RESEARCH ARTICLE

Ethanolic Extract from *Derris scandens* Benth Mediates Radiosensitzation via Two Distinct Modes of Cell Death in Human Colon Cancer HT-29 Cells

Arunee Hematulin[1]*, Kornkanok Ingkaninan[2], Nanteetip Limpeanchob[3], Daniel Sagan[1]

Abstract

Enhancing of radioresponsiveness of tumors by using radiosensitizers is a promising approach to increase the efficacy of radiation therapy. Recently, the ethanolic extract of the medicinal plant, *Derris scandens* Benth has been identified as a potent radiosensitizer of human colon cancer HT29 cells. However, cell death mechanisms underlying radiosensitization activity of *D scandens* extract have not been identified. Here, we show that treatment of HT-29 cells with *D scandens* extract in combination with gamma irradiation synergistically sensitizes HT-29 cells to cell lethality by apoptosis and mitotic catastrophe. Furthermore, the extract was found to decrease Erk1/2 activation. These findings suggest that *D scandens* extract mediates radiosensitization via at least two distinct modes of cell death and silences pro-survival signaling in HT-29 cells.

Keywords: Radiosensitizer - *Derris scandens* benth - radiotherapy - apotosis mitotic catastrophe

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Introduction

Radiotherapy is the most common treatment for human cancers worldwide (Balcer-Kubiczek, 2012). Its efficacy depends on the tumor's responsiveness to the fatal effects of radiation (Coventry and Ashdown, 2012). However, several malignancies are relatively radioresistant, leading to treatment failure (Gavriel et al., 2011; Hanson et al., 2012; Mannino and Chalmers, 2011). Radiosensitizers are agents that enhance the sensitivity of cancer cells towards radiotherapy (Girdhani et al., 2005; Moding et al., 2013). The enchantment of radioresponsiveness of tumors by using radiosenstizers is suggested to be a promising strategy to improve radiotherapy efficiency (McGinn and Lawrence, 2001; De Schutter and Nuyts, 2009; Katz et al., 2009). Since decades, numerous studies have been attempting to search for novel radiosensitizers. Nevertheless, only very few substances are clinically used due to lack of therapeutic efficacy (Wilson et al., 2006; Verheij et al., 2010; Habr-Gama et al., 2011).

Frequently, cancer cells encompass multiple defects in failsafe mechanisms such as cell cycle arrest and cell death pathways leading to the resistance of cancer to radiation-induced cell death (Schmitt, 2003; Weaver and Cleveland, 2005). Indeed, lethality of cancer cells in response to radiation damage processes via different modes of cell death including apoptosis and mitotic catastrophe (Schmitt, 2003; Weaver and Cleveland, 2005; Vakifahmetoglu et al., 2008; Vitale et al., 2011; Balcer-Kubiczek, 2012). It is a challenging strategy to search for substances for combined use with radiation to trigger those programs of cell death that are still intact in cancer cells. Interestingly, the radiosensitizing potential of numerous botanicals and their derivatives is reported in several studies for cancer cells (Chendil et al., 2004; Park and Shin, 2004; Hara et al., 2008; Kim et al., 2008; Sandur et al., 2009). Notably, their radiosensitizing activities are found to process via several mechanisms including disturbing of cell cycle regulation, cell survival or cell death pathways. Since the radioresponsiveness of a tumor is influenced by multiple factors, further studies are needed to not only search for new radiozensitizers, but also to provide essential information of the mechanisms of their actions.

The Asian plant *Derris scandens* Benth *(D scandens)* belongs to the family of Leguminosae. The major active constituents of *D scandens* stem extracts are benzyls and isoflavones, including genistein, coumarins, scandinone, scandenin, prenylated isoflavones, and isoflavone (Rukachaisirikul et al., 2002; Laupattarakasem et al., 2004; Rao et al., 2007). *D scandens* stem extracts has been widely used in traditional medicine for the treatment of several diseases such as arthritis, muscular pain, and inflammation (Tiangburanatham, 1996).

