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The radioprotective potential of *Centotheca lappacea* (L) desv. extract in human endothelial cell

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ABSTRACT

Background: Radiation-induced vascular injury in normal tissue is a common adverse effect of radiation therapy. Radioprotectors can reduce the adverse effects of radiation-induced vascular injury. However, therapeutic applications of current radioprotectors are limited due to toxicity. Natural compounds derived from medicinal plants are less toxic and favorable for developing of radioprotectors. *Centotheca lappacea* has been long-term used for medicinal purposes and contains a variety of biological active compounds. Thus, the ethanolic extract from *Centotheca lappacea* may possibly have the potential to act as radioprotector.

Objectives: To determine radioprotective property of the ethanolic extract from *Centotheca lappacea* in human endothelial cell (EA.hy 926).

Materials and methods: The effect of the ethanolic extract from *Centotheca lappacea* on cell viability was assessed by MTT assay. Cell cycle distribution was determined by flow cytometry. Nuclei morphology was determined by fluorescence microscopy. The levels of Akt and phospho-Akt were determined by Western blot analysis.

Results: Pretreatment of cells with 0.2 µg/ml *Centotheca lappacea* extract for 3 hrs prior to irradiation demonstrated a radioprotective effect on EA.hy926 cells by significantly increasing cell viability and decreasing abnormal nuclei formation. Treatment of the cells with the extract in combination with radiation clearly increased the level of Akt phosphorylation. The extract is not toxic and does not interfere with cell cycle progression of EA.hy926 cells.

Conclusion: The ethanolic extract from *Centotheca lappacea* possesses radioprotective activity. No toxic impact and no impact on cell cycle progression in EA.hy926 cells were observed. The results indicate that *Centotheca lappacea* extract is an ideal resource of radioprotector for protecting radiation-induced damage to human endothelial cells.

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Introduction

Radiotherapy is one of the most effective cancer treatment modalities. The goal of radiotherapy is to maximize the killing effect of radiation on tumor cells and to minimize radiation damage to normal surrounding tissues.[1, 2] Although advanced technologies provide a greater precision of radiation delivery to tumor lesions, radiation toxicity in normal tissues remains problematic.[2] Radiation–induced vascular injury is one of the most common adverse effects of cancer radiotherapy. Radiotherapy primarily damages endothelial cells of blood vessel within the irradiation field.[3, 4] Such damage initiates vascular events like stenosis, thrombotic or fibrotic occlusion, which leads to the reduction of blood flow to normal organs of the damaged area.[3-5] The severity of clinical manifestation from vascular injury depends on organ functions and the extent of the injury. Frequently, quality of life of patients decreases due to a partial loss of function of an effected organ. Life-threatening complications such as stroke and aortic rapture can occasionally develop.[3-6]

Radioprotective agents can be used in combination with radiation to reduce radiation damage in normal tissues. These agents are given to cancer patients before or at the time of irradiation.[7] However, therapeutic application of radioprotectors is limited due to their toxicity.[7, 8] Natural compounds derived from plants have been reported to have radioprotective activities, low toxicity and do not manifest adverse effects.[9] Therefore, it can be expected that some Thai local medicinal plants may have a radioprotective potential against radiation–induced vascular injury. *Centotheca lappacea*, a perennial tufted grass, belongs to the family of *Poaceae*.[10] It has been long term used in traditional medicine in Thailand and many Southeast Asia countries for wound healing and muscle tightening in post labor women.[11, 12] Drinking of an infusion prepared by boiling of *Centotheca lappacea* in water has been purposeful for body nourishing.[11, 12] *Centotheca lappacea* extract contains phenolic compounds, such as catechin, gallate, rosmarinic acid, epigallocatechin, gallate and gallic acid.[13] It also contains flavonoids, such as flavonoids fatty acids, triterpenes and phytosterols, which have antioxidative and anti-inflammatory properties.[14] Moreover, it contains 4-coumaric acid, which has been reported to protect rabbit corneal cells from UVB-induced DNA damage.[14, 15]

Summarizing, *Centotheca lappacea* extract contains a variety of biological active components, such as anti-oxidants and anti-inflammatory substances, which may facilitate cellular repair activity and tissue regeneration. It can be expected that it may have radioprotective activity against radiation-induced vascular injury. Therefore, the radioprotective potential of *Centotheca lappacea* extract on human endothelial EA.hy926 cells is evaluated in this study.

