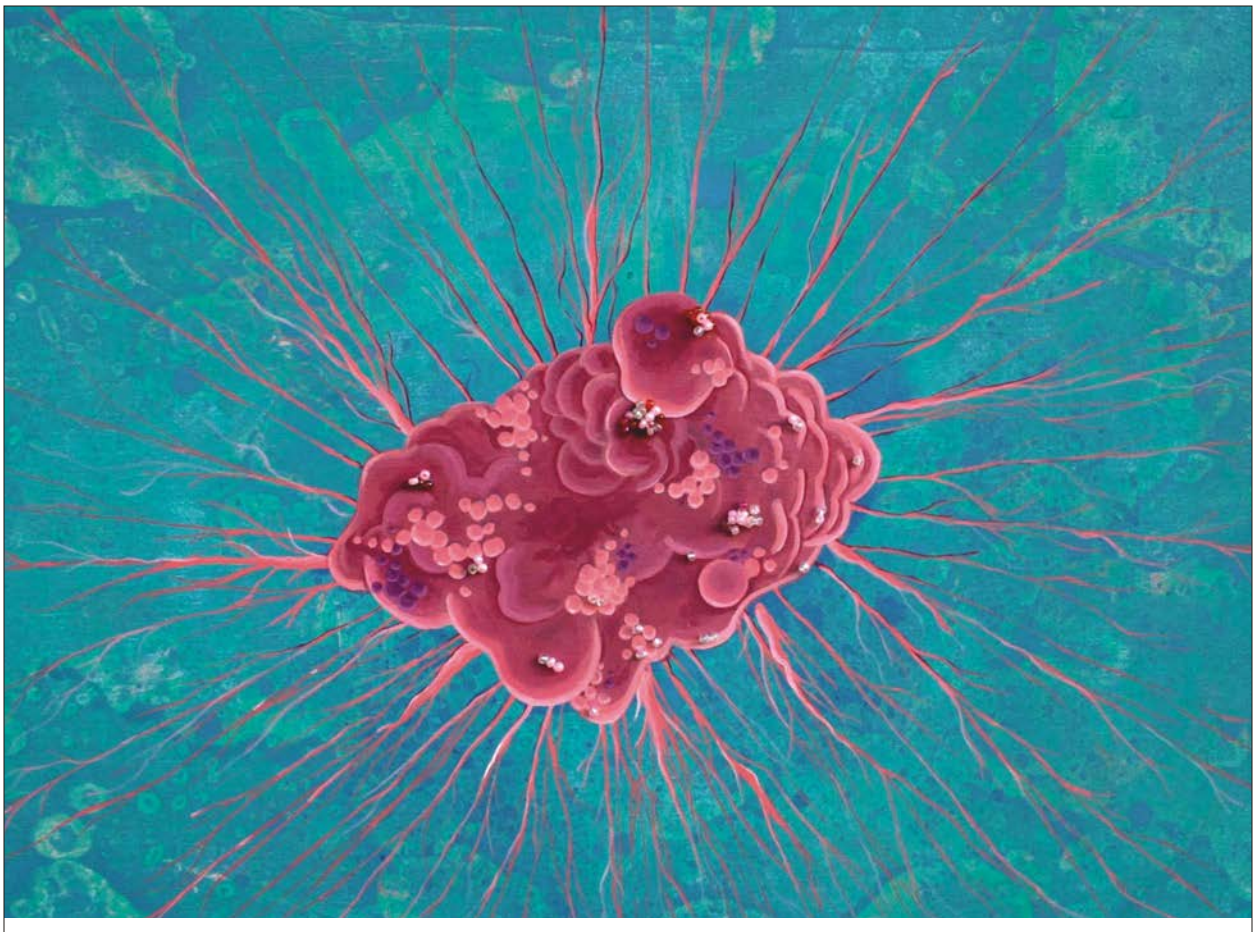

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Research Article
Radiosensitizing Efficacy of Diosmin- Hesperidin Complex Against Ehrlich Solid Carcinoma in Mice, A Potential Role of Histone Deacetylase and Pro-angiogenic Chaperones Targeting

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Abstract

Background and Objective: Adaptation to hypoxic microenvironment is critical for tumor survival and metastatic spread. Hypoxia inducible factor-1α (HIF-1α) plays a key role in this adaptation by stimulating the production of proangiogenic factors and inducing enzymes necessary for anaerobic metabolism. Histone deacetylase inhibitors (HDACIs) produce a marked inhibition of HIF-1α expression and are currently in clinical trials partly based on their potent antiangiogenic effects. Thus, the current study was conducted to evaluate the possible effect of diosmin-hesperidin complex (daflon®) as a radiosensitizing and anti-angiogenic natural product against Ehrlich solid carcinoma (ESC) in mice as a model for solid tumor. **Materials and Methods:** Mice were treated with diosmin-hesperidin complex (daflon® D; 100 mg kg^{-1} b.wt., orally for 4 weeks after ESC induction) and exposed to a total body γ-radiation (R; 2 Gy/week for 4 weeks up to a total dose of 8 Gy). Cytotoxicity of D was assessed by MTT assay and animal 30 days survival after tumor induction was determined. Histone deacetylase (HDAC) activity, HIF-1α, heat shock protein-90 and -70 (HSP-90 and 70), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS) and survivin levels were determined in tumor tissue. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Holm-Sidak as a post-hoc test estimated by Sigma Plot 11. **Results:** The obtained results showed a significant increase ($p<0.05$) in tumor tissue levels of VEGF, HIF-1α, HSP-90 and -70 and survivin as well as HDAC and iNOS activities in E group. Combination of D with R in ESC bearing mice resulted in a remarkable decrease in the aforementioned biochemical parameters in tumor tissue, which was indicated by significant tumor volume regression and mice survival. **Conclusion:** This study confirms the anti-angiogenic and radiosensitizing effect of D mediated via inhibiting HDAC activity and disruption of HIF-1α-HSP-70/90 signaling axis in ESC xenograft model.

