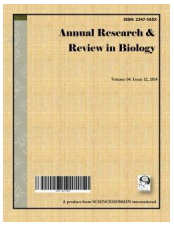

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Protective Effect of *Artemisia judaica* against Doxorubicin-Induced Toxicity in Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author ESA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author WKBK performed the statistical analysis. Authors DMM and MMH managed the analyses of the study. Author DMM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study aimed to investigate the protective effect of *Artemisia Judaica (A. Judaica*) against doxorubicin (DOX)-induced toxicity in male mice.
Place and Duration of Study: Department of Cell Biology, Genetic engineering and biotechnology division, National research centre, Egypt, between March 2016 and February 2017.
Methodology: Male mice were divided into 7 groups (n=10) and treated as follow: the control group, the group treated with DMSO, the group injected (i.p.) with DOX , the groups treated with low and high dose of *A. judaica* extract and the groups injected (i.p.) with DOX and treated with low and high dose of *A. judaica* extract. Femur, testes and liver samples were collected for different analyses.
Results: Our data showed that *A. judaica* significantly reversed the DOX-induced elevation of DNA fragmentation rate and MDA level in liver tissue, as well as declined chromosomal aberrations (CAs) either in the bone marrow cells or in the spermatocyte cells. Meanwhile, the expression of

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apoptosis-related genes (Bax and Caspase-3) in liver tissues was analyzed by quantitative real-time PCR (qRT-PCR) and results revealed that genes expression were up-regulated in DOX treated mice however; the administration of *A. judaica* didn't alter such increase.
Conclusion: Overall, the findings indicated that *A. judaica* may attenuate the DOX-induced toxicity. However; further studies are required to confirm the protective effect of *A. judaica* extract against toxicity caused by DOX drug.

Keywords: Artemisia judaica; doxorubicin; apoptosis; toxicity; Mice.

1. INTRODUCTION

Up to date the cancer disease is considered as the most important disease causing high rate of mortality globally which leads to in 2012 about 14,000 000 cases and it is expected to increase by 70% in the next two decades [1,2]. As a result of developments in cancer treatment with increased possibility of recovery from cancer, in the last decade, attentions have focused on the protection of life from chemotherapeutic agents induced toxicity [3]. DOX, an anthracycline antibiotic, is a wide spectrum antineoplastic agent, used for the treatment of an array of cancer types [4]. Like any other anticancer agent, DOX is associated with several undesirable side effects on some organs [5]. The semiquinone form of doxorubicin is a toxic short-lived metabolite which interacts with molecular oxygen and embarks a cascade of reactions, producing reactive oxygen species. Reactive oxygen species generation and lipid peroxidation were suggested to be responsible for doxorubicin-induced toxicity [6].

Natural agents and herbal products can enhance the actions and diminish the toxicity of conventional chemotherapeutic drugs [7]. Artemisia is one of the large and diverse genera of Asteraceae family with various important medicinally valuable essential oils and secondary metabolites [8]. The genus is used in many parts of the world either alone or with other plants as herbal remedies for a range of human diseases [9]. Furthermore, Artemisia has anti-tumor [10], anti-hepatotoxic [11], anti-inflammatory [12] and antioxidant [13] effects.

A. judaica is a perennial fragrant shrub which grows extensively on the Sinai Peninsula in Egypt, and is a very common anthelmintic drug in many Middle-Eastern and North African countries where its Arabic name is "shih balady" [14]. The distinct scent of *A. Judaica* is owing to the high volatile oil content contributing to the anti-inflammatory, anti-tumor, anti-microbial [15,16] and antioxidant activity [17].

As before mentioned, it was proposed that if doxorubicin toxicity is related to free radical formation and oxidative stress, an antioxidant such as *A. judaica* may protect against doxorubicin-induced toxicity, hence the present study was conducted to investigate the effect of *A. judaica* extract on doxorubicin-induced toxicity.

2. MATERIALS AND METHODS

2.1 Chemicals

DOX was purchased from Sigma Aldrich (Saint Louis, MO 63103, USA). RNAse-free DNAse kit, TRIzol Reagent and SYBR Green master mix were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). RevertAid™ First Strand cDNA Synthesis Kit was purchased from Fermentas (Thermo Fisher Scientific Inc., Canada). All other chemicals used throughout the experiments were of the highest analytical grade available.

