RESEARCH COMMUNICATION

Derris scandens Benth Extract Potentiates Radioresistance of Hep-2 Laryngeal Cancer Cells

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Abstract

The use of herbal products as radiosensitizers is a promising approach to increase the efficacy of radiotherapy. However, adverse effects related to the use of herbal medicine on radiotherapy are not well characterized. The present study concerns the impact of *Derris scandens* Benth extract on the radiosensitivity of Hep-2 laryngeal cancer cells. Pretreatment with *D. scandens* extract prior to gamma irradiation significantly increased clonogenic survival and decreased the proportion of radiation-induced abnormal nuclei of Hep-2 cells. Furthermore, the extract was found to enhance radiation-induced G2/M phase arrest, induce Akt activation, and increase motility of Hep-2 cells. The study thus indicated that *D. scandens* extract potentiates radioresistance of Hep-2 cells, further demonstrating the importance of cellular background for the adverse effect of *D. scandens* extract on radiation response in a laryngeal cancer cell line.

Keywords: Radioresistance - radiosensitivity - *Derris scandens* benth - herbal medicine - cancer

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Introduction

Radiotherapy is one of the most potent approaches for treatment of cancer. The success of this strategy counts on the sensitivity of tumor cells to the lethal effect of radiation. Although the radiosensitivity of tumor cells is primary an intrinsic property of the cell, it nevertheless can be altered by radiosensitzers and radioprotectors (Katz et al., 2009; Habr-Gama et al., 2010; Verheij et al., 2010). Radiosensitizers are agents that enhance the fatal effect of radiation, whereas radioprotectors decrease its lethal effect. Radiosensitizers escalate radiation damage in tumor cells via various mechanisms such as increase in initial DNA damage, interfere or inhibit DNA damage repair, cell cycle control, cell survival or cell death pathways (Girdhani et al., 2005; Javvadi et al., 2008; Shin et al., 2008; Katz et al., 2009; Verheij et al., 2010). Radioprotectors are compounds that diminish damaging effects of radiation to normal cells mostly via their ability to scavenge free radicals generated by ionizing radiation (Maurya et al., 2006; Sagar, 2010). Since radiation response of tumors is controlled by multiple factors, including individual genetic background, tumor type, stage, and type of mutations present (Verheij et al., 2010), more studies are needed to not only to identify novel radiozensitizers or radioporetectors, but also to provide essential information of the mechanisms of their actions. Although the use of radiomodulators for improving radiotherapy efficiency has been of interest of investigators for decades, only very few compounds have been approved for clinical application because of non-specificity and unacceptable toxicity (Katz et al., 2009; Habr-Gama et al., 2010; Kim et al., 2011). It has been proposed that the use of naturally occurring compounds derived from herbal medicinal plants as radiomodulators is less toxic within certain doses (Nambiar et al., 2011). Numerous herbal extracts and their derivatives have been continually reported for their potential to modify radiosensitivity of tumor cells. In addition, various polyphenolic compounds and flavonoids found in medicinal plant such as curcumin, genistein, gossypol, and resveratrol have been reported to have anti-oxidant ability which protect normal cells from radiation damage. In addition, they also possess radio-sensitizing effect in several cancer cells (Goel & Aggarwal, 2010; Sagar, 2010; Nambiar et al., 2011).

Derris Scandens Benth (*D. scandens*), a woody vine growing plant, belongs to the family of Leguminosae. A variety of biological active compounds have been identified from the extract of this plant. The major active constituents of *D. scandens* are benzyls and isoflavones, including genistein, coumarins, scandinone, scandenin, prenylated isoflavones, and isoflavone glycosides (Rukachaisirikul et al., 2002; Laupattarakasem et al., 2004; Mahabusarakam et al., 2004; Rao et al., 2007). The main compounds showing intestinal α-glucosidase inhibitory and free radical scavenging activity are

