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PRECLINICAL STUDIES

Acetylamine derivative of diospyrin, a plant-derived binaphthylquinonoid, inhibits human colon cancer growth in *Nod*-Scid mice

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Summary Anticancer activity of diospyrin and its derivatives (**1**–**5**) was evaluated against thirteen human cell lines. Compared to diospyrin (**1**), the acetylamine derivative (**4**) exhibited increase in cytotoxicity, particularly in HT-29 colon cancer cells, showing GI_{50} values of 33.90 and 1.96 μM, respectively. Also, enhanced toxicity was observed when cells, pre-treated with compound **4**, were exposed to radiation. In vivo assessment of **4** was undertaken on tumour-bearing *Nod*-Scid mice treated at 4 mg/kg/day. Significant reduction in relative tumour volume (~86–91 %) was observed during the 12th–37th days after drug treatment. Increased caspase-3 activity and DNA ladder formation was observed in HT-29 cells after treatment with **4**, suggesting induction of apoptotic death after drug treatment. Moreover, flow cytometric determination of Annexin V- FITC positive and PI negative cells demonstrated 17.4, 26.4, and 27.9 % of early apoptosis, respectively, upon treatment with 5, 10 and 25 μM of **4**. HT-29 cells after treatment with **4** (1–25 μM) revealed ~2.5- 3- folds generation of ROS. Furthermore, concentration dependent decrease of mitochondrial trans-membrane potential ($\Delta\psi_m$), and expression of Bcl-2/Bax and other marker proteins suggested involvement of mitochondrial pathway of cell death. Overall, our results demonstrated the underlying cell-death mechanism of the plant-derived naphthoquinonoid (**4**), and established it as a prospective chemotherapeutic 'lead' molecule against colon cancer.

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Introduction

Colon cancer is one of the causes of highest mortality among cancer-related deaths, mainly in the developed countries [1]. At an early stage, the strategic treatment involves surgical resection, followed by adjuvant chemo or radiotherapy. Unfortunately, the available therapeutic options often fail at an advanced stage of colon cancer, as about fifty percent of the patients die due to metastasis-related complications [2]. Therefore, attempts are going on to design small molecules capable of modulating cellular differentiation, or apoptosis in cancer cells [3, 4].

At present, nearly 50 % of available drugs are based on the molecules derived from natural products, the most exclusive source of medications since antiquity [5]. In the case of cancerous diseases this proportion surpasses 60 %, including quinonoids of the anthracycline family representing one of the largest groups of modern anticancer therapeutics, such as daunorubicin, doxorubicin and their analogues [6]. Quinonoids, in general, act as potent inhibitors of electron transport, uncouplers of oxidative phosphorylation, bioreductive alkylating agents, and as producers of reactive oxygen radicals [7]. Incidentally, critical analysis could validate the customary ethnomedical prescriptions based on certain medicinal plants with quinonoid constituents [8]. For example, some of the plant-derived naphthoquinonoids, like plumbagin, shikonin, β-lapachone, juglone, etc., are under investigation for their significant anticancer property [9–11]. Thus, there is an increasing interest to search and develop new drugs based on quinonoid molecules, particularly those originating from traditional medicinal plants [12–15].

In our laboratory, diospyrin, a binaphthylquinonoid compound, was isolated from the stem bark of *Diospyros montana* Roxb. (Family: Ebenaceae) and identified as an antitumour agent showing inhibitory activity against Ehrlich ascites carcinoma in mouse. Subsequently, careful modification of diospyrin template was carried out to obtain several analogues with enhanced efficacy against human cancer cell lines [16–18]. On further studies, a diethylether derivative of diospyrin was found to generate intracellular reactive oxygen species (ROS), and to induce apoptosis in human breast cancer (MCF-7) cell line [19].

Presently, we tested the dimethyl and diethyl ethers, aminoacetyl and diepoxide derivatives of diospyrin against a broad panel of thirteen human cancer cell lines of different histological origin for the first time in order to compare their anti-proliferative efficacy vis-à-vis diospyrin. The results showed that structural modification of the mother compound diospyrin could effectively enhance the cytotoxic property, more or less, against all the tested cell lines. However, the acetylamine derivative was found to be most promising, and was selected to evaluate the in vivo growth inhibitory activity against a solid tumour, using *Nod*-Scid mouse xenograft model of HT-29, a human colon adenocarcinoma cell line. Subsequently, the mode of action of this compound on HT-29 cells was studied through a number of fluorimetric approaches including intracellular ROS generating property, alteration of mitochondrial transmembrane potential, caspase activation, cell cycle analysis, externalization of membrane-associated phosphatidylserine, and fragmentation of intracellular DNA, considered as the hallmark event of apoptotic cell death. Further, the expression profiles of different apoptotic marker proteins were checked in the drug-treated cells through western blot analysis. In addition, the radiosensitization potential of the selected derivative was evaluated against HT-29 cells for the first time.

Materials and methods

Compounds

Diospyrin (**1**), isolated from the stem bark of *D. montana* Roxb. (Ebenaceae), was converted into its dimethyl ether (**2**), diethyl ether (**3**), aminoacetyl (**4**), and diepoxide (**5**) derivatives, as described previously [17, 18]. The compounds **1**–**5** (Fig. 1) were dissolved in sterile dimethyl sulfoxide (DMSO; Analytical grade) to prepare 10 mM stocks, and diluted with respective culture medium so that the active concentration of DMSO did not exceed 0.1 % (*v*/*v*) in the experiments.

Cytotoxicity of diospyrin and derivatives against human cancer cell lines

Diospyrin (**1**) and its four derivatives (**2**–**5**) were evaluated for tumour inhibitory activity against a panel of thirteen human cancer cell lines (Table 1) available at the 'Anticancer Drug Screening Facility' at ACTREC, Tata Memorial Centre, Mumbai, through sulforhodamine B (SRB) assay. Briefly, cells (5×10^3cells/well; 90 μl) were inoculated into 96-well plates and incubated at 37 °C in humidified 5 % CO_2 atmosphere for 24 h. Aliquots of 10 μl drug dilutions were added to the appropriate wells, incubated for 48 h, and the assay was terminated with cold TCA. After 60 min, SRB solution (50 μl) at 0.4 % (*w*/*v*) in 1 % acetic acid was added to each of the wells, and the plates were incubated for 20 min at room temperature. Subsequently, the bound stain was eluted with 10 mM Tris base, and the absorbance was read on a plate reader at a wavelength of 540 nm, with 690 nm as the reference wavelength. GI_{50} value (concentration inhibiting 50 % cell growth) for each compound against each of the cell lines was determined from the respective dose-response curve [20, 21]. Each assay was performed in triplicate, and the results were expressed as the mean of three independent experiments.

