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Evaluation of Radiosensitizing and Anti-Angiogenesis Activity of *Chelidonium majus* Extract in Ehrlich Ascites Carcinoma Transplanted Mice

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ABSTRACT

The use of a radiosensitizing and anti-angiogenic agents might augment the efficiency of cancer radiotherapy. Therefore, the anti-angiogenic and radiosensitizing potency of *Chelidonium majus* (*C. majus*; CM) ethanolic extract was examined in the current study. Ehrlich ascites Carcinoma (EAC) cells were inoculated into mice to induce an experimental model of solid tumors. Mice with well developed solid tumor were treated with *C. majus* (100 mg/kg b.wt, i.p) and exposed to a whole body γ- irradiation (6 Gy). The pro-angiogenic mediators (platelet activating factor; PAF and vascular endothelial growth factor; VEGF) matrix Metalloproteinases-2 and 9 (MMP-2 and 9) activity were evaluated in serum of different mice groups. The expression of epidermal growth factor receptor (EGFR) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) were determined using RT-PCR. The results obtained showed that the observed increase in serum PAF, VEGF level and MMP-2&9 activities are associated with significant elevation in EGFR and suppression TIMP-1 expression in EAC and/ or R treated mice. Combination of CM extract with R in EAC bearing mice exhibited a significant reduction in PAF, VEGF and MMP-2 and 9 serum level with up-regulated TIMP-1 and down-regulated EGFR expression, which is indicated by significant tumor volume regression. It could be concluded that CM extract possessed an anti-angiogenic and radiosensitizing potential against EAC in mice.

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PAF

INTRODUCTION

Tumor angiogenesis is the proliferation of blood vessels penetrating the cancerous growth for the supply of nutrients and oxygen. It starts with the release of molecules by tumor cells that send signals to the surrounding normal host tissue, activates certain genes to make protein that encourage growth of new blood vessels. The molecular basis of this mechanism may be increased production of angiogenic factors or loss of angiogenesis inhibitors. Thus, the switch to an angiogenic phenotype is regulated by a change in the equilibrium between positive and negative regulators of angiogenesis (Yadav *et al*., 2015). They include the vascular endothelial growth factor (VEGFs), epidermal growth factor (EGF) and matrix Metalloproteinases (MMPs) which act as effectors of angiogenesis (Mousa *et al*., 2015).

The VEGF is a family of growth factors and their receptors form an important pathway in signaling tumor angiogenesis. VEGF also plays an important role in tumor metastasis by inducing the construction of abnormal blood vessels. Regarding the vital role of VEGF in promoting different cancers, its signaling pathway has been considered to be an attractive target for cancer therapy (Ranjbar *et al*., 2015). The epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors is another target which has been considered for cancer immunotherapy. Since EGFR is involved in different parts of cancer growth such as tumor initiation, angiogenesis and metastasis, it represents an attractive target for therapeutic interventions. This process is mediated through a group of ligands and receptors that work in tight regulation (Ranjbar *et al.*, 2015).

Platelet-activating factor (PAF), which is produced by a variety of inflammatory cells, is a potent lipid mediator involved in cellular activation, fertilization, intracellular signaling, apoptosis, and a myriad of inflammatory reactions. PAF also augments angiogenesis by promoting various angiogenic factors via activation of NF-κB. As NF-κB activity has been associated with the regulation of protein levels of MMP-9, but not MMP-2, NF-κB-dependent MMP-9 may play a role in PAF-induced angiogenesis (Ko *et al*., 2005).

Natural products have been increasingly used worldwide to treat various diseases, including cancer. *Chelidonium majus* (CM) commonly known as swallow-wort, rock poppy or greater celandine belongs to Family-Papaveraceae and is widely distributed in Europe and Western Asia (Nawrot *et al*., 2008). The plant contains, as major constituents, isoquinoline alkaloids (such as sanguinarine, chelidonine,

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chelerythrine, berberine, protopine and coptisine), flavonoids, and phenolic acids. Both crude extracts of *C. majus* and purified compounds derived from it exhibit a wide variety of biological activities (anti-inflammatory, antimicrobial, immunomodulatory, antitumoral, choleretic, hepatoprotective, analgesic, etc.) which are in concordance with the traditional uses of *C. majus* (Gilca *et al*., 2010). In fact, owing to its lower costs and greater compatibility, herbal medicine has received a great attention in recent decades (Dkhil *et al*., 2015). Radiosensitizers are agents that enhance the sensitivity of cancer cells towards radiotherapy. The enchantment of radio-responsiveness of tumors by using radiosenstizers is suggested to be a promising strategy to improve radiotherapy efficiency (Hematulin *et al*., 2014). A precise control of the mode of action of the radiation is important in order to achieve the maximum effect on tumor tissue, while minimizing the effect on normal tissue (Jagetia and Enkatesha, 2005). Interestingly, the radiosensitizing potential of numerous botanicals and their derivatives is reported in several studies for cancer cells (Hematulin *et al*., 2014). Therefore, the current investigation aimed to evaluate the radiosensitizing and anti-angiogenic potential of *C. majus* extract in EAC bearing mice and to unveil its effect on pro-angiogenic mediators and tumor vasculature remodeling during angiogenesis.

