Egypt. J. Chem. **Vol. 65,** No. SI:13B pp. 1097 - 1112 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Modulation of *Nrf2*, *CBR1*, and *TNF-a* Expressions and Antioxidant Activity Mediated The Cardioprotective Effects of *Zingiber Officinale* and *Moringa Oleifera* Extracts Against Doxorubicin-Induced Toxicity

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Abstract

This work proposed the cardioprotective mechanisms of *Zingiber officinale* (Ginger) rhizome and *Moringa oleifera*'s leaf and seed methanolic extracts against doxorubicin (DOX)-mediated cardiotoxicity. Mice were distributed into groups: CNT group (normal control); DOX group (cardiotoxic control, 15 mg/kg BW); D+G, D+ML, and D+MS groups (pre-cotreated, 200 mg/kg BW); and G, ML, and MS groups (extracts' controls). The extracts' phytochemicals and antiradical activities were studied. Expression levels of cardiac nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), carbonyl reductase 1 (*CBR1*), and tumor necrosis factor-alpha (*TNF-α*), and oxidative stress were assessed. Serum lipids, cardiac enzymes, and cardiac histopathology were evaluated. The results showed that the extracts contain different phytochemicals and radicals scavenging abilities. DOX significantly increased the cardiac enzymes and induced cardiac redox disturbance and increased lipids as compared with the CNT group. Additionally, DOX significantly decreased the *Nrf2* and *CBR1* expression levels, but it significantly increased the *TNF-α* expression level. Conversely, the pre-cotreatments significantly prevented the DOX effects. Where, the expression levels of the tested genes, redox status, enzymes' activities, lipids, and histopathological changes were recovered. In conclusion, the extracts' cardioprotective potentials may be due to their modulation effects on the important regulator of the cellular redox homeostasis (*Nrf2-CBR1*), inflammatory cytokine (*TNF-α*), serum lipids, and their direct antioxidant activities.

Keywords: Doxorubicin cardiotoxicity; Oxidative stress; *Zingiber officinale*; *Moringa oleifera*; Phenolic and flavonoid; Gene expression; Antioxidant

1. Introduction

Doxorubicin (DOX) is one of the frequently prescribed chemotherapies because of its high anticancer activity, but initiation of cardiotoxicity limits its medical usage **[1]**. The possible ways that were proposed to clarify the DOX-induced injury include oxidative stress (OS), minerals dysregulation, modifications in cardiomyocytes structure and expressions of important genes, mitochondrial disorder, cardiomyocytes apoptosis, and endothelial cells and blood vessels toxicity **[2, 3]**. As known, DOX induces cardiomyocytes apoptosis via activation of cellular death mediators, such as tumor necrosis factor-alpha (TNF-α) **[4]**. Recent gene-chemotherapy interaction studies have revealed that changes in some genes' activities are involved in chemotherapy-induced cardiotoxicity **[5]**. For example, nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) codes for important redox-sensitive transcription factors and is stimulated in response to OS to maintain cellular redox homeostasis and prevent the subsequent oxidative injury **[6]**. Besides, the carbonyl reductase 1 (*CBR1*) gene, is one of the dehydrogenase/reductases family that catalyzes the reduction reactions of the active carbonyl substrates in many biological and pharmacological processes **[7]**. In consequence, the up-regulation of *Nrf2* and *CBR1* may avert OS-induced cardiac toxicity.

Usage of the chemicals that were developed to protect the heart is frequently associated with unwanted side effects, such as weight gain, hypertension, gastric ulcer, and insulin resistance **[8]**. On the other hand, consumption of vegetables and fruits has been associated with abundant health benefits, as a result of their high nutritious content and therapeutic abilities **[9]**. For instance; *Zingiber officinale* (Ginger) has an elongated history in herbal medicine to treat a diversity of disorders including

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Receive Date: 11 August 2022, Revise Date: 20 August 2022, Accept Date: 31 August 2022
DOI: 10.21608/EJCHEM.2022.155718.6734

vomiting, indigestion, pain, and cold-induced symptoms. In addition, it possesses anti-clotting, anti-inflammatory, analgesic, and anti-cancer activities **[10]**; and *Moringa oleifera* is the most broadly distributed species, mainly in Asian countries, and is recognized for numerous pharmacological properties. Leaves, flowers, roots, seeds, and immature pods of *M. oleifera* are believed to act as anti-pyretic, anti-tumor, anti-epileptic, anti-ulcer, anti-inflammatory, diuretic, anti-hypertensive, anti-diabetic, hepatoprotective, anti-fungal, and anti-bacterial activities **[11, 12]**.

However, the earlier cardioprotective effects of *Z. officinale* and *M. oleifera* crude extracts have been reported, their protective mechanisms are still not widely understood. Therefore, this paper attempts to provide a more detailed investigation regarding the cardioprotective effects of the hydro methanolic 80% (v/v) extracts of *Z. officinale* rhizome and *M. oleifera*'s seed and leaf against DOX-induced cardiotoxicity in mice. The preliminary bioactive profiles and antioxidant capacities of the extracts were studied. Additionally, the cardiac OS markers and the expression levels of *Nrf2*, *TNF-α*, and *CBR1* genes, serum cardiac enzymes' activities, and lipids levels were evaluated. In the meantime, the physiological and cardiac histopathological changes were evaluated.

2. Experimental

2.1. Chemicals and kits

Doxorubicin hydrochloride injectable saline solution (Adricin®) was manufactured by Hikma Specialized Pharmaceuticals, Cairo, Egypt. Total protein (LOT 180420), AST (CAT NO: GOT112050), and CK-MB (CAT NO: CKM108025) kits were obtained from Egy-Chem for lab technology, Cairo, Egypt. Kits for LDH (CAT NO: LDHK0109018), total cholesterol (CAT NO: CHOL0109020-2) and triglycerides (CAT NO: TRIG0211020) were obtained from Egyptian Company for Biotechnology, Cairo, Egypt. Catalase (CAT NO: CA2517), SOD (CAT NO: SD2521), NO (CAT NO: NO2533), MDA (CAT NO: MD2529) kits, as well as hematoxylin and eosin stains, Folin Ciocalteau reagent, sodium carbonate, sodium nitrite, sodium hydroxide, potassium persulphate, aluminum chloride, formalin, absolute methanol, physiological saline, sodium phosphate buffer, and the chemicals that were used for the qualitative phytochemicals analysis were obtained from Biodiagnostic Co., Cairo, Egypt. Gallic and ascorbic acids, rutin, DPPH, and ABTS were bought from Sigma-Aldrich, USA. RNA safeguard reagent (CAT NO: BSC54M1) was prepared by Hangzhou Bioer Technology Co., Ltd., China. RNA extraction kit (RNeasy® mini kit, CAT NO: 74104) obtained from Qiagen GmbH, Germany. The *Nrf2*, *CBR1*, *TNF-α*, and *GAPDH* primers were prepared by Beijing SBS Genetech, China. A quantitative reverse transcriptase-PCR Sensifast SYBR low-ROX one-step kit was purchased from Enzynomics, Daejeon, Korea.

2.2. Plant materials and authentication

Fresh leaves and seeds of the *M. oleifera* (NCBI: taxid3735) and *Z. officinale* rhizomes (NCBI: taxid94328) were obtained from and authenticated at the Plant Protection Research Institute, Agricultural Research Center (ARC), Dokki, Giza, Egypt. The plants were collected in October 2019.

2.3. Preparation of the extracts

For extracts preparations, the method of **Anwar *et al.* [13]** was conducted. First, the *moringa*'s leaves and seeds kernel (seeds were removed from the plant pods and the peels were removed), and ginger rhizomes were dried in the air with protection from sunlight for two days and dried in an oven for 24 hours at 37ºC. Then, they were separately ground in an electric grinder to be fine powders and extracted by hydro methanol 80% (v/v) as 1:5 (w/v). At room temperature, the mixtures were left for 48 hours to allow the plants' ingredients to be completely extracted. The extracts were filtered by Whatman filter paper of 150 mm in diameter. The extracts were dried by using a rotary evaporator (Heidolph rotary evaporator) at 45ºC and 138 rpm. The resultant dried extracts were then weighed and stored at 4°C. Eventually, the yield percentage of each extract was calculated [(weight of dried extract/weight of dried plant's powder before the extraction)*100].