Key words: Diosmin, hesperidin, γ-radiation, ehrlich solid carcinoma, histone deacetylase, heat shock proteins

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Angiogenesis describes the formation of new blood vessels from the existing vasculature and is required for the promotion of fundamental physiological processes including embryonic development, fertility and tissue repair[1]. While angiogenesis has strong implications in homeostasis, it also has the potential to promote tumor growth and metastasis[2]. Within tumors, new blood vessel formation can occur by sprouting from pre-existing vasculature which may be assisted by the recruitment of circulating cells such as bone marrow derived endothelial progenitor cells, macrophages and fibroblasts[3,4]. These cells along with malignant cells are able to secrete pro-angiogenic factors including vascular endothelial growth factor (VEGF), which induce tumor blood vessel formation[5]. The transcription factor hypoxia-inducible factor 1-alpha (HIF-1α), regulates the expression of numerous genes involved in various cellular signaling pathways including angiogenesis via the increased expression of VEGF[6]. Over-expression of VEGF mediated by the stabilization of HIF-1α has been identified in multiple malignancies[7] and for this reason targeting the tumor vasculature via the inhibition of VEGF either directly or indirectly has become an attractive target in novel anti-cancer drug development[8].

Histone acetyltransferases and deacetylases (HDACs) play a central role in chromatin structure modification and gene expression[9]. Histone acetyltransferase inactivation is associated with tumorigenesis[10]. Aberrant HDAC activity is proposed to be the key event in tumor formation, since excessive HDAC activity inappropriately repressed transcription of tumor suppressor genes[11,12]. These compounds are well known to affect gene expression through the hyperacetylation of histones. However, their effects are not restricted to these proteins: Nε-acetylation of transcription factors is emerging as an important mechanism of regulating transcription factor activity by affecting their DNA-binding affinity, transactivation activity, stability, or subcellular localization[13,14]. The role of acetylation in controlling HIF-1α function is a matter of controversy. It was proposed that acetylation of HIF-1α would increase its affinity for von Hippel-Lindau (VHL) and thereby enhance HIF-1α ubiquitination and proteasomal degradation[14].

The heat shock protein (HSP) family includes a group of molecular chaperones that appear to play important roles during protein folding[15] and conformational maturation[16]. HSP-90 is critically involved in the function and stability of many oncogene products and cell-signaling molecules. Client proteins downregulated by 17DMAG, a well proved HSP-90 inhibitor; include Raf-1, cdk4 and Akt[17], which is noteworthy in part because Akt regulates cell survival via phosphorylation of multiple substrates involved in the regulation of apoptosis[18]. The fact that such molecules are recoverable from heterocomplexes containing HSP-90 suggests that destabilization of protein-HSP-90 multi-molecular complexes likely causes several important signaling proteins to undergo rapid degradation via the ubiquitin-proteasome system[19]. Interestingly, expression of HSP-90 is upregulated in tumors, compared with normal tissues[20] and tumor cells are particularly sensitive to HSP-90 inhibition[21], as well as, the ability of HSP-90 inhibitors to enhance the radiosensitivity of tumor cells[22,23] and the anticancer efficacy of chemotherapy[24].

HSP-70 is known to be a potent negative regulator of cell death and an essential factor supporting tumor cell survival and tumor growth; moreover, elevated levels of HSP-70 within tumors correlate with a poor prognosis in cancer patients[25]. Depletion of HSP-70 not only increases tumor cell death, but also selectively sensitizes malignant cells to chemotherapeutic agents[26]. Indeed, down regulation of HSP-70 reverses cancer cell drug resistance, probably through its ability to inhibit apoptosis both upstream and downstream of mitochondrial signaling[27]. It is well documented that HSP-90 inhibition leads to compensatory over expression of HSP-70[28], which can diminish the incidence of cell death otherwise induced by HSP-90 inhibitors and thus decrease their anti tumor efficacy[29]. Moreover, Kim *et al.*[30] elucidated the crucial role of HSP-70 as a pro-angiogenic regulator. Therefore, the dual inhibition of HSP-90 and 70 might potentiate the anti-tumor and anti-angiogenic efficacy of drugs[31]. Herein, the present investigation was performed to evaluate the probable anti-angiogenic and radiosensitizing effects of diosmin-hesperidin complex in Ehrlich solid carcinoma xenograft model. Particularly, through targeting HDAC activity and subsequent disruption of HIF-1α -HSP-90/ 70 signaling axis. The anti-angiogenic and radiosensitizing potency of diosmin-hesperidin complex as perceived through assessing VEGF and survivin levels was also studied.

MATERIALS AND METHODS

The study was conducted in the Biochemistry Laboratory in the Radiation Biology Department, National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt. All of the study laboratory procedures have taken place during the time between January and March, 2016.

Animals: Female Swiss albino mice weighing 20-25 g were obtained from the breeding unit at the National Centre for

Radiation Research and Technology (NCRRT). The animals were housed (10 animals/cage) and maintained under proper environmental conditions *i.e.*, controlled air, temperature and relative humidity. They were provided with pelleted diet and free access to water. Animal experimentations were consistent with the guidelines of ethics by Public Health Guide for the Care and Use of Laboratory Animals (National Research Council)[32] 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.

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. Diosmin-hesperidin was obtained in a commercial form marketed as Daflon® (Servier Egypt Industries Limited, 6th October City, Giza, Egypt), consisting of 90% diosmin and 10% hesperidin, was dissolved in isotonic (0.9% NaCl) saline solution immediately before use.