2.2 Experimental Animals

Seventy adult Swiss albino male mice (22-27 g), purchased from the Animal House Colony, Giza, Egypt, were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water adlibitum at the Animal House Laboratory, National Research Centre, Dokki, Giza, Egypt. After an acclimation period of 1 week, animals were divided groups (10 mice/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23 ± 1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All mice received human care in compliance with the guidelines of the Animal Care and Use Committee of National Research Centre, Dokki, Cairo, Egypt and the National Institutes of Health (NIH publication 86e23 revised 1985).

2.3 Plant Extract Preparation

Plant extract was prepared according to the procedure described by Moustafa et al. [18] with slight modification. Fresh whole of A. judaica plant was collected from South of Sinai in Egypt. The plant was dried at ambient temperature and stored in a dry place prior to use. The plant was washed well with water, dried at room temperature in the dark, and then ground in an electric grinder to give a coarse powder. The powder (150 ml Methanol for 50 g powder) was soaked in solvent at room temperature for 24h. The plant extract was collected dropwise and filtered using Whatman No.1 filter paper. The residues soaked at 150 ml of methanol for 28h and filtered it again. The extract was then dried and finally placed in glass vials and stored frozen at -20ºC and the residue was resuspended in dimethyl sulphoxide (DMSO) 1% before testing.

2.4 Experimental Design

Animals were divided into 7 groups (10 animals each) as follow: Group 1: Control animals were treated with physiological saline, Group 2: Animals treated with DMSO, Group 3: Animals injected intraperitonealy (i.p.) with Doxorubicin (DOX, 10 mg/kg) twice per week for one month, Group 4: Animals treated (orally) with low dose of *A. judaica* extract (0.5 mg/kg bw) for one month, Group 5: Animals treated with high dose of *A. judaica* extract (5mg/kg bw) for one month, Group 6: Animals treated with DOX + low dose of *A. judaica* extract (0.5 mg/kg bw) for one month and Group 7: Animals treated with DOX + high dose of *A. judaica* extract (5mg/kg bw) for one month.

2.5 Chromosomal Aberrations Analysis

Mice were sacrificed 24 h after treatment termination for chromosomal aberration analysis. One hour and half or two hours before sacrifice, mice were injected with 4 mg colchicine / kg. b.w. Femurs were removed and the bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared using the method of Preston et al. [19]. Fifty metaphase spreads per animal were analyzed, for scoring the different types of chromosome aberrations. For spermatocyte cells, chromosomal preparations were made according to the air-drying method of Evans et al. [20]. Briefly, after killing the animals, the epididymides excised and minced in isotonic sodium citrate solution (2.2%), dispersed and filtered to exclude large tissue fragments. The slides were stained with Giemsa dye in phosphate buffer (pH 6.8).Fifty primary spermatocytes/mouse at diakinesis-metaphase I were scored. Abnormalities recorded included univalents (x–y univalent and autosomal univalent), chains, rings, N±1 and polyploidy.

2.6 Determination of Malondialdehyde (MDA) Level

Liver tissues were homogenized and the supernatant was chemically treated and centrifuged at 10000 rpm for 3 min for quantitative measurement of lipid peroxidase malondialdehyde (MDA) according to the method of Ohkawa et al. [21]. Briefly, about 0.5 ml of organ homogenates, 0.5 ml physiological solution and 0.5 ml 25% trichloroacetic acid and centrifuge at 2,000 rpm for 20 min. A 1 ml of protein-free supernatant was mixed with 0.25 ml 0.5% thiobarbituric acid and heated at 95°C for 1 h. After cooling, the intensity of pink colour of the end fraction product was determined at 532 nm.