2.4. The preliminary phytochemical investigations

2.4.1. Bioactive profile

Standard chemical methods were performed for the identification of glycosides, carbohydrates, alkaloids, terpenoids, flavonoids, tannins, saponins, and anthraquinones in the extracts **[14]**.

2.4.2. Determination of total phenolic content

Folin Ciocalteau assay was used to detect the total phenolic content **[15]**. Briefly, 1 ml of each extract methanolic solution (1 mg/ml) was added to 9 ml of distilled water and 1 ml of Folin-Ciocalteau phenol reagent. Then, after 5 minutes, 10 ml of 7% (w/v) sodium carbonate solution was added to the mixture. The mixture was diluted by adding distilled water to a volume of 25 ml. The absorbance against the reagent blank was measured at 765 nm (JASCO V-730 spectrophotometer) after incubation for 90

minutes at room temperature. Samples were analyzed in triplicate and the total phenolic concentrations were detected from the gallic acid calibration curve. The mean values were reported in gallic acid equivalent (GAE) using the unit of µg/mg of dried extract.

2.4.3. Determination of total flavonoid content

Total flavonoid content was determined using rutin as standard **[16]**. Concisely, 250 µl of each extract methanolic solution (1 mg/ml) was added to 1.25 ml of distilled water and 75 µl of 5% (w/v) sodium nitrite solution and incubated at room temperature for 6 minutes. Then, 150 µl of 10% (w/v) aluminum chloride solution was added and 500 µl of sodium hydroxide solution (1 M) was added after 5 minutes. The mixture was made up to 2.5 ml by adding distilled water. The absorbance was immediately measured at 510 nm (JASCO V-730 spectrophotometer) against the reagent blank. Test samples were analyzed in triplicate and each extract's total flavonoid content was expressed in µg rutin equivalent (RE)/mg of dried extract.

2.4.4. *In-vitro* radical scavenging activities of the extracts

ABTS assay: The antioxidant activities were determined using ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)] method that was described by **Re *et al.* [17]**. In brief, fresh ABTS stock solution was prepared by adding 2.6 ml of ABTS absolute methanolic solution (7 mM) with 2.6 ml of potassium persulphate water solution (2.5 mM) for 24 hours at room temperature in dark, then the absorbance of the solution was adjusted to 0.7 ± 0.02 at 734 nm (AG lab spectrophotometer). Afterward, 100 µl of each extract methanolic solution (0.1 mg/ml) was added to 900 µl of diluted ABTS stock solution by absolute methanol (1:10, v/v) and the absorbance was immediately measured at 734 nm against absorbance of the sample blank (100 µl of the extract solution was added to 900 µl absolute methanol). An ascorbic acid aqueous solution (0.03 mg/ml) and absolute methanol were used as the positive and negative controls, respectively. The extracts' scavenging activities against ABTS are calculated from the following formula: ABTS inhibition % = $[(A\text{-}A_S)/A]*100$, where, A is the absorbance of the diluted ABTS stock solution, and A_S is the absorbance of the remaining ABTS radical after adding the extract against sample blank. The assay was carried out in triplicate.

DPPH assay: The assay depends on the extract's antioxidant molecules scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) radical activity **[18]**.

In concise, 50 µl of different concentrations of each extract (5, 2.5, 1.25, and 0.5 mg/ml) was added to 250 µl of DPPH absolute methanolic solution (0.1 mM) in a micro-well plate. The mixtures were incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517 nm against sample blank (250 µl hydro methanol 80% (v/v) + 50 µl sample) (Biochrom EZ Read 800 micro-well plate reader). An ascorbic acid aqueous solution (0.3 mg/ml) and hydro methanol 80% (v/v) were used as positive and negative controls, respectively. The DPPH inhibition (%) is calculated by using the equation: DPPH radical inhibition % = $[(A_D - A_T)/A_D]*100$. Where; A_D is the absorbance of DPPH solution and A_T is the absorbance of DPPH after adding the sample against the sample blank.

Determination of extracts IC_{50} and ARP: The extracts concentrations that inhibit 50% radical (IC_{50}) were determined from the linear equation that resulted from the curve fit between extract concentration as mg/ml (X-axis) and DPPH scavenging % (Y-axis). Antiradical power (ARP) is used to explain the specific antioxidant activity of an antioxidant and is well-defined as a reverse of IC_{50} ($1/IC_{50}$) **[19]**.

Table 1: Group treatment of experimental animals for the study

Group	Treatments		
	1st week	**2nd** week	**3rd** week
CNT	No treatment	Three equal physiological **saline** intraperitoneally injections (500 µl, with 4 days intervals)	
DOX	No treatment	Three equal **DOX** intraperitoneally injections (500 µl, 5 mg/kg BW each injection, with 4 days intervals)	
D+G	**GE** administration*	**DOX** injection with continuous **GE** administration	
D+ML	**MLE** administration*	**DOX** injection with continuous **MLE** administration	
D+MS	**MSE** administration*	**DOX** injection with continuous **MSE** administration	
G	**GE** administration*****		
ML	**MLE** administration*****		
MS	**MSE** administration*****		

CNT (normal control), DOX (doxorubicin), GE (*Z. officinale* rhizomes 80% methanolic extract), MLE (*M. oleifera* leaves 80% methanolic extract), MSE (*M. oleifera* seeds 80% methanolic extract), and * (the dose at 200 µl of 200 mg/kg BW/day, orally).

2.5. Animals

Male albino mice aged between 8-10 weeks and their body weight (BW) between 20-25 g were purchased from Nile Company for Drugs and

Chemicals Industry, Egypt, and maintained at the animal house of Medical Technology Center, Alexandria University, Egypt. The animals were housed in cages with a wood chip floor in constant conditions (temperature 21±2°C, relative humidity 55±5%, and 12-hour light/dark cycle). Water and standard mice diet (protein 18.9%, carbohydrate 4.7%, and fat 3.5%) were accessible *ad libitum*.

2.6. Acute oral toxicity test of the extracts

The acute oral toxicity study of each extract was conducted according to the Organization of Economic Co-operation and Development Guidelines (OECD Guideline 425) for animal toxicity studies for medicines **[20]**. The limit test at 2000 mg/kg BW was performed to minimize the number of animals required to estimate the acute oral toxicity, where five mice were tested for each extract. At the first, one mouse was administered 2000 mg/kg BW, and no signs of mortality or abnormal performance up to 48 hours were noticed. Then, four extra mice were also dosed with no abnormal signs observed. As per the results of the acute oral toxicity test, the LD_{50} of each extract is bigger than 2000 mg/kg BW. On this basis, the dose of 200 mg/kg BW for each extract was selected for treatment.

2.7. Study design

After one week of adaptation, the mice were arbitrarily distributed into eight equal groups (6 mice each). The studied groups were prepared as follows (Table 1);

The normal control group (CNT group): the mice were intraperitoneally (IP) injected with three equal physiological saline injections (500 µl) at 4 days intervals for two weeks, starting on the first day of the second week.

The cardiotoxic control group (DOX group): the mice were IP injected with three equal DOX injections (the injected volume was adjusted to 500 µl by physiological saline at a dose of 5 mg/kg BW) at 4 days intervals for two weeks, starting at the first day of the second week (the total cumulative dose: 15 mg/kg BW).

The (D+G), (D+ML), and (D+MS) groups: the pre-cotreated groups, where mice were administered with 200 µl (the extracts were dissolving in distilled water at a dose of 200 mg/kg BW) of freshly-prepared GE, MLE, and MSE solutions, respectively, through an intragastric feeding tube every day for three weeks with DOX injection as mentioned in the DOX group.

