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Screening of bioactive components in *Ferula assafo* dried oleo-gum resin and assessment of its protective function against cadmium-induced oxidative damage, genotoxicity, and cytotoxicity in rats

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ABSTRACT

Cadmium (Cd) is among the most ecologically harmful heavy metals. The purpose of this work was to identify the biologically active components in dried oleo-resin-gum of *Ferula assafo* extract (FAE) and assess their preventive efficacy against oxidative damage caused by Cd in rats. The biologically active components were identified using HPLC and GC-MS. Six groups of female Sprague-Dawley rats were randomly assigned and received oral treatment for two weeks. They consisted of the control group, the groups that got FAE at low or high doses (150 and 250 mg/kg b.w.), the group that received $CdCl_2$ (2 mg/kg b.w.), and the groups that received $CdCl_2$ + FAE at the low or high dose. Tissues and blood samples were collected for different assays and pathological examinations. The HPLC detected 11 polyphenol compounds, whereas the GC-MS identified 24 bioactive compounds. The *in vivo* study revealed that $CdCl_2$ alone disrupted all biochemical indices, oxidative indicators, cytokines, antioxidant enzymes, pro and anti-apoptotic mRNA gene expression, increased DNA fragmentation percentage, and caused pathological alterations in hepatic and renal sections. FAE plus $CdCl_2$ therapy considerably improved all indicators and the histological architecture of the kidney and liver, with the higher dose being more effective in improving all of the measured parameters. Therefore, FAE is a promising option for food and pharmaceutical applications to protect against oxidative damage caused by Cd exposure.

1. Introduction

Cadmium (Cd) is a serious environmental pollutant that people are exposed to when they consume contaminated drinking water and food [1,2], alcoholic beverages, and cigarette smoke in industrialized countries [3], while occupational exposure to it during mining or manufacturing of batteries and pigments [4]. The EFSA [5] determined the tolerable daily intake level of Cd as 0.36 mg/kg of b w. /day and Tolerable Weekly Intake as 2.5 mg/kg of b.w./week. Cd is the most toxic transition metal element and has a long half-life in the human body that causes oxidative stress and serious damage to a variety of systems and organs including the gastrointestinal tract, liver, nervous system, kidney, mucous tissues, heart, and testis depending on the exposure, time and dose [6,7]. Cd is a cytotoxic, mutagenic metal and it was categorized as a class I human carcinogen [8].

Although the mechanism of its adverse impact remains unknown, some studies showed that Cd induces oxidative damage and depletion in the antioxidant enzyme activities in the testes and kidneys of rats. These studies pointed to three major hypothesizes for its toxicity: (1) the direct interaction with proteins leads to attachment to enzyme active sites, cell structural components, and cell transport proteins [9]; (2) substitution of metal elements such as iron in erythrocytes or calcium in bones with metals leading to damage [10]; (3) involvement in boosting the production of the reactive oxygen forms and modifying the activity of

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antioxidant system [11]. Currently, natural compounds with various bioactivities and medicinal potential are now being employed as new antioxidant agents to mitigate Cd-induced oxidative stress and organ damage [12].

Natural antioxidants have been shown to have the ability to detect free radicals and neutralize the negative effects of heavy metal toxicity [13]. Plant-derived bioactive metabolites have been employed as therapeutic agents for heavy metal-induced tissue damage [14]. In this regard, resveratrol efficiently reduces hepatic oxidative damage caused by Cd in rats [15]. *Portulacae oleracea* has been shown to reverse Cd-induced hepatorenal toxicities in mice [16], while *Catharanthus roseus* extract has potential antioxidant action against ROS-mediated DNA damage caused by Cd poisoning [17]. Hussein et al. [18] found that *Urtica pilulifera* leaf extract reduces cadmium-induced hepatotoxicity by modulating antioxidants, Nrf-2 signaling, and inflammatory markers in mice.

Ferula asafetida is the oleo-gum-resin extracted from carrot-shaped roots, a perennial *Ferula* plant belonging Umbelliferae family. It is distributed widely in Central Asia, Iraq, Afghanistan, Turkey, Eastern Iran, North Africa, and Europe [19]. It is extensively consumed in folk medicine to treat several ailments including epilepsy, stomachache, asthma, flatulence, bronchitis, weak digestion, influenza, and intestinal parasites [20]. Several biological and pharmacological researches have indicated that *Ferula* possesses numerous biological properties including antioxidant activity [21], antiviral [22], antifungal [23], anti-diabetic [24], anticonvulsant [25], cancer chemopreventive [26], antispasmodic and hypotensive [27], molluscicidal [28], and anticytotoxic [29]. The methanolic extract has shown iron ion chelating power, DPPH radical, and nitric oxide scavenging activity because of its rich phenols and phenolic compounds [21]. Thus, this work was done to screen the bioactive components in the aqueous extract of *F. asafetida* (L.) and evaluate the protective activity against the cellular and oxidative damage of Cd in rats.

2. Materials and methods

2.1. Chemicals, reagents, and kits

Cadmium chloride ($CdCl_2$) was supplied by Sigma (MO, USA). Kits for bilirubin (T. BIL and D. BIL), Cholesterol (Cho), high and low-density lipoprotein (HDL and LDL), triglycerides (TriG), urea, creatinine, total protein (TP), and albumin (Alb) were obtained from FAR Diagnostics (Via Fermi, Italy). The kits for transaminases (AST and ALT) were acquired by Diagnostic Systems GmbH Co. (Holzheim, Germany). Kit for malondialdehyde (MDA) was supplied by Oxis Research TM Co. (USA). Kits for glutathione peroxidase (GPx) glutathione reductase (GSH), superoxide dismutase (SOD), and nitric oxide (NO) were obtained from Eagle Diagnostics Co. (TX, USA). However, alpha-fetoprotein (AFP) ELISA kit was obtained from Biochem Immuno Systems (Montreal, Canada). A tumor necrosis factor-alpha (TNF-α) kit was obtained from Orgenium (Helsinki, Finland). The kit for carcinoembryonic antigen (CEA) was purchased from Biodiagnostic (Cairo, Egypt). TRIZOL reagent was supplied by Invitrogen TM (Carlsbad, CA, USA). Other available high-analytical grade chemicals were selected in this work.

2.2. Plant material and extract preparation

The plant was botanically identified by the Botany Dept, Faculty of Science, Arish University, Arish, North Sinai, Egypt. The powder of dried oleo-resin-gum of *F. asafetida* was accurately weighed and soaked overnight at room temperature in a predetermined amount of distilled water before being filtered to eliminate any deposits. The filtrate was kept at −20 °C until use in different experiments.

2.3. Determination of bioactive components using HPLC and GC-MS

The total polyphenols were determined using an Agilent 1260 series HPLC with a C18 column (4.6 mm×250 mm i.d., 5 μm) as described in detail in our previous work [29]. However, the volatile hydrocarbon compounds were determined using a GC-MS (Hewlett-Packard model 5890) as detailed in our earlier work [30]. Hydrocarbons (C7-C20, Aldrich Co.) served as references for calculating the retention indices (Kovats index) of the isolated volatile components [31].

2.4. Experimental animals

Two-month-old female Sprague-Dawley rats (150–170 g) were acquired from the Faculty of Science at Arish University in North Sinai, Egypt. The rats were kept in pairs in filter-top polycarbonate cages with free access to food and water in a thermally regulated and artificially lighted environment (20–24 °C and 12 h of dark/light cycle) at Arish University's Faculty of Science. Before the trial began, the animals were held for a week of acclimation. The animals were fed conventional rodent feed acquired from Meladco Feed (Cairo, Egypt). All animals were treated humanely following the Animal Care and Use Committee guidelines of the Faculty of Science, Arish University, North Sinai, Egypt (permission # ARU/SF.10) and all animal studies were performed in compliance with the ARRIVE guidelines and the National Health Institutes for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978).