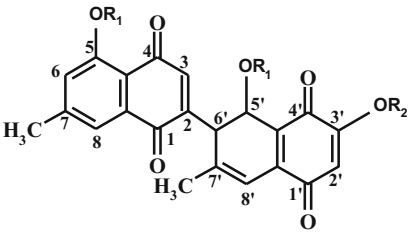
Colon cancer cell culture

Human colon adenocarcinoma (HT-29) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured and subsequently maintained in DMEM medium supplemented with 10 % heat-inactivated fetal bovine serum and antibiotics (streptomycin 200 μg/ml, gentamicin 200 μg/ml, and penicillin 100 units/ml) at 37 °C in humidified 5 % CO_2 atmosphere (CO_2 incubator; Nuaire 5500E, USA). The medium was removed after 24 h of plating the cells, and replenished with fresh medium containing either the tested compounds at different concentrations, or DMSO as the vehicle control.

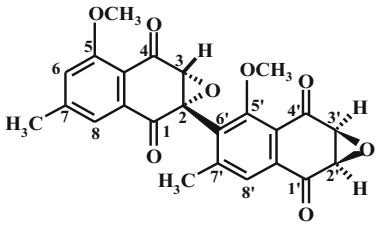
Clonogenic assay

For clonogenic survival assessment, HT-29 cells (5×10^5) were plated and incubated overnight, followed by treatment with the test compound (10 μM). After 24 h, cells were harvested, counted, and approximately 500 cells per plate were seeded in new 100 mm culture dishes and kept at 37 °C in CO_2 incubator [22]. After 2 weeks, the plates were rinsed with phosphate buffer saline (PBS), fixed with methanol and stained with Giemsa. Colonies consisting of 50 or more cells were counted to compare with the untreated control group in the experiment.

Fig. 1 Chemical structures of compounds **1**–**5**



Compounds 1-4



Compound 5

Compounds	R_1	R_2
1	-H	-H
2	$-CH_3$	-H
3	$-C_2H_5$	-H
4	$-CH_3$	$-NHCOCH_3$

Tumour xenograft study in vivo

HT-29 tumours were grown in the dorsal right hind limb of each experimental male *Nod*-Scid mouse. Briefly, tumour from the donor mouse was cut into small pieces of approximately 2 mm with the help of forceps and scalpel blade, and transplanted aseptically into experimental mice. Once the tumors attained a measurable growth, the mice were included in the testing groups comprising six mice in each group. The 'treated' group was administered with the test compound by intraperitoneal injection at a dose of 4 mg/kg/day for 5 days, and kept under observation for 37 days. At the end of the experiment, tumour volume was measured. The ratio of the tumour volume of 'treated' group (T) and the same of tumor-bearing untreated 'control' sets (C) was calculated and expressed as T/C value. T/C ≤ 0.2 is considered to demonstrate activity. The experiments were conducted in accordance with the approved protocol of the Institutional Animal Ethics Committee.

Determination of intracellular ROS generation

Intracellular ROS generation in HT-29 cells was measured with fluorescence probe DCFH-DA (2′, 7′-dichlorodihydrofluorescin diacetate) [23]. Briefly, trypsinized tumour cells (2×10^5 cells/ml) were incubated overnight at 37 ° C for attachment. After 24 h, the cells were treated with different concentrations (1, 5, 10 and 25 μM) of the tested sample diluted in culture medium and again incubated for 24 h under the same condition. Cells were then harvested, washed with sterile PBS (pH 7.4), and incubated with DCFH-DA (10 μM) for 1 h. The fluorescent intensity of each sample was measured at 520 nm using an excitation wavelength of 490 nm. Each assay was performed in triplicate.

Table 1 Cytotoxicity of diospyrin and derivatives against 13 human cancer cell lines in terms of GI_{50} values (μM)

Cancer cell lines	Breast			Colon			CNS	Neuro-blastoma	Oral	Prostate		Lung	Melanoma
Compounds	MDA-MB-435	MCF-7	ZR-75-1	HCT-15	HT-29	Colo-205	U373	IMR32	KB	DU- 145	PC-3	A549	SK-MEL-2
1	0.18	2.54	2.10	2.11	33.90	0.13	2.07	2.23	0.17	2.46	0.20	2.45	2.21
2	0.19	2.30	0.16	0.17	2.28	0.14	0.18	0.18	0.17	2.04	0.18	2.36	0.18
3	0.19	2.25	0.15	0.18	2.19	0.13	0.19	0.18	0.18	0.19	0.19	2.32	0.18
4	0.17	2.25	0.15	0.17	1.96	0.13	0.17	0.18	0.18	0.17	0.18	2.17	0.16
5	0.20	2.27	0.21	0.23	2.38	0.14	0.18	0.20	0.17	2.38	0.19	38.50	0.23
Adriamycin	0.13	0.17	0.10	0.17	0.14	<0.10	<0.10	<0.10	<0.10	0.14	0.16	0.12	0.13

Change in mitochondrial transmembrane potential ($\Delta\psi_m$)

Alteration in mitochondrial transmembrane potential ($\Delta\psi_m$), following drug treatment, was estimated fluorimetrically using JC-1 probe. Briefly, trypsinized cells (2×10^5 cells/ml) were incubated overnight at 37 °C for attachment and treated with different concentrations (10 and 25 μM) of the test sample. After incubation at 37 ° C, cells were harvested, washed with sterile PBS (pH 7.4), and incubated with JC-1 (10 μg/ml). After 30 min, fluorescence intensity was measured at 530 and 590 nm in a fluorescence spectrophotometer (Perkin Elmer LS55) using an excitation wavelength of 485 nm. The ratio of intensity values obtained at 590 and 530 nm was plotted as the relative fluorescence intensity (R.F.I.) for each tested concentration. Each assay was performed in triplicate.