MATERIALS AND METHODS

Chemicals

All chemicals utilized in the present investigation were of analytical grade and purchased from Sigma Chemical Company, St. Louis, U.S.A. 0.9% NaCl (pyrogen free normal saline) was obtained from Otsuka Pharmaceutical Co, Japan.

Preparation of the C. majus *ethanolic extract*

The air-dried powdered aerial parts of *C. majus* were prepared from the whole plant obtained from Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. The plant was taxonomically authenticated by Abdel-Halim Mohamed, Flora and Phyto-taxonomy Researches Department, Horticulture Research Institute, Egypt. The air-dried aerial parts of *C. majus* (500gm) were crushed to coarse powder and macerated in 1500 ml 70% ethanol for 72 hours in a glass container, then filtered. The filtrate was then exhaustively concentrated by using rotary vacuum evaporator and dried to yield a dark brown viscous mass (62.8 gm; 12.56 %). The crude ethanolic extract was kept in airtight containers under deep freeze (4°C) until the time of further use. The concentrated crude extract of *C. majus* was dissolved in 2% Tween-80 solution in 0.9% saline to prepare 100 mg/kg b.wt. prior to administration.

Animals

Female outbreed Swiss albino mice originally obtained from National Cancer Institute (NCI) (20-25g) were used as experimental animals throughout the experiment period. The animals were housed under standard laboratory condition in polypropylene cages in a well-ventilated room maintained at 25±2°C and fed a balanced diet with free access to water *ad libitum*. Animal experimentations were consistent with the guidelines of ethics by Public Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) in accordance with the recommendations for the proper care and use of laboratory animals approved by Animal Care Committee of the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Ehrlich ascites carcinoma (EAC) cell line inoculation

EAC (*Mus muscles*), which is a fibroblast –like in shape (Sato *et al*., 1961), were obtained from National Cancer Institute (NCI) , Cairo university, Giza, Egypt. EAC cells is originated from breast cancer which was modified to grow in female Swiss albino mice and maintained by intraperitoneal inoculation of carcinoma cells in the mice (Gupta *et al*., 2004). Viable EAC cells ($2x10^6$/ mice) were inoculated into the right thigh in each mouse.

Irradiation procedures

Whole body γ-irradiation was performed using a Canadian Cs^{137} Gamma Cell-40 at the NCRRT. Cairo, Egypt. Mice were exposed to a single dose of 6 Gy according to experimental design at a dose rate of 0.61 Gy/min.

Selection of the optimum radiosensitizing C. majus *ethanolic extract dose*

A separate experimental set (pilot study) was performed to determine the optimum radiosensitizing dose of *C. majus* (CM) ethanolic extract in parallel with the optimum irradiation (R) dose. The drug dose was selected according to standard protocol recommended by the Drug Evaluation Branch, Drug Research and Development, NIH, U.S.A (Geran *et al*., 1972). Ten days after tumor inoculation, mice were divided into different groups including mice treated with different doses of CM ethanolic extract (50, 100 and 150 mg/kg b.wt.) combined with different irradiation doses (R) (4, 5 and 6 Gy) and cross matched as each dose of CM extract tested against each dose of radiation. The results obtained

revealed that the optimum dose of CM extract was 100 mg/kg b.wt. against the most efficient dose of R (6 Gy) as indicated in terms of tumor volume regression and these doses were selected as the original experiment tested doses.

Experimental design

Mice were divided into 8 equal groups (15 mice/group) as follows: (1) control group (C): animals that received intrapretoneal 0.2 ml of 2% Tween-80 solution in saline along with experimental time course, (2) Ehrlich EAC: animals were inoculated intramuscularly with 0.2 ml in the right thigh once, (3) Irradiated group (R): animals were exposed to 6 Gy gamma radiation as a single shot at 17^{th} day post EAC inoculation, (4): *Chelidonium majus* ethanolic extract treated group (CM): animals that received 100mg/kg b.wt. of *C. majus* ethanolic extract in 2% Tween 80- saline solution intraperitoneally for 15 days post tumor initiation, (5) EAC + R (ER): animals were irradiated after 7 days from tumor initiation, (6)R+ CM (RCM): animals irradiated with total body 6Gy gamma radiation after 7 days from CM extract administration, (7) EAC+ CM (ECM): animals that received CM extract for 15 days from tumor induction, (8) Combination group (ERCM): animals injected with EAC cells, then after 10 days (induction period) they received CM extract for 7 days, then irradiated (6 Gy) at 8^{th} day, and re-administered CM extract for another 7 days.

