

EVALUATION OF PRO-APOPTOTIC ACTIVITY OF *Chelidonium majus* L. AND/OR GAMMA-RADIATION AGAINST EHRlich AS CARCINOMA TRANSPLANTED IN MICE

AVALIAÇÃO DA ATIVIDADE PRÓ-APOPTÓTICA DA *Chelidonium majus* L. E/OU RADIAÇÃO GAMA CONTRA O TUMOR ASCÍTICO DE EHRlich TRANSPLANTADO EM RATOS

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ABSTRACT: This study was to evaluate, the pro-apoptotic activity of ethanolic *Chelidonium majus* L. (CM) leaf extract and/or gamma-radiation in Ehrlich ascites carcinoma (EAC) bearing mice by measuring tumor volume, apoptotic factors (caspase-3, Bcl-2 and Bax proteins, tumor necrosis factor-alpha (TNF- α), angiogenesis factors (matrix metalloproteinase (MMP-2 and MMP-9); tissue inhibitor of metalloproteinases (TIMP-1), DNA fragmentation, besides histopathological examination. After tumor inoculation, mice received CM (100 mg/body weight/day) on the 10th day and were exposed to whole body γ -radiation (6Gy) on the 17th day. The data obtained reveals that combining CM with γ -radiation exposure induce significant regression in tumor growth, up-regulate Bax, caspase-3, TIMP-1 and inhibit Bcl-2, TNF- α , MMP-2 and 9). Cytotoxicity is substantiated by increased DNA-fragmentation (comet assay and DNA content) and histological examination. It could be suggested that combining CM with gamma-radiation increased apoptosis of tumor cells which would help to increase the lifespan of mice.

KEYWORDS: Caspase-3. Bcl-2. TIMP-1. DNA fragmentation. Tumor growth.

INTRODUCTION

Cancer is one of the major causes of death, the general conventional treatment and standard care for clinical oncology remains surgery followed by radiation and/or systemic chemotherapy (ABD EL-WAHAB; FOUUDA, 2009). Apoptosis is a mode of cell death used by multicellular organisms to remove cells in diverse physiological and pathological settings (BHATTACHARYYA et al., 2003). Modulation of signal transduction pathways, gene expression and arrest in cycle progression or apoptosis suppress tumorigenesis (CHATTERJEE et al., 2013).

Radiation therapy is considered to be one of the most popular and important tools to cure cancer. The killing action of ionizing radiation (X-rays, γ -rays) is through free radicals produced from the radiolysis of water in the cells. Free radicals by interacting with critical targets like DNA and membranes bring about irreversible damage leading to cell death (HANAFI; MANSOUR, 2010). The study of radio-sensitizers has emerged as a persistent hot spot in radiation oncology (SU et al., 2014).

The herbal drugs have gained attention and popularity because of their negligible toxicity and possibly with a ray of hope that they may replace some of the available toxic antineoplastic drugs. The

Celandine (*Chelidonium majus* L., CM) is a plant of the Papaveraceae family, known for its richness in specific alkaloids such as sanguinarine, chelidonine, chelerythrine, coptisine or berberine. The plant contains also several secondary metabolites for example isoquinoline. Caffeic acid derivatives, several flavonoids and phenolic acids are present. The plant was shown to possess antitumor, anti-inflammatory, anti-arthritic and antimicrobial properties which influence diverse cellular process (NADOVA et al., 2008).

The aim of this study was to evaluate the pro-apoptotic potential of ethanolic extract of CM leaves and/or gamma radiation in Ehrlich ascites carcinoma (EAC) bearing mice. This goal was achieved by measuring tumor volume, apoptotic factors such as caspase-3, Bcl-2 proteins, Bax proteins and tumor necrosis factor-alpha (TNF- α), angiogenesis factors including matrix metalloproteinase (MMP-2 and MMP-9) and tissue inhibitor of metalloproteinases (TIMP-1), DNA fragmentation and histopathological examination.

MATERIAL AND METHODS:

Preparation of *Chelidonium majus* L. leaves extract

Chelidonium majus L (CM) leaves obtained from local market in Cairo, Egypt were left to dry at

20 °C in a dark place. After drying, the material was fragmented and two gm of dried CM leaves were extracted with 50 ml of ethyl acetate using a Soxhlet apparatus. After 24 h, the infusions were filtered through Whatman No.1 filter paper and the residue re-extracted with 50 ml of ethyl acetate. After 48 h, the process was repeated. The combined supernatants were evaporated to dryness under vacuum at 40 °C using Rotatory evaporator and stored in sterile sample tubes in a refrigerator at 4 °C (JAKOVLJEVIĆ et al., 2013).

Cytotoxicity Assay:

The MTT assay to determine the growth and survival of Ehrlich Ascites Carcinoma cells (EAC) was performed *in vitro* according to Freimoser et al. (1999). The method is based on the reduction of MTT (3-(4, 5 -dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) a water soluble salt to non-water-soluble violet formazan crystals. The amount of these crystals can be determined spectrophotometrically and serve as an estimate for the mitochondrial activity and hence the number of living cells in the sample.

