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Photodynamic apoptosis and antioxidant activities of *Brassica napus* extracts in U937 and SK-HEP-1 cells

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Abstract *Brassica napus* is the most common feedstock for biodiesel production, and its cultivation area has been rapidly increased. Thus, *B. napus* residues left in the field after harvest are valuable resources. However, there have been few studies on biologically active substances from *B. napus* plant. The objective of this study is to evaluate cytotoxicity/photodynamic activity and antioxidant activity of *B. napus* plant extracts. *B. napus* plants were sequentially extracted with organic solvents (hexane, chloroform, ethanol, and water) and then screened for antioxidant activity and cytotoxicity against leukemia U937 and human liver cancer SK-HEP-1 cells. Among the solvent extracts, the cytotoxicity was the highest when cells treated with chloroform extract and irradiated. Degree of apoptosis substantially increased in both cell types in concentration-dependent manner, and non-irradiated cells showed similar results as the control cells. For the highest concentrations (100 µg/ml), toxicity effect in U937 and SK-HEP-1 cells was $94.62 \pm 0.15\%$ and $74.16 \pm 1.54\%$, respectively. We observed the number of cells significantly decreased, and vesicles were floating in *B. napus* chloroform extract (BNCE) and light condition. BNCE induced DNA laddering pattern (between 300 and 1000 bp) and caspase-3/7 activation in both U937 and SK-HEP-1 cells. Total apoptotic U937 and SK-HEP-1 cells following BNCE 100 µg/ ml and light treatment were significantly increased ($92.62 \pm 2.07\%$ and $59.71 \pm 4.38\%$, respectively) compared with control. Our results showed that U937 cells were more sensitive than SK-HEP-1 cells. For the antioxidant activity, *B. napus* ethanol extract was the highest ($IC_{50} = 0.52$ mg/ml).

Keywords Apoptosis · *Brassica napus* · Caspase · Cell toxicity · Photodynamic

Introduction

Nowadays, photodynamic therapy (PDT) is being spotlighted in advanced nations as new cancer therapy, as it can minimize side effects as well as improve the quality of life of patients [1]. In PDT, photosensitizer injected into the body is selectively accumulated in tumor tissue and then activated by irradiating light of a particular wavelength, resulting in selective destruction of cancer cells. Currently, PDT has been clinically applied in cancer and general skin disease therapy [2, 3]. But, it rarely causes cutaneous phototoxicity, and patients receiving photosensitizers are advised to stay away from direct sunlight and bright ambient light until the photosensitizer concentration in the skin tissues decreases to safe level to avoid cutaneous phototoxicity [4].

Reactions to PDT can be classified as type I or type II. In a type I reaction, the photosensitizer directly reacts with sites of photosensitizer localization to generate free radicals, causing cell toxicity through an oxidation reaction. In a type II reaction, photosensitizer in triplet state directly delivers energy to oxygen to generate singlet oxygen, resulting in damage to cell structures, including lipids and membranes, nucleic acids, and proteins [5]. Most of the

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photosensitizers either approved for commercialization or undergoing various stages of clinical trials are related to tetrapyrrolic system (porphyrins, chlorins, bacteriochlorins, corroles, and phthalocyanines) or the extended ring systems (texaphyrin). Significant effort is now being employed in the development of derivatives with improved activity and minimal side effects [6, 7].

Brassica napus is Brassicaceae yearly plant originated from China. It has been prescribed for the treatment of dystocia, contraception, and menstrual irregularity for hundreds of years in Korea. In addition, it contains rich amount of vitamins and is ingested as an herbal treatment for spring fever and fatigue [8]. Until now, research has demonstrated the eco-friendly control of soil nematodes using *B. napus* rapeseed [9], effective weed control using rapeseed green manure [10], and the anticancer activity of brassinolide separated from pollen [11]. However, there has been insufficient research into the biological activity of *B. napus*. Recently, usage of plant residue after harvesting has increased due to enlarged cultivation areas of biodiesel material and ornamental grass. In this study, we confirmed the photodynamic and antioxidant activities of *B. napus* extract and provide basic data for the development of an anticancer drug and antioxidant based on its functionality.

Materials and methods

Chemicals and reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin (P/S), and trypsin/EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Actinomycin D and colcemid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caspase-3/7 activity assay kit and Cell viability assay kit were purchased from Promega (Madison, WI, USA). Annexin V and Dead cell kit was purchased from Merck Millipore (Darmstadt, Germany). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from TCI Co. (Tokyo, Japan).

Preparation of extracts from *B. napus*

Brassica napus was purchased from an agricultural and marine products market. Dried powder of *B. napus* was extracted three times with hexane, chloroform, ethanol and water for 4 h. Solvent extraction was performed sequentially from hexane with low polarity to H_2O with high polarity. Extracts were filtered through Advantech No. 5C filter paper (Toyo Roshi Kaisha, Ltd., Japan) and concentrated using a rotary evaporator and stored at 4 °C.