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scandinone, scadenone, scandenin A, scandenin B, and 4', 5', 7-trihydroxybiprenyl isoflavone (Rao et al., 2007). The extract from the stem of this plant has been long term used in traditional remedies throughout Southeast Asia. It has been used for the treatment of numerous diseases such as arthritis, muscular pain, diabetes, inflammation, and hypertension (W, 1996). It was reported that *D. scandens* extracts were effective and safe for the treatment patients with knee osteoarthritis (Kuptniratsaikul et al., 2011). Furthermore, the ethanolic extract of *D. scandens* has been found to have antimigration activity in hepatoma cell line (HepG2), breast cancer cell line (MCF-7), and cholangiocarcinoma cell lines (KKU-M139 and KKU-M213) (Pisamai Laupattarakasem, 2007). Recently, we showed that the stem extract of *D. scandens* could abolish G2/M checkpoint proficiency of colon cancer cell line (HT-29), resulting in strongly increased radiosensitivity of HT-29 cells. Remarkably, it had no effect on the radiosensitivity of normal retinal pigment epithelial cells (RPE) (Arunee Hematulin, 2011) .

D. scandens extract has been reported to have both, radiosensitizing activity, and antioxidant capacity. Thus, it can be postulated that its effect as a radiomodular strongly depends on the cell type. Therefore, this study investigates the effects of the extract on the radiosensitivity of Hep-2 laryngeal cancer cells.

Materials and Methods

Chemicals and antibodies

Dulbecco's Modified Eagle Medium and fetal bovine serum were purchased from Gibco (Invitrogen, USA). RNAase 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 following companies: actin (I-19 sc-1616) and alpha tubulin (sc-5286) from Santacruz Biotechnology (California, USA), Akt1 (2967), phospho-Ser473-Akt (9271), phosphor-Thr68-Chk2 (2661) from Cell signaling (Beverly, MA)

Cell culture

The laryngeal carcinoma cell line Hep-2 was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 0.25% sodium bicarbonate, 40 units/ml penicillin G, and 40 μg/ml streptomycin. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Preparation of D. scandens extract

The stem of *D. scandens* was collected at Bangkratum province, Phitsanulk, Thailand. The stem was chopped into small pieces. The sample was dried at 60 °C then powdered in a mixer grinder. The extraction process was performed by macerated the dried powder in 95% ethanol for 3 days. The extracts were subsequently filtered, evaporated till dryness under reduced pressure. The TLC fingerprint of the extract was made and kept as a reference. The plants extract were kept for further experiments at -20 °C.

Cell treatment and irradiation

For cell treatment, the medium was aspirated from the 6 well plates and replaced with 2 ml of fresh medium containing 10 or 20 μg/ml of *D. scandens* extract diluted in DMSO. The treated cells were incubated for 24 h prior gamma irradiation. For cell irradiation, a Cobalt-60 source (Theratron phoenix) with the dose rate of 2.2 Gy/min was used to irradiate cells at a single dose of 0, 2, 4, or 6 Gy at room temperature. The source to sample distance was 80 cm. After irradiation, cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. The treated cells were collected at different time points for cell cycle analysis, western blot analysis, and fluorescence microscopy.

Clonogenic cell survival assays

An appropriate number of exponential growing cells were seeded into 6-well plates and then cultivated for 12 h to allow the cells attach to the plate. The number of cells per plate initially seeded varies with the dose, so that the number of colonies surviving is in a range that can be counted conveniently. The cells pre-treated with or without 10 or 20 μg/ml of *D. scandens* extract for 24 h were irradiated with a single dose of 0, 2, 4, or 6 Gy at room temperature. After that, the cells were allowed to grow for 10-14 days until the surviving cells produced macroscopically visible colonies that can be counted easily. The cells were fixed with 95% ethanol for 10 min and then stained with Giemsa for 10 min. Colonies containing more than 50 cells were counted and survival fractions were calculated as the ratio of colonies formed to the cells plated corrected for plating efficiency.

Cell cycle analysis by flow cytometry

At each indicated time point, the treated cells were trypsinized and pooled with the floating cells in the culture medium. The cells were collected by centrifugation at 300 ×g for 5 min. The cell pellet was mildly resuspended in a solution containing 584 μg/ml NaCl, 1,000 μg/ml Na-citrate, 10 μg/ml RNAase A, 0.3 μg/ml Nonidet P-40, and 50 μg/ml propiodium iodide (PI). The cell suspensions were incubated for 30 min in the dark at room temperature, followed by the addition of a solution containing 15 mg/ml citric acid, 0.25 mM sucrose, and 50 μg/ml PI. 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).