Western blot analysis of apoptotic proteins

Total cellular proteins derived from treated as well as untreated cells were prepared as described previously [24]. Briefly, tumour cells (2×10^6 cells/ml) treated with 10 and 25 μM of compound **4** and incubated at 37 ° C temperature in a humidified 5 % CO_2 incubator for 24 h. The cells were scraped out from the culture plates, washed twice in cold PBS, and boiled for 5 min with lysis buffer [20 mM Tris-HCl, pH 7.5, 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.1 % (*v*/*v*) β mercaptoethanol] mixed with SDS-PAGE Laemmli buffer (4×) [20 mM Tris-HCl, pH 6.8, 4 % SDS, 20 % β-mercaptoethanol, 2 mM EDTA, 0.004 % bromophenol blue, 40 % glycerol]. The cell lysate from each sample (25 μg protein) was separated on SDS-PAGE (12 %) and observed for the expression of Bcl-2, Bcl-XL, Bax, Mcl-1 and α-tubulin.

Measurement of caspase activity

Trypsinized cells (2×10^5 cells/ml) were treated with tested compound at different concentrations (5, 10, 25 μM) and cultured for 24 h at 37 °C in CO_2 incubator. Caspase-8 activity in harvested cells was assessed using a colorimetric assay kit (Sigma, St Louis, MO, USA) as per manufacturer's instructions. Results were expressed as relative optical density, which is the ratio of O.D. values of respective treated cells and untreated control measured at 405 nm in an ELISA reader (Bio-rad Model 680, USA). Caspase-3 activity in similarly treated cells was assessed using a fluorimetric kit (Sigma, St Louis, MO, USA) as per manufacturer's instructions. Results were expressed in terms of relative fluorescence intensity (R.F.I.), which is the ratio of fluorescence intensity of treated cells and untreated control measured at 460 nm in a fluorescence spectrophotometer (Perkin Elmer LS55).

DNA laddering by agarose gel electrophoresis

Trypsinized tumour cells (1×10^6 cells/ml), cultured for 24 h in the presence or absence of different concentrations (5, 10, 25, and 50 μM) of drugs, were harvested and re-suspended in digestion buffer [500 μl; 10 mM EDTA, 50 mM Tris–HCl (pH 8.0) and 0.5 % sodium lauryl sulfate] to which 0.5 mg/ml proteinase K was added. The mixture was incubated for 3 h at 37 ° C in the presence of 0.1 mg/ml RNase A. The DNA material was extracted by phenol/chloroform (1:1) treatment and precipitated by adding 3 M sodium acetate and ice-cold ethanol (100 %) to the aqueous phase. Following overnight incubation at −20 °C, the material was centrifuged and the pellet was collected, air-dried and re-suspended in Tris–EDTA buffer (50 μl, pH 8.0). DNA aliquots (10 μg) were electrophoresed on 0.8 % agarose gel containing ethidium bromide (0.5 μg/ml), using Tris–acetate–EDTA (pH 8.0) running buffer, observed and photographed under UV light.

Flow cytometric analysis of cell cycle

Trypsinized cells (1×10^6 cells/ml), untreated or treated with different concentrations of drug (5, 10, 25 μM) were incubated for 24 h at 37 °C. Cells were then harvested, washed with PBS, and fixed in 70 % ethanol and incubated overnight at −20 °C. Then, the cells were re-suspended in PBS (pH 7.4), and ethidium bromide (50 μg/ml) and RNase A (50 μg/ml) were added before incubation at room temperature in darkness for 20 min. The fluorescence intensity was recorded and analyzed with a flow cytometer (Becton Dickinson, San José, CA, USA) using a 595 nm filter. The results were recorded as histogram to show the shift in percentage of treated cells in sub-G1 region with respect to the untreated control.

Determination of Annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) binding

Cells (1×10^6 cells/ml) were treated with or without tested sample at different concentrations (5, 10, 25 μM) and incubated at 37 °C. After 24 h, cells were harvested, washed with PBS, and re-suspended in binding buffer (1x). After incubation for 1 h, FITC and PI were added to each set and kept at room temperature for 20 min in darkness. Subsequently, treated and untreated cells were analysed on a flow cytometer (Becton Dickinson, San José, CA, USA) using a 515 nm filter for FITC fluorescence (FL-1H) and a 623 nm filter for PI detection (FL-2H), and a dot plot of FL-1H (X-axis; FITC fluorescence) versus FL-2H (Y-axis; PI fluorescence) was recorded.

Cell proliferation in combination with radiation

The antiproliferative effect of the compound, alone, and in combination with 2 and 5Gy radiation on HT 29 cell line was

assessed in vitro by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reduction assay [25]. Briefly, trypsinized cells (1×10^4/well) were incubated overnight in 96-well plate at 37 °C for attachment. Cells were then treated with drugs at different concentrations in a final volume of 200 μl. 2Gy and 5Gy radiation was applied to respective sets 1.5 h after drug treatment. Wherever required, cells were irradiated at room temperature using ^{60}Co teletherapy machine Bhabhatron-II (Panacea Medical Technologies, Bangalore, India) at a dose rate of 1Gy/min. The culture plate was incubated for 48 h, followed by addition of MTT (0.5 mg/ml, in PBS), to each well. The plates were then further incubated for 3 h at 37 °C. Thereafter, the cell pellet was re-suspended in DMSO (100 μl) and absorbance (O.D.) was measured at 570 nm in an ELISA reader (Bio-rad Model 680, USA). A parallel study was performed on the normal human intestinal cell line, INT 407, procured from NCCS, Pune, India. The percentage of cell death was calculated with respect to untreated control using the following formula:

$$\%\ \text{Cell death} = \frac{\text{Control O.D.} - \text{Treated O.D.}}{\text{Control O.D.}} \times 100\%$$

