Suppression of Doxorubicin Apoptotic, Histopathologic, Mutagenic and Oxidative Stress Effects in Male Mice Bone Marrow and Testis Tissues by Aqueous Rosemary Leaves Extract

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Abstract

In the present set of investigations, the anti-mutagenic and anti-cytotoxic effects of aqueous rosemary leaves extract (RE) beside the dose dependency of these effects on male mice bone marrow and germ cells have been evaluated using in vivo cytogenetic, histopathologic and apoptotic assays, as well as biochemical analysis. Doxorubicin (DXR), a well-known mutagen and cytotoxic agent, was given at a single dose of 25 mg/kg b. wt. intraperitoneally at the fifteenth day. 25, 125, 250 and 375 mg/kg b. wt. of RE were given through oral intubation once a day/three days for 15 days prior to DXR administration. The animals of the positive control group (DXR alone) showed significant increase in the mutagenic effect in bone marrow cells, histological damage, incidence of apoptotic cells (TUNEL-positive cells), level of lipid peroxidation and activity of superoxide dismutase in testis. Though, the activities of the other antioxidant enzymes such as glutathione peroxidase, catalase and glutathione reduced form beside the serum level of testosterone and the rate of primary spermatocytes' transformation to spermatids were significantly declined ($P < 0.001$). The ratio of dismutase to glutathione peroxidase and/or catalase was significantly elevated. Pretreatment with each dose of RE showed significant reduction in these frequency of chromosomal aberrations and mitotic index of bone marrow cells and the level of peroxidation, the ratio of SOD/ GPX or CAT, the histological damage and the incidence of apoptotic cells in testes. Also, it caused increase in the levels of some antioxidant enzymes (GSH, CAT and GPX), the level of testosterone and returned the semineferous tubular cell populations' ratio to the control distribution.The protective efficacy of the RE was much pronounced following pretreatment with 125 mg/kg b. wt.

Keywords: doxorubicin- cytogenetic- histopathology- apoptosis- oxidative, stress- cell dynamics- rosemary- bone marrow – testis

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Introduction

Rosemary (Rosmarinus officinalis Labiatae) herb and oil are commonly used as spice and flavoring agents in food processing for their desirable flavor and high antioxidant activities [23, 34]. Rosemary contains flavonoids, phenols, volatile oil and terpenoids [42, 20].

Leaves of R. officinalis possess a variety of bioactivities; including antioxidant[55], antitumor [61] and anti-inflammatory actions [2].These bioactivities of the rosemary leaves extract are comparable with known antioxidants constituents, such as carnosic acid, carnosol, rosemarinic acid, ursolic acid, butylated hydroxyanisole and butylated hydroxytoluene, without the cytotoxic and carcinogenic risk of synthetic antioxidants[26,52,1]. Among the antioxidant compounds in rosemary leaves, ~90% of the antioxidant activity can be attributed to carnosol and carnosic acid[34]. Recently, reports indicated that carnosol is active in anti- inflammation and is an active anti-metastatic against malignant melanocytes [25, 34, 45]. Carnosol was stated to inhibit nitric oxide (NO) production in activated macrophages through modulation of the nuclear factor NF-α [16, 34]. Also, rosmarinic acid was widely studied for its antimicrobial and complement inhibition properties [53]. Additional studies have revealed that rosemary extracts, carnosic acid and carnosol strongly inhibited phase I enzyme, CYP 450 activities and induced the

expression of the phase II enzyme, glutathione S-transferase (GST) [36]. These results gave insight into the different mechanisms involved in the chemopreventive actions of aqueous rosemary leaves extract (RE).

Doxorubicin (DXR) is an anthracycline antibiotic used as an antitumor agent against human malignancies such as leukemia, lymphomas and many solid tumors [13, 47, 33]. Though, previous investigations indicated that DXR has the ability to induce mutations and chromosomal aberrations in normal and malignant cells [60,29,51] in addition to a wide variety of toxic side effects on organs including testis [65,47]. DXR was recognized to alter sperm development, production, structural integrity and motility rates in association with increased cellular apoptosis in spermatogonia and spermatocytes, induction of spermatid micronuclei [62, 31, 30, 47] and oligozospermia [56].

The cytotoxic action of DXR may be exerted by various mechanisms, such as DNA binding, oxygen free radicals formation, membrane composition differentiation and function alteration [4, 64,5].

The reduction of oxidative DNA damage by antioxidants has been evaluated as a chemotherapeutic approach for reducing damage caused by chemotherapy agents such as doxorubicin [51].So, recent studies hypothesized that the combination of the chemotherapeutic drug together with a potent antioxidant may be the appropriate approach to reduce the toxic side effects of anticlastogenic agents. Though, some properties of synthetic antioxidant drugs limited their therapeutic application [65].Thus, the use of plant extracts and food supplements which augment the major cellular endogenous antioxidants following their administration have been recently preferred to combat the oxidative stress associated with different diseases.

Therefore, the present investigation was undertaken to 1)-test the anticlastogenic or clastogenic effects of aqueous rosemary leaves extract (RE) on bone marrow and testis tissues of mice, 2)- determine this extract modulating effect on chromosomal damages, oxidative stress, histopathological alterations and apoptosis induced by DXR and 3)-examine the dose-dependency of these effects.

Materials and Methods

Chemicals

Doxorubicin (Adriblastina® produced by Carlo Erba) was purchased from a local pharmacy in a form of 10 mg/ampoule. The in situ cell death (TUNEL assay) detection kit was purchased from (POD; Roche Molecular Biochemicals). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Rosemary leaves Extract preparation

Rosemary leaves (Rosmarinus officinalis) were obtained and identified by a well known botanist of Botany Department, Beni-Sueif University, Faculty of Science. Leaves were cleaned, shade dried, powdered and extracted. The extract was prepared by refluxing leaves with bi-distilled H2O for 36 hours (12 hours X 3). The cooled liquid extract was then transformed to powder by evaporating its liquid contents. The powder was redissolved in bi-distilled water just before oral administration [28].

Animals

160 male albino mice (Mus musculus), aged 6 weeks and weighing ~28 g, were used. The animals were obtained from the Ophthalmology Research Centre. They were housed in stainless-steel cages at room temperature (25-30 °C) and provided with food and water ad-libitum.

Doses and treatment

The dose of DXR in this study was selected as 25 mg/kg body wt. This dose was previously reported to induce an increase in the frequency of chromosomes, tissues and cells damage in mammalian system [4, 47]. The animals were treated with DXR by the intraperitoneally (i. p.) route since this mode of administration permits a marked exposure of bone marrow and testis cells to the agent tested Preston et al, 1987 [48]. The chosen dose of DXR was adjusted 0.2 ml/25 g body wt in sterile water prior to use and was given once after 15 days of the onset of the experiment.

