Suppression of doxorubicin
apoptotic, histopathologic, mutagenic and oxidative
stress effects in mice bone marrow and testis tissues by
aqueous rosemary leaves extract

Supressão de doxorubina opoptótica,
histopatológica, mutagênica e efeito de estresse
oxidativo em medula óssea e tecido testicular de ratos
por extrato aquoso de folhas de alecrim

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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 (Ho *et al.*, 1994; Lo *et al.*, 2002). Rosemary contains flavonoids, phenols, volatile oil and terpenoids (NEWALL, 1996; COLLIN AND CHARLES, 1987).

Leaves of *R. officinalis* possess a variety of bioactivities; including antioxidant (RICHEIMER *et al.*, 1996), antitumor (SINGLETERY *et al.*, 1996) and anti-inflammatory actions (ALTINIER *et al.*, 2007). 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 (HUANG *et al.*, 1994; RICHEIMER *et al.*, 1996; RAMIREZ *et al.*, 2006; ALMELA *et al.*, 2006). Among the antioxidant compounds in rosemary leaves, ~90 % of the antioxidant activity can be attributed to carnosol and carnosic acid (Lo *et*

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al., 2002). Recently, reports indicated that carnosol is active in anti-inflammation and is an active anti-metastatic against malignant melanocytes (HUANG, 2000; LO *et al.*, 2002; PENG *et al.*, 2007). Carnosol was stated to inhibit nitric oxide (NO) production in activated macrophages through modulation of the nuclear factor NF- κ (CHAN *et al.*, 1995; LO *et al.*, 2002). Also, rosmarinic acid was widely studied for its antimicrobial and complement inhibition properties (RAMPART *et al.*, 1986). 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) (MACE *et al.*, 1998). 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 (BOOSER AND HORTOBAGYI, 1994; PRAHALATHAN *et al.*, 2006; LEBRECHT *et al.*, 2007). Though, previous investigations indicated that DXR has the ability to induce mutations and chromosomal aberrations in normal and malignant cells (SINGAL *et al.*, 2000; JUNG AND RESZKA, 2001; QUILES *et al.*, 2002) in addition to a wide variety of toxic side effects on organs including testis (YAGMURCA *et al.*, 2004; PRAHALATHAN *et al.*, 2006). 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 (SJOBLOM *et al.*, 1998; KATO *et al.*, 2001; KANG *et al.*, 2002; SUOMINEN *et al.*, 2003; PRAHALATHAN *et al.*, 2006) and oligospermia (SAWADA *et al.*, 1994).

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 (ANTUNES AND TAKAHASHI, 1998; TAVARES *et al.*, 1998; ANTUNES *et al.*, 1999).

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 (QUILES *et al.*, 2002). 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 (YAGMURCA *et al.*, 2004). 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 at the Botany Department, Faculty of Science, Beni-Sueif University. 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 (JINDAL *et al.*, 2006).

ANIMALS — 60 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 (ANTUNES AND TAKAHASHI, 1998; PRAHALATHAN *et al.*, 2006). 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). 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 (ARUOMA *et al.*, 1992). 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 pretreated with 0.2 ml of water one time/three days for 15 days before injection with a single i.p. dose of DOX. In groups three, four, five and six (G3, G4, G5 and G6) were orally given 25, 125, 250 and 375 mg/kg b. wt. of rosemary extract while those of G7, G8, G9 and G10 were pretreated with 25, 125, 250 and 375 mg/kg b. wt. of rosemary extract respectively, one time/three days for 15 days before DOX treatment (ARUOMA *et al.*, 1992) which was given two hours after the last dose of RE as a single i.p. dose of 25 mg/kg b. wt.

PREPARATION OF THE MICE BONE MARROW CELLS — Bone marrow cell preparations for the analysis of chromosomal aberrations and mitotic indices were conducted by the colchicine–hypotonic technique. After completion of the treatment period, five animals from each group were scarified 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.wt. 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). For each group, slides were stained with Giemsa staining method and 50 well spread metaphase plates/animal were analyzed for chromosomal aberrations and the incidence of aberrant cells. The mitotic index was obtained by counting the number of mitotic cells in 1000 cells/animal. The percentage of suppressed aberrant cells was calculated according to SHUKLA & TANEJA, (2002) as follows: $100 - [\% \text{ of aberrant cells in G7-G10} / \% \text{ aberrant cells in G2 (positive control group)}] \times 100$.

