



Protective effect of *Cupressus macrocarpa* and *Dioon edule* against gentamicin-induced nephrotoxicity in rats

[Efecto protector de *Cupressus macrocarpa* y *Dioon edule* contra la nefrotoxicidad inducida por gentamicina en ratas]

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Abstract

Context: It has been reported that *Cupressus macrocarpa* Hartw. ex Gordon (*Cupressaceae*) and *Dioon edule* Lindl. (*Zamiaceae*) contain compounds with antioxidant and anti-inflammatory effects; therefore, they may exert a nephroprotective effect.

Aims: To explore the renoprotective effect of *C. macrocarpa* and *D. edule* and the underlying molecular mechanisms.

Methods: Ten male Wister rats were used for each group of control (1% CMC in saline), gentamicin (GM, 40 mg/kg), *D. edule* leaves methanol extract (DLM), *C. macrocarpa* leaves combined petroleum ether and methanol extract (CLEM) and *C. macrocarpa* roots methanol extract (CRM). Rats were orally administered the extracts (250 mg/kg) daily concomitantly for 9 days with GM. Evaluation of kidney function, histopathology and measuring renal AMPK- α 1, NF- κ B and iNOS genes expression were carried out. There were no symptoms of systemic adverse effects in any treated group.

Results: Blood urea nitrogen (mg/dL) was significantly reduced by CLEM (24.0 ± 1.205) and CRM (22.889 ± 1.250). Creatinine (mg/dL) was significantly decreased by CLEM (0.59 ± 0.022) and CRM (0.580 ± 0.029). Gene expression of AMPK- α 1 enhanced by CLEM (16.717 ± 0.430), CRM (19.18 ± 0.871), DLM (0.417 ± 0.020). iNOS was downregulated by CLEM (0.9 ± 0.056), CRM (1.53 ± 0.044), and DLM (1.642 ± 0.050). NF- κ B was decreased by CLEM (1.148 ± 0.076), CRM (0.918 ± 0.065), and DLM (1.783 ± 0.071). CRM and CLEM exhibited normal to mild renal degeneration and mild to moderate expression of iNOS.

Conclusions: *C. macrocarpa* extracts exhibited significant renoprotective effect and could be considered as a new promising strategy against renal complications.

Keywords: AMPK- α 1; blood urea nitrogen; creatinine; iNOS; NF- κ B.

Resumen

Contexto: Se ha informado que *Cupressus macrocarpa* Hartw. ex Gordon (*Cupressaceae*) y *Dioon edule* Lindl. (*Zamiaceae*) contienen compuestos con efectos antioxidantes y antiinflamatorios; por lo tanto, pueden ejercer un efecto nefroprotector.

Objetivos: Explorar el efecto renoprotector de *C. macrocarpa* y *D. edule* y los mecanismos moleculares subyacentes.

Métodos: Se utilizaron diez ratas Wister macho para cada grupo de control (1% de CMC en solución salina), gentamicina (GM, 40 mg / kg), extracto metanólico de hojas de *D. edule* (DLM), extracto combinado de éter de petróleo y metanol de hojas de *C. macrocarpa* (CLEM) y extracto metanólico de raíces de *C. macrocarpa* (CRM). A las ratas se les administraron por vía oral los extractos (250 mg/kg) diariamente, de forma concomitante con GM, durante 9 días. Se evaluaron función renal, histopatología y medición de la expresión de genes AMPK- α 1, NF- κ B e iNOS renales. No hubo síntomas de efectos adversos sistémicos en ningún grupo tratado.

Resultados: El nitrógeno ureico en sangre (mg/dL) se redujo significativamente por CLEM ($24,0 \pm 1,205$) y CRM ($22,889 \pm 1,250$). La creatinina (mg/dL) disminuyó significativamente en CLEM ($0,59 \pm 0,022$) y CRM ($0,580 \pm 0,029$). Expresión génica de AMPK- α 1 aumentó para CLEM ($16,717 \pm 0,430$), CRM ($19,18 \pm 0,871$) y DLM ($0,417 \pm 0,020$). iNOS fue regulado negativamente por CLEM ($0,9 \pm 0,056$), CRM ($1,53 \pm 0,044$) y DLM ($1,642 \pm 0,050$). NF- κ B se redujo en CLEM ($1,148 \pm 0,076$), CRM ($0,918 \pm 0,065$) y DLM ($1,783 \pm 0,071$). CRM y CLEM exhibieron degeneración renal normal a leve y una expresión leve a moderada de iNOS.

Conclusiones: Los extractos de *C. macrocarpa* exhibieron un efecto renoprotector significativo y podrían considerarse como una nueva estrategia prometedora contra las complicaciones renales.

Palabras Clave: AMPK- α 1; creatinine; iNOS; NF- κ B; nitrógeno ureico sanguíneo.

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Abbreviations: AMPK- α 1: Adenosine monophosphate-activated protein kinase alpha 1-subunit; CLEM: *Cupressus macrocarpa* leaves combined petroleum ether and methanol extract; CMC: Carboxymethyl cellulose; CRM: *Cupressus macrocarpa* roots methanol extract; Ct: Threshold cycle; DLM: *Dioon edule* leaves methanol extract; GM: Gentamicin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase is a housekeeping gene; iNOS: Inducible nitric oxide synthase; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; RCN: Relative copy number.

INTRODUCTION

The awareness of complementary and alternative medicine (CAM) has increased over the past decade. The argument about the efficiency of alternative methods has attracted a great deal of attention among many medical professionals (Ali, 2015). Different studies have proposed that numerous extracts of natural and dietary antioxidants could protect the kidney against oxidative stress injuries affecting glomerular and tubular functions (Vargas et al., 2018).

Cupressus macrocarpa Hartw. ex Gordon. (Cupressaceae) and *Dioon edule* Lindl. (Zamiaceae) are gymnosperms (Singh, 2010). *C. macrocarpa* or Monterey cypress is an evergreen monoecious tree with a narrow and pyramid shape when young that elongates with age to reach a height of 20 m. Twigs emanate a scent of citronella when pressed. The leaves are scale-like, opposite and decussate, densely crowded, rhombic-ovate, and 1-2 mm long (Gut, 2008). This plant contains monoterpenes, sesquiterpenes, diterpenes and biflavonoids (Carman and Sutherland, 1979; Cool, 2005; Al-Sayed and Abdel-Daim, 2014; Salem et al., 2018). *D. edule* is a dioecious palm-like shrub with aerial or subterranean pachycaul, cylindrical stems, and usually many leaves. Their leaf bases are persistent or shedding to leave smooth bark in different species (Singh, 2006). Both plants were suggested to have a nephroprotective activity. The present study tested the nephroprotective effect of the total extract of *C. macrocarpa* leaves, which contain major compounds of volatile oil (monoterpenes and sesquiterpenes) as well as flavonoids and biflavonoids. The total extract of roots was tested for the first time and there is no report about its active constituents.