2.5. Study design

After an acclimation period of one week, all rats were divided at random into 6 groups (10 rats/ group) and received daily oral gavage for 2 weeks as shown in the following diagram:

Following the last treatment (on day 15), blood samples were taken from the retroorbital venous plexus of each animal under isoflurane anesthesia. The serum was separated by centrifugation under refrigeration and kept at −20 °C until utilized to determine the liver and kidney function (ALT, AST, Alb, TP, D. Bil. T. Bil, creatinine, uric acid, and urea), lipid profile (Cho, TriG, HDL and LDL), NO, TNF-α, AFP, and CEA with a spectrophotometer following the instructions of kits. After collecting blood samples, the rats were euthanized, and then a sample of the hepatic and renal tissue was dissected from each animal, weighed, and homogenized using phosphate buffer (pH 7.4). The supernatant was used to measure MDA, GSH, GPx, CAT, and SOD using a spectrophotometer, as previously reported [32]. Other samples of both organs were excised, fixed in normal formalin, rehydrated in ethanol at increasing grades, cleared in xylene, and embedded in paraffin. Histological examination was performed on 5-μm thick sections stained with hematoxylin and eosin (H&E) according to Bancroft et al. [33]. Scheme 1

2.6. Gene expression analysis

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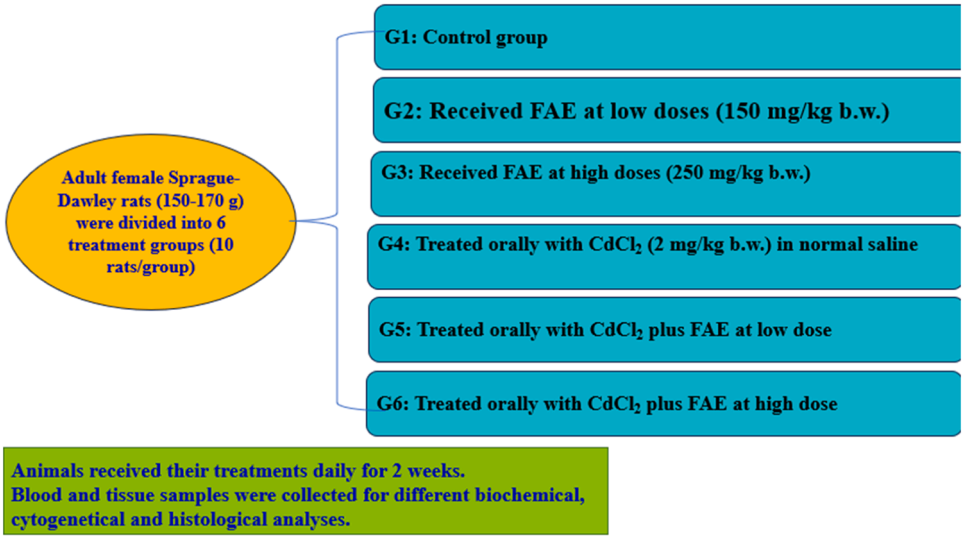
2.7. Total RNA isolation

The entire genomic RNA of each animal's hepatic tissue was extracted using TRIzol®. The RNA pellet was stored in DEPC-treated water before being processed with an RNAse-free DNAse kit to digest any possible DNA residues. Aliquots of RNA were utilized to do reverse transcription [34].

2.8. Quantitative real-time PCR (qRTPCR) and reverse transcription reaction

The First Strand cDNA Synthesis Kit (iNtRON Biotechnology, Korea) was used to synthesize the cDNA copy of hepatic tissue by reverse



Scheme 1. Schematic diagram showing treatment schedule and experimental design.

transcription reaction (RT) as described in detail previously [35]. The SYBR® Premix Ex TaqTM kit (TaKaRa, Biotech. Co. Ltd.) was utilized for the qRTPCR studies using the produced copies of cDNA from the hepatic tissues. A melting curve profile for each reaction was generated. The primers chosen were derived from published Gen Bank sequences. Table 1 displays primers sequences of SOD, GPx, CAT, Bcl-2, Bax, *β*-actin, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as well as the annealing temperature for RT-PCR. The target genes' quantitative values were standardized based on the expression of the housekeeping genes. The $2^{-\Delta\Delta CT}$ technique was used to calculate the quantitative values of individual genes to GAPDH and β-actin [36]. The target's relative quantification to the reference was measured using the $2^{-\Delta\Delta CT}$ technique, as shown below:

$\Delta C_{T(test)} = CT_{(target,\ test)} - T_{(reference,\ test)}$,

$\Delta CT\ (calibrator) = CT_{(target,\ calibrator)} - CT(_{reference,\ calibrator)}$,

$\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}$. The relative expression was computed as $2^{-\Delta\Delta CT}$.

2.9. DNA fragmentation percentage (DPA assay)

The colorimetric determination of DNA content was performed as described previously [43]. Following DNA extraction with acid, both the pellet and supernatant were utilized for the DPA test. The proportion of DNA fragmentation was expressed using the following formula:

$$\%\ \text{DNA fragmentation} = \frac{\text{O.D. of the supernatant}}{\text{O.D. of the pellet} + \text{O.D. of the supernatant}} \times 100$$

2.10. Agarose gel electrophoresis and DNA fragmentation

DNA was extracted from liver tissue using the technique described by Kuo et al. [44]. Each pair of cell lysates was then mixed with 0.4 ml of saturated NaCl, and incubated for 5 min on ice before being centrifuged for 30 min at 2000 rpm. Chilled ethanol was utilized to precipitate the DNA, which was then separated by centrifugation. After the extracted DNA was suspended in TAE buffer (1 mM EDTA and 40 mM Tris-acetate), it was electrophoresed on a 1 % agarose gel containing 0.5 μg/ml ethidium bromide. The bands of DNA were spotted and captured on camera using a UV transilluminator.

2.11. Statistical analysis

SPSS for Windows was used to statistically analyze the data and presented as mean ± SE for matched samples. ANOVA (or one-way analysis of variance), was used to compare the means, and Tukey's post-hoc multiple comparison test was used to evaluate group differences. P-values were considered statistically significant if they were < 0.05.

Table 1
Primer sequences used for real-time PCR.

Gene	Nucleotide sequence 5´–3`	Accession no.	Product size (bp)	Annealing (°C)	Reference
Glutathione peroxidase 1 (Gpx1)	CTCTCCGCGGTGGCACAGT CCACCACCGGGTCGGACATAC	NM_030826.4	288 bp	61	[37]
Superoxide dismutase 1 (SOD)	GCAGAAGGCAAGCGGTGAAC TAGCAGGACAGCAGATGAGT	NM_017050.1	447 bp	60	[38]
Catalase (CAT)	GCGAATGGAGAGGCAGTGTAC GAGTGACGTTGTCTTCATTAGCACTG	NM_012520.2	652 bp	61	[39]
GAPDH	CAAGGTCATCCATGACAACTTTG GTCCACCACCCTGTTGCTGTAG	NM_017008.4	496 bp	58	[40]
β-actin	CCACCATGTACCCAGGCATT CGGACTCATCGTACTCCTGC	NM_031144	189 bp	60	[41]
Bax	AGGATGATTGCTGATGTGGATAC CACAAAGATGGTCACTGTCTGC	NM_017059.2	300 bp	60	[34]
Bcl−2	GCTACGAGTGGGATACTGGAGA AGTCATCCACAGAGCGATGTT	NM_016993.2	446 bp	59	[42]

3. Results

The HPLC results of polyphenols in FAE (Table 2) showed the presence of 11 polyphenolic compounds. The main components were gallic acid (190.21 μg/g), taxifolin (92.37 μg/g), naringin (35.78 μg/g), catechin (12.78 μg/g), and methyl gallate (11.26 μg/g). Ferulic acid, vanillin, rutin, coffeic acid, chlorogenic acid, and syringic acid were detected in lesser amounts (3.46, 2.15, 1.67, 1.11, and 0.9 μg/g, respectively). The GC-MS detected 24 compounds (Table 3) with the largest compounds being 3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-, and 9-Octadecenamide, (*Z*)- (52.51 and 32.56 %, respectively). Palmitic acid-TMS derivative, Linolelaidic acid-trimethylsilyl ester, erucylamide, and myristic acid were found in low concentrations (2.11, 1.35, 1.27, and 1.19 %, respectively). Other compounds, however, were found at concentrations less than 1 %.