*[1]Radiobiology Research Unit, Department of Radiation Technology, Faculty of Allied Health Sciences, [2]Department of Pharmaceutical Chemistry and Pharmacognosy, [3]Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand *For correspondence: aruneeh@nu.ac.th, hematulin_a@yahoo.com*

Moreover, *D scandens* extract has been reported to inhibit α-glucosidase activity, scavenge free radicals, and inhibit migration of several cell lines (Laupattarakasem, 2007; Rao et al., 2007). Recently, we demonstrated that *D scandens* extract powerfully increases the radiosensitivity of human colon cancer HT-29 cells, while not influencing the radiosensitivity of normal retinal pigment epithelial cells (Hematulin et al., 2011). However, the cell death mechanisms underlying this radiosensitzation activity of *D scandens* extract has not been clearly identified so far. In this study, we demonstrate that the plant extract sensitizes HT-29 cells towards radiation-induced cell death via apoptosis and mitotic catastrophe.

Materials and Methods

Chemicals and antibodies

Dulbecco's Modified Eagle Medium (DMEM/F12) and fetal bovine serum were purchased from Gibco (Invitrogen, USA). RNase A, Hoechst 33342, and Giemsa were purchased from Sigma-Aldrich (St. Louis MO, USA). Amersham ECL Plus™ was purchased from GE Healthcare (Buckinghamshire, England). Antibodies were obtained from the following companies: actin (I-19 sc-1616) and alpha tubulin (sc-5286) from Santa Cruz Biotechnology (California, USA), Erk1/2 (4695), phospho-T202/Y204-Erk1/2 (9101), phospho-T68-Chk2 (2661), phospho-Y15-Cdc2 (9111), and phospho-S15-p53 (9284) from Cell Signaling (Beverly, MA).

Cell culture

HT-29 human colon cancer cells (ATCC, HTB-38) were maintained at 37°C, 5% CO_2 in DMEM/F12, containing 2.5mM L-glutamine, 10% fetal bovine serum, 0.25% sodium bicarbonate, 40units/ml penicillin G, and 40μg/ml streptomycin.

*Preparation of **D scandens** extract*

The stem of *D scandens* was collected at Bangkratum province, Phitsanulok, Thailand. The plant authenticity was identified by Dr. Pranee Nangngam, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok. The voucher specimen (no. 3464) is kept at PNU Herbarium, Department of Biology, Faculty of Science, Naresuan University. The stem was sliced into small pieces, dried at 60°C, then ground with a mixer grinder. The dried powder was macerated in 95% ethanol for 3 days. The extract was subsequently filtered, evaporated till dryness under reduced pressure. The % yield of the extract comparing to dried stem was approximately 10%. A thin layer chromatography fingerprint of the extract was made and kept as a reference. The plant extracts were kept at -20°C.

*Treatment of cells with **D scandens** extract and gamma-irradiation*

For *D scandens* extract treatment, the medium was aspirated from the culture plates and then replaced with fresh medium containing 5 or 15μg/ml of *D scandens* extract diluted in DMSO. The treated cells were incubated for 24h before gamma-irradiation. For gamma-irradiation, a Cobalt-60 source (Theratron Phoenix) with a dose rate of 2.1Gy/min was used for irradiating cells with a single dose of 0, 2, 4, or 6Gy at room temperature. The source to sample distance was 80cm. After irradiation, cells were maintained at 37°C in a 5% CO_2 humidified atmosphere and collected at different time points for further experiments.

Clonogenic cell survival assays

Exponentially growing cells were seeded in duplicate into 6-well plates. The cell number seeded per plate varied with the radiation dose, so that the colonies could be counted conveniently. The seeded cells treated with or without 5 or 15μg/ml of *D scandens* extract for 24h were irradiated with a single dose of 0, 2, 4, or 6Gy at room temperature. The cells were allowed to grow for 10-14 days until the surviving cells produced macroscopically visible colonies that could be counted easily. The cells were fixed with 95% ethanol for 10 minutes and then stained with Giemsa for 10 minutes. Colonies containing more than 50 cells were counted and survival fractions were calculated as ratio of the amount of colonies formed from treated cells and untreated cells, corrected for plating efficiency.

Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay

HT-29 cells were seeded onto sterile glass cover slips and cultured in 6-well plates overnight. The cells were treated with 15μg/ml of *D scandens* extract alone or combined treatment with 6Gy gamma-irradiation. Forty-eight hours after irradiation, a TUNEL assay was performed according to manufacturer's protocol (Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay, Invitrogen). The stained cells were visualized by fluorescence microscopy (Zeiss). For each treatment condition, the number of TUNEL-positive stained cells was counted from randomly selected fields and expressed as a percentage of the total number of nucleated cells.

