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Original Article

Cytotoxicity of Selenium-Enriched Chinese Kale (*Brassica oleracea* var. alboglabra L.) Seedlings Against Caco-2, MCF-7 and HepG2 Cancer Cells

Vijitra Luang-In[1,*], Worachot Saengha[1], Benjaporn Buranrat[2], Anut Chantiratikul[3], Nyuk Ling Ma[4]

ABSTRACT

Background: The Selenium-enriched Chinese kale (*Brassica oleracea* var. alboglabra L.) seedlings (Se-KS) have been known for its antioxidant activities, however its cytotoxic effects on various cancer cells are yet to be reported. **Objective:** The objective of this work was to study the cytotoxic effects of Se-KS on Caco-2, MCF-7 and HepG2 cancer cells. **Materials and Methods:** Freeze-dried seedlings were ground and incubated in 0.1 M citrate phosphate buffer pH 7.0 for 1 h at 37°C and extracted with dichloromethane to obtain total isothiocyanate (ITC) content which was quantified using the 1,2-benzenedithiole (BDT)-based cyclocondensation assay. The extracts from fresh seedlings were used to determine the cytotoxic effect on Caco-2, MCF-7 and HepG2 cancer cells. **Results:** Se-KS was found to contain total ITC content at 1.02 mmol/100 g dry weight (DW) which was significantly lower than that of 7-day old broccoli microgreens (1.60 mmol/100 g DW) as reference Cruciferous vegetables. In addition, Se-KS extract exhibited cytotoxic effects in a dose- and time-dependent manners. The lowest IC50 value of 82.83 µg/mL at 72 h was derived from HepG2 cells and the highest IC50 value of 164.00 µg/mL at 72 h was from MCF-7 cells suggesting that the Se-KS extract was most effective against HepG2 cells. Cancer cells showed signs of apoptotic bodies over 72 h and DNA fragmentations at 24 h indicating that the Se-KS extract was able to induce apoptosis in cancer cells in addition to cytotoxic effect. **Conclusion:** Thus, Se-KS could be a novel source of organo selenium with chemopreventive benefits for functional food development.

Key words: Kale, Caco-2, HepG2, Isothiocyanate, MCF-7, Selenium.

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INTRODUCTION

About 10% of the world's vegetable production is derived from Cruciferae species.[1] Vegetables in the Cruciferae family are well-known to produce the glucosinolates; S-containing compounds with a plethora of evidence in human health benefits in especially their hydrolysis products called isothiocyanates (ITCs).[2,3] Mainly, glucosinolates are responsible for plant communicating and signaling pertaining in response to external or environmental stimuli such as plant defense against insects, some food bacteria, and against some fungi.[2,3] Interestingly, Cruciferous species are able to accumulate Selenium (Se) and synthesize seleno-amino acids, selenoproteins as well as selenoglucosinolates. The breakdown products of selenoglucosinolates such as phenylalkyl isoselenocyanates confer more chemopreventive effects compared to their S-containing analogs.[4,5] The antioxidant selenoproteins have been presumed to have counter effect against oxidative stress.[6] Previously, it was shown that consumption of Se-enriched broccoli containing these seleno-compounds alongside others may have also a beneficial implication in the human immune response.[7]

In Thailand, Chinese kale (*Brassica oleracea* var. alboglabra L.) represents the most popular vegetable consumed and belongs to the family of Cruciferae.[8] Chinese kale can reduce the toxicity of inorganic Se because it transforms inorganic Se to several organic species of Se.[8] Recently, it has been reported that the highest antioxidant activities of Se-enriched Chinese kale seedlings (Se-KS) were found in seedlings grown on 30 mg·Se/L, 15 days after germination.[8] Total Se content and antioxidant activities were strongly correlated suggesting that Se could enhance antioxidant capacities. In addition, Se-KS did not create any toxicological signs or abnormal pathological changes in the liver, kidney, and heart or mortality in rats.[8] Therefore, the Se-KS could be an alternative source of organo Se as a novel Se supplementary functional food. However, the cytotoxicity of Se-KS on human cancer cells is yet to be reported. Thus, the objective of this work was to study the cytotoxic effects of Se-KS on Caco-2, MCF-7 and HepG2 cancer cells.

MATERIALS AND METHODS

Cultivation of Se-KS

Seeds of Chinese kale (*Brassica oleracea* var. alboglabra L.) were obtained from Chia Tai Company Limited (Thailand). Authentication of the seeds was done by Assoc. Prof. Dr. Anut Chantiratikul from Mahasarakham University using seed morphology checking and cultivation trial in

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prior to this work. Briefly, the Chinese kale seeds were soaked in tap water for 15 h before planting. After that, the seeds were cultivated on a wet sponge (35 × 40 × 30 cm) at room temperature in the dark for 3 days until the seeds began to germinate. The covers of the plastic pots were released, and the germinated Chinese kale was subjected to light from a fluorescent lamp (36 W) with a 12 h dark/light cycle and watered with tap water daily for 4 days as previously described.[9] Afterwards, the germinated seedlings were cultivated in the adapted modified Hoagland's solution containing 30 mg·Se/L in the form of sodium selenite under light intensity of 200 µmol m^{-1} sec^{-1} for 12 h per day. After 15 days of cultivation, the Se-Chinese kale (Se-KS) seedlings were harvested, washed thoroughly with deionized water, freeze-dried, and stored in a freezer at −20°C until analysis. Broccoli microgreens of 7-day old without Se supplementation were used as reference. Broccoli seeds (Now Foods, USA) were grown according to the modified method of Wu *et al.* (2019).[10] Briefly, broccoli seeds were soaked in distilled water for 8 h and germinated on vermiculite at 25 °C (12 h light/12 dark cycle) and were sprayed with 20 mL of deionized water daily for 7 days till harvested by cutting the shoots above the roots, freeze-dried and stored at -20 °C.