Experimental animals

Adult female Swiss Albino mice (22-25g) were obtained from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Cairo) and used in this study. The animals received a commercial standard pellet diet and water *ad libitum*. Animal care and handling were performed according to the guidelines set by the world health organization, Geneva, Switzerland and approved from the committee for animals care at National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority.

Tumor transplantation:

A cell line of EAC was used in this study. The parent line was supplied from the Egyptian National Cancer Institute (NCI), Cairo University. The EAC cells originated from breast cancer which were adjusted to grow in female Swiss albino mice and maintained by intraperitoneal injection (IP) of 2.5×10^6 EAC cells in the mice. The EAC cells were counted before their IP administration to mice using the bright line haemocytometer and diluted with physiological sterile saline solution. To induce Ehrlich Solid Tumor (EST), 0.2 ml (2.5×10^6 EAC cells) was inoculated intramuscularly in the right thigh of the lower limb of female mouse (GUPTA et al., 2004). The day of EAC inoculation was referred to as day zero of experiment.

Radiation process:

Whole body γ -radiation was performing using a Canadian gamma cell-40 (^{137}CS) at the NCRRT, Cairo, Egypt. Mice were exposed to 6 Gy applied in one shot dose at a dose rate of 0.49 Gy/min, on the 17th day after EAC inoculation.

In vivo study:

70 Female Swiss albino mice were divided randomly into 5 groups. *Normal group*: 10 mice considered as normal control, *E group*: 15 mice bearing EAC, *E+R group*: 15 mice bearing EAC and exposed to γ -radiation, *E+CM group*: 15 bearing EAC mice treated with CM extract at a dose of 100 mg/kg b.wt (ZAREI et al., 2014) on the 10th day after EAC inoculation and *E+CM+R group*: 15 bearing EAC mice treated with CM extract and exposed to γ -radiation. On the 24th day, animals were fasted for 16 hrs prior to sampling. Then the animals were sacrificed and blood samples and tumor tissues collected for further biochemical and histopathological examinations.

Tumor volume monitoring

Tumor growth analysis was performed by measuring tumor volume, using vernier calliper on the 8th, 16th and 23rd day. The volume of solid tumor was calculated using the formula $[A \times B^2 \times 0.52]$, where A and B are the longest and the shortest diameter of tumor, respectively (O'REILLY et al., 1997).

Experimental parameters:

Detection of caspase-3 and TIMP-1 gene expression by quantitative real time PCR (qRT-PCR):

RNA isolation and reverse transcription: RNA was extracted from the tumor tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. Genomic DNA was removed by DNase-on-column treatment supplied with the kit. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. One μg of RNA was used in the subsequent cDNA synthesis reaction, using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70 °C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl_2 (25mM), RTase buffer (10X), dNTP

mixture (10mM), oligod(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/ μ l). This mixture was incubated at 42°C for 1 h .

Quantitative real time PCR: qRT-PCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions of 40 cycles of 15 s at 95°C and 60 s at 60°C after an initial denaturation step at 95°C for 10 min. Each 10 μ l reaction mixture contained 5 μ l SYBR Green Master Mix (Applied Biosystems), 0.3 μ l gene-specific forward and

reverse primers (10 μ M), 2.5 μ l cDNA and 1.9 μ l nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalizing to the endogenous control GAPDH (LIVAK; SCHMITTGEN, 2001).

Table 1. Primer sequences used for RT-PCR

Primer	Sequence
Caspase-3	Forward:5'-ATGGACAACAACGAAACCTC -3' Reverse:5'-TTAGTGATAAAAAGTACAGTTCTT -3'
TIMP-1	Forward primer 5'-GCATCTGGCATCCTCTTGTT-3' Reverse primer 5'-TGGGGAACCCATGAATTTAG-3'
GAPDH	Forward: 5'- CTCCATTCTTCCACCTTTG-3' Reverse: 5'- CTTGCTCTCAGTATCCTTGC-3'

Determination of Serum MMP-2 and -9

Determination of MMP-2 and MMP-9 was carried out using polyacrylamide gel electrophoresis according to Birkedal-Hansen and Taylor (1982). MMP-2 and MMP-9 protein levels are quantitatively assessed by vertical slab SDS-PAGE containing 1 mg/ml type B gelatine as a substrate for the enzymes. MMP-2 and MMP-9 detected on the SDS gel as transparent bands. The serum samples were used without boiling, MMP-2 (72 kDa) and MMP-9 (92 kDa) proteins can be defined on slab gel by their gelatinolytic enzyme activity, which appeared as bands on background and compared to standard human purified collagenase IV (Sigma Co., USA) and a protein marker (Sigma Co., USA) the gel system adapted as described by Davis (1964).