The results of $CdCl_2$ alone or with FAE on biochemical indices (Table 4) revealed that $CdCl_2$ alone significantly raised AST, ALT, D.BIL, T.BIL, urea, creatinine, uric acid, and NO, while decreasing Alb and TP. Animals given FAE (LD) or FAE (HD) exhibit no significant changes in serum biochemical markers, except TP, which was found to be raised, and NO, which was found to be lowered in a dose-dependent pattern. Co-administration of $CdCl_2$ plus FAE (LD) or FAE (HD) showed a substantial improvement in all the evaluated parameters towards the control levels, and FAE (HD) was more efficient than the low dosage since it could normalize AST and ALT and considerably reduce the levels of creatinine, urea, uric acid, and NO compared to the group received $CdCl_2$ alone. Table 5 depicts the effect of different treatments on lipid profile and shows that $CdCl_2$ dramatically increased Cho, TriG, and LDL levels while decreasing HDL levels. Administration of FAE (LD) or FAE (HD) alone causes a significant drop in Cho and TriG but does not affect HDL or LDL levels. Administration of $CdCl_2$ with FAE (LD or HD) dramatically improved lipid profile parameters in a dose-dependent manner, whereas FAE (HD) normalized LDL and HDL levels.

The current results also showed that the rats treated with $CdCl_2$ induced a significant elevation in TNF-α, AFP, and CEA (Table 6). Animals that received FAE (LD) or FAE (HD) had significantly lower levels of TNF-α, AFP, and CEA than the controls. Co-administration with $CdCl_2$ plus FAE at the two tested dosages improved these cytokines toward the control levels but did not normalize them except AFP in the groups that received $CdCl_2$ plus FAE (LD) or FAE (HD) which was similar to those in the control.

Table 7 shows the impact of $CdCl_2$ and/or FAE at the two investigated dosages on the activity of antioxidant enzymes and the levels of MDA in the liver and kidneys. $CdCl_2$ induces a significant reduction in the levels of GSH, GPx, CAT, and SOD in the liver and kidneys, along with a substantial rise in MDA in both tissues. Except for CAT in the FAE (LD)-treated group, which was similar to the control level, the administration of FAE alone dramatically reduced the levels of hepatic and renal MDA and significantly elevated the antioxidant indices in a dose-dependent manner. Administration of $CdCl_2$ with FAE dramatically improved all antioxidant enzyme activity and MDA levels in both tissues in a dose-dependent manner, although none of these parameters were normalized by any of these treatments.

The current data showed that $CdCl_2$ induced down-regulation of mRNA of GPx (Fig. 1A), SOD (Fig. 1B), and CAT (Fig. 1C) in the liver tissue by 3.3, 3.12, and 3.6-fold, respectively. FAE at the two tested doses doesn't affect mRNA expression of these genes except GPx mRNA which was up-regulated in the liver of the animals that received FAE (HD). The expression transcript levels of the target genes in the groups that received $CdCl_2$ plus FAE showed a considerable improvement in mRNA expression of these genes and FAE (HD) was more efficient than FAE (LD) regarding GPx, but no significant difference was observed between the two tested doses in term of SOD and CAT.

To investigate the preventative impact of FAE against $CdCl_2$-induced hepatic apoptosis, Bax and bcl-2 as the pro and the anti-apoptotic genes were tested using RT-qPCR. The current results showed that $CdCl_2$ increased the hepatic Bax mRNA expression by 2.1-fold (Fig. 2A) and down-regulated bcl-2 mRNA by 2.6-fold compared to the control level (Fig. 2B). FAE (LD) administration had no effect on the expression of mRNA for both bcl-2 and Bax; however, FAE (HD) administration dramatically reduces Bax mRNA expression while increasing the expression of bcl-2 mRNA above the control level. The combined treatment of $CdCl_2$ and FAE at the two tested dosages enhanced the expression of both genes, with no significant difference in the expression of Bax mRNA between FAE (LD) and FAE (HD).

The impact of various treatments on the percentage of DNA fragmentation (Table 8) showed that $CdCl_2$ administration considerably enhanced the proportion of DNA fragmentation. Meanwhile, there was no significant difference in the proportion of DNA fragmentation between rats given low or high doses of FAE. When $CdCl_2$ and FAE were administered together at the two tested dosages, the percentage of DNA fragmentation was much lower than when $CdCl_2$ was administered alone. For the groups that got the low and high dosages of FAE, the percentage of inhibition in DNA fragmentation was 44.3 and 45.8 %, respectively.

The data in Fig. 3 showed that agarose gel electrophoresis of the DNA validated the results of the colorimetric tests of DNA fragmentation and verified the altered amounts of gene transcripts. When compared to the negative control and FAE-treated groups, the liver of rats treated with $CdCl_2$ revealed a smear (the hallmark of necrosis) of DNA fragmentation with no ladder formation, indicating random DNA degradation. Treatment with $CdCl_2$ with either a low or high dosage of FAE significantly reduced DNA fragmentation, although DNA remained localized at the starting location. Furthermore, no significant difference was found between the DNA electrophoretic patterns of FAE (LD) or FAE (HD) and control groups.

Microscopic analysis of liver sections from control animals or those given FAE (LD) or FAE (HD) revealed normal histological architecture (Fig. 4A, B, and C). Rat liver sections treated with CdCl2 revealed significant vacuolar degeneration, as well as monocyte infiltration, congestion, and necrosis (Fig. 4D). Animals treated with $CdCl_2$ with FAE (LD) or FAE (HD) had significantly improved hepatic architecture (Fig. 4E,F). The kidney sections of the control rats (Fig. 5A), rats that got FAE (LD) (Fig. 5B), or those that received FAE (HD) (Fig. 5C) revealed normal renal architectures. However, kidney sections from rats treated with $CdCl_2$ (Fig. 5D) exhibited localized vacuolar degeneration in the epithelial lining of renal tubules with pyknotic nuclei and shrinkage in mesangial glomeruli. Rats treated with $CdCl_2$ plus FAE (LD) showed minor improvements in tubules in their kidney sections (Fig. 5E); however, rats treated with $CdCl_2$ plus FAE (HD) showed mild intratubular congestion and fibrous tissue (Fig. 5F).

Table 2
HPLC analysis of total polyphenols in FAE aqueous extract.

Polyphenol	Area	Conc. (μg/g)
Gallic acid	1894.52	190.21
Chlorogenic acid	14.34	1.11
Catechin	56.94	12.78
Methyl gallate	580.84	11.26
Caffeic acid	49.86	1.67
Ferulic acid	135.47	4.72
Syringic acid	22.95	0.90
Rutin	17.49	2.15
Vanillin	128.13	3.46
Naringenin	662.02	35.78
Taxifolin	806.60	92.37

4. Discussion

The HPLC analysis of FAE sowed the identification of a total of 11 phenolic compounds and organic acids including gallic acid, the bulk of which were gallic acid and taxifolin. In this concern, Deveci et al. [45]

Table 3
GC-MS analysis of FAE (Accession No. KP317994.1).