Four doses of rosemary leaves extract (25, 125, 250 and 375 mg/kg b. wt.) were administrated prior to doxorubicin administration according to the literature data [7].Every dose was given one time/three days by gastric incubation for 15 days.

Organization of experimental groups

Experimental groups were organized as 10 groups including 16 animals each. In each group ten animals were used for cytogenetic analysis while the rest of animals (six mice) were used for biochemical, histopathological and TUNEL analysis. The animals of group one (G1) were used as a negative control group treated with water. The animals of group two (G2) were served as positive control and was given DOX 24 h and 48 h before sacrifice. In groups three,

four, five and six (G3, G4, G5 and G6), 25, 125, 250 and 375 mg/kg b. wt. of rosemary extract respectively were given to the animals. Animals of groups G7, G8, G9 and G10 were pretreated with 25, 125, 250 and 375 mg/kg b. wt. of rosemary extract respectively through oral incubation one time/three days for 15 days consecutively and DOX post-treatment was given after two hours of the last dose of RE on the 15th day, as a single dose of 25 mg/kg b. wt. intraperitoneally.

Preparation of the mice bone marrow cell system

Bone marrow cell preparations for the analysis of chromosomal aberrations and mitotic index were produced by the colchicine–hypotonic technique.

After completion of the treatment period, five animals from each group were scarified at sampling time of 24 h and 48 h post-injection with H2O, DXR or RE, by cervical dislocation. Colchicine was given at the dose of 4 mg/Kg b.w. intraperitoneally at 22 and 46 h prior to sacrificing the animals. The bone marrow smears of animals in each group were prepared according to Preston et al, 1987 protocol [48]. For each group, slides were stained with Giemsa and 50 well spread metaphase plates/animal were analyzed for chromosomal aberrations and the incidence of aberrant cells (in percentages). The mitotic index was obtained by counting the number of mitotic cells in 1000 cells/animal. While the percentage of suppressed aberrant cells was calculated according to Shukla and Taneja, (2002) as follows: 100 – [% of aberrant cells in G7-G10 / % aberrant cells in G2 (positive control group)] x 100

Biochemical analysis

A part of testis (0.5g) was ice-cooled and homogenized in 5 ml 0.9% NaCl (10% w/v) using Teflon homogenizer (Glas-Col, Terr Haute, USA). The homogenate was centrifuged at 30000g for 15 minutes at 4°C. The supernatant was collected and preserved at -20°C till used for oxidative stress and antioxidant defense system measurements. On the other hand, Blood samples were obtained from cervical vein and allowed to coagulate at room temperature. Sera were separated by centrifugation at 3000 r.p.m. for 15 minutes. The clear non haemolysed sera were quickly removed and stored in deep freezer at -40°C till being used for testosterone measurements. Serum testosterone level was measured by radioimmunoassay (RIA) as described in the instructions provided with the assay kits (Diagnostic Products Corporation, USA).

In testes homogenates, lipid peroxidation (LPO) was determined by measuring the thiobarbituric acid reactive substances (TBARS) according to method of Pressus et al, 1998 [49]. Superoxide dismutase (SOD) activity was measured in the first supernatant part according to the method of Arthur and Boyne, 1985 [6]. Glutathione reduced form (GSH) level was measured colourmetrically as protein-free sulfhydryl content using Ellman reagent [12]. Catalase (CAT) activity was analyzed according to the method of Cohen et al, 1970 by monitoring the enzyme-catalyzed decomposition of hydrogen peroxide using potassium permanganate. Glutathione peroxidase (GPX) was assayed according to the method of Pinto and Bartley, 1989 [19,46].

Histopathological and TUNEL studies

Pieces of testis were fixed in 10% neutral buffered formalin for 24 hours. After dehydration, tissue samples were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin for histopathological examination or mounted on super-frost plus slides (Thermofisher Scientific, Pittsburg, PA) to detect apoptotic cells using the TUNEL technique [9,41].

Haematoxylin and eosin-stained paraffin sections of testes were, also, exposed to examination to determine the relative percentages of different germ cell types in 10 seminefrous tubules/ animal for six animals/ group.

The Tdt-mediated dUT nick-end labeling (TUNEL) assay was applied using the in situ cell death detection kit (POD; Roche Molecular Biochemicals) according to the manufacturer instructions. The apoptotic index (the percentage of dark brown to black-stained cells) was determined at 10-random locations within each seminefrous tubule. Ten seminefrous tubules for each animal were recorded for six animals/ group.

Statistical analysis

Statistical analyses for the difference in the mean number of chromosomal aberrations and mitotic index between groups were obtained by using student-t-test ($P < 0.05$ was considered significant) while the biochemical and the immunohistochemical results were analyzed using PC-STAT one-way analysis of variance [54].

Table 1: *Suppressive effect of RE pretreatment on DXR induced structural and numerical chromosomal aberrations in mouse bone marrow cells at 24h sampling time.*

Groups	Number of examined cells	Structural Chromosomal Aberrations							Numerical Chromosomal Aberrations		
		Chromatid Breakage	Centric Attenuation	Centric Fusion	End to end Association	Total of Structural Aberrations	Number of cells with one aberr.	Number of cells with more than one aberr.	Polyploidy	Endomitosis	Total of Numerical Aberrations
G1	**250**	18 (0.072)	2 (0.008)	1 (0.004)	2 (0.008)	23 (0.092)	23 (0.092)	---	---	8 (0.032)	8 (0.032)
G2	**250**	82 (0.328)	1 (0.004)	15 (0.060)	12 (0.048)	110 (0.440)	64 (0.256)	46 (0.184)	6 (0.024)	20 (0.080)	26 (0.104)
G3	**250**	22 (0.088)	7 (0.028)	---	1 (0.004)	30 (0.120)	27 (0.108)	3 (0.012)	---	11 (0.044)	11 (0.044)
G4	**250**	22 (0.088)	10 (0.040)	---	---	32 (0.128)	30 (0.120)	2 (0.008)	2 (0.008)	4 (0.016)	6 (0.024)
G5	**250**	26 (0.104)	4 (0.016)	3 (0.012)	1 (0.004)	34 (0.136)	27 (0.108)	7 (0.028)	2 (0.008)	9 (0.036)	11 (0.044)
G6	**250**	31 (0.124)	5 (0.020)	2 (0.008)	2 (0.008)	40 (0.160)	36 (0.144)	4 (0.016)	4 (0.016)	9 (0.036)	13 (0.052)
G7	**250**	60 (0.240)	---	5 (0.020)	4 (0.016)	69 (0.276)	59 (0.236)	10 (0.040)	7 (0.028)	7 (0.028)	14 (0.056)
G8	**250**	61 (0.244)	3 (0.012)	5 (0.020)	5 (0.020)	74 (0.296)	67 (0.268)	7 (0.028)	2 (0.008)	9 (0.036)	11 (0.044)
G9	**250**	52 (0.208)	1 (0.004)	12 (0.048)	4 (0.016)	69 (0.276)	61 (0.244)	8 (0.032)	6 (0.024)	20 (0.080)	26 (0.104)
G10	**250**	69 (0.276)	---	7 (0.028)	4 (0.016)	80 (0.320)	66 (0.264)	14 (0.056)	6 (0.024)	14 (0.056)	20 (0.080)