Tumor size determination

Animals in each trial were checked daily and checked for any adverse clinical symptoms and deaths were recorded. After 7 days post inoculation with EAC, tumor size was measured weekly using calipers and determined as described by Papadopoulos *et al*. (1989) according to the following equation:

$4/3\pi\ (A/2)^2x\ (B/2) = 0.52A^2B,$

where A is minor tumor axis and B is major tumor axis.

In vitro *cytotoxicity assay (MTT assay)*

Cytotoxicity profile of *C. majus* ethanolic extract (CM) was performed using MTT reagent (3- (4,5-dimetylthiazol-2-yl) - 2, 5- diphenyltetrazolium bromide) (Sigma, Germany) according to Burton (2005) with modification. Viable EAC ($2.5x\ 10^{-6}$) cells were inoculated into a 24-well flat bottom plates as control. CM extract was added at several concentrations 50, 75,100 and 150 µg/ml. After 24h, the supernatants were removed and cell layers were washed with phosphate buffer saline (PBS, Invitrogen Gibco) and incubated with 300 µl MTT/well solution (0.5 mg MTT/ml) in 5% Co_2 incubator for 4 h, then cells were pelleted by centrifugation (15,000Xg) for 5 min. The media were removed and 500 µL of iso-propanol /HCl mixture was added (2 ml of 0.1 N HCl in 23ml iso-propanol). Subsequently, the samples were vortexed vigorously and the O.D was measured at 560 nm. The absorbance of untreated cells was considered as 100%. Each extract and control was assayed in triplicate in three independent experiments. Percent growth (%) of viable cells exposed to treatments was calculated as follows:

% viable = sample abs /control abs x 100.

Blood and tissue samples collection

After 24 h of the last dose of CM extract treatment and 16 h fasting, animals of each group were sacrificed, blood was collected *via* cardiac acupuncture and serum obtained by centrifugation at 3000 rpm for 10 min for biochemical analysis. Tumor tissues were dissected immediately and a portion of it homogenized in lysis buffer [(40 mM HEPES, 50 mM KCl, 1% Triton X-100, 1 mMNa3VO4, 50 mM NaF, 5 mM EDTA,1 mM benzamidine and 1% Triton-X (Sigma)] using a potter-Elvehjem homogenizer for MMP-2 and 9 activity assessment. The remaining part of the tumor tissues were submerged in liquid N_2 for further RT-PCR processing.

Biochemical assays

Measurement of VEGF

Serum VEGF was measured by using a commercial mouse ELISA kit (quantikine R&D system, USA) according to the manufacturer`s instructions. VEGF was expressed in pg/ml.

Determination of serum PAF

Serum PAF was determined by using a commercial mouse ELISA kit (My Bio Source, USA) according to the manufacturer`s instructions. PAF was expressed in ng/ml.

Quantitative reverse transcriptase real time PCR (qRT-PCR)

Relative expression levels of genes for TIMP-1 and EGFR were determined by qRT-PCR. RNA was extracted from the tumor tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions.The RNA concentration was determined spectrophotometrically at 260 nm using the Nano Drop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on. (1 µg) of RNA were used in the subsequent cDNA synthesis reaction, which was performed 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 MgCl2 (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo d (t) primers, RNAse inhibitor (20 U) and AMV reverse transcriptase (20 U/µl). This mixture was incubated at 42°C for 1 h.

Quantitative real time PCR

qPCR 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 min 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C). Each 10 µl reaction contained 5 µl SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5 µl cDNA and 1.9 µl nuclease-free water. The sequences of PCR primer pairs used for each gene (TIMP-1 and EGFR) are shown in Table I. 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). For each target gene, relative expression was calculated using the comparative threshold cycle method. All values were normalized to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene signal as a housekeeping gene, generating Δ cycle threshold (C_t) value (ΔC_t = C_t target gene – C_t reference gene) (Muller *et al*., 2002). The relative gene expression was calculated according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt experimental setting – ΔCt control setting (Livak and Schmittgen, 2001).

Table I.- Primer sequences used for RT-PCR

Primer*	Sequence
TIMP-1	Forward primer
	5′-GCATCTGGCATCCTCTTGTT-3'
EGFR	Reverse primer
	5′-TGGGGAACCCATGAATTTAG-3'
	Forward primer
	5′- CGAGGGCAAATACAGCTT-3'
	Reverse primer
	5′- AAATTCACCAATACCTATT-3'
GGAPDH	Forward:
	5′- CTCCCATTCTTCCACCTTTG-3′
	Reverse:
	5′- CTTGCTCTCAGTATCCTTGC-3′

*TIMP-1, Tissue inhibitor of matrix metalloproteinase-1; EGFR, Epidermal growth factor receptor; GAPDH, Glyceraldehyde-3 phosphate dehydrogenase.