Western blot analysis and antibodies

At each indicated time point, the whole cell lysates of treated cells were prepared as described previously (Hematulin et al., 2008). Thirty μg of protein from each sample were separated by electrophoresis in SDS-polyacrylamide gels and electro-blotted onto a PVDF membrane. The membrane was blocked in TBS-T containing 5% nonfat skim milk for 1 h at room temperature. Then, the membrane was probed overnight at 4 °C with a primary antibody diluted in 3% BSA in TBS-T. After washing 3 times with TBS-T, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody diluted in blocking buffer for 1 h.

The membrane was washed 3 times with TBS-T and then developed using chemiluminescence detection on X-ray films.

Immunostaining and Fluorescence microscopy

Approximately 8×10^4 cells were seeded into sterile glass cover slips and cultured in 6-well plates overnight. The cells were treated with 20 µg/ml of *D. scandens* extract alone or combined treatment with 6 Gy gamma irradiation. At each indicated time point, the cells on cover slips were washed briefly with PBS and fixed in -20 ºC methanol for 5 minutes. The cells were washed in three changes of PBS and incubated with 3% BSA in PBS for 30 minutes. The cells were washed again with PBS and incubated with an antibody against α- Tubulin diluted in 1.5% BSA for 60 minutes. The cells were washed with three changes of PBS for 5 minutes each, and then incubated for 45 minutes in the dark with a Rhodamine-conjugated secondary antibody diluted in PBS with 1.5% BSA. After washing with three changes of PBS, the cell nuclei were stained with Hoechst 33342 in the last washing. Cells were mounted with 90% glycerol for microscopy analysis (Zeiss). Abnormal nuclei were counted and expressed as a percentage of the total number of nuclei.

Scratch assay

Cell migration was performed by scratch assay. Approximately 10^6 cells were seeded into 6-well plates and cultured until reaching confluence monolayer. The cell surface was scratched by using 200 μl pipette tip and then washed twice with PBS. After that the cells were treated with 20 µg/ml of *D. scandens* extract while the control cells were left untreated. The scratched areas were monitored over the time course of 30 h by inverted microscope with 100x magnification (Zeiss). The images were taken and measured the migration distances by AxioVision software.

Statistical analysis

The data are presented the mean ± standard error of at least three independent experiments. Standard errors and p-values were calculated by comparing means by an independent-samples T-test using SPSS.

Results

D. scandens extract increases radio-resistance of Hep-2 cells

The effect of *D. scandens* extract on the radiosensitivity of Hep-2 cells was analyzed by a colony forming assay. Hep-2 cells were pretreated with 10 or 20 ug/ml *D. scandens* extract or left untreated. 24 h later, the cells were gamma-irradiated with 0, 2, 4, or 6 Gy. Treatment of the cells with *D. scandens* extract only, did not impact clonogenic survival of Hep-2 cells (Figure1). However, at the radiation doses of 2 and 4 Gy, *D. scandens* extract pretreatment slightly enhanced the survival fraction of Hep-2 cell. Remarkably, at the radiation dose of 6 Gy, pretreatment of the cell with 20 ug/ml of the extract significantly increased the survival fraction of Hep-2 cells by a factor of 1.5 ($p<0.05$). These results suggest that *D. scandens* extract escalates radio-resistance of Hep-2 cells.

D. Scandens extract restores the ability of Hep-2 cells to halt the cell cycle at G2/M phase in response to radiation damage

The impact of *D. Scandens* extract on cell cycle regulation is one of the most probable mechanisms that may be involved in increasing clonogenic survival of Hep-2 cells following ionizing irradiation. Thus, this postulate was further elucidated by cell cycle analysis. Flow cytometry revealed that cell treatment with *D. scandens* extract alone did not alter cell cycle distribution when compared to untreated control cells (Figure 2 upper panel). It is well known that radiation damage can induce cell cycle arrest of mammalian cells at G2/M phase (Deckbar et al., 2011). Therefore, cell cycle distribution