Table 2: *Suppressive effect of RE pretreatment on DXR induced structural and numerical chromosomal aberrations in mouse bone marrow cells at 48h sampling time.*

Groups	Number of examined cells	Structural Chromosomal Aberrations							Numerical Chromosomal Aberrations		
		Chromatid Breakage	Centric Attenuation	Centric Fusion	End to end Association	Total of Structural Aberrations	Number of cells with one aberr.	Number of cells with more than one aberr.	Polyploidy	Endomitosis	Total of Numerical Aberrations
G1	**250**	18 (0.072)	2 (0.008)	---	2 (0.008)	22 (0.088)	22 (0.088)	---	2 (0.008)	8 (0.032)	10 (0.040)
G2	**250**	89 (0.356)	9 (0.036)	7 (0.028)	7 (0.028)	112 (0.448)	87 (0.348)	25 (0.100)	8 (0.032)	14 (0.056)	22 (0.088)
G3	**250**	22 (0.088)	7 (0.028)	---	1 (0.004)	30 (0.120)	27 (0.108)	3 (0.012)	1 (0.004)	11 (0.044)	12 (0.048)
G4	**250**	21 (0.084)	10 (0.040)	---	---	31 (0.124)	28 (0.112)	3 (0.012)	---	6 (0.024)	6 (0.024)
G5	**250**	26 (0.104)	5 (0.020)	3 (0.012)	2 (0.008)	36 (0.144)	27 (0.108)	9 (0.036)	2 (0.008)	10 (0.040)	12 (0.048)
G6	**250**	30 (0.120)	6 (0.024)	---	4 (0.016)	40 (0.160)	30 (0.120)	10 (0.040)	5 (0.020)	9 (0.036)	14 (0.056)
G7	**250**	68 (0.272)	1 (0.004)	7 (0.028)	9 (0.036)	85 (0.340)	73 (0.292)	12 (0.048)	6 (0.024)	4 (0.016)	10 (0.040)
G8	**250**	65 (0.260)	---	3 (0.012)	8 (0.032)	76 (0.304)	64 (0.256)	12 (0.048)	2 (0.008)	10 (0.040)	12 (0.048)
G9	**250**	53 (0.212)	---	3 (0.012)	3 (0.012)	59 (0.236)	52 (0.208)	7 (0.028)	4 (0.016)	17 (0.068)	21 (0.084)
G10	**250**	65 (0.260)	1 (0.004)	7 (0.028)	1 (0.004)	74 (0.296)	65 (0.260)	9 (0.036)	5 (0.020)	9 (0.036)	14 (0.056)

Table 3: *Effects of aqueous rosemary leaves extract on mitotic index, Incidence of aberrant cells, Number of aberrations/Cell and percentage of suppressed aberrant cells in doxorubicin-injected mice.*

Groups	Mitotic Index [a]		Incidence of aberrant cells [a] (%)		Number of aberrations/Cell [a]		Suppressive effect (%)	
	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
G1	82.839 ± 5.977	83.752 ± 4.859	12.40 ± 1.496	12.80 ± 2.993	0.0250 ± 0.003	0.0260 ± 0.006	---	---
G2	47.28 ± 9.294 [b]	47.51 ± 5.641 [b]	45.60 ± 3.441 [b]	42.00 ± 6.324 [b]	0.556 ± 0.091 [b]	0.528 ± 0.118 [b]	---	---
G3	68.51 ± 4.566	66.60 ± 3.748 [b]	13.20 ± 4.489	14.30 ± 4.070	0.164 ± 0.071	0.166 ± 0.062	---	---
G4	68.48 ± 4.084 [b]	67.10 ± 3.745 [b]	14.80 ± 2.713	14.00 ± 2.412	0.152 ± 0.032	0.150 ± 0.031	---	---
G5	65.80 ± 4.014 [b]	65.35 ± 3.504 [b]	15.60 ± 4.800	15.40 ± 4.119	0.180 ± 0.065	0.191 ± 0.066	---	---
G6	64.77 ± 2.805 [b]	68.22 ± 2.658 [b]	20.20 ± 3.487	19.40 ± 2.490	0.212 ± 0.034	0.216 ± 0.041	---	---
G7	59.25 ± 4.433 [c]	63.30 ± 4.792 [c]	27.60 ± 4.271 [c]	33.20 ± 3.919 [c]	0.333 ± 0.048 [c]	0.384 ± 0.046 [c]	39.474	20.952
G8	64.14 ± 2.391 [c]	65.27 ± 4.420 [c]	30.00 ± 3.347 [c]	30.00 ± 4.000 [c]	0.340 ± 0.049 [c]	0.360 ± 0.063 [c]	34.211	28.571
G9	56.15 ± 4.483	54.87 ± 5.253	36.00 ± 3.347 [c]	31.60 ± 1.959 [c]	0.388 ± 0.041 [c]	0.340 ± 0.022 [c]	21.053	24.762
G10	53.07 ± 4.228	60.51 ± 1.079 [c]	37.20 ± 4.118 [c]	32.80 ± 3.487 [c]	0.424 ± 0.034 [c]	0.356 ± 0.034 [c]	18.421	21..905

[a] Values represent mean ± SE of five animals.
[b] Significantly different from untreated controls (G1) $P < 0.05$.
[c] Significantly different from positive controls (G3) $p < 0.05$.