2.7 Molecular Analysis

2.7.1 DNA fragmentation assay

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml lysis buffer containing 10mM tris-HCL (PH.8), 1 mM EDTA, 0.2 % triton X-100, centrifuged at 10000 r.p.m. (Eppendorf) for 20 minutes at 4ºC. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and supernatants (S), 1.5 ml of 10 % trichloroacetic acid (TCA) was added and incubated at 4ºC for 10 minutes. The samples were centrifuged for 20 minutes at 10000 r.p.m. (Eppendorf) at 4ºC and the pellets were suspended in 750 µl of 5 % TCA, followed by incubation at 100ºC for 20 minutes. Subsequently, to each sample 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde] was added and incubated at room temperature for 24 hour [22]. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

DNA fragmentation = [OD of fragmented DNA (S) / OD of fragmented DNA (S) +OD of intact DNA (P)] X100

2.7.2 Quantitative Real-Time PCR (qRT-PCR)

2.7.2.1 Isolation of total RNA

TRIzol® Reagent was used to extract total RNA from liver tissues according to the manufacturer's instructions. The total RNA samples were pretreated using RNase-free™ DNase to remove any possible genomic DNA contamination. The concentration and purity of RNA was measured at 260/280 nm using ultraviolet spectrophotometer (ratios fell between 1.75 and 1.9, indicating very pure RNA in all cases). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

2.7.2.2 Reverse Transcription (RT) Reaction

Total RNA (1µg) was reverse transcribed into cDNA using RevertAid^{TM} First Strand cDNA Synthesis Kit. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 95°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real Time- polymerase chain reaction (qRT-PCR).

2.7.2.3 qRT-PCR

StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the liver cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR green master mix , 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 9.5 µL distilled water, and 1 µL of cDNA template. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used in this study are listed in Table 1. Data from the real-time PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method [23].

2.8 Statistical Analysis

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System followed by Scheffé-test to assess significant differences between groups. The software was used is SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). The values were expressed as mean±SEM. All statements of significant were based on probability of $P < 0.05$.

3. RESULTS

Table (2) shows the effect of A. judaica extract and /or DOX on DNA fragmentation and MDA levels in mice. Treatment of mice with DOX increased the DNA fragmentation and MDA levels in liver tissue. However, the DNA fragmentation rates and MDA levels in male mice treated with different doses of *A. judaica* extract were low and relatively similar to that in control group. Additionally, treatment of male mice treated with DOX and *A. judaica* extract decreased significantly the rate of DNA fragmentation and MDA levels compared with DOX treated group.

The results revealed that DOX induced highly significant increase in chromosomal aberrations in both bone marrow (Table 3) and spermatocyte (Table 4) cells. The DOX treatment caused high percentage of cells contained chromosome aberrations. The break aberration is the main type of chromosomal aberrations in bone marrow cells. X-Y and autosomal univalents are the main types of chromosomal aberrations in spermatocyte cells treated by DOX. However, the treatment with *A. judaica* extract plus DOX significantly decreased all types of chromosomal aberrations in both bone marrow and spermatocyte cells.

Table 1. Sequence of the primers used in the qRT-PCR analysis

Gene name	Sequence (5'-3')	Reference
Bax	F: AGGATGATTGCTGATGTGGATAC	van der Hoeven et al [24]
	R: CACAAAGATGGTCACTGTCTGC	
Caspase -3	F: AAATTCAAGGGACGGGTCAT	Liu et al [25]
	R: ATTGACACAATACACGGGATCTGT	
GADPH	F: CAAGGTCATCCATGACAACTTTG	Ahmed et al [26]
	R: GTCCACCACCCTGTTGCTGTAG	

Expression of apoptosis related genes (Caspase-3 and Bax) in male mice treated with DOX and/or *A. judaica* extract are summarized in Figs. 1 and 2, respectively. Treatment of mice with DOX drug increased the expression of Caspase-3 and Bax gene in liver tissue to 2.54 ± 0.15 and 4.1 ± 0.18 fold, respectively when compared to control. However, the expression of Caspase-3 and Bax gene in mice treated with low and high doses of *A. judaica* extract was relatively similar to that in control group. In addition, the expression of Caspase-3 and Bax in mice treated with DOX and supplemented with low dose or high dose of *A. judaica* extract was decreased but this decrease is unnoticeablely when compared with DOX treated mice.

4. DISCUSSIONS

DOX-based chemotherapy is associated with severe side effects to non-tumorous tissues restricting its clinical applications [27]. Doxorubicin-induced release of free radicals induces oxidative stress, resulting in DNA damage and cell death [28]. In the present work, mice treated with DOX suffer from oxidative stress as indicated by the increase of DNA fragmentation rate and lipid peroxidation expressed as MDA. Also, DOX caused high percentage of structural and numerical chromosome aberrations. These results are similar to the previous studies which provided evidence that DOX induced hepatotoxicity and chromosomal aberrations [29-31].