Cell cycle analysis by flow cytometry

Approximately 8×10^4 cells of exponentially growing HT-29 cells were seeded into 6-well plates and cultivated for 12h. Twenty-four hours prior irradiation, the cells were treated with or without 15μg/ml of *D scandens* extract. Subsequently, the treated cells were gamma-irradiated with a single dose of 0 or 6Gy. The treated cells were trypsinized and collected 24h after irradiation and DNA staining was performed as described previously (Hematulin et al., 2008). The suspension of PI stained isolated nuclei were mixed and kept in the dark at 4°C before flow cytometric measurement. The cell cycle distributions were analyzed on a FACScan (Becton-Dickinson).

Immunostaining for analysis of nuclear morphology

Approximately 8×10^4 cells of exponentially growing HT-29 cells were seeded onto sterile glass cover slips and cultured overnight. Then, the cells were treated with or without 15μg/ml of *D scandens* extract for 24h. Subsequently, the cells were gamma-irradiated with a

single dose of 0 or 6Gy. Forty-eight hours after irradiation, immunostaining against α-tubulin followed by staining with a rhodamine-labelled secondary antibody was performed. Nuclei were counterstained with Hoechst 33342 as described previously (Hematulin et al., 2012). The stained cells were visualized by fluorescence microscopy.

Western blot analysis

Total protein was extracted from HT-29 cells at indicated time points after each treatment as described previously (Hematulin et al., 2008). Thirty μg of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis and electro-blotted onto PVDF membranes. The membranes were blocked in TBS-T containing 5% nonfat skim milk for 1h at room temperature. After that, the membranes were probed with a primary antibody diluted in 3% BSA in TBS-T overnight at 4°C. After washing thrice with TBS-T, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody diluted in blocking buffer for 1h. The membranes were washed thrice with TBS-T and bands were detected by chemiluminescence on X-ray films.

Statistical analyses

The mean±standard error was calculated from at least three independent experiments. Synergistically effects of both factors on cellular survival were tested by two-way ANOVA analysis of the ln-transformed values of surviving fractions (SPSS, version 17.0). In addition, combination indices (CI) were calculated based on the methods of Chou and Talalay (Chou and Talalay, 1984; Chou, 2006) using CompuSyn software (Chou and Matin, 2005). CI values of less than 1 indicate synergistic effects, equal to 1 indicate additive effects, and greater than 1 indicate antagonistic effects.

Results

D scandens *extract synergistically sensitizes HT-29 cells to radiation-induced cell death*

Clonogenic survival of HT-29 cells after irradiation is decreased by pre-treatment of the cells with *D scandens* extract at concentrations of 5μg/ml and 15μg/ml (Figure 1). Consequently, treatment of the cells with plant extract (without irradiation) slightly reduces cellular survival to 97% and 89% at a dose of 5 and 15μg/ml, respectively, compared to untreated control cells. Plant extract treatment and gamma-radiation synergistically impact cellular survival (p=0.03) (Slinker, 1998). Moreover, the combined effect of *D scandens* extract and radiation on cellular survival was determined from CI values (Table 1). A synergistic effect of both factors on HT-29 colony formation was revealed by CI values lower than 1. Thus,

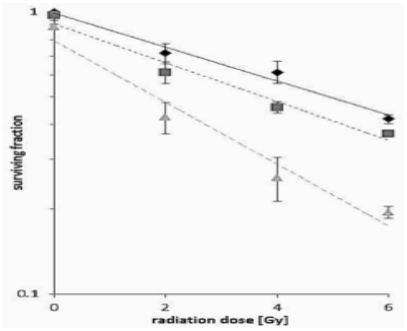

Figure 1. *D Scandens* Extract and Gamma-radiation Synergistically Induce Cell Death in HT-29 Colon Cancer Cells. Cellular radiosensitivity of untreated HT-29 cells (diamond), cells treated with 5μg/ml of *D scandens* extract (square) or treated with 15μg/ml of *D scandens* extract (triangle). Cells were seeded into 6-well plates and pre-treated with or without *D scandens* for 24 hours before gamma-irradiation. Clonogenic survival of gamma-irradiated cells was determined at day twelve after gamma-irradiation. The plot shows the mean surviving fraction±standard error from three independent experiments. Significant effects of gamma-radiation, plant extract treatment and combined treatement were tested by two-way ANOVA (see text)