***In vitro* cytotoxicity assay of diosmin-hesperidin complex (MTT assay):** Cytotoxicity profile of diosmin-hesperidin complex (daflon) was performed using MTT reagent (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, Germany) according to Burton[33] with modification. Briefly, about (2.5×10^6) viable EAC cells were inoculated into a 24-well flat bottom plates as control. Diosmin-hesperidin complex was added at several concentrations 25, 50, 75, 100 and 125 µg mL^{-1}. After 24 h, 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^{-1}) in 5% CO_2 incubator for 4 h, then cells were pelleted by centrifugation (15,000 rpm) (Thermo Fisher, USA) for 5 min. The media were removed and 500 µL of iso-propanol /HCl (Sigma-Aldrich, USA) mixture was added (2 mL of 0.1 N HCl in 23 mL iso-propanol). Subsequently, the samples were vortexed vigorously by a vortex mixer (Boeco, Germany) and the O.D was measured at 560 nm (721-VIS-spectrophotometer, China). The absorbance of untreated cells was considered as 100%. Each drug and control was assayed in triplicate in three independent experiments. Percent growth (%) of viable cells exposed to treatments was calculated as follows:

$$\text{Growth (\%)} = \frac{\text{Sample}_{abs}}{\text{Control}_{abs}} \times 100$$

Ehrlich ascites carcinoma (EAC) cell line and induction of solid carcinoma: Ehrlich ascites carcinoma (EAC) cell line was obtained from Pharmacology and Experimental Oncology Unit of the National Cancer Institute (NCI), Cairo University, Giza, Egypt. The tumor cell line was maintained in the experimental female Swiss albino mice by weekly intraperitoneal injection of 2.5 million cells/mouse. EAC cells are of mammary origin. The viability of the cells was 99% as judged by trypan blue exclusion assay. The xenograft model of ehrlich solid carcinoma (ESC) was induced in female mice Swiss albino mice by viable EAC cells about (2.5×10^6)/mice in 0.2 mL isotonic saline implanted subcutaneously (s.c.) into the right thigh of the hind limb of each mouse[34,35]. The tumor was developed in 100% of the mice with a palpable solid tumor mass ($\geq$1000 mm^3) was achieved within 7 days post-implantation. A day of tumor implantation was assigned as day 0. On 7th day, animals were randomized into five experimental groups.

Survival rate analysis: The day of implantation was considered as zero point of the experiment, mice were monitored for recording and analysis of the survival rate daily for 30 days by registering mortalities occurred in ESC-bearing untreated and treated groups according to Abdin *et al.*[36].

Irradiation procedures: Whole body γ-irradiation was performed with a Canadian Cs^{37} Gamma Cell-40 biological irradiator at the NCRRT. Cairo, Egypt, at a dose rate of 0.61 Gy min^{-1}. After tumor inoculation, mice were irradiated with 2 Gy/week up to a total dose of 8 Gy according to the experimental design.

Experimental design and treatment protocols: Seventy five mice were divided into five equal groups (15 mice/group) as follows: (1) Normal control group (C): Animals received 2 mL kg^{-1} 0.9% saline, orally at corresponding times along with experimental time course, (2) Ehrlich solid carcinoma group (E): Animals were inoculated intramuscularly with 0.2 mL in the right thigh once and received 2 mL kg^{-1} 0.9% saline, orally at corresponding times along with experimental time course, (3) Irradiated ESC-bearing mice group (ER): Animals were inoculated intramuscularly with 0.2 mL in the right thigh once and received 2 mL kg^{-1} 0.9% saline, orally at corresponding times along with experimental time course and exposed to whole body γ-irradiation (2 Gy/week for 4 weeks up to a total dose of 8 Gy)[37], (4) Diosmin-hesperidin complex (daflon) treated ESC-bearing mice group (ED): ESC-bearing mice were gavaged diosmin-hesperidin

complex (daflon) at dose of 100 mg kg^{-1} b.wt.,/day[38] for 4 weeks, (5) Diosmin-hesperidin complex (daflon) treated and irradiated ESC-bearing mice (EDR): ESC-bearing mice were administered diosmin-hesperidin complex (daflon) at dose of 100 mg kg^{-1} b.wt.,/day; orally for 4 weeks and exposed to fractionated whole body γ-radiation at a dose level 2 Gy once a week for 4 weeks.

Tumor size monitoring and tumor growth inhibition determination: The volume of solid tumor was measured by using microcaliper (Vernier, Shanghai, China) after 7, 14, 21 and 28 days from inoculation of EC. The tumor volume was calculated by the following Eq:

$$\text{Tumor volume } (mm^3) = ½ \times [(\text{length } (mm) \times \text{width } (mm)]^2$$

According to the method of Jensen *et al.*[39], where length is the greatest longitudinal diameter and the width is the greatest transverse diameter. Tumor growth inhibition rate (%) was determined according the following Eq:

$$\text{Tumor growth inhibition rate } (\%) = \frac{\text{Average tumor volume of control group-Average tumor volume of treated group}}{\text{Average tumor volume of control group}} \times 100$$

Tumor tissue samples collection and preparation: After 30 days and 16 h fasting, animals of each group were sacrificed under gentle ether anesthesia and tumor tissues were dissected immediately and weighted, then homogenized in lysis buffer [(40 mM HEPES, 50 mM KCl, 1% Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM EDTA, 1 mM benzamidine and 1% Triton-X (Sigma)] using a potter-Elvehjem homogenizer for biochemical analyses. Protein concentration was determined for all unknown sample tissue extract using a Bicinchoninic Acid (BCA) Assay Kit (Thermo Scientific Pierce; Rockford, IL) adapted for use in a 96-well plate according to the manufacturers' instructions. Extracts were diluted in reagent diluent to a final protein concentration of 0.5 or 1 µg $µL^{-1}$ for the assay and further diluted if necessary.