Gelatin zymography of tissue matrix metalloproteinases (MMPs)

Gelatinase (MMP-2 and MMP-9) activities in tumor tissues were analyzed under non-reducing conditions using gelatin-SDS-polyacrylamide gel electrophoresis (zymography) according to Toth and Fridman (2001). Briefly, 50 µg of lysate samples were mixed with loading buffer, and then applied to a 10% SDS-polyacrylamide gel copolymerized with 1 mg/ml (0.1%) gelatin (Sigma, France) and electrophoresed at constant voltage. The gels were washed in 2.5% Triton X100 (Sigma, France) for 1 h to remove SDS and placed in an incubation buffer (50 mM Tris–HCl, 5 mM CaCl 2 pH 7,6) for 24 h at 37◦C. Then, the gels were washed with deionized water and stained with 0.25% (w/v) Coomassie Brilliant Blue G (Sigma, France) in 40% methanol, 7% acetic acid water (5:2:5 v/v/v) for 15 min, and destained in 5% methanol, 7% acetic acid water. The gelatinase activities appeared as clear bands against a blue background. 2µl of 20 µg recombinant human MMP-2 and 9 standard (Chemicon) as positive control recognized as the standard of MMP-2 and MMP-9 molecular weight were run in each zymogram. The zymograms were analyzed using densitometry images analysis software (Image Master VDS).

Statistical Analysis

The results were expressed as the mean value ± SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison as a *post-hoc* test estimated by SPSS software version 21. P- values < 0.05 were considered to be statistically significant.

RESULTS

Cytotoxicity profile of C. majus *ethanolic extract (MTT assay)*

The effect of different doses of *C. majus* ethanolic extract was investigated in *vitro* using MTT assay. MTT solution was used at dose ranges from 50-150 µg/ml of *C. majus* on EAC cells; EAC cells were considered as control group with 100% viability. The in *vitro* cytotoxic activities of all doses are shown in Figure 1. 100 µg/ml was chosen regarded to the least number viable cells recorded.

EAC tumor size monitoring

The size of the left thigh of mice was measured four times along the experiment starting from the inoculation of Ehrlich carcinoma cell in control group. The diminish of tumor size in mice treated with ethanolic extract of *C. majus* at dose 100 mg/kg b.wt either alone or combined

with 6Gy γ-radiation is shown in Figure 2. As noticed the tumor bulb was a formed after 10 days of inoculation (2.5 x 10^{-5} cell) in EAC group in volume of 0.3283mm^3 with a significant increase as compared to control group (0.0315mm^3). After administration of *C. majus* (CM group) at 16th and 23thday the tumor volume was significantly decreased either alone (0.2367mm^3 and 0.4383 mm^3, respectively) or in combination with R (0.3283 mm^3 and 0.32 mm^3, respectively) when compared to EAC group (0.4433 mm^3, 0.7433 mm^3).

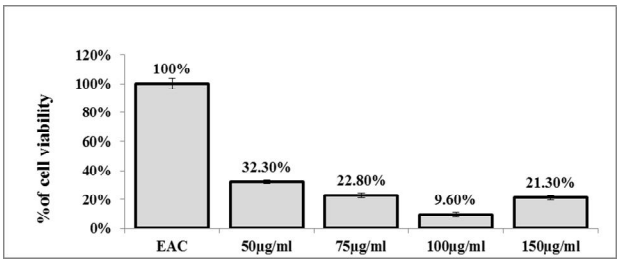

Fig. 1. Cell viability (%) of EAC incubated with different concentrations of *C. majus* ethanolic extract.

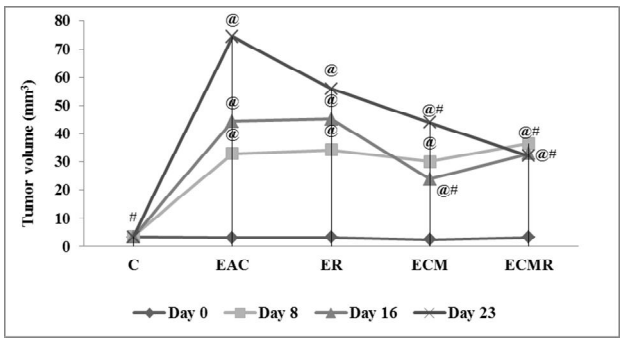

Fig. 2. Effect of *C. majus* and/or γ-radiation on tumor size in different time intervals. Each value represents the mean ± SE (n=15). Significance level at p< 0.05, where @ significant vs control (C) and # significant vs ehrlich tumor bearing group (EAC).

Combination of C. majus *ethanolic extract with radiation suppress serum VEGF level in EAC transplanted mice*

Data depicted in Figure 3A showed the effect of *C. majus* ethanolic extract and/or R on VEGF serum level in EAC bearing mice. It was clear that EAC developed mice recorded a significant increase ($p<0.05$) in serum VEGF level when compared to control group with a concentration value of 121.37±1.04 and 96.7±0.82 in R exposed mice. Whereas, it exhibited a significant decrease ($p<0.05$) in mice treated with *C. majus* alone (65.8 ±0.8). In addition, treatment of EAC bearing mice with *C. majus* (ECM) produced a significant reduction ($p<0.05$) in VEGF serum level as compared to EAC group. Also, ER group revealed a significant increase ($p<0.05$) in VEGF serum level as compared to control mice. However, the group of EAC bearing mice treated with *C. majus* and irradiated with 6 Gy (ECMR) VEGF serum level was significantly decreased and has no significant change from control (43.4±1.06) and (39.48±0.5), respectively.