Cupressufalvone is a biflavonoid isolated from *C. macrocarpa*, which exerted hepato- and nephroprotective effects in mice against carbon tetrachloride-induced toxicity (Al-Sayed and Abdel-Daim, 2014).

D. edule leaves contain phenolic compounds and flavonoids with antioxidant activity (Moawad and Al Amir, 2016), which in turn may exhibit a nephroprotective effect through acting against reactive oxygen species (ROS) in the diseased kidneys.

Gentamicin (GM) is an aminoglycoside antibiotic that is commonly used against gram-negative bacteria and is an existing therapeutic agent against *Pseudomonas*, *Proteus* and *Serratia* microorganisms that are resistant to other antibiotics. It can cause ototoxicity and nephrotoxicity, which can severely restrict its use (Krause et al., 2016). All aminoglycosides, including GM, are freely filtered across the glomerulus. They are partly taken up by the proximal tubular cells where they are concentrated, leading to renal damage (Lopez-Novoa et al., 2011).

A pronounced mechanism of GM nephrotoxicity is the cumulative production of mitochondrial reactive oxygen species (ROS), resulting in a deficiency in intrinsic antioxidant enzymes. High levels of ROS are capable of causing injury to many cellular molecules, including proteins, lipids, and nucleic acids, thus impairing cell function and leading to cell death (Bustos et al., 2016). In addition, GM nephrotoxicity participates in mesangial and vascular contraction and contributes to inflammation, which is identified by the infiltration of various inflammatory cells such as monocytes and macrophages. These immune cells release different pro-inflammatory cytokines, such as IL-1 and TNF- α , and trigger the nuclear factor kappa B (NF- κ B) pathway (Jaikumkao et al., 2016).

Therefore, the current study was conducted to investigate the reno-protective role of different extracts of leaves and roots of *C. macrocarpa* and leaves of *D. edule* against gentamicin-induced nephrotoxicity in rats. The underlying molecular mechanisms of these natural extracts regarding the expression of adenosine monophosphate-activated protein kinase alpha 1-subunit (AMPK α 1), nuclear factor kappa-light-chain-enhancer of activated B cells

(NF- κ B) and inducible nitric oxide synthase (iNOS) as inflammatory regulators should be clarified.

MATERIAL AND METHODS

Plant material and preparation of extracts

Cupressus macrocarpa leaves, branchlets and roots were obtained from a nursery at Shebin Elkom City (30°33'2.02"N, 31°0'45.21"E), while *Dioon edule* leaves were collected from Shebin el-Kanater of Qalyubia Governorate (30°18'47.88"N, 31°19'17.04"E). They were collected in May 2017. Both species were identified by Prof. Dr. Mohammed Ibrahim Fotoh, Professor of Ornamental Horticulture and Landscape Design. Voucher specimens (PG00411) and (PG00412) were kept at Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Tanta University. The maceration method was used to obtain all extracts. The dried leaves with branchlets (2 kg) of *C. macrocarpa* were extracted first with 6 L of petroleum ether for 3 days (7.5% yield) and then with 5 L of 95% methanol for another 3 days (6.2% yield). Both extracts were evaporated under reduced pressure. Roots (1 kg) of *C. macrocarpa* and leaves of *D. edule* (2 kg) were percolated in 3 and 5 L of methanol 95%, respectively, for 3 days and then concentrated under reduced pressure to give a crude dry extract of 7.25% and 8.47% yield, respectively.

Preparation of leaf ether extract of young and older trees of C. macrocarpa

Dried and powdered leaves (100 g) of two and seven years old trees of *C. macrocarpa* were macerated at room temperature in diethyl ether (250 mL). Then, the supernatant was evaporated under reduced pressure, leaving volatile compounds and other components. Both extracts were subjected to GC-MS analysis.

GC-MS analysis

GC-MS analysis of the leaves ether extract of the young and older trees of *C. macrocarpa* was carried out using a Perkin-Elmer model (PerkinElmer, Waltham, Massachusetts, USA) with acquisition parameters as follows: Oven with initial temperature 50°C for 3 min, ramp 7°C/min to 280°C, hold 5 min,

inj = 280°C, volume = 1; injector volume 1 μ L, split = 20:1, carrier gas = He, solvent delay = 3.00 min, transfer temperature = 280°C, source temperature = 200°C, scan: 50 to 600 Da, Rxi-5SilMS column 30 m, 0.25 mm ID, 0.25 μ m.

Chemicals

Gentamicin was purchased from EIPICO (Egypt). Saline solution was obtained from Al Mottahedda Pharma Co. Egypt. Carboxymethyl cellulose, diethyl ether, hematoxylin, eosin, xylene, streptavidin-biotin and diaminobenzidine were purchased from Sigma -Aldrich Co., USA. Methanol 95 %, ethanol, petroleum ether (60-80°C) and hydrogen peroxide were obtained from El-Nasr Pharmaceutical Co., Egypt.

Animals and experimental design

The experimental protocol was approved by the Research Ethical Committee of the Faculty of Pharmacy, Tanta University, Egypt (FPTU-REC, 103/2018/420). Fifty adult male Wistar rats weighing 180 - 200 g were purchased from the animal facility of Giza Institute of Ophthalmology Cairo, Egypt. Rats were housed in wire cages for 2 weeks under identical environmental conditions for adaptation and allowed *ad libitum* access to a balanced laboratory diet and water. Rats were fed standard pellet chow (El- Nasr Chemical Company, Cairo, Egypt), and were housed in normal temperature and normal dark/light cycle.

After the acclimatization period, rats were divided into five equal groups. Group 1 (control group) rats received orally 1 mL/rat of the vehicle (1% carboxymethyl cellulose in saline solution). Group 2 (GM group) rats were injected intraperitoneally daily with 1 mL of gentamicin 40 mg/kg body weight for 9 successive days (El-Ashmawy et al., 2018b). The induction of nephrotoxicity was adjusted by a pilot study using different doses of gentamicin. Different extracts, including methanol extract of *D. edule* leaves (DLM, Group 3), leaves combined petroleum ether and methanol extracts of *C. macrocarpa* (CLEM, Group 4) and methanol extract of *C. macrocarpa* roots (CRM, Group 5), were prepared in 1% carboxymethyl cellulose in saline. Rats

were orally administered the different extracts suspensions at 250 mg/kg body weight in 1 mL/rat daily for 9 days simultaneously with injection of GM. The suitable dose was determined by a pilot experiment using different doses of the different extracts.

Sample collection

At the end of the experiment, rats were anesthetized under light diethyl ether, and then blood was withdrawn *via* cardiac puncture. The serum was collected and stored at -80°C until analysis. Rats were sacrificed by cervical dislocation, and the kidneys were removed immediately. Right kidneys were fixed in 10% buffered formalin solution at room temperature for histopathological and immunohistochemical studies. Left kidneys were sectioned and stored at -80°C under liquid nitrogen for gene expression analysis.