Table 4: *Effects of aqueous rosemary leaves extract on testicular cell population dynamics of doxorubicin-treated animals.*

Groups	Germ cell types					Interstitial cells	
	Spermatogonia (2C)	**Primary spermatocytes (4C)**	**Secondary spermatocytes (S-phase)**	**Spermatids and sperms (1C)**	**4C/2C ratio**	**Intact leydig cells**	**Degenerating Leydig cells**
G1	14.11± 0.04^{d}	11.55±0.13^{c}	10.44±0.09^{g}	65.37±0.12^{b}	0.81± 0.01^{ab}	81.3±0.23^{ab}	15.25±0.02^{j}
G2	12.56 ± 0.12^{f}	8.48±0.11^{d}	30.45±0.12^{a}	44.18±0.05^{g}	0.68± 0.01^{e}	52±0.82^{e}	45.77±0.28^{a}
G3	12.19±0.16^{g}	9.34±0.14^{d}	10.46±0.14^{g}	63.41±o.16^{c}	0.77± 0.03^{d}	77.7±0.61^{abc}	21.27±0.13^{f}
G4	17.33±0.16^{c}	12.43±0.09^{c}	12.45±0.12^{e}	66.41±0.12^{a}	0.80± 0.07^{bc}	86.3±0.23^{a}	13.37±0.14^{j}
G5	13.43±0.11^{e}	11.39±0.13^{c}	12.27±0.06^{e}	65.39±0.13^{b}	0.78± 0.01^{cd}	81.7±0.24^{ab}	18.3±0.11^{h}
G6	13.38±0.18^{e}	10.81±0.16^{d}	11.38±0.13f	65.08±0.54^{b}	0.81± 0.01^{ab}	81±0.69^{ab}	20.2±0.14^{g}
G7	18.16±0.11^{b}	12.2±0.18^{d}	12.26±0.09^{e}	50.04±0.17^{f}	0.67± 0.02^{e}	68±0.41^{d}	31.37±0.14^{b}
G8	19.36±0.11^{a}	16.43±0.13^{a}	19.40±0.16^{b}	56.47±0.37^{d}	0.83± 0.02^{a}	78±0.41^{abc}	22.23±0.10^{e}
G9	18.41±0.11^{b}	14.42±0.14^{b}	17.38±0.12^{c}	51.87±0.13^{e}	0.81± 0.02^{ab}	74.6±0.23^{bcd}	23.57±0.2^{d}
G10	18.28±0.09^{b}	12.56±0.18^{c}	13.56±0.16^{d}	50.13±0.55^{f}	0.69± 0.01^{e}	71±0.41^{cd}	28.17±0.27^{c}
F-probability	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001
LSD at 5%	0.356	1.59	0.346	0.841	0.002	9.41	0.711
LSD at 1%	0.479	2.15	0.466	1.133	0.003	12.84	0.970

Data are expressed as mean ± standard error.
For each parameter, values share the same superscript letter are not statistically significant.

Table 5: *Effects of aqueous rosemary leaves extract on oxidative stress parameters, antioxidant defense system, testosterone and apoptotic index in doxorubicin-injected mice.*

Groups	Oxidative stress and antioxidant defense system parameters					SOD/ GPX	SOD/CAT	Testosterone (ng/mol)	Apoptotic index
	LPX	GSH	GPX	CAT	SOD				
G1	33.63±0.31^{i}	42.38±0.65^{a}	152.033±0.03^{g}	33.18±0.28^{abc}	283.7 ± 1.13^{d}	1.78±0.02^{e}	8.5±0.02^{h}	3.37±0.06^{a}	2.78±0.03^{g}
G2	53.81±0.18^{a}	32.84±0.98^{d}	112.9±2.5^{f}	23.34± 0.25^{e}	398.65±0.98^{a}	3.3±0.06^{a}	17.01±0.07^{a}	1.3±0.04^{c}	39.1±0.27^{a}
G3	37.67±0.21^{g}	34.31±0.78^{d}	128.49±0.69^{abc}	27.09± 0.28^{d}	316.98±1.09^{bcd}	2.38±0.08^{b}	11.45±0.08^{b}	2.6±0.04^{b}	2.2±0.04^{c}
G4	45.73±0.27^{c}	42.26±0.57^{a}	174.49±0.89^{a}	36.14± 0.18^{a}	345.05±2.1^{b}	1.85±0.02^{de}	9.3±0.07^{f}	3.43±0.02^{a}	3.2±0.1^{f}
G5	42.89±0.25^{d}	37.0±0.97^{b}	155.39±1.02^{b}	35.14± 0.22^{ab}	334.79±0.77^{bc}	2.3±0.05^{bc}	9.38±0.14^{ef}	3.4±0.11^{a}	2.56±0.02^{gh}
G6	39.29±0.18^{f}	36.03±0.48^{c}	134±0.55^{d}	34.21± 0.29^{acd}	326.41±1.02^{bcd}	2.35±0.05^{b}	8.9±0.04^{g}	2.63±0.15^{b}	2.45±0.02^{gh}
G7	34.17±0.24^{i}	32.11±0.45^{d}	125.43±0.9^{e}	30.74± 0.22^{bcd}	292.4±1.06^{cd}	2.2±0.09^{bc}	9.8±0.03^{c}	2.47±0.12^{b}	27.5±0.09^{b}
G8	44.17±0.11^{c}	41.61±0.22^{a}	155.12±1.02^{b}	34.45± 0.13^{ab}	323.38±0.74^{bcd}	1.9±0.05^{d}	9.1±0.06^{fg}	3.1±0.04^{a}	20.5±0.08^{e}
G9	41.28±0.27^{e}	37.67±0.64^{b}	144.34±0.68^{c}	32.17± 0.24^{abc}	313.41±0.51^{bcd}	2.17±0.01^{c}	9.6±0.05^{cd}	2.57±0.06^{b}	23. 5±0.04^{d}
G10	36.39±0.24^{h}	33.88±2.6^{cd}	132.19±0.65^{d}	31.12± 0.18^{bcd}	303.22±0.6^{bcd}	2.23±0.06^{bc}	9.5±0.08^{de}	2.5±0.06^{b}	24.63±0.1^{c}
F-probability	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001	< 0. 001
LSD at 5%	0.65	2.9	5.3	4.9	45.8	0.16	0.21	0.3	0.4
LSD at 1%	0.88	2.93 3.9	2.9 7.1	6.7	61.76	0.21	0.28	0.4	0.6

Data are expressed as mean ± standard error.
For each parameter, values share the same superscript letter are not statistically significant.