Single cell gel electrophoresis (SCGE) Comet assay

The alkaline comet assay was performed on tumor tissue. Tissues were dissected and homogenized in ice-cold buffer (0.075 M NaCl and 0.024 M Na₂ EDTA; 1 g tissue / 1 ml buffer). The comet assay was carried out under alkaline conditions, as described by Singh et al. (1988). The slides were stained with ethidium bromide (20 Ig / ml) and examined using a 250 X magnification fluorescence microscope (Zeiss, Jena, Germany) equipped with an excitation filter of 515– 560 nm and a barrier filter of 590 nm. A computer-based image analysis system (Comet Assay II; Perceptive Instruments Ltd., Suffolk, UK) was used. A total of

50 comets per sample per animal were scored, and data were pooled (250 comets per group). Tail intensity (% DNA in the comet tail) and tail length (expressed in micrometres) were evaluated. The values of tail length were used for the determination of the long-tailed nuclei count as described previously (BENKOVIC'et al., 2009).

Histopathological Estimation of Tumor

Portions of the formalin-fixed tumor were setting in paraffin, sectioned and stained with hematoxylin and eosin stain (H&E) for histopathological examination under a light microscope (BANCROFT et al., 1996).

DNA content

Total protein was detected by mercury-bromophenol blue stain (MAZIA et al., 1953) and DNA content was examined by Feulgen reaction (PEARSE, 1985).

Immunohistochemistry

Immunostaining technique of TNF- α , Bcl-2 and Bax proteins were tested by the method of Teerds and Dorrington (1995).

Statistical Analysis

The data were statistically analyzed using SPSS computer Program (v.17). The results were presented as Mean \pm SE. The differences between mean values were analysed by one way analysis of variance (ANOVA test) followed by LSD as a post

hoc test for multiple comparisons. All statements of significance were based on probability of $P \leq 0.05$.

RESULTS

Cytotoxicity Assay:

To show the effect of CM on cell viability, EAC cells were incubated with CM at different concentration (50, 75, 100 and 150 $\mu\text{g/mL}$) for 48 hours. The results revealed that, CM at a dose of 100 $\mu\text{g/mL}$ was the most efficient in decreasing the viability of EAC cells (Table 2).

Table 2. Effect of different concentrations of CM extract on the cell viability of EAC cells

Groups	EAC	CM (50 μg)+EAC	CM (75 μg)+EAC	CM (100 μg)+EAC	CM (150 μg)+EAC
Means \pm SE	1.75 \pm 0.036	0.57 \pm 0.01 ^a	0.4 \pm 0.018 ^a	0.17 \pm 0.017 ^a	0.37 \pm 0.014 ^a
% of cell viability	100%	32.57%	22.86%	9.71%	21.14%

Values were represented as mean \pm SE. a: Significant compared to EAC group ($P < 0.05$)

Tumor Volume:

The results revealed that, treatment of EAC bearing mice with CM and/or gamma-radiation

caused a marked decrease in tumor volume compared to E-group (Figure 1).

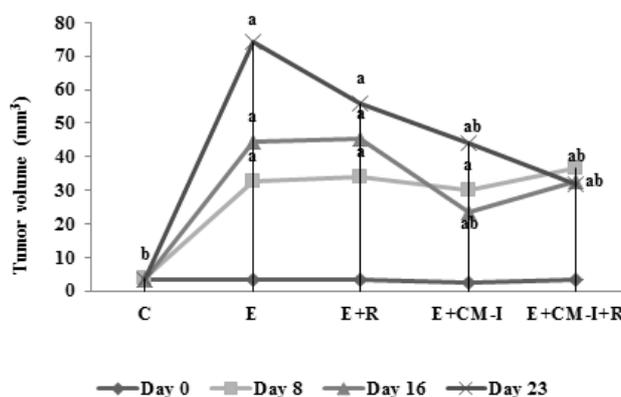


Figure 1. Effect of administration of CM (100 mg/kg b.wt) and/or exposed to γ -radiation on tumor volume in E treated mice. Values were represented as mean \pm SE (n=10). a: Significant compared to the control group ($P < 0.05$). b: Significant compared to E group ($P < 0.05$).

Detection of Caspase-3 and TIMP-1 Gene Expression:

In the present study, the results showed that in the control group the gene expression of caspase-3 and TIMP-1 was 1 \pm 0.13 fold and 5.03 \pm 0.59 fold, respectively. In E-group, the data revealed non-significant change in gene expression of caspase-3 mRNA compared with control. But in EAC mice

treated with CM and/or exposed to γ -radiation, it was significantly higher compared to E-group ($P < 0.05$) (Table 3). On the other hand, the results displayed a significant decrease ($P < 0.05$) in TIMP-1 mRNA in E-group compared with control group. Meanwhile, a significant increase in TIMP-1 mRNA was recorded in E+CM and E+CM+R groups compared to E-group ($P < 0.05$) (Table 3).

Table 3. Effect of CM and/or γ -radiation on gene expression of caspase-3 and TIMP-1

Group	Caspase-3	TIMP-1
C	0.98 \pm 0.027 ^a	5.03 \pm 0.240 ^a
E	1.22 \pm 0.069 ^a	2.43 \pm 0.164 ^b
E+ R	5.85 \pm 0.103 ^b	2.90 \pm 0.141 ^b
E+CM	4.67 \pm 0.198 ^c	8.65 \pm 0.329 ^c
E+CM+ R	11.75 \pm 0.290 ^d	10.18 \pm 0.181 ^d

Values are means \pm SE (n = 10); Values with different superscripts significantly different, at $P \leq 0.05$; Values with same superscripts not significantly different, at $P \leq 0.05$.