BIOCHEMICAL ANALYSIS — A part of testis (0.5 g) 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 30000 g 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. In testes homogenates, lipid peroxidation (LPX) was determined by measuring the thiobarbituric acid reactive substances (TBARS) according to method of PRESSUS *et al.* (1998). Superoxide dismutase (SOD) activity was measured in the first supernatant part according to the method of Arthur and BOYNE (1985). Glutathione reduced form (GSH) level was measured colourmetrically as protein-free sulfhydryl

content using Ellman reagent (BEUTLER *et al.*, 1963). 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).

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).

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 (BANCROFT & GAMBLE, 2002) for histopathological examination, or mounted on super-frost plus slides (Thermofisher Scientific, Pittsburg, PA) to detect apoptotic cells using the TUNEL technique (NEGOESCU *et al.*, 1996). Haematoxylin and eosin-stained paraffin sections of testes were also examined to determine the relative percentages of different germ cell types in 10 seminiferous 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 seminiferous tubule. Ten seminiferous 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 indices 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 (RAO *et al.*, 1985).

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 phases of the 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. The chromatid breakages were the most frequent chromosomal aberration. Other structural and numerical aberrations increased significantly at $P < 0.05$ over the control group (G1), while the mitotic index decreased significantly to 47.28, compared to the control value of 82.84 ($P < 0.05$), 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 per cell and percentage of incidence of aberrant cells, the G3, G4, G5 and G6 groups displayed no significant differences ($P > 0.05$), Confirming its non- mutagenicity (Table 3). The mean total numbers of structural and numerical chromosomal aberrations increased gradually with increasing the dose of RE (Tables 1 & 2). The RE was also found to be non-cytotoxic at the given four doses (25, 125, 250 and 375 mg/kg b. wt.) as indicated by the low significant decrease in the mitotic index from that of G1 and its significant increase over G2 (Table 3).

In DXR groups pretreated with RE (G7, G8, G9 and G10), data obtained during first phase of cell cycle (24 h sampling time), revealed a significant decrease in rates of clastogenetic changes compared with the DXR treated group (Table 1 & 3). All types of chromosomal aberrations induced by DXR including chromatid breakages (breaks or clear terminal deletion), 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 to 59.25, 64.14, 56.15 and 53.07, respectively, indicating its anti-cytotoxicity towards DXR (Table 3). The percentage of aberrant cells in DXR-treated animals (45.60 %) was reduced to 27.60 %, 30.00 %, 36.00 % and 37.20 % ($P < 0.05$) 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. of RE, respectively (Fig. 1).

During the second phase of the cell cycle (48 h sampling time) the incidence of different types of chromosomal damage caused by DXR declined at all four dose levels of RE pre-treatment (Tables 2 & 3). The incidence of aberrant cells percentage was found to be 42.00 in G2 and declined to 33.20, 30.00, 31.60 and 32.80 ($P < 0.05$) in G7, G8, G9 and G10, respectively (Table 2). Mitotic indices of these groups when compared with that of DXR-treated group (G2), were found to be increased to 63.30, 65.27, 54.87 and 60.51 ($P < 0.05$) in groups G7, G8, G9 and G10, respectively (Table 3). 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. 1).

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 seminiferous 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 4). 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