Determination of kidney biomarkers

The following biomarkers were assessed using commercial kits obtained from Biodiagnostic Co. (Egypt). Blood urea nitrogen (BUN) levels were measured colorimetrically with the urease-Berthlot method (Fawcett and Scott, 1960). Serum creatinine levels were assayed colorimetrically according to a method described by (Slot, 1965).

Gene expression of AMPK α 1-subunit, iNOS and NF- κ B by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from frozen renal tissues using a miRNeasy Mini Kit (Qiagen®, USA) according to the manufacturer's instructions. The concentration and purity of the obtained RNA were detected spectrophotometrically at 260/280 nm using a ScanDrop Nano-volume spectrophotometer (Analytik Jena®, Italy). The extracted RNA was then reverse transcribed into complementary DNA (cDNA) using Applied Biosystems Inc.® USA. The obtained cDNA was amplified using a SensiFAST™ SYBR No-ROX kit (Bioline, USA) as described by the manufacturer.

Sequences of primers (Invitrogen Co., USA) used for RT-qPCR were as follows: adenosine monophosphate-activated protein kinase α 1 subunit (AMPK α 1) gene forward primer: (5'-GGTCCTGGTGGTTTCTGTTG-3'), reverse primer: (5'-ATGATGTCAGATGGTGAATT-3'); inducible nitric oxide synthase (iNOS) gene forward primer: (5'-GATGAGAAGCTGAGGCCAG-3'), reverse primer: (5'-TAGCGCTTCCGACTTTCCTG-3'); nuclear factor kappa B (NF- κ B) gene forward primer: (5'-GAACTTGTGGGGAAGGACTG-3'), reverse primer: (5'-GGGGTTATTGTTGGTCTGGA-3'); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward primer: (5'-TGGACCACCCAGCCAGCAAG-3'), reverse primer: (5'-GGCCCCTCCTGTTGTTATGGGGT-3').

The PCR program was adjusted to start with initial activation for 2 min at 94°C, followed by 45 cycles (94°C for 5 sec, 62°C for 10 sec, and 72°C for 20 sec) using a Pikoreal 5100 RT-qPCR system (Thermo Fisher Scientific Co., Finland). Each sample was analyzed and normalized to the level of the reference gene (GAPDH) and expressed as a relative copy number (RCN). The threshold cycle (Ct) values of the samples were calculated, and transcript levels were analyzed by the $2^{-\Delta\Delta C_t}$ method (Rao et al., 2013).

Histopathological examination of kidney tissue

Kidney tissues were cut into 3- μ m-thick sections using a microtome (Leica RM 2135, Germany), deparaffinized and hydrated in a descending series of ethanol. Sections were stained with hematoxylin and eosin (H&E), dehydrated and cleared in xylene (Anan et al., 2016). The slides were then inspected for tubular and glomerular structures by a blinded expert pathologist using a light microscope (Olympus Electron Microscope, Olympus, Japan) and photographed. A minimum of six fields for each kidney section was examined and categorized by the severity of changes (brush border loss, tubular degeneration and glomerular atrophy) by an observer blinded to the treatments of the animals using scores of not detected (-), mild (+), moderate (++) and severe (+++).

Immunohistochemical staining of iNOS

Paraffin blocks were deparaffinized in xylene, dehydrated in a graded alcohol series, and incubated in a solution of 3% hydrogen peroxide (H₂O₂) for 10 min to block endogenous peroxidase. Tissue sections were incubated with primary antibody for iNOS (Lab Vision, Fremont, CA, USA). The antigen-antibody complex was visualized by a labeled streptavidin-biotin followed by diaminobenzidine as a chromogen. All sections were examined blindly by a pathologist using a photomicroscope (Leica DM 500, Heerbrugg, Switzerland) with an image analysis system under 200× magnification. For semi-quantitation of iNOS, histo-score (H-score) was applied according to (Samaka et al., 2014). The slides were inspected by a light microscope (Olympus Electron Microscope, Olympus, Japan) and photographed by a digital camera (Olympus DP27, Japan) at 200× magnification.

Statistical analysis

Analysis of data was performed using the Statistical Package for Social Science (SPSS) software version 22 (Duricki et al., 2016). Data are presented by the mean $\pm$ standard error of the mean (SEM). Significance was set at $p < 0.05$ with $n = 10$ per group. Statistical comparison among groups was performed by one-way analysis of variance (ANOVA) using Fisher's least-significant differences (LSD) method for comparison between two groups.

RESULTS

GC-MS analysis

GC-MS analysis was performed to the diethyl ether extract of leaves, and the major active constituents were recognized. The active compounds in GC-MS analysis were abietane diterpenes as 13-isopropyl-podocarpa-6, 13-diene (cupressene) and 13-methyl-13-vinyl podocarp-7-en-3-one. Kaurane diterpenes were found as kaur-16-ene, kaur-15-ene and 2,3-dihydro-5,6-dimethoxy-3-methyl-1H-indene-1-one. Ferruginol, a phenolic diterpene, was

detected and monoterpenes as γ -terpinene, limonene, α -phellandrene, camphor, α -terpinene, in addition to the presence of citronellol and citronellyl butyrate.

They are listed in Tables 1 - 2 and demonstrated in Figs. 1 - 3.

Effect of treatment on kidney biomarkers

Serum blood urea nitrogen (BUN) and creatinine levels, markers of kidney injury, were increased significantly ($p < 0.05$) to 32.333 ± 2.699 mg/dL and 0.700 ± 0.015 mg/dL, respectively, in the GM group compared to those in the control group (26.000 ± 0.633 mg/dL and 0.547 ± 0.019 mg/dL, respectively). The CLEM and CRM treated groups exhibited significant reductions in BUN to 24.000 ± 1.205 mg/dL and 22.889 ± 1.250 mg/dL ($p < 0.05$) and creatinine levels to 0.590 ± 0.022 mg/dL ($p < 0.05$) and 0.580 ± 0.029 mg/dL ($p < 0.001$), respectively, compared to the GM group (Fig. 4). Serum BUN and serum creatinine in the CLEM- and CRM-treated groups were not significantly different from their corresponding values in the control group. On the other hand, treatment with methanol extract of *D. edule* leaves non-significantly decreased BUN (28.333 ± 0.500 mg/dL, $p < 0.05$) and creatinine levels (0.673 ± 0.032 mg/dL, $p < 0.05$) compared to the gentamicin treatment.

Effect on AMPK α 1-subunit, iNOS and NF- κ B gene expression in kidney tissue

Fig. 5A shows that GM significantly ($p < 0.05$) decreased AMPK α 1-subunit gene expression compared to the control. On the other hand, AMPK α 1-subunit gene expression was increased significantly at (16.717 ± 0.43) and (19.18 ± 0.871) of CLEM and CRM extracts, respectively compared to that of GM-treated rats. Treatment with DLM extract was non-significantly upregulated AMPK α 1-subunit gene expression compared to GM treatment.

