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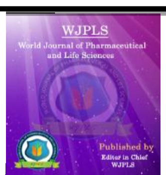

PROTECTIVE EFFECT OF *CALENDULA OFFICINALIS* AGAINST DOXORUBICIN-INDUCED GENOTOXICITY, BIOCHEMICAL AND HISTOPATHOLOGICAL CHANGES IN MALE MICE

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ABSTRACT

Doxorubicin (DXR) is a chemotherapy drug for malignant tumors. However, its clinical utility is limited by severe genotoxicity, nephrotoxicity and hepatotoxicity, DNA damage and free radical stress. In this study, the protective role of *Calendula officinalis* extract (COE) against Doxorubicin (DXR) induced genotoxicity, biochemical and histopathological changes has been evaluated in male albino mice. We have evaluated the protective effects of *Calendula officinalis* 70 % ethanolic extract on cytogenetic, DNA damage, biochemical and histopathological changes induced by DXR administration in male mice. Swiss albino male mice were divided into six groups for sub-acute treatment (daily for 14 consecutive days) before euthanasia. The 1st group served as negative control. The 2nd group served as positive group and received DXR (16 mg/kg bw) on day 15 of treatment. The 3rd group received COE (250mg/kg bw by gavage) for 14 days and 0.9% NaCl on day 15. The 4th group received COE (250mg/kg bw) for 14 days and DXR (16mg/ Kg bw) on day 15. The 5th group received COE (500mg/kg bw) for 14 days and 0.9% NaCl on day 15. The 6th group received COE (500mg/kg bw) for 14 days and DXR (16mg/ kg bwip) on day 15. Chromosomal abnormalities of both germ and somatic cells, formation of micronuclei (MNs) in bone marrow cells in male mice. As well DNA damage, biochemical and histopathological changes have been determined. The results of the present study showed a significant decrease in the frequencies of chromosomal aberrations in both somatic and germ cells, percentages of DNA damage and in the frequencies of micronucleated cells in all groups received COE and/or DXR, especially with the high dose of the extract (500mg/kg bw) compared to DXR treated group only. The administration of COE 14 days prior to DXR injection induced significant decrease in malondialdehyde (MDA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine but increase in albumin level in both concentrations (250,500 mg/kg bw) when compared to DXR only injected group. Histopathological observations revealed that DXR-intoxication resulted in massive structural and functional impairment of liver, kidney and spleen. However, oral administration of COE significantly attenuated DXR-induced oxidative damage in these specimens. In conclusion, its important to use a protective agent possess multiple pharmacological activities such as COE to prevent toxicities induced by DXR and this may be mediated by its potent antioxidant effects.

KEYWORDS: *Calendula officicnalis*, Doxorubicin, DNA damage, genotoxicity, biochemical, histopathology, mice.

1. INTRODUCTION

Doxorubicin (DXR) is an anthracycline group of antibiotic available for the treatment of broad spectrum of human cancers. However, its clinical use is limited because of several side effects like genotoxicity, cardiotoxicity, nephrotoxicity and hepatotoxicity.[1] Mechanisms behind the DXR-induced toxicities are increased level of oxidative damage, DNA fragmentation, inflammation and apoptosis.[2] The therapeutic potential of DXR is achieved through the processes of intercalating into DNA, inhibiting topoisomerase II, preventing DNA and RNA synthesis.[3]

Metabolism of DXR in our body significantly increased the production of free radicals and the occurrence of lipid peroxidation,[4] as well as depletion of antioxidants and sulfhydryl groups,[5] and due to great importance of DXR in cancer chemotherapy, it is essential to reduce its toxicity to normal cells, a goal that can be achieved by

administration of free radical scavenging agents such as antioxidants.[6]

A significant increase in chromosomal aberration and DNA damage were observed in rat, as a rodents model, treated with adriamycin (DXR).[7] In addition, DXR administration, exhibited a distinct histological difference when compared to control, moreover, the concentration of MDA was increased.[8] DXR also was found to induced genotoxicity by increasing, MNs, frequency, structural chromosomal aberrations (CAs) such as chromatid breaks, dicentrics, acentric fragments, and gaps. DXR induced a pronounced histopathological changes, as well a significant increase in MDA content in male albino mice and rats.[9,10]

In complementary and alternative medicine, the toxins were removed from the body with the using of herbal treatments.[11] Modern research has shown that a wide range of plants can neutralize or detoxify toxins and protect the body from the toxic effects of drugs and chemicals. These plants included: CO with its detoxification ability especially hepato-and nephro-protective effects of this medicinal plant.[12] Pharmacological studies of different COE have shown anti-viral,[13] anti-inflammatory,[14] anti-genotoxic,[15] antioxidant and anticlastogenic activities.[16] COE effectively scavenged free radicals. These radicals are generated inside the body during the normal metabolism or in presence of xenobiotics.[16] Antioxidants have direct effects on inhibition of DNA strand-breaks which can reduce the mutagenicity and further carcinogenicity.

The hepatoprotective effect of calendula extracts on oxidative stress, genotoxicity and alteration of gene expressions were reported and when male rats treated with the extracts, it showed a significant decrease in oxidative damage markers, micronucleated cells, and DNA fragmentation.[17] Therefore, the aim of the present study was to explore the protective effect of COE against DXR- induced genotoxicity, biochemical and histopathological changes in male mice.

2. MATERIALS AND METHODS

2.1. Plant: Calendula officinalis linn. flower

The flowers were collected from Faculty of Pharmacy Farm, Cairo University, Egypt and identified by Botany Department, Faculty of Science, Cairo University, Egypt, then air dried, pulverized and stored tightly closed in glass container till subjected to further studies.