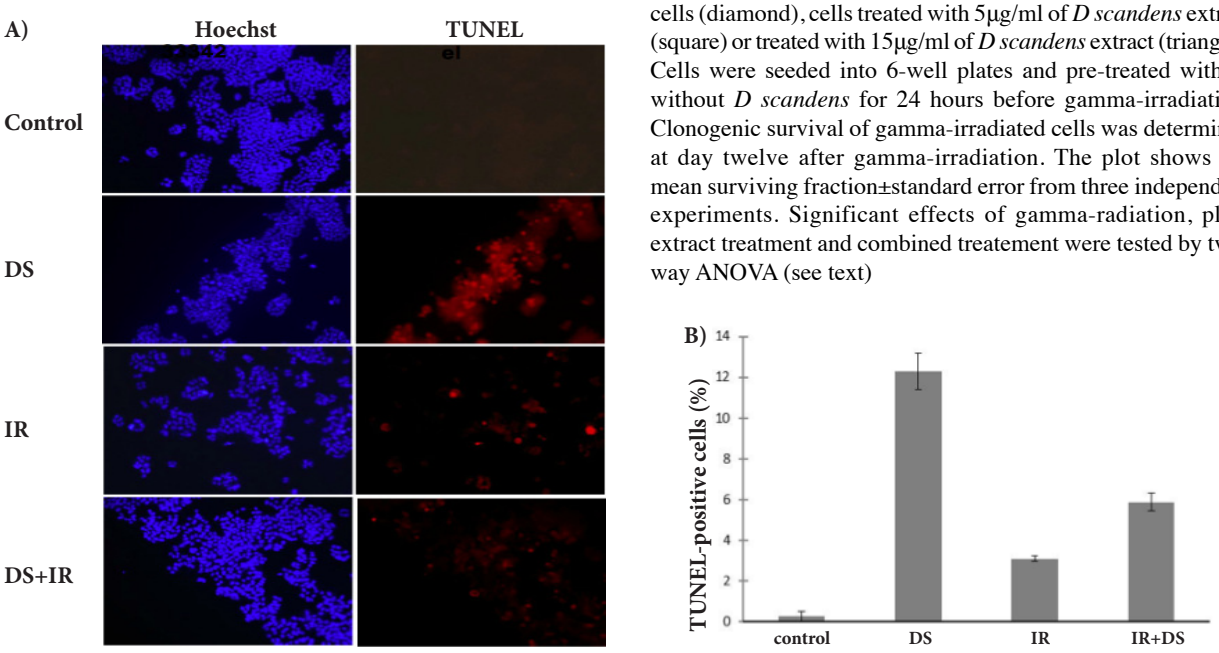

Figure 2. *D scandens* Extract Predominantly Induces Apoptosis in HT-29 Colon Cancer Cells. HT-29 cells were pre-treated with or without *D scandens* extract for 24 hours before gamma-irradiation. Forty-eight hours after irradiation, TUNEL imaging was performed to detect apoptotic DNA fragments (red). Nuclei were stained with hoechst 33342 (blue). Representative fluorescence microscopic images of control cells that received no treatment (control), 15μg/ml of *D scandens* extract treated cells (DS), 6gy gamma-irradiated cells (IR), 15μg/ml of *D scandens* extract treated and 6gy gamma-irradiated cells (IR+DS) (**A**). the percentages of TUNEL-positive cells counted from a minimum of 800 cells are shown as mean±standard error (**B**)

the data strongly indicate that *D scandens* extract exerts a synergistic effect with radiation to induce cell death in HT-29 cells.

A D37 value of 7.1Gy (radiation dose at which 37% of cells survive compared to untreated cells) was observed for cells that received no plant extract treatment prior to irradiation. Plant extract treatment with concentrations of 5, and 15μg/ml reduced the D37 value to 5.7Gy, and 3.0Gy, respectively. Thus, plant extract radiosensitizes the cells by an enhancement ratio of 1.2 and 2.4 for concentrations of 5 and 15μg/ml of plant extract, respectively.

***D scandens** extract induces apoptosis in HT-29 cells*

Apoptosis is one of the most common forms of cell death induced by ionizing radiation. To investigate whether *D scandens* extract induces apoptosis in HT-29 cells, treated cells were analyzed by TUNEL assay. TUNEL-positive cells were determined by fluorescence microscopy. Treatment of the cells with *D scandens* extract alone for 48h significantly induces 12±1% of the cells to undergo apoptosis, whereas 6Gy of gamma-radiation induce apoptosis in 3.1±0.1% cells, only. Treatment with 6Gy of gamma-irradiation in combination with *D scandens* extract induces apoptosis in 5.9±0.4% of the cells (Figure 2A, B). Thus, *D scandens* extract is a more potent inducer of apoptosis than ionizing radiation.

