

Derris scandens Benth Extract Radiosensitizes HT-29 Colon Cancer Cells

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ABSTRACT

Objective: To evaluate the radiosensitizing potential of *Derris scandens* extract in a human colon cancer cell model.

Methods: The radiosensitizing effect of *D. scandens* extract on human colon cancer cells (HT-29) and non-cancerous human retinal epithelial cells (RPE) was determined by cell survival and micronuclei analysis after irradiation. Cell cycle distribution was analyzed by a flow cytometer.

Results: *D. scandens* extract powerfully increases the radiosensitivity of HT-29 cells, but is less toxic and does not affect the radiosensitivity of RPE cells. Furthermore, the extract was found to reduce radiation-induced G2/M phase arrest in HT-29 cells, but not in RPE cells.

Conclusion: *D. scandens* extract may have potential as a radiosensitizer for cancer treatment.

Keywords: Radiosensitizer, *Derris scandens* Benth, radiotherapy, medicinal plant, cancer

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Radiotherapy is the most general treatment for human cancer. Its capability to cure cancer or relieve cancer symptoms depends on the response of cancer cells to radiation damage. Nevertheless, a large number of tumor types are weakly responsive to radiation. The enhancing of radioresponsiveness of tumors by using radiosensitizers is a promising approach to increase the radiotherapy efficacy. However, the success of this approach is limited by normal tissue damage.¹⁻⁵ Therefore, it is imperative to search for novel radiosensitizers that are highly selective and effective to damage tumor cells, but are less toxic to normal cells.

The use of plant extracts in combination with radiation has been reported for the potential enhancement of the radiosensitivity of tumor cells in several studies.^{2,6-9} The natural compounds enhancing radiosensitivity are supposed to act via different mechanisms, including increasing DNA damage, inhibiting cellular damage repair processes, and interfering with cell cycle progression.^{2,6,8,9}

The medicinal plant *Derris scandens* Benth (*D. scandens*) belongs to the Leguminosae. Its stem has been widely used in Thai traditional medicine.¹⁰ It has been reported that hydro-alcoholic extract from the stem of *D. scandens* provides antimicrobial, immunostimulating, and anti-inflammatory activity.¹¹⁻¹³ Moreover, *D. scandens* contains a variety of biologically active compounds with free radical scavenging, antibacterial, hypertensive, and anti-diabetic activities.¹⁴⁻¹⁶

A wide use of the stem extract of this plant for medicinal purposes and its variety of biological active compounds, raised the possibility that it may have the potential property of a radiosensitizer. Therefore, the radiosensitizing potential of *D. scandens* extract is evaluated in this study.

MATERIALS AND METHODS

Preparation of *D. scandens* extract

D. scandens was supplied by Bangkratum Hospital, Phitsanulok, Thailand. The stem was diced into small pieces, dried at 60°C and powdered in a mixer grinder. The dried powder was macerated with 95% ethanol for

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3 days. The extracts were subsequently filtered, evaporated until dry under reduced pressure. The plant extracts were kept at -20°C . The thin layer chromatography fingerprint of the extract was made and kept as a reference.

Cell culture

HT-29 (ATCC, HTB-38) and RPE (ATCC, CRL-2302) cells were cultured in Dulbeccos Modified Eagle Medium (DMEM/F12) containing 2.5 mM L-glutamine, 10% fetal bovine serum, 0.25% sodium bicarbonate, 40 units/ml penicillin G, and 40 $\mu\text{g}/\text{ml}$ streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell irradiation

A linear accelerator (Siemens, Mevatron, 6 MV) was used to irradiate cells at a single dose of 0, 2, 4, 6, or 9 Gy at room temperature. The source to sample distance was 94 cm. After irradiation (IR), cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for further experiments.

Cell viability assay

Cell viability was assessed by using trypan blue staining. Exponential growing cells were prepared by seeding 4×10^4 cells into 6-well plates containing DMEM/F12 medium and then cultivated for 12 h., and 24 h prior to irradiation, and the cells were treated with or without 5 or 15 $\mu\text{g}/\text{ml}$ of *D. scandens* extract diluted in DMSO. The cells were irradiated with a single dose of 0, 2, 4, or 6 Gray (Gy) at room temperature. Forty eight hours later, the cells were harvested by trypsinization, stained with 0.2% trypan blue and counted.