2.1. 1. Preparation of flower extract

In a glass jar dried *Calendula officinalis* Linn. flowers powder were extracted according to the Egyptian pharmacopeia.[18] by percolation several times till exhaustion with 70 % ethanolic solution then filtered and most of the solvent was removed using rotatory evaporator apparatus attached with vacuum pump and low temperature 50 ºC.

2.2. Animals and treatment

Adult male albino mice each weighting (30gm) served as experimental animals. The mice were housed in plastic cages at an environmentally controlled room (constant temperature 25-27 °C) with 12 hr. light/dark cycle for one week prior to starting the experiment, and they were provided with water and feed *ad libitum*. Mice were divided into six groups (5 animals/ group) as follow: The 1st group served as negative control and were administered orally a distilled water (10 ml/kg bw/day by gavage) for 14 days prior to treatment with 0.9% NaCl by intraperitoneal (IP) injection. The 2nd group served as positive control and received distilled water (10 ml/kg bw/ day by gavage) for 14 days, and were i.p treated on day 15 with DXR (16 mg/kg bw). The 3rd group received COE orally (10 ml/kg bw/day by gavage) prepared at a dose 250 mg/kg for 14 days prior to treatment with 0.9% NaCl on day 15.The 4th group received COE orally (10 ml/kg bw/day by gavage) prepared at a dose 250 mg/kg for 14 days prior to treatment with DXR (16mg/ Kg bw). The 5th group received COE orally (10 ml/kg bw/day by gavage) prepared at a dose 500 mg/kg for 14 days prior to i.p treatment with 0.9% NaCl on day 15. The 6th group received COE orally (10 ml/kg bw/day by gavage) prepared at a dose 500 mg/kg for 14 days prior to treatment with DXR (16mg/ Kg bw). All animal experiments were approved by the ethics committee of our Institute and that informed consent was obtained.

2.3. Experimental procedures

2.3.1. Cytogenetic and DNA damage analysis

2.3.1.1. Chromosomal analysis in somatic cells

Mice were subjected to cytogenetic analysis from bone marrow cells using the method of.[19] Briefly, mice were injected with Colchicine (0.05 mg/kgbw) for two and a half hours before sacrifice. Animals were sacrificed and femoral bone marrow cells were flushed with isotonic solution (0.9% NaCl). Hypotonic solution (0.56% KCl) was added to the cell pellet and incubated at 37°C for 30 minutes and the solution was fixed, slides were air dried and stained with 10% Giemsa stain for 20 minutes. 50 metaphases were studied per animal scoring different types of structural and numerical aberrations in bone marrow cells.

2.3.1.2. Chromosomal analysis in germ cells

Spermatocyte cells were prepared according to.[20] Chromosomes were spread on clean glass slides by the gradual fixation/air-drying method. The preparations were stained with 20% Giemsa (Merck, Darmstadt, Germany) in PBS (pH 6.8) for 10 min for conventional chromosome analysis. Aberrations are scored in metaphase chromosomes of dividing cells. Fifty metaphase spreads per animal were analyzed for studying the structural and numerical chromosomal aberrations.

2.3.1.3. DNA fragmentation

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), 1mM EDTA, 0.2 %

triton X-100, centrifuged at 10000 r.p.m. (Eppendorf) for 20 minutes at 4°C. The pellets were re-suspended 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 4°C for 10 minutes. The samples were centrifuged for 20 minutes at 10000 r.p.m. (Eppendorf) at 4°C and the pellets were suspended in 750µl of 5 % TCA, followed by incubation at 100°C for 20 minutes. Subsequently, to each sample, 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150µl of sulfuric acid and 60µlacetaldehyde] was added and incubated at room temperature for 24 hour.[21] The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula: DNA fragmentation =

$$\frac{\text{OD of fragmented DNA(S)}}{\text{OD of fragmented DNA (S)} + \text{OD of intact DNA (P)}}$$

2.3.1.4. Comet Assay
Isolated blood cells of all groups of male mice were subjected to the modified single-cell gel electrophoresis or comet assay.[22] To obtain the cells, the pellet of blood cells was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm3 pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline (to remove red blood cells), the blood cells were dispersed into single cells using a pipette. In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at × 40 magnifications. For each experimental condition, about 100 cells (about25 cells/animal) were examined to determine the percentage of cells with DNA damage that appear like comets. The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus.[23] A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

2.3.1.5. Micronucleus assay
Bone marrow slides were prepared according to the method described by.[24] The bone marrow were washed with 1ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 minutes, followed by staining in 5% Giemsa stain for 5 minutes then washed in distilled water and mounted. For each animal 1000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei.

2.3.2. Biochemical Analysis
2.3.2.1. Analysis of oxidative enzyme
Liver tissues were homogenized in 20mm Tris-HCI (pH 7.4). Homogenates were centrifuged at 6000g for 30 min. MDA levels in the supernatants were determined using a spectrophotometric assay kit according to the manufacturer's instruction. The absorbance of the resultant pink product was measured at 534 nm according to.[25] The lipid peroxidation values were expressed as nmol MDA/g tissue.

2.3.2.2. Blood serum analysis
2.3.2.2.1. Liver Function
Blood samples were collected from each animal in a clear sterile centrifuge tube without anticoagulant obtaining serum for determination of AST and ALT .The tubes were left at room temperature. After 1 hour the tubes centrifuged at 2500 rpm for 10 min and the supernatant (serum) was subjected to assay for (ALT and AST) using Ranox kit following Standard method of.[26]

2.3.2.2.2. Kidney Function
Serum was obtained from control and treated mice of each group. The urea and uric acid concentration was determined using the modified Berthelot method by.[27] and creatinine concentration by alkaline picture method using standard method of.[28]

2.3.3. Histopathological investigations
Liver, kidney and spleen specimens from all animals were dissected immediately after death, and fixed in 10% neutral-buffered formal saline for 72 hours at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin; serial sections of 6 Mm thick were cut and stained with haematoxylin and eosin according to.[29] for histopathological investigation.