Detection of serum MMP-(2 and 9) by gel electrophoresis:

The experimental data for MMP-(2 and 9) in different mice groups is presented in Table 4 and Figure 2. The concentrations of MMP-2 and MMP-9 in active form were 117.12 ± 1.26 and 86.35 ± 0.431 U/mg proteins in control group, respectively. A significant increase in MMP-(2 and 9) concentration was observed in samples isolated from the serum of E-groups as compared to control group. The

treatment with CM and/or γ -radiation induced a remarkable modulation in MMP-(2and9) concentration as compared to E-group. The qualitative analysis of the zymographs showed that the majority of E-groups serum showed gelatinolytic bands for both the active enzymatic forms of MMP-2 and MMP-9 when compared with normal serum (Figure. 2).

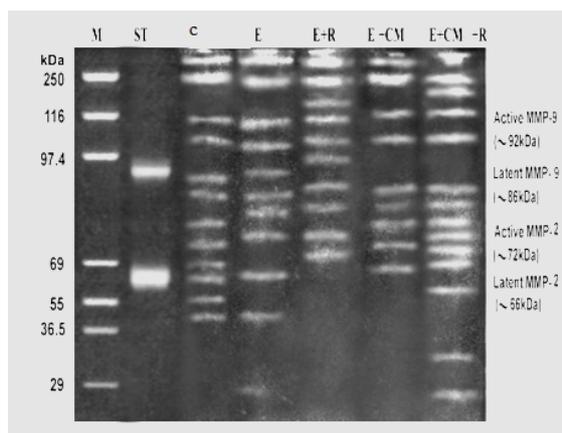


Figure 2. Zymograph of Matrix metalloproteases (Gelatinase A; MMP-2 and Gelatinase B; MMP-9) active and inactive forms (lane M) Marker proteins (<300Ka); (lane ST) Standards of purified MMP-2 and MMP-9; (C) Serum of normal control mice; (E) Serum of EAC mice; (E+R) Serum of EAC irradiated mice; and (E+CM) Serum of EAC treated with CM mice; (E+CM+R) Serum of EAC treated with CM irradiated mice.

Table 4. Effect of CM and/or γ -radiation on serum MMP-(2 and-9) (U/mg protein) in active forms analyzed by Gelatin zymography

Group	MMP2-active	MMP9-active
C	117.12 ± 1.26^a	86.35 ± 0.431^a
E	244.19 ± 1.25^b	195.68 ± 0.815^b
E+R	192.48 ± 1.38^c	167.75 ± 0.811^c
E+CM	175.30 ± 0.62^d	157.60 ± 0.611^d
E+CM+ R	127.58 ± 0.43^e	114.33 ± 0.745^e

Legend as Table 3

Comet Assay:

The results displayed the DNA strand breaks by CM or ionizing radiation, in isolated lymphocyte cells, whereas damaged nuclei consist of a head with DNA migrating into the tail region because of extensive single-strand breaks. The results showed normal nuclei in the control group

(Figure 3-C), different levels of DNA damage in E-group (Figure 3-1), and apoptotic nucleus in tumor cells in E+R, E+CM and E+CM+R groups (Figure 3-2, 3-3 and 3-4), respectively. Average background DNA damage in lymphocytes was significantly higher in the E+CM+R group than those of E-group (Table 5).

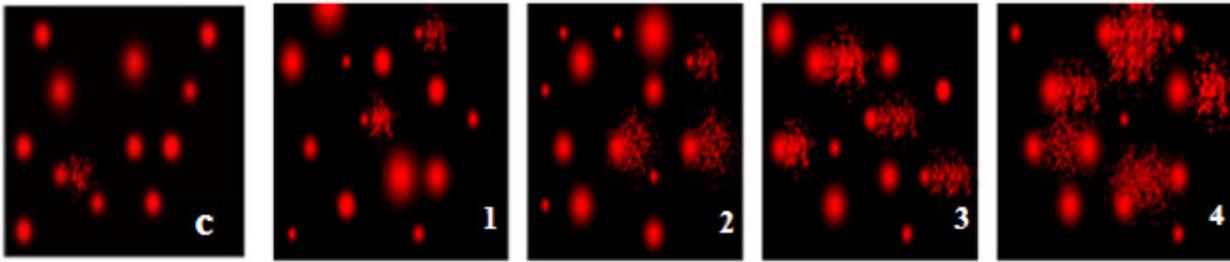


Figure 3. Appearances of comet in different types of mice tumour tissue. All photomicrographs are acquired by image analysis system Comet Assay II (Perceptive Instruments Ltd., Suffolk, UK) under magnification 250x. Stained with ethidium bromide.