Statistical analysis

All data are represented as the mean \pm standard error of the mean of three independent experiments. For cell survival analysis, the mean relative survival (SF) of three independent experiments was fitted to the function $SF(d) = C \cdot e^{-\alpha \cdot d}$ where d is the radiation dose, C is a constant, reflecting the amount of living cells in an unirradiated sample and α is a measure for radiosensitivity. Standard errors and p-values were calculated by comparing means, and an independent-samples t-test using SPSS.

Micronuclei measurement and cell cycle analysis by flow cytometry

DNA staining of isolated nuclei for micronuclei measurement and cell cycle analysis was performed using a modified method according to Nüsse et al.^{17,18} Briefly, exponential growing cells were prepared by seeding 4×10^4 cells into 6-well plates containing DMEM/F12 medium and then cultivated for 12 h., and 24 h., prior to irradiation, and the cells were treated with or without 5 or 15 $\mu\text{g}/\text{ml}$ of ethanolic extract of the stem of *D. scandens* diluted in DMSO. Subsequently, the treated cells were irradiated using a single dose of 0, or 9 Gy at room temperature. Forty hours later, the treated cells were collected and gently resuspended in 500 μl of solution containing 584 mg/l NaCl, 1,000 mg/l Na-citrate, 10 mg/l RNAase, 0.3 mg/l Nonidet P-40, and 50 $\mu\text{g}/\text{ml}$ Propidiumiodide (PI). The cell suspensions were incubated for 30 minutes at room temperature. Subsequently, 500 μl of solution containing 15 g/l Citric acid, 0.25 mM Sucrose, and 50 $\mu\text{g}/\text{ml}$ PI was added. The cell suspensions were stored at 4°C before flow cytometric measurement. The micronuclei and

cell cycle distributions were analyzed with a FACScan (Becton-Dickinson). Flow cytometry was used to measure micronucleus (NM) formation of PI stained isolated nuclei and reveal cell cycle phase distribution over DNA content.

RESULTS

D. scandens extract enhances radiosensitivity of human colon cancer cells

To determine the radiosensitizing effects of *D. scandens* extract on colon cancer cells HT-29 and retinal epithelial cells RPE, cell survivals after exposure to IR were investigated. Pretreatment with *D. scandens* extract significantly enhances the radiation sensitivity of HT-29 cells, represented by significantly different α -values of the survival curves after plant extract treatment compared with that of curves without plant extract treatment ($p < 0.05$ (5 $\mu\text{g}/\text{ml}$), $p < 0.01$ (15 $\mu\text{g}/\text{ml}$), Fig 1A). The survival fraction \pm standard error at a dose of 2 Gy (SF2) reduces from 0.80 ± 0.04 for cells without plant extract treatment to 0.50 ± 0.06 and 0.34 ± 0.20 for cells that were treated with plant extract at a concentration of 5 $\mu\text{g}/\text{ml}$ or 15 $\mu\text{g}/\text{ml}$, respectively. The radiation doses to reduce survival to a rate of 0.37 (D37) are 9.1 Gy \pm 0.6 Gy, 4.9 Gy \pm 0.3 Gy, and 2.8 Gy \pm 0.2 Gy for plant extract concentrations of 0, 5 or 15 $\mu\text{g}/\text{ml}$, respectively. However, this effect was not observed for RPE cells, although they are more sensitive to IR than HT-29 cells. The surviving fraction at a dose of 2 Gy is approximately 0.5 and D37 is approximately 5 Gy independently of plant extract treatment (Fig 1B). The results from this experiment indicate that pretreatment with *D. scandens* extract enhances the radiosensitivity of HT-29 colon cancer cells. For a concentration of 5 $\mu\text{g}/\text{ml}$ the enhancement ratios are 1.6 calculated by SF2 and 1.8 calculated by D37. For a concentration of 15 $\mu\text{g}/\text{ml}$ these are 2.4 and 3.3 calculated by SF2 or D37, respectively.

