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Circulating microRNAs as novel biomarkers for measuring the potency of ginger extract against cyclophosphamide toxicity in rat renal tissues: molecular and histopathological study

S.A. GABR[1], W.M. ELSAED[1], M.A. ELADL[2], G.A. GHONIEM[3], M. EL-SHERBINY[4], K.S. EL-BAYOUMI[1,5], H. ABOUHISH[6], A.M. DESOUKY[7], M.M. ABDEL-AZIZ[8], M. ELDESOQUI[1,4], M. ELSHAFEY[1,9], H.A. EBRAHIM[10], N.S. NOSSEIR[11,12], A.M.R. EL-SAYED[8]

[1]Department of Anatomy and Embryology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[2]Department of Basic Medical Sciences, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates
[3]Department of Food Industries, Faculty of Agriculture, Mansoura University, Mansoura, Egypt
[4]Department of Basic Medical Sciences, College of Medicine, AlMaarefa University, Riyadh, Saudi Arabia
[5]Department of Basic Medical Sciences, Ibn Sina University for Medical Sciences, Amman, Jordon
[6]Department of Clinical Pharmacology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[7]Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[8]Gastrointestinal Surgery Center, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[9]Department of Physiological Sciences, Fakeeh College for Medical Sciences, Jeddah, Saudi Arabia
[10]Department of Basic Medical Sciences, College of Medicine, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia
[11]Department of Anatomy and Embryology, Faculty of Medicine, Suez University, Suez, Egypt
[12]Department of Biomedical Sciences, College of Medicine, Gulf Medical Universty (GMU), Ajman, United Arab Emirates

Abstract. – OBJECTIVE: This study aims to explore underlying molecular variations in the expression of miRNAs in kidney tissues of ginger-treated and non-treated cyclophosphamide (CP)-intoxicated rats.

MATERIALS AND METHODS: A total of 40 adult male Wistar rats were randomly divided into four groups of 10 each: Group I (control: received normal food and water), Group II (received ginger at a dose of 300 mg/kg), Group III (received CP 75 mg/kg, i.p.), and Group IV (received the same dose of CP and ginger extract). Rats received a single injection of 75 mg/kg CP on days 3, 4, 5, 19, 20, and 21. In CP-intoxicated rats, the treatment with ginger extract at a dose of 300 mg/kg was received by oral gavage starting seven days before CP and continuing throughout the duration of the experiment for four weeks. Molecular variations in the expression of miRNAs, apoptotic genes, histological kidney damage, and abnormal kidney function in control, ginger, and CP-intoxicated rats were identified by using real-time RT-PCR Analysis, immunohistochemical, and colorimetric assays. In addition, HPLC analysis and liquid chromatography spectrophotometry analysis using Diphenyl-1-picrylhydrazyl (DPPH) radical, and B-Carotene-linoleic acid reagents were applied respectively for *in-vitro* screening of phytoconstituents and antioxidant activity for ginger extract.

RESULTS: The kidney tissues of CP-intoxicated rats displayed an increase in lipid peroxidation marker malonaldehyde (MDA), DNA damage, and fibrosis markers like hyaluronic acid (HA) and hydroxyproline Hypx) with a decrease in the superoxide dismutase (SOD) and total antioxidant capacity (TAC).

In addition, molecular expressions of mRNA fibrotic genes such as collagen, type 1, alpha 1 (COL1A1), and α-smooth muscle actin (αSMA). Molecular expressions of levels of B-cell lymphoma 2 (BCl-2) mRNA gene were down-regulated, and the expression of mRNA apoptotic; BCL2 associated X gene (Bax), caspase-3, Bax/BCl-2 ratio genes were significantly up-regulated respectively. Moreover, cellular oxidative genes, erythroid 2-related factor (Nrf2), and heme oxygenase-1 (HO-1) were down-regulated, respectively. The miR-155-5p, miR-34a-5p, miR-21-5p significantly increased while the miR-193b-3p, miR-455-3p, and miR-342-3p significantly decreased. Ginger also increased the expression of Nrf2, HO-1, and BCl-2 genes in the kidneys of rats induced with CP. In addition, active phytoconstituents, particularly 6]]-shogaol

Corresponding Author: Sami A. Gabr, MD; e-mail: dr.samigabr@gmail.com; samiali@mans.edu.eg

and 6]]-gingerol, were significantly identified in ginger extract using HPLC analysis. Antioxidant activity of these active metabolites were shown to be higher against *in vitro* free radicals (DPPH and B-Carotene-linoleic acid), suggesting the potential antioxidant and antiapoptotic properties of ginger against CP-toxicity.

CONCLUSIONS: Treatment with ginger in rats induced with CP resulted in significant improvement in the expression of certain molecular miRNAs. The kidney tissues of these rats showed a marked decrease in the expression of miR-155-5p, miR-34a-5p, and miR-21-5p, while the levels of miR-193b-3p, miR-455-3p, and miR-342-3p were observed to increase significantly. In conclusion, ginger can protect rats from CP-induced nephrotoxicity.

Key Words:
Ginger, miRNAs, Apoptosis, Phytoconstituents, Cyclophosphamide, Kidney.

Introduction

Cyclophosphamide (CP) refers to certain alkylating drug agents that biologically act on DNA cross-linking, and cause cell death (apoptosis) by preventing cell division[1,2]. Even with the established toxicity of CP, previous studies[3-6] reported the use of CP in many clinical trials for treating several neoplastic and non-neoplastic diseases, particularly leukemia, and breast and pulmonary cancers, in addition to other forms of systemic vasculitides[6] and systemic inflammatory diseases[7-10].

CP showed to be involved in the treatment trials of several renal diseases such as nephropathy, steroid-resistant nephrotic syndrome, proliferative lupus nephritis, and progressive IgA-nephropathy[11,12].

Many cytotoxic side effects for CP administration were reported[13-15] in multi-organs, particularly cellular oxidative stress, bone marrow toxicity, and severe opportunistic infections. However, secondary hematologic malignancy, sterility, and teratogenicity are related to latent CP cytotoxic effects[13,14]. Accordingly, the use of CP greatly produces toxicity for both the kidneys and urinary bladder by producing the form of hemorrhagic cystitis and bladder cancer[16-18]. Although treatment with CP in non-neoplastic diseases is dose-dependent, longer uses of the CP need Mesna (2-mercaptoethanol sulfonate) in combination with the drug to reduce bladder toxicity and other related adverse effects[13,18-20].

Previously, it was noticed[21] that microRNA (miRNA) regulatory roles of certain genes are greatly affected by exposure to toxic substances. Thus, miRNA expression might be involved as predictive biomarkers for the toxic effects of different toxicants in animal and human tissues, respectively[21]. In addition, adverse outcome pathways of specific toxicants could be verified by alteration in the expression levels of miRNAs[22].