Table 5. Comet assay expressed as tail length

Group	Tail length (µm)
Control	0.78±0.005 ^a
E	1.13±0.044 ^b
E+ R	3.12±0.030 ^c
E+CM	3.57±0.018 ^d
E+CM+R	4.27±0.064 ^e

Legend as Table 3;

Histological Studies:

Solid tumor in muscle tissue

Histopathological examination of the solid tumor in muscle section of E-group, showed tumor cells characterized by their large, round and polygonal with polymorphic shape, basophilic cytoplasm, hyper-chromatic nuclei and mitosis (Figure 4A) and many islets of tumor cells surrounding muscular fibres (Figure 4B). It was observed also that, islets of tumor cells were enclosed by necrotic rupture muscle fibres replacing

the normal fibres (Figure 4C) and such islets were invaginated by newly formed blood capillaries “angiogenesis” surrounded by a thick zone of tumor cells (Figure 4D). Nevertheless, solid tumor of E+R and E+CM groups showed small regression in volume of islets of tumor cells that were surrounded by muscular tissue capsule (Figure 5A and 5B, respectively). While, the solid tumor of E+CM+R group, showed more regression in tumor volume than the previous groups (Figure 5C).

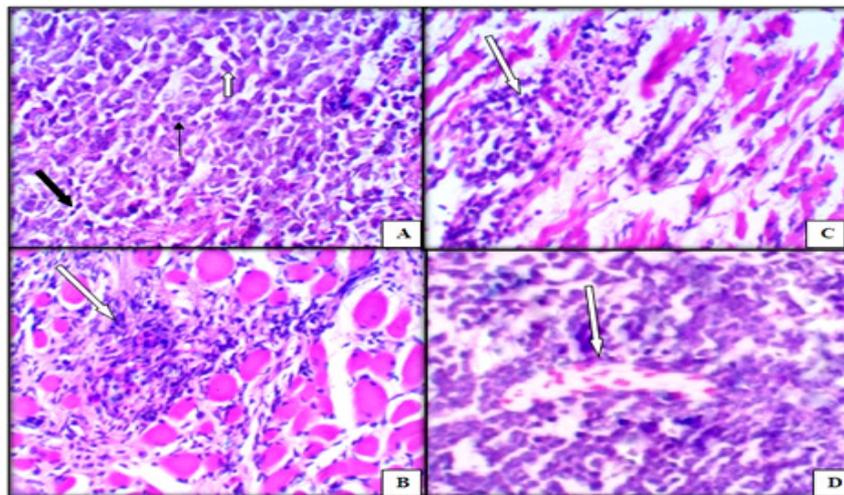


Figure 4. Photomicrograph of solid tumour section showing: (A) the tumour cells characterized by their large, round (arrow) and polygonal structure (white arrow) with polymorphic shape, basophilic cytoplasm, hyperchromatic nuclei and mitotic activity (black arrow) (H&E× 400). (B): Numerous islets of tumour cells (white arrow) surrounded muscular fibers (H&E× 200). (C): islets of tumour cells(white arrow)were enclosed by necrotic ruptured muscle fibers replacing the normal fibres. (D): islets of tumour cells were invaginated by newly formed blood capillaries “angiogenesis” (white arrow) surrounded with a thick zone of tumour cells (H&E× 400).

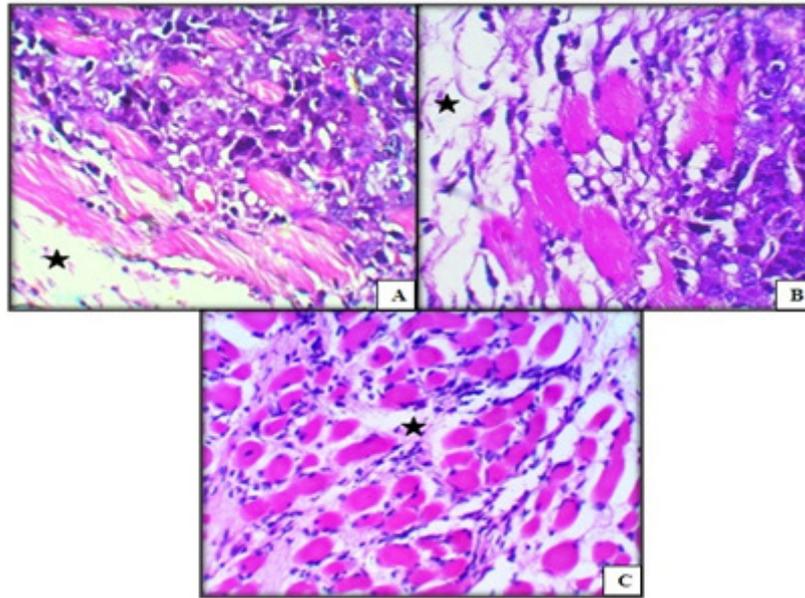


Figure 5. Photomicrograph of solid tumour section treated with γ -radiation (A) or CM (B) showed small regression in volume of islets of tumour cells (black star) that were surrounded by muscular tissue capsule (H&E \times 400). (C): group treated with γ -radiation and CM showed more regression in tumour volume (black star) (H&E \times 400).

DNA content in solid tumor:

Solid tumor sections of E-group showed DNA chromatin forms well-defined irregular clumps with variable sizes, shapes and sharp pointed projections as well as tumor nuclei with multiple

nucleoli and irregular nuclear membrane (Figure 6A). On the other hand, E+CM+R group revealed a few neoplastic cells and some apoptotic cells identified by DNA fragmentation (Figure 6B).