Materials and methods

Chemicals and antibodies

Dulbecco's Modified Eagle Medium and fetal bovine serum, penicillin G, and streptomycin were purchased from Gibco (Invitrogen, USA). RNAase A , Hoechst 33342, and Propidium Iodine 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: alpha tubulin (A01410) from Gen Script (New Jersey, USA), Akt1 (2967), phospho-Ser473-Akt (9271), and anti-rabbit IgG HRP-linked (7074) from Cell signaling (Beverly, MA).

Cell culture

Human endothelial cells EA.hy926 (ATCC® CRL-2922™) were kindly provided by Dr. Piyanuch Thitiwuthikiat. The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin G (40 units/mL), streptomycin (40 µg/mL), and 0.25% Sodium bicarbonate. The cells were maintained in a humidified 5% CO_2 incubator at 37°C.

Preparation of the ethanolic extract from *Centotheca lappacea*

The dried aerial part of *Centotheca lappacea* was provided from Chaopraya Abhaiphubejhr hospital, Prachinburi, Thailand. The size was reduced by grinder and pass through the 60 mm mesh size sieve. Then the powder was macerated in 95% ethanol for one day under shaking and filtered through filtered paper (repeated two times). The filtrates were combined and evaporated under reduced pressure until dry. The extract was kept in -20 °C until analysis. The fingerprint was made and kept as a reference. The percent yield of the extract was 3.3%.

Cell treatment and irradiation

For cell treatment, fresh media containing 0.2 µg/mL of *Centotheca lappacea* extract was added to each well. The cells were incubated with the extract for 3 hrs prior to X-irradiation.

For cell irradiation, the cells were irradiated with 6 MV x-rays using linear accelerator (Varian 2100CD, Varian Medical Systems, Palo Alto, CA, USA). The x-rays was delivered with the dose rate of 600 MU/min at room temperature. The source to sample distance was 100 cm.

Cell viability assay

EA.hy926 cells were seeded into 96-well plates with a seeding density of 6x103 cells per well and cultured for 24 hrs. After that, the cells were treated with or without 0.2 µg/ml *Centotheca lappacea* extract for 3 hrs. The cells were then irradiated with a single dose of 2 Gy at room temperature. At 24 or 48 hrs after irradiation, the culture medium was replaced with 100 µl serum free medium and 30 µl MTT solution, and the plates were kept in the dark for 4 hrs. Afterwards, the medium was discarded and the formazan crystals were dissolved in 100 µl DMSO. The plates were kept in the cell culture incubator for 15 min to complete solubilization of the purple formazan crystals. The absorbance at 540 nm was then read using a reference wavelength of 630 nm on a microplate reader (EnSpire™ Multimode Plate Reader; PerkinElmer, USA). All experiments were performed in triplicate and the percentage of cell viability was calculated from three independent experiments.

Cell cycle analysis by flow cytometry

EA.hy926 cells were seeded into 6-well plate with a seeding density of $6x10^4$ cells per well and cultured for 24 hrs. After that, the cells were treated with or without 0.2 µg/mL *Centotheca lappacea* extract for 3 h prior to

X-irradiation. At the indicated time points, the cells were trypsinized and collected by centrifugation. The isolated nuclei were stained with propidium iodide (PI) as described previously.[16] Briefly, the cell pellet was resuspended in a solution containing 584 µg/mL NaCl, 1,000 µg/mL Na-citrate, 10 µg/mL RNase A, 0.3 µg/mL Nonidet P-40 and 50 µg/mL PI and incubated in the dark at room temperature for 30 min. After that, a solution containing 15 mg/mL citric acid, 0.25 mM sucrose and 50 µg/mL PI was added. The suspension of PI-stained isolated nuclei was kept at 4 °C in the dark before cell cycle distribution measurement using flow cytometry (Cytomics FC500 MCL with CXP 2.2 software; Beckman Coulter, Indianapolis, USA)