In injured kidney tissues, the expression of cellular miRNAs was significantly highly upregulated, like miR-21-5p, miR-155-5p, and miR-18a-5p. This confirms the strength, reproducibility, and specificity of the miRNA response in distinguishing normal and diseased kidney tissues[23,24]. Thus, in therapeutic trials[24] of herbal origin, the expression of miRNAs in treated and non-treated injured kidney tissues is considered a useful potential biomarker for treatment, prevention, and protection as well.

Ginger (*Zingiber officinale*), a known flavoring spice used worldwide, is known to have several biological activities[25]. Ginger has been used to treat many human diseases, and its effectiveness against targeted diseases mainly depends on the number of polyphenolic components it presents[26]. The presence of a variety of antioxidants like terpenoids, ascorbic acid beta-carotene, alkaloids, and polyphenols, such as flavonoids, flavones glycosides, and rutin[27], greatly represents the antioxidant, dominative protective effect on DNA, anti-microbial, antidiabetic as well as tumor preventing activities[25-29].

This study aimed to administer cyclophosphamide (CP) to induce nephrotoxicity in rats and then investigate changes in miRNA expression profiles. The study explored their predictive role and their link to fibrosis, cellular apoptosis, and oxidative stress in kidney tissues. Furthermore, the protective and curative effects of ginger extracts against CP-induced renal toxicity were assessed in rats, along with *in-vitro* screening of ginger's phytoconstituents, antioxidant properties, and free radical scavenging activity.

Materials and Methods

Chemicals

CP was purchased from Baxter Oncology GmbH, Frankfurt, Germany. The fresh rhizomes of *Zingiber officinale* were obtained from the local market and identified by the pharmacognosy staff in the Faculty of Pharmacy, at Mansoura University, Egypt.

Assessment of Ginger Extract

The assessment of ginger extract was done as previously reported in the literature[30-33].

Analysis of Phytochemical and Phytoconstituents in Ginger Extract

Analysis of phytoconstituents

In this method, a liquid chromatography HPLC System (Hewlett Packard HP 1050 Series, Agilent Technologies Inc, East Lyme, CT, USA) was used to estimate the proposed phytoconstituents, like total phenolic compounds present in 300 was used to estimate the proposed phytoconstituents, like total phenolic compounds present in 200 mg of the ginger extract as previously mentioned[30-34].

Analysis of phytochemical

Ginger extract was screened for the presence of alkaloids, flavonoids, Tannin, glycosides, triterpenoids, carbohydrates, steroids, saponins, and p- Hydroxy benzoic acid (PHBA), as previously reported in the literature[35-38].

Estimation of Total and Flavonoid Contents

In this test, total polyphenols were estimated in the ginger extract by the Folin-Ciocalteu method, as previously described[28-38]. In addition, the total flavonoids were estimated according to the Dowd method as previously adopted[28-38].

Assessment of In-vitro Antioxidant Activity of Ginger Extract

Diphenyl-1-picrylhydrazyl (DPPH) radical estimation

The radical scavenging ability against DPPH was performed in various concentrations of the ginger extract by Spectrophotometric analysis as previously described[30-41].

β-Carotene-linoleic acid estimation

In this test, spectrophotometric analysis was used to identify the antioxidant activity of the ginger extract against B-Carotene-linoleic as previously reported[29-41].

Animals and Sampling

Forty adult male Wistar rats (220±30 g) were randomly divided into four groups of 10 each. Animals with historical surgery, infection complications, and other medical interventions were excluded from this experimental study. The animals were housed and subjected to normal feeding, drinking, and health care mechanisms for two weeks before starting any research procedures.

The first group (group I) was considered the control group and only received water and food. The second group (group II) received ginger extract (a dose of 300 mg/kg) by oral gavage. The third group (group III) received CP (75 mg/kg, i.p.), through intraperitoneal administration on days 3, 4, 5, 19, 20, and 21 of the study[41]. The fourth group (group IV) received the same dose of CP and ginger extract (a dose of 300 mg/kg) by oral gavage starting seven days before CP and continuing throughout the duration of the experiment. After four weeks of CP injection, all animals were subjected to light intraperitoneal pentobarbital anesthesia (50 mg/kg) and killed by cervical dislocation. Blood and kidney tissue samples were collected in sterile tubes and containers. Then, serum samples were liquated in smaller containers and stored at -80°C until assaying. The kidney tissues were fixed in a relaxed state after emptying under 50 mg/kg intraperitoneal pentobarbital anesthesia (as an initial dose, a smaller amount was added when necessary)[38-41]. The fixed samples were dehydrated in graded levels of ethanol, cleared in xylene, and embedded in paraffin. The 5-µm-thick sections were stained with hematoxylin and eosin and were examined by a light microscope and photographed.

In addition, the cellular expression of *caspase-3* was identified in the kidney tissues by immunohistochemical staining analysis. In this test, kidney tissue sections with 5 µm-thick were deparaffinized and rehydrated. Then, the sections were treated with hydrogen peroxide (3%) to block the activity of endogenous peroxidase and were incubated with BSA (1%) for one hour. Finally, the sections were incubated overnight at 4°C with anti-cleaved *caspase-3* primary antibodies at a dose of 1:100 (ab2302, Abcam, Cambridge, UK).

Slides were immersed in horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) (37°C, 0.5 h), labeled streptavidin-biotin for 30 min (DETHP 1000, Sigma-Aldrich). The slides were counterstained using hematoxylin.

Acute Toxicity Test

In this test, to measure the cytotoxicity of ginger, a healthy group of rats (10 rats) were subjected to gradual concentrations of ginger (50 mg to 600 mg/rat) as previously reported in many toxicity studies[42,43].

Assessments of Kidney Function

Parameters of renal function like creatinine, creatinine clearance, and urinary albumin were identified according to previously reported routine lab methodology[38-43].

Determination of Renal DNA Damage, Antioxidant Enzymes, and Oxidative Free Radicals

Assessment of cellular DNA damage

To estimate DNA in liver tissue samples, a diphenylamine reagent was used as an organic solvent for extraction, and DNA concentration was estimated as previously reported[44,45].

Assessments of renal antioxidant enzyme status

The superoxide dismutase (SOD) activity of control and treated groups were assayed by the modified spectrophotometric method previously reported[45,46].

Determination of Renal Malonaldehyde (MDA) as Oxidative Free Radical

MDA was estimated in homogenized kidney tissue samples by the thiobarbituric acid method as previously reported[27,30,31].

Assessments of Renal Fibrosis Markers

Hyaluronic acid (HA) and hydroxyproline (Hypx) were measured in kidney tissues using commercially available bioassays[41-49].