Fig. 5B shows that GM significantly ($p < 0.05$) up-regulated iNOS gene expression by a 2.2-fold increase compared to the control.

Table 1. Major compounds in GC-MS analysis of leaves ether extract of *Cupressus macrocarpa* (two years old tree).

RT	Peak area %	Compound name
6.985	1.338	α -Phellandrene
7.160	0.653	α -Pinene
8.130	1.144	γ -Terpinene
8.521	0.403	Myrcene
9.381	4.271	O-Cymene
9.491	7.998	Limonene
10.181	21.685	α -Terpinene
14.343	0.692	Carvone
19.185	0.435	Methyl carbamate phenol
24.507	0.727	Octadecyne
26.273	0.510	1,3-Dimethyladamantene
28.334	1.253	3,7,11,15-Tetramethyl-2-hexadecan-1-ol
30.039	1.297	Kaur-16-ene
30.595	4.878	2,3-Dihydro-5,6-dimethoxy-3-methyl-1H-Indene-1-one
31.155	3.948	Ferruginol
32.615	4.610	Podocarp-7-en-3-one-13-methyl-13-vinyl
33.67	1.290	Kauren-18-ol
36.107	0.717	Cholesterol
36.967	0.603	Urs-12-ene-28-ol
38.488	1.302	1-(Ethenyloxy)-octadecane
40.539	5.032	Docosene

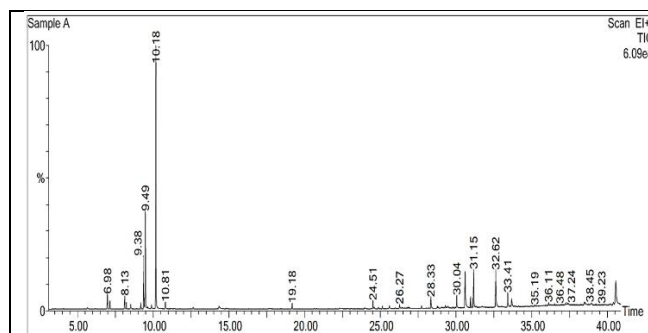
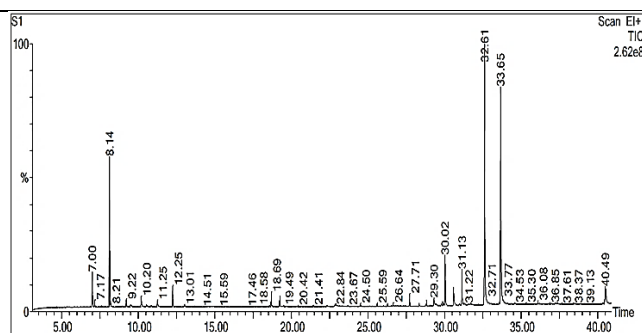
**Figure 1.** GC-MS analysis of leaves ether extract of *Cupressus macrocarpa* (two years old).**Figure 2.** GC-MS analysis of leaves ether extract of older tree of *Cupressus macrocarpa* (seven years old).

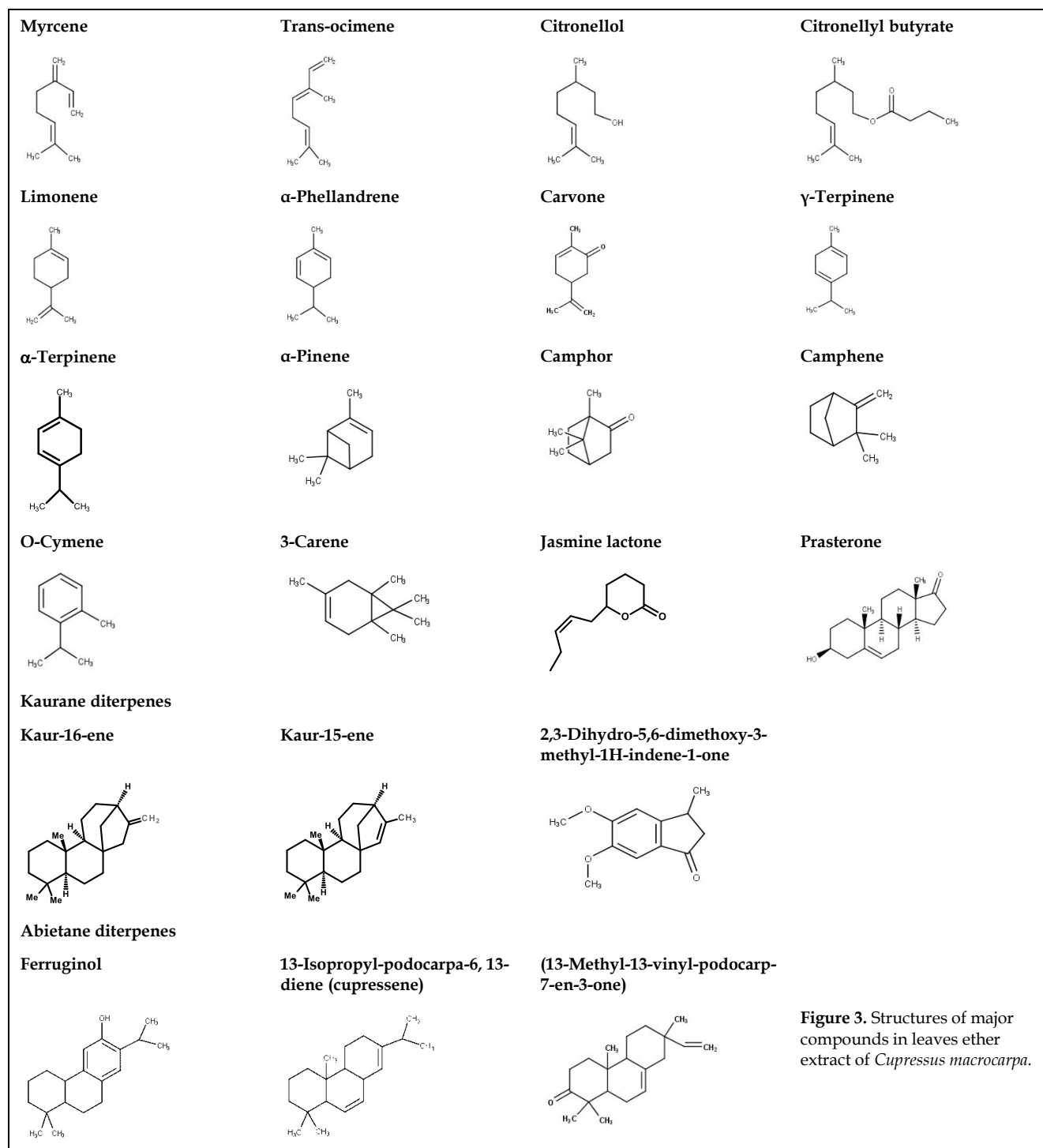
Table 2. Major compounds in GC-MS analysis of leaves ether extract of *Cupressus macrocarpa* (seven years old tree).