Biochemical assays

Estimation of HDAC activity in tumor tissue: HDAC extraction was carried out according to the method previously described by Baidyaroy *et al.*[40]. HDAC activity was evaluated using colorimetric HDAC activity assay Kit of Biovision. Briefly, 10 µg of nuclear extract was incubated with HDAC assay buffer and HDAC assay substrate buffer at 37°C for 1 h. A total 40 µg of quenching solution was added to stop the activator solution at room temperature for 15 min. Activity was measured using ELISA micro plate reader at 405 nm. HDAC activity was expressed as µM mg^{-1} tissue protein.

Determination of tumor tissue HIF-1α level: The level of HIF-1α was determined in tumor tissue using a commercial mouse total HIF-1 α ELISA kit (MyBiosource, USA) according to the manufacturers' instructions. HIF-1 α was expressed as ng mg^{-1} tissue protein.

Assessment of HSP-70 and HSP-90 levels in tumor tissue: Tumor tissue levels of HSP-70 and HSP-90 were determined using a commercial mouse ELISA kit (DuoSet, R and D systems, USA) according to the manufacturers' instructions. HSP-70 and -90 were expressed as pg mg^{-1} tissue protein.

Measurement of tumor tissue VEGF level: The level of VEGF in tumor tissue was measured by using a commercial mouse ELISA kit (quantikine R and D systems, USA) according to the manufacturers' instructions. VEGF level was expressed in pg mg^{-1} tissue protein.

Estimation of iNOS activity in tumor tissues: Activity of iNOS was measured according to the method of Ryoyama *et al.*[41], whereby L-arginine and molecular oxygen were catalyzed by NOS to generate nitric oxide (NO). The rate of NO production by NOS in 1 min was determined with the Griess reaction. The iNOS activity was determined by spectrophotometric assay at 540 nm. iNOS activity was expressed as U mg^{-1} protein.

Survivin level in tumor tissue: Survivin levels were determined by using a commercial mouse survivin ELISA kit (Biocompare, USA) according to the manufacturers' instructions. Survivin level was expressed as pg mg^{-1} tissue protein.

Statistical analysis: The results were expressed as the mean value±SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Holm-Sidak as a post-hoc test estimated by Sigma Plot 11 (Germany). p-values<0.05 were considered to be statistically significant. The paired-samples t-test was applied to detect difference in the tumor volume monitored at the start point and at the end point of the experiment. The cumulative survival curve was plotted and the overall and pairwise comparisons of survival rate were analyzed by Log-rank and Gehan-Breslow-Wilcoxon tests using Kaplan-Meier method.

RESULTS

***In vitro* cytotoxicity of different concentrations of D (MTT assay):** To demonstrate the cytotoxicity of D, ESC cells were treated with different concentrations of D *in vitro*. 25, 50, 75, 100 and 125 μg mL^{-1} of D could inhibit viability of ESC cells in dose-dependent manner, with inhibition rate of 28.2, 48.4, 76, 90.6 and 98.1% (Fig. 1, $p<0.01$, $p<0.001$, respectively). The IC_{50} dose of D was 43.051 μg mL^{-1}, suggested strong cytotoxicity of D in EAC cell lines.

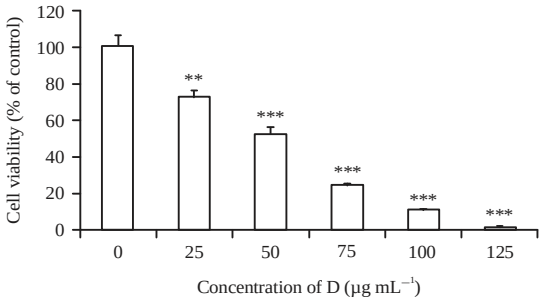

Fig. 1: Effect of different concentrations of D on viability of EAC cells

Cells were treated with 25, 50, 75, 100 and 125 μg mL^{-1} of D and incubated with 2.5×10^6 viable EAC cells for 24 h. **p<0.01 vs control and ***p<0.001 vs control

Combination of D with γ-radiation improves survival rate and induces tumor volume regression in ESC-bearing mice: The effect of D administration to ESC-bearing mice at dose of 100 mg kg^{-1}/day for 4 weeks and/or exposed to fractionated whole body γ-radiation at a dose level 2 Gy once a week for 4 weeks on survival rate and tumor regression was depicted in Fig. 2a-d. With regard to survival, the overall survival comparison among groups was significant ($p<0.05$). The pairwise comparisons showed significant difference between the untreated E group (group II) and ESC-bearing mice treated with D (ED; group IV) as well as ESC-bearing mice treated with D and exposed to fractionated doses of γ-radiation (EDR; group V). Treatment of ESC-bearing mice with D prolonged survival rate (66.7%) compared to E group (33.3%) and delayed 1st death event to be occurred at day 12 compared to E group which occurred at day 8 post ESC inoculation (Fig. 2a). Interestingly, combination of D treatment with R as observed in EDR (group V) exhibited a significant improvement in mice survival rate (80%) as compared to E and ER groups and delayed the 1st death event to be occurred at day 16 compared to E, ER and ED groups in which the 1st death event occurred at days 8, 9 and 12, respectively, suggesting the remarkable therapeutic outcome elicited by D and R combination in ESC-bearing mice. The cumulative survival function of the different studied groups was displayed in Fig. 2a. ESC volume monitoring is illustrated in Fig. 2b. It is obvious that the inoculation of 2.5 million of EC cells in the thigh region of healthy normal female mice produced a tumor with a mean size of 1112.3 ± 98.6 mm^3 on the 7th day post tumor inoculation. ESC volume proceeds along the experimental period reaching 2979 ± 223.4 mm^3 on day 28 post tumor inoculation (Fig. 2b). Treatment of ESC-bearing mice with D combined with R 7 days after EC tumor transplantation induced marked suppression ($p<0.05$) in tumor volume and weight when compared to E, ER and ED groups (Fig. 2b and c) and recorded the highest tumor growth inhibition percentage (57.5%) compared to 28.5 and 42.1% in ER and ED groups, respectively (Fig. 2d).