The (G), (ML), and (MS) groups: the extracts' control groups, where mice were administered with freshly-prepared GE, MLE, and MSE solutions (200 µl, 200 mg/kg BW), respectively, through a feeding gastric tube every day for three weeks.

The DOX dose and route of administration were chosen according to the standard method that is used to induce cardiotoxicity by DOX in the mice model and was provided by **Singal *et al.* [21]**. The weights of mice were recorded throughout the study.

2.8. Blood sampling

On day 4 after the last DOX injection, the mice were permitted free access to water only for 12 hours and the blood samples were got from the retro-orbital plexus of the inner eye canthus under diethyl ether anesthesia. The collected blood samples were kept for 10 minutes at room temperature and centrifuged at 4000 rpm for 10 minutes. The resulting clear sera were kept at -20°C for the biochemical analyses.

2.9. Bodyweight change and heart weight index calculation

After anesthesia, the mice were euthanized by cervical dislocation and the hearts were rapidly harvested, rinsed with cold physiological saline, dried, and weighted to calculate heart weight index (HWI) to evaluate the cardiac hypertrophy, where HWI = heart weight (mg)/body weight (g) **[22]**. Bodyweight change percentages (BWC %) were calculated by using the formula: [(Final weight-Initial weight)/Initial weight]*100.

2.10. Serum parameters

Creatine phosphokinase cardiac isoenzyme (CK-MB), aspartate transaminase (AST), and lactate dehydrogenase (LDH) activities were measured by using the kits that depend on **Young *et al.* [23]**, **Bergmeyer *et al.* [24]**, and **Van der Heiden *et al.* [25]**, respectively. The enzymes' activities were expressed as U/L. As well, the total cholesterol (TC) and triglycerides (TG) levels were measured by the kits according to the colorimetric methods described by **Richmond [26**], and **Bucolo and David [27]**, respectively. The levels of TC and TG were expressed as mg/dl.

2.11. Tissue sampling

Each heart was dissected into three parts; part for RNA extraction: same part of each heart was immediately incubated in 5 volumes of RNA safeguard reagent at 4°C overnight, then stored at -20°C till used; part for cardiac biochemical analyses: same part of each heart was stored at -20°C till used; and part for histopathological examination: in 10% formalin solution, the same part of each heart was fixed at room temperature for 48 hours and stored at -20°C till used.

2.12. Cardiac biochemical analyses

The frozen heart samples were thawed and homogenized in the ice-cold medium of sodium phosphate buffer (pH 7, 0.1 M) to produce 10% (w/v) homogenate utilizing ultrasonic homogenization (Vibra-cell sonicator) and then centrifuged at 4000 rpm for 10 minutes at 4°C (Labofuge 400R cooling centrifuge). The aliquot of the supernatant of each sample was stored at -80°C till used. The total protein (TP), nitric oxide (NO), and malondialdehyde (MDA) levels in the heart homogenate were measured according to the procedures that were described by **Gornall *et al.* [28]**, **Montgomery and Dymock [29]**, and **Ohkawa *et al* [30]**, respectively. The kits' instructions were followed and the results of TP were expressed as mg/g tissue, whereas the results of NO and MDA were expressed as nmol/mg protein. In addition, the activities of superoxide dismutase (SOD) and catalase (CAT) were measured following the instructions of the kits depending on the methods that were described by **Nishikami *et al.* [31]**, and **Aebi [32]**, respectively. SOD activities were expressed in U/mg protein; where 1.5 U of the purified enzyme produced 80% inhibition, and CAT activities were also measured in U/mg protein; where one enzyme unite equal 1 µmol H_2O_2 decomposed/min at 25°C.

2.13. Molecular study

Total RNA extraction: By following the RNA extraction kit's instructions, the cardiac total RNA was isolated. The purity (A260/A280 ratio) and the concentration of the extracted RNA were measured by a spectrophotometer (BioDrop µLite, Australia).

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR): To assess the expression levels of *Nrf2*, *CBR1*, and *TNF-α* genes, the RT-qPCR technique was performed by following the instructions of the manufacturer. Briefly, 1 µl of the enzyme mixture was mixed on ice with 1 µl of the RNA (500 ng/µl), 1 µl of the forward primer (10 µM) and 1 µl of the reverse primer (10 µM) were added to 10 µl of the 2X SYBR mixture, and the sterile RNase free water up to 20 µl. The RT-qPCR cycle included one cycle of reverse transcription for 30 minutes at 50˚C, 40 cycles of initial denaturation at 95˚C for 10 minutes, 5 seconds of denaturation at 95˚C, and 30 seconds of annealing and elongation at 60˚C. The expression levels were calculated from the cycle threshold (CT), the number of cycles it took to detect a real signal from the samples. The relative fold expression of the target genes was obtained using the comparative CT (ΔΔCT) method using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene. The ΔCT was calculated by (CT of the target gene - CT of *GAPDH*), whereas ΔΔCT was obtained by (ΔCT of the experimental group - ΔCT of the calibrator). The expression fold changes were calculated from the following formula: Expression fold change = $2^{-\Delta\Delta CT}$ **[33]**. Following primer sequences were used (Table 2): *Nrf2* (forward, 5′-TAGTGCCCCTGGAAGTGTCA-3′ and reverse, 5′-TTGGGATTCACGCATAGGAG-3′) **[34]**, *CBR1* (forward, 5′-GGTGCTAACAAAGGAATC-3′ and reverse, 5′-CTCTGCTTGAATGTGGAA-3′) **[35]**, *TNF-α* (forward, 5′-ACTCAACAAACTGCCCTTCTGAG-3′ and reverse, 5′- TTACAGCTGGTTTCGATCCATTT-3′) **[36]** and *GAPDH* (forward, 5′-TGCATCCTGCACCACCAACT-3′ and reverse, 5′-TGCCTGCTTCACCACCTTC-3′) **[35]**.

Table 2: The primers in qRT-PCR analysis

Gene	Forward primer (5′-3')	Reverse primer (5′-3')
Nrf2	TAGTGCCCCTGGAAGTGTCA	TTGGGATTCACGCATAGGAG
CBR1	GGTGCTAACAAAGGAATC	CTCTGCTTGAATGTGGAA
TNF-α	ACTCAACAAACTGCCCTTCTGAG	TTACAGCTGGTTTCGATCCATTT
GAPDH	TGCATCCTGCACCACCAACT	TGCCTGCTTCACCACCTTC

Nrf2 [nuclear factor (erythroid-derived 2)-like 2], *CBR1* (carbonyl reductase 1), *TNF-α* (Tumor necrosis factor-alpha) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase).

2.14. Cardiac tissue histopathological study

The fixed samples were dehydrated in a gradient sequence of alcohol, embedded in paraffin blocks, sectioned at 5 µm thickness, and stained with hematoxylin and eosin **[37]**.

2.15. Statistical analysis

All data were given as mean ± SD. Comparisons between means of the studied groups were performed by Tukey's test (ANOVA) using the statistical software IBM SPSS (version 26.0) and the differences were considered significant at ($P \leq 0.05$).

3. Results

3.1. Description of the extracts

The GE, MLE, and MSE were brown, dark greenish-brown, and yellow semi-solids, respectively, and the yield percentages by this extraction process were 13.4%, 15.5%, and 14.0% (w/w), respectively.

3.2. The total phenolic and flavonoid contents

As can be seen from Table (3) the GE and MLE contain high phenolic and flavonoid content which are expressed as µg GAE/mg extract and µg RE/mg extract, respectively.