Combination of C. majus *ethanolic extract with radiation modulate TIMP-1 and EGFR expression in EAC transplanted mice*

The expression profile of EGFR and TIMP-1genes in ehrlich bearing mice is presented in Figures 3B,C. Our data revealed that EGFR gene was up-regulated in EAC, R and ER groups as compared to their expression in the corresponding control values. In groups of EAC, R and ER, the expression of EGFR gene significantly elevated ($p<0.05$) with a fold change values 3.5, 2.6 and 3.9, respectively. However, EAC bearing mice treated with *C. majus* ethanolic extract (ECM group) as well as EAC bearing mice treated with *C. majus* ethanolic extract and irradiated with 6 Gy (ECMR) showed a significant decrease ($p<0.05$) with 1.5 and 2.05 fold change when compared to its corresponding non-treated EAC transplanted mice (EAC group). On the other hand, TIMP-1 gene expression was markedly down-regulated ($p<0.05$) in EAC, R and ER groups as compared to their expression in its respective control group. In addition, the suppression in TIMP-1 gene expression in these groups (EAC, R and ER) recorded a fold change values 2.1, 1.3 and 1.7, respectively as compared to control. Worthwhile, EAC bearing mice treated with *C. majus* ethanolic extract (ECM group) and EAC tumorized mice received C. *majus* ethanolic extract and exposed to 6 Gy (ECMR) exhibited a significant rise ($p<0.05$) in TIMP-1 expression with 3.6 and 4.2 fold change as compared to its corresponding EAC group.

Combination of C. majus *ethanolic extract with radiation decrease serum PAF level in EAC transplanted mice*

Data presented in Figure 3D showed the effect of *C. majus* ethanolic extract and/or R on PAF serum level in EAC bearing mice. Inoculation of EAC cells and/or irradiation of mice resulted in a significant increase ($p<0.05$) in serum PAF level as shown in EAC and ER groups when compared to their respective control (Fig.3D). The recorded PAF serum level was 96.6 and 86 ng/ml in EAC and ER groups, respectively. However, EAC bearing mice treated with *C. majus* extract (ECM) and EAC bearing mice treated with *C. majus* extract and irradiated with 6 Gy (ECMR) markedly diminished