Table 1. Suppressive effect of RE pretreated on DXR induced structural and numerical chromosomal aberrations in mouse bone marrow cells at 24 h 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 (3.60±1.200)	2 (0.40±0.489)	1 (0.20±0.400)	2 (0.40±0.489)	23 (4.60±1.356)	23	---	---	8 (1.60±0.489)	8 (1.60±0.489)
G2	250	82 ^a (16.40±8.006)	1 (0.20±0.400)	15 ^b (3.00±0.632)	12 ^b (2.40±1.200)	110 ^a (22.00±2.366)	64	46	6 ^b (1.20±0.748)	20 ^b (4.00±1.549)	26 ^b (5.20±1.166)
G3	250	22 (4.40±2.245)	7 ^a (1.40±1.020)	---	1 (0.20±0.400)	30 ^a (6.00±2.898)	27	3	---	11 (2.20±0.979)	11 (2.20±0.979)
G4	250	22 (4.40±1.019)	10 ^a (2.00±1.095)	---	---	32 ^a (6.40±1.019)	30	2	2 (0.40±0.800)	4 (0.80±0.400)	6 (1.20±0.979)
G5	250	28 ^a (5.20±2.315)	4 (0.80±0.748)	3 (0.60±0.489)	1 (0.20±0.400)	34 ^a (6.80±2.993)	27	7	2 (0.40±0.489)	9 (1.80±0.400)	11 (2.20±0.400)
G6	250	3 ^a (6.20±1.166)	5 (1.00±0.894)	2 (0.40±0.489)	2 (0.40±0.489)	40 ^a (8.00±1.095)	36	4	4 (0.80±0.979)	9 (1.80±0.748)	13 (2.60±1.625)
G7	250	60 ^a (12.0±2.000)	---	5 ^c (1.00±0.632)	4 ^c (0.80±0.748)	69 ^a (13.80±1.720)	59	10	7 (1.40±1.019)	7 ^c (1.40±0.800)	14 ^c (2.80±1.166)
G8	250	6 ^a (12.20±1.900)	3 (0.60±0.489)	5 ^c (1.00±0.632)	5 ^c (1.00±0.000)	74 ^a (14.80±2.039)	67	7	2 ^a (0.40±0.489)	9 ^c (1.80±1.326)	11 ^c (2.20±1.166)
G9	250	52 ^a (10.40±1.959)	1 (0.20±0.400)	12 (2.40±0.489)	4 ^c (0.80±0.979)	69 ^a (13.80±2.856)	61	8	6 (1.20±1.166)	20 (4.00±1.414)	26 (5.20±1.166)
G10	250	69 ^a (13.80±2.039)	---	7 ^c (1.40±0.800)	4 ^c (0.80±0.748)	80 ^a (16.00±1.673)	66	14	6 (1.20±0.979)	14 ^c (2.80±1.469)	20 ^c (4.00±1.265)

/values in brackets represent mean ± SD /animal.

^a Chromatid breakage includes chromatid breaks and terminal deletions.^b Significantly different from untreated controls (P < 0.05).^c Significantly different from positive controls (P < 0.05).

Fig. 1: The suppressive effect of aqueous rosemary leaves extract (RE) in bone marrow cells of mice at sampling times 24 and 48 hours.

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

Groups	Number of examined cells	Structural Chromosomal Aberrations							Numerical Chromosomal Aberrations		
		Chromatid ^d 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 (3.60±0.800)	2 (0.40±0.489)	---	2 (0.40±0.489)	22 (4.40±1.356)	22	---	2 (0.40±0.800)	8 (1.60±0.489)	10 (2.00±1.095)
G2	250	89 ^b (17.80±5.912)	9 ^b (1.80±1.469)	7 ^b (1.40±0.489)	7 ^b (1.40±0.489)	112 ^b (22.20±5.192)	87	25	8 ^b (1.60±0.800)	14 ^b (2.80±2.785)	22 ^b (4.40±2.870)
G3	250	22 (4.40±1.356)	7 ^b (1.40±1.020)	---	1 (0.20±0.400)	30 ^b (6.00±2.457)	27	3	1 (0.20±0.400)	11 (2.20±0.768)	12 (2.40±1.170)
G4	250	21 (4.20±1.391)	10 ^b (2.00±1.120)	---	---	31 ^b (6.20±1.213)	28	3	---	6 (1.20±0.748)	6 (1.20±0.748)
G5	250	28 ^b (5.20±1.166)	5 (1.00±0.894)	3 (0.60±0.489)	2 (0.40±0.800)	36 ^b (7.20±1.691)	27	9	2 (0.40±0.800)	10 (2.00±1.120)	12 (2.40±1.200)
G6	250	30 ^b (6.00±2.898)	6 ^b (1.20±0.456)	---	4 (0.80±0.979)	40 ^b (8.00±1.095)	30	10	5 (1.00±0.800)	9 (1.80±0.487)	14 (2.80±2.246)
G7	250	68 (13.60±2.939)	1 ^c (0.20±0.400)	7 (1.40±0.489)	9 (1.80±1.166)	85 ^b (17.00±2.449)	73	12	6 (1.20±0.748)	4 ^c (0.80±0.979)	10 ^c (2.00±1.095)
G8	250	65 ^b (13.60±1.496)	---	3 ^c (0.60±0.800)	8 (1.60±0.489)	76 ^c (15.80±1.600)	64	12	2 ^c (0.40±0.800)	10 ^c (2.00±2.280)	12 ^c (2.40±2.059)
G9	250	53 ^b (10.60±1.356)	---	3 ^c (0.60±0.800)	3 ^c (0.60±0.489)	59 ^c (11.80±0.979)	52	7	4 ^c (0.80±1.166)	17 (3.40±1.019)	21 (4.20±1.469)
G10	250	65 ^c (13.00±2.097)	1 ^c (0.20±0.400)	7 (1.40±1.019)	1 ^c (0.20±0.400)	74 ^c (14.60±2.416)	65	9	5 (1.00±1.265)	9 ^c (1.80±1.166)	14 ^c (2.80±1.782)

^a Values in brackets represent mean±SE /animal.^d Chromatid breakage includes chromatid breaks and clear deletions.^b Significantly different from untreated controls (TP < 0.05).^c Significantly different from positive controls (SP < 0.05).