Combination of D with γ-radiation suppresses HDAC activity, represses HIF-1α level and modulates HSP-70 and -90 levels in ESC transplanted mice: The effect of D and/or R treatment on the HDAC activity, HIF-1α, HSP-70/90 levels in tumor tissue of ESC bearing mice as revealed in Fig. 3. In E group, there was a significant rise ($p<0.05$) in HDAC activity paralleled by a significant elevation ($p<0.05$) in HIF-1α level as well as HSP-70 and -90 levels in tumor tissue as compared to normal control group (Group I) as shown in Fig. 3a-d. Treatment of ESC-bearing mice with D and/or R exhibited a remarkable reduction ($p<0.05$) in HDAC activity coupled with a significant decline ($p<0.05$) in HIF-1α, HSP-70 and -90 levels in tumor tissue of ER, ED and EDR groups as compared to untreated E group set (group II) as presented in Fig. 3a-d. There was a notable synergism between D and R as seen in EDR group (group IV) regarding their effect on HDAC activity, destabilization of HIF-1α and reduction of the pro-angiogenic chaperones HSP-70 and -90 levels denoting their pronounced anti-tumor and anti-angiogenic activity in solid tumors.

Combination of D with γ-radiation modulates iNOS activity and diminishes VEGF and survivin levels in ESC transplanted mice: The effect of D and/or R on iNOS activity, VEGF and survivin levels in ESC bearing mice as depicted in Fig. 4. Implantation of EC in mice induced a significant increase ($p<0.05$) in iNOS activity concomitantly with remarkable elevation ($p<0.05$) in VEGF and survivin levels in tumor tissue of E group (group II) as compared to normal control group (group I) as shown in Fig. 4a-c. Treatment of EAC-bearing mice

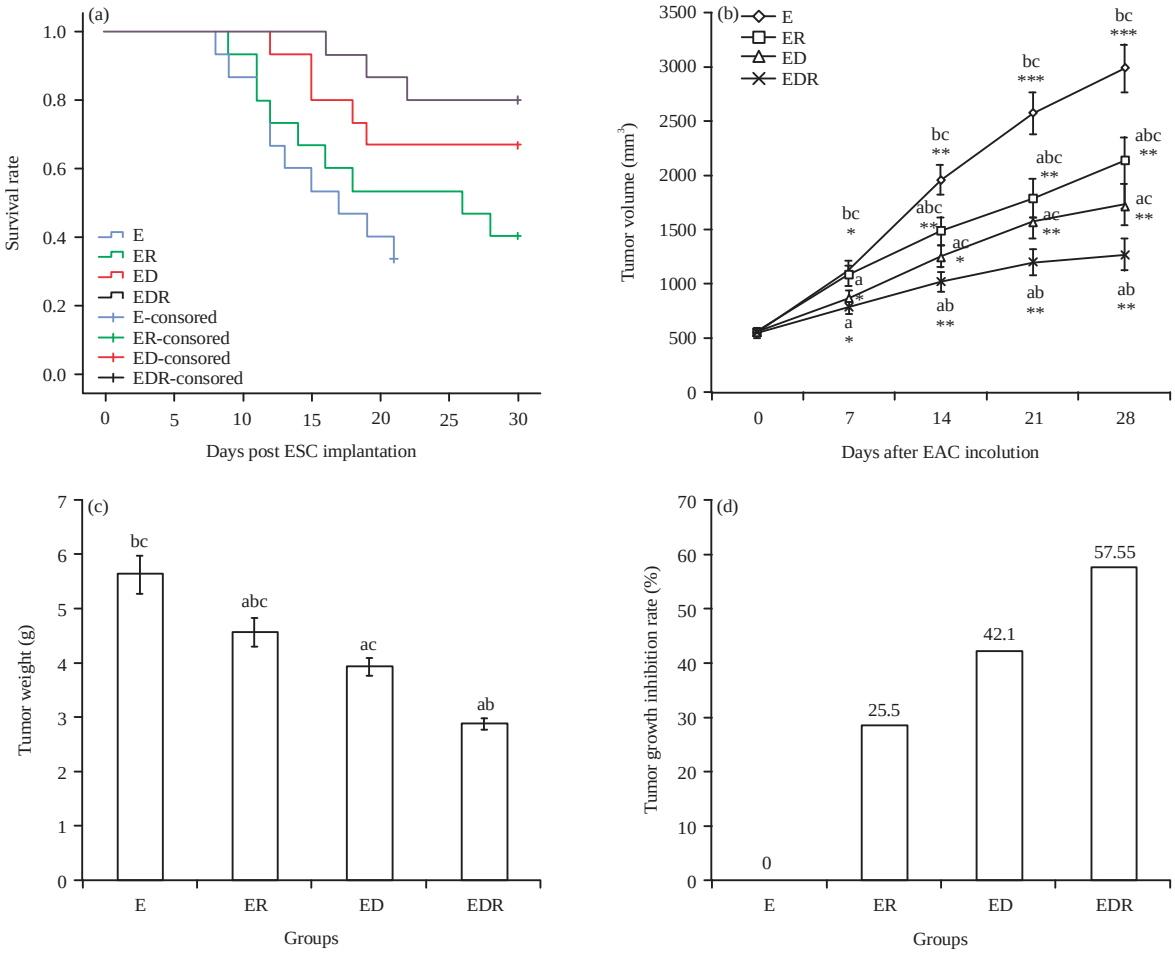

Fig. 2(a-d): Effect of D treatment combined with whole body exposure to fractionated doses of γ-radiation in ESC transplanted mice, (a) Survival rate, (b) Tumor volume, (c) Tumor weight and (d) Tumor growth inhibition rate