Nuclei staining and fluorescence microscopy analysis

Approximately 12×10^4 EA.hy926 cells were seeded onto sterile glass cover slips and cultured in 6-well plates overnight. Cells were pre-treated with or without 0.2 µg/mL of *Centotheca lappacea* extract for 3 h before X-irradiation with a single dose of 2 Gy. At 24 or 48 hrs after irradiation, the cells on cover slips were washed briefly with PBS and fixed in ice-cold methanol for 10 min. The cells were washed with PBS thrice and stained the cell nuclear with Hoechst 33342 for 20 min. The cells were washed thrice with PBS and mounted with anti-fading solution for fluorescence microscopy analysis (Zeiss HBO100 microscope Illuminating System Axiovision Rel 4.8; Carl Zeiss AG, Oberkochen, Germany. Abnormal nuclei were scored from cells containing condensed nucleus, fragmented nucleus, micronucleus, and multilobulated nucleus. Abnormal nuclei were expressed as a percentage of the total of nuclei.

Western blot analysis

Whole cell lysates of treated cells were prepared at the indicated time points as described previously.[16] The protein from each sample was subjected to electrophoresis on 8-15% SDS-polyacrylamide gels and electro transferred to a PVDF membrane. The membrane was blocked in TBS-T containing 2% nonfat skim milk for 1 h at room temperature prior to antibody treatment. The membrane was probed with primary antibody diluted in 2% BSA in TBS-T at 4 °C overnight. The membrane was washed thrice with TBS-T and then probed with the secondary antibody diluted in blocking buffer for 1 h at room temperature. The membrane was washed thrice with TBS-T and the protein bands were detected with chemiluminescence using a digital phosphorimager (Chemi Doc™ XRS+Image Lab™ 5.1 software, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The data are presented as Mean±standard error of three independent experiment. The differences in each experiment group were analyzed by independent sample student's t-test at 95% confidence level. A p value less than 0.05 was considered as statistically significant.

Results

Radioprotective effect of *Centotheca lappacea* extract on human EA.hy926 endothelial cells

The radioprotective effect of *Centotheca lappacea* extract was assessed by MTT assay. EA.hy926 cells were pretreated with 0.2 µg/mL of *Centotheca lappacea* extract or left untreated. Three hrs later, the cells were irradiated with a single dose of 2 Gy of X-rays. Treatment of the cells with only *Centotheca lappacea* extract, did not affect cell viability of EA.hy926 cells (Figure 1). Cell viability of EA.hy926 cells significantly reduced to 68.0% after irradiation as compared to that of control untreated cells ($p<0.05$). Remarkably, treatment of the cells with *Centotheca lappacea* extract prior to irradiation significantly increased cell viability of EA.hy926 cells to 92.6% as compared to that of cells that were treated with only irradiation ($p<0.05$). These results clearly indicate a protective effect of *Centotheca lappacea* extract against radiation-induced toxicity in human endothelial cells EA.hy926.

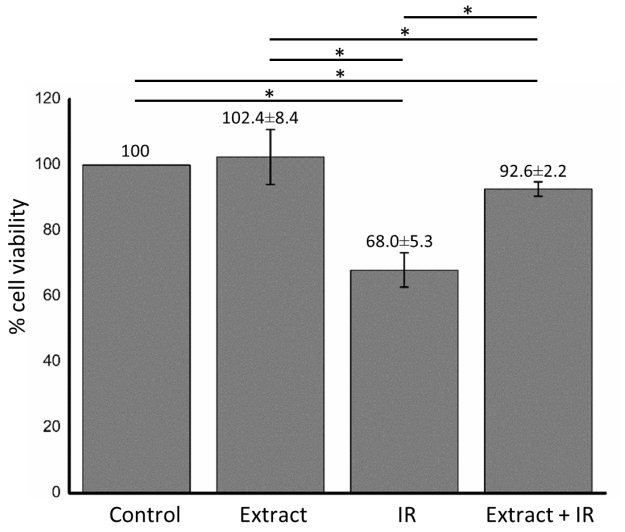

Figure 1. *Mean±standard error of cell viability at 24 hrs after irradiation of EA.hy926 cells pretreated with or without 0.2 µg/mL of Centotheca lappacea extract for 3 hrs prior to 2 Gy X-irradiation (Control: untreated cells, Extract: cells treated with only extract, IR: cells irradiated only, Extract+IR: cells treated with extract prior to irradiation).*