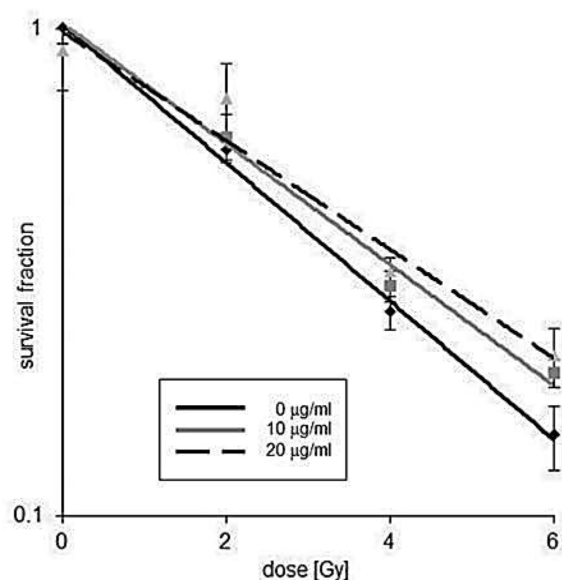


Figure 1. Survival Curves of Cells Untreated or Treated with 10 or 20 μg/ml of *D. scandens* Extract, Black Bar, Light Grey Bar, or Dash Black Bar, Respectively. Twenty four hours after treatment, the cells were irradiated with a dose of 0, 2, 4 or 6 Gy. Clonogenic survival of gamma-irradiated cells was determined at the day twelfth after irradiation. The dose survival curves were presented as the mean ± standard error of three independent experiments

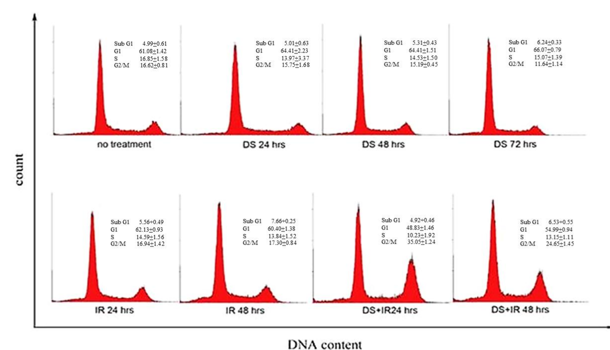
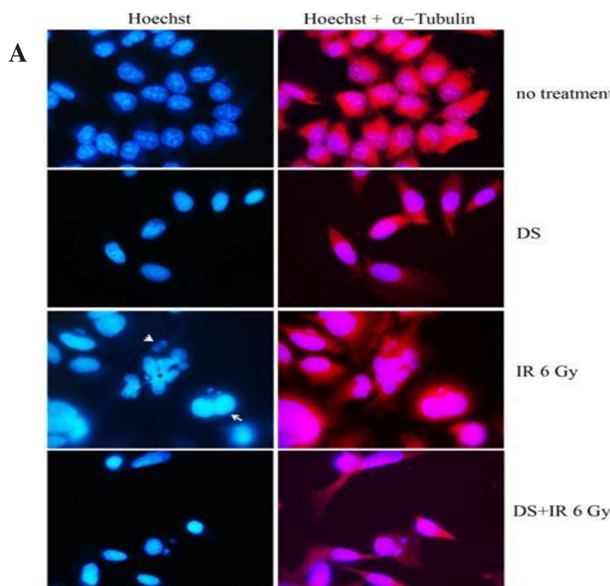


Figure 2. Histograms of Cell Cycle Distribution of Cells Untreated or Treated with 20 μg/ml of *D. scandens* (DS) Extract. Twenty four hours later, the untreated or treated cells were irradiated with a dose of 0 or 6 Gy and then collected at the indicated time points for cell cycle analysis by flow cytometry. The percentages of the cells in each phase of the cycle are presented as the mean ± standard error of three independent experiments