Table 2. Effect of *A. judaica* extract and /or DOX on DNA fragmentation and MDA levels in mice

Treatment	DNA fragmentation	MDA (nmol MDA/g wet weight)
Control (-Ve)	6.80 ± 0.37^{e}	2.70 ± 0.44^{d}
DMSO	8.30 ± 0.53^{de}	3.92 ± 0.72^{cd}
DOX (+Ve)	39.80 ± 0.86^{a}	12.36 ± 1.12^{a}
A. Judaica(LD)	9.20 ± 0.58^{d}	4.16 ± 0.83^{c}
A. Judaica(HD)	10.30 ± 0.30^{d}	4.80 ± 1.25^{c}
A. Judaica(LD) +DOX	28.50 ± 0.44^{b}	8.64 ± 0.49^{b}
A. Judaica(HD) +DOX	19.60 ± 0.50^{c}	5.28 ± 0.83^{c}

The results are expressed as mean ± SE, each value is the average of three duplicate reading values.[abcd] Means in the same column followed by different lowercase letters are significantly different (P = 0.05), DOX: Doxorubicin; LD: low dose of A. judaica extract; HD: high dose of A. judaica extract

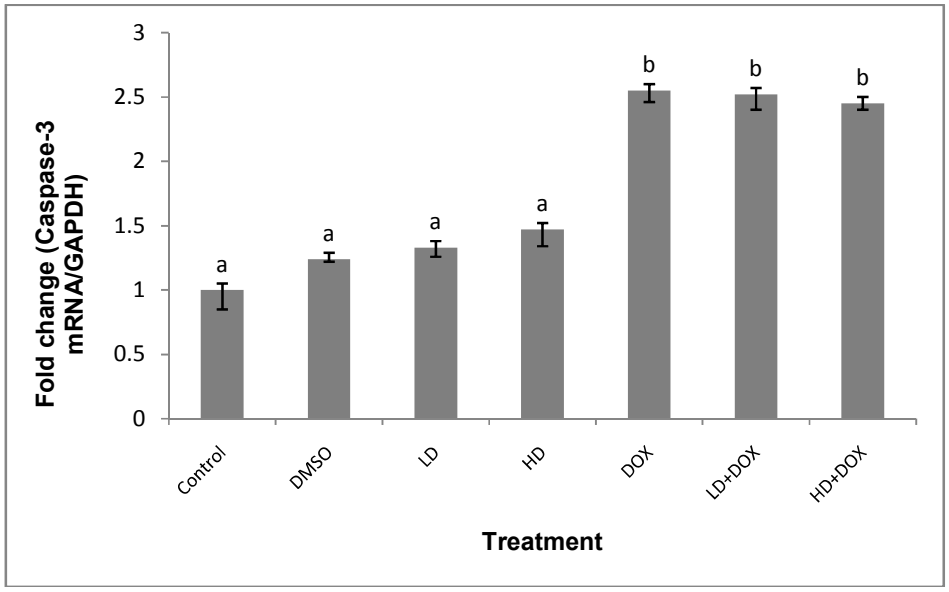

Fig. 1. Expression of Caspase-3 mRNA in liver tissue of male mice treated with doxorubicin and/or *A. judaica* extract analyzed by quantitative real-time polymerase chain reaction. a,bMean with different letters, within the tissue, differ significantly (p≤0.05)

Table 3. Mean parentages of chromosomal aberration in male mice bone marrow cells treated with DOX and/or *A. judaica* extract