Cell line and cell culture

U937 human leukemia cells and SK-HEP-1 human hepatic cancer cells were obtained from the Korean Cell Line Bank (KCHB30052®, KOREA). U937 cells were cultured in RPMI medium, and SK-HEP-1 cells were cultured in DMEM medium. Medium consisted of 10% (v:v) FBS and 1% (v:v) penicillin–streptomycin. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere. Cells were passaged every 36 h to maintain an appropriate number.

### Determination of the photodynamic activity

The final concentration of DMSO did not exceed 1% (v/v) in all experiments. Cultured cells were treated with various concentrations of chloroform extract of *B. napus* (BNCE). After 4 h of incubation, cells were irradiated at 3250 Lux for 10 min with daylight fluorescent lamps having wavelengths of 440, 550, and 620 nm or kept in the dark. After 6 h of incubation, all assays were performed. ADCL (actinomycin D and colcemid) common apoptosis agent was treated as positive control and not light-dependent apoptosis induction [12]. The combination of ADCL concentration was 0.1 mM actinomycin D and 0.1 mM colcemid. It mixed equal volume and treated 2 μl in a well.

Monitoring of morphologic changes

To confirm photodynamic activity in cancer cells, we observed morphological changes in the light and dark groups. Cells were seeded at a density of 2×10^4 cells/well in 96-well plate and incubated for 6 h. BNCE was added at concentrations of 50 and 100 μg/ml to each well. After inducing photodynamic activity, various morphologic changes during apoptosis were observed by an IX51 inverted microscope (Olympus America Inc, Center Valley, PA, USA) (200× magnification).

Cell viability assay

To determine whether or not BNCE inhibits growth of cancer cells, cell viability was determined by Cell viability Glo 2.0 assay (Promega). Briefly, cells were seeded at a density of 1×10^4 cells/well in 96-well white plate. After 6 h of incubation, cells were treated with different concentrations of BNCE (10, 25, 50, and 100 μg/ml) in each well. After inducing photodynamic activity, cells were mixed with an equal amount of CellTiter-Glo® 2.0 reagent for 10 min. Luminescence was measured with a GloMax™ Multi+ microplate multimode reader (Promega).
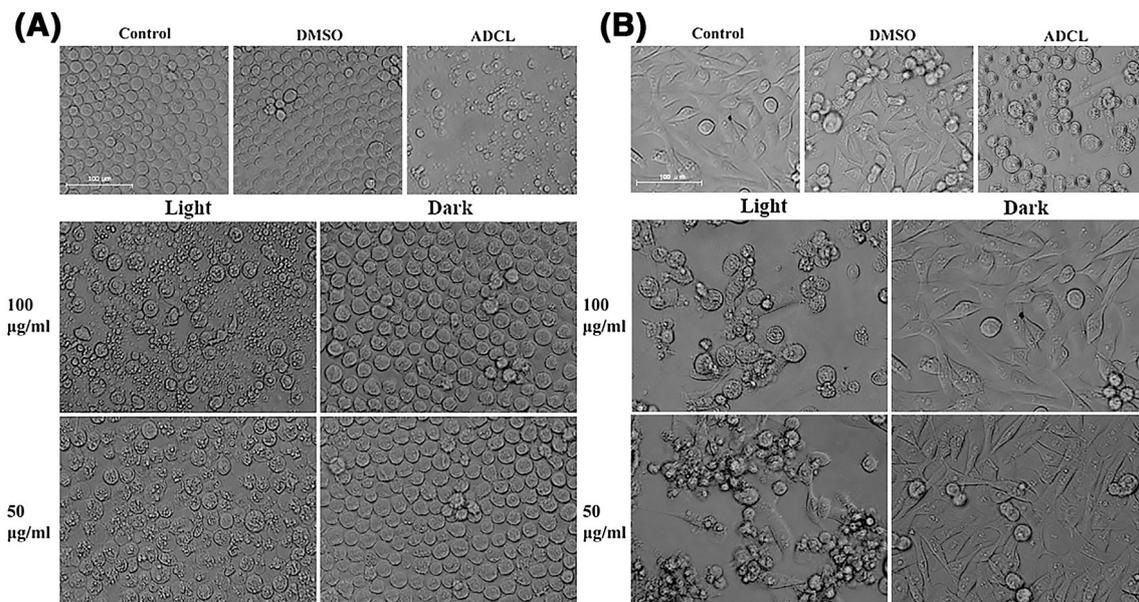
Fig. 1 Morphological changes in U937 and SK-HEP-1 cells treated with BNCE and light. Cells were treated with BNCE 50 and 100 µg/ml concentrations and then irradiated at 3250 Lux for 10 min with daylight fluorescent lamps (wavelengths: 440, 550, and 620 nm) or kept in the dark. After 6 h, cells were visualized under an inverted microscope. Cell death occurred only in the well treated with BNCE and light, the number of cells significantly decreased, and vesicles were floating. Magnification, ×200. (**A**) U937 cells, (**B**) SK-HEP-1 cells

Caspase activity assay

To evaluate changes in caspase activity by BNCE treatment, caspase activity was measured using Caspase-Glo 3/7 assay (Promega). Cells were seeded at a density of 1×10^4 cells/well in 96-well white plate and incubated for 6 h. BNCE was added at different doses of 10, 25, 50, and 100 µg/ml to each well. After inducing photodynamic activity, an equal volume of CellTiter-Glo® reagent was added, plates were shaken for 1 h, and luminescence measured with a GloMax™ Multi+ microplate multimode reader (Promega).