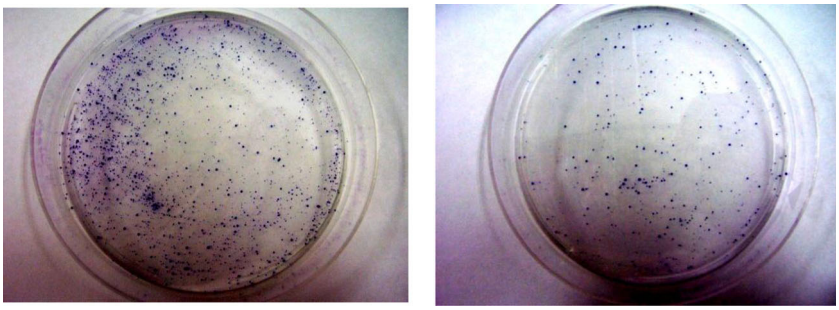
Results and discussion

In our earlier studies, diospyrin and its semisynthetic analogues have been found to exhibit variable cytotoxicity against human cancer cell lines [16–18]. Presently, we carried out a comprehensive assessment of the antitumour efficacy of diospyrin (**1**) and four of its derivatives (**2**–**5**) (Fig. 1) against a panel of thirteen cancer cell lines by sulforhodamine-B (SRB) semi-automated assay protocol. Previously, the aminoacetyl derivative of diospyrin (compound **4**) was reported for its marked inhibitory activity against A375 and Hep2 cells, as well as Ehrlich ascites carcinoma in vivo [18]. Furthermore, it also exhibited a fairly tolerable toxicity against human lymphocytes (PBMC) indicating a selectivity index in its favour. In the present study also, the compound **4** demonstrated notable cytotoxicity in comparison to the standard drug adriamycin (GI_{50}=~0.1 μM, or less) against the tested cell lines originating from breast, colon, neuronal, oral, prostate, lung and skin cancers (Table 1). It was found that only this derivative (**4**) could exhibit consistent enhancement of activity (GI_{50}=~0.13–2.25 μM) against all the thirteen cell lines when compared to diospyrin (**1**) itself (GI_{50}=~0.17–34 μM). Recently, Kawamura, et al. [15] also reported substantial improvement in the anti-cancer activity of plumbagin through acylation. Presently, we observed that the enhancement of activity exhibited by the aminoacetyl derivative (**4**) was most pronounced against the HT-29 colon cancer, showing GI_{50}= 1.96 μM, which was more than 17-fold stronger in comparison to **1** (GI_{50}=33.90 μM). Therefore, compound **4** was envisaged for detailed investigation against HT-29. Actually, this is the first time that diospyrin and its derivatives were found to be effective against three colon cancer cell lines (HCT-15, HT- 29 and Colo-205) endowed with distinct biological characters. In fact, HT-29 is known to be one of the most challenging solid tumours in view of its chemo- and radio- resistant properties [26, 27]. Therefore, the present endeavour was to evaluate compound **4** against a colorectal tumour model of HT-29 in vivo, and to elucidate the relevant cell death mechanism in vitro. A preliminary assessment of the radio-sensitising potential of this compound against HT-29 tumour cells was also undertaken.

To start with, an MTT assay was performed to estimate the anti-proliferative effect of compound **4** on HT-29 cells, and the IC_{50} value was found to be 5.7±0.2 μM, which was essentially comparable to the GI_{50} value obtained from the SRB-assay. In a separate experiment, compound **4** demonstrated reasonable toxicity against normal human intestinal cells (INT 407) showing IC_{50} value >25 μM (data not shown). Here, it may be mentioned that HT-29 cell proliferation was reportedly inhibited by plumbagin, another naphthoquinonoid constituent of medicinal plants, and the IC_{50} value (62.5 μM) obtained by MTT assay was more than 10-fold greater than compound **4** [28]. Further, clonogenic assay was performed with compound **4** (10 μM) to corroborate its tumour-inhibitory property. The result demonstrated 82 % reduction in clone number in comparison to the untreated control set (Fig. 2).

Next, a group of *Nod*-Scid mice bearing human HT-29 xenograft were treated with compound **4** (4 mg/kg/day; i.p.). After 15 days, a minimum T/C ratio of 0.09 was observed indicating 90.9 % inhibition of tumour growth compared to the untreated control group (T/C ≤ 0.2 means positive activity) [29]. After 37 days of observation, the T/C value was found to be 0.14 [Table 2; Fig. 3]. In a separate experiment, a group of normal mice treated with compound **4** did not show any visible toxicity in the liver, spleen, or intestine after 30 days of observation (data not shown).

The promising result obtained from the in vivo study led us to investigate the cell death mechanism of compound **4** in HT-29 cell line. It has been postulated that the commonly used drugs for the treatment of human malignancies induce apoptosis in the cancer cells. Hence, it was our aim to determine the apoptosis inducing potential of compound **4** against HT-29 cells in vitro. By all accounts, the most important cytotoxic mechanism of quinonoid class of compounds involves bioreduction, followed by reaction with molecular oxygen and generation of ROS [7]. Eventually, ROS- induced DNA damage and associated intracellular modifications lead to apoptotic mode of cell death [30]. In fact, the role of reactive oxygen species (ROS) in mediating the apoptotic process is



Untreated Control Treated with 10μM compound 4

Fig. 2 The inhibition of colony formation in HT-29 cells exposed to compound **4**. After 2 weeks, the colonies (greater than 50 cells) were counted under the dissecting microscope, and the data were expressed as percent inhibition compared to untreated control set

being increasingly recognized as a key mechanism of anticancer activity exhibited by quinonoid compounds [31].

Earlier studies showed that diospyrin analogues could induce cell death through mitochondria-mediated generation of ROS [19, 23]. Therefore, we measured the endogenous ROS level in HT-29 cells, following treatment with different doses of **4**, by using the fluorimetric probe, DCFH-DA. The data presented in Fig. 4a indicated substantial amount of ROS generation at 10 and 25 μM concentrations (~2.5- 3- fold) of **4** in cultured HT-29 cells. The compound also demonstrated a concentration dependent drop of mitochondrial transmembrane potential (Δψm), which is considered to be a crucial event for initiating programmed cell death pathway. The study was performed by using the fluorescent probe JC-1 [Fig. 4b]. Here, R.F.I. values for the cells treated with 10 and 25 μM of compound **4** were found to be ~2.5 and 1.5, respectively, in comparison to the untreated control set (R.F.I. value ~6.0).

Mitochondrial integrity is known to be regulated by pro- and anti-apoptotic members of the Bcl-2 group of proteins, such as Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic). Hence, we investigated the Bcl-2 and Bax expression along with other associated apoptotic marker proteins in HT-29 cells following treatment with compound **4** by western blot. As shown in Fig. 5, moderate inhibition of Bcl-2 at 10 μM occurred in response to treatment with compound **4**. Modulations in the expression of Bax, Mcl-1 and Bcl-X proteins have also been observed after treatment (Fig. 5).

In the present study, fluorimetric assessment clearly showed a dose-dependent increase (~2-fold) in downstream caspase-3 activity up to 25 μM of compound **4** [Fig. 6a], which confirmed its involvement in programmed cell death in HT-29. However, according to Fig. 6b, no change in the activity of caspase-8 was observed within a dose range of 5–25 μM of compound **4**, indicating that the extrinsic apoptotic pathway was probably not involved in this process. Here, it is to be noted that previous workers also observed the induction of apoptosis through activation of caspase-3 and cytochrome-c in HT-29 cells treated with 75 μM of plumbagin [28].

