing and staining with hematoxylin and eosin (H&E) according to a standard protocol.[25]

2.5. Statistical analyses

Statistical analyses of the data were performed by using GraphPad prism version 6.0 (Graphpad Software, Inc., San Diego, USA). Analysis of group differences was carried out using one-way analysis of variance (ANOVA) followed by Tukey's *t*-test for multiple comparisons. The level of significance was accepted with $p < 0.05$ and all relevant results were displayed as mean ± SEM.

3. Results

3.1. Methanolic and ethyl acetate fractions of C. pentandra show strong antioxidant activity

The *C. pentandra* 80% aqueous methanol extract and its obtained fractions (Experimental Section) were submitted for antioxidant activity assessment by evaluating their $DPPH^{\bullet}$ scavenging activities. The assay results revealed that the methanolic and ethyl acetate fractions of *C. pentandra* showed antioxidant activities comparable to a standard natural antioxidant (ascorbic acid) and the standard synthetic antioxidant (BHA) (Fig. 1). In contrast, the dichloromethane and *n*-butanol fractions showed weaker antioxidant activities than all other fractions (Table S6, Supplementary material).

3.2. Ethyl acetate fraction of C. pentandra is non-toxic in rats

Next, the oral toxicity of the ethyl acetate fraction was evaluated in rat. No deaths and no behavioural, physiological or toxicological changes were observed in rats at doses up to 5000 mg/kg for the 14 days that the experiment lasted ($LD_{50} > 5000$ mg/kg). According to that, the ethyl acetate fraction of *C. pentandra* was believed to be safe in rats.

3.3. C. pentandra and silymarin attenuate MTX-induced body-weight loss

Once we assured that the ethyl acetate fraction of *C. pentandra* was safe in rats, we evaluated its efficacy against MTX-induced

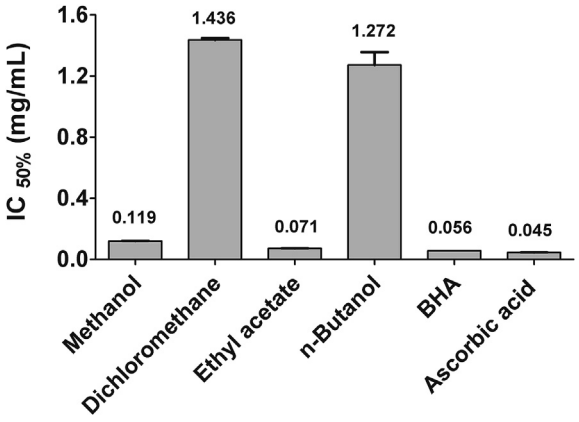

Fig. 1. DPPH• radical scavenging activity (IC_{50}) of different fractions of *C. pentandra*. Data are expressed as the mean ± SEM of three independent experiments. Ascorbic acid and BHA were used as positive natural and synthetic antioxidant controls, respectively.

nephrotoxicity. To this end, rats (10 per group) were given either the ethyl acetate fraction of *C. pentandra* or silymarin, daily by oral gavage for 10 days, and a single i.p. injection of MTX on day 5. The control group received the CMC vehicle daily by oral gavage, whereas the untreated group received the MTX injection on day 5 only. Two mortalities were recorded in the MTX group, on the 2nd and 4th days after MTX injection.

Animals in the healthy control (vehicle) group remained alert throughout the experiment and gained weight (~23%), whereas those in the MTX group appeared lethargic and lost weight (~10.7%), compared to their corresponding weights on the day of MTX injection. This MTX-induced weight loss was not observed in animals treated with either silymarin or *C. pentandra*; animals in both these groups seemed alert and gained weight (~2% and ~6.6%, respectively) compared to the corresponding weight at the time of MTX administration (Fig. 2).

3.4. *C. pentandra and silymarin prevent MTX-induced renal impairment*

We then examined the renal functions in the four groups of rats. The MTX group showed significantly higher levels of serum creatinine, BUN, cystatin C, microalbuminuria, and urinary KIM-1 than the healthy control group (Table 1) which indicates impaired renal functions. Administration of either *C. pentandra* or silymarin prevented this MTX-induced renal impairment, as indicated by significantly lower levels of serum creatinine, BUN, cystatin C, microalbuminuria, and urinary KIM-1 in these groups than in the MTX group, with a slight superiority of *C. pentandra* over silymarin (Table 1).

3.5. *C. pentandra and silymarin protect renal tissue against MTX-induced oxidative stress*

Since production of reactive oxygen species has been related to MTX-induced nephrotoxicity,[2] we next evaluated the activity of renal oxidant/antioxidant enzymes in treated vs. untreated rats. As expected, the MTX group showed a pronounced imbalance in the renal oxidant/antioxidant system, as indicated by significantly lower renal tissue activities of the antioxidant enzymes SOD and CAT, and lower renal tissue content of GSH than the healthy control group, as well as significantly higher levels of renal MDA and NO (Table 2). Both *C. pentandra* and silymarin protected against these signs of MTX-induced oxidative stress, as demonstrated by significantly higher activities of SOD and CAT and higher levels of GSH than in the MTX group, as well as significantly lower levels of renal MDA and NO, with no difference in efficacy between *C. pentandra* and silymarin (Table 2).

3.6. *C. pentandra and silymarin attenuate MTX-induced alterations in inflammatory markers and apoptotic modulators*

Next, we examined production of inflammatory markers and apoptotic modulators, since this have also been implicated in MTX-induced renal damage.[3] The MTX group showed significantly greater mRNAs renal expression of the proapoptotic caspase-3 (Fig. 3A) and weaker mRNAs expression of antiapoptotic *Bcl-2* (Fig. 3B) than the healthy control group, as detected by RT-PCR. Notably, these effects were attenuated in the groups that received *C. pentandra* and silymarin (Fig. 3A and B).