Peak	RT	Name	Formula	Area	Area Sum %
1	9.817	Pentane, 2,2,3,4-tetramethyl-	C9H20	68977.99	0.48
2	10.768	Propanoic acid, hexyl ester	C9H18O2	56844.2	0.39
3	11.712	1,3-Hexanediol, 2-ethyl-	C8H18O2	39957.67	0.28
4	12.155	Octanoic acid, TMS derivative	C11H24O2Si	67731.06	0.47
5	12.37	Decane, 2,3,5,8-tetramethyl-	C14H30	57033.61	0.4
6	12.982	1-Octadecanesulphonyl chloride	C18H37ClO2S	57579.55	0.4
7	14.696	Decane, 2,3,5,8-tetramethyl-	C14H30	52101.13	0.36
8	15.203	3-Furanacetic acid, 4-hexyl−2,5-dihydro−2,5-dioxo-	C12H16O5	7578799.3	52.51
9	17.54	Dodecane, 2,6,10-trimethyl-	C15H32	73179.64	0.51
10	17.913	2-Hexenal, 2-ethyl-	C8H14O	76891.23	0.53
11	17.995	2-Piperidinone, N-[4-bromo-n-butyl]-	C9H16BrNO	58164.97	0.4
12	18.147	2-Hexenal, 2-ethyl-	C8H14O	39174.48	0.27
13	18.963	Myristic acid, TMS derivative	C17H36O2Si	172399.75	1.19
14	19.715	Dodecane, 2,6,10-trimethyl-	C15H32	76088.49	0.53
15	20.84	Palmitic Acid, TMS derivative	C19H40O2Si	304197.14	2.11
16	21.679	2-methyltetracosane	C25H52	51607.7	0.36
17	22.332	Linoelaidic acid, trimethylsilyl ester	C21H40O2Si	194835.45	1.35
18	22.367	Oleic Acid, (*Z*)-, TMS derivative	C21H42O2Si	124053.79	0.86
19	22.571	Stearic acid, TMS derivative	C21H44O2Si	69641.01	0.48
20	23.754	9-Octadecenamide, (*Z*)-	C18H35NO	4699618.8	32.56
21	25.427	8-Methyl−6-nonenamide	C10H19NO	134088.28	0.93
22	26.866	Erucylamide	C22H43NO	183997.14	1.27
23	31.203	Silane, diethylheptyloxyoctadecyloxy-	C29H62O2Si	56058.2	0.39
24	33.039	Methyldopa, 4TBDMS derivative	C34H69NO4Si4	54670.85	0.38

Table 4
The impact of FAE on serum biochemical markers in rats treated with $CdCl_2$.

Groups Parameter	Control	FAE (LD)	FAE (HD)	$CdCl_2$	$CdCl_2$ + FAE (LD)	$CdCl_2$ + FAE (HD)
ALT (U/L)	52.87 ± 3.44^a	53.33 ± 3.31^a	53.57 ± 4.29^a	$78.37 \pm 4.6b^b$	58.07 ± 2.30^c	54.70 ± 1.36^d
AST (U/L)	199.27 ± 4.93^a	197.17 ± 4.57^a	198.00 ± 3.31^a	243.83 ± 3.66^b	200.07 ± 3.67^a	196.83 ± 7.84^a
Alb (mg/dl)	3.80 ± 0.06^a	3.82 ± 0.15^a	3.81 ± 0.14^a	1.61 ± 0.06^b	3.80 ± 0.12^a	3.92 ± 0.18^a
TP (g/dl)	7.07 ± 0.32^a	7.72 ± 0.35^b	7.64 ± 0.18^c	4.59 ± 0.17^d	7.38 ± 0.03^b	7.41 ± 0.18^b
T.BIL (mg/dl)	0.06 ± 0.01^a	0.06 ± 0.01^a	0.06 ± 0.01^a	0.09 ± 0.01^b	0.07 ± 0.01^c	0.07 ± 0.01^c
D.BIL (mg/dl)	$0.018 \pm .002^a$	0.017 ± 0.004^a	0.019 ± 0.002^a	0.060 ± 0.002^b	0.017 ± 0.001^a	0.015 ± 0.001^c
Creatinine (mg/dl)	0.70 ± 0.03^a	0.71 ± 0.10^a	0.70 ± 0.04^a	0.96 ± 0.01^b	0.61 ± 0.04^c	0.58 ± 0.02^c
Urea (mg/dl)	49.80 ± 2.19^a	50.70 ± 1.10^a	51.93 ± 3.06^a	66.67 ± 2.47^b	54.90 ± 3.95^c	44.33 ± 2.37^d
Ulric Acid (mg/dl)	1.63 ± 0.12^a	1.62 ± 0.10^a	1.63 ± 0.12^a	2.44 ± 0.22^b	1.77 ± 0.07^c	1.43 ± 0.12^d
NO (μmol/L)	17.80 ± 0.21^a	16.50 ± 0.35^b	15.73 ± 0.90^c	31.90 ± 2.05^d	20.23 ± 2.85^e	19.60 ± 3.26^f

Means superscripts with different letters within each row indicate significant differences ($P < 0.05$).

Table 5
Impact of FAE on parameters of the lipid profile in rats given $CdCl_2$.

parameter Groups	Cho (mg/dl)	Tri G (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	53.67 ± 3.84^a	78.33 ± 3.71^a	38.97 ± 2.88^a	15.47 ± 2.24^a
FAE (LD)	47.33 ± 0.88^b	71.33 ± 6.69^b	39.47 ± 4.22^a	15.07 ± 1.28^a
FAE (HD)	40.00 ± 0.58^c	68.67 ± 4.06^c	41.77 ± 2.73^a	14.50 ± 0.35^a
$CdCl_2$	80.00 ± 4.51^d	114.00 ± 1.20^d	23.70 ± 1.17^b	21.67 ± 0.88^b
$CdCl_2$ + FAE (LD)	63.67 ± 1.86^e	92.67 ± 14.48^e	35.07 ± 2.14^c	17.53 ± 1.12^c
$CdCl_2$ + FAE (HD)	55.33 ± 1.86^c	81.00 ± 4.51^f	41.33 ± 1.70^a	14.73 ± 1.70^a

Means superscripts with different letters within each row indicate significant differences ($P < 0.05$).

Table 6
Effects of FAE on tumor markers in rats given $CdCl_2$.

parameter Groups	TNF-α (ng/ml)	AFP (ng/ml)	CEA (ng/ml)
Control	0.33 ± 0.01^a	1.40 ± 0.20^a	1.15 ± 0.17^a
FAE (LD)	0.23 ± 0.02^b	1.27 ± 0.14^b	1.03 ± 0.27^b
FAE (HD)	0.26 ± 0.02^c	1.55 ± 0.12^c	1.03 ± 0.33^b
$CdCl_2$	0.81 ± 0.03^d	3.34 ± 0.25^d	4.83 ± 0.38^c
$CdCl_2$ + FAE (LD)	0.40 ± 0.02^e	1.38 ± 0.08^a	2.46 ± 0.31^d
$CdCl_2$ + FAE (HD)	0.35 ± 0.01^a	1.05 ± 0.01^e	1.92 ± 0.06^e

Means superscripts with different letters within each row indicate significant differences ($P < 0.05$).

identified 16 compounds and reported that protocatechuic acid was the dominant phenolic compound followed by catechin hydrate and fumaric acid. Moreover, Niazmand and Razavizadeh [46] reported that vanillic acid, ferulic acid, coumaric acid, umbelliprenin, kamolonol, karatavicinol, and galbanic acid were identified by HPLC in the aqueous extract. The differences between our data and those in the previous reports may be due to several environmental elements including plant variety, altitude, climate, and water availability [25].