Table 2 Antitumour activity of compound **4** against HT-29 xenograft in *Nod*-Scid mice

Days	Tumour volume (c.c.)		Relative tumour volume			
	Control	Treated [a]	Control (C)	Treated (T) [a]	T/C [b]	Percent reduction in relative tumour volume [c]
1	0.06±0.01	0.04±0.01	1	1	1	0
5	0.16±0.09	0.03±0.11	2.58±1.07	0.81±0.27	0.31	68.6
9	0.29±0.19	0.06±0.03	4.86±3.00	1.41±0.79	0.29	71.0
12	0.34±0.17	0.03±0.01	5.74±2.40	0.71±0.31	0.12	87.6
15	0.45±0.21	0.03±0.02	7.82±3.78	0.71±0.44	0.09	90.9
18	0.46±0.21	0.05±0.06	8.07±3.69	1.07±1.29	0.13	86.7
22	0.47±0.21	0.05±0.06	8.14±3.69	1.09±1.34	0.13	86.6
26	0.55±0.28	0.06±0.07	9.76±5.58	1.35±1.62	0.14	86.2
30	0.68±0.41	0.07±0.06	12.44±7.90	1.67±1.31	0.13	86.6
37	0.70±0.42	0.07±0.07	12.84±8.09	1.76±1.48	0.14	86.3

[a] Mice (n=6) were treated at a dose of 4 mg//kg /day, i. p., for 5 days

[b] T/C ≤0.2 is considered to demonstrate activity

[c] Percent reduction in relative tumour volume = (Relative tumour volume of control - Relative tumour volume of treated / Relative tumour volume of control)×100

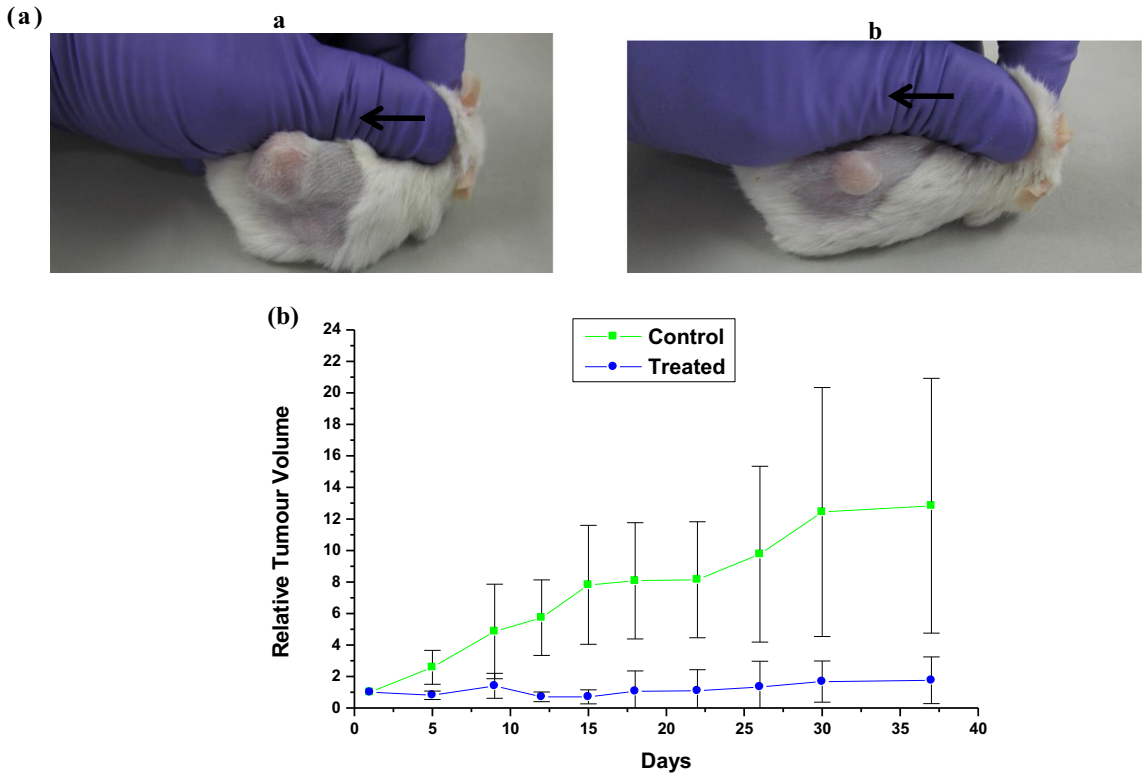


Fig. 3 **a** Suppression of tumor growth in *Nod*-Scid mice xenograft of HT-29 cells, following treatment with compound **4** (4 mg/kg/day for 5 days; i.p.). Photographs of one representative mouse from: (*a*) 'untreated control' (n=6), and (*b*) 'treated' (n=6) groups on day 37 post treatment; **b** Graphical representation of relative tumour volume of 'treated' as well as 'untreated control' groups of mice during the in vivo experiment

Further, oligonucleosomal DNA fragmentation pattern was observed in gel electrophoresis performed on HT-29 cells following treatment with 5, 10, 25 and 50 μM of compound **4** (Fig. 7). These DNA fragments appear as sub-G1 peak in the DNA content frequency histogram. Distribution of cells in different phases of cell cycle in terms of the cellular DNA content was quantified by flow cytometry using ethidium bromide (EtBr) as the labeling agent. EtBr crosses the plasma membrane, binds to cellular DNA, and the DNA fragments in apoptotic cells give a fluorescence signal at lower than G1 region (sub-G1). Following 24 h incubation with 5, 10 and 25 μM of compound **4**, respectively, 8.8, 10.8, and 8.3 % of the cells were found in the sub-G1 region, as shown in Fig. 8a.