Values are the Mean±SEM. a, b and c indicate a statistically significant difference of $p<0.05$ as compared with the E, ED and EDR groups (one-way ANOVA followed by Holm-Sidak as a *post hoc* test. Paired-samples t-test: Significant difference of tumor volume between start point and end point in each group, *$p<0.05$, **$p<0.01$ and ***$p<0.001$. E: Mice injected with EAC cells, ER: Mice injected with EAC cells and exposed to a total dose of 8 Gy, ED: Mice injected with EAC cells and received D, EDR: Mice injected with EAC cells, received D and exposed to 8 Gy

with D and/or R caused a significant reduction ($p<0.05$) in iNOS activity and VEGF level in tumor tissue of ER, ED and EDR group as compared to untreated ESC-bearing mice (Fig. 4a-c). In addition, combination of D and R treatment in ESC-bearing mice (EDR group) significantly decreased ($p<0.05$) survivin tumor tissue level as compared to E, ER and ED groups, suggesting abrogation of tumor angiogenesis regulators and pro-survival oncoproteins.

DISCUSSION

The most effective antitumor treatment is currently achieved by chemotherapeutic agents that abrogate tumor cells. Despite this, chemotherapy is virtually without influence on life expectancy of patients with certain cancers. With bearing this in mind, novel strategies for treating malignancies are being developed in experiments and applied in clinical setting[37,42]. Combining chemotherapy with radiotherapy represents a key oncology strategy for a more comprehensive attack toward cancers. Combination of chemoradiotherapy has been shown to improve treatment outcome for various solid tumor malignancies. Systemic chemotherapy complements local primary tumor control provided by radiotherapy. In addition, a number of chemotherapeutic drugs exhibit radiation-sensitizing activity and are capable of enhancing efficacy of radiotherapy targeting at primary tumor[43].

It is well established that solid tumor growth is dependent upon the growth of new blood vessels which is known as angiogenesis. Several studies have reported that the degree

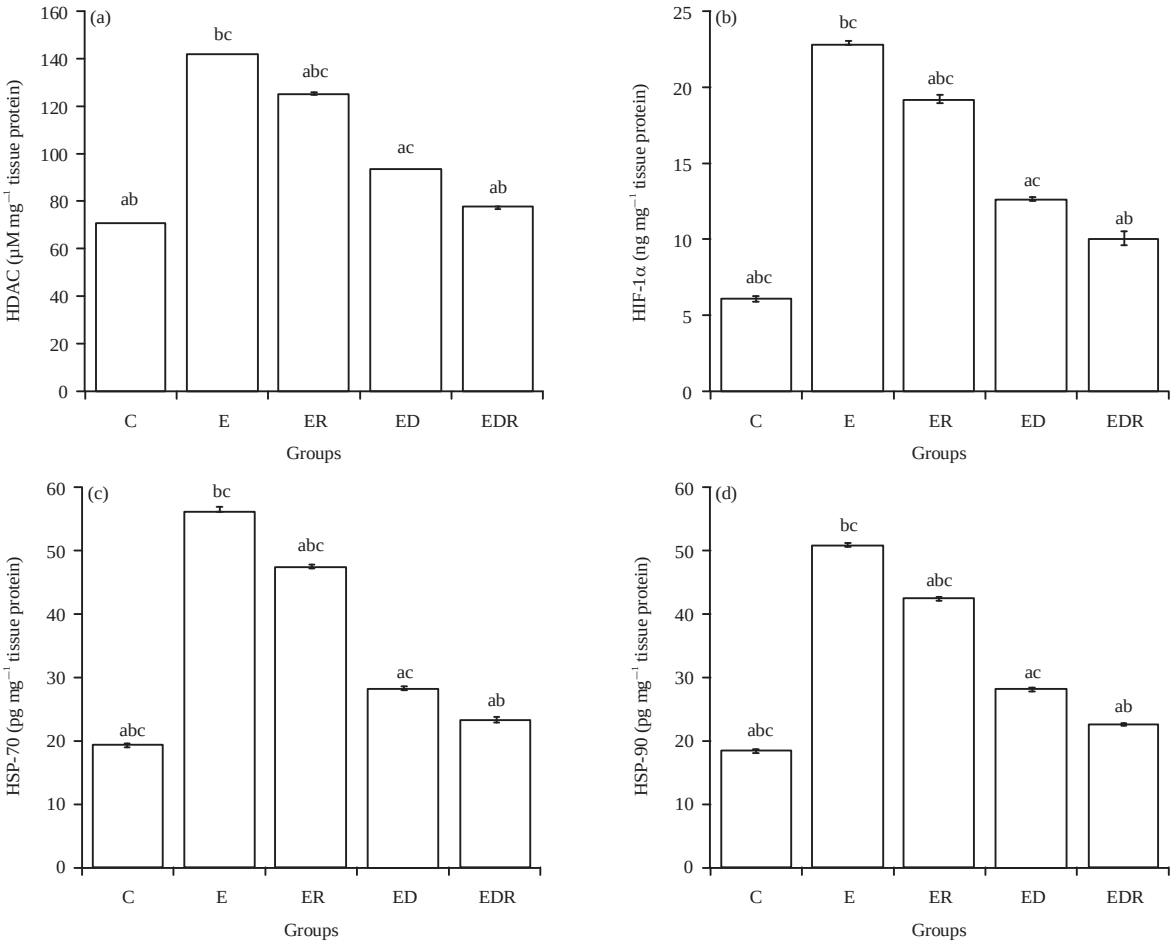

Fig. 3(a-d): Effect of D treatment combined with whole body exposure to fractionated doses of γ-radiation in ESC transplanted mice, (a) HDAC activity, (b) HIF-1α level, (c) HSP-70 level and (d) HSP-90 level in tumor tissue