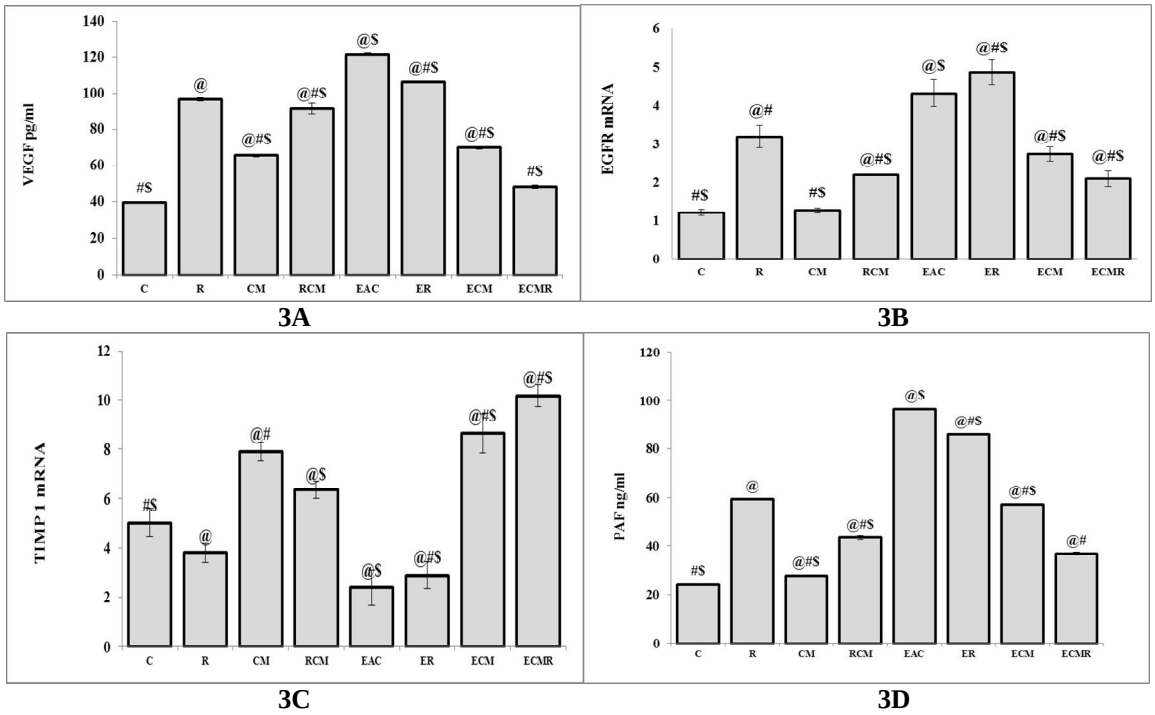

Fig. 3. Effect of *C. majus* and/or γ-radiation on (A) VEGF serum level in EAC bearing mice, (B) EGFR expression, (C) the expression of MMP inhibitor, TIMP-1. (D) PAF serum level in EAC bearing mice. Data from real time PCR are expressed as the fold increases over control level. Each value represents the mean ± SE (n=15). Significance level at p< 0.05, where @ significant vs control (C), # significant vs ehrlich tumor bearing group (EAC) and $ significant vs R group.

($p<0.05$) serum PAF level with serum level of 57.2 and 36.75 ng/ml, respectively as compared to EAC group. Interestingly, ECMR group revealed the greatest effect in reducing serum PAF level among all treated groups and showed comparable serum PAF level to normal control.

Combination of C. majus *ethanolic extract with radiation reduce tumor vascular remodeling and invasiveness*

Matrix metalloproteinases (MMP-2 and 9) are known to be involved in the degradation of extracellular matrix facilitating tumor cell migration and local invasion along with activation of vascularization in the tumor stroma. Therefore, we evaluated the expression of MMP-2 and 9 specifically, by gelatin zymography, in the tumor tissue lysate based on its enzymatic activity. The activity of MMP-2 and 9 were detected in zymography (Fig. 4; panel A) and their concentration in Figure 4; panel B. The basal activity of MMP-2 and 9 were 117.125±1.26 and 86.35±0.4, respectively. In EAC and/or R groups, a significant increase ($p<0.05$) has occurred compared to control group and recorded an increase their activities with 243.86±2.39 and 190.15±1.55 for MMP-2 as well as 197.12±1.16 and 161.07± 2.67 for MMP-9. In addition, tumor tissue activities of MMP -2 and 9 were significantly reduced ($p<0.05$) in EAC transplanted mice and treated with *C. majus* extract (ECM) and EAC bearing mice administered *C. majus* extract and irradiated with 6 Gy (ECMR) as compared to EAC and/or ER groups reflecting their potency in abrogating MMP-2 and 9 activities and tumor invasion. It is apparent that combination of CM extract with the applied dose of radiation produced a superior effect on MMP-2 and 9.

DISCUSSION

Tumor angiogenesis is crucial for the proliferation, survival and metastases of all malignancies. The response of the tumor microvasculature to ionizing radiation can be modified to improve tumor control in preclinical mouse models of cancer (Lu *et al.*, 2003). The effect of radiation on tumor tissue can be optimized by introducing radiosensitizing agents, in order to achieve a greater degree of tumor damage than expected from the additive effect of each modality (Jagetia and Venkatesha, 2005). The results of the present study unequivocally demonstrated the radiosensitizing and anti-angiogenic activity of the ethanolic extract of *C. majus* against Ehrlich ascites carcinoma (EAC) in mice.