Externalisation of the inner membrane-associated phospholipid phosphatidylserine (PS), a characteristic of early apoptotic cells, was studied by Annexin-V-FITC binding assay following treatment with different concentrations of compound **4** [32]. Simultaneous application of the DNA binding dye propidium iodide (PI) and analysis of the stained cells by flow cytometry was used to discriminate the necrotic or late

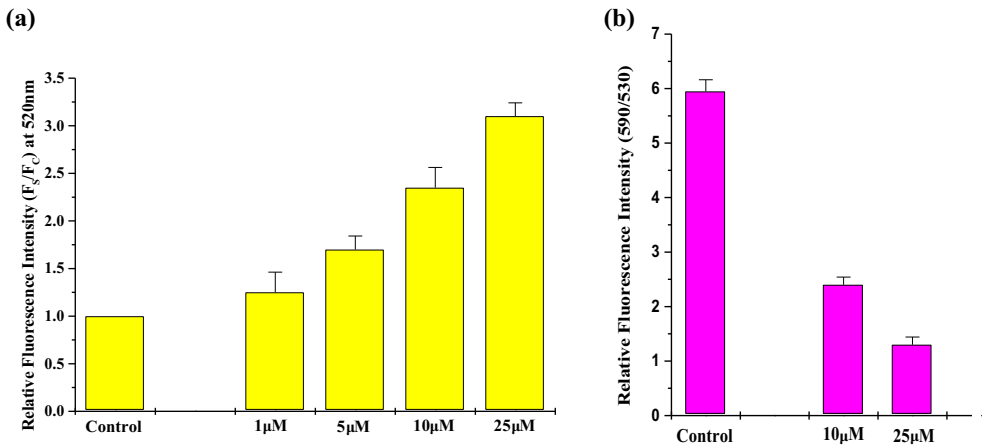


Fig. 4 **a** Analysis of the intracellular ROS generation in HT-29 cells treated with 1, 5, 10 and 25 μM of compound **4** using the fluorimetric probe DCFH-DA; **b** Analysis of the mitochondrial membrane potential of HT-29 cells, treated with 10 and 25 μM of compound **4**, stained with the potentiometric probe JC-1

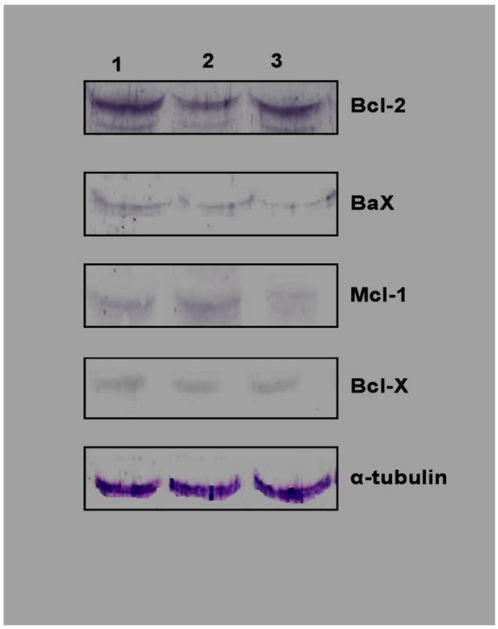


Fig. 5 Effect of compound **4** (10 and 25 μM) on Bcl-2, Bax, Mcl-1, and Bcl-x protein expression in HT-29 cells. *Lanes* 1, 2 and 3 represent untreated control, treated with 10 and 25 μM of compound **4**, respectively

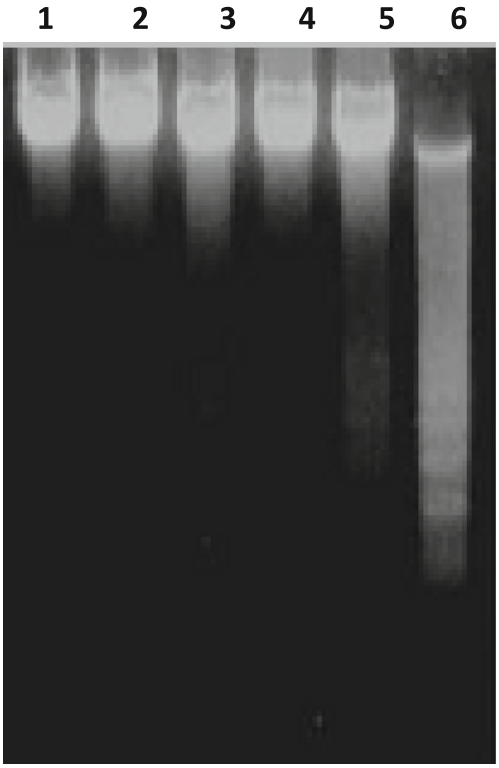


Fig. 7 Determination of apoptotic cell death by DNA fragmentation in HT-29 cells. Lane 1 and 2: DNA from untreated control set; Lane 3–6: DNA from HT-29 cells treated with 5, 10, 25 and 50 μM of compound **4**, respectively

apoptotic cells from the early apoptotic ones. The percentage of early apoptotic cells (Annexin V-FITC positive and PI negative) increased from 0.0 % in untreated control to 17.4, 26.4, and 27.9 %, following treatment with 5, 10 and 25 μM of compound **4**, respectively [Fig. 8b].

HT-29 is known to be a relatively radio-resistant cell line, although the mechanism behind it remains unclear [27]. Nevertheless, to explore the potential of compound **4** to enhance the sensitivity of HT-29 tumor cells towards radiation, a combinatorial study was performed with different concentrations of this compound in presence of radiation exposures. The results showed that exposure to 2 and 5Gy radiation alone resulted in ~27 to 38 % cell death, respectively, whereas a combination treatment with compound **4** (2.0 μM) could effectively enhance the cytotoxicity up to ~64 % in both the cases. As already mentioned above, compound **4** could reduce 50 % of the cell proliferation at a concentration of 5.7 μM in MTT assay. However, administration of the drug at almost 3-fold lower dose along with radiation was found to decrease the viability of the cancer cells by more than 60 %. This would indicate the prospective radio-sensitizing efficacy of compound **4** for treatment of colon cancer.