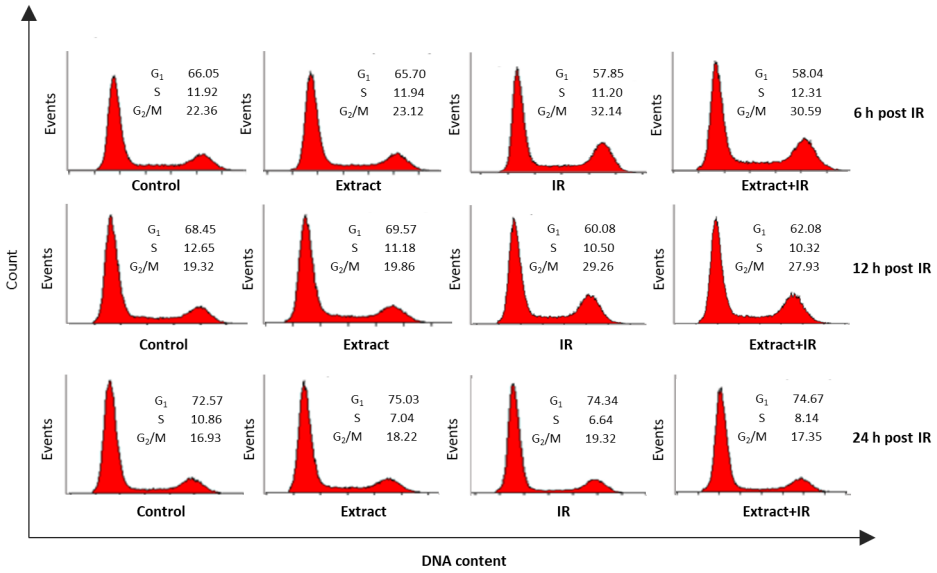

Figure 2. *Cell cycle distribution profiles of EA.hy926 cells at indicated time points after 2 Gy X-irradiation. (Control: untreated cells, Extract: cells treated with 0.2 µg/mL of Centotheca lappacea extract only, IR: cells irradiated only, Extract+IR: cells treated with 0.2 µg/mL of Centotheca lappacea extract prior to irradiation).*

Centotheca lappacea extract has no impact on cell cycle distribution of EA.hy926 cells.

The impact *Centotheca lappacea* extract on cell cycle progression might be involved in its radioprotective activity. Therefore, this hypothesis was elucidated by cell cycle analysis. Flow cytometry shows that cells treatment with 0.2 µg/mL of *Centotheca lappacea* extract did not alter cell cycle distribution as compared to that of control untreated cells (Figure 2). It is widely known that radiation can induce cell cycle arrest of mammalian cells at G2/M phase. Therefore, cell cycle distribution of EA.hy926 cells pretreated with or without *Centotheca lappacea* extract was determined at 6, 12 or 24 hrs after irradiation. As expected, radiation induced G2/M arrested was demonstrated in EA.hy926 cells that were not treated with the extract by an increase of the G2/M population from 22.36% to 32.14% at 6 hrs after irradiation. After that, the G2/M population of EA.hy926 cells slightly decreased to 29.26% at 12 hrs after irradiation, which remained evidently higher than that of control untreated cells. Similar pattern of radiation induced G2/M arrested was demonstrated in EA.hy926 cells that were pretreated with the extract prior to irradiation. The G2/M population of *Centotheca lappacea* extract pretreated cells increased from 23.12% to 30.59% at 6 hrs after irradiation. Afterward, the G2/M population slightly declined to 27.93% at 12 hrs after irradiation. Remarkably, radiation induced G2/M arrested in EA.hy926 cells completely abolished within 24 hrs after irradiation. Taken together, these results suggest that radioprotective mechanism of *Centotheca lappacea* extracts does not involve in the cell cycle progression of EA.hy926 cells.