Table 1. *D scandens* Extract Synergistically Enhances the Radiosensitivity of Human Colon Cancer HT-29 Cells

Radiation dose (Gy)	*D scandens* extract (μg/ml)	Surviving fraction	CI values	IR-DS combination effect
0	0	1.00	-	-
	5	0.97	-	-
	15	0.89	-	-
2	0	0.72	-	-
	5	0.62	0.71	synergism
	15	0.43	0.46	synergism
4	0	0.62	-	-
	5	0.46	0.74	synergism
	15	0.27	0.4	synergism
6	0	0.42	-	-
	5	0.37	0.77	synergism
	15	0.20	0.4	synergism

*Mean surviving fractions were calculated from three independent experiments. Combination Index (CI) values of less than 1 for *D scandens* (DS) extract and radiation (IR) indicate synergistic effects

Mitotic catastrophe is a predominant form of cell death in HT-29 cells as response to irradiation

Mitotic catastrophe is another main form of cell death induced by ionizing radiation (Vakifahmetoglu et al., 2008). The most prominent morphological characteristic of mitotic catastrophe is the appearance of giant cells with gross nuclear alterations such as micronuclei, multinucleated, and multilobulated nuclei (Vitale et al., 2011). To investigate whether mitotic catastrophe might be a major mode of cell death in HT-29 cells in response to radiation damage, nuclear morphology of the cells was analyzed. Cells pre-treated with or without 15μg/ml of *D scandens* extract were irradiated with a single dose of 6Gy. Two days later, the cells were stained with an α-tubulin antibody and Hoechst 33342 and visualized by fluorescence microscopy. Treatment of the cells with radiation alone predominantly induces mitotic catastrophe in HT-29 cells. This is indicated by the presence of micronuclei and giant cells with multiple nuclei. Treatment of cells with plant extract alone induces apoptosis reflected by the presence of fragmented or condensed nuclei, while mitotic catastrophe was not observed. As expected, cell populations that received a combined treatment with plant extract and irradiation, contained cells with features of mitotic catastrophe as well as apoptotic cells (Figure 3). This observation indicates that mitotic catastrophe is the predominant mode of cell death of HT-29 cells induced by irradiation.