2.3.4. Statistical analysis
Statistical analysis was performed with SPSS for Windows, version 11.5 (SPSS, Inc.). Data were analyzed using one-way analysis of variance (ANOVA). The results were shown as mean ± SE and the differences were considered significant at $P \leq 0.05$.

3. RESULTS
3.1. Cytogenetic and DNA damage analysis
3.1.1. Chromosomal aberrations in bone marrow cell (somatic cells)
Cytogenetic analysis in the present study (Table 1) showed that there were a significant increase in both structural and numerical chromosomal aberrations in DXR treated group compared to control and all treated groups. That is for all types of structural aberrations which includes, gaps, breaks, deletion, fragments, centromeric attenuations and

endomitosis, as well for total structural aberrations (24.40±1.36 ^{e} and 0.80 ± 0.37^{a} for total structural aberrations in DXR and control groups, respectively). However, both doses (250 and 500 mg/kg bw) of COE decrease significantly all types of these aberrations when compared to control specially with the high dose of Calendula (24.40±1.36 ^{e}, 17.60 ±1.63^{d} and12.40 ±0.24^{c}, for total structural aberration in DXR, COE 250mg/kg +DXR, COE 500 mg/kg +DXR, treated groups, respectively.). The same findings were found concerning numerical variations, which consisted of hyperploidy, hypoploidy, polyploidy and total numerical variations, where, there was a significant increase in total variations in DXR treated group compared to control and/ or COE treated groups (9.20 ± 0.3 and 0.40±0.40 for total numerical variations in DXR and control groups, respectively). However, both doses (250 and 500 mg/kg bw) of COE decrease significantly all types of these aberrations when compared to control specially with the high dose of Calendula in a dose dependent manner (9.20 ± 0.3 ^{e}, 6.60 ±0.24^{d} and4.60 ±0.40^{c}, for total numerical variations in DXR, COE 250mg/kg +DXR and COE 500 mg/kg +DXR, treated groups, respectively).

Table 1: Mean frequency of chromosomal aberrations in bone marrow cell of mice treated with Calendula and /or DXR.

Groups	Structural aberrations						Total structural aberrations	Numerical aberrations			Total number of aberrations
	Gap	break	Fragments	Deletions	Centermetric attenuation	Endometosis		Hypopolidy (N-1)	Hyperploidy (N+1)	Polyploidy	
C –ve	0.60 ± 0.24	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.20 ± 0.20	0.00 ±0.00	0.80 ± 0.37^{a}	0.20 ±0.20	0.00 ±0.00	0.20 ±0.20	0.40 ± 0.40 ^{a}
C +ve (DXR)	2.40 ± 0.40	7.80 ± 0.37	3.40 ±0.24	3.60 ±0.24	3.60 ± 0.24	3.40 ±0.50	24.40±1 .36 ^{e}	6.20 ±0.20	1.60 ±0.24	1.40 ±0.24	9.20 ± 0.3 ^{e}
COE 250mg/kg	0.40 ± 0.24	1.00 ±0.00	0.40 ±0.24	0.60 ±0.24	0.40 ± 0.24	0.20 ±0.20	3 ±0.31^{b}	1.20 ±0.20	0.00±0. 00	1.00±0. 00	2.2 ± 0.20 ^{b}
COE 250mg/kg +DXR	1.80 ±0.37	4.60 ± 0.50	2.40 ±0.40	3.60 ±0.67	3 ± 0.31	2.20 ±0.20	17.60 ±1.63^{d}	4.60 ±0.24	1.00 ±0.00	1.00±0. 00	6.60 ±0.24^{d}
COE 500mg/kg	0.20 ± 0.20	0.40 ± 0.24	0.80 ±0.20	0.20 ±0.20	0.60 ± 0.24	0.20 ±0.20	2.40 ± 0.87^{b}	0.60 ±0.24	0.0± 0.00	1.00± 0.00	1.60 ±0.24^{b}
COE 500 mg/kg +DXR	1.60 ± 0.24	3.40 ± 0.24	1.80 ±0.20	2.00± 0.00	1.60 ± 0.24	2.00 ±0.00	12.40 ±0.24^{c}	2.0 ±0.00	1.0 ± 0.00	1.80 ±0.20	4.60 ±0.40^{c}
Data were expressed as mean ± SE , means with different superscript letters (a, b, c, d, e) are significantly different at P≤0.05											

3.1.2. Chromosomal Aberrations in sperm cells (germ cells)

The results presented in table (2) revealed that there was a significant decrease in the frequencies of structural aberrations (x-y univalent, autosomal, fragments and total structural aberration) (28^{f} ***, 18.8^{e} **, 13.6^{d}* and 0.8^{a} for DXR, COE 250mg/kg +DXR, COE 500 mg/kg +DXR and control, groups, respectively), as well as numerical variation (hypo, hyper, polyploidy and total numerical) in all COE treated groups (250 and 500mg /Kg bw) when compared with DXR treated and control groups(12.4^{f}, 8.0^{e} , 6.4^{d} ,and 0.4^{a} for DXR, COE 250mg/kg +DXR, COE 500 mg/kg +DXR and control groups, respectively), and that decrease was dose-dependent.

Table 2: Mean percentage of chromosomal aberrations in male mice spermatocytes treated with Calendula and /or DXR.