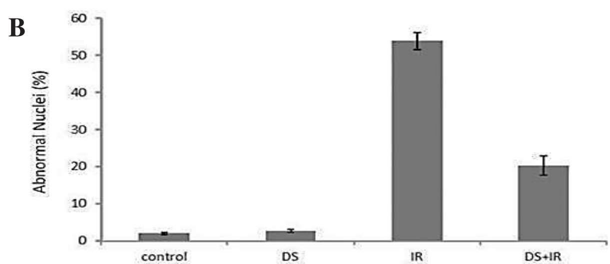


Figure 3. A) Fluorescence Microscopy Images of Nuclear Staining with Hoechst 33342 (blue), Counterstained with Rhodamine-conjugated Antibody Against Alpha-tubulin (red). The cells were untreated or treated with 20 μg/ml of D. scandens extract for 24 h, then irradiated with the dose of 0 or 6 Gy. Immunostaining was performed at 72 h after irradiation. Representative examples of normal nuclei of control cells no treatment (no D. scandens extract treatment, no irradiation), multiple micronuclei (arrowheads), multinucleated cells (arrows), and multilobuated nuclei (black asterisk) of irradiated cells. B) Histogram of abnormal nuclei frequency quantified from fluorescence microscopy of Hoechst 33342 nuclear stained cells. The cells were untreated or treated with 20 μg/ml of D. scandens extract for 24 h, and then irradiated with a dose of 0 or 6 Gy (control: untreated cells, DS: cells treated with plant extract only, IR: cells irradiated only, DS+IR: cells treated with plant extract prior to irradiation). Hoechst staining was performed at 72 h after irradiation. The percentages of abnormal nuclei were presented as the mean ± standard error of three independent experiments. More than 800 cells were counted for each determination

of cells treated with or without *D. scandens* extract was determined 24 or 48 hours after irradiation. Notably, treatment of Hep-2 cells with radiation alone did not significantly alter proportions of the cells in any phase of cell cycle when compared to control cells (Figure 2 lower panel). These results clearly demonstrated that DNA damaged checkpoint predominantly G2 checkpoint of Hep-2 cells were not effective in response to radiation. However, when cells were irradiated after pretreatment with *D. scandens* extract, the population of the cells pretreatment with the extract exhibited a remarkably increasing in G2/M phase population increased 24 h after irradiation from about 16% in samples that were irradiated without plant extract pretreatment to 34% in

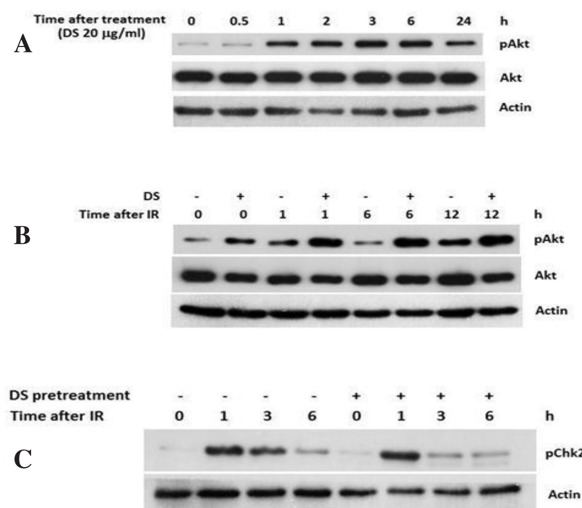


Figure 4. The Impact of *D. scandens* Extract (DS) on the Activation of Akt and Chk2 in Hep-2 Cells in Response to Irradiation. The treated cells were collected at different time points for protein extraction. The levels of proteins were determined by Western blot analysis. A) The level of Akt and p-Ser437-Akt in cells that were treated with 20 μg/ml of *D. scandens* extract. The level of Akt and p-Ser437-Akt (B) or p-Thr68-Chk2 (C) in cells that were untreated (-) or treated (+) with 20 μg/ml of D. scandens extract for 24 h followed by 6 Gy irradiation. Detection of actin was used as a loading control. Each result is representative of two independent experiments

samples that were pretreated with *D. scandens* extract before irradiation. Forty-eight hours after irradiation, the G2/M population declined in the pre-treated samples, but still was significantly bigger than the G2/M population measured in cells that were irradiated only. These results indicate that *D. scandens* extract restores the ability of Hep-2 cells to arrest at the G2/M phase in response to radiation induced damage.

D. Scandens extract lessens radiation-induced mitotic catastrophe in Hep-2 cells

The previous findings indicate a defective G2/M arrest of Hep-2 cells which can be overcome by *D. scandens* extract treatment. Plant extract treatment restores the ability of Hep-2 cells to arrest the cells with damaged DNA at G2/M phase after irradiation. To investigate further consequences of this effect, nuclear morphology of the cells was analyzed. The cells pre-treated with or without *D. scandens* extract were irradiated with a dose of 6 Gy. Three days later, the cells were stained with Hoechst 33342 and an antibody against α-tubulin. The nuclear morphology of the cells was visualized by fluorescence microscopy. Treatment of the cells with the extract alone did not alter nuclear morphology (Figure 3A). Irradiation of the cells with a dose of 6 Gy strongly induced mitotic catastrophe in Hep-2 cells, characterized by accumulation of multiple micronuclei, multinucleated cells, and multilobuated nuclei (Vakifahmetoglu et al., 2008). Remarkably, treatment of the cells with *D. scandens* extract before irradiation significantly reduced proportion of abnormal nuclei. Moreover, micronuclei were the predominantly morphological nuclear abnormalities *D. scandens* extract pretreated cells, while multinucleated cells and multilobuated nuclei were barely detected. The