Groups	Structural aberrations						Total structural aberrations	Numerical variation		Total
	Gap	Break	Chromatid gap	Fragment	Deletions	Endowments		N-1	N+1	
Control	0.40 ± 0.24^{c}	0.20 ± 0.20^{d}	0.20 ± 0.20^{d}	0.40±0.24^{c}	0.20 ± 0.20^{c}	0.20 ± 0.20^{d}	1.60 ± 0.34^{f}	0.20 ± 0.10^{c}	0.20 ± 0.10^{c}	0.40 ± 0.10^{d}
DMSO	0.40 ± 0.24^{c}	0.60 ± 0.24^{cd}	0.60 ± 0.24^{cd}	0.60±0.24^{bc}	0.80 ± 0.20^{bc}	0.40±0.24^{cd}	3.40 ± 0.24^{e}	0.60± 0.24^{bc}	0.80 ± 0.37^{bc}	1.40 ± 0.21^{c}
LD	1.00 ± 0.31^{b}	0.80 ± 0.20^{c}	0.80 ± 0.20^{c}	1.20±0.20^{b}	0.80 ± 0.20^{bc}	0.80 ± 0.20^{c}	5.40 ± 0.37^{e}	1.20 ± 0.20^{b}	1.20 ± 0.20^{b}	2.40 ± 0.40^{b}
HD	2.00 ± 0.31^{ab}	1.20 ± 0.20^{c}	1.20 ± 0.20^{c}	1.80±0.37^{ab}	1.60 ± 0.24^{b}	1.40 ± 0.40^{c}	9.20 ± 0.37^{d}	1.40 ± 0.24^{b}	2.00 ± 0.31^{a}	3.40± 0.50^{ab}
DOX	3.40 ± 0.74^{a}	5.20 ± 0.73^{a}	5.20 ± 0.73^{a}	2.80±0.66^{a}	4.40 ± 0.60^{a}	4.60 ± 0.92^{a}	25.60 ± 0.44^{a}	2.40 ± 0.50^{a}	2.80 ± 0.37^{a}	5.20 ± 0.37^{a}
LD + DOX	2.60 ± 0.40^{ab}	4.20 ± 0.37^{ab}	4.20 ± 0.37^{ab}	1.80±0.37^{ab}	3.80 ± 0.73^{ab}	4.20 ± 0.91^{a}	20.80 ± 0.37^{b}	2.00 ± 0.31^{a}	2.20 ± 0.37^{a}	4.20 ± 0.24^{a}
HD + DOX	1.00 ± 0.31^{b}	3.00 ± 0.31^{b}	3.00 ± 0.31^{b}	1.00±0.31^{b}	2.00 ± 0.31^{b}	3.00 ± 0.70^{b}	13.00 ± 0.37^{c}	2.20 ± 0.58^{a}	1.80 ± 0.37^{a}	4.00 ± 0.23^{a}

The results are expressed as mean ± SE, each value is the average of three duplicate reading values.^{abcd} Means in the same column followed by different lowercase letters are significantly different (P = 0.05)DOX: Doxorubicin; LD: low dose of A. judaica extract; HD: high dose of A. judaica extract

Table 4. Mean parentages of chromosomal aberration in male spermatocyte cells treated with DOX and/or *A. judaica* extract

Treatment	No. of examined cells	Structural aberrations								Total aberration		Numerical variation				Total numerical variation	
		x-y univalent		Autosomal univalent		Chain		Ring				N-1		N+1			
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Control	250	0	0.0	1	0.4	0	0.00	1	0.4	2	0.8	0	0.0	1	0.4	1	0.4
DMSO	250	3	1.2	2	0.8	2	0.80	1	0.4	8	3.2	3	1.2	3	1.2	6	2.4
LD	250	2	0.8	2	0.8	3	1.2	3	1.2	10	4.0	4	1.6	4	1.6	8	3.2
HD	250	3	1.2	3	1.2	3	1.2	4	1.6	13	5.2	4	1.6	5	2.0	9	3.6
DOX	250	11	4.4	10	4.0	10	4.0	13	5.2	44	17.6	14	5.6	13	5.2	27	10.8
LD +DOX	250	8	3.2	7	2.8	7	2.8	8	3.2	30	12.0	9	3.6	8	3.2	17	6.8
HD+DOX	250	5	2.0	6	2.4	5	2.0	6	2.4	22	8.8	7	2.8	7	2.8	14	5.6

DOX: Doxorubicin; LD: low dose of A. judaica extract; HD: high dose of A. judaica extract