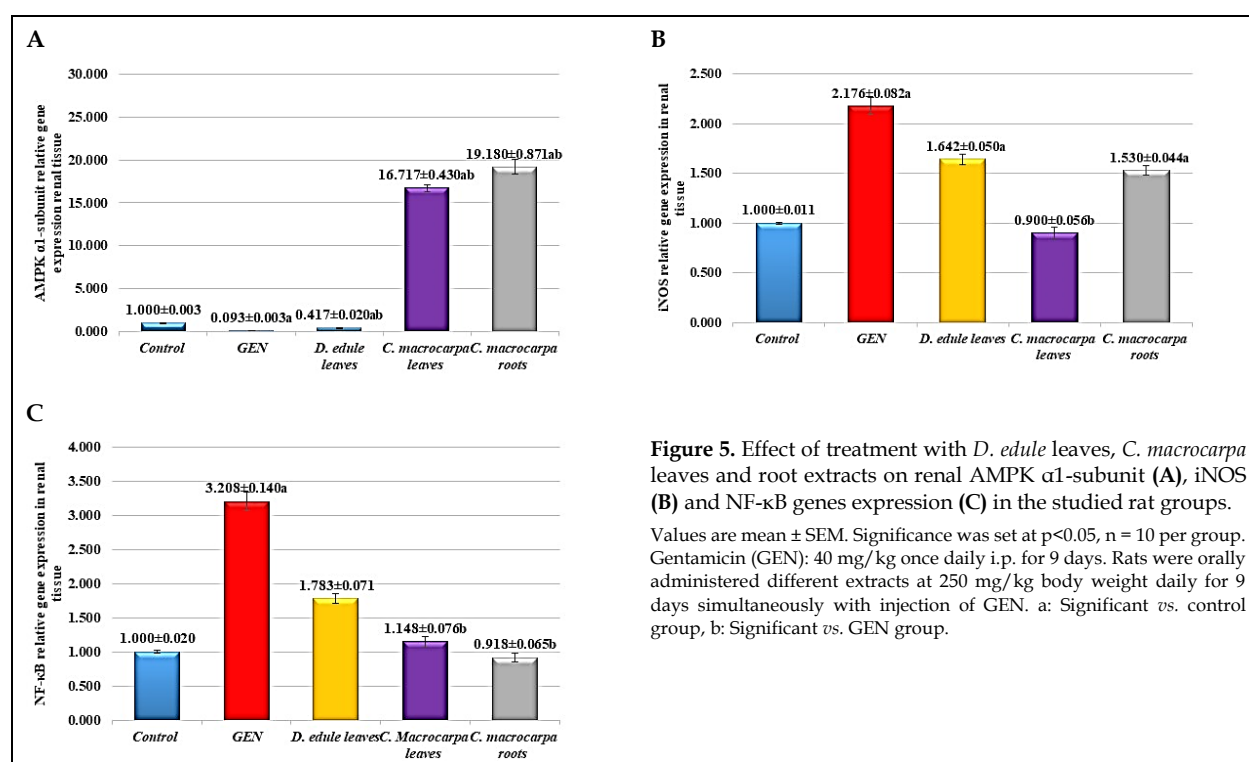
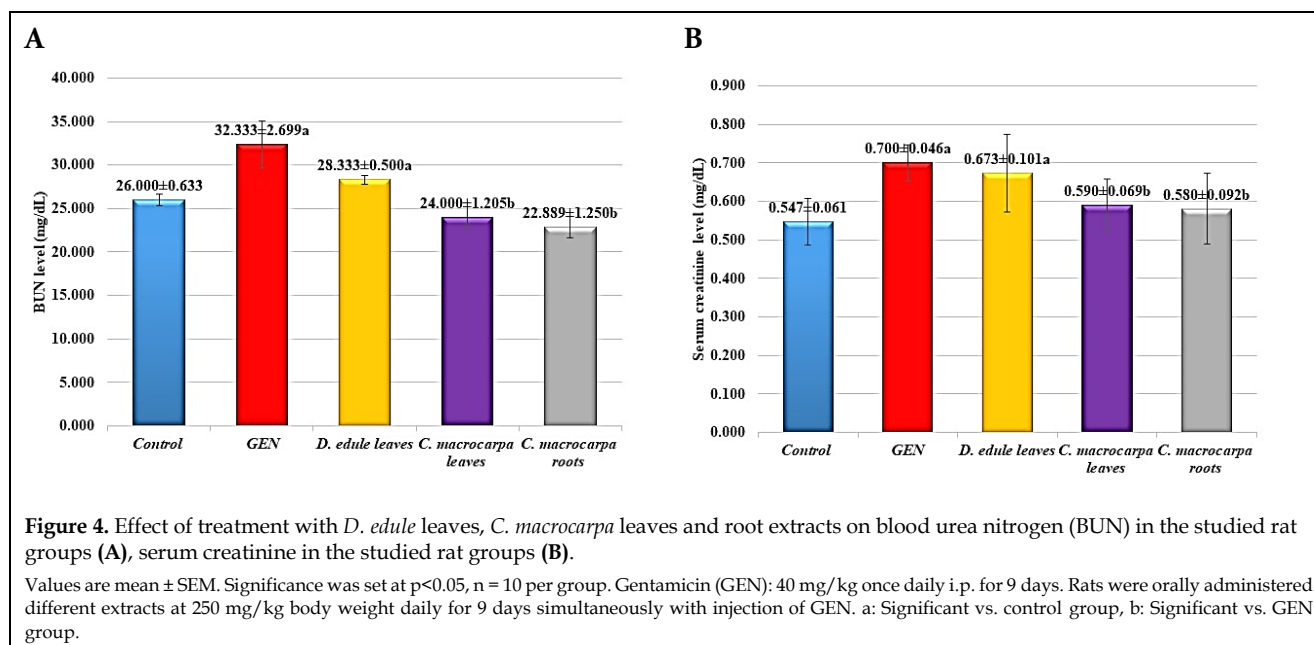
RT	Peak area %	Compound name
7.005	2.652	α -Phellandrene
7.170	0.324	Trans-ocimene
8.135	9.625	γ -Terpinene
9.221	0.526	Limonene
10.196	0.876	α -Terpinene
12.252	1.111	Camphor
18.690	0.814	Camphene
24.497	0.166	Citronellol
25.593	0.177	3-Carene
26.638	0.229	Citronellyl butyrate
27.713	0.674	13-Isopropyl-podocarpa-6,13-diene
28.809	0.374	Kaur-16-ene
29.304	0.844	3,7,11,15-Tetramethyl hexadeca-1,3,6,10,14-pentaene
29.464	0.203	Jasmine lactone
29.874	0.194	Kaur-15-ene
30.024	3.337	Naphthalene derivative
30.575	1.971	2,3-dihydro-5,6-dimethoxy-3-methyl-1H-Indene-1-one
31.135	2.377	Ferruginol
32.610	20.354	Podocarp-7-en-3-one-13-methyl-13-vinyl
33.651	19.542	Prasterone
36.077	0.199	Cholesterol

Table 3. Semi-quantitative scoring of renal lesions within different treatment groups.