Table 3. Effect of aqueous rosemary 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(%)		Number of aberrations/Cell		Suppressive effect (%)	
	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs	24hrs	48hr
G1	82.839_±5.977	83.752_±8.859	12.40_±1.496	12.80_±2.993	0.0250_±0.003	0.0260_±0.006	---	---
G2	47.28_±9.294 ^b	47.51_±5.64 ^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_±1.070	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_±1.119	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 (TP < 0.05).^c Significantly different from positive controls (SP < 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	11.55±0.13	10.44±0.09	65.37±0.12	0.81±0.01 ^{ab}	81.3±0.28 ^b	15.25±0.02
G2	12.56±0.12	8.48±0.11 ^f	30.45±0.12	44.18±0.05	0.68±0.01 ^e	52±0.82	45.77±0.28
G3	12.19±0.16	9.34±0.14	10.46±0.14	63.41±0.16	0.77±0.03	77.7±0.61 ^{bc}	21.27±0.13
G4	17.33±0.16	12.43±0.09	12.45±0.12	66.41±0.12	0.80±0.07 ^c	86.3±0.23	13.37±0.14
G5	13.43±0.11	11.39±0.13	12.27±0.06	65.39±0.13	0.78±0.01 ^d	81.7±0.24 ^b	18.3±0.11
G6	13.38±0.18	10.81±0.16	11.38±0.13 ^f	65.08±0.54	0.81±0.01 ^{ab}	81±0.69 ^b	20.2±0.14
G7	18.16±0.11	12.2±0.16	12.26±0.09	50.04±0.17	0.67±0.02	68±0.41 ^f	31.37±0.14
G8	19.36±0.11	16.43±0.13	19.40±0.16	56.47±0.37	0.83±0.02	78±0.41 ^{bc}	22.23±0.10
G9	18.41±0.11	14.42±0.14	17.38±0.12	51.87±0.13	0.81±0.02 ^{ab}	74.6±0.23 ^{cd}	23.57±0.2
G10	18.28±0.09	12.56±0.18	13.56±0.16	50.13±0.55	0.69±0.01 ^f	71±0.41 ^d	28.17±0.27
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 sharing the same **superscript** 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	42.38±0.65	152.03±0.08	33.18±0.28 ^{bc}	283.7 ± 1.13	1.78±0.02	8.5±0.02	3.37±0.06	2.78±0.03
G2	53.81±0.18	32.84±0.98	112.9±2.5	23.34±0.25 ^f	398.65±0.98	3.3±0.08	17.01±0.07 ^f	1.3±0.04	39.1±0.27
G3	37.67±0.21 ^f	34.31±0.78	128.49±0.69 ^{bc}	27.09±0.28	316.98±1.09 ^{cd}	2.38±0.08	11.45±0.08	2.6±0.04	2.2±0.04
G4	45.73±0.27 ^f	42.26±0.57 ^f	174.49±0.89	36.14±0.18	345.05±2.1	1.85±0.02 ^{ab}	9.3±0.07 ^f	3.43±0.02	3.2±0.1
G5	42.89±0.25	37.0±0.97	155.39±1.02	35.14±0.22 ^f	334.79±0.77 ^f	2.3±0.05 ^c	9.38±0.14 ^f	3.4±0.11	2.56±0.02 ^f
G6	39.29±0.18	36.03±0.48	134±0.55	34.21±0.28 ^{cd}	326.41±1.02 ^{cd}	2.35±0.05	8.9±0.04	2.63±0.15	2.45±0.02 ^f
G7	34.17±0.24	32.11±0.45	125.43±0.9	30.74±0.22 ^{cd}	292.4±1.08 ^f	2.2±0.09 ^c	9.8±0.03	2.47±0.12	27.5±0.09
G8	44.17±0.11	41.61±0.22	155.12±1.02	34.45±0.13 ^f	323.38±0.78 ^{cd}	1.9±0.05	9.1±0.09	3.1±0.04	20.5±0.08
G9	41.28±0.27 ^f	37.67±0.64	144.34±0.68	32.17±0.24 ^{bc}	313.41±0.51 ^{cd}	2.17±0.01 ^f	9.6±0.05 ^f	2.57±0.06	23.5±0.04
G10	36.39±0.24	33.88±2.6 ^f	132.19±0.65	31.12±0.18 ^{cd}	303.22±0.8 ^{cd}	2.23±0.06 ^f	9.5±0.06 ^f	2.5±0.06	24.63±0.1
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	3.9	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 sharing the same **superscript** are not statistically significant.
LPX: lipid peroxidation GSH: Glutathione reduced form GPX: Glutathione peroxidase CAT: Catalase SOD: Superoxide dismutase