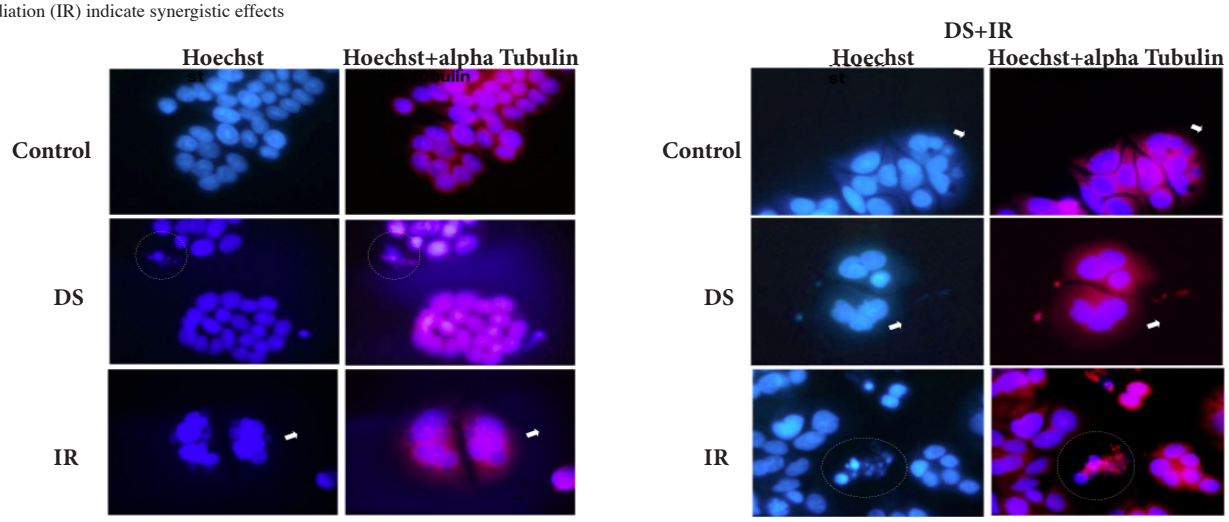

Figure 3. Mitotic Catastrophe is a Predominant Form of Cell Death in HT-29 Cells in Response to Irradiation. HT-29 cells were pre-treated with or without *D scandens* extract for 24 hours before irradiation. Forty-eight hours after 6Gy of gamma-irradiation, nuclear morphology was assessed by fluorescence microscopy. Nuclei were stained with Hoechst 33342 (blue), alpha-tubulin was stained by appropriate antibodies (red). Representative fluorescence microscopic images of normal nuclei of control cells that received no treatment (control), 15μg/ml *D scandens* extract treated cells (DS), 6Gy gamma-irradiated cells (IR), 15 μg/ml of *D scandens* extract treated and 6Gy gamma-irradiated cells (IR+DS). A Cell with fragmented nuclei (circle), a giant cell with multiple micronuclei and multilobulated nuclei (arrowheads) are shown

D scandens *extract decreases phosphorylation of Erk1 and Erk2 (Erk1/2)*

Erk1/2 are effector proteins of the MAP3 kinase pathway that mediate cell survival through inhibition of the apoptosis cascade (Cagnol and Chambard, 2010). Immunoblotting of total lysates of HT-29 cells was performed to examine the impact of *D scandens* extract on the activity of Erk1/2. Treatment of HT-29 cells with *D scandens* extract strongly decreased the phospholylation level of Erk1/2 within 1h after treatment and the level remained lesser than that of control cells during the observation period of 24h after treatment (Figure 4A). The phosphorylation level of Erk1/2 significantly increased in HT-29 cells that were treated with radiation alone. In contrast, increasing of phosphorylation level of Erk1/2 was not observed in cells that were treated with *D scandens* extract before irradiation (Figure 4B).

HT-29 cells do not arrest at G2/M in response to radiation damage

In response to ionizing radiation-induced DNA damage, activation of DNA damage checkpoint signaling is crucial for human cells to prevent cells with damaged DNA from entering mitosis (Sancar et al., 2004). To investigate the efficiency of DNA damage checkpoints of HT-29 cells in response to gamma-irradiation, the cell cycle and the levels of phospho-p53, phospho-Chk2 kinase, and phospho-Cdc2 kinase were analyzed. The G2/M population of HT-29 cells upon 6Gy irradiation was slightly increased as compared to unirradiated cells. Treatment of the cells with *D scandens* extract had no impact on cell cycle distribution of HT-29 cells (Figure 5A). Induction of phospho-p53 and phospho-Chk2 kinase were clearly observed in response to gamma-irradiation, whereas the level of phospho-Cdc2 was only slightly altered (Figure 5B). Notably, treatment of the cells with *D scandens* extracts alone or in combination with irradiation had no effect on the activation of p53, Chk2 kinase, or Cdc2.

Taken together, these finding indicate that the G2 checkpoint of HT-29 cells in response to radiation damage is not fully effective.

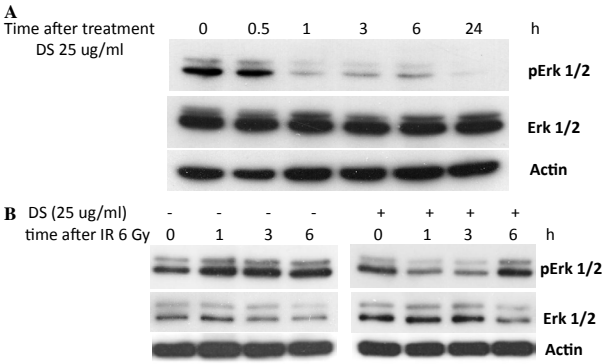

Figure 4. Impact of *D scandens* Extract on the Activation of Erk1/2. Treated cells were collected at different time points for total protein extraction. The levels of proteins were determined by Western blot analysis. Levels of phospho-Erk1/2 and total Erk1/2 in cells that were treated with 15μg/ml of *D scandens* extract (**A**). Levels of phospho-Erk1/2, total Erk1/2 and phospho-Chk2 in cells that were treated with (+) or without (-) 15μg/ml of *D scandens* extract for 24h followed by 6Gy of irradiation (**B**). Detection of actin was used as a loading control

Discussion

In the present study we clearly demonstrate that *D scandens* extract synergistically potentiates radiation-induced cell death of human colon cancer HT-29 cells. Radiosensitation was dose dependent as demonstrated by a radiation enhancement ratio (for D37) of 1.2 and 2.4 for 5 and 15μg/ml of *D scandens* extract, respectively. This long-term survival result obtained by clonogenic survival is consistent with the finding generated from a short-term cell viability assay (trypan blue staining) in our previous study (Hematulin et al., 2011).