Centotheca lappacea extract lessens radiation-induced abnormal nuclei formation of human EA.hy926 endothelial cells

To investigate whether *Centotheca lappacea* extract can reduce radiation-induced abnormal nuclei formation, nuclear morphology of EA.hy926 cells was analyzed. Cells pretreated with or without 0.2 µg/mL of *Centotheca lappacea* extract were irradiated with a single dose of 2 Gy. Twenty-four or 48 hrs later, cells were stained with Hoechst 33342 and nuclei were visualized by fluorescence microscopy. The percentages of abnormal nuclei in control untreated cells were 7.75+0.09% and 8.80+0.49% as determined at 24 and 48 hrs after mock irradiation, respectively (Figure 3). While percentages of abnormal nuclei in *Centotheca lappacea* extract treatment cells were 8.35+0.23% and 9.93+0.49% as determined at 24 and 48 hrs after mock irradiation, respectively. There were no statistically significant differences in the proportions of abnormal nuclei between *Centotheca lappacea* extract treatment and control untreated cells (p>0.05). In contrast, treatment of cells with 2 Gy of X-rays strikingly increased abnormal nuclei formation to 22.77+0.91% and 25.35+0.80% as determined at 24 and 48 hrs after irradiation, respectively. The portions of abnormal nuclei were 15.18+1.22% and 18.25+0.38% as determined at 24 and 48 hrs after irradiation, respectively, in cells that were treated with the extract in combination with radiation. Irradiation significantly increased the percentages of abnormal nuclei in cells that were pretreated with plant extract and in cells that were left untreated before irradiation as compared to control untreated cells (p<0.05). Yet, a significant lower percentage of abnormal nuclei was observed in cells that were pre-treated with extract before irradiation as compared to cells that were left untreated before irradiation (p<0.05). These observations indicate radioprotective effect of *Centotheca lappacea* extract against radiation-induced abnormal nuclei formation in EA.hy926 cells.