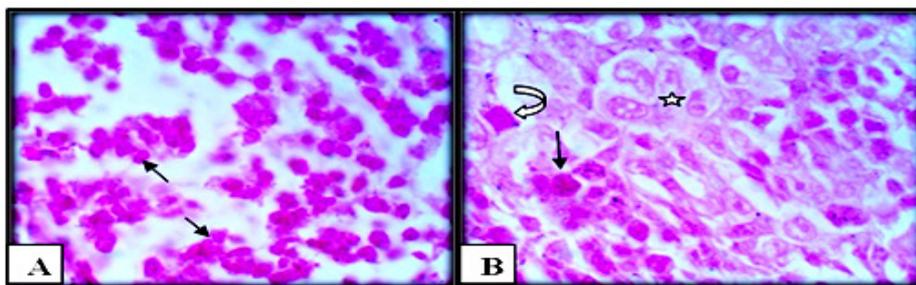


Figure 6: Photomicrograph for the determination of DNA content in solid tumour tissue (A) showed DNA chromatin forms well-defined irregular clumps with variable sizes, shapes and sharp pointed projections (arrow) and tumour nuclei with multiple nucleoli and irregular nuclear membrane (arrow) in (E) group. (B) Showed some neoplastic cells (round arrow), some apoptotic cells illustrated by DNA fragmentation and some dead cells (white star) in E+CM+R.

Immunohistochemistry

TNF- α

Immunostaining of TNF- α was identified by the brown coloration in the cytoplasm of tumor tissue. Its overexpression elevation is clearly indicated in E-group (Figure 7A and 7B). Whereas, in E+R (Figure 7C and 7D) and E+CM (Figure 7E

and 7F) groups, TNF- α expression was slightly reduced in the cytoplasm of solid tumor cells compared to the previous group. While, in E+CM+R group (Figure 7G and 7H) TNF- α expression was highly reduced compared to the previous groups.

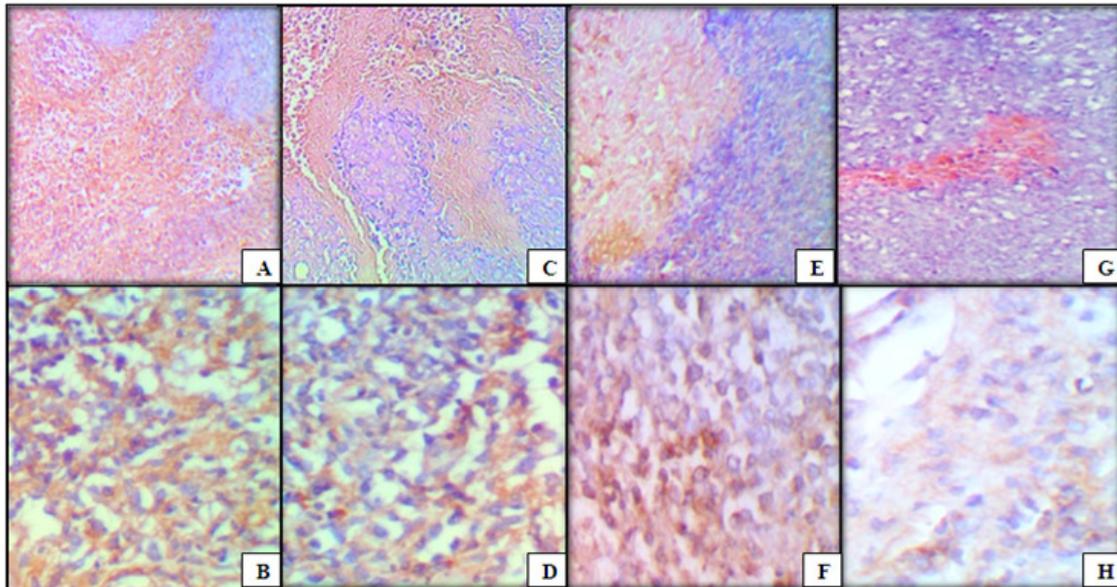


Figure 7. Immunohistochemical staining with TNF- α : (A and B) in E-group, (C and D) in E+R group, (E and F) in E+CM group and (G and H) in E+CM+R group. (A), (C), (E), (G) (magnification $\times 100$). (B), (D), (F), (H) (magnification $\times 1000$).

Bax

Accumulation of Bax protein was identified by a brown coloration in the cytoplasm of tumor tissue. Down expression of Bax protein in the E-group are detected (Figure 8A and 8B). While, the E+R (Figure 8C and 8D) and E+CM (Figure 8E and

8F) groups revealed slightly elevation in Bax protein compared with the previous group. Whereas, the E+CM+R group (Figure 8G and 8H) showed overexpression of Bax protein in the cytoplasm of tumor tissue compared with the previous groups.