The MTX group also showed significantly higher levels of the circulating inflammatory markers TNF-α and CRP than the control group (Table 1). Additionally, RT-PCR analysis revealed markedly higher mRNA expression of the proinflammatory cytokine IL-18 in

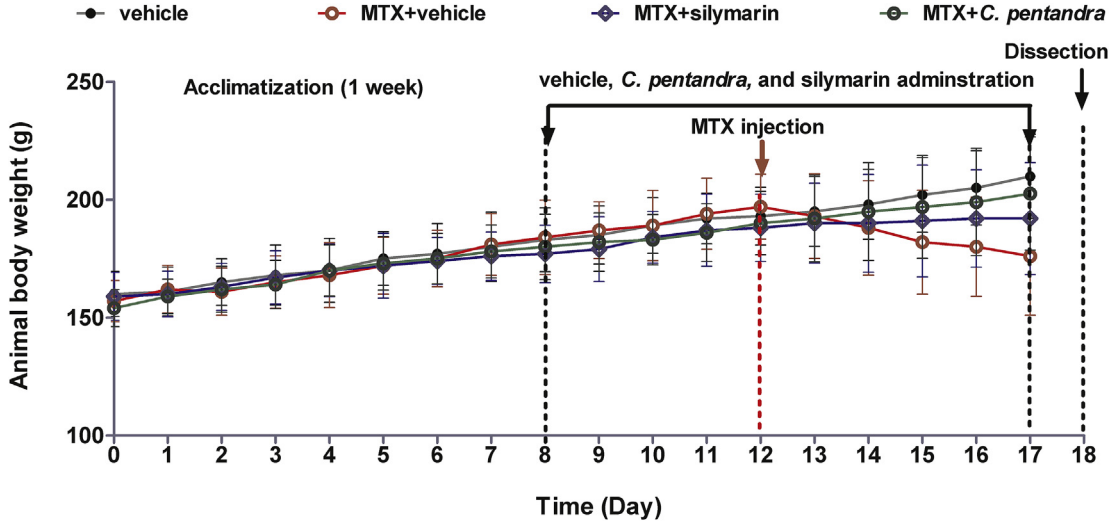

Fig. 2. Animal body weight changes during the experimental period. Data are presented as the mean ± SEM (n = 10). Intended for color reproduction on the Web

Table 1
Effects of the tested compounds on kidney function tests, apoptosis, and inflammatory markers.

Group	vehicle	MTX + vehicle	MTX + silymarin	MTX + *C. pentandra*
Creatinine (mg/dL)	0.58 ± 0.05	1.86 ± 0.05***	0.85 ± 0.06$^{*,○○○}$	0.79 ± 0.07$^{○○○}$
BUN (mg/dL)	10.68 ± 0.49	21.96 ± 1.01***	14.07 ± 1.11 $^{○○○}$	12.09 ± 0.89$^{○○○}$
Cystatin C (mg/L)	1.49 ± 0.13	3.73 ± 0.20***	2.40 ± 0.15$^{**,○○○}$	2.20 ± 0.15$^{*,○○○}$
KIM-1 (pg/mL)	5.41 ± 0.62	61.40 ± 6.60***	30.00 ± 2.50$^{***,○○○}$	15.11 ± 1.99$^{○○○,\$}$
Microalbuminuria (mg/L)	13.50 ± 2.44	153.30 ± 15.68***	87.00 ± 5.01$^{***,○○○}$	80.63 ± 5.77$^{***,○○○}$
TNF-α (ng/mL)	0.88 ± 0.13	2.55 ± 0.18***	1.83 ± 0.22$^{**,○}$	1.67 ± 0.16$^{*,○○}$
CRP (mg/L)	1.30 ± 0.25	6.45 ± 0.65***	4.24 ± 0.54$^{**,○}$	3.22 ± 0.31$^{*,○○○}$

Data are presented as the mean ± SEM (n = 10). *, ○, and $ indicate significant differences with the vehicle, (MTX + vehicle), and (MTX + silymarin) groups, respectively. *, ○, $ indicate significance at p < 0.05; **, ○○, and $$ indicate significance at p < 0.01; ***, ○○○, and $$$ indicate significance at p < 0.001. BUN: Blood urea nitrogen; CRP: C-reactive protein; KIM-1: Kidney injury molecule-1; MTX: Methotrexate; TNF-α: Tumor necrosis factor alpha.

Table 2
Effects of the tested compounds on oxidative stress markers in kidney tissue homogenates.

Group	vehicle	MTX + vehicle	MTX + silymarin	MTX + *C. pentandra*
SOD (U/g protein)	0.137 ± 0.016	0.043 ± 0.012***	0.113 ± 0.015$^{○○}$	0.107 ± 0.009$^{○}$
CAT (U/mg protein)	0.042 ± 0.007	0.003 ± 0.000**	0.030 ± 0.006$^{○}$	0.031 ± 0.008$^{○}$
GSH (μmol/g protein)	7.57 ± 0.79	2.77 ± 0.29***	5.48 ± 0.88$^{○}$	5.89 ± 0.68$^{○}$
MDA (nmol/g protein)	16.14 ± 1.29	44.99 ± 5.09***	28.43 ± 2.43$^{*,○○}$	21.98 ± 1.79$^{○○○}$
Nitric oxide (μmol/g protein)	0.67 ± 0.08	5.69 ± 0.49***	3.16 ± 0.52$^{***,○○○}$	3.07 ± 0.36$^{***,○○○}$