D. scandens extract enhances radiation-induced micronucleus formation in human colon cancer cells

The micronucleus end point is a sensitive marker for measuring cellular responses to various genotoxic agents including IR.¹⁹ Treatment with *D. scandens* alone does not increase micronuclei-formation in both cell lines (Fig 2A, B). Cell treatment with IR alone with a dose of 9 Gy increased the micronuclei population of HT-29 cells

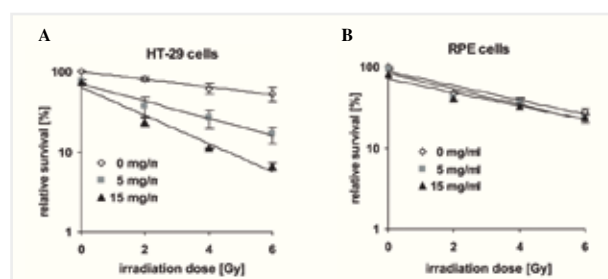


Fig 1. HT-29 cells and RPE cells were left untreated or treated with 5 or 15 μg of *D. scandens* extract. Twenty-four h later, the cells were irradiated with a dose of 0, 2, 4 or 6 Gy and surviving cells were counted as trypan blue-free cells. Relative survival is given in percent \pm standard error, relative to the fraction of living cells of a sample that was neither treated with plant extract nor with irradiation. The radiosensitizing effect of the plant extract is visualized by the distinct slopes of the lines (A). The plant extract had no radiosensitizing effect on the RPE cells as visualized in lines with identical slopes (B).

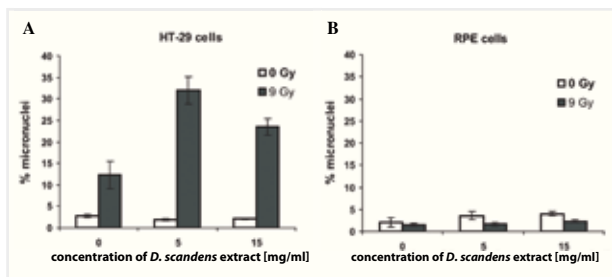


Fig 2. HT-29 cells were untreated or treated with 5 or 15 µg of *D. scandens* extracts. 24 h later, the cells were irradiated with a dose of 0 or 9 Gy (white or grey bars, respectively) and the micronuclei fraction was calculated relative to the total amount of cells ± standard error (A). RPE cells were treated and results calculated like described for HT-29 cells (B).

by a factor of approximately 4 (Fig 2A). The combined treatment of cells with 5 or 15 µg/ml of *D. scandens* extract and irradiation increased the micronuclei fraction of HT-29 cells by a factor of approximately 10 as compared to control cells that received no radiation and were not treated with the extract. In contrast, micronuclei-formation could not be induced by IR or by *D. scandens* extract in RPE cells (Fig 2B). The extract of *D. scandens* radiosensitized HT-29 cells but not RPE cells.

The impact of *D. scandens* extract on cell cycle phase distribution

To further investigate the mechanism by which *D. scandens* extract enhances radiosensitivity, cell cycle phase distribution was analyzed. At the time point for irradiation, 24 h after treatment with *D. scandens* extract (5 or 15 µg/ml), the cell cycle distribution of HT-29 and RPE cells was altered slightly. As compared to untreated control cells, the numbers of cells in the S or G2/M phase slightly increased (Fig 3). A radiation-induced G2/M block was clearly demonstrated by the increase of the G2/M population from 8% to 66% in RPE control un-irradiated cells and 9 Gy irradiated cells, respectively (Fig 4). However, there was no additional G2/M arrested cells after a combined cell treatment with *D. scandens* extract and IR. The relative amount of G2/M phase cells slightly decreased if the cells were treated with *D. scandens* extract before irradiation.