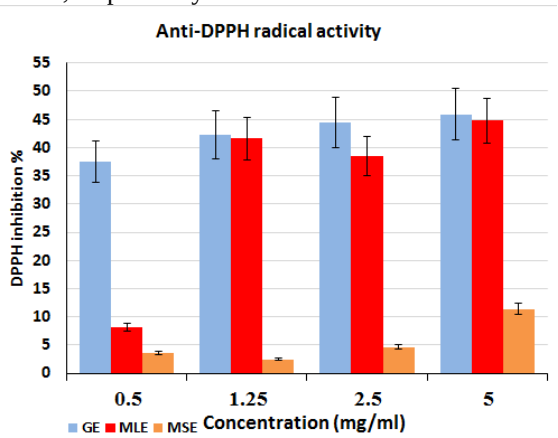

Fig. (1): The DPPH inhibition (%) of the extracts at different concentrations. GE (*Z. officinale* rhizomes 80% methanolic extract), MLE (*M. oleifera* leaves 80% methanolic extract), MSE (*M. oleifera* seeds 80% methanolic extract), and DPPH (2,2-diphenyl-1-picrylhydrazyl).

3.3. Radical scavenging activity and bioactive profile

The data in Fig. (1) and Table (3) reveal that the extracts showed different antiradical activities and ARP. The results of bioactive compounds screening in Table (4) showed that tannins, flavonoids, alkaloids and/or nitrogenous bases, triterpenoids, and carbohydrates and/or glycosides were shown in all extracts. Saponins were not identified in the MLE, but they were detected in the other extracts. In addition, anthraquinones were not detected in all extracts.

3.4. The effect of the treatments on body weight, heart weight index, and cardiac total protein

Results of the BW, HWI, and TP are summarized in Table (5). Compared to the healthy mice (CNT group) that there weight increased by about 30%, a weight loss was observed in the DOX group by about 21% ($P < 0.01$). In comparison with the DOX group, the D+ML and D+MS groups showed a lower weight loss (about 6% and 11%, respectively), whereas the D+G group showed an increase in BW by about 4% ($P < 0.01$). Additionally, the BW of G group,

Table 3: Total phenols and flavonoids, and antioxidant properties of the extracts

Extract	Total phenols (µg GAE/mg extract)	Total flavonoids (µg RE/mg extract)	ABTS inhibition (%)	IC_{50} (mg/ml)	ARP
GE	454.4 ± 4.1	155.0 ± 11.0	75.5 ± 4.0	6.9 ± 0.7	0.15 ± 0.01
MLE	305.4 ± 2.1	148.7 ± 8.2	34.9 ± 3.8	11.2 ± 2.7	0.09 ± 0.00
MSE	58.6 ± 3.1	4.1 ± 0.3	29.3 ± 1.8	38.1 ± 2.3	0.03 ± 0.00

GE (*Z. officinale* rhizomes 80% methanolic extract), MLE (*M. oleifera* leaves 80% methanolic extract), MSE (*M. oleifera* seeds 80% methanolic extract), GAE (gallic acid equivalent), RE (rutin equivalent), ABTS [2,2-azinobis-(3-ethylbenzothiazolinicale-6-sulphonate)], IC_{50} (extract's concentration that inhibit 50 % of DPPH radical), and ARP (anti-radical power, $1/IC_{50}$).

Table 4: The bioactive profile of the tested extracts

Phytochemical constituent	Test	GE	MLE	MSE
Alkaloids and/or nitrogenous bases	Wagner's test	+	+	+
Carbohydrates and/or glycosides	Molisch's and Borntrager's tests	+	+	+
Tannins	Ferric chloride test	+	+	+
Flavonoids	General lab test	+	+	+
Saponins	Froth test	+	-	+
Triterpenoids	General lab test	+	+	+
Anthraquinones	General lab test	-	-	-

GE (*Z. officinale* rhizomes 80% methanolic extract), MLE (*M. oleifera* leaves 80% methanolic extract), MSE (*M. oleifera* seeds 80% methanolic extract), + (detected), and – (not detected).

ML, and MS groups increased by about 23%, 25%, and 28%, respectively.

In comparison with the CNT group, HWI results revealed that the DOX group showed higher HWI ($P < 0.01$). However, the ML and MS groups showed a non-significant change when compared with the CNT group, the G group showed a lower HWI ($P < 0.01$). In comparison with the DOX group the D+G, D+ML, and D+MS groups showed a significant ($P < 0.01$) decrease in the HWI. In addition, cardiac TP results revealed that there was a non-significant change between all studied groups.

3.5. Different treatments improved the DOX-induced cardiac injury

Activities of CK-MB, LDH, and AST of all studied groups are presented in Fig. (2). Where the DOX injection resulted in a significant ($P < 0.01$) increase in activities of the enzymes in comparison

with the normal control (CNT group). In contrast, the activities of the enzymes in the groups that were pre-cotreated (D+G, D+ML, and D+MS groups) showed a significant ($P < 0.01$) decrease as compared with the DOX group. Meanwhile, the G, ML, and MS groups showed non-significant alterations in the activities of the enzymes when compared with the CNT group.

Table 5: The changes in body weight during the experimental period, heart weight index and cardiac total protein

Group	Body weight (g)		BWC%	HWI	TP
	Initial	Final			(mg/g tissue)
CNT	25.8 ± 2.0	33.5 ± 3.9	29.8 ± 3.3	5.1 ± 0.3	80.1 ± 3.2
DOX	32.5 ± 3.1	25.7 ± 1.9	-20.9 ± 3.3*	6.3 ± 0.2*	82.9 ± 5.8
D+G	25.7 ± 3.1	26.8 ± 1.5	4.3 ± 1.3$^{*,\#}$	5.1 ± 0.4$^{\#}$	90.1 ± 8.1
G	25.6 ± 4.0	31.6 ± 2.7	23.4 ± 2.3$^{\#}$	4.8 ± 0.3$^{*,\#}$	86.0 ± 5.7
D+ML	30.0 ± 2.9	28.2 ± 1.0	-6.0 ± 2.0$^{*,\#}$	5.0 ± 0.4$^{\#}$	85.3 ± 8.3
ML	27.2 ± 1.2	34.0 ± 2.9	25.0 ± 3.2$^{\#}$	5.1 ± 0.2$^{\#}$	88.4 ± 12.2
D+MS	30.2 ± 0.9	26.9 ± 2.0	-10.9 ± 2.4$^{*,\#}$	5.6 ± 0.5$^{*,\#}$	90.6 ± 4.5
MS	23.8 ± 2.4	30.4 ± 2.5	27.7 ± 2.3$^{\#}$	5.0 ± 0.1$^{\#}$	91.3 ± 6.8

CNT (control group), DOX (Doxorubicin group), D+G (pre-cotreated with GE), G (GE control group), D+ML (pre-cotreated with MLE, ML (MLE control group), D+MS (pre-cotreated with MSE), MS (MSE control group), BWC% (the body weight change percentage, HWI (heart weight index), TP (cardiac total protein), * (significant against the CNT group), and # (significant against the DOX group , at $P \leq 0.05$). All data are presented as mean ± SD

3.6. Different treatments attenuated the DOX-induced cardiac oxidative disturbance

The results in Fig. (3) demonstrate that DOX injection significantly ($P < 0.01$) decreased the SOD activity, but it significantly ($P < 0.01$) increased the CAT activity, and the NO and MDA levels in comparison with the CNT group. In contrast, in the pre-cotreated groups the SOD activities were increased (significantly, $P < 0.01$, in the G+ML group, and non-significantly in the D+G and D+MS groups). Additionally, the pre-cotreatments significantly ($P < 0.01$) decreased the CAT activities, and the NO and MDA levels, except for the D+ML group, which showed a non-significant decrease in its NO level in comparison with the DOX group. Meanwhile, non-significant changes were observed in the SOD activities of the G, ML and MS groups when compared with the CNT group. However, GE and MSE administrations to normal mice significantly ($P < 0.01$) increased the CAT activities when compared with the CNT group, but the MLE administration showed no change. In addition, the G and MS groups showed non-significant changes in the NO levels, but the ML group showed a significant elevation. Moreover, the G, ML, and MS groups showed a reduction in the levels of MDA (significant in the G group, $P < 0.01$, and the ML group, $P < 0.05$, but non-significant, $P = 0.057$, in the MS group) when compared with the CNT group.