Data are presented as the mean ± SEM (n = 10). *, ○, and $ indicate significant differences with the vehicle, (MTX + vehicle), and (MTX + silymarin) groups, respectively. *, ○, $ indicate significance at p < 0.05; **, ○○, and $$ indicate significance at p < 0.01; ***, ○○○, and $$$ indicate significance at p < 0.001. CAT: Catalase; GSH: Reduced glutathione; MDA: Malondialdehyde; MTX: Methotrexate; SOD: Superoxide dismutase.

the MTX group than in the healthy control group (Fig. 3C). However, administration of *C. pentandra* or silymarin led to significant alleviation in the serum levels of both TNF-α and CRP, and a marked decrease in IL-18 gene expression compared to the MTX group; *C. pentandra* showed a slight superior effect than silymarin (Table 1 and Fig. 3C).

3.7. *C. pentandra* and silymarin prevent MTX-induced histopathological changes in kidneys

Kidneys of rats in the healthy control group showed a normal histological structure of the renal parenchyma (Fig. 4A). In contrast, MTX administration caused marked deteriorations in the kidney tissue architecture in the form of renal blood vessel congestion, focal interstitial nephritis, atrophy of the glomerular tuft, and focal necrosis of renal tubules associated with inflammatory cell infiltration (Fig. 4B). The kidneys of rats from the silymarin group showed vacuolation and congestion of the glomerular tuft and intertubular renal blood vessels (Fig. 4C). Kidneys of rats in the *C. pentandra* group showed no histological differences from those in the control group except for congestion of renal blood vessels in some examined sections (Fig. 4D).

3.8. Isolation and identification of phenolic compounds from the ethyl acetate fraction of *C. pentandra*

Because of the auspicious antioxidant results of the ethyl acetate fraction, it was submitted for chromatographic investigation. Repeated column chromatography using silica gel, RP-18, Sephadex LH-20, MCI-gel CHP–20P and occasionally HPLC purification led to the isolation of six compounds. On the basis of physicochemical and spectral analyses, as well as comparison with data from the literature, we identified the isolated compounds (Fig. 5) as quercetin-3-*O*-α-L-rhamnoside (quercitrin, **1**, 11 mg),[26] cinchonains Ia (**2**, 2.3 mg)[27] and Ib (**3**, 4 mg),[27] N-(3,4-dihydroxy-*cis*-cinnamoyl)-3-(3′,4′-dihydroxyphenyl)-L-alanine (*cis*-clovamide, **4**, 12 mg),[28] N-(3,4-dihydroxy-*trans*-cinnamoyl)-3-(3′,4′-dihydroxyphenyl)-L-alanine (*trans*-clovamide, **5**, 1.8 mg),[28] and dehyrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (glochidioboside, **6**, 11 mg).[29] This is the first report on the isolation of these compounds from the family Bombacaceae.

From the pharmacological point of view, these compounds are either nephroprotective themselves (**1**) or structurally related (**2**–**6**) to the reported anti-inflammatory nephroprotective compounds silymarin (Fig. 5A) and curcumin (Fig. 5B) and the anti-inflammatory woorenoside 1 (Fig. 5C).[30–34] These compounds could be thus responsible totally or in part for the determined antioxidant and nephroprotective effects of *C. pentandra*.

4. Discussion

Till now, attenuation of the MTX-induced multi-organ toxicity, especially renal damage, is considered as an unmet target to improve its therapeutic index in the treatment of several types of human malignancies and autoimmune diseases.[2,35]

Some traditional herbal medicines partially preserve renal structure and function, probably through their antioxidant properties.[4] *C. pentandra* is an important traditional medical plant with multiple pharmacological effects.[9] Our evaluation of the total methanol extract of the aerial parts of *C. pentandra*, and its solvent fraction, indicated that the ethyl acetate fraction acts as a potent antioxidant.

To our knowledge, no previous studies have been conducted to evaluate the effects of *C. pentandra* against MTX-induced nephrotoxicity. We selected rats as a convenient model in which to study these effects of the ethyl acetate at the histopathological, biochemical and molecular levels.

In harmony with previous reports, administration of MTX caused a marked decrease in body weight, attributed to intestinal mucositis that resulted in decreased food consumption in addition to diarrhea,[36] both of which we noticed in the current study. Administration of *C. pentandra*, and to a lesser extent silymarin, mitigated this MTX-forced body weight loss, and the animals seemed alert and even gained weight after MTX administration. This prevention of weight loss could reflect the previously reported activities of *C. pentandra* and silymarin as anti-inflammatory,