The GC-MS analysis identified 25 bioactive compounds, 3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo-and 9-Octadecenamide, (*Z*)- were the main compounds (52.51 and 32.56 %, respectively). Niazmand and Razavizadeh [46] reported that (*Z*)-b-ocimene, (*E*)-1-propenyl sec-butyl disulfide (*E*)-b-ocimene and b-pinene were the major compounds; however, Baser et al. [47] stated that nonane, a-pinene, and germacrene B were the main constituents. Moreover, Kavoosi and Rowshan [48] reported that (*E*)-1-propenyl sec-butyl disulfide, (*Z*)-1-propenyl secbutyl disulfide, 10-epi-c-eudesmol, α, and β-pinene were the main components identified by GC-MS. In this concern, Fokou et al. [49] reported that chemical composition may be changed from plant to plant even in the same species due to several abiotic factors such

Table 7
The impact of FAE on antioxidant enzymes activity and MDA in rats treated with $CdCl_2$.

Parameter Groups	MDA (nmol/g tissue)		GSH (μmol/g protein)		GPx (U/mg protein)		SOD (U/mg protein)		CAT (U/mg protein)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Control	0.67 ± 0.03[a]	0.56 ± 0.02[a]	1.07 ± 0.05[a]	1.33 ± 0.08[a]	28.00 ± 1.04[a]	23.65 ± 0.58[a]	1.01 ± 0.02[a]	0.79 ± 0.04[a]	15.91 ± 0.41[a]	12.51 ± 0.23[a]
FAE (LD)	0.58 ± 0.03[b]	0.51 ± 0.04[b]	1.26 ± 0.04[b]	1.47 ± 0.04[b]	32.92 ± 2.92[b]	27.44 ± 0.54[b]	1.21 ± 0.05[b]	0.94 ± 0.03[b]	15.88 ± 0.52[a]	14.07 ± 0.45[a]
FAE (HD)	0.52 ± 0.02[c]	0.47 ± 0.03[c]	1.63 ± 0.14[c]	1.86 ± 0.02[c]	35.06 ± 0.13[c]	31.00 ± 0.44[c]	1.70 ± 0.18[c]	1.36 ± 0.07[c]	22.34 ± 1.33[b]	17.54 ± 0.45[b]
$CdCl_2$	1.43 ± 0.11[d]	1.38 ± 0.05[d]	0.25 ± 0.03[d]	0.40 ± 0.04[d]	12.87 ± 0.31[d]	10.54 ± 0.89[d]	0.17 ± 0.03[d]	0.10 ± 0.01[d]	7.71 ± 0.23[c]	6.30 ± 0.06[c]
$CdCl_2$ + FAE (LD)	1.03 ± 0.05[e]	1.06 ± 0.03[e]	0.46 ± 0.03[e]	0.53 ± 0.02[e]	15.06 ± 0.13[e]	16.92 ± 0.81[e]	0.39 ± 0.04[e]	0.30 ± 0.03[e]	9.05 ± 0.40[d]	8.13 ± 0.12[d]
$CdCl_2$ + FAE (HD)	0.85 ± 0.05 [f]	0.76 ± 0.04 [f]	0.90 ± 0.01 [f]	1.04 ± 0.05 [f]	21.05 ± 0.89 [f]	20.22 ± 0.61 [f]	0.72 ± 0.04 [f]	0.57 ± 0.02 [f]	13.41 ± 0.79[e]	10.85 ± 0.91[e]

Means superscripts with different letters within each row indicate significant differences ($P < 0.05$).

as soil hydrology, salinity, pH, and the microclimate in which the plants are growing [50]. Besides, other biotic factors affect the chemical composition of the plant such as the soil organisms and microorganisms [51]. Postharvest treatments also can affect the chemical components such as the drying of the plant materials which may affect the reaction between the secondary metabolites [52]. Additionally, the method of extraction was reported to induce a significant influence on the chemical composition of the plants [53].

Cd is a prevalent environmental toxic pollutant with a 20–30 years biological half-life in humans, and accumulates in the human tissue due to the low excretion rate from the body [1,6]. After the absorption, Cd is distributed to different organs in the body through blood circulation but accumulates in the target organs mainly the kidneys and liver [54]. ATSDR and WHO have classified Cd as the most dangerous element that harms organs such as the liver, kidney, and nervous system [7,55]. In our *in vivo* study, the preventive role of FAE was evaluated against $CdCl_2$-induced oxidative damage, disturbances in gene expression, and DNA fragmentation in rats. The $CdCl_2$ dose was determined based on our previous study [56]; however, the FAE dose was determined based on the literature [57].

Administration of $CdCl_2$ alone increased significantly the liver and kidney function markers, Cho, TriG, LDL-Cho, TNF-α, AFP, CEA, MDA in the hepatic and renal tissue, and Bax mRNA expression along with a dramatic decrease in HDL-Cho, the antioxidant enzymes in the liver and kidney and the expression of their mRNA. The elevation of liver enzymes in rats who received $CdCl_2$ alone is similar to the previous results which indicated that $CdCl_2$ impairs the liver and acts as a crucial biomarker of its function [56]. The elevation of the serum transaminases suggested their leakage in the bloodstream as a result of the severe hepatocyte membrane damage [58]. Additionally, the deficiency of TP and Alb revealed an overexpression of the high molecular mass proteins [6]. $CdCl_2$ administration also impairs the function of kidneys as evidenced by the increase in uric acid, creatinine, and urea levels as suggested by Yan and Allen [5]. The initial indicator of renal dysfunction is an increase in urea; however, the most accurate indicator is an increase in creatinine, which occurs when the kidneys sustain any significant damage. Zhu et al. [59] showed a similar significant elevation in serum creatinine and urea levels in laying hens exposed to $CdCl_2$. Additionally, Cd may induce renal injury in a dose-dependent manner, which can be attributed to disrupted $Fe2+$ absorption, redox imbalance, and apoptosis in the kidney [60].

The current data showed that oral administration of $CdCl_2$ disturbs the level of lipid profile compared with the non-exposed rats. These results confirmed the previous findings of other investigators who suggested that exposure to Cd generates oxidative stress and produces excessive amounts of ROS resulting in several pathophysiological processes, disturbance in the oxidant/ pro-oxidant balance, damage to the cell function, unfavorable biological reactions leading to dyslipidemia and disease development [6,61].

Oral administration of $CdCl_2$ also showed a dramatic increase in TNF-α, AFP, and CEA which agree with the previous reports [62]. The overproduction of these cytokines is probably owing to the immunomodulatory action of $CdCl_2$ on the immune cells as a consequence of oxidative stress [62]. Our findings supported the actions of $CdCl_2$ on M1-type macrophages, which produce pro-inflammatory cytokines to trigger an inflammatory response [63]. Kany et al. [64] indicated that the activated macrophages produce TNF-α in response to infections and damaging stimuli, which is a key element in systemic and local inflammations. Additionally, this cytokine prolongs and amplifies the inflammatory reactions by triggering the release of IL-1β, ROs, and NO by other cells and hence promotes the damage and inflammation of the tissue [63].

It is well documented that Cd-induced hepato-nephrotoxicity is mainly through the generation of ROS, lipid peroxidation, and cell inflammation along with the diminishing of antioxidant defense mechanisms such as GSH, CAT, SOD, and GPx [65]. The GSH depletion is mainly caused by the excess free radicals' production [66], and agrees with the previous data suggesting that this metal interacts with SH-groups in glutathione as the major intracellular defense resulting in the status of oxidative stress [66]. In addition, the obtained data confirm the elevation of MDA through the lowering of the level of GSH during Cd exposure [6]. ROS such as superoxide radicals' hydrogen peroxide, and hydroxyl affect several cell components mostly lipids, carbohydrates, and proteins which leads to the inconsistency in metabolic dysfunction and cell integrity [67]. MDA is the master participant in lipid peroxidation; its malignant activity causes parenchymal cell damage, interferes with several biomolecules like acetaldehyde, and DNA, and advances the glycation end product that constitutes the integrity of the cell [68]. Thus, the increased levels of oxidative markers (MDA and NO) in the liver and kidney, as well as the decrease in antioxidant enzymes in these organs, result in the development of subsequent pathological conditions due to the prolonged retention in the organs [69].