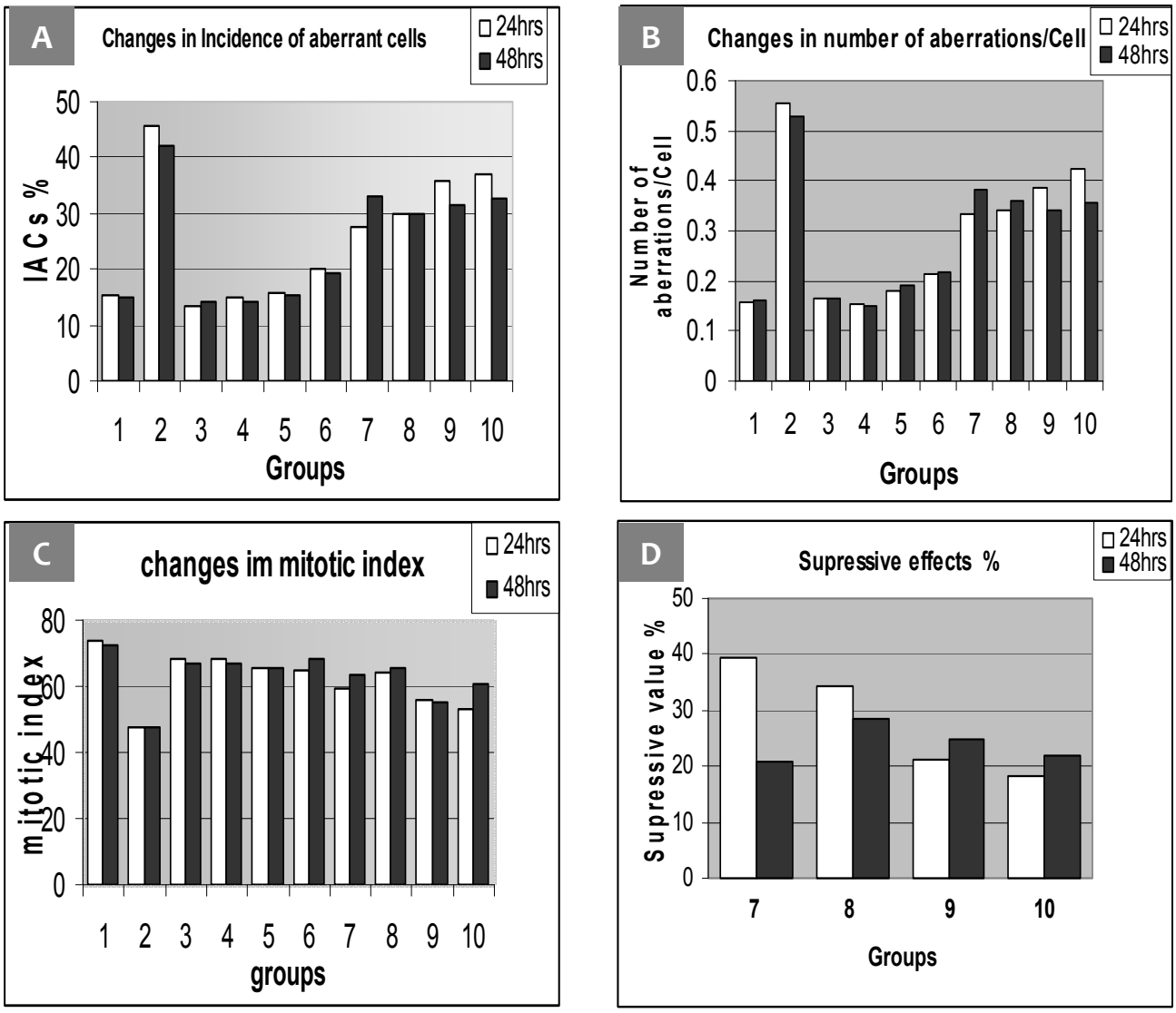


Figure 1: *Illustrates the antimutagenic activity of aqueous rosemary leaves extract (RE) in bone marrow cells of mice at sampling times 24 and 48 hours. (a) Incidence of aberrant cells, (b) Changes in number of aberrations/cell. (c) Changes in mitotic index, (d) Suppressive effect of RE on chromosomal aberrations*

Results

According to the cytogenetic results illustrated in tables (1, 2 and 3), six structural and numerical chromosomal aberrations were determined in the control and the experimental groups. The results, in first phase of cell cycle (24 h sampling time), revealed that doxorubicin (DXR) when given at a single dose of 25 mg/kg b. wt. (G2) caused a high frequency of chromosomal aberrations in bone marrow cells of mice when compared with the control (G1) group (Tables 1 & 3). The chromatid breaks were the most frequent chromosomal aberrations. The mitotic index was decreased by 47.28%, over the control value ($P < 0.05$), indicating bone marrow cytotoxicity (Table 3). When the aqueous rosemary extract (RE) treated groups (G3, G4, G5 and G6) were compared with the control group (G1) in terms of the mean total number of chromosomal aberrations and percentage of incidence of aberrant cells the G3 and G4 groups displayed no significant differences ($P > 0.05$), whereas in the G5 and G6 groups the mean total number of chromosomal aberrations and percentage of incidence of aberrant cells was non-significantly higher ($P < 0.05$) confirming its non-mutagenicity (Fig. 1a). The RE was also not found to be cytotoxic at the given four doses (25, 125, 250 and 375 mg/kg b.w.), as there were low significant decrease in mitotic index over G1 and