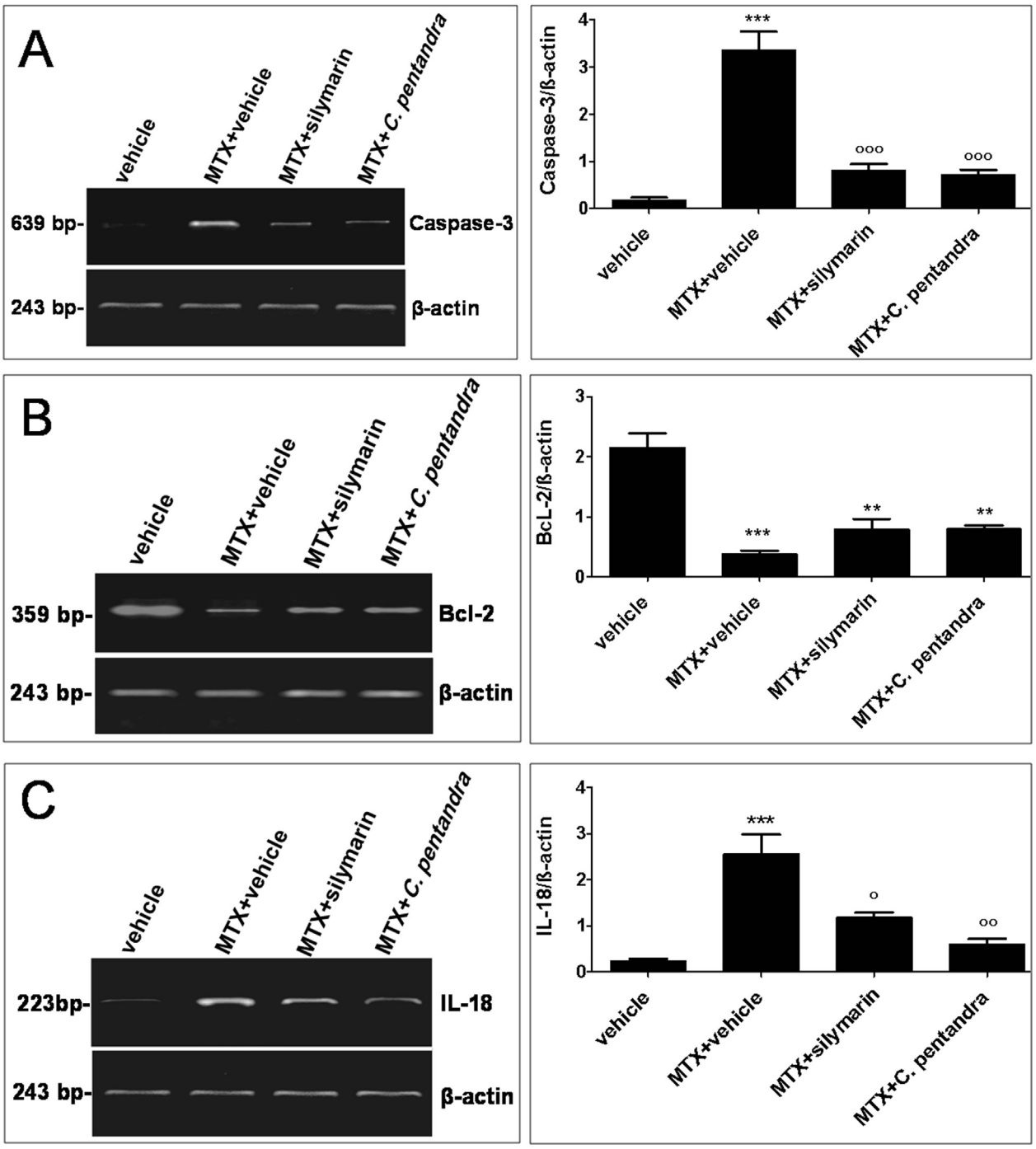

Fig. 3. Expression of caspase-3 (A), *Bcl-2* (B) and IL-18 (C) in renal tissue homogenates of the vehicle (control), MTX + vehicle, MTX + silymarin and MTX + *C. pentandra* groups at the end of the experiment, assessed at the mRNA level by RT-PCR. Expression of the β-actin gene was detected in parallel as an internal control. The right panels represent corresponding quantification of each gel analysis measured by ImageJ software and expressed as a β-actin ratio. Data are presented as the mean ± SD (n = 3). * and ○ indicate significant differences with the control and MTX + vehicle, respectively. * and ○ indicate significance at $p < 0.05$; ** and ○○ indicate significance at $p < 0.01$; *** and ○○○ indicate significance at $p < 0.001$.

antioxidant and antidiarrheal agents that inhibited the direct toxicity of MTX to the animal intestinal mucosa. We also proposed that *C. pentandra* and silymarin hindered the indirect adverse effects of MTX on the gastrointestinal tract (inadequate food intake and exhaustive energy expenditure), leading to a decreased internal hormones secretion and resulting in diminished trophic effects to the mucosa.[9,37]

We observed numerous signs of MTX-induced nephrotoxicity, in agreement with previous results, including a marked perturbation in biochemical nephrocyte injury markers.[38] For example, compared to healthy control rats, those exposed to MTX showed more than twofold higher levels of serum creatinine, cystatin C, and BUN, and a more than tenfold higher level of microalbuminuria, suggesting significant renal impairment. *C. pentandra* administration ameliorated these signs of MTX-induced renal impairment, as indicated by the marked decreases in the overall estimated