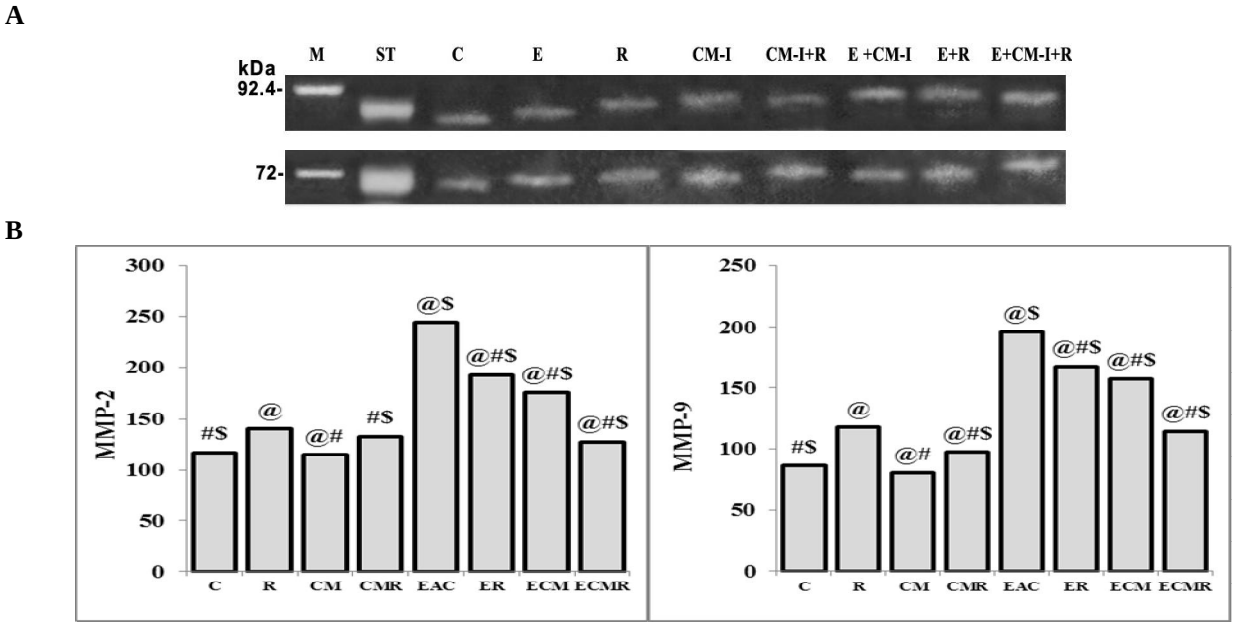

Fig. 4. Effect of *C. majus* and /or γ-radiation on animal bearing EAC tumor. (A) Gelatin zymography analysis shows the effect of *C. majus* and /or γ-radiation treatment on MMP-2 and MMP-9 activities (U/mg protein). (B)Quantitative activities of MMP-2 amd 9 of different treated groups. Each value represents the mean ± SE (n=15), where @ significant vs control (C), # significant vs ehrlich tumor bearing group (EAC) and $ significant vs R group. Standard for purified active MMP-2 (~ 72 KD) and active MMP-9 (~ 92 KD).

MTT assay is a well established *in vitro* model used to test cytotoxicity of compounds against cancer cell lines as well as screening of compounds with potential anti-tumor properties (Kondo *et al*., 2000). The death of the cells caused by *C. majus* extract might be due to the loss of mitochondria which is one of the hallmarks of the apoptotic pathway (Christopher, 1992).

The development of new blood vessels by the tumor is regulated by the production of angiogenic stimulators including vascular endothelial growth factor (VEGF). Since VEGF is a key regulatory factor in the prognosis of various cancers, inhibition of VEGF production is a promising therapeutic approach for treating cancer (Thapa *et al*., 2011). The experimental data of the current study revealed a significant increase ($p<0.05$) in tumor tissue level of MMP (2 and 9) (Fig. 4), EGFR mRNA expression (Fig. 3B), PAF (Fig. 3D) and VEGF (Fig. 3A) as well as a significant decrease ($p<0.05$) in tumor tissue mRNA level of TIMP-1 (Fig. 3C) in mice bearing tumor (EAC group) as compared to their values in normal control group. The imbalance between angiogenic activators and inhibitors was observed in mice bearing tumor in our findings. EGFR is a member of the HER/erbB family of receptor tyrosine kinases (Arteaga, 2001). Overexpression of EGFR signaling pathways are suggested mechanisms that promote a malignant phenotype and has been observed in many solid tumors (Laskin and Sandler, 2004). This pathway is triggered by EGFR dimerization promoted through the binding of a specific set of ligands to the extracellular domain of the receptor (Ellis, 2004). Dimerization of the EGFR receptor triggers the activation of intracellular signaling pathways, which leads to cytoplasmic tyrosine kinase enzyme activation, causing autophosphorylation of intracellular tyrosine residues (Tabernero, 2007). Phosphorylated tyrosines serve as docking sites for a number of signal transducers and adaptor molecules that initiate further signaling pathways. Activation of EGFR signaling can up-regulate the production of VEGF in human cancer cells (Cascio *et al*., 2009).

Tumors derived from many human tissues secrete VEGF and its receptors are highly expressed on the endothelial cells of tumor-associated blood vessels (Kolch *et al*., 1995). VEGF binds to 2 major receptor tyrosine kinases, VEGFR1 and VEGFR2 (Ferrara *et al*., 2003). Upon VEGF binding, VEGFR2 activates a number of downstream mediators enabling endothelial cells to proliferate, migrate, invade and differentiate to form capillary-like structures (Sato *et al*., 2000). Nuclear factor- kappa B (NF-$_K$B) signaling mediates numerous cellular processes and positively regulates VEGFR2 expression (Ronicke *et al*., 1996; Dong *et al*., 2014). Furthermore, PAF, a potent pro-inflammatory phospholipid, has been found to trigger tumor growth and

angiogenesis through its G-protein coupled receptor (PAFR) (Sun *et al*., 2015). Recently, PAF have shown to promote angiogenesis through a NF-$_K$B- dependant pathway *via* regulating expression of genes directly involved in VEGF expression (Ko *et al*., 2006). Moreover, Ko *et al*. (2005) demonstrated that PAF augment angiogenesis through enhancing MMP-9 expression and activity in a NF-$_K$B- dependant manner.