Groups	Renal degeneration and necrosis	Tubular regeneration	Inflammation	Fibrosis	Extent of lesions
Control	-	-	-	-	-
GM	+++	+	++++	+++	+++
<i>D. edule</i> leaves extract	++	++	+++	++	++
<i>C. macrocarpa</i> leaves extract	+	+++	+	+	++
<i>C. macrocarpa</i> roots extract	+	+++	+	+	+

A minimum of six fields was examined in each kidney section and categorized by the severity of changes by an observer blinded to the treatments of the animals using scores of means no detectable lesions (-), mild lesions (+), moderate lesions (++), severe focal lesions (+++), and severe diffuse lesions (++++). Gentamicin (GM): 40 mg/kg once daily i.p. for 9 days. Rats were orally administered different extracts at 250 mg/kg body weight daily for 9 days simultaneously with injection of GM.





On the other hand, iNOS gene expression in rats treated with DLM and CRM extracts was significantly ($p < 0.05$) increased by 1.6- and 1.5-fold at values of (1.642 ± 0.05) and (1.53 ± 0.044), respectively, compared that in the control rats. The highest significant downregulation of iNOS gene expression was found in treated rats with CLEM (0.9 ± 0.056)

compared to GM-treated rats (2.17 ± 0.082). Treatment with DLM extract non-significantly downregulated iNOS gene expression compared to GM treatment.

Fig. 5C shows that GM significantly ($p < 0.05$) up-regulated NF-κB gene expression by a 3.2-fold increase compared to the control. On the other hand,

NF- κ B gene expressions in rats treated with DLM, CLEM and CRM were downregulated compared to that in GM-treated rats. Significant downregulation of NF- κ B gene expression was found in CLEM (1.148 ± 0.076) and CRM (0.918 ± 0.065)-treated rats compared to that in the GM-treated rats (3.208 ± 0.14).

Histopathology of kidney tissue

Kidney sections from the control group showed a normal histological structure of the glomeruli and renal tubules (Fig. 6A). Kidney sections from the GM group showed hypercellular lobulated glomeruli with wide Bowman space, degenerated tubules and cloudy swelling. Moreover, interstitial diffuse dense mononuclear cellular infiltration was observed in the GM group, which indicated interstitial nephritis (Fig. 6B). Kidney sections from rats treated with DLM extract showed a moderate degree of interstitial nephritis associated with periglomerular and peritubular fibrosis (Fig. 6C). The kidneys from the CLEM extract-treated group showed a mild degree of renal tubular epithelial degeneration and normal Bowman space and cellularity (Fig. 6D). On the other hand, kidney sections from the CRM extract-treated group showed normal structure of the glomeruli and renal tubules without mononuclear cellular infiltration in the interstitial space (Fig. 6E). A semi-quantitative assessment of the histopathological findings within the treated groups is demonstrated in Table 3.

Immunohistochemical staining of iNOS

Positive staining for iNOS, a marker of oxidative stress, was identified by a brown color. The degree of staining for iNOS in kidney sections from the normal control group (Fig. 7A) was comparable to that in sections from the CRM extract-treated group (Fig. 7E), where both showed mild expression of iNOS within the renal tubules. iNOS staining of kidney sections in the GM group was significantly more evident than that in normal controls with a marked expression of iNOS within the renal tubules (Fig. 7B). Kidney sections from the DLM and CLEM extract groups showed interrupted moderate and mild positive staining, respectively (Fig. 7C for DLM and Fig. 7D for CLEM), with a reduction