Values are the mean±SEM. a, b and c indicates a statistically significant difference of $P < 0.05$ as compared with the E, ED and EDR groups (one-way ANOVA followed by Holm-Sidak as a post hoc test). C: Control (mock) mice, E: Mice injected with EAC cells, ER: Mice injected with EAC cells and exposed to a total dose of 8 Gy, ED: Mice injected with EAC cells and received D, EDR: Mice injected with EAC cells, received D and exposed to 8 Gy

of tumor vascularity correlates positively with disease stage and metastasis. A potent endothelial mitogen, VEGF correlates with vascular permeability and also induces B-cell lymphoma-2 (Bcl-2), which is vascular survival promoting agent[44,45]. Therapy against angiogenesis has been approved as an effective cancer treatment strategy and more than 120 novel antiangiogenic agents are in clinical trials[44]. Activation of VEGF as a pro-angiogenic factor is mediated by HIF-1α to induce tumor angiogenesis[4]. Hypoxic conditions have been demonstrated to regulate HDAC function both directly and indirectly through mediating HDAC involvement in oxygen regulated gene expression and hypoxia-induced angiogenesis[46]. One of the important findings in this study is that combination of D treatment with exposure to whole body γ-radiation (EDR group) significantly suppressed HDAC activity, repressed HIF-1α and modulated HSP-70 and -90 levels in tumor tissue of ESC-bearing mice (Fig. 3a-d). This finding sets up diosmin-hesperidin complex as a promising anti-tumor agent. In support of our findings, Kahali *et al.*[47] demonstrated that diosmin and hesperidin were able to restore Brahma (BRM) protein in BRM-deficient cancer cell lines and attributed this effect principally to HDAC inhibition induced by these flavonoids and this effect contributes to their anti-tumor potency. In addition, hesperidin was found to inhibit HIF-1α expression in human HMC-1 mast cell line[48].

Earlier studies have reported that degradation of HIF-1α is mediated by its interactions with the VHL protein, a tumor suppressor that acts as an ubiquitin ligase (E3)[49,50]. This interaction explains the high level of HIF-1α expression and the high degree of vascularization observed in VHL-deficient

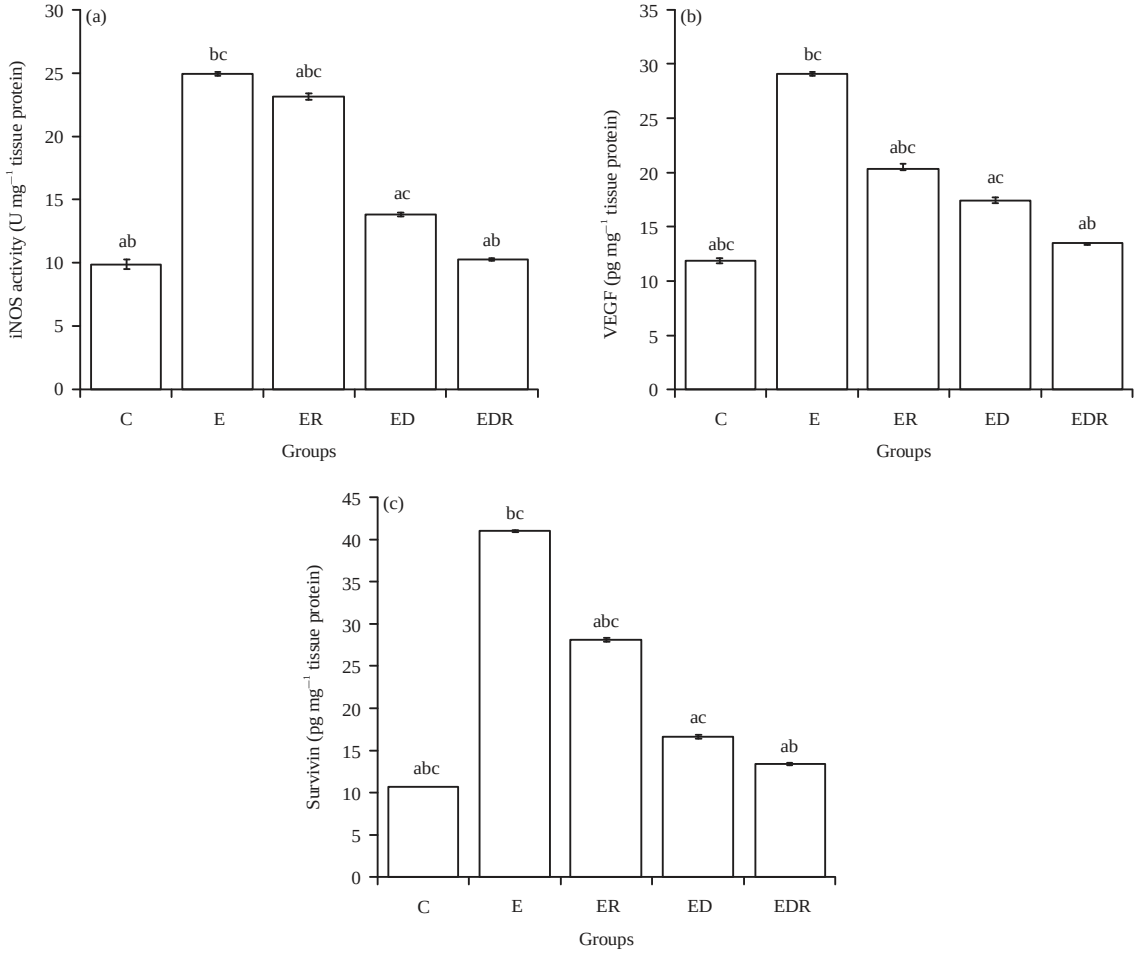

Fig. 4(a-c): Effect of D treatment combined with whole body exposure to fractionated doses of γ-radiation in ESC transplanted mice, (a) iNOS activity, (b) VEGF level and (c) Survivin level in tumor tissue