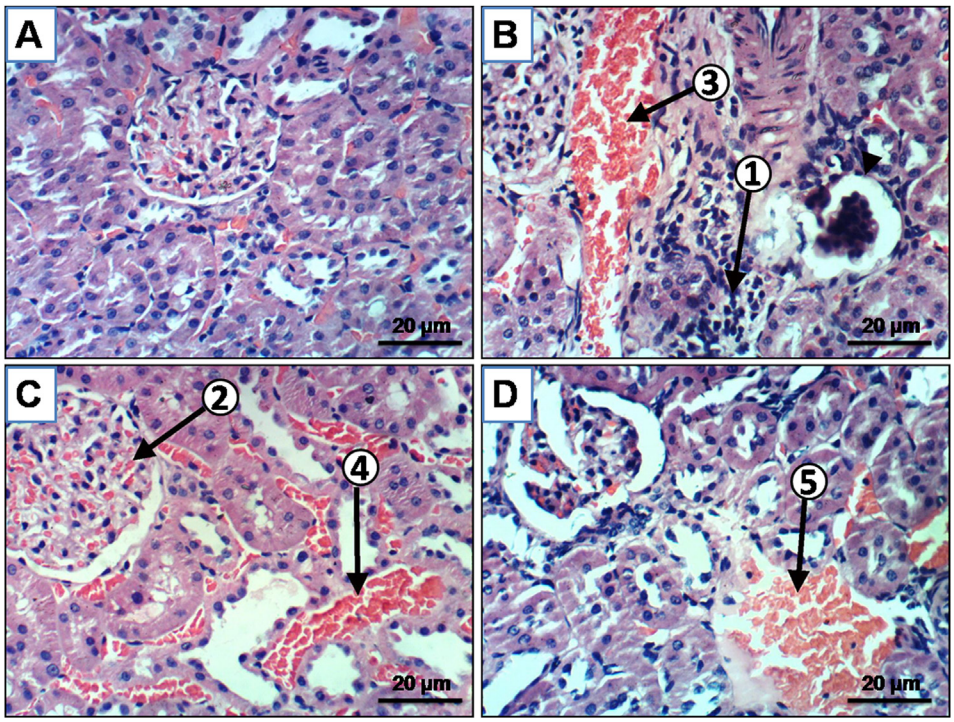

Fig. 4. Representative H&E stained renal tissue sections from (A) control, (B) MTX + vehicle, (C) MTX + silymarin, and (D) MTX + *C. pentandra* groups, (scale bar 400×). Arrows refer to histopathological findings: (1) focal interstitial nephritis and atrophy of glomerular tuft; (2) congestion of glomerular tufts; (3–5) congestion of renal blood vessel. Intended for color reproduction on the Web.

biochemical indices of renal functions, with a nonsignificant superiority over silymarin (Table 1). No significant changes were recorded in the examined parameters between those animals which received very high doses of the extract and a corresponding healthy control which received only the suspending CMC vehicle (data not shown).

Our findings were consistent with the results of several previous reports concerning the possible involvement of oxidative stress in the pathogenesis of MTX-induced renal damage. MTX administration strongly reversed the oxidant/antioxidant balance in renal tissue, manifesting as a significant increase in the lipid peroxidation products MDA and NO with a marked depletion in GSH level and the antioxidant enzymes SOD and CAT. These effects may be attributed to MTX-induced mitochondrial dysfunction and oxidative stress via interaction with molecular oxygen, that initiates a cascade of consecutive reactions that liberates reactive oxygen and nitrogen species; these alterations deplete the tissue antioxidants and consequently result in disturbed oxidant/antioxidant balance, resulting in tissue injury.[39]

C. pentandra improved renal tissue antioxidant capacity and reduced the concomitant oxidative and nitrosative stress, as indicated by elevations in GSH levels and SOD activity, the tenfold increase in CAT activity, and the decreases in the tissue levels of MDA and NO compared to those of MTX-treated rats (Table 2). Results of our *in vitro* DPPH$^{\bullet}$ radical-scavenging assay strongly supported the antioxidant findings, as the ethyl acetate fraction of *C. pentandra* demonstrated robust antioxidant potential with a comparable IC_{50} value compared to those of ascorbic acid (a standard natural antioxidant) and BHA (a standard synthetic antioxidant). This result could be attributed to the isolated polyphenols (**1**–**6**, Fig. 5), which exert robust antioxidant and radical-scavenging capacities.[26–34,40] The effects of *C. pentandra* were comparable to those of silymarin, which has extensively been reported as a strong natural antioxidant.[2,32]

In addition to its disturbance of the oxidant/antioxidant equilibrium, MTX may aggravate inflammation and cause apoptotic cell death via free radical oxidation.[3,36] As expected, MTX administration, in the present study, initiated an inflammatory response, as illustrated by the approximately five- and threefold increases in the circulating levels of CRP and TNF-α, respectively (Table 1). MTX also increased the mRNA levels of the proinflammatory cytokine IL-18 and the proapoptotic protein caspase-3 in kidney homogenates with a concomitant diminished renal expression of the anti-apoptotic gene *Bcl-2* (Fig. 3). These inflammatory and apoptotic trends could be attributed to the MTX-induced oxidative stressors that have been reported to trigger the intrinsic mitochondrial-dependent apoptotic pathway, leading to membrane phospholipids damage and loss of mitochondrial membrane potential.[3,35]

Interestingly, the MTX-induced alterations in inflammatory and apoptotic modulators were strongly attenuated in the *C. pentandra* group and, to a lesser extent, in the silymarin group (Fig. 3 and Table 1). These findings are consistent with other studies that indicated that herbal formulations containing polyphenols, similar to silymarin and those isolated from *C. pentandra* (**1**–**6**, Fig. 5), have anti-inflammatory and antiapoptotic activities, either directly by affecting the inflammatory and apoptotic signaling pathways or indirectly through their strong antioxidant potential.[9,40]

Moreover, the present study provides an additional evidence for the apoptosis-linked nephroprotective activity of *C. pentandra*. Urinary KIM-1 is a type I transmembrane protein that is minimally detectable in healthy kidney tissue but is strongly overexpressed in dedifferentiated proximal tubule epithelial cells after ischemic or toxic insults.[41] In agreement with other studies that identified KIM-1 as a specific and sensitive biomarker of early proximal tubule injury caused by a variety of xenobiotics, including MTX,[41] we showed that MTX administration resulted in substantially higher urinary levels of KIM-1 than those found in the healthy controls, suggesting considerable tubular injury (Table 1). This could be