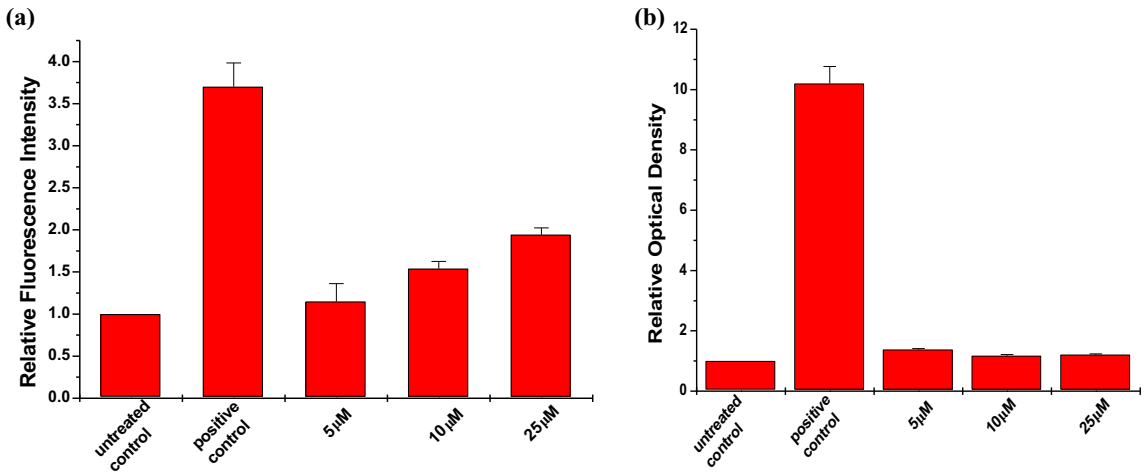


Fig. 6 Effect of compound **4** (5, 10, 25 μM) on (**a**) caspase 3, and (**b**) caspase 8 expression in HT-29 cells

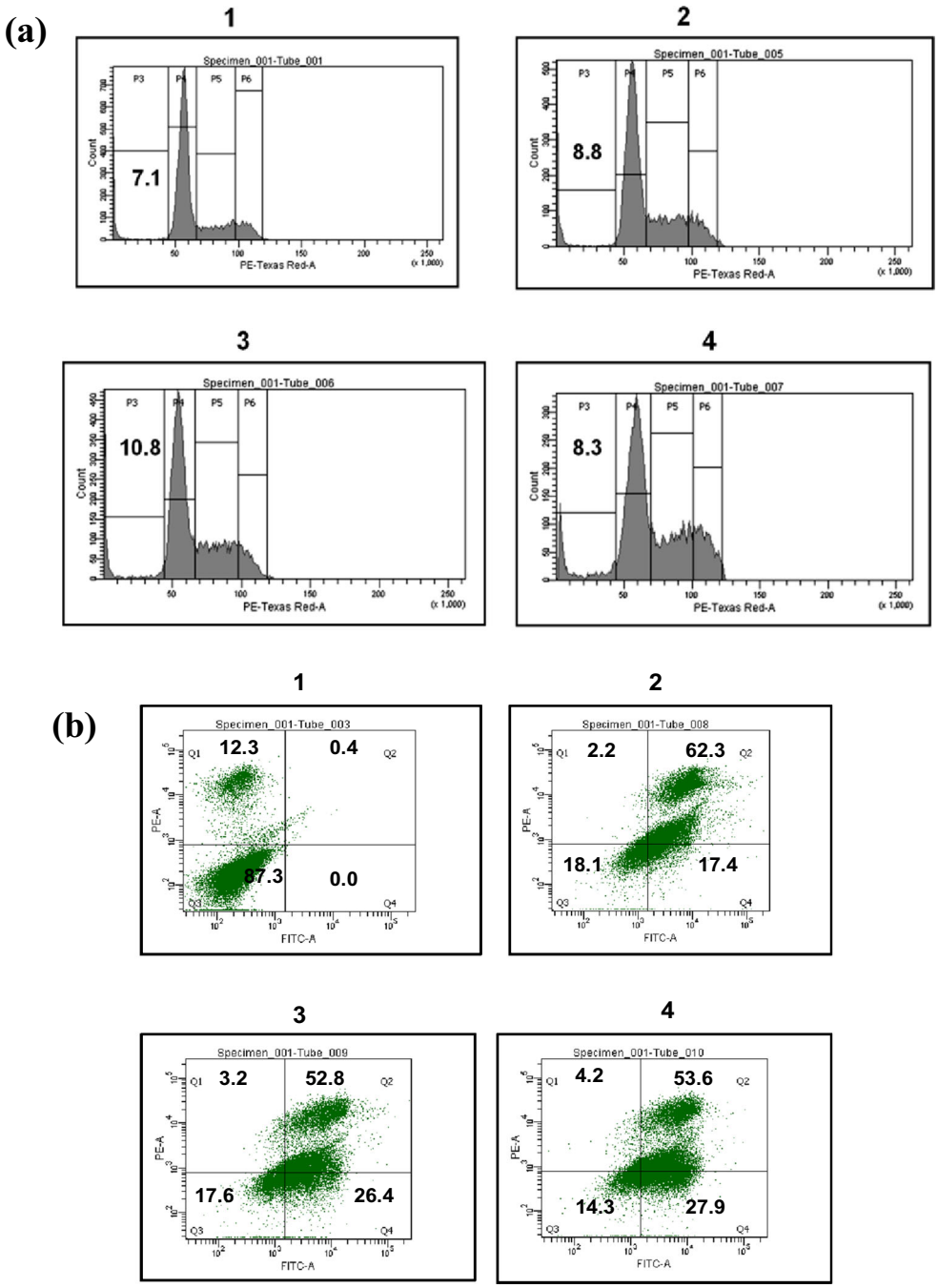


Fig. 8 **a** Determination of sub-G1 peak of HT-29 through cell cycle analysis: cells were treated with (*2*) 5 μM, (*3*) 10 μM and (*4*) 25 μM of compound **4** for 24 h, along with (*1*) untreated control; **b** Flow cytometry analysis of phosphatidyl serine externalisation of HT-29 cell. Cells treated with (*2*)5 μM, (*3*)10 μM and (*4*) 25 μM of compound **4** are presented in a *dot-plot* analysis along with untreated controls. The unstained population is also shown as (*1*) internal intensity control