DNA fragmentation

To observe DNA fragmentation, which is biochemical hallmark of apoptosis, cells were seeded at a density of 1×10^6 cells/ml in 6-well plate and incubated for 6 h. BNCE was then added at concentrations of 50, 100, and 200 µg/ml. After inducing photodynamic activity, cells were harvested and washed with ice-cold PBS. Cell pellets were suspended in Lysis buffer (wizard SV Genomic DNA Purification system, Promega) at 4 °C for 10 min and centrifuged at 4 °C 14,000 rpm for 20 min. After collection, the supernatant was incubated with 2 µl of RNase A (10 mg/ml, Ambion) and 10 µl of proteinase K (20 mg/ml, Roche) at 37 °C for 1 h, separately. DNA was precipitated with nine volumes of isopropyl alcohol at 4 °C overnight and centrifuged at 14,000 rpm for 20 min at 4 °C. Pellets were dissolved with 0.5% TE buffer. Electrophoresis was performed on 1.6% agarose containing ethidium bromide. DNA bands were examined using a UV Transilluminator Imaging System.

Flow cytometer analysis

We used a Muse™ Annexin V and Dead cell kit (Millipore, Billerica, MA, USA) for quantitative analysis of apoptosis in cancer cells treated with BNCE. Cells were seeded at a density of 1×10^6 cells/ml in 6-well plate. After 6 h of incubation, BNCE was added at concentrations from 50, 100 µg/ml. After inducing photodynamic activity, cells were harvested, washed with ice-cold PBS, and stained according to the manufacturer's protocol. Briefly, cell suspensions were adjusted to 2×10^4 cells/100 µl and mixed an equal volume of solution for 20 min at room temperature in the dark. The reactions are measured using a Muse™ Cell Analyzer (Millipore).

Antioxidant activity

Hydrogen-donating ability may serve as significant indicator of antioxidant activity. We assessed the antioxidant activity of each extract using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and compared results with that of vitamin C as a positive control. Each
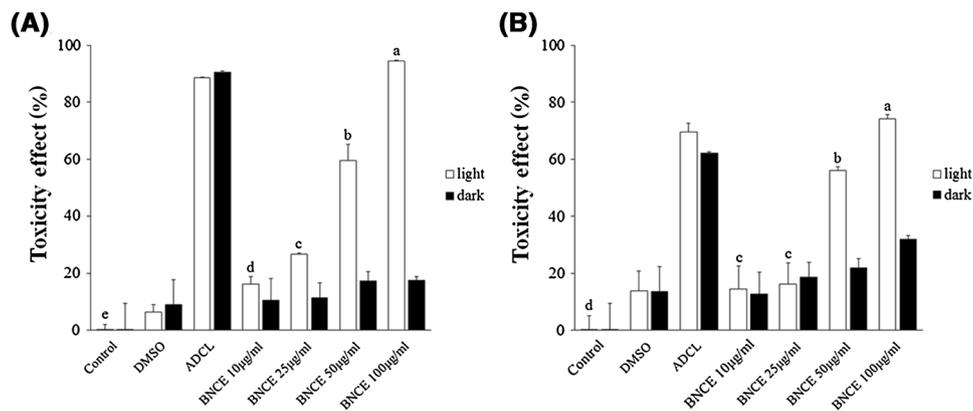
Fig. 2 Growth inhibitory effects of U937 and SK-HEP-1 cells by light treatment. Cells were treated with BNCE (10, 25, 50, and 100 μg/ml) for 4 h. Cells were irradiated with light for 10 min or kept in the dark, followed by incubation for 6 h. Toxicity effect was determined by CellTiter-Glo 2.0 assay. Results are expressed as the mean ± SD of three independent experiments. Statistical analysis was analyzed using one-way ANOVA $p < 0.001$, dark conditions not significant. *Different letters* denote significant differences between groups ($p < 0.001$). (**A**) U937 cells, (**B**) SK-HEP-1 cells

extract was applied at concentrations of 250, 500 μg, 1 mg, and 2 mg/ml in 96-well plate, followed by the addition of an equal volume of 0.2 mM DPPH solution. Plate was kept in the dark for 30 min for reaction. The absorbance was measured by an Epoch microplate reader (BioTek Instruments, Inc., USA) at 517 nm. The percentage inhibition of DPPH free radical was calculated as:

$$\text{Inhibition } (\%) = [1 - \{(S - S_0)/(C - C_0)\}] \times 100$$

where S is the absorbance of the sample containing DPPH, S_0 is the absorbance of the sample containing ethanol, C is the absorbance of the sample solvent containing DPPH, and C_0 is the absorbance of the sample solvent containing ethanol. The IC$_{50}$ value was defined as the concentration of extracts required to inhibit 50% of radical scavenger activity.