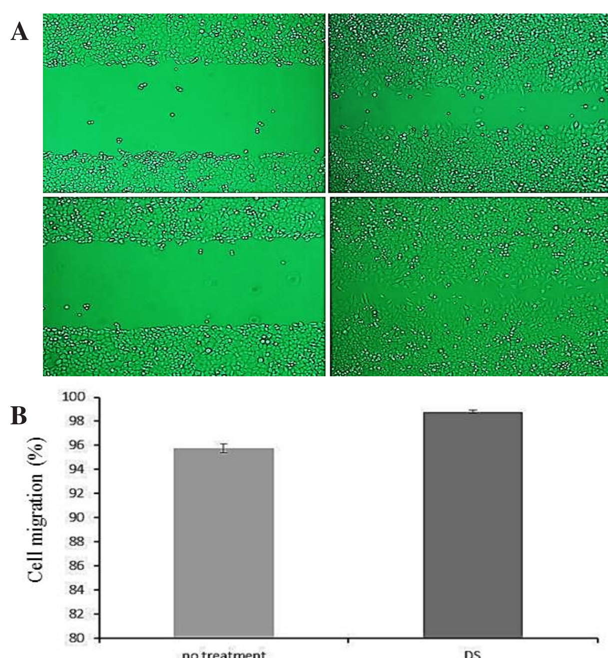


Figure 5. The Impact of *D. scandens* Extract (DS) on the Motility of Hep-2 Cells. The migration distances were monitored at 0 h and 30 h after treatment. A) Representative images of control cells no treatment (upper panel) and the cells treatment with 20 ug/ml *D. scandens* extract (lower panel). B) Histogram of the percentages of cell migration distances presented as the mean ± standard error calculated from cell migration distances (at 30 h) relative to their initiation boundaries (0 h)

percentages of abnormal nuclei in control and *D. scandens* extract treated cells were 2.0 and 2.7, respectively (Figure 3B). Upon gamma irradiation, the proportion of abnormal nuclei was significantly increased to 54%. Pretreatment of the cells with *D. scandens* extract significantly reduced the proportion of radiation-induced abnormal nuclei from about 54% to 20%.

D. scandens extract increases phosphorylation of Akt

The molecular pathways that might mediate the radioprotective activity of *D. scandens* extract were investigated by western blot analysis. Since the Akt pathway is a prominent pro-survival pathway involved in both, apoptosis and mitotic catastrophe, phosphorylation of Akt was determined (Hirose et al., 2005; Hemstrom et al., 2006; Zhang et al., 2011). Upon treatment of Hep-2 cells with *D. scandens* extract, the phosphorylation level of Akt at serine 473 significantly increased within one hour after treatment and remained higher than that of control cells during the observation period until 24 hour after treatment (Figure 4A). After irradiation, the phosphorylation level of Akt significantly increased. However, even higher levels of phosphorylated Akt were observed in cells treated with *D. scandens* extract before irradiation (Figure 4B). Chk2 kinase is the major effector for activating the radiation-induced DNA damage checkpoint. Phosphorylation of Chk2 was strongly increased one hour after irradiation and significantly declined within 6 hours after irradiation. However, pretreatment of the cells with *D. scandens* extract did not significantly alter radiation-induced phosphorylation of Chk2 (Figure 4C).

D. scandens extract increases motility of Hep-2 cells

Migration of cancer cells is a necessary step for initiation of tumor metastasis and can be stimulated by several agents including phytoestrogens from plants (Brownson et al., 2002). Since phytoestrogen genistein is a major active constituent of *D. scandens* extract, the impact of this extract on motility of Hep-2 cells was determined. The percentages of cell migration were calculated from cell migration distances as observed at 30 h after treatment of the cells with 20 ug/ml *D. scandens* extract relative to their initiation boundaries. The result showed that treatment of the cells with the extract significantly increases motility of Hep-2 cells as compared to that of control untreated cells ($p<0.05$) (Figure 5A and B).