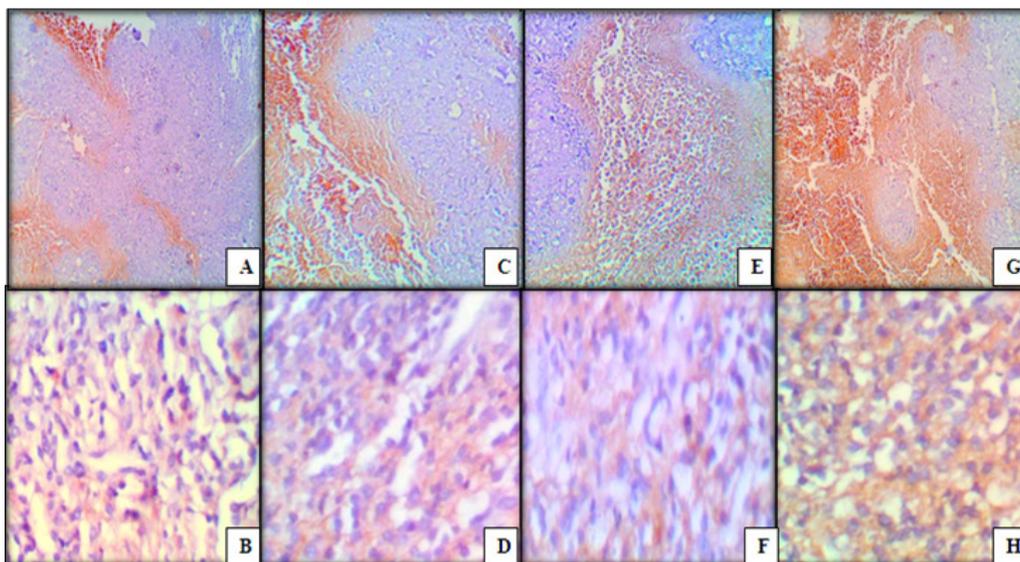


Figure 8. Immunohistochemical staining with Bax: (A and B) in E-group, (C and D) in E+R group, (E and F) in E+CM group and (G and H) in E+CM+R group. (A), (C), (E), (G) (magnification $\times 100$). (B), (D), (F), (H) (magnification $\times 1000$).

Bcl2

The accumulation of Bcl-2 protein is indicated by a brown coloration in the cytoplasm of tumor tissue. Strong immune reactivity for Bcl-2 was detected in the E-group (Figure 9A and 9B). While, the E+R (Figure 9C and 9D) and E+CM

groups (Figure 9E and 9F) revealed weak expression in Bcl-2 protein compared with the previous group. On the other hand, strong down expression of Bcl-2 protein was observed in the E+CM+R group (Figure 9G and 9H) compared with the previous groups.

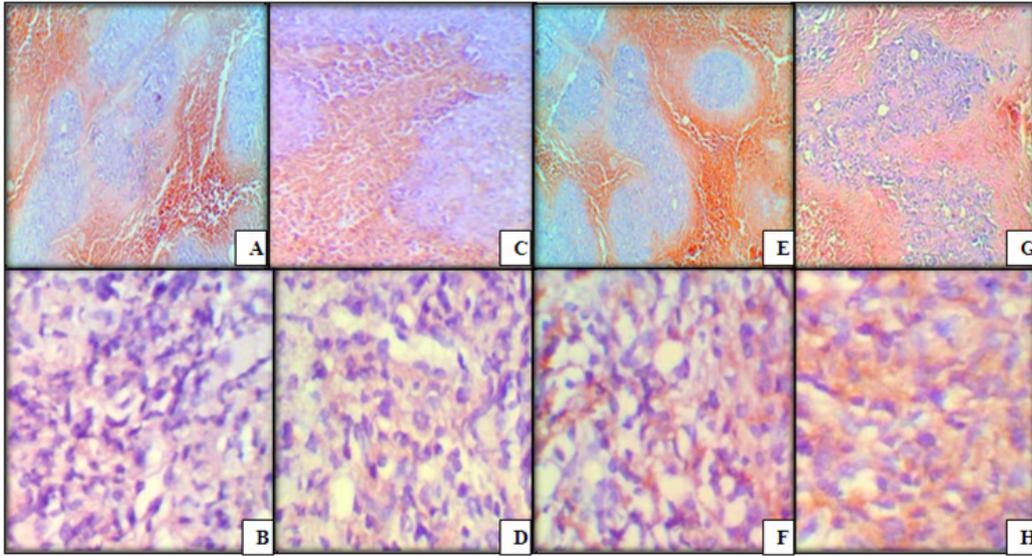


Figure 9. Immunohistochemical staining with Bcl-2: (A and B) in E-group, (C and D) in E+R group, (E and F) in E+CM group and (G and H) in E+CM+R group. (A), (C), (E), (G) (magnification $\times 100$). (B), (D), (F), (H) (magnification $\times 1000$).