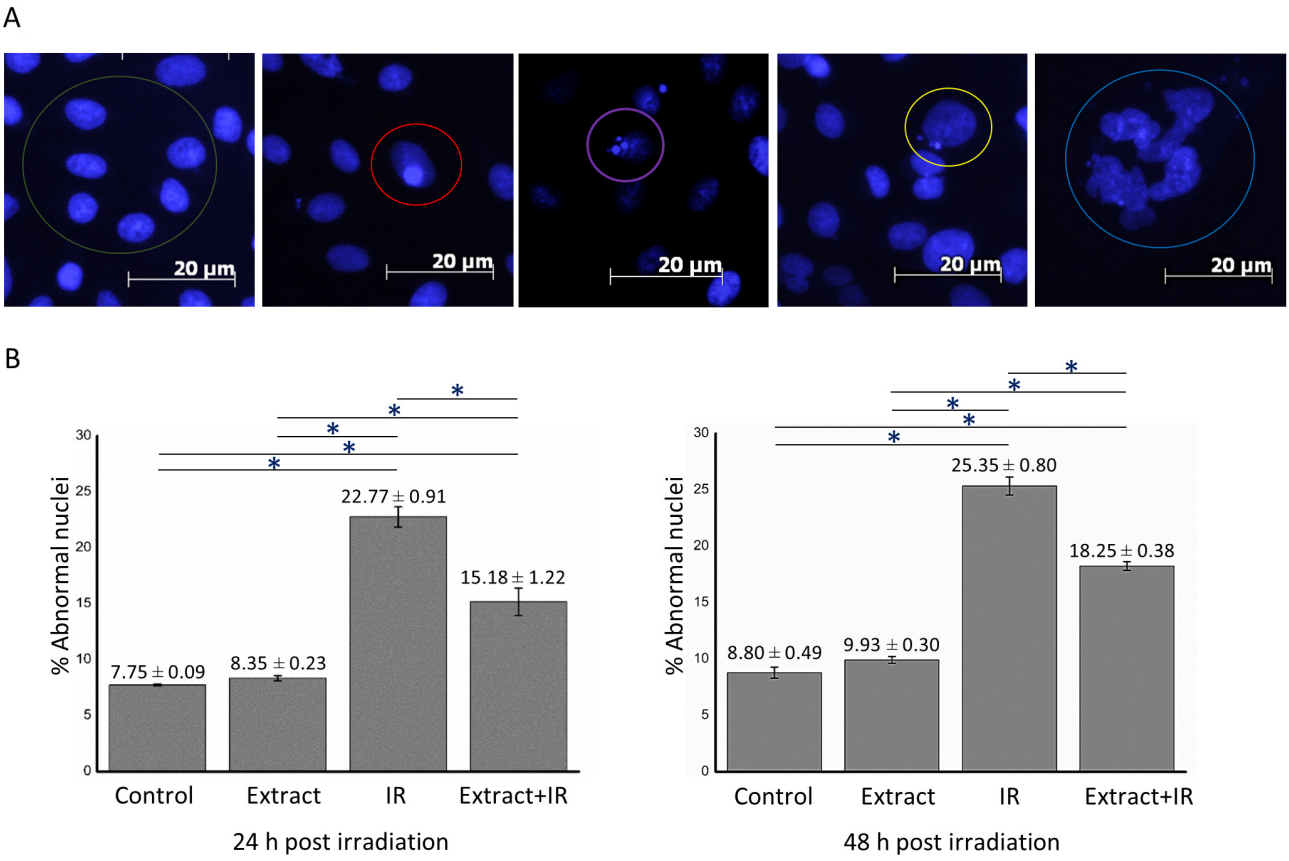

Figure 3. *Nuclei staining and fluorescence microscopic images of EA.hy926 cells. Cells were left untreated or treated with 0.2 µg/mL of Centotheca lappacea extract for 3 hrs. Subsequently, they were irradiated with a single dose of 2 Gy or left unirradiated. Hoechst staining was performed at 24 and 48 hrs after irradiation or mock irradiation. A) Representative fluorescence microscopic images of normal nuclei (green circle), condensed nucleus (red circle), fragmented nucleus (pink circle), micronuclei (yellow circle), and multilobulated nuclei (blue circle) are shown. B) Abnormal nuclei frequencies quantified from fluorescence microscopy of Hoechst 33342 nuclear stained cells. The percentages of abnormal nuclei are presented as the mean ± standard error of three independent experiments. More than 500 cells were counted for each determination. (Control: untreated cells, Extract: cells treated with 0.2 µg/mL of Centotheca lappacea extract only, IR: cells irradiated only, Extract+IR: cells treated with 0.2 µg/mL of Centotheca lappacea extract prior to irradiation).*

Centotheca lappacea extract effects Akt activity in EA.hy926 cells

Akt is a key protein in the PI3K-Akt pathway. It plays a role in controlling cell survival in response to DNA damage.[17-18] Immunoblotting of total lysates of EA.hy926 cells was performed to observe the effect of *Centotheca lappacea* extract on the activity of Akt. The level of Akt before and after irradiation did not alter in cells that were treated or untreated with the extract as compared to that of control untreated cells (Figure 4A). In contrast, the level of Akt phosphorylation (pAkt) was noticeably lower in cells that were treated with the extract as compared to untreated cell (Figure 4A and 4B). One h after irradiation, the level of pAkt clearly increased in cells that were pre-treated with plant extract and in cells that were left untreated before irradiation as compared to control untreated cells. Yet, a remarkably higher of pAkt level was observed in cells that were pretreated with extract before irradiation as compared to cells that were left untreated before irradiation. Three hours after irradiation, the level of pAkt was found to decline in cells that were treated with extract or were left untreated before irradiation. These results indicate an impact of *Centotheca lappacea* extract on Akt activity in EA.hy926 cells.