Fig. 2 [a-f]. Photomicrographs of haematoxylin and eosin-stained testis sections. X 165 — a, illustrating the normal control testicular histological structure; the seminiferous tubules (ST) with their lumen (LU), the interstitial cells (IC) in the tubular spaces, the spermatogenic lineages and the Sertoli cells (""); b, showing atrophied seminiferous 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; c, indicating normal seminiferous 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); d, illustrating 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 (""); e, showing hyperplastic interstitial cells (IC) and normal seminiferous tubules in the testes of mice given 250 mg/kg b. wt. of the rosemary aqueous extract; f, illustrating reduction in spermatogenic lineage recorded in the 375 mg/kg b. wt. rosemary leaves aqueous extract-treated group.

Fig. 2. [g-j]. Photomicrographs of haematoxylin and eosin-stained testis sections. X 165 — g, showing 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; h, indicating normal testicular structure, observed in mice treated with aqueous rosemary leaves extract (125 mg/kg b. wt.) before doxorubicin administration; i, illustrating some normal seminiferous 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 ("!"); j, showing 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.

Fig. 3 [a-j]. Photomicrograph of TUNEL-stained testis sections indicating: a, the apoptotic index variability (dark-brown nuclei) recorded in the testis sections among the control group; b, the doxorubicin-injected group, c, d e and f, 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 g, h, i and j, 375 mg/ kg b. wt. of rosemary aqueous extract prior to doxorubicin administration, respectively. [X 165].

5), reversed the alterations in androgenesis (testosterone level) (Table 5) and increased the number of intact leydig cells (Table 4) as compared to the doxorubicin group.

These doses, also, 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

Numerous investigations indicated that, some 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 (ANTUNES *et al.*, 1999; SHUKLA & TANEJA, 2002). Some of these protection studies have shown beneficial effects of these extracts against DXR toxicity (CABRERA, 2000; QUILLES *et al.*, 2002).

Doxorubicin (DXR) is an antineoplastic drug which is cell cycle specific for the S phase of cell division (KUSYK & HSU, 1976). The majority of the mutagenic/carcinogenic compounds act by generating electrophilic intermediates by cellular enzymatic reactions causing mutagenic and cytotoxic effects. However, several mechanisms seemed to account for the effects of the anthracycline; doxorubicin both in term of anticancer action and other organ toxicities (DIEMER *et al.*, 2003; GÜLKAÇ *et al.*, 2004). 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 (BOSE *et al.*, 1995; LORENZO *et al.*, 2002; QUILLES *et al.*, 2002; ASHIKAWA *et al.*, 2004). Previous studies also, indicated the DXR effect on reproductive performance, and attributed this effect to the destruction of meiotic and early spermatogenic stages (MEISTRICH *et al.*, 1985; OZAKI *et al.*, 1989; KATO *et al.*, 2001).

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 (Barton *et al.*, 2003). 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 (ANDERSON *et al.*, 1995; SHINODA *et al.*, 1999; MIZUTANI *et al.*, 2005; MISHRA & BHIWGADE, 2007).

In the current study, administration of aqueous rosemary extracts abated the oxidative stress, chromosomal damages, histopathological lesions and apoptosis produced by 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. The current 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. But adverse effects on all preceding variables were recorded at the dose of 500 mg/kg b. wt. Also, SERDAROGLU & YILDIZ-TURP (2004) reported that rosemary extract slow down effectively the lipid peroxidation in chicken. 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- β -picrylhydrazyl (DPPH) free radicals produced from Fenton reaction.