Discussion

In this study, a radioprotective effect of *D. scandens* extract is clearly demonstrated. The extract decreases radiation-induced cell death and abnormal nuclei formation of Hep-2 cells. In addition, treatment of cells with *D. scandens* extract prior irradiation restores the ability of Hep-2 cells to halt the cell cycle at G2/M phase. This result is contradictory to our previous report for radiosensitizing potential of *D. Scandens* extract on colon cancer HT-29 cells, where extract treatment abolishes G2/M cell cycle arrest (Hematulin, 2011). The G2 checkpoint is a mandatory checkpoint that arrest cells at G2/M phase following radiation-induced DNA damage (Kastan & Bartek, 2004; Sancar et al., 2004; Deckbar et al., 2011). The function of this checkpoint is to prevent cells with damaged chromosomes from entering mitosis where unrepaired chromosomal damage might lead to cell death (Morrison & Rieder, 2004; Bucher & Britten, 2008). Restoration of G2/M arrest by *D. scandens* extract most probably prevents DNA-damage-induced mitotic catastrophe in Hep-2 cells. Whereas abolishing G2/M arrest by the extract promotes DNA-damage-induced cell death in HT-29 cells.

Treatment of Hep-2 cells with *D. scandens* extract alone had no effect on cell cycle distribution showing that the extract is involved in modulation of pathways activated by radiation. Thus, the molecular mechanisms for the involvement of *D. scandens* extract on G2/M arrest in response to irradiation were further analyzed. G2/M arrest is typically mediated by Chk1 and Chk2 kinases (Deckbar et al., 2011). However, in Hep-2 cells Chk1 was not activated after irradiation, regardless of treatment with *D. scandens* extract prior to irradiation or not (data not show). Although Chk2 was found to activate after irradiation, treatment of Hep-2 cells with the extract did not alter radiation-induced the activation of Chk2 kinase. It has been reported that Akt activation inhibits radiation-induced cells death via apoptotic or mitotic catastrophe pathways (Hirose et al., 2005; Hemstrom et al., 2006; Zhang et al., 2011). Highly active Akt in non-small-cell lung carcinoma (NSCLC) was found to promote cellular survival and resistant to radiation (Brognard et al., 2001). Furthermore, inhibition of PI3K-kinase/Akt pathway was reported to induce mitotic catastrophe in NSCLC cells (Hemstrom et al., 2006). We found that

phosphorylation levels of Akt were strongly increased after treatment of Hep-2 cells with *D. scandens* extract. Although, irradiation alone also induced phosphorylation of Akt, an even higher induction of phosphorylated Akt was clearly observed in the cells treated with plant extract prior to irradiation. Activation of the pro-survival protein Akt was found to be induced by *D. scandens* extract in this study. However, the precise connection between this activation and the enhancement of radioresistance of Hep-2 cells accompanied by an enhanced G2/M arrest requires further investigation.

The enhancement of motility of Hep-2 cells is an additional adverse effect of *D. scandens* extract that could be demonstrated in this study. Genistien is a major active constituent of *D. scandens* extract (Laupattarakasem et al., 2004). It was reported for its potential activity as antioxidant and anti-cancer agent (Khan et al., 2011; Nambiar et al., 2011). However, several reports have demonstrated that it can stimulate proliferation and migration of breast cancer cells (Brownson et al., 2002; Lucki & Sewer, 2011). Therefore, it is possible that increasing motility of Hep-2 cells after *D. scandens* extract treatment is probably resulted from the activity of its active constituent genistein.

In conclusion, numerous botanicals were claimed to have radio-therapeutic potential for treatment of cancer (Sagar, 2010; Nambiar et al., 2011). However, the exact mechanism for their action so far has not been fully identified. It has been continuously reported that most of herbals and their derivative compounds were found to have both radioprotecting and radiosensitizing properties (Goel & Aggarwal, 2010; Kim et al., 2011; Nambiar et al., 2011). The results of this and the former study indeed indicate that *D. scandens* extract acts as a radioprotector or as a radiosensitizer, depending on the cellular context. Thus, it should be assured that medical use of this extract in cancer patients does not impact cancer treatment regarding efficacy and adverse events depending on the type of cancer. Proposed by the results given here, usage of *D. scandens* extract might reverse therapeutic efficiency of laryngeal cancer patients.

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