Assessment of Molecular Changes in Kidney Tissues

RNA isolation and cDNA preparation

RNA from rat kidney tissue was isolated using TRIzol Reagent (Invitrogen Life Technologies, California, USA), and subjected to cDNA preparation and real-time PCR analysis proceeded in a triplicate with coefficients of variation greater than 5%[50].

Real-Time RT-PCR Analysis of Apoptotic, Fibrosis, and Antioxidant Markers

Cellular apoptotic genes like *Bcl-2 (B-cell lymphoma 2), Bax (Bcl-2-associated X protein), and caspase-3* were identified in control, ginger-treated, and non-treated CP-intoxicated kidney tissues by using the Maxima SYBR Green Rox qPCR master mix kit (Thermo Scientific Fermentas PCR, science house, Corston Bath, UK) as mentioned previously in the literature[51,52].

In this test, real-time PCR reactions were performed using the Steponeplus (Applied Biosystem, Waltham, MA, USA) with corresponding primers of the respective selected genes *bcl-2*, *Bax, caspase-3*, *α-SMA*, *Col-1a1*, *Nrf2*, *heme oxygenase 1 (HO-1)*, and *GAPDH* (*Glyceraldehyde3-phosphate dehydrogenase*) gene was applied as standard endogenous control primer. (Table I)

Real-Time RT-PCR Analysis of miRNAs

The miRNAs from kidney tissues were analyzed using miRCURY LNA Universal RTmicroRNA PCR reagents with miRCURY LNA microRNA set of primers (Invitrogen Life Technologies, California, USA)[50-54].

Calculation of miRNA Fractional Rank

The Roche LC software (Roche Diagnostics Corporation, Indianapolis, IN, USA) was used to analyze the amplification curves of miRNAs. Both the crossing point (Cp) value and the melting

Table I. Distribution of diagnoses of patients receiving narrowband UVB phototherapy.

	Primer sequences	
Primer ID	**Forward sequence**	**Reverse sequence**
miR-155-5p	5′-UUAAUGCUAAUUGUGAUAGGGGU-3′	5′-ACCCCUAUCACAAUUAGCAUUAA-3′
miR-34a-5p	5′-GGACTTGGCAGTGTCTTAGCTG-3′	5′-GTGCAGGGTCCGAGGTATTC-3′
miR-21-5p	5′-GGGTAGCTTATCAGACTGA-3′	5′-GTGCAGGGTCCGAGGT-3′
miR-193b-3p	5’- CGCGCCCTGAAACACCC -3’	5’- AGTGCAGGGTCCGAGGTATT-3’
miR-455-3p	5’- TAAGACGTCCATGGGCAT-3’	5’- GTGCAGGGTCCGAGGT-3’
miR-342-3p	5′-GGGTCTCACACAGAAATCGC-3′	5′-CAGTGCGTGTCGTGGAGT-3′
GAPDH gene	5`-AAGCTCATTTCCTGGTATG-3`	5`-CTTCCTCTTGTGCTCTTG-3`
Bcl-2 gene	5`-ATCGCCCTGTGGATGACTGAGT-3`	5`-GCCAGGAGAAATCAAACAGAGGC-3`
Bax gene	5`-ATG GAC GGG TCC GGG GAG CA-3	5`-CCC AGT TGA AGT TGC CGT CA-3`
Caspase-3 gene	5`-CAACAACGAAACCTCCGTGG-3`	5`-ACACAAGCCCATTTCAGGGT-3
α-SMA gene	5`- GTGATCACCATCGGGAATGA -3`	5`- CAGCAATGCCTGGGTACATG -3`
Col-1a1 gene	5`- AACCCCAAGGAGAAGAAGCA-3`	5`- AGCGTGCTGTAGGTGAATCG-3`
NRF2 gene	5`- TTGTAGATGACCATGAGTCGC-3	5`- TGTCCTGCTGTATGCTGCTT-3
HMOX1	5`- GTAAATGCAGTGTTGGCCCC-3	5`- ATGTGCCAGGCATCTCCTTC-3

Table II. Phytoconstituents screening, antioxidant activity of ginger extract (mg/300 mg).

Item	Ginger mg/300 mg
Percentage yield	31.7%
Phytochemical screening (+/-):	
Alkaloids	+
Flavonoids	+
Tannins	+
Glycosides	+
Triterpenoids	+
Carbohydrates	+
Steroids	+
Saponins	-
p-Hydroxy benzoic acid (PHBA)	+
Phytochemical constituents (M ± SD)	
Total polyphenolic content[1]	34.57±3.8 mg
Total flavonoid content[2]	89.1±0.86 mg
The most abundant phytochemical compounds	Ginger mg/300 mg
Pyrogallol	18.6
Catechein	6.7
Reversetrol	7.4
p-coumaric	6.1
Citral	18.9
[6]-shogaol	22.9
[6]-gingerol	29.1
Antioxidant capacity	
Radical scavenging activity (BCLA; %)	
At cons. of 500 µg/mL	92.5%
At cons. 1,000 µg/mL	98.4%
Total antioxidant activity (DPPH; %)	87.5%

(+/-) presence or absence of phytoconstituents; phytochemical constituents represented as mean ± SD ($n = 3$). [1]Expressed as mg of gallic acid equivalents (GAE)/300 g of the dry extract. [2]Expressed as mg of quercetin equivalents (QE)/300 g of the dry extract.

curve analysis for each miRNA were estimated by the 2nd derivative method[50-54].

Statistical Analysis

Experiments were repeated thrice, and data were expressed as mean ± standard deviation (SD). Statistics were assessed to be statistically significant when differences had a p-value *<0.05 **p<0.01, and ***p-value<0.001 as compared to control.

Results

Screening of Active Constituents

The results of this experiment showed that ethanolic ginger (300 mg) contains approximately 31.7% w/w of active phytoconstituents. Alkaloids, flavonoids, glycosides, tannins, triterpenoids, carbohydrates, steroids, and P-hydroxy benzoic acid were shown to be the most common phytoconstituents in ginger extract (Table II).

Total Phenolic (TPC) and Flavonoid Contents

The ginger extract showed considerable amounts of phenolic and flavonoid compounds. In this test, the values of TPC and flavonoid were calculated as gallic acid equivalents (GAE/300 g) and quercetin equivalents (mg/300 g) of dry ginger weight. The total amounts of phenolic and flavonoid compounds in ginger extract (300 mg) were found to be 34.57±3.8 of gallic acid equivalents (mg/300 g) and 89.1±0.86 of quercetin equivalents of (mg/300 g), respectively (Table II). The most abundant polyphenolic compounds present in ginger were pyrogallol, catechin, resveratrol, P-coumaric, citral, shogaol-[6], and gingerol-[6] (Table II).