Statistical analysis

All statistical analyses were performed with SPSS (SPSS Inc, Chicago, IL, USA). Significant differences between groups were assessed by one-way analysis of variance (ANOVA). A $p < 0.001$ was considered to be significant. Results are expressed as the mean ± SD (standard deviation) of three independent experiments.

Results and discussion

BNCE induces morphological change of cancer cells

Cell death is mainly classified into apoptosis and necrosis, which are distinguished according to cell shape and
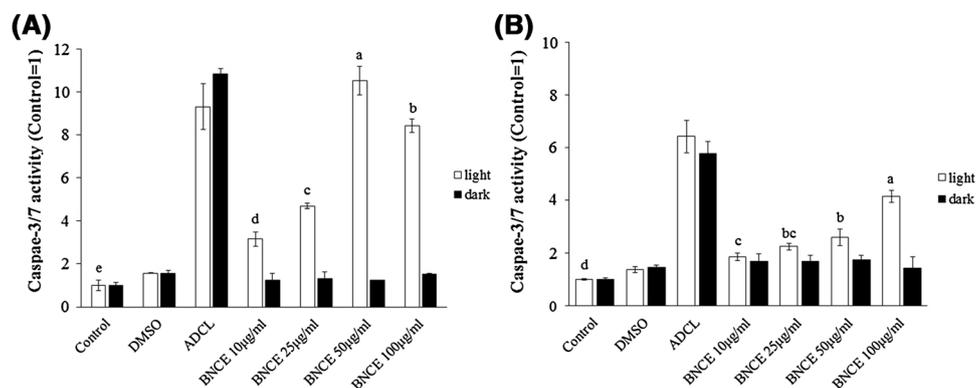
Fig. 3 Measurement of caspase-3/7 activity in U937 and SK-HEP-1 cells by BNCE and light treatment. Cells were treated with BNCE (10, 25, 50, and 100 μg/ml) for 4 h. Cells were irradiated with light for 10 min or kept in the dark, followed by incubation for 6 h. Caspase activity was determined by Caspase-Glo 3/7 assay. Results are expressed as the mean ± SD of three independent experiments. Statistical analysis was analyzed using one-way ANOVA $p < 0.001$, dark conditions not significant. *Different letters* denote significant differences between groups ($p < 0.001$). (**A**) U937 cells, (**B**) SK-HEP-1 cells

biochemical characteristics. Apoptosis is an active process controlled by genes. On the contrary, necrosis causes inflammation by destroying the cell membrane, resulting in cell swelling and spillage of intracellular materials into the surrounding environment [13]. Apoptotic cell death is morphologically characterized by cell shrinkage, membrane blebbing, cell budding, and formation of apoptotic bodies without plasma membrane breakdown [14]. In this study, we observed photodynamic-mediated cell death by introverted microscopy. As shown in Fig. 1, U937 showed a monocyte shape during normal growth, whereas SK-HEP-1 cells implanted in the well plate were epithelial form [15]. BNCE-treated cells irradiated with light showed apoptotic bodies, and the number of cells was significantly reduced. Floating small vesicles were observed in U937 cells (Fig. 1A). SK-HEP-1 cells showed loss of adherence and cell floating after apoptosis (Fig. 1B). Further, there was no difference in cell morphology between non-irradiated BNCE-treated cells and control cells. The cytotoxicity appeared only in cells irradiated with light after BNCE treatment. Other sequentially solvent extracts did not affect morphology (data not shown). The results of the examination of the UV–Vis absorbance of BNCE showed maximum absorption band at 420, 460, and 660 nm like chlorophyll a. The BNCE may possibly contain considerable amount of porphyrin derivatives.

BNCE has toxicity effects in dose-dependent manner

To evaluate the effects of BNCE treatment on growth inhibition in U937 and SK-HEP-1 cells, toxicity effect assay was performed. As shown in Fig. 2A, B, toxicity effect was increased in U937 and SK-HEP-1 cells, depending on light and dose. Compared to the control group, the toxicity effect increased from $16.37 \pm 2.60\%$ at a concentration of 10 µg/ml BNCE to $94.62 \pm 0.15\%$ at 100 µg/ml BNCE in U937 cells, whereas the effect increased from $14.41 \pm 8.21\%$ at 10 µg/ml BNCE to $74.16 \pm 1.54\%$ at 100 µg/ml BNCE in SK-HEP-1 cells. A significant toxicity effect was seen above 50 µg/ml BNCE in U937 and SK-HEP-1 cells ($59.55 \pm 5.75\%$ and $56.07 \pm 1.39\%$, respectively). For the low concentrations (10 and 25 µg/ml), toxicity effect is similar to DMSO treatment in SK-HEP-1 ($14.41 \pm 8.21\%$ and $16.25 \pm 7.36\%$, respectively). Results of our experiment revealed that the BNCE exhibits relatively high toxicity effect in U937 cells against SK-HEP-1 cells. This corresponds to a previous study reporting that the effects of photosensitizers depend on tissue distribution, affinity for certain cell types, penetration into various subcellular compartments, and accumulation [16]. Further, there was the difference between cells due to the various mechanisms involved in cell death [3, 17, 18]. The phototoxicity at 100 µg/ml of BNCE was higher than ADCL. U937 and SK-HEP-1 cells were not cytotoxic in the absence of light irradiation (Fig. 2).