Values are the mean±SEM. a, b and c indicate a statistically significant difference of $p<0.05$ as compared with the E, ED and EDR groups (one-way ANOVA followed by Holm-Sidak as a post hoc test). C: Control (mock) mice, E: Mice injected with EAC cells, ER: Mice injected with EAC cells and exposed to a total dose of 8 Gy, ED: Mice injected with EAC cells and received D, EDR: Mice injected with EAC cells, received D and exposed to 8 Gy

tumors[51]. Moreover, Kim *et al.*[46] suggested that HDAC inhibitors down regulated HIF-1α by upregulating p53 and VHL, thus promoting HIF-1α degradation. However, Kong *et al.*[14] re-established this concept and elucidated that HDAC inhibitors (HDACIs) induced the proteasomal degradation of HIF-1α by a mechanism that is independent of VHL and p53 and did not require the ubiquitin system. This degradation pathway involved the enhanced interaction of HIF-1α with HSP70 and is secondary to a disruption of the HSP70/HSP90 axis function. Therefore, focused this research on HIF-1α and HSP70/HSP90 detection in ESC xenograft model and it's targeting by diosmin-hesperidin complex through D treatment in combination with R as a consequence of HDAC inhibition by D and R as observed in EDR group (Fig. 3a-d).

Although, HSP-90 is the most abundantly expressed HSP[52], HSP-70 at low levels acts to maintain cellular activities under stress conditions and safeguards cells from death induced by various pro-apoptotic insults[53]. Thus, their level is upregulated in tumor cells which mediates radioresistance[54]. It is generally known that one of the side effects of Hsp90 inhibition is the compensatory induction of Hsp70[55]. So, dual targeting of HSP-70 and HSP-90 improve therapeutic potential of radiotherapy[31]. In this context, our data confirmed that combined treatment of D with R in ESC-transplanted mice (EDR group) significantly decreased HSP-70 and -90 in tumor tissue (Fig. 3c and d). The antioxidant activity of diosmin and hesperidin is well documented in previous studies[56-58]. Thus, the effect of diosmin-hesperidin complex on HSP-70 and -90 could be interpreted in the view that treatment of ESC-bearing

mice with D induced the pre-conditioning of tumor cells against oxidative stress delivered through radiation mediated via their antioxidant effect and thus disable tumor cytoprotective machinery during irradiation which contributes to the radiosensitizing effect of diosmin-hesperidin complex on EC cells.

Interestingly, combined treatment of D with R in ESC-transplanted mice (EDR group) significantly depressed VEGF and survivin levels as well as iNOS activity in tumor tissue (Fig. 4a-c). This effect could be attributed to molecular targeting of pro-angiogenic chaperones (HSP-70/90) and proteasomal degradation of HIF-1α induced by diosmin-hesperidin. In EDR group, the observed suppressive effect on survivin levels is in consistence with previous findings[59]. The exerted pro-apoptotic effect is attributed to downregulation of specificity protein-1 (SP-1) by hesperidin, which in turn, leads to modulation of its downstream target proteins including survivin. The iNOS-derived NO has been recognized as one of the most versatile players in immune system and pathogenesis of various diseases including cancer[60]. In consistent, the current results revealed a significant elevation in iNOS activity in tumor tissues of the untreated ESC-bearing mice (E group) associated with tumor progression as shown in Fig. 4a, suggesting importance of inhibiting iNOS activity as a new target in tumor therapy. Noteworthy, Abdin *et al.*[36] found that there was a positive correlation between iNOS activity and ESC tumor progression. The elicited modulation in iNOS activity in tumor tissue of EDR group is attributed to the anti-inflammatory potency of diosmin and hesperidin. This is in agreement with previous reports[61,62]. In this study, disable of molecular mechanisms contributes to tumor survival and angiogenesis and subsequently metastasis was reflected on improved mice survival rate and tumor regression which was more pronounced in EDR group (Fig. 2a-d), suggesting the remarkable synergism between D and R in ESC xenograft model. Meanwhile, the plausible mechanism by which radiation affect tumor growth rate is mediated through damaging the DNA within the tumor cells, making them unable to divide and grow[63].

CONCLUSION

Taken together, the data sheds light on the radio sensitizing efficacy of diosmin-hesperidin complex (Daflon®) in ESC xenograft model in mice which is mediated through inhibiting HDAC activity, repressing HIF-1α level, disrupting HSP-70/90 response and subsequently decreased pro-angiogenic regulator VEGF and pro-survival oncoprotein, survivin in tumor tissue. This molecular effect was reflected on animal survival and tumor regression. Based on this study, diosmin-hesperidin complex might be a promising radiosensitizer in radiotherapy protocols.

SIGNIFICANCE STATEMENTS

This study discovers a novel mechanistic insight associated with the anti-angiogenic and radiosensitizing effects of diosmin-hesperidin complex in Ehrlich solid carcinoma in mice xenograft model through targeting pro-angiogenic mediators and HDAC activity in tumor micro environment. This study will help researchers to further uncover significant molecular targets of this drug. This drug complex could be used as a cheap, safe and effective adjuvant therapy in the treatment of a wide array of solid carcinoma.

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