In Vitro Antioxidant and Free Radical Scavenging Activity Of Ginger Extract

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene-linoleic acid test were efficiently

used, respectively, to estimate both the free radical scavenging and antioxidant activities of ginger. The ginger extract showed a significant free radical scavenging activity (92.5-98.4%) against the DPPH reagent. In addition, its antioxidant activity against β-carotene-linoleic acid was 87.5% (Table II).

Effect of Ginger Extract on Renal Oxidant - Antioxidant Function

TAC, SOD, MDA, and DNA damage as markers of renal oxidant-antioxidant function were estimated in all groups.

In the kidney homogenate of rats treated with ginger (GII) at a dose of 300 mg/kg, TAC, SOD, MDA, and DNA damage as parameters of cellular oxidative stress significantly (p-value=0.01) improved compared to healthy control rats (GI), signifying the potential role of ginger when used alone as a protective agent in healthy targets. When rats were treated with CP (GIII) at a dose of 75 mg, their homogenates showed a significant (p-value=0.01) reduction in the levels of SOD and TAC activity with an increase in the levels of cellular DNA damage and MDA as a marker of oxidative lipid peroxidation compared to normal and ginger-treated rats. Whereas, in CP-ginger-treated rats (GIV), the oxidative damage induced by CP-intoxication was restored following administration of the ginger extract at a dose of 300 mg/kg. A significant increase (p-value=0.001) in both TAC and SOD enzyme activity with a decline in the levels of both cellular DNA damage content and MDA was reported in ginger-treated rats compared with those of CP-treated rats.

Effect of Ginger Extract on the Expression of Antioxidant Signaling Genes Nrf2/ HO-1

The gene expression levels of Nrf2 and *HO-1*, transcription factors controlling the expression of antioxidant enzymes, were significantly (p-value<0.001) down-regulated in the kidney of CP-intoxicated rats (GII) at a dose of 75 mg when compared with the control (GI) and ginger-treated (GII) rats. Moreover, the expression levels of *Nrf2* and *HO-1* in the kidney tissues of rats treated with ginger as a protective agent (GII) were significantly up-regulated, which indicates the antioxidant protective activity of ginger in healthy status. In addition, the kidneys of CP-intoxicated rats (GIV) showed a significant upregulation (p-value<0.001) in *Nrf2* and *HO-1* mRNA expression levels when treated with ginger at a dose of 300 mg/kg, respectively.

Effect of Ginger Extract on Renal Fibrosis

In this experiment, hyaluronic acid (HA) and hydroxyproline (Hypx) were estimated in kidney tissues as protein markers of kidney fibrosis in all studied rats. In CP-intoxicated, there was a significant (p-value=0.01) increase in the levels of HA and Hypx compared to normal controls. In addition, healthy rats that received ginger at a dose of 300 mg/kg showed significant improvement in the identified fibrotic proteins, HA and Hypx, respectively, identifying the protective role of ginger against kidney fibrosis. Whereas, in ginger-treated CP-intoxicated rats, a significant (p-value=0.001) reduction in fibrotic markers, HA, and Hypx, were obtained in comparison with CP-intoxicated rats. The data support the anti-fibrotic activity of ginger against CP-induced fibrosis in kidney tissues of rats.

Effect of Ginger Extract on the Expression of Fibrotic Genes a-SMA and Col-1a1

The expression levels of both *a-SMA* and *Col-1a1* fibrotic genes significantly upregulated (increased) in CP-intoxicated (GIII) rats compared to healthy (GI) and ginger-protective (GII) rats, respectively. When CP-intoxicated rats were treated with ginger at a dose of 300 mg/kg, the expression levels of fibrosis genes *a-SMA* and *Col-1a1* significantly (p-value=0.001) downregulated, which signifies that the use of ginger in healthy and intoxicated renal humans or animal models might improve the lifespan of the fibrosis models without toxicity.

Effect of Ginger Extract on Cellular Apoptosis Induced by CP-Intoxication

In this study, to identify the potential antiapoptotic activity of ginger, we identified its effect on the gene expression levels of *BCL-2*, *BAX*, and *caspase-3* in the kidney tissues of ginger-treated and non-treated CP-intoxicated rats (Figure 1). The results showed that the expression levels of mRNAs of *BCL-2* gene significantly reduced and the expression of mRNAs of *Bax*, *Bax/BCl-2* ratio, and *caspase* genes significantly increased in CP-intoxicated rats (GIII) compared to those of the control group (GI), respectively (Figure 1A-D). Moreover, healthy rats treated with ginger (GII) at a dose of 300 mg/kg significantly improved cellular apoptotic proteins. *BCL-2* as an antiapoptotic gene significantly increased, and the other apoptotic inducing genes, *Bax*, *Bax/ BCL-2* ratio, and *caspase-3*, significantly reduced

(Figure 1A-D). In addition, CP-intoxicated rats (GIV) treated with ginger at a dose of 300 mg/kg, showed a significant reduction in the expression levels of *Bax, Bax/BCL-2* ratio, and *caspase-3* inducing apoptosis in the kidney tissues compared to that of CP-non-treated group of rats (GIII) (Figure 1A-D).

Differential Expression of miRNAs in Kidney Tissues

The expression of cellular miRNAs was differentially estimated in kidney tissues of rats intoxicated with cyclophosphamide (CP) and treated with ginger extract at a dose of 300 mg. In CP-treated rats, differential increases in the expression of cellular miR-155-5p, miR-34a-5p, miR-21-5p, and a decrease in the expression of cellular miR-193b-3p, miR-455-3p, and miR-342-3p were reported in kidney tissues of CP-intoxicated rats compared to that obtained in healthy control rats as shown in Figure 2. Little insignificant (p-value=0.12) change (improvement) was reported in healthy rats treated with ginger at a dose of 300 mg/kg, signifying a protective and non-toxic ginger dose. Moreover, when CP-intoxicated rats were treated with ginger at a dose of 300 mg/kg, there was a significant improvement in the expressed cellular miRNAs in the kidney tissues of the rats. The results showed that differential decreases in the expression of cellular miR-155-5p, miR-34a-5p, miR-21-5p, and an increase in the expression of cellular miR-193b-3p, miR-455-3p, and miR-342-3p was reported in CP-rats following treatment with ginger (Figure 2).