BNCE induces apoptosis via caspase-3/7 activation in U937 and SK-HEP-1 cells

Caspase is an important apoptosis inducer that exists as an inactive pro-form on the outer membranes of nuclei and mitochondria, regulated by the balance between anti-apoptotic (Bcl2, Bcl-xL) and pro-apoptotic members (Bax,
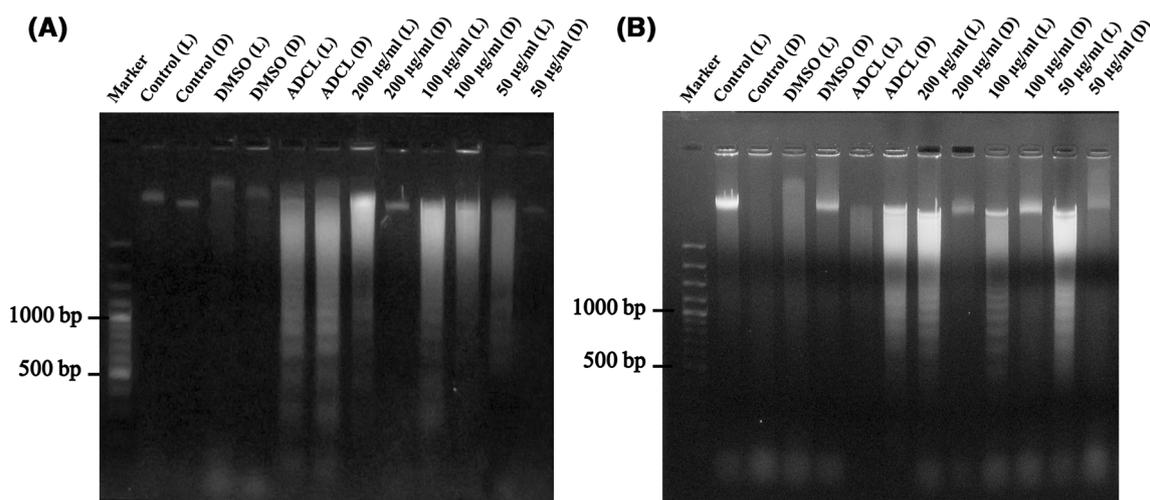
Fig. 4 Detection of DNA fragmentation in U937 and SK-HEP-1 cells by BNCE and light treatment. Cells were treated with BNCE (50, 100, and 200 µg/ml) for 4 h. Cells were irradiated with light for 10 min or kept in the dark, followed by incubation for 6 h. Cells were harvested, after which DNA was extracted and determined using electrophoresis on 1.6% agarose gel. (A) U937 cells, (B) SK-HEP-1 cells (L light, D dark)
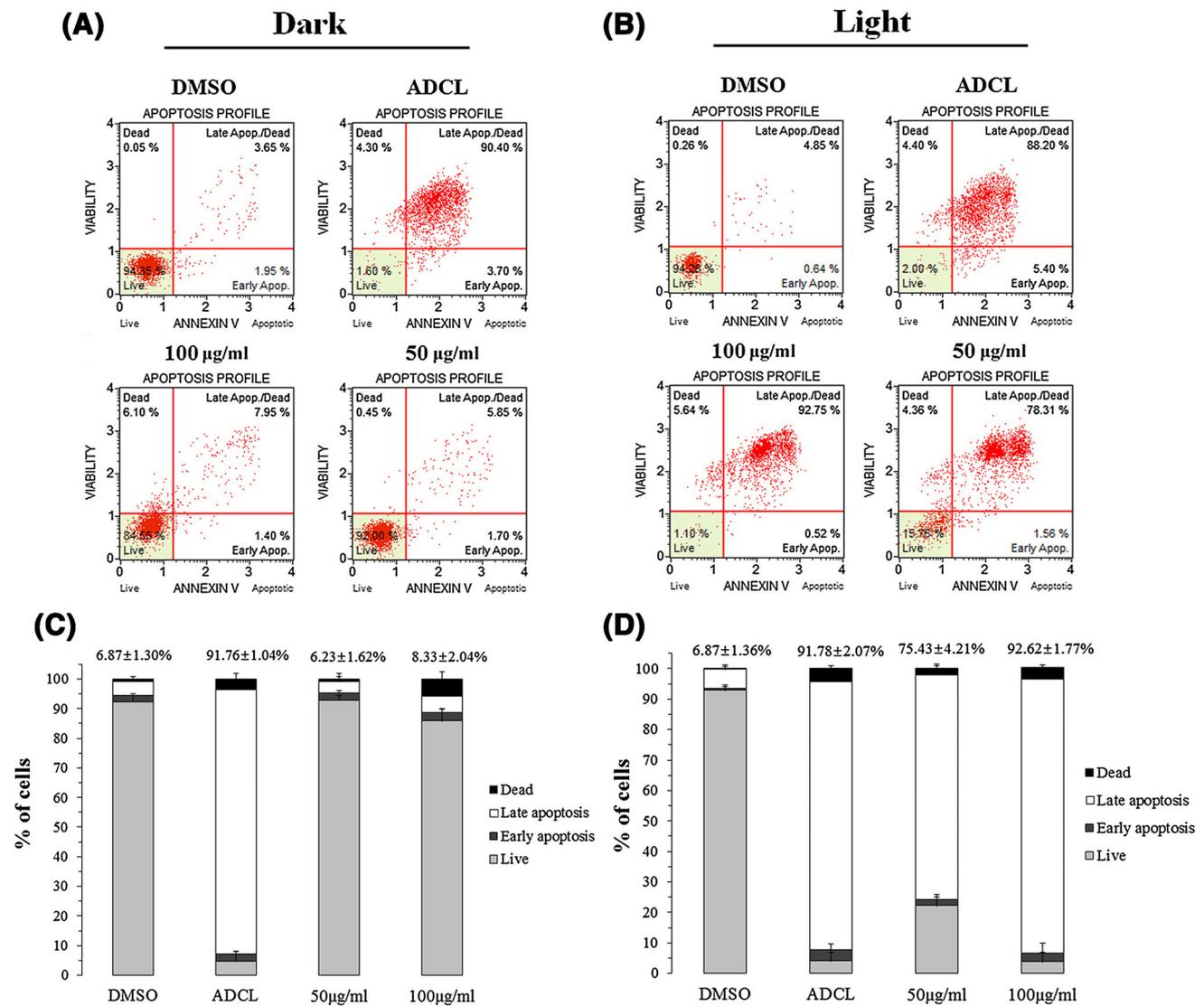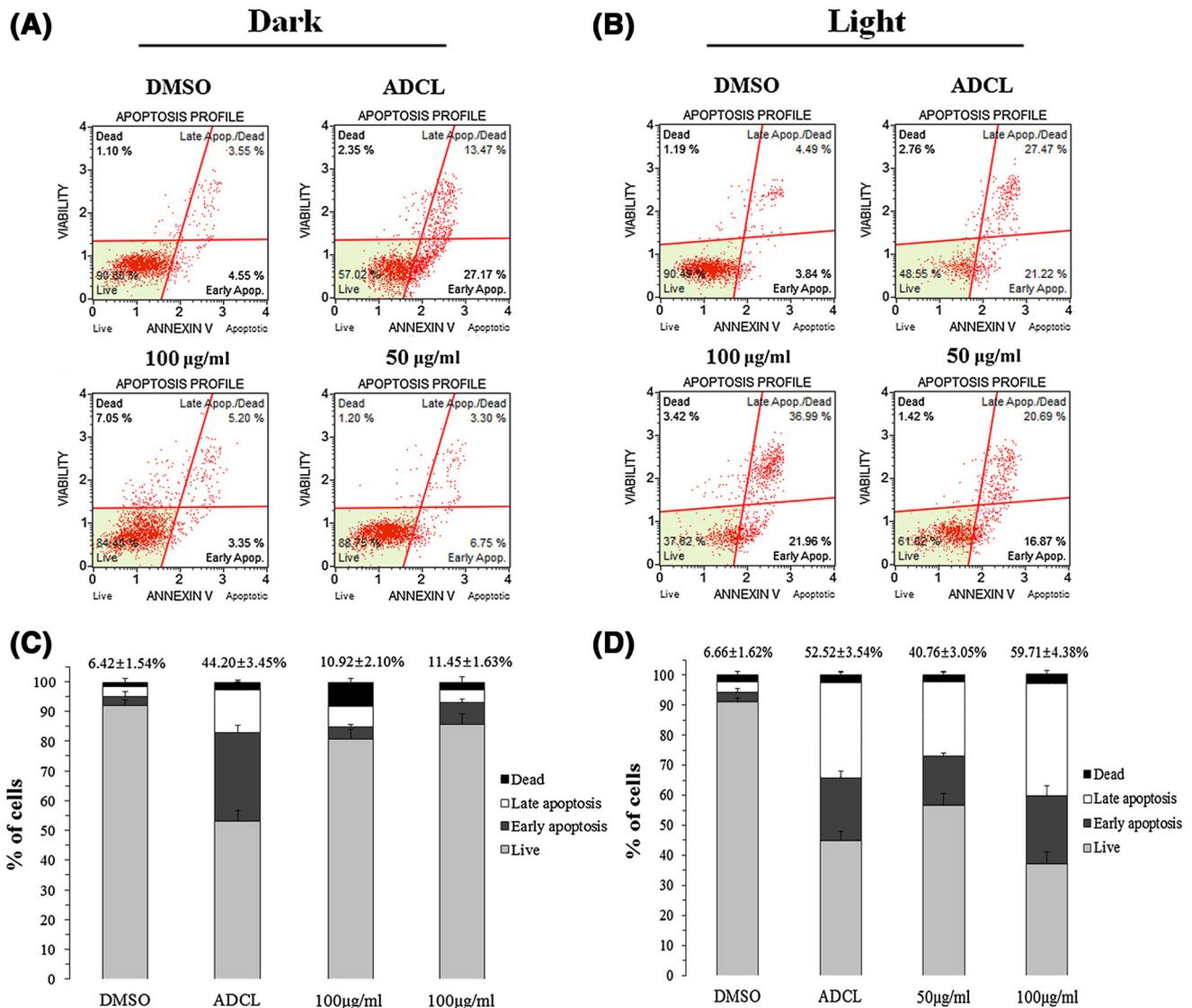
Fig. 5 U937 cells viability by BNCE treatment and light irradiation. Cells were treated with BNCE (50 and 100 μg/ml) for 4 h. Irradiated with light for 10 min or kept in the dark, followed by incubation for 6 h. Cells were stained using a Muse^TM Annexin V and Dead cell kit for 20 min and analyzed using a Muse^TM Cell Analyzer. Results show a dose-dependent increase in the number of apoptotic U937 cells compared to dark conditions. (**A**), (**B**) Scatter plots showing distribution of Annexin V and 7-AAD staining (UL: dead cells, UR: late apoptotic/dead cells, LL: live cells, and LR: early apoptotic cells). The (**C**), (**D**) represent % of cells and the summary mean percentages ± SD of apoptosis (early and late apoptosis) of three independent experiments in dark and light. Statistical analysis was analyzed using one-way ANOVA