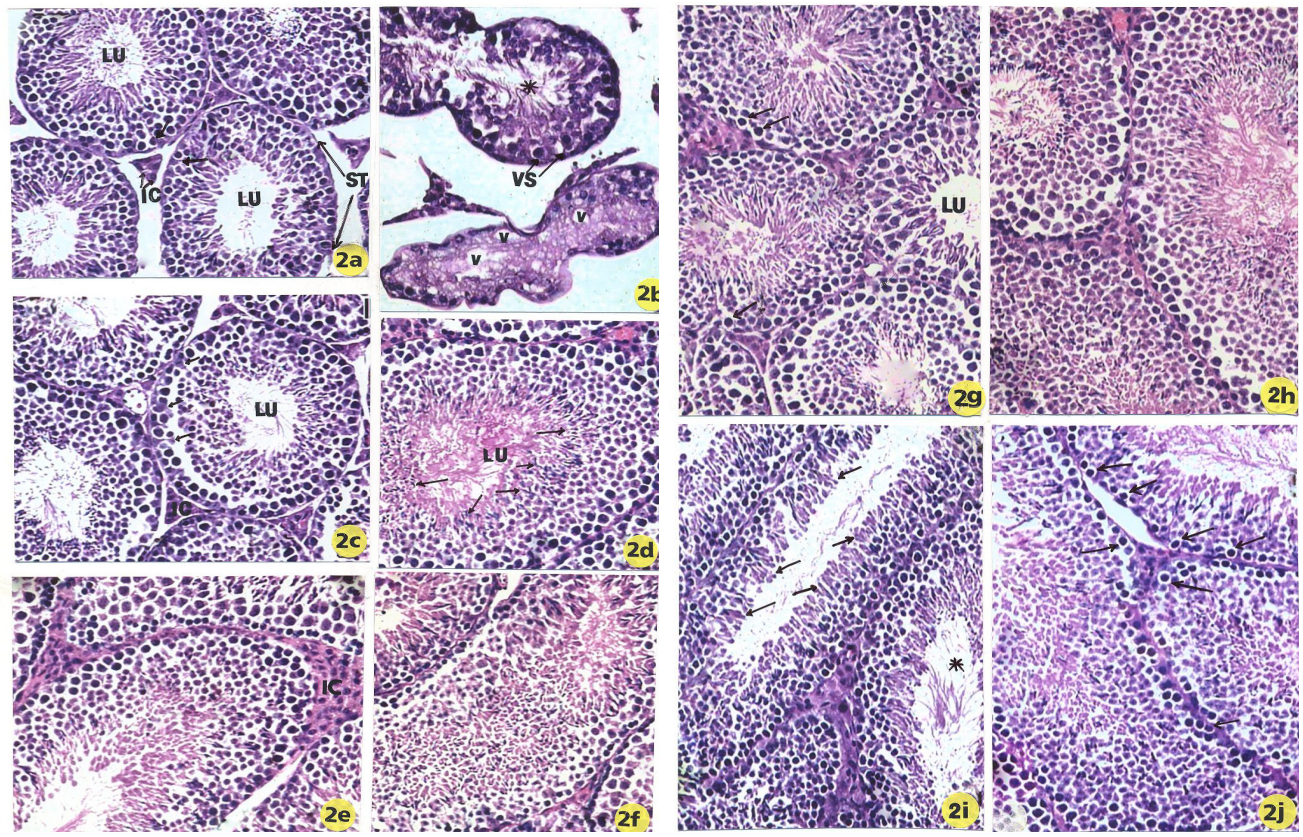
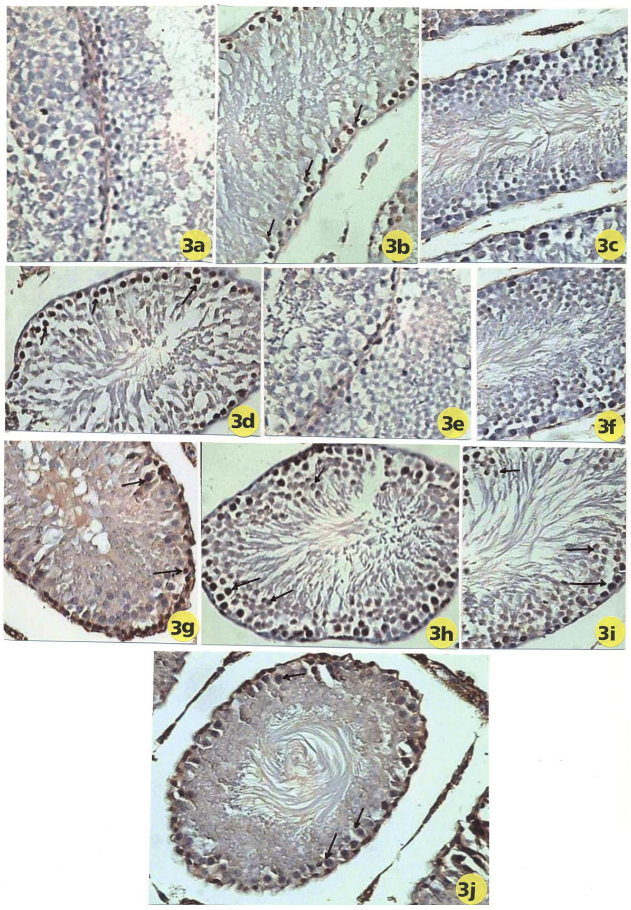


Figure 2: *Figures from 2a-j represent haematoxylin and eosin-stained testis sections. X 400*

Fig. 2a *illustrates the normal control testicular histological structure; the seminefrous tubules (ST) with their lumen (LU), the interstitial cells (IC) in the tubular spaces, the spermatogenic lineages and the Sertoli cells (↓).* ***Fig. 2b*** *showed atrophied seminefrous tubules (ST) after doxorubicin injection with severe destruction of the spermatogenic lineage and tubular vacuolations (V). Some tubules showed great reduction of sperms in the lumen (*), vacuolar degenerative changes of spermatogonia (VS) and great reduction in their size.****Fig. 2c*** *indicated normal seminefrous tubules with their lumen (LU), spermatogenic lineage and Sertoli cells (↓) recorded in low dose (25 mg/ kg b. wt) rosemary leaves aqueous extract treated group. Note the interstitial cells (IC).* ***Fig. 2d*** *illustrated normal histological structure of a testis of mice administered 125 mg/ kg b. wt. of the aqueous rosemary extract. Note: The lumen (LU) is occupied with numerous sperms (↓).* ***Fig. 2e*** *showed hyperplastic interstitial cells (IC) and normal seminefrous tubules in the testes of mice given 250 mg/kg b. wt of the rosemary aqueous extract.* ***Fig. 2f*** *illustrated reduction in spermatogenic lineage recorded in the 375 mg/kg b. wt. rosemary leaves aqueous extract-treated group.* ***Fig. 2g*** *showed vacuolar degenerative changes in spermatogonia (↓) associated with reduction in the spermatogenic lineage and sperms recorded in the doxorubicin-injected mouse administered the low dose (25 mg/ kg b. wt.) of the aqueous rosemary extract. Fig. 2h indicated normal testicular structure, observed in mice treated with aqueous rosemary leaves extract (125 mg/kg b. wt.) before doxorubicin administration.* ***Fig. 2i*** *illustrated some normal seminefrous tubules and others (*) suffering from great reduction in the spermatogonia, reported after administration of the aqueous rosemary leaves extract in a dose of 250 mg/kg b. wt. prior to doxorubicin injection. Note the clumps of sperms (↓).* ***Fig. 2j*** *showed vacuolar degenerative changes of spermatogonia (↓), recorded in doxorubicin-pretreated group with the high dose of the aqueous rosemary leaves extract in a dose of 375 mg/kg b. wt.*

significant increase in mitotic index over G2 (Table 3). In DXR groups pretreated with RE (G7, G8, G9 and G10), there was a significant decrease in rates of clastogenetic changes compared with the DXR treated group (Tables 1 & 3). All types of chromosomal aberrations induced by DXR including breaks, end to end association, centric fusion, centromeric attenuation, and other multiple damages were found to be reduced by RE. Also, doses of 25, 125, 250 and 375 mg/kg b. wt. of RE increased the status of the mitotic index by 59.25%, 64.14%, 56.15% and 53.07% respectively, indicating its anti-