3.7. Different treatments reduced the DOX-induced serum lipids disorder

The results in Fig. (4) indicate that DOX injection showed a significant ($P < 0.01$) increase in the levels of TC and TG in comparison with the CNT group. Whereas, the TC levels in the groups that were pre-cotreated were significantly ($P < 0.01$) decreased when compared with the DOX group. As well as, the levels of TG in these groups were decreased (significantly, $P < 0.01$, in the D+G and D+ML groups, but non-significantly in the D+MS group). Meanwhile, the levels of TC and TG in the G, ML and MS groups showed non-significant changes when compared with the CNT group.

3.8. The effect of different treatments on the expression levels of the cardiac *Nrf2*, *CBR1*, and *TNF-α* genes

It can be seen in Fig. (5) that the DOX administration resulted in a significant ($P < 0.01$) decrease in the expression of *Nrf2* and *CBR1* genes, but the expression of the *TNF-α* gene was significantly ($P < 0.01$) increased as compared with the normal control group. On the other hand, the expression levels of *Nrf2* and *CBR1* genes in all pre-cotreated groups were significantly ($P < 0.01$) increased, whereas the expression levels of the *TNF-α* gene were significantly ($P < 0.01$) decreased as compared with the DOX group. In comparison with the CNT group, all extracts administration to the normal mice resulted in non-significant changes in the expression levels of *Nrf2*, *CBR1*, and *TNF-α* genes, excluding the MLE administration resulted in a significant ($P < 0.01$) increase in the *Nrf2* and *CBR1* expression levels.

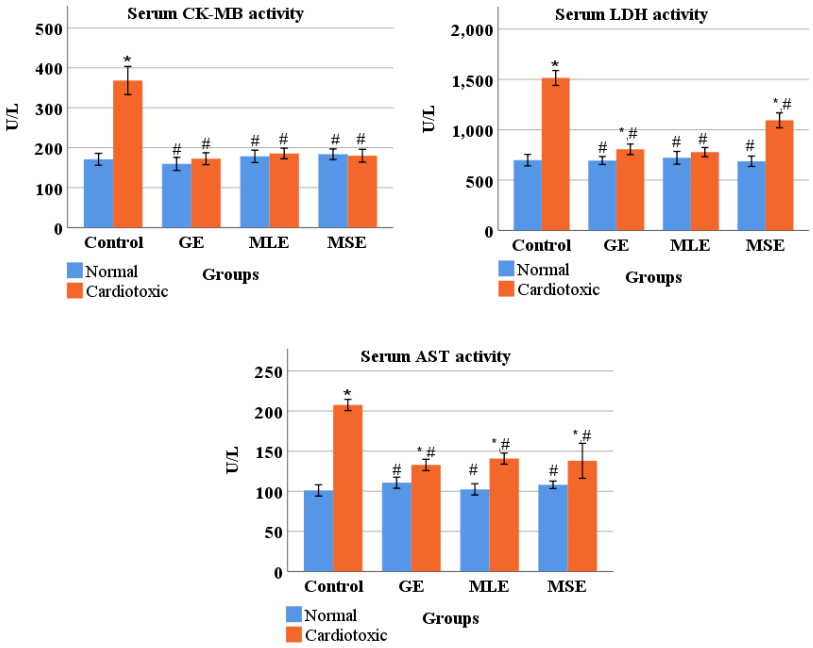

Fig. (2): The effect of DOX and the extracts on CK-MB, LDH, and AST activities. **Control** [Normal (CNT group) and Cardiotoxic (DOX group)], **GE** [Normal (G group) and Cardiotoxic (D+G group), **MLE** [Normal (ML group) and Cardiotoxic (D+ML group)], **MSE** [Normal (MS group) and Cardiotoxic (D+MS group)], CK-MB (Creatine phosphokinase cardiac isoenzyme), LDH (Lactate dehydrogenase) and AST (Aspartate transaminase), * (significant against the CNT group), and # (significant against the DOX group, at $P \leq 0.05$). All data are presented as mean ± SD.

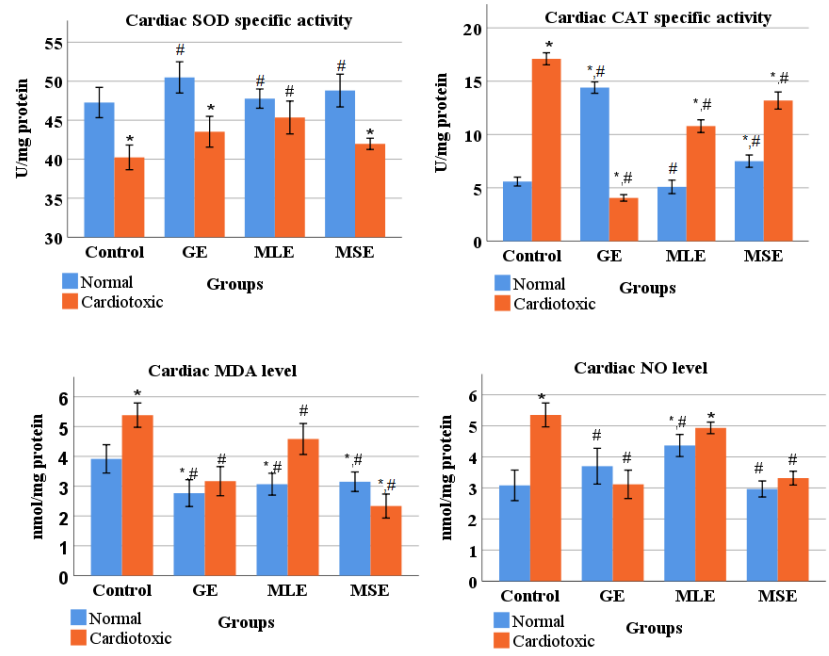

Fig. (3): The effect of DOX and the extracts on activities of the antioxidant enzymes, and the levels of lipid peroxidation and nitric oxide. **Control** [Normal (CNT group) and Cardiotoxic (DOX group)], **GE** [Normal (G group) and Cardiotoxic (D+G group), **MLE** [Normal (ML group) and Cardiotoxic (D+ML group)], **MSE** [Normal (MS group) and Cardiotoxic (D+MS group)], SOD (Total superoxide dismutase, 1.5 U of the purified enzyme produced 80% inhibition), CAT (Catalase, one enzyme unite equal 1 µmol H_2O_2 decomposed per min at 25°C), MDA (malondialdehyde) and NO (nitric oxide), * (significant against the CNT group), and # (significant against the DOX group, at $P \leq 0.05$). All data are presented as mean ± SD.

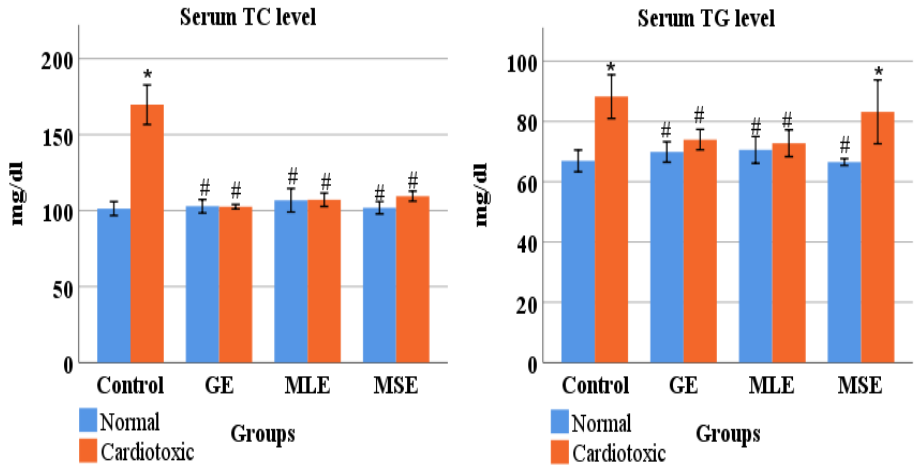

Fig. (4): The effect of DOX and the extracts on the level of serum lipids. **Control** [Normal (CNT group) and Cardiotoxic (DOX group)], **GE** [Normal (G group) and Cardiotoxic (D+G group), **MLE** [Normal (ML group) and Cardiotoxic (D+ML group)], **MSE** [Normal (MS group) and Cardiotoxic (D+MS group)], TC (Total cholesterol) and TG (Triglycerides), * (significant against the CNT group), and # (significant against the DOX group, at $P \leq 0.05$). All data are presented as mean ± SD.