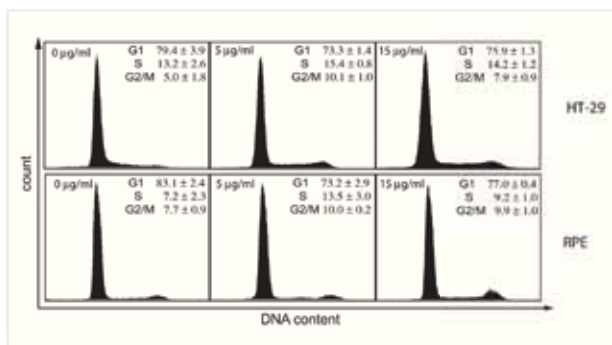


Fig 3. Cell cycle distribution of cells left untreated or treated with 5 or 15 µg of *D. scandens* extracts. The treated cells were collected at 24 h after treatment for monitoring cell cycle phase of individual cells by measuring DNA content by flow cytometer using PI staining, as described in materials and methods. The cell cycle phase distribution of HT-29 cells is presented on the upper row and that of RPE cells is presented on the lower row.

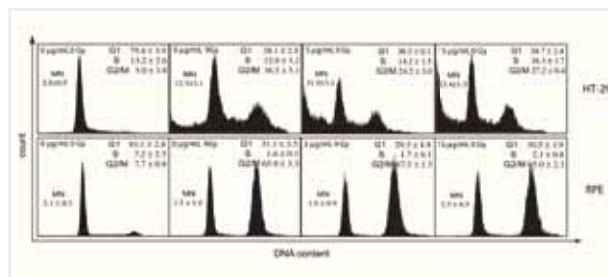


Fig 4. Cell cycle distribution of HT-29 (upper row) or RPE (lower row) cells left untreated or treated with 5 or 15 µg of *D. scandens* extracts. 24 h after treatment, the cells were irradiated with a dose of 0, or 9 Gy and then collected at 48 h later for cell cycle analysis by flow cytometer.

Upon irradiation, the G2/M population of HT-29 cells increased from 5% to 36% in unirradiated and irradiated (9 Gy) cells, respectively (Fig 4). Combined treatment of HT-29 cells with IR and *D. scandens* extract reduced the amount of cells in the G2/M population. Pre-treatment of the cells with a concentration of 5 µg/ml *D. scandens* extract resulted in a decrease to 24% of G2/M cells compared to 36% of G2/M cells which received irradiation without pre-treatment with the plant extract. After irradiation of cells that were pre-treated with a concentration of 15 µg/ml of plant extract, 27% of cells were arrested in the G2/M phase (Fig 4).

This result demonstrates a failure of HT-29 cells to halt the cell cycle at the G2/M phase in response to IR-induced damage, especially if irradiation happens after pretreatment with *D. scandens* plant extract.

DISCUSSION

We evaluated the radiosensitizing potential of the extract of the stem of *D. scandens* on HT-29 cells and RPE cells. Pre-treatment with *D. scandens* extract enhances the radiosensitivity of HT-29 cells. This is reflected by an increase of micronuclei formation and enhanced cell death upon irradiation. In contrast, the radiosensitizing activity of the extract on non-cancerous RPE cells was not observed. This finding demonstrates the selectivity of the plant extract on the synergistic cell killing of plant extract pre-treated cancer cells by radiation.

The probable mechanism that may be involved in the radiosensitizing activity of *D. scandens* extract was further elucidated by cell cycle distribution analysis. Pre-treatment with *D. scandens* extract alone slightly increases the S and G2/M population of HT-29 and RPE cells. Treatment of RPE cells with radiation alone results in an exaggerated G2/M phase arrest. In general, the G2/M checkpoint is activated upon DNA damage.^{20,21} Obviously, this checkpoint is very effective in RPE cells. In contrast, HT-29 cells possess an only partially active G2/M checkpoint. Upon irradiation, the G2/M population increased only moderately. A combination of irradiation and *D. scandens* extract pre-treatment of HT-29 cells further decreased the G2/M population. However, no effect on the G2/M population was observed in RPE cells upon combined treatment with irradiation and plant extract. The function of the G2/M checkpoint is to avoid cells with damaged chromosomes from attempting the mitosis process.²² In HT-29 cells, this checkpoint seems to be blocked by pre-treatment with *D. scandens* extract. It is likely that the less effective G2/M

checkpoint of HT-29 cells is responsible for a reduced cell survival after irradiation.

In conclusion, our study demonstrates that *D. scandens* extract pre-treatment sensitizes cancer HT-29 cells to radiation-induced cell killing, but not in non cancer RPE cells. This finding provides information for developing a novel radiosensitizer with potent efficacy for cancer treatment.

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