The perturbation in mRNA expression of Bax and Bcl-2 (pro- and anti-apoptotic genes), as well as the decrease in CAT, SOD, and GPx (antioxidant enzymes) and the increase in the percentage of DNA fragmentation, support the hypothesis that Cd catalyzes its toxicity through oxidative damage, as previously suggested [70,71]. The histological examination of the liver and kidney sections reported herein is consistent with previous reports, indicating that Cd exposure resulted in serious pathological changes in both organs, the majority of which were caused by oxidative damage [72].

In the current study, we demonstrated the hepato-nephroprotective role of FAE against $CdCl_2$-induced oxidative damage in rats. Interestingly, FAE did not induce any deleterious effects at the tested doses, and both doses induced a marked protective effect against changes in oxidative stress markers, lipid profiles, gene expression, biochemical

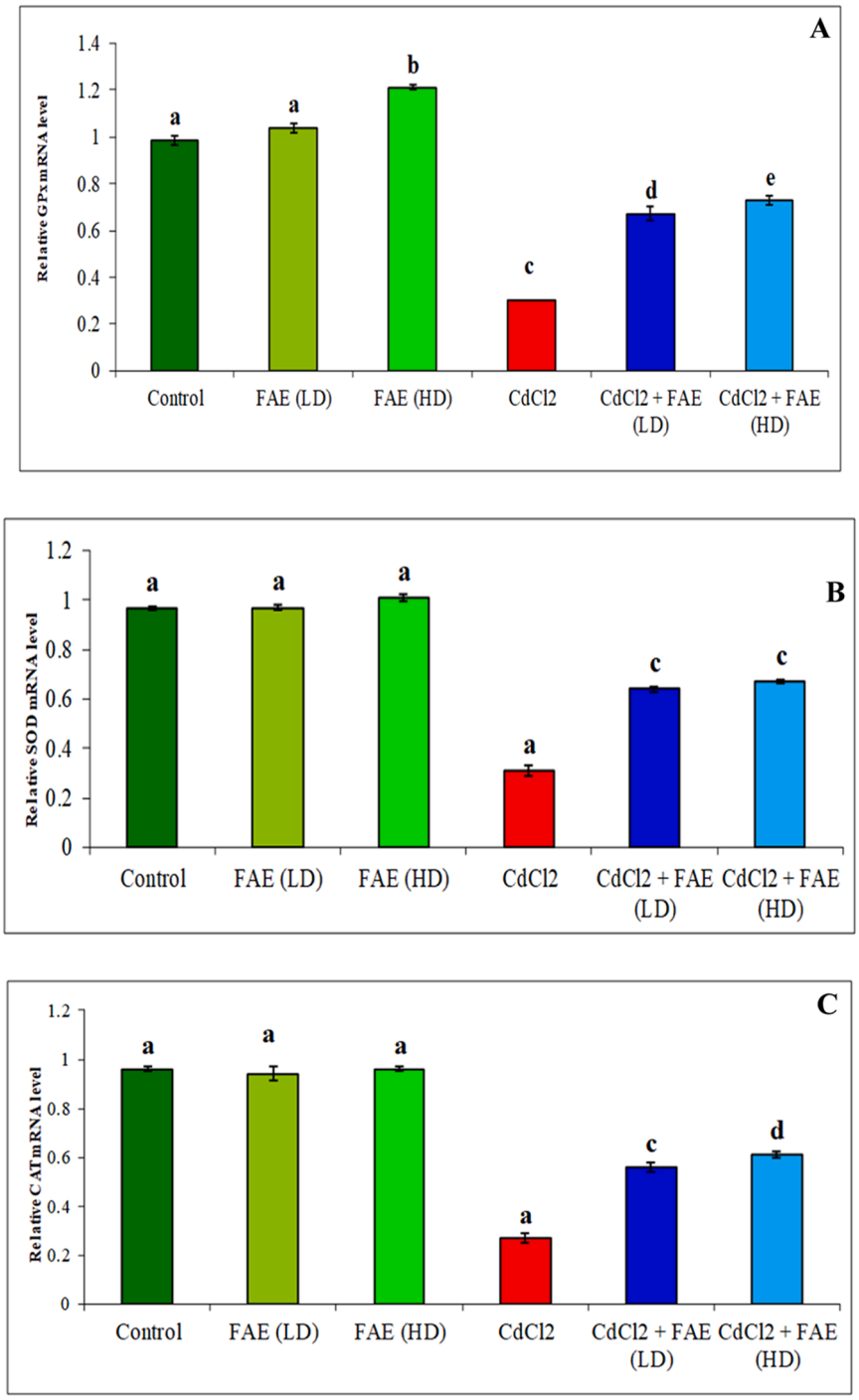


Fig. 1. Effect of FAE on the relative gene expression of (A) GPx, (B) SOD and (C) CAT in liver of rats with treated with $CdCl_2$. Data represent the mean ± SD of three replicates in same group. Column superscripts with different letter are significantly differences at $P \leq 0.05$.

parameters, cytokines, liver and kidney histology, and lipid profiles. This protective effect is mainly due to the rich content of phenolic and other bioactive compounds determined by HPLC and GC-MS analysis which possess a strong antioxidant activity. The antioxidant of phenolic compounds is well documented previously, for instance, the extract is rich in gallic acid, taxifolin, naringenin, catechin, methyl gallate, vanillin, rutin, caffeic acid, chlorogenic acid, and syringic acid which are well known to have antioxidant activities. Gallic acid and methyl gallate reduce offensive agents, increase mucosal defense, activate antioxidant mechanisms, and inhibit toxic oxidative mechanisms [73]. They also increase the activity of the antioxidant enzymes such as SOD and CAT and/or increase the nonenzymatic antioxidant markers such as GSH [74]. Taxifolin, rutin, and vanillin showed high reducing ability, marked antioxidant, free radical scavenging, and metal-chelating properties [75]. These phenolic compounds were reported to inhibit MDA formation, enhance CAT, GPx, and SOD activities, and induce GSH formation [42]. Naringenin and catechin also are potent antioxidants and free radicals scavengers, able to efficiently chelate trace elements such as Cd, which enhances ROS generation, hydroxyl oxidation, and superoxide radicals by hydrogen atom donation [76]. Additionally, caffeic acid and ferulic acid possess antioxidant activity and promote neutral sterol and its acidic form, leading to the decrease of Cho and promoting the fecal