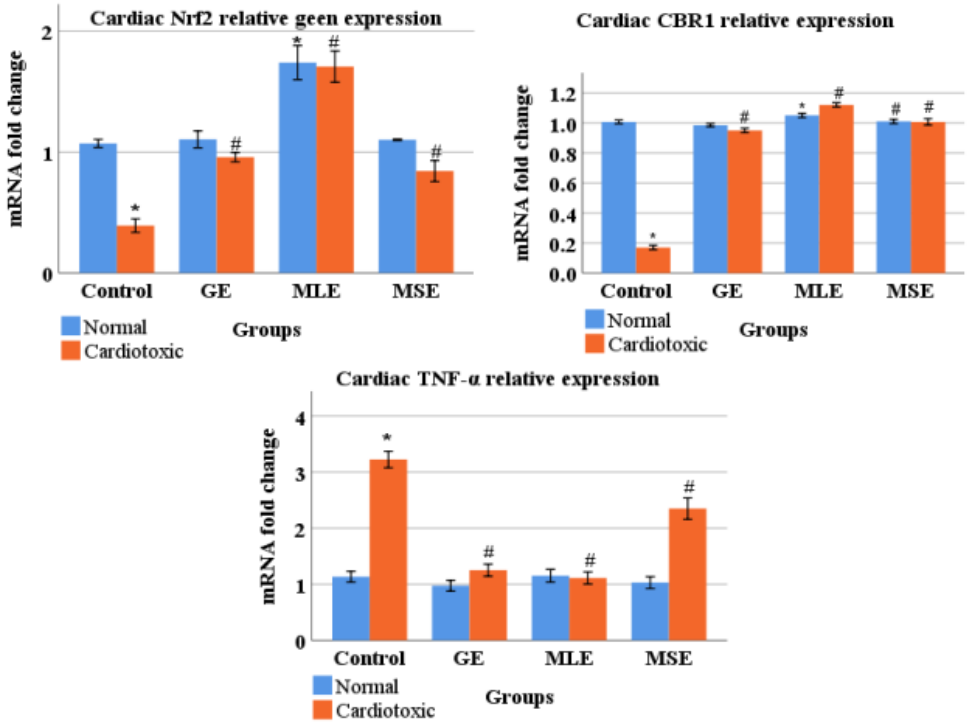

Fig. (5): The effect of DOX and the extracts on expression of *Nrf2*, *CBR1*, and *TNF-α*. **Control** [Normal (CNT group) and Cardiotoxic (DOX group)], **GE** [Normal (G group) and Cardiotoxic (D+G group), **MLE** [Normal (ML group) and Cardiotoxic (D+ML group)], **MSE** [Normal (MS group) and Cardiotoxic (D+MS group)], *Nrf2* [nuclear factor (erythroid-derived 2)-like 2], *CBR1* (carbonyl reductase 1), and *TNF-α*, tumor necrosis factor-alpha. The results were normalized to *GAPDH* mRNA expression, * ($P < 0.05$ relative to the CNT group), and # ($P < 0.05$ relative to the DOX group). All data are presented as mean ± SD