Effect of Ginger Extract on the Histology of the Kidney Tissues

Histological examination showed the normal histological structure of the glomeruli and renal tubules in both control rats (Figure 3A) and rats who received ginger at a dose of 300 mg/kg (Figure 3B). On the other hand, several structural alterations in the H&E-stained kidney sections of CP-intoxicated rats, including vacuolation of the cytoplasm of the endothelium of the renal tubules, acidophilic depositions of collagen, and some tubules have hyaline cast in their lumens with a remarkable degree of congestion (Figure 3C). However, H&E-stained kidney sections of CP-intoxicated rats treated with ginger at a dose of 300 mg/kg (Figure 3D) revealed remarkable improvement in the histological appearance of the glomeruli and renal tubules with interstitial hemorrhage noticed in the rats received 300 mg/kg ginger extract.

Effect of Ginger Extract on the Expression of Apoptotic Markers Cleaved Caspase-3 in Renal Tissues

Immunostaining of the renal tissues for apoptosis and necroptosis was detected by cleaved *caspase-3*. Kidney sections of CP-intoxicated rats showed marked expression of cleaved *caspase-3*

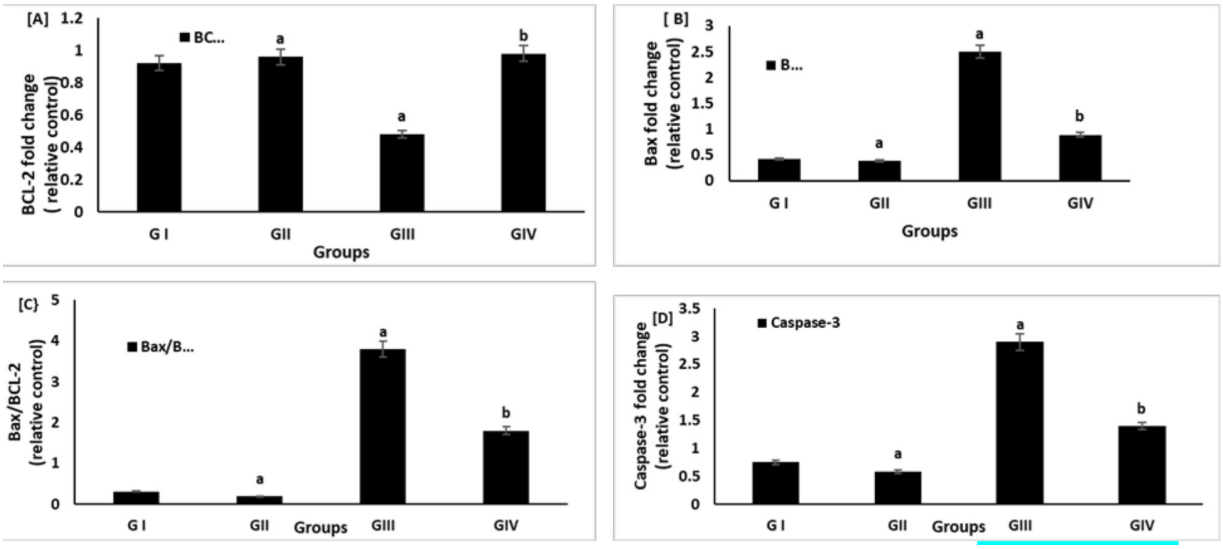

Figure 1. Effect of ginger on renal apoptosis profiles induced by CP-intoxication, *BCL-2* (**A**), *Bax* (**B**), *Bax/BCL-2* ratio (**C**), and *caspase-3* (**D**).

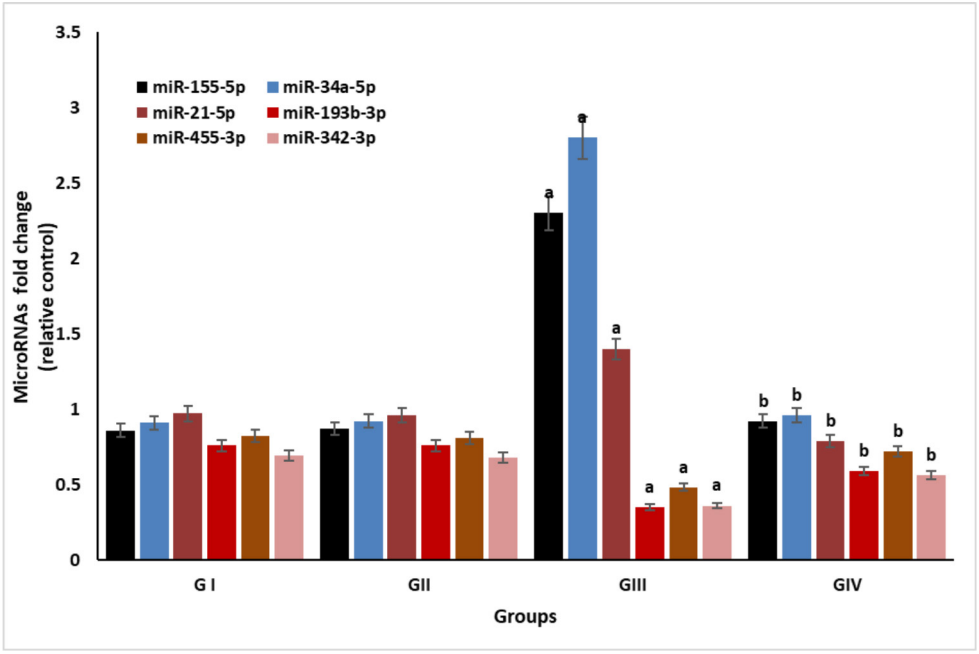

Figure 2. Effect of ginger on the expression of renal miRNAs induced by CP-intoxication in ginger-treated and non-treated CP-intoxicated rats at a dose of 300 mg/kg for 4 weeks, measured by Real-Time RT-PCR Analysis. Normalized levels of expressed miRNAs are expressed as the fractional rank.