Groups	Number of examined cells	Structural aberrations										Numerical aberrations							
		Chain		Ring		Autosomal univalent		X-Y univalent		Total structural aberrations		Hypopolidy (N-1)		Hyperpolidy (N+1)		Polyploidy		Total numeric aberrations number	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
C-ve	250	1	0.4	0	0	0	0	1	0.4	2	0.8^{a}	0	0	0	0	1	0.4	1	0.4^{a}
C+ve (DXR)	250	24	9.6	19	7.6	10	4	17	6.8	70	28^{f} ***	10	4	5	2	16	6.4	31	12.4^{f}
COE 250 mg/ kg	250	2	0.8	2	0.8	2	0.8	3	1.2	9	3.6^{f}	4	1.6	4	1.6	2	0.8	10	4^{b}
COE 250mg/kg + DXR	250	13	5.2	12	4.8	11	4.4	11	4.4	47	18.8^{e} **	9	3.6	8	3.2	3	1.2	20	8^{e}
COE 500 mg/kg	250	2	0.8	1	0.4	1	0.4	3	1.2	7	2.8^{b}	4	1.6	3	1.2	1	0.4	8	3^{b}
COE 500 mg/ kg + DXR	250	9	3.6	9	3.6	6	2.4	10	4	34	13.6^{d}*	5	2	4	1.6	7	2.8	16	6.4^{d}

Data were expressed as mean $\pm$ SE, means with different superscript letters (a, b, c, d, e) are significantly different.
* Significance at P<0.05 ** Significance at P < 0.01 *** Significance at P <0.001

3.1.3. DNA fragmentation

The percentages of the DNA fragmentation in mice treated with COE and /or DXR are shown in table (3). The results revealed that rate of DNA fragmentation decreased significantly in mice treated with COE compared with DXR and control groups. Administration of COE in a dose of 250 and 500 mg/Kg prior to DXR administration decreased significantly DNA fragmentations especially with the high dose of calendula (.35.8 ± 0.58^{e}, 27.60 ± 0.24^{d}, $20.80\pm0 .37^{c}$, 7.20 ± 0.58^{a} for DXR, COE 250mg/kg bw+DXR, COE 500 mg/kg bw +DXR and control, groups, respectively), and that decrease is in a dose-dependent manner.

Table 3: Mean frequency of the DNA fragmentation in mice treated with Calendula and /or DXR (mean ± SE).

Groups	DNA fragmentation
C-ve	7.20 ± 0.58^{a}
C+ve (DXR)	35.8 ± 0.58^{e}
COE 250mg/kg	11.20 ± 0.37^{b}
COE 250mg/kg +DXR	27.60 ± 0.24^{d}
COE 500mg/kg	9.40 ± 0.50^{b}
COE 500mg/kg +DXR	$20.80\pm0 .37^{c}$

Another way to detect DNA damage is a comet assay. The results of that assay are presented in table (4). Comet assay in the tail length in peripheral blood of male mice indicated a significant decreases in tail length in DXR-treated mice, which received 250 mg/kg and 500 mg/kg of COE indicating the antigenotoxic effect of COE especially at high dose level (17.2^{e}, 11.8^{d}, 8.8^{c}, 4.4^{a} for DXR, COE 250mg/kg bw+DXR, COE 500 mg/kg bw +DXR and control, groups, respectively), and that decrease is dose-dependent.

Table 4: Visual score of DNA damage in male mice treated with calendula and /or DXR.

Groups	Number of animals	Number of cells		Class of comet				DNA damage in cells (%)
		Analyzed	Total comets	0	1	2	3	
C-ve	5	500	22	478	17	3	2	4.4[a]
C+ve (DXR)	5	500	86	414	36	26	24	17.2[e]
COE 250mg/kg	5	500	27	473	12	8	7	5.4[b]
COE 250mg/kg +DXR	5	500	59	441	23	19	17	11.8[d]
COE 500mg/kg	5	500	25	475	18	4	3	5[b]
*COE*500mg/kg +DXR	5	500	44	456	16	15	13	8.8[c]

Means with different superscript letters (a, b, c, d, e) are differ significantly at P≤0.05
Class o =no tail , 1 = Tail length < diameter of nucleus ;2=Tail length between 1x and 2x the diameter of nucleus ;3= Tail length> at the diameter of nucleus (*): No of cells analyzed

3.1.4. Micronucleus assay

The effect of COE on micro nucleated polychromatic erythrocytes (MNPE) in the bone marrow cells of male mice are given in table (5). The results showed that DXR treated group present a higher significant values when compared to negative control group (27.20 ± 1.303[e] vs. 6.20 ± 0.836[a] for DXR and control, groups, respectively).COE 250 mg/kg and 500 mg/Kg doses have demonstrated low values of micro-nucleated cells, showing that these compounds did not have a mutagenic nor cytotoxic effect on mouse bone marrow cells i.e., no statistically significant difference in the frequency of MN PCE or the ratio of PCE to NCE among these groups. However, when COE was administered prior to DXR injection, a significant decrease in the frequency of DXR-induced MNPCE was observed in mice treated with both doses of COE compared to DXR treated group (27.20 ± 1.303[e] , 21.40 ± 1.140[d], 15.20± 0.836[b]and 6.20 ± 0.836[a] for DXR,COE 250mg/kg +DXR, COE 500mg/kg +DXR and control, groups, respectively).

Table 5: Micro-nucleated polychromatic erythrocytes (MNPCES) of male mice treated with DXR and /or COE (mean ± SE).