The results of the present study revealed a significant suppression ($p<0.05$) in EGFR mRNA (Fig. 3B), PAF (Fig. 3D), MMP (2 and 9) (Fig. 4) and VEGF serum level (Fig. 3A) in parallel with a significant increase ($p<0.05$) in tumor tissue level of TIMP-1 mRNA (Fig. 5) in *C. majus* ethanolic extract treated (CM group) and *C. majus* in combination with R (CMR) treated groups. This effect might be axial in augmenting the antiangiogenic and radiosensitizing activity of the extract. Berberine, one of the major alkaloids isolated from *C. majus*, showed antiangiogenic activity in previous studies (Jie *et al*., 2011; Hamsa and Kuttan, 2012). The effect of berberine on invasion, migration, metastasis, and angiogenesis is mediated through the inhibition of focal adhesion kinase (FAK), NF- *κ*B, urokinase-type plasminogen-activator (u-PA) and down-regulation of hypoxia-inducible factor- 1 α (HIF-1α) mediated VEGF upregulation (Hamsa and Kuttan, 2012). In addition, Wang *et al*. (2013) reported that berberine down-regulated EGFR expression through enhancing Cb1 ligase activity, an ubiquitin ligase belongs to E3 ubiquitin ligases family, which is tyrosine phosphorylated, resulting in ubiquitinylation of EGFR and its translocation to endosomal compartment. Moreover, the anti-inflammatory activity of berberine was proved to involve inhibition of mitogen activated protein kinases (MAPKs) and NF- *κ*B signaling pathways (Li *et al*., 2014). This effect might contribute to the suppressive effect of *C. majus* on PAF.

Sanguinarine; is a benzophenanthridine alkaloid isolated from *C. majus;* shows antiangiogenic and anti-invasive effects, and overcomes P-glycoprotein-mediated multi-drug resistance (MDR) phenotype (Weerasinghe *et al*., 2006; Choi *et al*., 2009). Moreover, Park *et al.* (2014) illustrated the antiangiogenic activity of sanguinarine *via* inhibiting MMP-9 expression through inducing TIMP-1 and TIMP-2 expression, as endogenous inhibitor of MMP-9 by modulating hemeoxygenase (HO-1) expression. Worthwhile, Xu *et al.* (2013) confirmed the antiangiogenic activity of sanguinarine and attributed this effect to its inhibitory activity on VEGF induced Akt and p38 activation.

According to the obtained results, it is obvious that mice irradiated with 6 Gy (R group) alone showed a marked elevation ($p<0.05$) in EGFR mRNA expression (Fig. 3B), MMP- (2 and 9) (Fig. 4), PAF (Fig. 3D) and VEGF serum level (Fig. 3A) associated with a significant decrease ($p<0.05$) in TIMP-1 mRNA level (Fig. 3C) as compared to control (C) group, suggesting its pro-angiogenic activity. Previous studies have demonstrated the deleterious effects of γ- radiation on vascular system leading to a broad range of vascular pathologies (Jurado *et al.*, 2008; Little *et al*., 2012). Emerging evidence indicated that γ- radiation promote angiogenesis and increase metastasis (Park *et al*., 2006; Sofia *et al*., 2010), often through up-regulation of pro-angiogenic mediators such as VEGF, HIF-1α and basic fibroblast growth factor (bFGF) in irradiated tissue (Grabham and Sharma, 2013). Furthermore, exposure of tumor cells to ionizing radiation may also increase EGFR phosphorylation and activation of MAPKs signaling pathways (Diaz-Miqueli and Martinez, 2013). Therefore, combination of EGFR inhibitors with γ- radiation might sensitize tumor vasculature to radiation and improve anti-proliferative outcome.

The results of the current study revealed that combination of *C. majus* ethanolic extract with the applied dose of radiation (6 Gy) showed a significant decrease ($p<0.05$) in tumor volume (Fig. 2) which may reflect the radiosensitizing effect of the extract. This effect could be interpreted in the view of proapoptotic activity of *C. majus* ethanolic extract. The obtained data are consistent with those obtained by Habermehl *et al.* (2006) who postulated that antitumoral efficacy of Ukrain (an approved anticancer; semi-synthetic derivative of *C. majus* alkaloids) is attributed to the proapoptotic activity of *C. majus* alkaloids. Since tumor angiogenesis is essential for tumor growth, the anti-angiogenic strategy has become a valuable approach in tumor therapy which leads to tumor apoptosis (Wang *et al.*, 2015). Hence, the exhibited anti-angiogenic activity of *C. majus* ethanolic extract might contribute to tumor regression.

CONCLUSION

Collectively, *C. majus* ethanolic extract possessed anti-angiogenic and radiosensitizing efficacy via suppression of pro-angiogenic mediators (EGFR, PAF and VEGF) and modulation of vascular remodeling (MMP-2 and 9 and TIMP-1). Down-regulation of EGFR might play a significant role in the radiosensitizing activity of *C. majus* extract as a novel mechanism for the anti-tumor property of the extract.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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