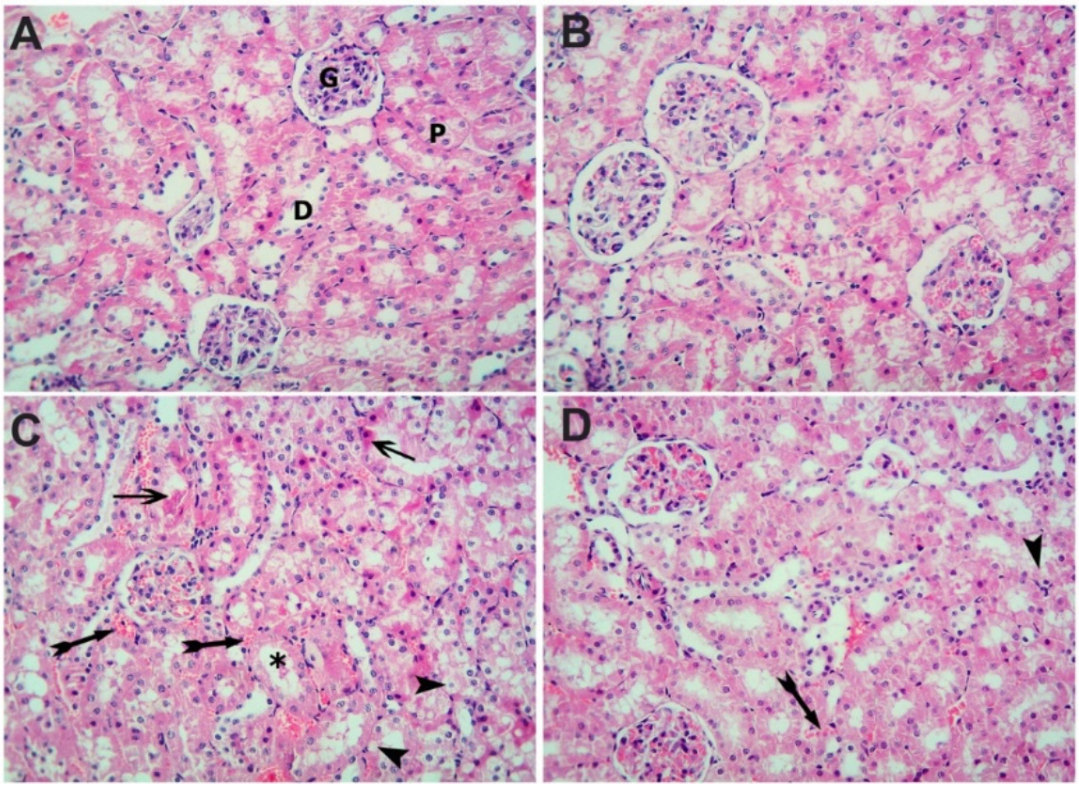

Figure 3. H&E-stained sections in the kidney of control (**A**), normally treated with ginger 300 mg/kg (**B**), CP-intoxicated rats (**C**), and ginger-treated CP-intoxicated rats (**D**) (H&E: X200).

(Figure 4C) compared to normal renal tissues in both control rats (Figure 4A) and rats that received ginger at a dose of 300 mg/kg (Figure 4B). The renal expression for cleaved *caspase-3* was markedly decreased in renal sections of CP-intoxicated rats treated with ginger at a dose of 300 mg/kg (Figure 4D). In addition, a quantification analysis of the immunoreaction of cleaved *caspase-3* in kidney sections was performed (Figure 4E). The results showed that the quantity of expressed cleaved *caspase-3* in the tissues of CP-intoxicated rats significantly (p=0.00001) increased compared to those of normal (group I) and ginger-treated rats (group II) (Figure 4E). However, in ginger-treated CP-intoxicated rats, the reduction in the quantity of the immune reaction patches of cleaved *caspase-3* in ginger-treated rats was significant (p=0.00001) compared to CP-non-treated rats, which supports the anti-apoptotic property of ginger against the cellular toxicity of CP.

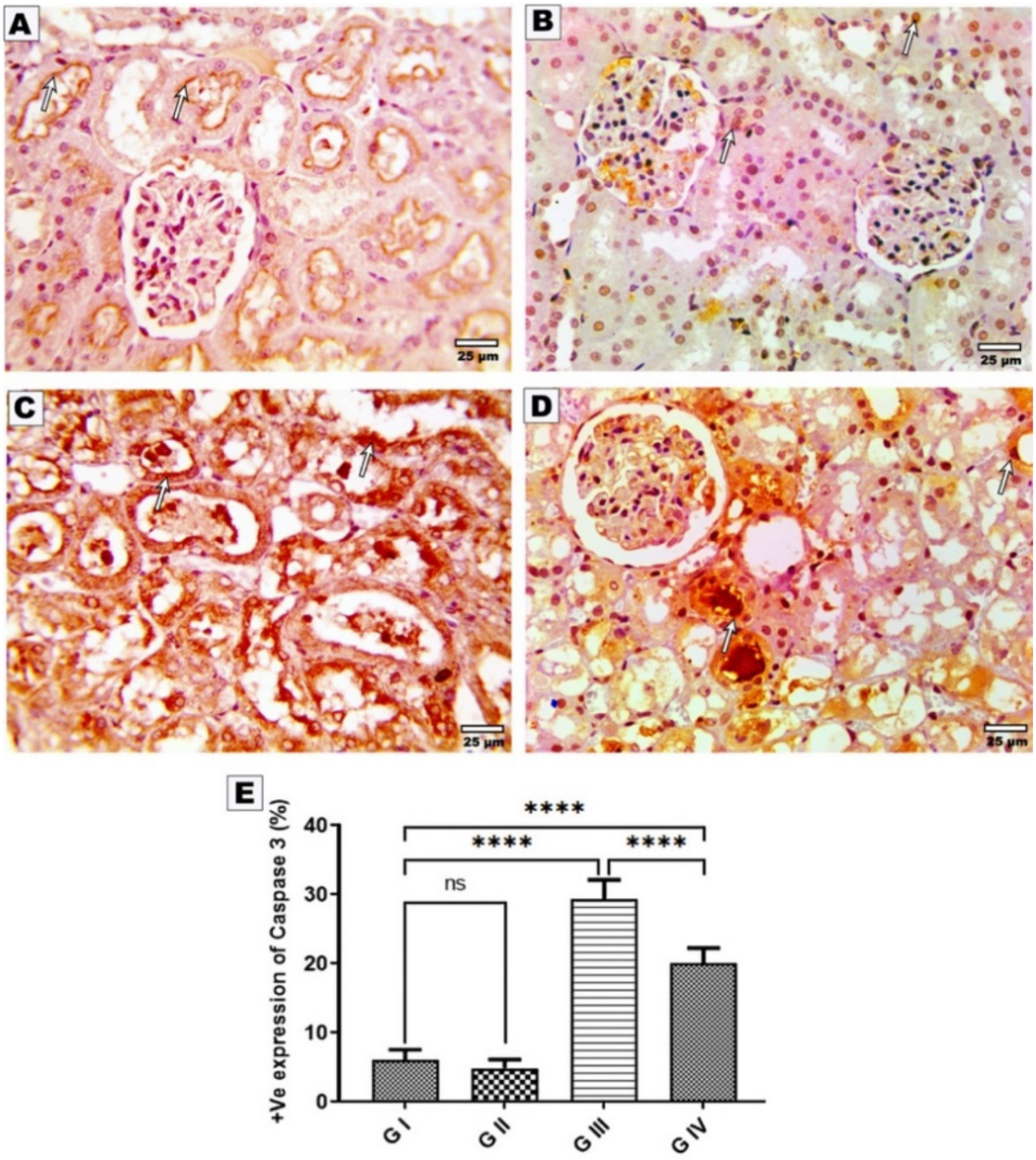

Figure 4. Kidney expression of cleaved caspase-3. **A-B**, Representative photomicrographs of immunohistochemically stained renal tissue against cleaved caspase-3. **C**, CP-intoxicated rats, (**D**) CP-intoxicated rats treated with ginger at a dose of 300 mg/kg, (**E**) Quantification of immunoreaction of cleaved caspase-3 in renal sections (X 200). **<0.01 $^{***}p$<0.001, and $^{****}p$-value<0.0001.