BASAGA *et al.* (1997) attributed the antioxidant activity of rosemary extract to its constituents of phenolic compounds. Also, earlier studies implicated its antimutagenic, anti-inflammatory and antioxidant effects to the presence of carnosic acid, carnosol, rosmarinic acid, ursolic acid, butylated hydroxyanisole and butylated hydroxytoluene compounds (ALMELA *et al.*, 2006; RAMIREZ *et al.*, 2006; CHANG *et al.*, 2008). 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 (CHEN and GOEDEL, 2002; LO *et al.*, 2002; JANG *et al.*, 2007). 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 (Ho *et al.*, 2000). Carnosic

acid, carnosol and rosmarinic acid have *o*-hydroxyl group and possessed high antioxidative activity.

In conclusion, the present 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.

RESUMO

O estudo (em rato) demonstrou a eficácia da utilização do extrato aquoso das folhas de alecrim na redução da taxa de aberrações cromossômicas e na divisão mitótica resultante da utilização de doxorubicina sobre a medula óssea. Também na melhora das mudanças celulares, na diminuição da "morte programada" e da taxa de SOD/CAF' SOD/GPX nos testículos dos ratos. Igualmente, o tratamento afetou a taxa de modificação das células espermáticas, o aumento do hormônio testosterona, e a taxa das enzimas anti-oxidantes como a CAT' GPX' GSF, em comparação com os animais injetados apenas com doxorubicina. A dose de 125 mg/kg de peso corpóreo, é a mais adequada para melhorar a sobrevida.

PALAVRAS CHAVE: doxorubicina; citogenética; histopatologia; apoptose; tensão-oxidativa; alecrim; medula-óssea, testículo.

SUMMARY

In the present set of investigations, the anti-mutagenic and anti-cytotoxic effects of aqueous rosemary leaves extract (RE) besides 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. Four different doses (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. Then, 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. The animals of the positive control group (DXR alone)

showed a significant increase in the mutagenicity in bone marrow cells with histological damage in the testis accompanied with increased incidence of apoptotic cells (TUNEL-positive cells), increase in the level of lipid peroxidation (LPX) and activity of superoxide dismutase (SOD). Though, the activities of the other antioxidant enzymes such as glutathione peroxidase (GPX), catalase (CAT) and glutathione reduced form (GSH) 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 resulted in a significant reduction in these adverse changes such as the 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), and in the level of testosterone and returned the seminiferous tubular cell populations' ratio to the control picture. The protective efficacy of the RE was much more pronounced following pretreatment with 125 mg/kg b. wt.

KEY WORDS: doxorubicin; cytogenetic; histopathology; apoptosis; oxidative-stress; rosemary; bone-marrow; testis

RÉSUMÉ

Cette étude a été faite pour examiner l'effet amélioré de l'utilisation de l'extrait hydraulique des feuilles du romarin sur le l'empoisonnement cellulaire, et l'effet bondissant résultant du doxorobissyne sur le tissu de la moelle épinière et le testicule des rats (males), et le degré de dépendance de cet effet sur le dosage. L'effet de cet extrait hydraulique a été expérimenté par l'utilisation de quatre différentes doses prises par la bouche (25, 125, 250, 375 mg/kg) du poids du corps, pour une période de 15 jours, une seule fois par jour chaque trois jours avant l'injection du doxorobissyne, puis le doxorobissyne a été injecté au quinzième jour au péritoine, par une dose qui s'élève à 25 mg/kg du poids du corps, après deux heures de la dernière dose. L'étude a prouvée l'efficacité de l'utilisation de l'extrait hydraulique des feuilles du romarin sur l'amoindrissement des taux de défiguration chromosomiques et la division mitotique résultant de l'utilisation du doxorobissyne dans la moelle épinière. Ainsi que l'amélioration des changements cellulaires, la diminution de la mort programmée et le taux de SOD/ CAF 'SOD/GPX aux testicules des rats. Egalement, le traitement a augmenté le taux de la modification des cellules spermatiques, et l'augmentation de l'hormone de testostérone, et les taux des enzymes de l'anti-oxydation comme

CAT'GPX 'GSF, en les comparant avec les animaux injectés avec le doxorobissyne seulement. La dose qui s'élève à 125 mg/kg du poids du corps, était la meilleure dose pour l'amélioration survenue.

MOTS CLÉS: doxorubicine; cytogénétique; hystopathologie; apoptoses; tension-oxidative; romarin; os-de-la-moelle, testicules

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