Figures from 3a-j *indicated the apoptotic index variability (dark-brown nuclei) recorded in the testis sections among the control group* ***(a)****, the doxorubicin-injected group* ***(b)****, the rosemary aqueous extract-treated groups with 25, 125, 250 and 375 mg/ kg b. wt.* ***(c, d, e and f)****, respectively and the treated groups with 25, 125, 250 and 375 mg/ kg b. wt. of rosemary aqueous extract prior to doxorubicin administration* ***(g, h, i and j)****, respectively (X 400)*

cytotoxicity towards DXR (Table 3). The percentage of aberrant cells in DXR treated animals (45.60 +3.441) was reduced to 27.60 +4.271, 30.00 +3.347, 36.00 +3.347 and 37.20 +4.118 ($P < 0.05$) by 25, 125, 250 and 375 mg/kg b. wt. dose of RE respectively (Table 3). A decrease in the number of aberrations per cell was, also, observed in RE pretreated and DXR post-treated groups. The calculated suppressive effect was 39.474%, 34.211%, 21.053% and 18.421% by 25, 125, 250 and 375 mg/kg b. wt. dose of RE respectively (Fig. 1d).

During second phase of cell cycle (48h sampling time) the incidence of aberrant cells DXR treated group (G2), was found to be relatively low than DXR treated group at 24h sampling time (42%, $P< 0.05$) but significantly higher than control (G1) group (Table 3, Fig. 1a). The cytotoxic potential of DXR was still evident in G2, as there was significant decrease in mitotic index (47.51%, $P< 0.05$). In G3, G4, G5 and G6, no significant increase in aberrant cells and a decrease in mitotic index were observed when compared to G1, indicating a non-mutagenic and non-cytotoxic response of RE at doses 25, 125, 250 and 375 mg/kg b. wt. (Table 3). Different types of chromosomal damage caused by DXR were declined at all four dose levels of RE pre-treatment (Tables 2 and 3). The incidence of aberrant cells was found to be 42.00 +6.324 in G2, but declined to 33.20 + 3.919, 30.00 + 4.000, 31.60 + 1.959 and 32.80 + 3.487 ($P < 0.05$) in G7, G8, G9 and G10 respectively (Fig. 1a). Mitotic index of these groups when compared with DXR treated group (G2) was found to be increased by 63.30%, 65.27%, 54.87% and 60.51% ($P < 0.05$) in groups G7, G8, G9 and G10 respectively (Fig. 1c). The inhibition efficacy using 25, 125, 250 and 375 mg/kg b. wt. of RE pretreatment against DXR induced cytogenetic damage was 20.952%, 28.571%, 24.762% and 21.905%, respectively (Fig. 1d).

Concerning the histopathological, biochemical and the apoptotic index investigations, the histological examination of the testes of animals in DXR-treated group (G2) showed that DOX treatment disrupted the cellular architecture of the normal testicular tissues (Fig. 2a and 2b) . The somniferous tubules showed decreased cellularity, drastic reduction in tubular diameter accompanied by atrophy evidenced by the appearance of cytosolic vacuoles, and apical sloughing and shedding of the cellular material in the lumen. The epithelium was disorganized resulting in increased inter-tubular spaces and the basement membrane became irregular and folded (Fig. 2b). In addition, the number of the intact leydig cells was reduced and the cells were almost atrophied (Table 4). Moreover, a significant depletion in the number of all tubular cell populations was noticed except for the secondary spermatocytes which showed significant elevation. The transformation of spermatogonia to spermatocytes (4C/2C ratio) was also significantly reduced (Table 5). This drop was accompanied by extensive DNA degradation (Fig. 3b, Table 5) represented by a significant increased incidence of apoptotic cells (TUNEL-positive cells) compared to the negative (distilled water) control (Fig. 3a) and aqueous rosemary extract (G3, G4, G5 and G6) groups (Figs. 3c, d, e and f). Spermatogonia and

spermatocytes appeared the target of DXR-induced DNA damage.

Also, DXR induced a highly significant increase ($P< 0.001$) in the level of lipid peroxidation and the activity of superoxide dismutase while the activities of the other antioxidant enzymes such as glutathione peroxidase, catalase and glutathione reduced form were significantly declined ($P< 0.001$). The ratio of dismutase to glutathione peroxidase and/or catalase was significantly increased (Table 5).

Furthermore, the serum level of testosterone was found to be significantly reduced in doxorubicin-injected group (G2) respective to the negative control group and aqueous rosemary extract groups (Table 5).

|Pre-treatment with all doses of aqueous rosemary extract of DXR- injected mice (G7, G8, G9 and G10) ameliorated the histopathological lesions, activated leydig cells proliferation (decreased intertubular spaces) (Figs. 2g, h, i and j), increased the transformation rate of spermatogonia to spermatocytes (4C/2C ratio) and the primary spermatocytes to spermatids and sperms (Table 4).

Furthermore, these doses of aqueous rosemary extract declined the DNA damage (the germ cell apoptotic index) (Figs. 3g, h, i and j, Table 5), reversed the alterations in androgenesis (testosterone level) (Table 5) and increase the number of intact leydig cells (Table 4) as compared to the doxorubicin group.

Also, they reduced lipid peroxidation and superoxide dismutase levels and increased the activity of catalase, glutathione peroxidase and reduced glutathione relative to the doxorubicin group (Table 5). The ratio of SOD/catalase or peroxidase was nearly returned to the control value.

Discussion

Doxorubicin (DXR) is an antineoplastic drug which is cell cycle specific for the S phase of cell division [32].The majority of the mutagenic/carcinogenic compounds act by generating electrophillic intermediates by cellular enzymatic reactions causing mutagenic and cytotoxic effects. Though, several mechanisms seemed to account for the effects of the anthracycline; doxorubicin both in term of anticancer action and other organ toxicities [22].DXR cytotoxicity and genotoxicity may be mediated by free radicals derived from this drug and its capability to induce apoptosis through a wide variety of mechanisms including production of reactive oxygen species (ROS), alkylation of cellular macromolecules, DNA intercalation and cross-linking, lipid peroxidation, cell membrane damage, ceramide production and p53 induction in various tissues [14,51,8]. Also previous studies indicated that DXR effect on reproductive performance attributed its effect to the destruction of meiotic and early spermatogenic stages [37].