Discussion

In this study, ginger extracts at doses of 300 mg/kg provide a curative and protective activity against CP-induced renal toxicity at a dose of 75 mg/kg. Changes in the expression of cellular miRNAs, damage of cellular DNA, abnormal body and kidney weights, renal function, cellular fibrosis, apoptosis, abnormal oxidative stress, and antioxidant status were significantly decreased towards the normal ranges following treatment with ginger extracts.

In vitro, the phytochemical analysis revealed that the ginger extract showed noticeable free radical scavenging (92.5-98.4%) and antioxidant activity (87.5%) against both DPPH and β-carotene-linoleic acid-free radicals, respectively. Our results were in accordance with others[30,55-58] who reported the potential free radical scavenging activity of ginger against superoxide, hydroxyl, and nitric oxide *in vitro*. In addition, the presence of considerable amounts of polyphenolic (345 mg) and flavonoid (89.1 mg) compounds in the used ginger was reported in a dose of 200 mg. The most abundant polyphenolic compounds present in ginger were pyrogallol, catechin, resveratrol, P-coumaric, citral, shogaol-[6], and gingerol-[6]. Our results, with others[30] recently, significantly support the curative and activity potency of ginger against CP-intoxication, whose higher amounts of gingerol-[6], shogaol-[6], citral, and pyrogallol were estimated in ginger extracts. In addition, the presence of zingerone, shogaols, and gingerols with other numerous active compounds efficiently supports the natural antioxidant activities of ginger as a chemopreventive agent of diseases[56-58]. The use of ginger, even in higher doses, was shown[59-60] to be very safe for humans, and our study revealed that ginger extract at doses of 300 mg/kg has no toxic effects (LD_{50}=0). This might be due to its contents of polyphenolic compounds that can suppress the chain reactions of lipid peroxidation *via* a free radical scavenging mechanism[56-60].

In CP-treated rats, the administration of ginger extract significantly improved cellular antioxidant status. TAC and SOD as markers of antioxidants significantly increased, while cellular DNA damage and MDA as a marker of peroxidation were significantly reduced when the ginger extract was applied at a dose of 300 mg. The used CP anticancer drug induces cellular oxidative stress with lower antioxidant enzymes, protein cross-linking, and DNA damage, collectively inducing cell death or apoptosis, as well as cytotoxic effects on normal cells[13,14,61,62].

Like others[30,37,60-71], our results support the potential protective effects of ginger phenolic and flavonoid compounds, which proceed *via* the antioxidant and nephroprotective pathways. The results in that part significantly support the importance of ginger as a therapeutic herbal remedy to manage renal function in patients with uremia, as previously reported in the literature[71].

Previous studies[55,63,72-74] have shown that CP-induced cellular toxicity in kidney tissues significantly increased the levels of lipid peroxidation (LPO), with a reduction in the markers of antioxidant statuses such as SOD and CAT. This collectively leads to severe renal toxicity, renal cell DNA damage, and subsequent pathogenesis of the kidney and nephrotoxicity[55,64-75], as measured by histopathological analysis in our study.

Previous studies[64-75] showed that the antioxidant and free radical scavenging efficiency of gingerols, shogaols, and other related bioactive phenolic compounds that exist in ginger, significantly nullify the toxicological effects of CP drug and prevent the pathogenesis of the kidney, as previously shown.

In addition, the molecular influence of ginger activity upon the expression of *Nrf2* and *HO-1* genes was identified in ginger-treated and non-treated CP-intoxicated rats. The results showed the expression levels of *Nrf2* and *HO-1*, transcription factors that control the expression of antioxidant enzymes, were significantly upregulated in the kidney tissues of CP-intoxicated rats treated with ginger at a dose of 300 mg/kg compared to CP non-treated rats. Moreover, healthy rats treated with the respective ginger dose showed upregulation in the expression levels of *Nrf2* and *HO-1* genes, which in turn signified that ginger might be used as a protective agent in healthy cases towards toxicity.

Previous studies[76-78] showed that the pro-oxidant nature of CP drug is significantly associated with the cellular production of cellular and molecular oxidative stress, inflammation, and apoptosis in rats. In intoxicated cells, the excessive production of cellular oxidative stress generated from exposure to endogenous or exogenous chemicals[79], inactivates the action of *Nrf2* in the cytosol by a set of proteins Kelch like-ECH-associated protein 1 (Keapl) and Cullin 3. When antioxidants of plant origin, such as ginger, were applied in intoxicated models with chemicals or drugs, *Nrf2* is activated and complexed with small Maf proteins

(Mafs) like the activator protein-1 (AP-1) protein in the nucleus, which consequently activates the transcription of antioxidant and cytoprotective genes such as *SOD* and *CAT* through binding to the antioxidant response elements (ARE)[80].

The effect of ginger extract on kidney fibrosis was also evaluated in this study. We investigated the effect of ginger on the production levels of fibrotic proteins, HA and Hypx, and molecular mRNA levels of *a-SMA* and *Col-1a1* fibrotic genes by calorimetry and Real-Time RT-PCR analysis. The treatment of CP-intoxicated rats with 300 mg of ginger significantly reduced the production of HA and Hypx proteins and the expression mRNA levels of *a-SMA* and *Col-1a1*, respectively, compared to non-treated CP-intoxicated rats.

It was reported[37,71,81-83] that the administration of CP anti-cancer drugs can significantly injure or damage normal tissues, which gives rise to numerous side effects. According to previous studies[81-87], CP compound produced some reactive metabolites that chemically cross-linked with cellular DNA, as well as protein that cause per-oxidative damage to the kidney and other vital organs by producing an overproduction of reactive oxygen species (ROS), which cause per-oxidative damage to the kidney and other vital organs. This, in turn, results in more expression of kidney fibrosis[2,88,89].