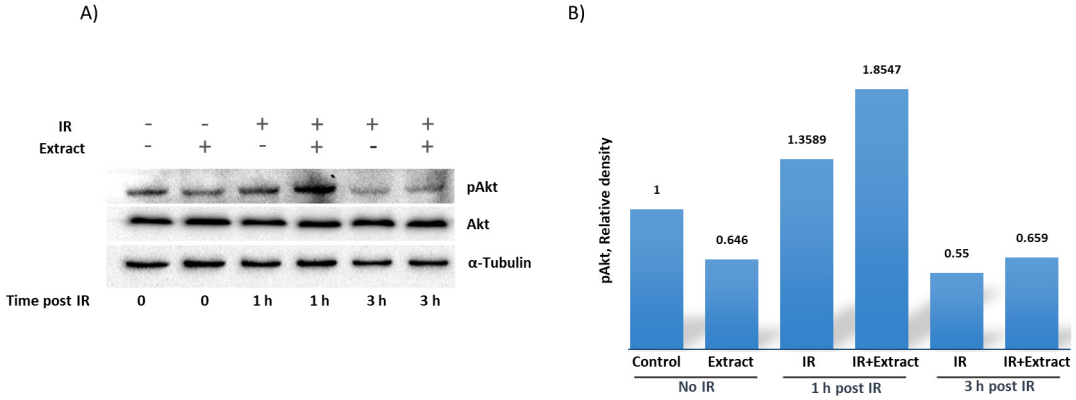

Figure 4. *EA.hy926 cells were collected at indicated time points for protein extraction. The levels of Akt and pAk were determined by Western blot analysis. The detection of tubulin was used as a loading control. (Control: untreated cells, Extract: cells treated with 0.2 µg/mL of Centotheca lappacea extract only, IR: cells irradiated only, Extract+IR: cells treated with 0.2 µg/mL of Centotheca lappacea extract prior to irradiation).*

Discussion

Over the past decades, investigators have been attempting to search for novel radioprotectors. Nevertheless, only very few compounds have been approved for clinical application due to high toxicity and side effects.[7, 19] In the present study, we clearly demonstrate that *Centotheca lappacea* extract decreases radiation-induced cytoxicity and abnormal nuclei formation in human endothelial cells EA.hy926. This finding reveals the radioprotective potential of *Centotheca lappacea* extract. Importantly, treatment of EA.hy926 cells with the extract prior to irradiation markedly increases the percentage of cell viability and lower the percentage of abnormal nuclei as compared to cells that were left untreated before irradiation. Whereas, treatment with the extract alone is not toxic to EA.hy926 cells. These findings indicate that *Centotheca lappacea* extract is a safe and potential candidate for the development of radioprotector reducing endothelial cell damage.

The protective effects of radioprotectors occur via different mechanisms, such as free radical scavenging, anti-inflammation, and facilitation of cellular repair activities.[8] *Centotheca lappacea* extract contains a variety of phenolic compounds and flavonoids, which have antioxidant and anti-inflammatory properties.[15-17] In addition, it contains p-coumaric acid, which has been reported to protect the rabbit corneal cells from UVB induced DNA damage.[15] Therefore, it is highly likely that radioprotective activity of *Centotheca lappacea* extract mediates its radioprotective effect via its antioxidant and anti-inflammatory properties. However, the precise mechanisms require further investigation.

Radiosensitivities of cells in each cell cycle phase are somewhat different. Cells are most radiosensitive in the G2/M phase, less sensitive in the G1 phase, and least sensitive in the S phase.[20] It has been reported that some plant extracts can alter radiosensitivity of the cells by interfering cell cycle regulation.[11, 12] However, we found no different in the pattern of cell cycle distribution before and after irradiation in cells that were treated or not with the extract. Therefore, the mechanism by which *Centotheca lappacea* extract mediates radioprotective effect does not involve cell cycle regulation.

Ionizing radiation has been shown to rapidly activate several cellular survival signaling cascades including PI3K-Akt pathway.[21] Activation of Akt is an intracellular stress response which has been found to promote cellular survival and inhibit radiation-induced cell death in several studies.[17,18, 21] In this experiment, we found that the level of phosphorylated Akt in EA.hy926 cells that were treated with *Centotheca lappacea* extract in combination with radiation was remarkably higher than that of irradiated cells and control untreated cells, as observed at 1 h after irradiation. This finding indicates that the radioprotective activity of *Centotheca lappacea* extract might mediate via the PI3K-Akt pathway. The extent of Akt activation depends on the magnitude of the damage caused by radiation.[22,23] In this study, we irradiated EA.hy926 cells with 2 Gy of x-ray. We found that, the level of phosphorylated Akt in irradiated cells strikingly declined as observed at 3 hrs after irradiation. It can be speculated that cellular stress induced by 2 Gy of x-ray in EA.hy926 cells was effectively eliminated within 3 hrs after irradiation.

Conclusion

Centotheca lappacea extract promoted cellular survival and inhibited radiation-induced abnormal nuclei formation with no toxic effect, no alteration of cell cycle regulation, and increase in Akt phosphorylation. The results of this study indicate that Centotheca lappacea extract is a promising candidate for radioprotector development to defend radiation-induced damage to endothelial cells.

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Conflict of interest

All authors declare no conflict of interest.

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