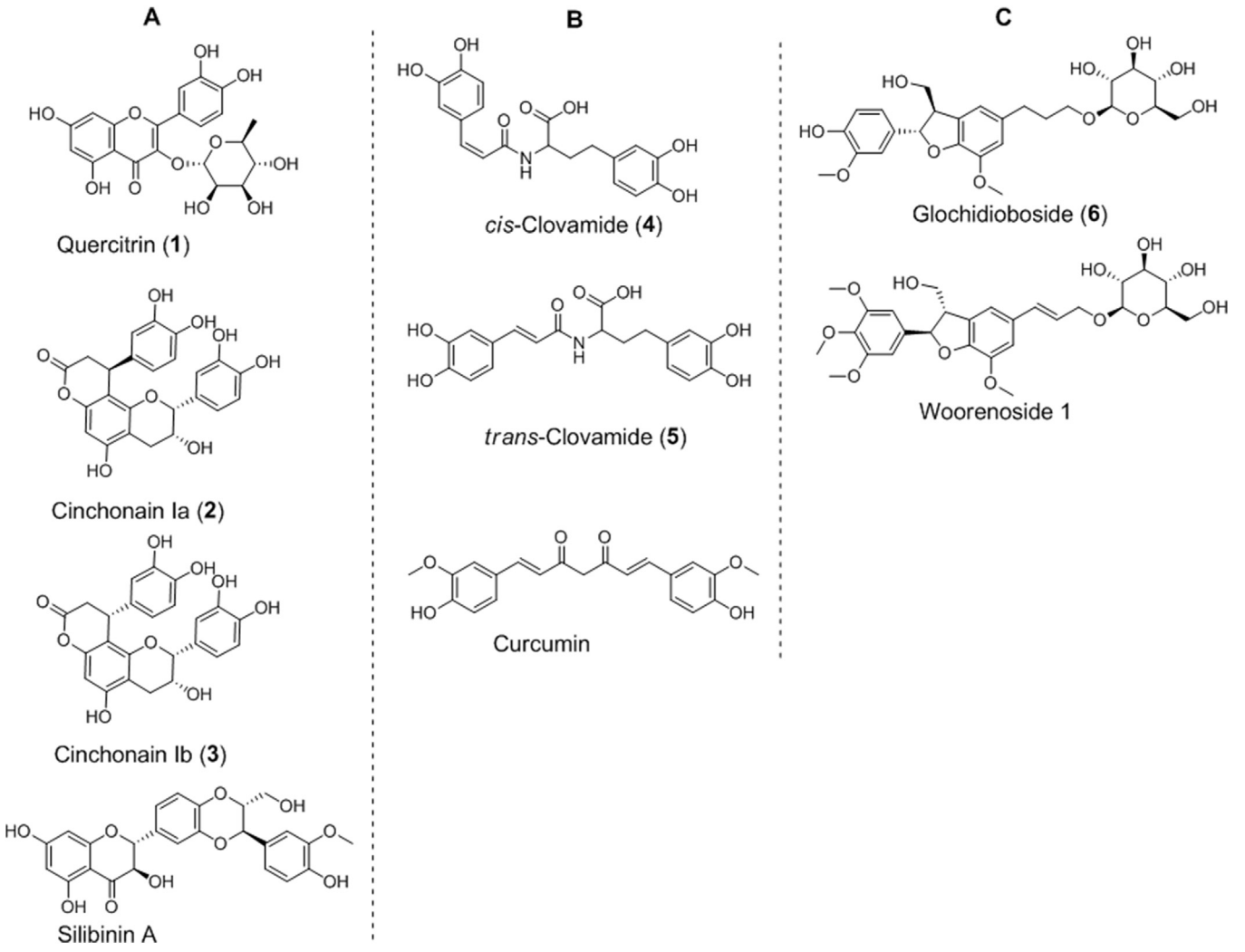

Fig. 5. (A) Structures of the isolated quercitrin (**1**) and the flavonolignans cinchonains 1a (**2**) and 1b (**3**) compared with silibinin A, the main component of the standard nephroprotective silymarin.[44] (B) Structures of the isolated phenylpropanoids *cis*-clovamide (**4**) and *trans*-clovamide (**5**) compared with the nephroprotective structural analogue curcumin.[45] (C) Structure of the isolated neolignane glochidioboside (**6**) compared with the anti-inflammatory structural analogue woorenoside 1.[46]

attributed to KIM-1 important roles in developmental remodeling, regulation of immune responses, and tissue homeostasis in acute renal injury via induction of phagocytosis of apoptotic and necrotic cells in the injured kidney tubules.[42] This process must be rapid and efficient to avoid the occurrence of secondary (postapoptotic) necrosis that leads to membrane disruption and leakage of proinflammatory intracellular contents into the tissue. During this event, the ectodomain of KIM-1 is proteolytically cleaved by metalloproteinase from damaged cells and released into urine at detectable levels.[42] *C. pentandra* markedly reduced KIM-1 urinary levels, significantly more than silymarin (Table 1). This reduction is most likely due to the more powerful direct antiapoptotic potential of *C. pentandra*, leading to the abolishment of post-apoptotic consequences such as phagocytosis of apoptotic and necrotic cells by KIM-1. However, these herbal products may also counteract the post-apoptotic consequences indirectly, blocking urinary KIM-1 release through scavenging oxidative stressors.

Our study also provides evidence for the nephroprotective effects of *C. pentandra*. MTX administration led to a marked deterioration in kidney tissue architecture. Similar histopathological alterations have been previously announced in acute MTX-induced nephrotoxicity.[43] Supplementation with *C. pentandra* substantially ameliorated the MTX-induced pathological alterations in the kidney and was more effective than silymarin, which did not significantly attenuate all the harmful effects of MTX; vacuolation and congestion of glomerular tufts and intertubular renal blood vessels remained observable in kidney tissues of this group (Fig. 4).