D scandens extract was found to induce apoptosis in HT-29 cells. Notably, the apoptosis-inducing potential of *D scandens* extract alone is stronger than that of gamma-irradiation or gamma-irradiation combined with *D scandens* extract treatment. On the other hand, data from clonogenic survival assay show that combination of the extract with irradiation is the most potent approach to persuade the death of HT-29 cells. Since the clonogenic survival assay measures the sum of all modes of cell death (Hall and Giaccia, 2006; Mirzayans et al., 2007), it is most likely that apoptosis is not the only mode of cell death in HT-29 cells in response to radiation damage generated in *D scandens* extract pre-treated cells.

Mitotic catastrophe is claimed to be the main form of cell death induced by ionizing radiation, especially in cancer cells that encompass checkpoint defects (Vakifahmetoglu et al., 2008; Verheij, 2008; Vitale et al., 2011). The result from our study indicates that the G2 checkpoint of HT-29 cells in response to radiation damage is not fully effective. This could be an explanation why

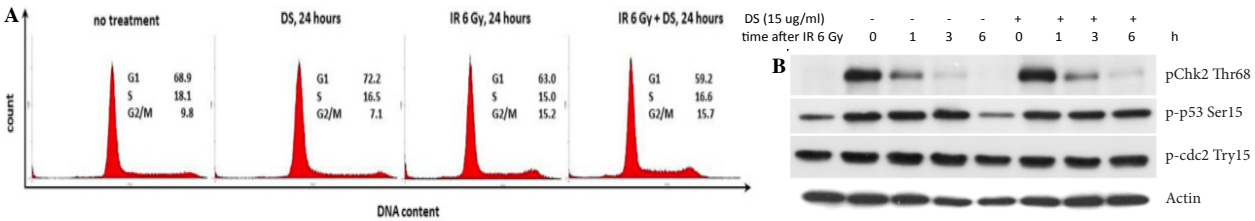

Figure 5. Impact of *D scandens* Extract on the Activation of DNA Damage Checkpoint Signaling. Treated cells were collected at different time points for cell cycle analysis and total protein extraction. Cell cycle distribution profiles were assessed by flow cytometry (**A**). Levels of proteins were determined by Western blot analysis. Levels of phospho-Chk2, phospho-p53, phospho-Cdc2 in cells that were treated with (+) or without (-) 5μg/ml of *D scandens* extract for 24h followed by 6Gy of irradiation (**B**). detection of actin was used as a loading control

mitotic catastrophe is a major form of cell death in HT-29 cells in response to gamma-irradiation. Mitotic catastrophe is predominantly induced by gamma-irradiation, whereas apoptosis is the predominant mode of cell death of HT-29 cells induced by *D scandens* extract. Thus, the combined application of irradiation and plant extract treatment leads to cell lethality by at least these two distinct modes of cell death.

The mechanism of interaction between *D scandens* extract and radiation remains to be elucidated. Activation of ERK cascade has been reported to promote cell survival by regulating the expression or activity of pro-apoptotic proteins and anti-apoptotic members (Ewings et al., 2007; Junttila et al., 2008; Kohno et al., 2011). The evidence that *D scandens* extract decreases Erk1/2 activation in HT-29 cells suggests an inhibitory activity of the extract on this pro-survival pathway. However, the result from our previous study showed that *D scandens* extract induce Akt activation but not effecting Erk1/2 activation in Hep-2 Cells (Hematulin et al., 2012). Hence, the effect of *D scandens* extract on the activation of survival pathway is most.

In conclusion, treatment of cells with *D scandens* extract prior to irradiation synergistically sensitizes HT-29 cells to radiation-induced cell death by enabling the cells to die by apoptosis and by mitototic catastrophe. In addition, plant extract treatment silences pro-survival signaling.

Acknowledgements

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