Groups	PCE screened	Mn PCEs /1000 PCE
C-ve	1000	6.20 ± 0.836[a]
C+ve (DXR)	1000	27.20 ± 1.303[e]
COE 250mg/kg	1000	11± 0.707[b]
COE 250mg/kg +DXR	1000	21.40 ± 1.140[d]
COE 500mg/kg	1000	9.80 ± 0.836[b]
COE 500mg/kg +DXR	1000	15.20 ± 0.836[c]

Data were expressed as mean + SE, means with different superscript letters (a, b, c ,d, e) are differ significantly at P<0.05

3.2. Biochemical Analysis

3.2.1. Malondialdehyde (MDA) level

Data in table (6) represented the effect of COE on MDA level in liver tissues in oxidative stress model. The treatment with DXR had a significant increase in MDA level compared to COE and /or DXR treated groups (34.4 0+0.67[e], 29 + 0.70[d], 21.20 +0.70[c] , 12.80 + 0.37[b] , 10.80 +0.37[b] , 7.60 ± 0.50[a], for DXR, COE 250mg/kg+ DXR, COE 500 mg/kg+ DXR, COE 250mg/kg, COE 500mg/kg and control, groups, respectively). The administration of male mice with (250 and 500 mg/kg) of COE for 14 consecutive days caused a significant decrease in MDA compared to C +ve (DXR treated group) especially with the high dose of Calendula.

Table 6: Mean frequency of MDA in male mice treated with COE and /or DXR.

Groups	(MDA) mmol /g tissues (M ± SE)
C –ve	7.60 ± 0.50[a]
C +ve (DXR)	34.40 ± 0.67[e]
COE 250mg/kg	12.80 ± 0.37[b]
COE 250mg/kg +DXR	29 ± 0.70[d]
COE 500mg/kg	10.80 ± 0.37[b]
COE 500mg/kg +DXR	21.20 ± 0.70[c]

Data were expressed as mean ± SE. Means with different superscript letters (a, b, c ,d, e) are differ significantly at P≤0.05

3.2.2. Liver and Kidney Functions

The results in table (7) represented the protective effect of COE on serum ALT and AST (liver function) and creatinine level, urea and albumin (kidney function) in male mice. COE *(*250,500 mg/kg bw)showed a significant decrease in AST, ALT level (liver function) and creatinine and urea (kidney function) compared with the DXR only treated group, indicating nephroprotective and hepatoprotective effect of COE, especially with the high dose. However, Calendula was found to increase significantly the albumin level compared to DXR treated group.

Table 7: Biochemical parameters of control, COE and/or DXR exposed mice.

Groups	ALT IU/L	AST IU/L	Creatinine mg/dl	Urea mg/dl	Albumin g/dl
C –ve	30±0.7 ^{a}	89±0.01^{a}	0.22±0.05 ^{a}	36±2^{a}	2.3±0.54 ^{a}
C +ve (DXR)	124.80±1.53^{e}	209.5±16.3^{e}	0.4±0.07 ^{e}	55±2.2^{e}	1.54± 0.45 ^{e}
COE 250mg/kg	62±1.58 ^{b}	128±1.87^{b}	0.3±0.07 ^{b}	42.4±1.68 ^{b}	2.6±0.47 ^{b}
COE 250mg/Kg+ DXR	94±1.4 ^{d}	177.8 ± 13.2^{d}	0.36±0.05 ^{d}	48.7±1.37^{d}	2.3±0.49 ^{d}
COE 500mg/kg	39 ±1.58 ^{b}	99±0.01^{b}	0.25±0.07 ^{b}	38.6±4.87^{b}	2.2±0.49 ^{b}
COE 500mg/Kg+ DX	94±1.92 ^{c}	153,4 ± 12.9^{c}	0.34±0.07^{c}	44±1.52 ^{c}	2.4±0.46 ^{c}

Data were expressed as mean ± SE. Means with different superscript letters (a, b, c ,d, e) are differ significantly at P≤0.05.

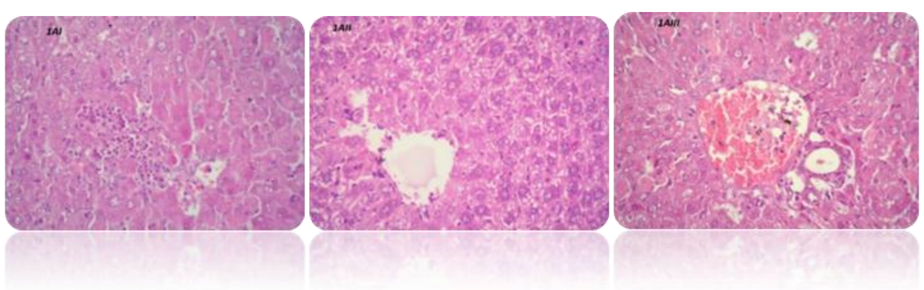
3.3. Histopathological findings

Histological features of the control and treated groups were captured and processed using Adobe Photoshop version 8.0.Figure (1A) indicate that DXR treated group showing, i) focal inflammatory cells, ii)vacuolar degeneration in hepatocytes, dilated central vein, and iii) congestion of central vein. However the control group revealed normal structure of the liver, i.e, no changes (1B). Meanwhile, Calendula (250 mg Kg bw) administration prior to DXR injection Fig. (1d: i & ii), showed hepatic cells are replaced by areas of hemorrhage and necrosis, and iii) ballooning degeneration of hepatic cells. However, the high dose of COE *(*500 mg Kg bw) administration prior to DXR injection showing that the hepatic cells return their architecture and becomes more or less normal except minimal evidence of edema (Fig. 1F).

Fig. (2a) shows a photomicrograph of a kidney section of DXR treated group tissue revealed area of hemorrhage and necrosis with degenerated tubules and intratubular debris with their epithelial lining shedding (H and E, ×100). While, Fig. (2 b) shows normal structure of the kidney and when COE (250 mg Kg bw) administered prior to DXR injection, a dilatation of tubules with some intratubular debris was observed (Fig. 2d), however, COE (500 mg Kg bw) administrated prior to DXR injection showed that renal tissue retune its normal architecture with focal area of hemorrhage (Fig. 2f).