Taken together, an acetylamine derivative of diospyrin dimethylether (**4**) could effectively inhibit the HT-29 cancer cells in vitro, as well as in a *Nod*-Scid mouse xenograft model. Further, HT-29 cells were found to demonstrate hallmark features of apoptosis after treatment with compound **4**. Preliminary observations in a combinatorial study performed in presence of radiation exposure indicated the prospective radiosensitizing property of this derivative of diospyrin. However, detailed investigation would be necessary to elucidate the mechanism of this event. In fact, all the aforesaid observations assumed added significance in view of the purported chemo- and radio-resistant character of HT-29 cells. Hence, this compound could be a promising 'lead' for development of a novel chemotherapeutic agent against colon cancer.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Haggar FA, Boushey RP (2009) Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg 22:191–197
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
3. Figarola JL, Weng Y, Lincoln C, Horne D, Rahbar S (2012) Novel dichlorophenyl urea compounds inhibit proliferation of human leukemia HL-60 cells by inducing cell cycle arrest, differentiation and apoptosis. Invest New Drugs 30:1413–1425
4. Senderowicz AM (2004) Targeting cell cycle and apoptosis for the treatment of human malignancies. Curr Opin Cell Biol 16:670–678
5. Kingston DGI (2011) Modern natural products drug discovery and its relevance to biodiversity conservation. J Nat Prod 74:496–511
6. Lown JW (1993) Anthracycline and anthraquinone anticancer agents: current status and recent developments. Pharmacol Ther 60: 185–214
7. Gewirtz DA (1999) A critical evaluation of the mchanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57:727–741
8. Hazra B, Das Sarma M, Sanyal U (2004) Separation methods of quinonoid constituents of plants used in Oriental traditional medicines. J Chromatogr B 812:259–275
9. Powolny AA, Singh SV (2008) Plumbagin-induced apoptosis in human prostate cancer cells is associated with modulation of cellular redox status and generation of reactive oxygen species. Pharm Res 25:2171–2180
10. Jang SY, Jang EH, Jeong SY, Kim JH (2014) Shikonin inhibits the growth of human prostate cancer cells via modulation of the androgen receptor. Int J Oncol 44:1455–1460
11. Pardee AB, Li YZ, Li CJ (2002) Cancer therapy with beta-lapachone. Curr Cancer Drug Targets 2:227–242
12. Kogan NM, Rabinowitz R, Levi P, Gibson D, Sandor P, Schlesinger M, Mechoulam R (2004) Synthesis and antitumor activity of quinonoid derivatives of cannabinoids. J Med Chem 47:3800–3806
13. da Silva EN, Jr CBC, Guimarães TT, Pinto Mdo C, Cabral IO, Pessoa C, Costa-Lotufo LV, de Moraes MO, de Andrade CK, Dos Santos MR, de Simone CA, Goulart MO, Pinto AV (2011) Synthesis and evaluation of quinonoid compounds against tumor cell lines. Eur J Med Chem 46:399–410
14. de Castro SL, Emery FS, da Silva Júnior EN (2013) Synthesis of quinoidal molecules: strategies towards bioactive compounds with an emphasis on lapachones. Eur J Med Chem 69:678–700
15. Kawamura M, Kuriyama I, Maruo S, Kuramochi K, Tsubaki K, Yoshida H, Mizushina Y (2014) Anti-tumor effects of novel 5-O-acyl plumbagins based on the inhibition of mammalian DNA replicative polymerase activity. PLoS One 9:e88736
16. Chakrabarty S, Roy M, Hazra B, Bhattacharya RK (2002) Induction of apoptosis in human cancer cell lines by diospyrin, a plant-derived bisnaphthoquinonoid, and its synthetic derivatives. Cancer Lett 188: 85–93
17. Das Sarma M, Ghosh R, Patra A, Hazra B (2007) Synthesis and antiproliferative activity of some novel derivatives of diospyrin, a plant-derived naphthoquinonoid. Bioorg Med Chem 15:3672–3677
18. Das Sarma M, Ghosh R, Patra A, Hazra B (2008) Synthesis of novel aminoquinonoid analogues of diospyrin and evaluation of their inhibitory activity against murine and human cancer cells. Eur J Med Chem 43:1878–1888
19. Kumar B, Kumar A, Pandey BN, Mishra KP, Hazra B (2009) Role of mitochondrial oxidative stress in the apoptosis induced by diospyrin diethylether in human breast carcinoma (MCF-7) cells. Mol Cell Biochem 320:185–195
20. Skehan P, Storeng R, Scudiero D, Monks A, McMohan J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107–1112
21. Vichai V, Kirtikara K (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 1:1112–1116
22. Sandhya T, Mishra KP (2006) Cytotoxic response of breast cancer cell lines, MCF 7 and T 47 D to Triphala and its modification by antioxidants. Cancer Lett 238:304–313
23. Kumar B, Kumar A, Pandey BN, Hazra B, Mishra KP (2008) Increased cytotoxicity by the combination of radiation and diospyrin diethylether in fibrosarcoma in culture and in tumor. Int J Radiat Biol 84:429–440
24. Kumar B, Kumar A, Ghosh S, Pandey BN, Mishra KP, Hazra B (2012) Diospyrin derivative, an anticancer quinonoid, regulates apoptosis at endoplasmic reticulum as well as mitochondria by modulating cytosolic calcium in human breast carcinoma cells. Biochem Biophys Res Commun 417:903–909
25. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
26. Dallas NA, Xia L, Fan F, Gray MJ, Gaur P, Van Buren G 2nd, Samuel S, Kim MP, Lim SJ, Ellis LM (2009) Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. Cancer Res 69: 1951–1957
27. Schwarz SB, Schaffer PM, Kulka U, Ertl-Wagner B, Hell R, Schaffer M (2008) The effect of radio-adaptive doses on HT29 and GM637 cells. Radiat Oncol 3:12
28. Subramaniya BR, Srinivasan G, Sadullah SS, Davis N, Subhadara LB, Halagowder D, Sivasitambaram ND (2011) Apoptosis inducing effect of plumbagin on colonic cancer cells depends on expression of COX-2. PLoS One 6:e18695
29. Kamal A, Reddy KS, Khan MN, Shetti RV, Ramaiah MJ, Pushpavalli SN, Srinivas C, Pal-Bhadra M, Chourasia M, Sastry GN, Juvekar A, Zingde S, Barkume M (2010) Synthesis, DNA-binding ability and anticancer activity of benzothiazole/benzoxazole-pyrrolo[2,1-c][1, 4]benzodiazepine conjugates. Bioorg Med Chem 18:4747–4761
30. Kayashima T, Mori M, Yoshida H, Mizushina Y, Matsubara K (2009) 1, 4-Naphthoquinone is a potent inhibitor of human cancer cell growth and angiogenesis. Cancer Lett 278:34–40
31. Lee JH, Yeon JH, Kim H, Roh W, Chae J, Park HO, Kim DM (2012) The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-5 kinase for ROS generation. PLoS One 7:e45023
32. Suzanne M, Steller H (2013) Shaping organism with apoptosis. Cell Death Differ 20:669–675