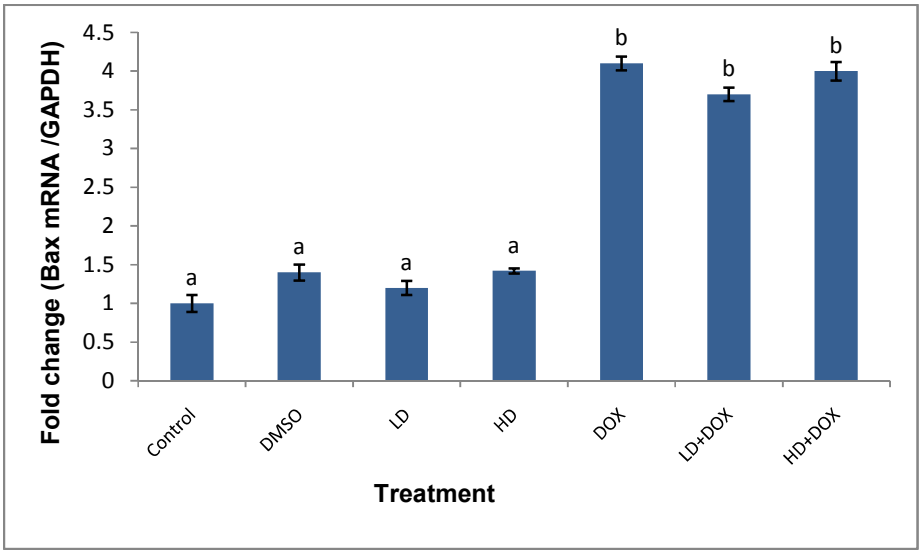

Fig. 2. Expression of Bax mRNA in liver tissue of male mice treated with doxorubicin and/or *A. judaica* extract analyzed by quantitative real-time polymerase chain reaction. a,bMean with different letters, within the tissue, differ significantly (p≤0.05)

On the other hand, Herbs are rich in various compounds such triglycerides, flavonoids, and polyphenols, that can defend against damages induced by toxic drugs [32]. The natural antioxidants act as a shelter against the offensives of free radicals that can induce harms to the cell [33]. *A. judaica*, one of the widespread species of the genus Artemisia, is a desert medicinal plant usually used as tea by population in Egypt Sinai [34]. In the traditional medicine of the Arabic area; *A. judaica* was used to treat skin disorders, weak immune system and gastro-intestinal disorders and also to decrease the risk of cancer [35,36]. In the current study, the administration of *A. judaica* in DOX treated mice significantly reduced the MDA levels in liver tissues. Chromosomal aberrations and DNA fragmentation rate findings supported our biochemical results and confirmed the protective effect of *A. judaica* against DOX toxicity. These findings are consistent with an earlier investigation [37] where the extract of *A. judaica* significantly decreased the DNA damage and the histopathological lesions induced by DOX treatment. The protective role of *A. judaica* may be ascribed to its powerful free radical scavenger activity [38,39]. Furthermore, *A. judaica* was revealed to have sesquiterpene lactones [36] flavonoids [35,40] and high phenolic contents [39,41,43]. These compounds were reported to have multiple biological effects including anti-inflammatory, antioxidant [43] chemopreventive and anticancer activities [44]. The results reported herein also revealed that both doses *A. judaica* extract had no toxic effect and this result is in line with a study conducted with Nofal et al. [45] who confirmed the safety of *A. judaica* extracts.

DOX-induced apoptosis is stimulated through two mechanisms: the death receptor pathway and the mitochondrial pathway [46]. The mitochondrial apoptotic pathway is regulated by various apoptosis-related genes, such as Bax [47]. Bax is a pro-apoptotic protein residing in the cytosol but translocates to the mitochondria upon the induction of apoptosis [48]. On the other hand, caspase-3 gene was identified as a key mediator of apoptosis in mammalian [49] and activated in the apoptotic cell by both pathways [50]. In the present study, DOX induced high expressions of caspase-3 and Bax genes in liver tissues. These results are similar to early studies that reported the DOX hepatotoxicity by apoptosis through up-regulation of caspase-3 and Bax [51,52]. Meanwhile, the administration of *A. judaica* did not alter the expression levels of apoptotic genes increased by DOX.

4. CONCLUSION

According to our study results, treatment with *A. judaica* may play a role in reducing the toxic effect of Doxorubicin and its antioxidant properties seem to mediate such protective effect. However, further studies on the mRNA

level are required to confirm the protective role of *A. judaica* against Doxorubicin-induced genotoxicity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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