Bcl-xs, Bid, Bad). Upon activation, caspase cleaves various cellular substrates such as actin, poly (ADP-ribose) polymerase, fodrin, and lamin [19]. Approximately 14 major caspases have been reported until now and are broadly categorized into initiators, effectors or executioners, and inflammatory caspases [20]. In this study, we investigated caspase-3/7 activity, which is an executioner type, according to BNCE and light treatment in U937 and SK-HEP-1 cells. In U937 cells, caspase activity increased until a concentration of 50 μg/ml in a light and BNCE dose-dependent manner. Especially, 50 μg/ml of BNCE resulted in 10.52 ± 0.67 caspase-3/7 activity higher than those of ADCL (9.30 ± 1.06). However, caspase activity was reduced at a BNCE concentration of 100 μg/ml (8.42 ± 0.32) (Fig. 3A). Reduced caspase activity at 100 μg/ml BNCE concentration was maybe due to cellular damage or caspase inactivation because of high BNCE concentration. These results are consistent with several reports that caspase-3/7 activation occurs later with lower doses of cisplatin, whereas caspase-3/7 activation occurs

Fig. 6 SK-HEP-1 cells viability by BNCE treatment and light irradiation. Cells were treated with BNCE (50 and 100 µg/ml) for 4 h. Irradiated with light for 10 min or kept in the dark, followed by incubation for 6 h. Cells were stained with Muse™ Annexin V and Dead cell kit for 20 min and analyzed using a Muse™ Cell Analyzer. Results show a dose-dependent increase in the number of apoptotic U937 cells compared to dark conditions. (**A**), (**B**) Scatter plots showing distribution of Annexin V and 7-AAD staining (UL: dead cells, UR: late apoptotic/dead cells, LL: live cells, and LR: early apoptotic cells). The (**C**), (**D**) represent % of cells and the summary mean percentages ± SD of apoptosis (early and late apoptosis) of three independent experiments in dark and light. Statistical analysis was analyzed using one-way ANOVA

earlier and decreases as time passes with higher doses of cisplatin [21]. Caspase activity in SK-HEP-1 cells increased in a dose-dependent manner and reached a maximum at 100 µg/ml of BNCE. On the other hand, caspase activity of non-irradiated BNCE-treated cells was similar to that of control cells (Fig. 3B). Results indicate that the growth inhibition activity of BNCE is associated with apoptosis via a caspase-dependent mechanism. When U937 and SK-HEP-1 cells were treated with the same concentration of BNCE, caspase activity was higher in

U937 cells. These results are due to differences in cell sensitivity.