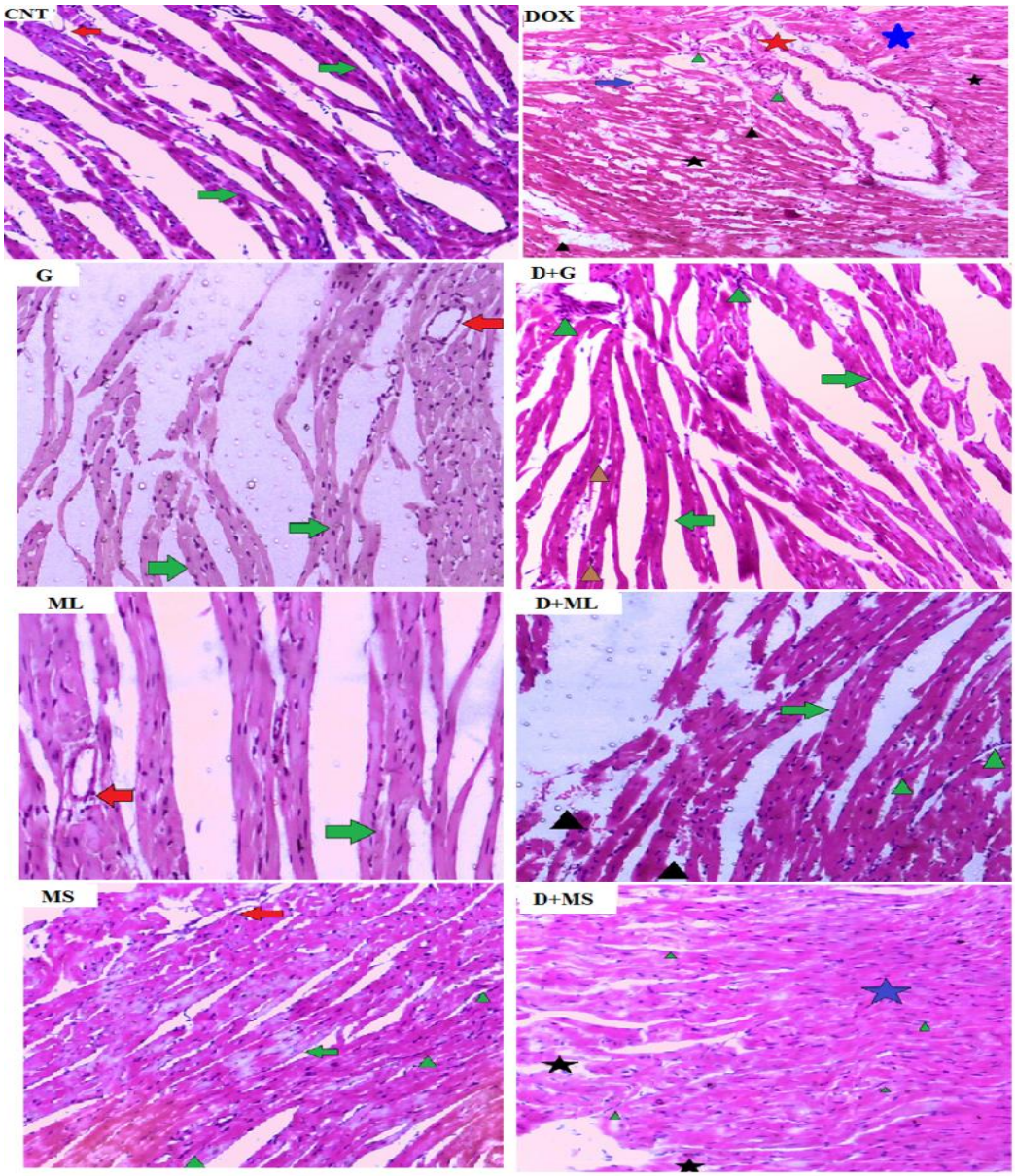

Fig. (6): The effect of the DOX and the extracts on mice myocardium (20X). **CNT** group illustrates the normal appearance of myocardial fibers (green arrow) with normal blood vessel (red arrow), **DOX** group demonstrates the disordered arrangement of myocardial fibers with vacuolization (blue arrow), blood vessel with thickened and elongated wall (red star), hyperemia (black arrow head), fibroblasts and lymphatic cells infiltration (green arrow head), moderate cardiocytes hyperplasia (blue star) and myocytolysis (black star), **D+G** group demonstrates the preserved normal arrangement of myocardial fibers (green arrow), few lymphatic cells (brown arrow head) and perivascular fibroblasts infiltration (green arrow head), **D+ML** group shows the preserved normal myocardial fibers (green arrow), few inflammatory cells infiltration (green arrow head), and hyperemia (black arrow head), **D+MS** group shows the moderate cardiocytes hyperplasia (blue star), few fibroblasts infiltration (green arrow head), and a low degree of myocytolysis (black star), **G** group determines the normal appearance of myocardial fibers (green arrow) with normal blood vessel (red arrow), **ML** group illustrates the normal appearance of myocardial fibers (green arrow) with normal blood vessel (red arrow), and **MS** group demonstrates the normal appearance of myocardial fibers (green arrow) with normal blood vessel (red arrow) and very few inflammatory cells infiltration (green arrow head).

3.9. Different treatments improved the heart histopathology induced by DOX

The results of the microscopic examination can be seen in Fig. (6), where the CNT group presented the normal appearance of the myocardial fibres with normal blood vessels. The DOX group showed that DOX injection resulted in histopathological changes, including the disordered arrangement of myocardial fibres with vacuolation, blood vessels with thickened

and elongated walls, hyperemia, fibroblasts and lymphatic cells infiltration, cardiocytes hyperplasia and myocytolysis. All the pre-cotreatments significantly reduced the DOX-induced histopathological changes. Since the D+G group showed a preserved normal arrangement of the myocardial fibres with very few lymphatic cells and perivascular fibroblasts infiltration. The D+ML group exhibited preserved normal myocardial fibres, with few inflammatory cells infiltration and hyperemia. The D+MS group indicated a moderate cardiocytes hyperplasia with few fibroblasts infiltration. Meanwhile, the G, ML, and MS groups showed a normal appearance of myocardial fibres with normal blood vessels.

4. Discussion

Extreme attention to phytochemicals has been stimulated because of their well-established health-promoting benefits due to their iron-chelating, radical-scavenging and biological activities **[9, 12]**. The ginger as well as the *moringa*'s leaves and seeds contain high amounts of many polyphenols, flavonoids, and other bioactive compounds with high anti-inflammatory and antioxidant activities that explain their potential use against many diseases. They also possess high antimicrobial, anti-tumor, hepatoprotective, and cardioprotective potentials **[10-12]**. Up till now, little is known about the cardioprotective mechanisms of the ginger and *moringa* extracts. In this regard, our study was prepared to study the protective mechanisms of GE, MLE, and MSE against DOX-induced cardiotoxicity in male albino mice. The 80% aqueous methanol (v/v) was used in the current extraction procedure because it can release more phytochemicals than other solvents as it is more effective against cell walls and releases intracellular ingredients **[38]**. In the present work, the mice were injected with 15 mg DOX/kg BW in three equal injections over two weeks to mimic chronic cardiotoxicity **[21]**. According to the current physiological results, DOX injection resulted in an obvious decrease in the BW as compared with the CNT group, which may be due to DOX diminishing appetite and protein synthesis, mucositis, and/or insufficient absorption of nutrients **[39]**. Myocardial hypertrophy is generally assessed by increasing the heart weight index (HWI) **[22]**. In tune with a previous report, the current study showed that the DOX injection caused myocardial hypertrophy that was revealed through HWI elevation as compared with the normal mice **[22]**.

As known, the cardiomyocytes injury is confirmed via increasing the serum activities of the cardiac enzymes (CK-MB, LDH, and AST) **[40]**. According to the present results, DOX injection resulted in a significant elevation of serum activities of the cardiac enzymes as compared with the normal control group, elucidated the cardiomyocytes disruption and damage. The DOX-induced cardiomyocyte damage is initiated in many ways, including the production of oxidants through the reduction of the quinone moiety of the DOX. This reduction is mediated via enzymatic pathways involving the NO synthases and NADPH oxidase of the mitochondrial electron transport chain to form semiquinone, which subsequently interacts with the molecular oxygen and generates superoxide radicals **[2, 41]**. Furthermore, the phospholipid of the mitochondrial inner membrane, cardiolipin, attracts the DOX resulting in DOX accumulation in the mitochondria, which can give rise to electron-transport chain disruption and result in the creation of the reactive oxygen and nitrogen species (ROS and RNS, respectively). Besides, doxorubicinol is a secondary metabolite of the DOX and supposed to be cardiotoxic via dysregulation of energy production, ions homeostasis, and movements of calcium that weaken the cardiac muscles' contraction and relaxation **[42, 43]**. Subsequently, the high ROS and RNS levels trigger the cytotoxic signalling in the cardiomyocytes leading to biomolecules oxidation and denaturation and cardiomyocyte damage that leads to an elevation in the serum cardiac enzymes' activities **[44]**.

The observed decline in the SOD activity after DOX injection in the current study as compared with the normal control group could be due to the DOX-induced mitochondrial impairment and consumption of the enzyme in the OS fighting. Where, SOD reduces the free radicals by converting superoxide radicals (O^{2-}) to hydrogen peroxide (H_2O_2) and prevents the formation of hydroxyl radicals through Fenton's reaction **[45]**. Also, the inhibition of SOD activity may be related to the oxidation of its cysteine residues, which are important in the enzyme's activity **[9]**. Whereas, the elevation of CAT activity after DOX injection could be a response to the elevation of the free radical generation, especially H_2O_2 **[46]**. These results are in harmony with previous results that showed a significant increase in CAT activity and a decrease in SOD activity by DOX (15 mg/kg BW) after DOX injection in mice **[47, 48]**.

From the molecular studies point of view, the *Nrf2* gene is an important regulator of the antioxidant system due to its regulation of many downstream genes such as *SOD* and *CBR1*, which subsequently relieve the OS and lipid peroxidation (LPO) **[6, 7]**. Under OS, the LPO is initiated by creating highly reactive products with carbonyl groups of aldehydes

and ketones **[7]**. CBR1 is a member of the short-chain dehydrogenase/reductases family that catalyzes the reduction of the active carbonyl substrates that prevent the initiation of the LPO and the toxicity to the cellular environment **[7]**. In parallel with previous studies, the current molecular investigations showed that the DOX injection significantly down-regulated the cardiac *Nrf2* gene expression as compared to the normal control group **[49, 50]**. This down-regulation was concomitant with significant elevations in cardiac OS markers (MDA and NO) and the gene expression of the inflammatory cytokine (*TNF-α*), and a significant decrease in the cardiac *CBR1* expression and the activity of SOD. In addition, the LPO is directly initiated by DOX via a non-enzymatic response, where DOX reduces Fe^{3+} and forms DOX-Fe^{2+} radical complexes that affect the cell function via changing membrane organization and penetrability **[51]**. Furthermore, the up-regulation of *TNF-α* gene expression was due to DOX administration inducing the expression and release of inflammatory mediators such as interleukins (IL-1β and IL-6) and TNF-α as a result of the OS and LPO products, which are directly implicated in cardiac pathogenesis and apoptosis **[3]**.

The synthesis of cardiac NO is mediated by the endothelial and inducible NO synthases (eNOS and iNOS, respectively) **[52]**. Our data showed that DOX injection resulted in a significant increase in the cardiac NO level in comparison with the normal mice. In agreement with the current study, **Aldieri *et al.* [53]** showed that the level of the NO was elevated in the cardiac cells after the DOX treatment. This elevation was connected with increasing the iNOS gene expression. In addition, **Pacher *et al.* [54]** established that following DOX administration to mice with null *iNOS* gene, the cardiac functions were well-maintained. The elevation in the serum TC and TG levels after DOX injection was due to the effect of DOX on lipid metabolism, which is one of the toxic effects of DOX on the cardiovascular system **[55]**.