In addition to that, intoxication with CP leads to a breakdown in the glomerular filtration barrier and the passage of excess protein. The produced proteinuria is toxic and leads to excess downstream tubular reabsorption of protein, resulting in tubular inflammation and fibrosis[90]. Also, CP-toxicity may increase the circulating uremic serum, which is considered a permissive factor in the systemic fibrogenesis of kidney tissues of intoxicated rats.[91-92] Whereas, in ginger-treated rats, kidney tissues improved and the production of both HA and Hypx significantly reduced with a reduction in the expression levels of both *a-SMA* and *Col-1a1* fibrotic genes, which supports the anti-fibrotic activity of ginger against CP-induced fibrosis in kidney tissues of rats. The regulatory effect of ginger extracts on kidney fibrosis depends mainly upon free radicals scavenging and antioxidant activity of ginger as a whole and its biological constituents[35,55,93-94], which could, in turn, normalize microsomes, lysosomes, mitochondria, and plasma membranes permeability and integrity, which lead to reduced expression of collagen fibrils in the kidney.

The treatments with CP drug significantly imbalance the expression of apoptotic proteins. The expression levels of the *BCL-2* gene were significantly reduced, and *Bax* and *caspase* genes, as well as *Bax/BCl-2* ratio, significantly increased in CP-intoxicated rats compared to those of the control group, respectively. Moreover, a significant reduction in the expression levels of Bax, Bax/BCL-2 ratio, and *caspase-3* inducing apoptosis with an increase in the expression levels of antiapoptotic *BCL-2* gene was reported in kidney tissues of CP-intoxicated rats treated with ginger at a dose of 300 mg/kg. The results of the present study showed that the protection of ginger against CP-cytotoxicity proceeds *via* an antiapoptotic pathway through upregulation of the expression of the *BCL-2* gene as a gene of antiapoptotic, and downregulation of respective apoptotic inducing genes like *Bax, Bax/BCL-2* ratio, and *caspase-3*. Thus, this was confirmed by an increase in the percentage of expressed antiapoptotic proteins BCL-2 within viable renal cells and decreased the apoptotic-inducing proteins *Bax* and *caspase-3* as measured by immunohistopathological analysis of ginger-treated and non-treated CP-kidney tissues.

The present data matched with those[93] that reported that ginger oils significantly lowered DNA damage and cell death induced by H_2O_2 *via* its ability to scavenge the reproduced oxygen radicals. In addition, several studies[94-96] showed that the active component of ginger, i.e., [6]-gingerol, can effectively protect as a scavenger to the active hydroxyl free radicals (˙OH), which leads to DNA damage and subsequent cellular kidney apoptosis. Generally, ginger and its active constituents, [6]-gingerol, at variable doses such as 100-200 mg/kg, may show a defensive impact against genotoxicity and reduce oxidative cell death induced by toxicants due to its anti-apoptotic, anti-oxidant, anti-inflammatory, and antifibrotic properties[25,97-101].

The effects of ginger extract and CP toxicity on the expression of cellular miRNAs were identified in ginger and CP-treated rats by real-time PCR analysis. Cellular miR-155-5p, miR-34a-5p, and miR-21-5p significantly increased, and cellular miR-193b-3p, miR-455-3p, and miR-342-3p significantly decreased in kidney tissues of rats with CP anticancer drug. The disturbance in the expression of miRNA correlated significantly with renal function, oxidative stress, lower cellular antioxidants, fibrosis, and apoptosis in CP-intoxicated kidney tissues. Previously, miR-21-5p, miR-155-5p, and miR-18a-5p were shown to be among the highest upregulated miRNAs in the kidney after injury. This confirms the robustness, reproducibility, and specificity of the miRNA re-

sponse in distinguishing normal and diseased kidney tissues[22,23]. MicroRNAs are short, non-coding RNAs that have demonstratedwith significant roles in the pathogenesis and prognosis of various diseases. Previous studies [79,80,102,103] showed that miRNAs are good regulators in diverse cellular processes, like mediating the metabolism and toxicity of xenobiotic compounds.

In kidney tissues injured by ischemia, toxicant exposure, hypertension, and fibrosis, abnormal increase in the expression of several miRNAs such as miR-21, miR-18a-5p, miR-132-3p, and miR-146b-5p were identified according to pathologic conditions of kidney diseases[24,102-104]. In addition, environmental toxicants and nephrotoxic therapeutics like cadmium chloride ($CdCl_2$) and cyclophosphamide, when increased in the tubules of the kidney, significantly affect the expression of cellular miRNAs, particularly the increase of those localized in the tubules of the kidney[88-105].

However, in CP-intoxicated rats treated with ginger extract, the expression of miR-155-5p, miR-34a-5p, and miR-21-5p was decreased, and the expression of miR-193-3p, miR-455-3p, and miR-342-3p was increased compared to rats treated with CP, respectively. In addition, expressed miRNAs significantly correlated with improved renal function, reduced oxidative effect, apoptosis, and fibrosis, and enhanced antioxidant status of kidney cells following ginger treatments.

Conclusions

This study's findings showed that ginger extracts at doses of 300 mg significantly alleviate the genotoxic, molecular, and cellular damage in kidney tissues induced by CP toxicity. The improvement in kidney parameters, as well as expressed in the levels of molecular miRNAs in kidney tissues, significantly related to antioxidant, anti-apoptotic, and anti-fibrotic activities of phenolic constituents estimated in the ginger extract. The data also suggested the potential use of molecular miRNAs as diagnostic biomarkers in kidney pathological states. However, the estimation of miRNAs in serum and urine and their correlation with certain genes, such as fibrotic, apoptotic, and oxidative genes, should be further addressed in subsequent studies.

Conflict of Interest
The authors declare that they have no conflict of interests.

Authors' Contributions
Conceptualization: S.A. Gabr, W.M. Elsaed, M.A. Eladl, G.A. Ghoniem. Methods: M. El-Sherbiny, K.S. El-Bayoumi. Data cleaning: H. Abouhish, A.M. Desouky, M.M. Abdel-Aziz. Investigation: M.B. Eldesoqui, M. Elshafey. Data collection: H. Abouhish, A.M. Desouky. Visualization: H.A. Ebrahim. Investigation: N.S. Nosseir. Manuscript writing: S.A. Gabr, W.M. Elsaed, M.A. Eladl, G.A. Ghoniem. Manuscript review: S.A. Gabr, W.M. Elsaed, A.M. El-Sayed.

Ethics Approval
The research design applied in our study meets Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. All the experimental procedures were carried out according to the Guidelines for the Care and Use of Laboratory Animals established by the National Institutes of Health of the United States (NIH) publication (No. 85-23, 1996). The study was approved by the Ethics Committee at the Faculty of Agriculture with ID No. MD.11.012.2022, Mansoura University, Mansoura, Egypt.

Informed Consent
Not applicable.

Data Availability
All data generated or analyzed during this study are presented in the manuscript. Please contact the corresponding authors to access the data presented in this study.

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ORCID ID
Sami A. Gabr: 0000-0001-5071-291X.

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