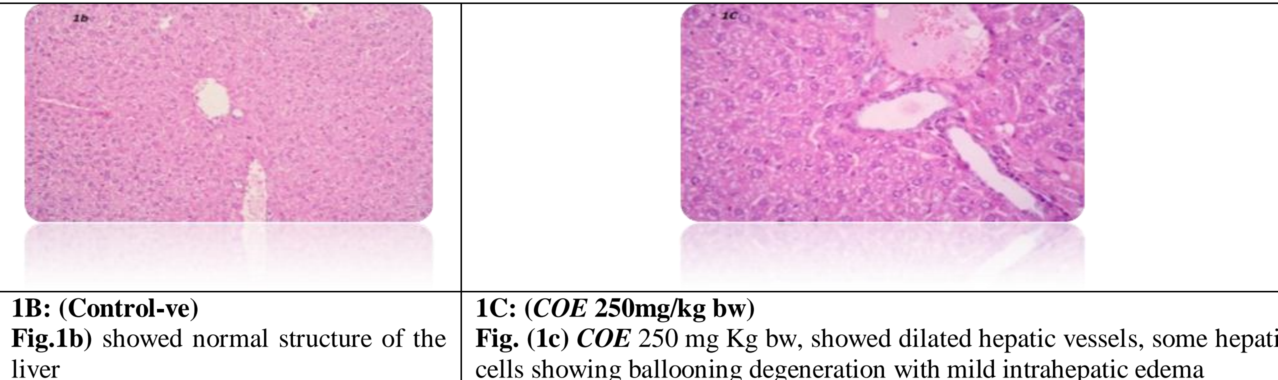
Concerning spleen tissues, a section of spleen tissue (Fig. 3 a) showed that administration of DXR in a dose of 16mg/Kg bw gives an increase in the capsule thickness (CT), trabeculae distributed in the spleen white pulp, a great decrease in the lymphocytic population with many pyknotic nuclei and relatively high degree of fibrosis (F), however, Fig (3b) showed normal structure of spleen in negative control group.

Although no clear protective effect on spleen was observed with the low dose of COE (250 mg Kg bw) when administered prior to DXR injection, where appearance of megakaryocytes was observed (Fig. 3d),the high dose of COE *(*500 mg /Kg bw) showed more protective effect when administrated prior to DXR injection, where, spleen tend to have normal structure confirming the protective effect of the high dose of Calendula (Fig. 3f).

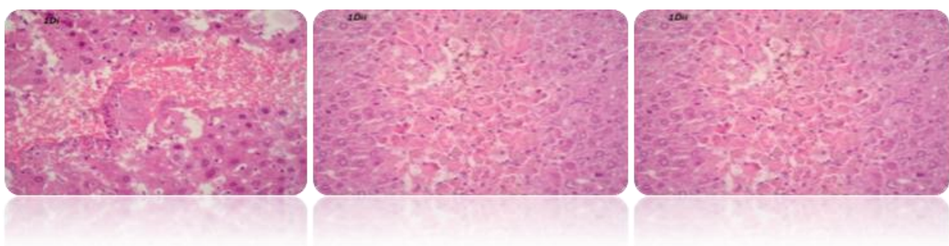


1A: (Control +ve).

Fig. 1a) C +ve is a photomicrograph of a section of liver tissue shows the effect of DXR in a dose of 16mg/Kg bw. **i)**focal inflammatory cells. **ii)** vacuolar degeneration in hepatocytes, dilated central vein. iii) congestion of central vein.

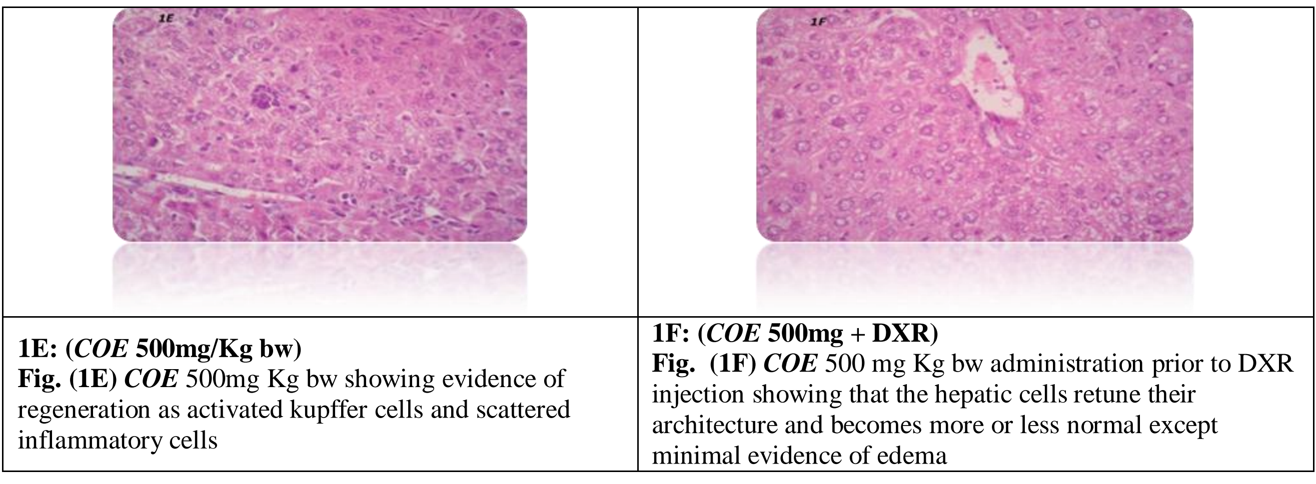


1B: (Control-ve) **Fig.1b)** showed normal structure of the liver	**1C: (*COE* 250mg/kg bw)** **Fig. (1c)** ***COE*** 250 mg Kg bw, showed dilated hepatic vessels, some hepatic cells showing ballooning degeneration with mild intrahepatic edema