On the other hand, the pre-cotreated groups in the current study as compared with the DOX group, showed a significant increase in the BW, reflecting the defensive properties of the extracts. As well as, the HWI of these groups were reduced, showing the extracts' abilities to maintain the normal integrity of the cardiomyocytes. The pre-cotreatments succeeded in preventing the disruption of the activities of antioxidant enzymes (CAT and SOD). Where these treatments significantly increased the SOD activities and decreased the CAT activities as compared with the DOX group. Moreover, the pre-cotreatments prevented the DOX toxic effects on the lipid metabolism and consequence of the cardiovascular system disorder. The pre-cotreatments exhibited a significant decrease in the cardiac NO levels, which indicates that the extracts prevented the DOX induction for *iNOS* and subsequent excessive NO production. The normal NO production adjusts the contractility of cardiomyocytes and the blood flow, but the excessive production is complicated in cardiovascular disorders such as dilated cardiomyopathy and congestive heart failure due to nitrosative stress and LPO **[56]**. In addition, the pre-cotreatments significantly reduced the CK-MB, LDH, and AST activities in serum as compared with the DOX group, signifying the ability of the extracts to preserve the normal cardiac muscle integrity and prevent DOX-induced myocardial damage. As well, the pre-cotreated groups showed a significant up-regulation of the cardiac *Nrf2* and *CBR1* genes expression, which was associated with an elevation of cardiac SOD activity and a decrease in OS markers. As documented, increasing the SOD activity considerably reduced the oxidative damage and enhanced the cardiac functions in mice that were injected with DOX **[50]**. Furthermore, the pre-cotreatments down-regulated the cardiac *TNF-α* gene expression, which reflected the reduction in the cellular oxidative damage and inflammatory circumstances.

The empirical findings of this study provide a new understanding of the protective mechanism of the *Z. officinale* rhizomes and *M. oleifera*'s leaves and seeds extracts in mice. We found that the up-regulation of the *Nrf2-CBR1* system by the extracts played an important role in attenuation of the DOX-induced heart toxicity, while the down-regulation of the *Nrf2-CBR1* system induced by DOX administration severely damaged the heart tissue. **Kwon *et al.* [7]** demonstrated that the up-regulation of CBR1 by *Nrf2* can reduce free radicals and prevent LPO in the hepatic cell line and the liver of mice after administration of sulforaphane, an activator of *Nrf2*. In addition to the modulation of the *Nrf2-CBR1* system, the protective role of the extracts was a result of their contents of antioxidants and biologically active components. Where the current in vitro radical scavenging activity study revealed that the extracts have antioxidant activity against DPPH and ABTS radicals. This antioxidant activity may be due to their phytochemical constituents such as flavonoids, tannins, alkaloids, and triterpenoids that have been reported in the current qualitative phytochemical study and by other previous studies **[40, 57, 58]**. In addition, the accumulated investigations have revealed the biologically active substances in the methanolic extract of the tested plants, where *M.*

oleifera leaves rich in rutin, gallic acid, hyperoside, kaempferol, quercetin, quercitrin and ascorbic acid, which are strong free radicals scavengers and prevent LPO **[12, 58, 59]**. As known from previous works, rutin inhibits the xanthine oxidase activity in vitro, which is a physiological source of superoxide radicals, and *moringa* seed methanolic extraction is rich in oleic and ascorbic acids **[60, 61]**. As well, the phenolic compounds that account for the several bioactivities of *Z. officinale* rhizomes are mainly gingerols, paradols and shogaols **[62]**.

The current histopathological result was in harmony with the biochemical and molecular interpretations. Where, DOX injection induced several toxic and degenerative changes in the cardiomyocytes, including the disordered arrangement of myocardial fibres with vacuolization, blood vessels with thickened and elongated walls, hyperemia, fibroblasts and lymphatic cells infiltration, moderate cardiocytes hyperplasia, and myocytolysis. The foremost reason is thought to be the degradation of the titin, a myofilament protein, and the integral fragment of the sarcomere, where the integrity of the titin is vital for the contractile movement of the heart. Moreover, DOX increases apoptosis in cardiomyocytes via increasing the expression of the death receptors on the cardiomyocytes cell surface **[63]**. In tune with one of the studies conducted by **Razmaraii *et al.* [64]** that showed DOX injection (cumulative dose of 12 mg/kg BW) produced histological modifications in cardiac tissues, for instance, interstitial oedema, vacuolization of the cytoplasm, degeneration, and necrosis. In contrast, the cardiac tissues of the animals that were pre-cotreated by the extracts showed a noticeable improvement in cardiac tissue integrity and structure. According to the histopathological analysis, the formerly declared lesions were prevented or less in frequency and severity, reflecting the prophylactic role of the extracts against DOX-induced cardiotoxicity. In addition, myocardial hypertrophy was obviously shown in the DOX group, but it was prevented or obviously decreased in the pre-cotreated groups by the extracts. The current histopathological, biochemical and molecular studies of the animals that were administered with the extracts only showed no toxic effects, verifying the extracts are safe to use. So, our results suggested that the down-regulation of the *Nrf2-CBR1* system plays an important role in the pathogenesis of DOX-induced cardiotoxicity via induction of oxidative and nitrosative stress, and LPO, whereas the positive modulation of this system attenuates the cellular injury through regulation of the cellular redox homeostasis and anti-inflammatory signalling.

5. Conclusion

The present study was designed to determine the cardioprotective role and mechanism of the *Z. officinale* rhizomes and *M. oleifera's* seeds and leaves hydro methanolic extracts against DOX-induced toxicity. Based on the present findings, we revealed that the extracts exhibit an effective cardioprotective potential against DOX-induced cardiomyopathy. The antioxidant and anti-inflammatory mechanisms of the extracts were attributed to suppressing the cardiac oxidative/nitrosative damage, and pro-inflammatory gene expression (*TNF-α*), thereby reducing myocardial myopathy and detrimental structural alterations. However, MSE in our study didn't show high antiradical activity as compared with the other extracts, it showed a cardioprotective activity mainly via up-regulation of *Nrf2* and *CBR1* expressions, increasing the activity of CAT enzyme, and down-regulation of *TNF-α* expression. So, for the first time, we showed that the cardioprotective roles of these extracts are not only due to their antioxidant activities but also due to their modulation effects on the important regulators of the cellular redox homeostasis (the *Nrf2-CBR1* system), inflammatory cytokine expression (*TNF-α*) and the serum lipids levels. Accordingly, the tested extracts (GE, MLE, and MSE) are highly recommended as adjuncts to prevent anthracyclines (DOX) cardiotoxic effects.

6. Ethics approval and consent to participate

The current study was permitted by the Faculty of Science, Mansoura University, 2022. The mice were treated according to the Guide of the Care and Use of Laboratory Animals, Faculty of Science, Mansoura University, 2022. The ethical Approval number is **Sci-Ch-Ph-2022-171**

7. Conflicts of interest

There are no conflicts to declare.

8. Funding: There is no funding

9. Authors' contributions:

Mohammad M. Zaki planned this study, designed, carried out the experimental part, performed the statistical analysis and illustrated the figures, and was the main supplier in writing and reviewing the manuscript, wrote, and reviewed the work. I. H., M. A., and O. Y. shared in proposing this study, and designed, planned and reviewed the manuscript. A. A. shared in planning and contribution to the sequence arrangement, and participated in reading and approving the final manuscript. The final manuscript has been approved by all authors.

10. Acknowledgments

The authors request to thank Dr. Hany Rafik Sedki, Pathology Department, Medical Research Institute, Alexandria University, Egypt, for his cooperation in the histopathological examination.

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