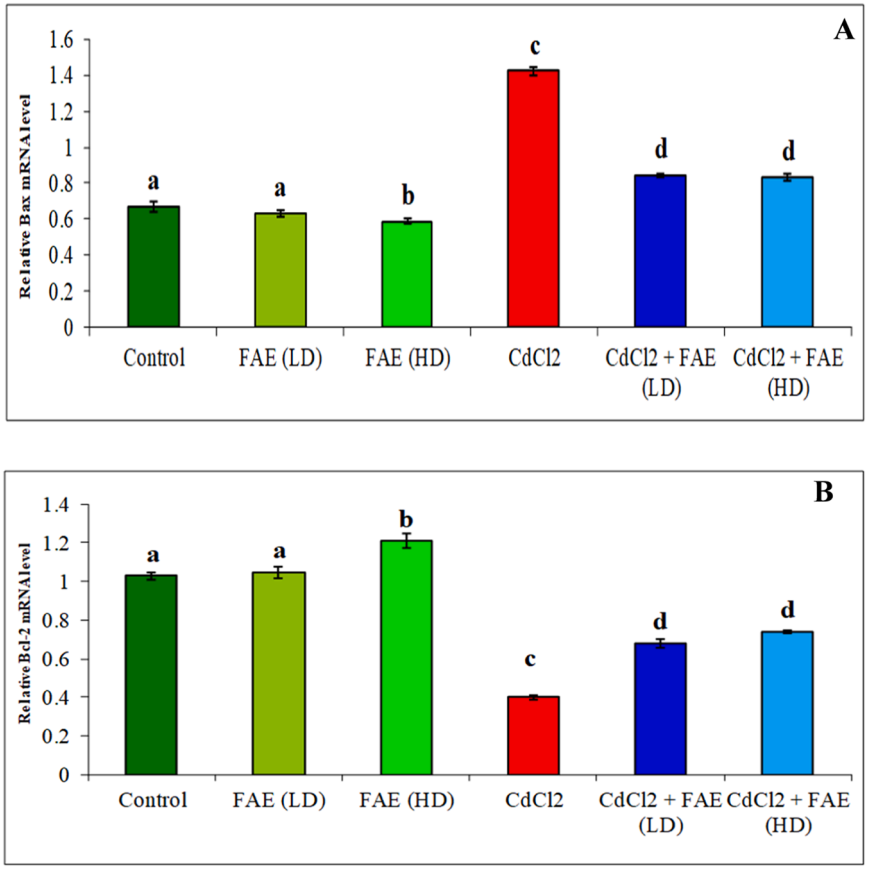


Fig. 2. Effect of FAE on the relative gene expression of (A) Bax and (B) Bcl-2 in the liver of rats treated with $CdCl_2$. Data represent the mean ± SD of three replicates in same group. Columns superscripts with different letters are significantly differences at $P \leq 0.05$.

Table 8
DNA fragmentation in liver cells of rats treated with FAE and $CdCl_2$ singly or in combination.

Treatment	DNA fragmentation Mean (% ± S.E)	DNA fragmentation inhibition (%)
Control	3.2 ± 0.05^{a}	
$CdCl_2$	7.9 ± 0.35^{b}	
FAE (LD)	2.9 ± 0.28^{a}	
FAE (HD)	3.1 ± 0.17^{a}	
$CdCl_2$ + FAE (LD)	4.4 ± 0.17^{c}	44.3
$CdCl_2$ + FAE (HD)	4.2 ± 0.29^{c}	45.8

excretion of sterols [77]. Moreover, chlorogenic acid showed antioxidant activity, reduced ER stress, and modulate the mRNA expressions of XBP1s GRP78, 17β-HSD, and 3β-HSD in the rats [78].

In addition to the polyphenolic compounds, the bioactive compounds detected by the GC-MS belonging to various biological classes that induce several activities such as antioxidant, anticancer, anti-inflammatory, immunomodulatory, antidiabetic, and free radical scavenging properties [28,79]. Generally, the preventive role of FAE against $CdCl_2$ is ascribed to its abundance of phenolic compounds and other bioactive components, which are well known to protect against oxidative stress through the free radicals scavenging activity, increasing the antioxidant capacity of the body, reducing the lipid peroxidation level and modulation the expression of several responsible gens.

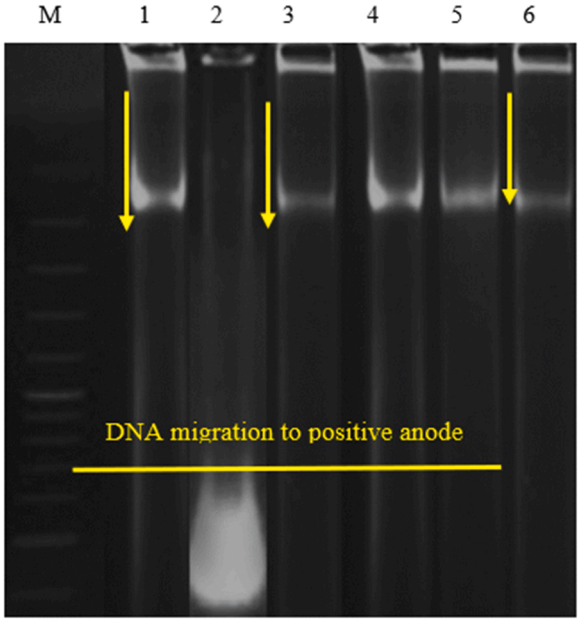


Fig. 3. Agarose gel electrophoresis of isolated DNA from the liver of rats. Lane M, 100 bp DNA ladder; Lane 1, untreated control group; Lane 2, $CdCl_2$-treated group; Lane 3, FAE (LD)-treated group; lane 4, FAE (HD); Lane 5, FAE (LD) + $CdCl_2$-treated group; and lane 6, FAE (HD) + $CdCl_2$-treated group. Yellow arrows indicate the direction of movement of DNA in agarose gel.