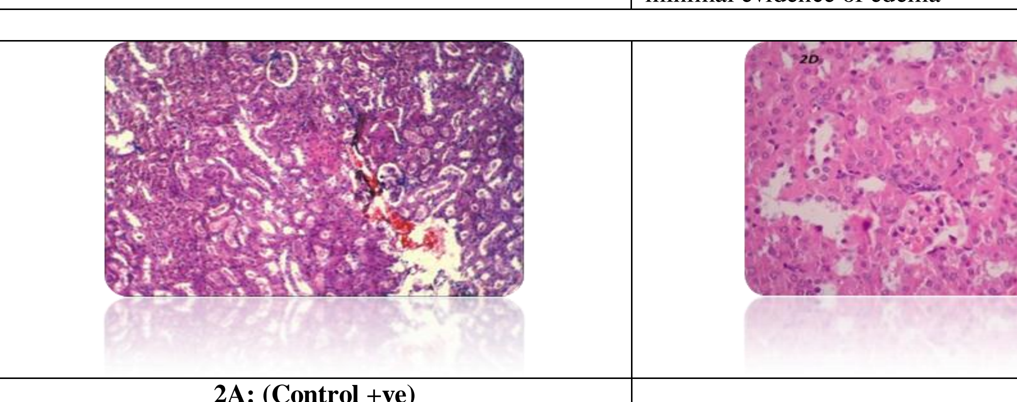


1D: (*COE* 250mg + DXR).

Fig. 1d: (i & ii) *COE* 250 mg Kg bw administration prior to DXR injection showing hepatic cells are replaced by areas of hemorrhage and necrosis ,iii) ballooning degeneration of hepatic cells.



1E: (*COE* 500mg/Kg bw) **Fig. (1E)** ***COE*** 500mg Kg bw showing evidence of regeneration as activated kupffer cells and scattered inflammatory cells	**1F: (*COE* 500mg + DXR)** **Fig. (1F)** ***COE*** 500 mg Kg bw administration prior to DXR injection showing that the hepatic cells retune their architecture and becomes more or less normal except minimal evidence of edema



2A: (Control +ve) **Fig. (2 a)** C+ve is a photomicrograph of a section of kidney tissue treated with DXR (16mg/Kg bw) showed area of hemorrhage and necrosis with degenerated tubules and intratubular debris with their epithelial lining shedding (H and E, ×100).	**2D: (*COE* 250mg/Kg bw +DXR)** **Fig.**(2d) ***COE*** 250 mg Kg bw administration prior to DXR injection showed dilatation of tubules with some intratubular debris

2B: (Control –ve) **Fig. 2 b)** shows normal structure of the Kidney	**2E: (*COE* 500mg/Kg B.Wt.)** **Fig. (2e)** ***COE*** 500mg Kg bw, showing renal tissue with normal architecture
2C: (*COE* 250 mg/Kg bw) **Fig.**(2c)***COE*** 250 mg Kg bw showed marked tubular edema with evidence of necrotic renal tissue	**Fig.** (2f)***COE*** 500 mg Kg bw administration prior to DXR injection, showing renal tissue retune its normal architecture with focal area of hemorrhage

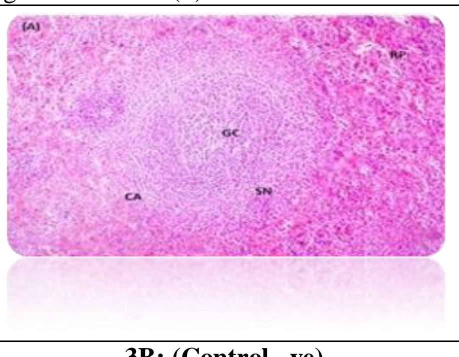
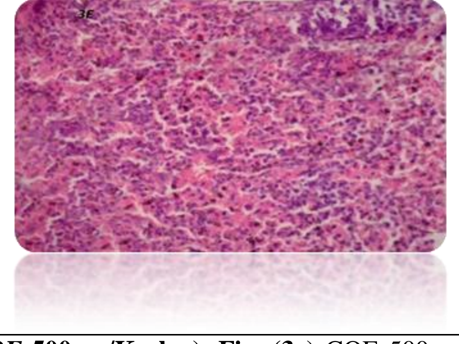
3A: (control +ve). Fig.(3 a) C +ve of spleen tissue treated with DXR(16mg/Kg bw) showed an increase in the capsule thickness (CT), trabeculae distributed in the spleen white pulp, a great decrease in the lymphocytic population with many pyknotic nuclei and relatively high degree of fibrosis (F)	**3D: (*COE* 250mg/Kg bw + DXR)Fig.(3d)*COE*** 250 mg Kg bw administration prior to DXR injection,showing appearance of megakaryocytes indicating no protection
	
3B: (Control –ve).	**3E: (*COE* 500mg/Kg bw). Fig. (3e)** COE 500mg Kg bw,

Fig. (3b) showed normal structure of spleen	showing areas of hemorrhage and necrosis
3C: (*COE* 250mg/Kg bw) Fig.**(3c)** ***COE*** 250 mg Kg bw showing that the splenic architecture has been replaced by areas of necrosis	**3F: (*COE* 500mg/Kg bw + DXR)** **Fig.(3f)** ***COE*** 500 mg /Kg bw administration prior to DXR injection showing that spleen tend to have normal structure

DISCUSSION

DXR is a chemotherapeutic drug. However, its clinical utility is limited by severe genotoxicity, nephrotoxicity and hepatotoxicity, cardiotoxicity, DNA damage and free radical stress.[30,31] Therefore, the present investigation was designed to evaluate the preventive role of COE on DNA damage, genetic, biochemical and histopathological alterations induced by DXR treated male mice.