The present study indicated that animals treated with a single dose of DXR showed several-folds increase in the frequency of aberrant cells, decrease in mitotic index, too high ratio of dismutase to glutathione peroxidase and/ or catalase, low serum levels of testosterone, inhibition of primary spermatocytes transformation to spermatids and increased DNA damage (apoptotic index). This agrees with the previous investigations which reported the ability of DXR to react with electron rich areas of susceptible molecules such as nucleic acid and proteins [10]. Therefore, DXR was suggested to target rapidly dividing cells, disrupting cell growth, mitotic activity, differentiation, and elevate the ratio of dismutase to glutathione peroxidase and/ or catalase which lead to increased H_2O_2 concentration [3,38].

Mizutani et al, 2005 suggested that H_2O_2 formed was considered the critical apoptotic trigger of doxorubicin via causing oxidative DNA damage and Shinoda et al, 1999 previously reported meiotically dividing spermatogonia and spermatocytes as vulnerable targets of doxorubicin-induced apoptosis [39,58]. Also, Mishra and Bhiwgade, 2007 reported that higher levels of H_2O_2 can be converted, in part, by Fenton reaction to $OH^{\cdot}$ which may lead to lipid peroxidation and DNA cross-linking. These results could explain our current observations related to doxorubicin-induced oxidative stress via increasing lipid peroxidation through the impairment of SOD/ GPX and/or catalase ratio [38]. It also gave reasons for chromosomal damages and histopathological complications recorded herein.

Also, Diemer et al, 2003 have shown that H_2O_2 was a potent oxidant that could inhibit steroidogenesis in Leydig cells [21]. Low serum levels of testosterone in the present work supported this assumption of the inhibition of the testicular androgenesis in doxorubicin-injected group.

This decrease of serum testosterone concentration was supposed to be the cause of diminished sperm noticed in the current study, as testosterone is the prime regulator of the spermatogenesis. Also, the significant decrease in haploid cells (spermatids and spermatozoa), as noticed in the present study, may be due to the inhibition of primary spermatocytes transformation to spermatids, the increased DNA damage (apoptotic index) in the spermatogonia and

spermatocytes or the drop recorded in the primary spermatocytes as indicated by our current results.

Moreover, the reported decrease of primary spermatocytes, in the present study, could be attributed to the toxic effect of doxorubicin on spermatogonia. This decrease in both primary spermatocytes (4C) and spermatogonia (2C) explained the lower 4C/2C ratio registered in the current study in doxorubicin-treated mice. Depletion of spermatogonia number and decreased number of stem cells were previously reported in doxorubicin-treated animals [31].Also, Ozaki et al, 1989 reported impaired sperm quality and morphological changes as a result of toxic doxorubicin effect on spermatogenesis [44].

In addition, the noticed elevation of S-phase cells after doxorubicin-treatment, in our work, may be attributed to the suppression of S-phase cells transformation to the subsequent populations.

Numerous investigations indicated that, some of plant natural product extracts may protect from harmful oxygen species and free radicals on electrophiles intermediates of anticancer drugs, which damage DNA and other cell targets[5,59]. Some of these protection studies have shown beneficial effects of these extracts against DXR toxicity [15, 51].

In the current study, administration of aqueous rosemary extracts abated the oxidative stress, chromosomal damages, histopathological lesions and apoptosis of doxorubicin toxicities via decreasing the incidence of aberrant cells, increasing mitotic index, elevating antioxidant enzymes like GSH, CAT, GPX, lowering the SOD/ GPX or SOD/ catalase ratio, decreasing extensive DNA damage (apoptotic index), diminishing lipid peroxidation, reversing the tubular cell populations' ratio to the control distribution or repairing the androgenesis.

Previous studies investigated the antioxidant efficiency and the anticytotoxic effect of RE. Our results are in accordance with those of Nusier et al. (2007) who reported that no significant changes in testicular cell population dynamics, sperm dynamics and testosterone level of male rats treated with aqueous extract of rosemary at a dose of 250 mg/kg b. wt respective to the control group [43]. Though, adverse effects on all preceding variables were recorded at the dose of 500 mg/kg b. wt. Also, Serdaroglu and Yildiz-Turp (2004) reported that rosemary extract slow down effectively the lipid peroxidation in chickens. Moreover, LO et al, 2002 reported that carnosol, a naturally occurring phytopolyphenol found in rosemary leaves, showed a potent antioxidative activity against α, α-diphenyl-ß-picrylhydrazyl (DPPH) free radicals produced from Fenton reaction[34].

Basaga et al, 1997attributed the antioxidant activity of rosemary extract to their constituents of phenolic compounds [11]. Also, earlier studies implicated its antimutagenic, anti-inflammatory and antioxidant effects to the presence of carnosic acid, carnosol, rosemarinic acid, ursolic acid, butylated hydroxyanisole and butylated hydroxytoluene compounds [1, 52, 17]. The mechanisms for protection of RE phenolic compounds involves scavenging potentially toxic and mutagenic electrophiles and free radicals that modulate activation of extra-cellular signaling protein, tumor necrosis factor (TNF), a major mediator of apoptosis and inflammatory response and enhance high antioxidative activity pathways [18,34,27]. It has been reported that the antioxidant activity of such RE phenolic compounds was related to their hydroxyl group in addition to the presence of a second hydroxyl group in the ortho or para position which is known to increase the antioxidative activity due to additional resonance stability [24].Carnosic acid, carnosol and rosmarinic acid have o-hydroxyl group and possessed high antioxidative activity.

In conclusion, our results demonstrated that 125 mg/kg b.wt. was the most potent dose of the aqueous rosemary leaves extract in significantly decreasing the chromosomal aberrations, increasing the mitotic index, reducing the DNA damage (apoptotic index), improving the histopathological lesions, decreasing the oxidative stress marker (lipid peroxidation), increasing the antioxidant enzymes activities of catalase, glutathione peroxidase and glutathione reduced form, raising the serum testosterone level and shifting the germ cell transformation ratio towards the negative control value. In Contrary, the less effective dose of this extract on the preceding parameters was the lowest dose (25 mg/kg. b. wt). The higher two doses (250, 375 mg/kg. b. wt) showed more or less similar effects but are of higher efficacy than the lowest.

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