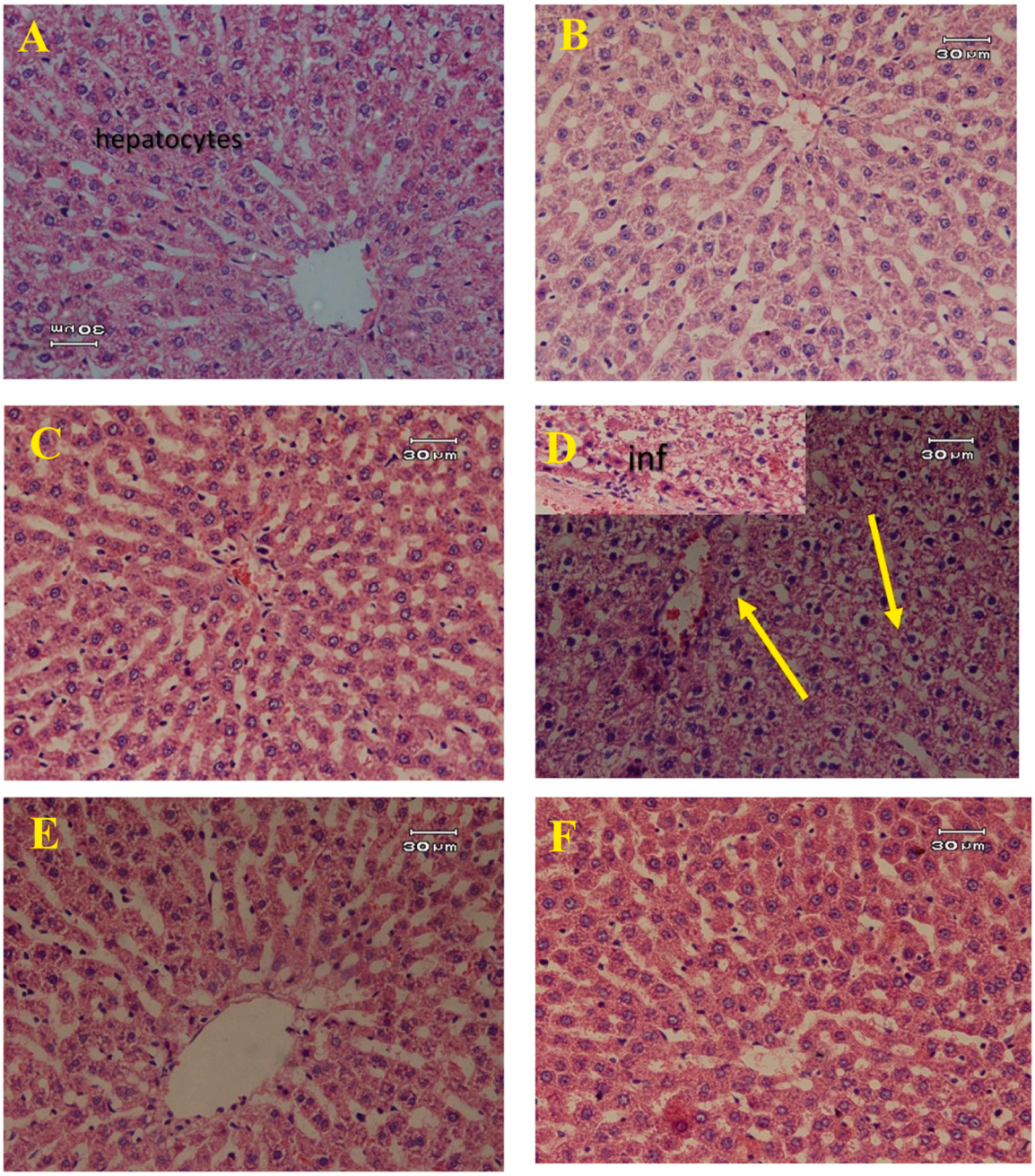


Fig. 4. Photomicrograph of the liver section of control animals (A), rats treated with FAE (LD), (B) and rats treated with the FAE (HD), (C) showing normal liver architecture. The liver section of rats treated with $CdCl_2$ showed severe vacuolar degeneration (yellow arrows), monocytes infiltration (inf), congestion and necrosis were also recorded (D). The liver of rats treated with $CdCl_2$ plus FAE (LD) or FAE (HD) showed a marked improvement in liver architecture and the sections appear simi normal (E, F).

5. Conclusion

The current study showed that FAE is rich in organic acids and phenolic compounds as well as other bioactive compounds belonging to monoterpenoids, fatty acids, fatty amides, alkanes, and isopropylidene derivatives. The results also confirmed that the toxicity of $CdCl_2$ was mainly due to oxidative damage and the disturbance of cell integrity in the liver and kidney. The FAE extract possesses potent protection against the oxidative damage of $CdCl_2$ through its antioxidant and free radical scavenging activity. The plant is promising to be used in several applications in medicine and food.

Ethical approval

The protocol of the current study was performed in compliance with the ARRIVE guidelines and the National Health Institutes for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978) and was approved by the ethics Animal Care and Use Committee of the Arish University, Arish, Egypt (approval # ARU/SF.10).

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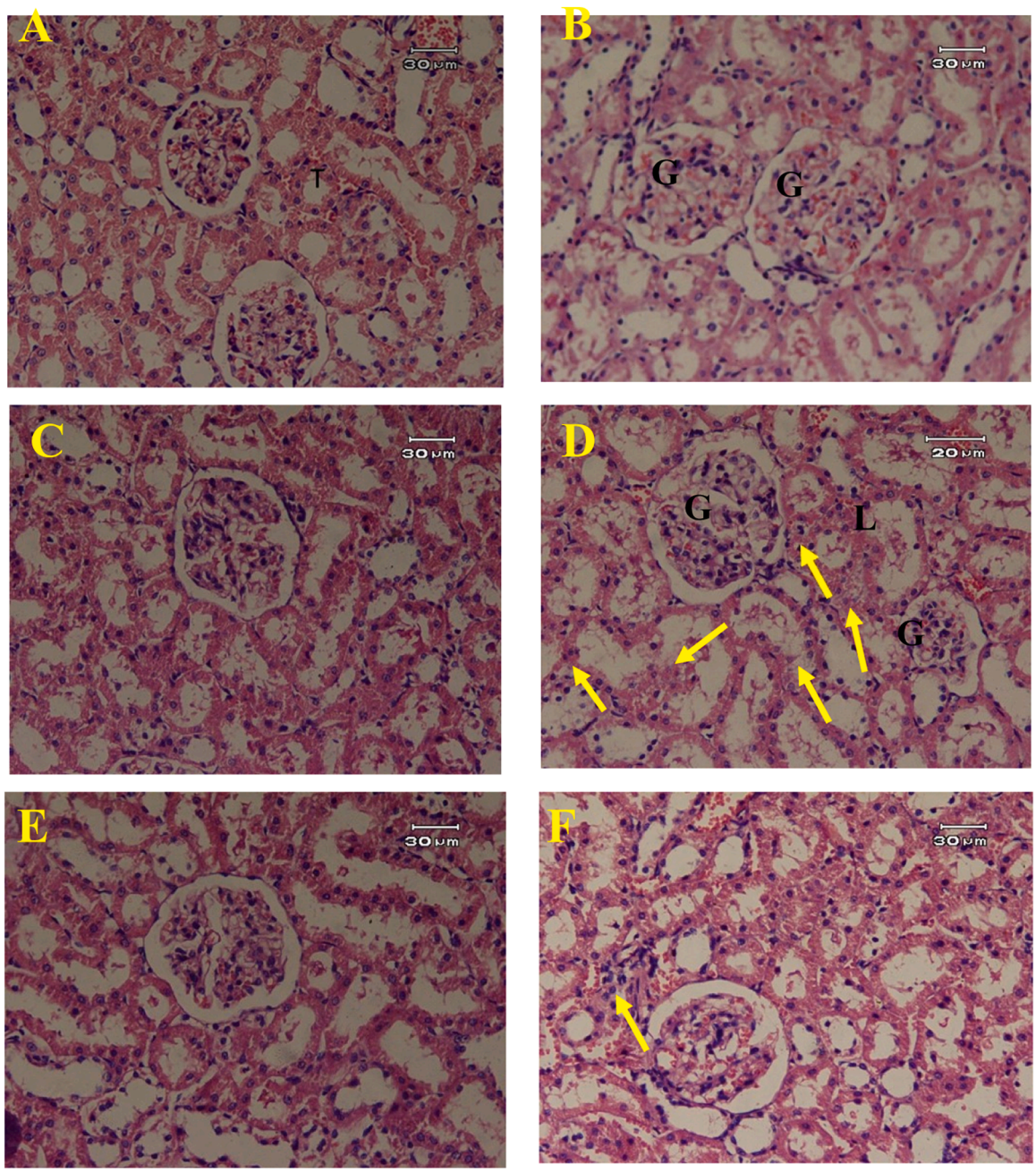

Fig. 5. Photomicrograph of kidney of **(A, B, and C)** control group, FAE (LD), and FAE (HD)-treated groups, respectively showing normal renal architectures; **(D)** kidney section of rats treated with $CdCl_2$ showing focal vacuolar degeneration in epithelial lining of renal tubules with pyknotic nuclei (arrows), shrunking in mesangial glomeruli (G); **(E)** kidney section of rats treated with $CdCl_2$ plus the FAE (LD) showing mild tubular improvement, and **(F)** rats treated with $CdCl_2$ plus the FAE (HD) showing mild intratubular congestion and fibrous tissue (arrow).

Author statement

This work was carried out in collaboration between all authors. Authors HE Mohammed, AA El-Nekeety, HM Rashwan and EE Hassan carried out the experimental work and the biochemical analysis. Author SH Abdel- Aziem carried out the genetic analysis. Author NS Hassan carried out the histological part. Author MA Abdel-Wahhab wrote the protocol, managed the project, managed the analyses of the study, performed the statistical analysis and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

CRediT authorship contribution statement

Hagar E. Mohammed: Methodology, Formal analysis, Data curation. **Aziza A. El-Nekeety:** Writing – original draft, Methodology, Investigation, Data curation. **Hanan M. Rashwan:** Methodology, Investigation, Data curation. **Sekena H Abdel-Aziem:** Methodology, Investigation. **Nabila S. Hassan:** Methodology, Investigation. **Mosaad A. Abdel-Wahhab:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Entesar E. Hassan:** Conceptualization, Formal analysis, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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