5. Conclusion

The present study demonstrates, for the first time, that *C. pentandra* alleviates MTX-induced kidney damage, and is likely effective as the established antioxidant silymarin. The nephroprotective effect of *C. pentandra* may be attributed to its antioxidant, antiapoptotic and anti-inflammatory activities. This is supported by the structural similarities of the six phenolics (**1**–**6**) isolated from *C. pentandra* to established antioxidant, anti-inflammatory and nephroprotective compounds [silymarin, curcumin and woorenoside 1, (Fig. 5). However, further studies are in demand to expand our understanding of this effect and to build a foundation from which to extend the cytoprotective capacity of *C. pentandra* to other organotoxicities.

Funding

We confirm that this research did not receive any specific grant

from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors thank Dr. Adel Kholoussy, Professor of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt for performing the histopathological examinations. Additionally, we acknowledge Dr. Essam Youssef, Associate Professor of Horticulture, Faculty of Agriculture, Assiut University, Egypt for his assistance in the identification and authentication of the supplied plant materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.08.006.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

1. Jerman S, Sun Z. Using zebrafish to study kidney development and disease. *Curr Top Dev Biol*. 2017;124:41–79.
2. Dabak DO, Kocaman N. Effects of silymarin on methotrexate-induced nephrotoxicity in rats. *Ren Fail*. 2015;37(4):734–739.
3. Vardi N, Parlakpinar H, Ates B, Cetin A, Otlu A. The protective effects of Prunus armeniaca L (apricot) against methotrexate-induced oxidative damage and apoptosis in rat kidney. *J Physiol Biochem*. 2013;69(3):371–381.
4. Heidari-Soreshjani S, Asadi-Samani M, Yang Q, Saeedi-Boroujeni A. Phytotherapy of nephrotoxicity-induced by cancer drugs: an updated review. *J Nephropathology*. 2017;6(3):254–263.
5. Gaikwad K, Dagle P, Choughule P, Joshi Y, Kadam V. A review on some nephroprotective medicinal plants. *Int J Pharm Sci Res*. 2012;3:2451–2454.
6. Rameshrad M, Razavi BM, Hosseinzadeh H. Protective effects of green tea and its main constituents against natural and chemical toxins: a comprehensive review. *Food Chem Toxicol*. 2017;100:115–137.
7. Chambers CS, Holeckova V, Petraskova L, et al. The silymarin composition... and why does it matter??? *Food Res Int*. 2017;100(Pt 3):339–353.
8. Neha Jaggi AS, Singh N. Silymarin and its role in chronic diseases. *Adv Exp Med Biol*. 2016;929:25–44.
9. Elumalai A, Mathangi N, Didala A, Kasarla R, Venkatesh Y. A Review on Ceiba pentandra and its medicinal features. *Asian J Pharm Techno*. 2012;2(3):83–86.
10. Refaat J, Desoky SY, Ramadan MA, Kamel MS. Bombacaceae: a phytochemical review. *Pharm Biol*. 2013;51(1):100–130.
11. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol*. 1995;28(1):25–30.
12. Lorke D. A new approach to practical acute toxicity testing. *Arch Toxicol*. 1983;54(4):275–287.
13. Hemeida RA, Mohafez OM. Curcumin attenuates methotraxate-induced hepatic oxidative damage in rats. *J Egypt Natl Cancer Inst*. 2008;20(2):141–148.
14. Alcaraz-Contreras Y, Mendoza-Lozano RP, Martinez-Alcaraz ER, et al. Silymarin and dimercaptosuccinic acid ameliorate lead-induced nephrotoxicity and genotoxicity in rats. *Hum Exp Toxicol*. 2016;35(4):398–403.
15. Bairwa NK, Sethiya NK, Mishra SH. Protective effect of stem bark of Ceiba pentandra linn. against paracetamol-induced hepatotoxicity in rats. *Pharmacogn Res*. 2010;2(1):26–30.
16. Bairwa NK, Sethiya NK, Mishra S. Protective effect of stem bark of Ceiba pentandra Linn. against paracetamol-induced hepatotoxicity in rats. *Pharmacogn Res*. 2010;2(1):26.
17. Elion Itou RDG, Morabandza CJ, Etou Ossibi AW, Louningou GF, Nsondé Ntandou FG, Abena AA. Possible mechanisms of anti-inflammatory and analgesic effects of the aqueous extracts of *Ceiba pentandra* Gaertn (Bombacaceae) and *Chromolaena odorata* L (King and Robinson) (Astéraceae) in rat. *Int J Herb Med*. 2018;6(1):20–25.
18. Itou RDGE, Sanogo R, Ossibi AWE, et al. Anti-inflammatory and analgesic effects of aqueous extract of stem bark of Ceiba pentandra Gaertn. *Pharmacol Pharm*. 2014;5(12):1113.
19. Muhammad HL, Kabiru AY, Busari MB, et al. Acute oral toxicity study of ethanol extract of Ceiba pentandra leaves as a glucose lowering agent in diabetic rats. *J Acute Dis*. 2016;5(3):237–243.
20. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem*. 1978;86(1):271–278.
21. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82(1):70–77.
22. Sun L, Peterson TE, McCormick ML, Oberley LW, Osborne JW. Improved superoxide dismutase assay for clinical use. *Clin Chem*. 1989;35(6):1265–1266.