It was found that COE had anti-genotoxic, anticlastogenic and antioxidant activity. This action was observed with the2 used doses of Calendula especially with the high dose (500mg/kg bw) compared to DXR administered and control groups. A significant decrease in the frequencies of chromosomal aberrations in (somatic and germ cells), percentages of DNA damage and in the frequencies of micro-nucleated cells at all doses levels was found. That coincide with those of,[16,17] who found anti-genotoxic, antioxidant and anticlastogenic activities of that extract. Antioxidants have direct effects on inhibition of DNA strand-breaks which can reduce the mutagenicity and further carcinogenicity. Calendula has been reported to contain flavonoids, triterpenoids and the alkaloid narcissi.[32] Calendula also is rich in carotenoids.[33] Moreover, it contains lycopene, *b*-carotene and Coumarins. These ingredients contained protective substances that decreasing damage to genetic materials and may contribute to the antioxidant and anticlastogenic potential of this extract.[34] Although,at the lower and medium end, ng/ml and mg/ml concentrations of Calendula extract conferred total protection against genotoxicity, at the higher end, g/ml concentrations produced genotoxic effects and induced unscheduled DNA synthesis (UDS) in rat liver cells.[15] That confirm our findings by using the medium concentration of COE.

Biochemical analysis in the liver and kidney of treated male mice showed that administration of DXR increased significantly biochemical alterations. However, COE, 14 days prior to DXR injection induced significant decrease in MDA, ALT, AST, urea, creatinine but increase in albumin level with both concentrations (250,500 mg/kg bw) when compared to DXR only injected group. Our observations are in agreement with those obtained by,[8,15,35] who stated that DXR induced biochemical changes, meanwhile, COE inhibited this toxicity. The elevated serum levels of urea and creatinine indicate reduced ability of the kidney to eliminate toxic metabolic substances.

Moreover, these results are in agreement with previously published reports,[36] who demonstrated that DXR caused deterioration in renal function as it significantly increased blood urea nitrogen (BUN), creatinine, compared to control, with distortion in normal renal histology. This inconsistent with,[37] who found that DOX-induced nephrotoxicity causing increased capillary permeability and glomerular atrophy.

In addition, the findings of,[38] revealed that DXR treatment in rats showed an increase in serum ALT, AST, and ALP concentration reflecting liver dysfunction. However, the serum total protein, albumin levels were decreased, and that support our findings. These results are in accordance with,[38] who attributed the increase in the serum enzyme levels to their increased leakage from damaged and necrotic hepatocytes as a result of toxicity. One of the most prevailing hypothesis of hepatic damage after DXR administration is the ability of the drug to produce reactive oxygen species (ROS) and suppress antioxidant defense mechanism. Also, the increased lipid peroxidation play a critical role in liver injury.[39-41] The improvement of liver and kidney function may be mediated *via* the antioxidant activity of Calendula. This is confirmed by the current study which revealed a significant decrease of MDA, ALT, AST, urea, creatinine and increase in albumin levels.

Histopathological results showed that DXR caused significant structural damage to liver, kidney and spleen tissue architecture (confirmed by 42), which were reversed with Calendula. Histopathological findings of liver tissue showed normal architecture (Fig. 1B)in the control group, whereas, focal inflammatory cells, vacuolar degeneration in hepatocytes, dilated central vein and congestion of central vein were observed in the DXR-treated group (Fig. 1A). Calendula supplementation significantly reversed the DXR-induced liver damage dose dependently. That in accordance with

those of,[1] who found that DXR caused significant structural damage to kidney and liver tissue architecture which were reversed with chrysin as a natural flavonoid antioxidant,[43]

It was clearly evident from the histopathological examination of kidney tissues that Calendula significantly prevented disruption of the normal renal architecture, which was distorted by DXR administration. Normal renal architecture was observed in control animals whereas renal lesions, inflammation, glomerular congestion and interstitial hemorrhage were observed in DXR-treated groups. The lesions were reduced significantly in Calendula treated group in a dose dependent manner. In accordance to our findings, the toxicity of DXR to rat renal tissues as conjugated dienes was confirmed.[42]

CONCLUSION

The co-administration of COE potentially prevented the deleterious effects of doxorubicin-induced toxicity in genetic materials, biochemical parameters and histology of liver, kidney and spleen. This improvement effect in organs injury maybe mediated *via* enhancement of the antioxidant defense system. In addition, ethanolic extracts of this plant especially the high dose (500 mg/Kg) showed anti-genotoxic effect determined by comet assay, where it has been repaired the DNA damage caused by DXR. At the cytogenetic analysis and micronucleus test, COE revealed a protective effect to genetic material. Histopathological changes further confirmed the biochemical results showing that DXR caused significant structural damage to kidney, liver and spleen tissue architecture and function which were reversed with Calendula. The results suggest that calendula attenuated genetic, nephro and hepatic damage induced by DXR due to their antioxidant property.

Abbreviations

DXR: Doxorubicin, COE: *Calendula officinalis* extract, AST: Aspartate aminotransferase, DNA: deoxyribonucleic acid, MDA: Malondialdehyde, ALT: Alanine aminotransferase, MNs: micronucleus, CAs: chromosomal aberrations, ANOVA: One-way analysis of variance, BUN: blood urea nitrogen.

Authors' contributions

HYE designed this work and participated in laboratory analysis. ESA designed this work and participated in laboratory analysis. SMG wrote with critical revision of the manuscript and analysis. HAHE completed the sample collection in the field and laboratory analysis and analysis. MMH completed the sample collection in the field and laboratory analysis and analysis. DMD completed the sample collection in the field and laboratory analysis. HYE, ESA and SMG were involved in reviewing the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that there are no conflict of interest.

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