DISCUSSION

In the present study, the results of E-group revealed increased angiogenesis and depressed apoptosis. The resistance of cancer cells to TNF- α induced death based on the fact that TNF- α induced survival signals such as nuclear factor- κ B (NF- κ B) that blunt the apoptotic pathway and activated the survival pathway (WAJANT et al., 2003). In a concurrent study, Abdel-Rahman and Kabel (2012) noted that, immune-staining of TNF- α and Bcl-2 showed over expression in the group of mice bearing tumour while down expression of Bax was observed in the same group. Meanwhile, Sun et al. (2010) reported that cancer cells down-regulate TIMP and produce extracellular MMP-inducer, a cell surface glycoprotein, which directly stimulates fibroblasts to produce MMP-1, 2, 3, 9 and 14. Changes occurring in the biochemical parameters were confirmed by the histopathological observation. The histopathological examination showed islets of tumor cells were invaginated by newly formed blood capillaries "angiogenesis". Active angiogenesis are identified by the presence of irregular blood capillaries to supply the tumours (GROSSMAN; MCNEIL, 2012). This leads to activation of NF- κ B in cancer cells, which regulate transcription of genes involved in cell proliferation, anti-apoptosis and invasion. Activation of NF- κ B induces tumour growth and reduces cytokines-induced apoptosis (AGGARWAL et al., 2006) which is supported by the previous findings of Salah (2011) and Hanafi (2012). The disappearance of dna

fragments in e-group, postulates loss of apoptosis ended by tumour development. Ghobrial et al. (2005) reported that types of cancers characterized by defects in apoptosis leads to immortal clones of cells.

CM and/or γ -radiation treatment, in the present study, revealed regression in tumour volume. It is well known that exposure of mammalian cells to ionizing radiation induces several types of damage to DNA, including double and single-strand breaks, base and sugar damage, as well as DNA-DNA and DNA-protein cross-links. During radiotherapy, ionizing radiation interacts with biological system and induces excessive oxygen free radicals or reactive oxygen species (ROS) which attack cellular DNA, proteins and membrane lipids. This leads to significant cellular damage by increasing apoptotic cells % and decreasing the mitochondrial membrane potential (BEGUM et al., 2012). Further, Kitada et al. (1996) showed that exposure of mice to γ -radiation results in a rapid elevation in the levels of the Bax protein in lymphoid cells. However, ionizing radiation induces mitotic cell death in dividing cells and activates pathways that lead to death by apoptosis in interphase cells and differentiated cells. In addition, Lawrence et al. (2008) reported that, γ -radiation therapy shrink tumour and kill cancer cells through damaging their DNA directly or creates free radicals within the cells to damage the DNA. Susskind et al. (2003) found that, radiation provokes a sharp decrease in MMP-9 within 2 weeks, without change in TIMP-1, and that decrease reflects reduced

neutrophil and macrophage involvement in the neoplastic process.

The modulation of tumor by CM treatment could be referred to its anti-inflammatory, antimicrobial, antiviral and antitumor activities. In addition, CM showed an anti-angiogenesis effect that was dependent on decreased activity of migration, inhibition of VEGF and MMP-2,-9 and increase of TIMP release (KIM et al., 2011). Beside and Park et al. (2015) found that, CM extract induce cell cycle arrest, activation of the caspase-3 dependent pathway, blocking of NF- κ B activation as well as significantly decrease the mRNA levels of Bcl-2, survivin and increase Bax expression. Moreover, sanguinarine isolated from CM,

suppresses NF- κ B and sensitizes cancer cells to apoptosis by inducing ROS generation and activate TNF-related apoptosis-inducing ligand (Gupta et al., 2010). In addition, Choi et al. (2009) found that, sanguinarine down regulates MMP-2 and MMP-9.

It is concluded that, CM as well as γ -radiation decrease tumor growth by initiating apoptosis, distribution of nuclear DNA fragmentation and histopathological investigations besides up-regulating Bax while down-regulating Bcl-2, by activation of caspase-3 and inhibition of TNF- α , by releasing TIMP-1 and preventing MMP-2,-9. The combination of both CM and γ -radiation has a better anti-tumor effect against tumour growth than CM or γ -radiation alone.

RESUMO: Este estudo foi realizado para avaliar a atividade pró-apoptótica do extrato etanólico da folha da *Chelidonium majus* L. (CM) e/ou radiação gama no tumor ascítico de Ehrlich tendo ratos para a medição do volume do tumor, fatores apoptóticos (caspase-3, proteínas Bcl-2 e Bax, fator de necrose tumoral (TNF- α)), fatores angiogênicos (metaloproteinase matriz (MMP-2 e MMP-9)); inibidor tecidual de metaloproteinase (TIMP-1), fragmentação de DNA, além de exame histopatológico. Depois da inoculação do tumor, os ratos receberam CM 100 mg/peso corporal/dia no 10º dia e tiveram o corpo completo exposto a radiação gama (6Gy) no 17º dia. Os dados obtidos revelam que combinar CM com exposição à radiação gama induz a uma regressão significativa no crescimento do tumor, regula positivamente Bax, caspase-3, TIMP-1 e inibe Bcl-2, TNF- α , MMP-(2 e 9). Citotoxicidade é substanciada pelo aumento da fragmentação do DNA (ensaio cometa e conteúdo de DNA) e exame histopatológico. Poderia ser sugerido que combinar CM com radiação gama aumentou a apoptose das células tumorais o que ajudaria a aumentar a expectativa de vida dos ratos.

PALAVRAS-CHAVE: Caspase-3. Bcl-2. TIMP-1. Fragmentação do DNA. Crescimento tumoral.

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