BNCE induces DNA fragmentation

Fragmentation of nuclear DNA into base pairs through activation of endogenous nucleases is a hallmark of apoptosis [22, 23]. Caspase activation is known to induce apoptosis, activate DNase, and cause DNA fragmentation [15]. To check whether or not inhibition of cancer cell
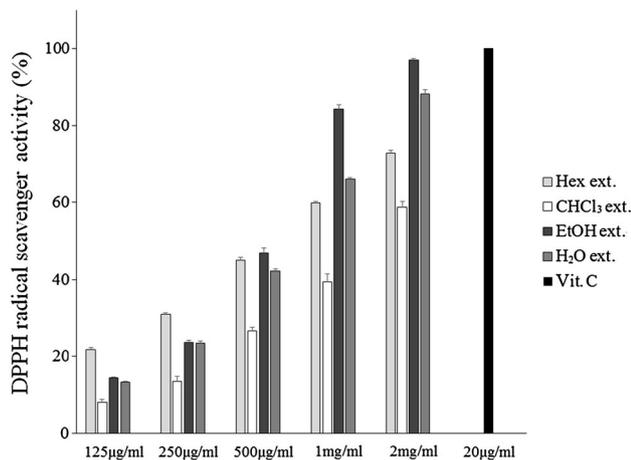
Fig. 7 Antioxidant activity of sequential solvent extracts from *B. napus*. Antioxidant activity was evaluated by measuring reduction in DPPH radical detected at 517 nm. Vitamin C was used as a positive control. Results are expressed as the mean ± SD of three independent experiments

proliferation by BNCE is related to induction of apoptosis, DNA fragmentation was observed by agarose gel electrophoresis using ethidium bromide staining and ultraviolet illumination. As shown in Fig. 4A, B, no DNA ladder pattern was observed in the control and dark. Only BNCE-treated and light-illuminated cells were markedly induced DNA laddering pattern (between 300 bp and 1000 bp) in both U937 and SK-HEP-1 cells, similar to ADCL treatment, indicating apoptotic cell death. However, U937 and SK-HEP-1 cells showed no internucleosomal (200 bp) DNA. Several research groups reported large DNA fragments during apoptosis and proposed that large DNA fragmentation occurs before internucleosomal DNA cleavage, and these large fragments serve as precursors for smaller DNA fragments [14].

BNCE increases apoptotic cell death

Phosphatidylserine (PS) is located in the inner leaflet of the plasma membrane. However during apoptosis, PS is translocated to the outer leaflet. Exposed PS is recognized and absorbed by phagocytes [24, 25]. External PS was stained by Annexin V and Dead reagent to quantitatively assess the degree of apoptosis using a cell analyzer. Simultaneous staining could distinguish between intact cells, early apoptosis, late apoptosis, or cell death. As a result, degree of apoptosis substantially increased in both cell types in a BNCE concentration-dependent manner, whereas non-irradiated treatment showed results similar to the control (Figs. 5A, 6A). We observed that BNCE induced cell death through apoptosis in both cell types. Total apoptotic U937 cells following BNCE (50 and

100 μg/ml) and light treatment were significantly increased (75.43 ± 4.21% and 92.62 ± 1.77%, respectively) compared with control (6.87 ± 1.36%) (Fig. 5D). For the highest BNCE concentration 100 μg/ml, total apoptosis of SK-HEP-1 cells increased 59.71 ± 4.38% compared to 6.66 ± 1.62% in control. The weakest effect (40.76 ± 3.05%) was observed in SK-HEP-1 cells treated with 50 μg/ml of BNCE for 6 h (Fig. 6D). The numbers of cells in late apoptosis and early apoptosis were similar (Fig. 6B). The total apoptosis of each cells following ADCL treatment was slightly lower than treatment 100 μg/ml BNCE and light (Figs. 5, 6). The results suggest that the relative apoptosis efficacy of 100 μg/ml BNCE in U937 cells within 6 h is substantially higher compared to 100 μg/ml BNCE in SK-HEP-1 cells. BNCE induced more late apoptosis in U937 cells than did SK-HEP-1 within 6 h. Therefore, U937 cells were more sensitive than SK-HEP-1 cells.

Brassica napus extracts show antioxidant activity

Although excessive ROS production destroys normal cells, it plays a key role in apoptosis in cancer cells [26]. The radical scavenging activity of vitamin C was 100% at a concentration of 20 μg/ml (data not shown). In measuring the antioxidant activity of each extract based on control, IC_{50} values in hexane and H_2O extracts were 0.63 ± 0.01 mg/ml and 0.64 ± 0.01 mg/ml, respectively. The IC_{50} value of EtOH extract was 0.52 ± 0.01 mg/ml and resulted in the highest activity. The IC_{50} value of BNCE was lower than those of other extracts at 1.47 ± 0.01 mg/ml. Antioxidant activity of H_2O, EtOH, hexane, and $CHCl_3$ extracts increased sequentially in dose-dependent manner (Fig. 7). BNCE, which most effectively suppressed cell proliferation, had the weakest antioxidant effect. Our results correspond to several reports that antioxidants increased cancer cell proliferation due to eliminating radical and ROS induced by apoptosis [27, 28]. In addition, these results were due to complex interactions among the various compounds contained in *B. napus*.

Apoptosis induction is the most effective means to control growth of cancer. In terms of cancer therapy, studies on the safety and efficacy of active materials derived from natural products can contribute to the development of new drugs. In this paper, we confirmed that chloroform extract of *B. napus* induced light-dependent apoptosis in U937 and SK-HEP-1 cells. Although BNCE is a crude extract, it has strong effect cell death at low concentrations. Further studies are needed to identify active compounds and evaluate its in vivo activity. Therefore, *B. napus* has sufficient potential as a photosensitizer and antioxidant source.

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