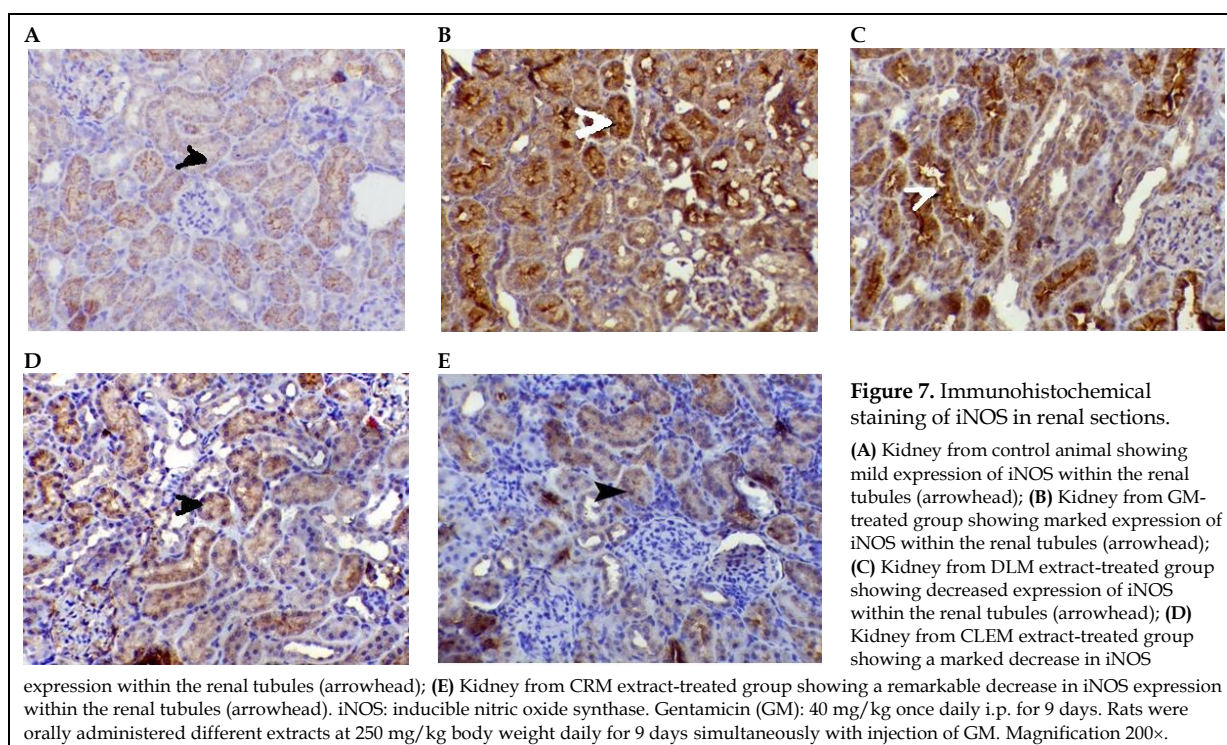
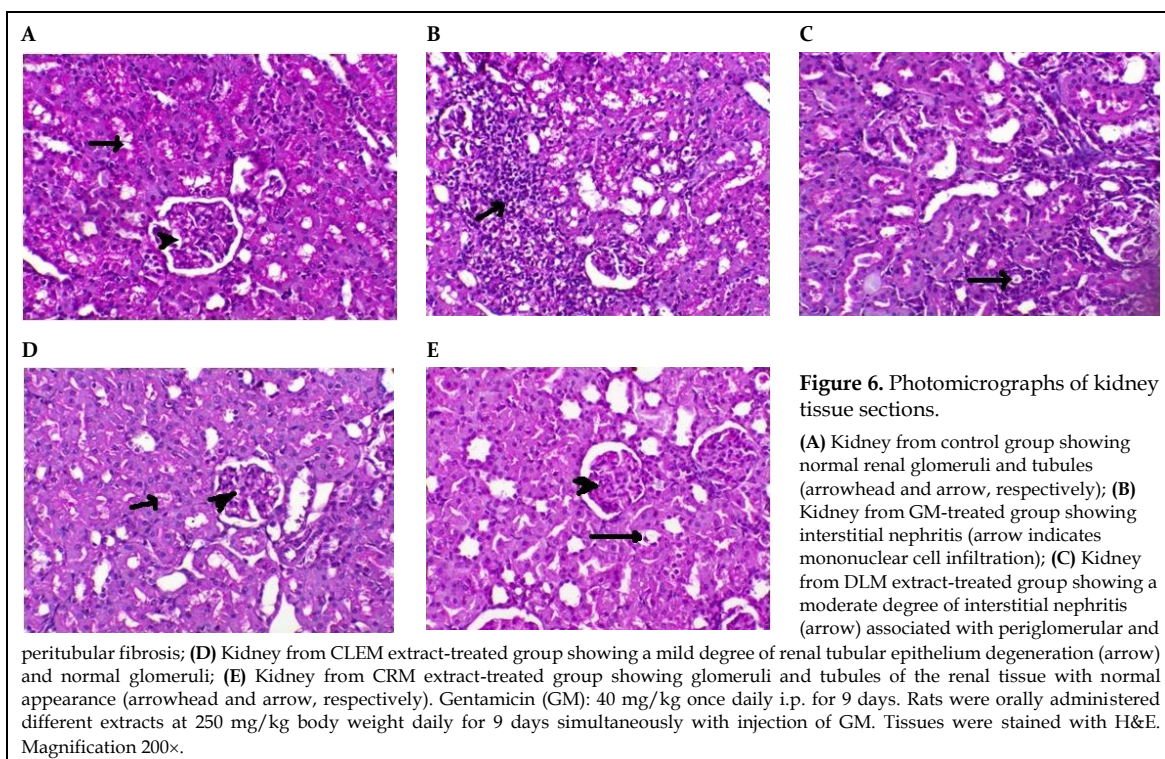
in the degree of iNOS staining compared to those from the GM group (Fig. 7B). A significant ($p < 0.05$) decrease in iNOS staining was found in the CRM and CLEM extract-treated groups compared to that in the GM group. A comparison of the degree of iNOS immunostaining among the studied groups is demonstrated in Fig. 8.

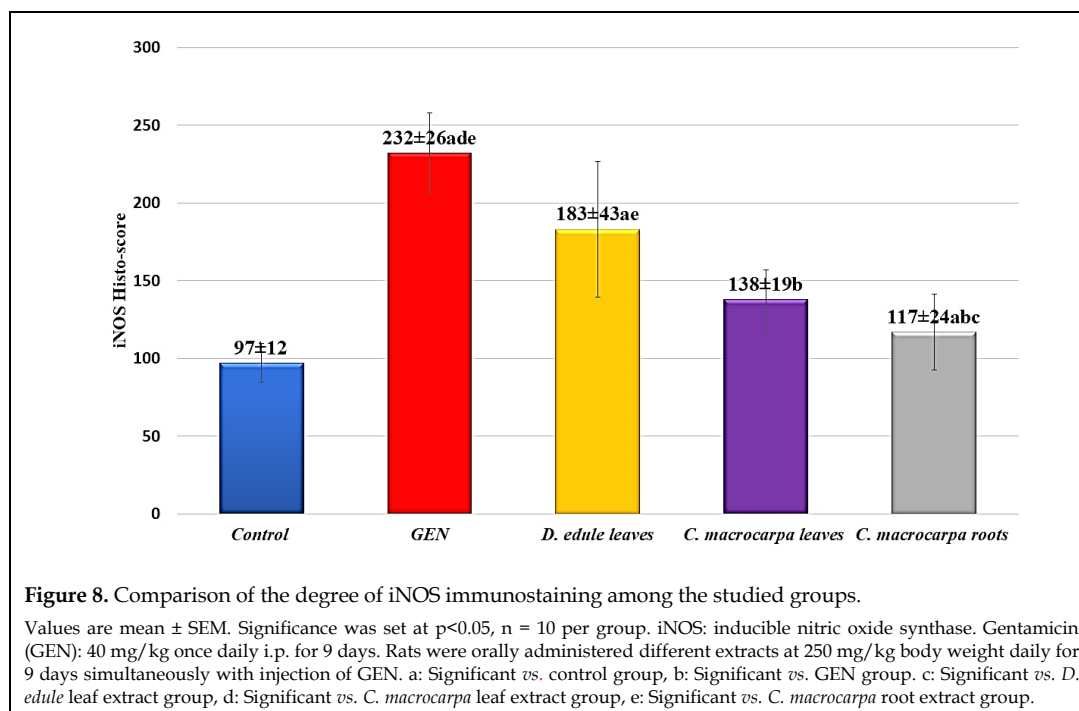
DISCUSSION

The kidney is more predisposed to toxicity than any other organ in the body. This process is a result of high levels of toxins being delivered to it. The use of natural products is increasingly encouraged in health care presently. Some studies showed that natural and dietary antioxidants could protect the kidney against oxidative stress damage to glomerular and tubular functions (Orr and Bridges, 2017). The current study compared the nephroprotective efficacy at the most effective dose of *Cupressus macrocarpa* leaf and root extracts as well as *Dioon edule* leaf extract against GM-induced nephrotoxicity in rats. Elucidation of the underlying molecular mechanisms was figured out for the first time. A pilot experiment was done using different doses of the different extracts, and it was found that the best effective dose was 250 mg/kg body weight of *C. macrocarpa* extracts.