23. Sinha AK. Colorimetric assay of catalase. *Anal Biochem*. 1972;47(2):389–394.
24. Montgomery H, Dymock J. *Determination of Nitrite in Water*. vol. 86. Science Park, Milton Rd: Royal Soc Chemistry Thomas Graham House; 1961:414–&. Cambridge CB4 0WF, Cambs, England.
25. Banchroft JD, Stevens A, Turner DR. In: *Theory and Practice of Histoloicl Techniques*. fourth ed. New york, London, San Francisco, Tokyo: Churchil Livingstone; 1996.
26. Bose S, Maji S, Chakraborty P. Quercitrin from Ixora coccinea leaves and its anti-oxidant activity. *J PharmaSciTech*. 2013;2(2):72–74.
27. Hsue-Fen C, Tanaka T, Nonaka G-I, Fujioka T, Mihashi K. Phenylpropanoid-substituted catechins from Castanopsis hystrix and structure revision of cinchonains. *Phytochemistry*. 1993;33(1):183–187.
28. Aderogba M, Kapche G, Mabusela W. Isolation and characterization of antioxidative constituents of ceiba pentandra (Kapok) leaves extract. *Niger J Nat Prod Med*. 2014;17(1):86–90.
29. Takeda Y, Mima C, Masuda T, Hirata E, Takushi A, Otsuka H. Glochidioboside, a glucoside of (7S, 8R)-dihydrodehydrodiconiferyl alcohol from leaves of Glochidion obovatum. *Phytochemistry*. 1998;49(7):2137–2139.
30. Choi Y-J, Kang J-S, Park JHY, Lee Y-J, Choi J-S, Kang Y-H. Polyphenolic flavonoids differ in their antiapoptotic efficacy in hydrogen peroxide–treated human vascular endothelial cells. *J Nutr*. 2003;133(4):985–991.
31. Osawa T. *Nephroprotective and Hepatoprotective Effects of Curcuminoids. The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease*. Springer; 2007:407–423.
32. Shahbazi F, Dashti-Khavidaki S, Khalili H, Lessan-Pezeshki M. Potential renoprotective effects of silymarin against nephrotoxic drugs: a review of literature. *J Pharm Pharm Sci*. 2012;15(1):112–123.
33. Zamperone A, Pietronave S, Colangelo D, et al. Protective effects of clovamide against H2O2-induced stress in rat cardiomyoblasts H9c2 cell line. *Food Funct*. 2014;5(10):2542–2551.
34. Zeng H, Locatelli M, Bardelli C, et al. Anti-inflammatory properties of clovamide and Theobroma cacao phenolic extracts in human monocytes: evaluation of respiratory burst, cytokine release, NF-kappaB activation, and PPARgamma modulation. *J Agric Food Chem*. 2011;59(10):5342–5350.
35. Hassanein EHM, Mohamed WR, Shalkami AS, Khalaf MM, Hemeida RAM. Renoprotective effects of umbelliferone on methotrexate-induced renal injury through regulation of Nrf-2/Keap-1, P38MAPK/NF-kappaB, and apoptosis signaling pathways. *Food Chem Toxicol*. 2018;116(Pt B):152–160.
36. de Araujo AA, Borba PB, de Souza FH, et al. In a methotrexate-induced model of intestinal mucositis, olmesartan reduced inflammation and induced enteropathy characterized by severe diarrhea, weight loss, and reduced sucrose activity. *Biol Pharm Bull*. 2015;38(5):746–752.
37. Sasu A, Herman H, Mariasiu T, et al. Protective effects of silymarin on epirubicin-induced mucosal barrier injury of the gastrointestinal tract. *Drug Chem Toxicol*. 2015;38(4):442–451.
38. Flombaum CD, Liu D, Yan SQ, et al. *Management of Patients with Acute Methotrexate Nephrotoxicity with High-Dose Leucovorin. Pharmacotherapy*. 2018.
39. Heidari R, Ahmadi A, Mohammadi H, Ommati MM, Azarpira N, Niknahad H. Mitochondrial dysfunction and oxidative stress are involved in the mechanism of methotrexate-induced renal injury and electrolytes imbalance. *Biomed Pharmacother*. 2018;107:834–840.
40. Crasci L, Lauro MR, Puglisi G, Panico A. Natural antioxidant polyphenols on inflammation management: anti-glycation activity vs metalloproteinases inhibition. *Crit Rev Food Sci Nutr*. 2018;58(6):893–904.
41. Bukowinski AJ, Mizuno T, Fukuda T, et al. Kidney Injury Molecule-1 and its association with delayed clearance and drug exposure in pediatric oncology patients treated with high dose methotrexate. *J Clin Oncol*. 2015;33(15_suppl l), 10034-10034.
42. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule–1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J Clin Investig*. 2008;118(5):1657.
43. Caglar Y, Ozgur H, Matur I, et al. Ultrastructural evaluation of the effect of N-acetylcysteine on methotrexate nephrotoxicity in rats. *Histol Histopathol*. 2013;28(7):865–874.
44. Gaedeke J, Fels L, Bokemeyer C, Mengs U, Stolte H, Lentzen H. Cisplatin nephrotoxicity and protection by silibinin. *Nephrol Dial Transplant*. 1996;11(1):55–62.
45. Nasri H, Abedi-Gheshlaghi Z, Rafieian-Kopaei M. Curcumin and kidney protection; current findings and new concepts. *Acta Persica*. 2018;1.
46. Cho JY, Baik KU, Yoo ES, Yoshikawa K, Park MH. In vitro antiinflammatory effects of neolignan woorenosides from the rhizomes of Coptis japonica. *J Nat Prod*. 2000;63(9):1205–1209.