GM-induced nephrotoxicity was demonstrated by a significant decline in renal function. GM significantly increased BUN and serum creatinine levels compared to the control. These biochemical alterations were supported by the histopathological results, which exhibited hypercellular, lobulated glomeruli with extensive Bowman spaces and diffuse dense mononuclear cellular penetration representing nephritis. The GM group also exhibited severe brush border loss, tubular degeneration, and glomerular atrophy.

In agreement with our results, several studies revealed that GM caused renal toxicity and elevated both serum creatinine levels and BUN in patients and animal models (El-Ashmawy et al., 2018b). It was found that GM disturbs the glomeruli, promoting an increase in their size, variation in their round shape and density, and a widespread swelling of the filtration barrier (Arif and Nihalani, 2013).





The present work showed that treatment with leaves combined petroleum ether and methanol extracts (CLEM) and roots methanol extract of *C. macrocarpa* (CRM) significantly improved kidney indices and alleviated kidney injury induced by GM injection. Both treatments decreased serum BUN and creatinine. The protective effect of *C. macrocarpa* leaves and roots extracts on kidney function, observed herein, were supported by the histopathological results, which indicated decreased brush border loss, tubular degeneration, glomerular atrophy and leukocytic infiltration in both treated groups. In this regard, *C. macrocarpa* root extract exhibited a better protective effect than *C. macrocarpa* leaf extract.

GC-MS analysis of two-years-old tree diethyl ether extract of dried leaves demonstrated that the major active constituents were α -terpinene, 13-methyl-13-vinyl-podocarp-7-en-3-one, limonene, Ocymene, ferruginol and α -phellandrene.

This study was designed to assess the nephroprotective effect of the dried leaf extract of seven-years-old trees of *C. macrocarpa*. The diethyl ether extract was analyzed with GC-MS to explore some of the major active constituents of leaves extract. The active compounds in GC-MS analysis were 13-

methyl-13-vinyl-podocarp-7-en-3-one, γ -terpinene, phellandrene, camphor, α -terpinene, limonene, ferruginol and 13-isopropyl-podocarpa-6, 13-diene, in addition to the presence of citronellol and citronellol butyrate.

The significant nephroprotective effect of *C. macrocarpa* could be due to cupressuflavone, abietane diterpenes as 13-isopropyl-podocarpa-6, 13-diene (cupressene) and 13-methyl-13-vinyl podocarp-7-en-3-one, phenolic diterpenes as ferruginol and monoterpenes as γ -terpinene and limonene. Cupressuflavone was isolated from methanol extract of leaves (Al-Sayed and Abdel-Daim, 2014), while other compounds were detected in the diethyl ether extract of leaves using GC-MS analysis. Cupressuflavone exerted an anti-inflammatory effect as well as a hepato-nephroprotective effect (Al-Sayed and Abdel-Daim, 2014; Al-Sayed et al., 2018). Abietane diterpenes were reported to have a nitric oxide inhibitory effect (Hu et al., 2018). Phenolic diterpenes were also reported to have anti-inflammatory and antioxidant effects (Hassani et al. 2016). γ -Terpinene and limonene exhibited strong antioxidant effects (Abou El-Soud et al., 2014).

One of the crucial regulators of the inflammatory protective mechanism when treating nephrotoxi-

city is AMPK (López et al., 2016). The AMPK signaling pathway plays an important role in cellular and organismal survival throughout stress by preserving metabolic homeostasis. Low-grade, chronic inflammation has a critical role in the pathogenesis of metabolic syndrome. AMPK can decrease nuclear factor- κ B (NF- κ B) system activation, a controller of innate immunity and inflammation (Salminen et al., 2011). To further focus on AMPK, the correlation between AMPK gene expression and NF- κ B gene expression was studied. In the present investigation, treatment with *C. macrocarpa* leaves or root extracts individually induced AMPK gene expression and suppressed NF- κ B gene expression, while the highest AMPK gene expression was obtained in *C. macrocarpa* root-treated rats. Our results showed that co-treatment with *D. edule* leaf extract down-regulated AMPK gene expression in renal tissue.

NF- κ B is an oxidative stress-sensitive transcription factor that triggers transcription of different pro-inflammatory genes, including COX-2, interleukin-6 (IL-6), IL-1b, and tumor necrosis factor- α (TNF- α) (El-Ashmawy et al., 2018a). Induced gene expression of NF- κ B found in the renal tissue of rats with GM-induced nephrotoxicity supports the role of GM-derived inflammation as a factor contributing to alteration of the kidney, favoring the development of nephrotoxicity (Abd-Elhamid et al., 2018). This finding could explain the data obtained in the current study, which showed the upregulation of NF- κ B in kidney tissue treated with gentamicin. Interestingly, inhibition of NF- κ B and activation of AMPK by *C. macrocarpa* extracts may potentiate the anti-inflammatory pathway and explain the observed improvement in the kidneys of rats treated with *C. macrocarpa* extracts in our study. This research is the first to study the impact of *C. macrocarpa* extracts on AMPK and NF- κ B gene expression.

In the present study, nephrotoxicity induction with GM significantly increased renal upregulation of iNOS gene expression. This effect were in accordance with (Morsy et al., (2014), who reported that GM could induce activation of the iNOS enzyme and increase the production of NO in renal tissue. However, simultaneous treatment with CLEM or CRM reversed the effect of GM. Arias-Salvatierra et

al. (2011) reported that blocking NF- κ B activation suppresses transcription of the iNOS gene. Administration of either CLEM or CRM as monotherapy suppressed iNOS expression and decreased nephrotoxicity scores. Furthermore, we examined the effect of DLM on GM-induced nephrotoxicity. Regarding the results obtained from biochemical and gene expression analysis, the histopathological and immunohistochemical analysis indicated that oral treatment of rats with DLM produced no significant effect on nephrotoxicity.

CONCLUSIONS

Cupressus macrocarpa leaves and root extracts were found to be the best examples for using natural extracts as nephroprotective agents. They exhibited a significant effect against gentamicin-induced nephrotoxicity, especially root extract. They increase the gene expression of AMPK and simultaneously reduce the expression of NF- κ B and iNOS, which explains the underlying possible molecular mechanism of action. It was found that abietane diterpenes, ferruginol, γ -terpinene, limonene, cupressuflavone present in leaves of *C. macrocarpa* could be responsible for the anti-inflammatory and antioxidant effects. Further clinical prospective studies are needed to study the effect of *C. macrocarpa* extracts in the treatment of nephrotoxicity, as well as extensive phytochemical investigations will be needed to explore the constituents responsible for the remarkable effect of roots.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Al-Ashmawy GM	El-Sherbeni SA
Concepts or ideas	x	x
Design	x	x
Definition of intellectual content	x	x
Literature search	x	x
Experimental studies	x	x
Data acquisition	x	x
Data analysis	x	x
Statistical analysis	x	
Manuscript preparation	x	x
Manuscript editing	x	x
Manuscript review	x	x

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