Quantification of the total ITC content

ITCs were extracted according to Luang-In *et al.* (2018).[11] The freeze-dried Se-KS (250 mg) or broccoli microgreens (250 mg) were ground in a sterile mortar with 4 mL of 0.1 M citrate-phosphate buffer (pH 7.0) and then the mixture was incubated at 37°C for 1 h with shaking at 250 rpm. Subsequently, dichloromethane (DCM) was added in a ratio of 1:1 to the mixture and incubated at 37°C for 30 min with shaking at 250 rpm. The mixture was then centrifuged at 10,000 g for 15 min and the lower layer of DCM phase was transferred to mix with 0.5 g $MgSO_4$ to rid of moisture and centrifuged again for 15 min. The supernatant was diluted with methanol in a ratio of 1:4 and dried using a rotatory evaporator at 40°C. Quantification of the total ITC content was carried out using the 1,2-benzenedithiole (BDT)-based cyclocondensation assay as previously described by Zhang (2012).[12] In theory, ITCs react with BDT and are fully converted to 1,3-benzodithiole-2-thione which can be detected at 365 nm using a spectrophotometer. Briefly, the ITC extract dissolved in methanol (10 µL) and methanol (90 µL) were added to the 96-well plate along with 0.1 M phosphate buffer pH 8.0 (90 µL) and 0.08 M 1,3-benzodithiole-2-thione (10 µL). The 96-well plate was incubated at 60°C for 2 h prior to measurement at 365 nm using a microplate reader (M965+, Mastertech, Taiwan). Direct spectrophotometric determination of the reacted solution at 365 nm allows measurement of as low as 1 nmol of ITC.[12] The total ITC content was recorded as mmol/100 g DW when calibrated to the ITC authentic standard, benzyl isothiocyanate (BITC) (Sigma-Aldrich, UK) with triplicate.

ITC extract preparation for cytotoxicity test

ITCs were extracted similar to the above method (Luang-In *et al.*, 2018)[11], however here fresh seedlings were used. Fresh Se-KS samples (50 g) were ground and homogenized with 50 mL of 0.1 M citrate phosphate buffer, pH 7.0 and incubated at 37°C for 1 h at 250 rpm using a shaking incubator (LSI-1005R, LabTech, Korea). Subsequently, DCM was added in a ratio of 1:1 to the mixture and incubated at 37°C for 30 min at 250 rpm using a shaking incubator (LSI-1005R, LabTech, Korea). DCM was a suitable solvent since ITCs were weakly polar and DCM led to higher ITC yields than when using ethyl acetate, acetonitrile or water.[13] The mixture was then centrifuged at 10,000 g for 15 min and the lower layer of DCM phase was transferred to mix with 5 g $MgSO_4$ to rid of moisture and centrifuged again for 15 min and clear supernatants were dried using a rotatory evaporator at 40°C.The ITC extract was then dissolved in dimethylsulfoxide (DMSO) at the concentration of 40 mg/mL and used as a stock solution and filtered through a 0.22 µm for cytotoxicity assay.

Cancer cell cultures

The human colorectal adenocarcinoma Caco-2 (ATCC® HTB-37™), human breast adenocarcinoma MCF-7 (ATCC® HTB-22™) and human hepatocellular carcinoma HepG2 (ATCC® HB-8065™) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cancer cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich)[14] supplemented with 10% (v/v) fetal calf serum and 100 U/mL of penicillin, 100 µg/mL of streptomycin at 37°C in an atmosphere of 5% CO_2/95% air at constant humidity. DMEM media for cell lines cultures were renewed every 2-3 days until 80% confluency was reached. Cultured cell lines were washed with phosphate-buffered saline (PBS), pH 7.2 before trypsinization with 0.25% Trypsin-EDTA.

Cytotoxicity on cancer cells

This was conducted as Buranrat et al. (2017).[14] Cancer cells were initially inoculated into a 96-well plate (at 5×10^3 cells/well). After incubation for 24 h, ITC extract from Se-KS (at a final concentration of 0-250 µg/ml) were subsequently added to the cancer cells, and then incubated for 24 -72 h and control wells were cancer cells treated with 0.5% DMSO in media. Thereafter, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (at a final concentration of 0.5 mg/mL) was added to each well and incubated for an additional 4 h. Culture media were discarded, 200 µL of DMSO was added and vibrated for 20 min. Absorbance was measured at 590 nm using a microplate reader (M965+, Mastertech, Taiwan). Cytotoxicity of ITC extract of Se-KS against cancer cells was measured as IC_{50} value. When % cytotoxicity was ≤ 50%, it represented non-cytotoxic effect and when % cytotoxicity was >50%, it represented cytotoxic effect. Cell morphology was also observed using an inverted microscope (NIB-100, Xenon, China).

DNA fragmentation analysis

The cancer cells ($2x10^5$ cells/mL) were seeded into 6 well plates and incubated at 37°C under 5% CO_2 for 24 h. ITC extract of Se-KS (250 µg/mL) were added and followed the same incubation condition for 48 h. Cells were washed with PBS buffer (pH 7.2) and genomic DNA was then extracted using GF-1 Plant DNA Extraction Kit (Vivantis, Malaysia) according to the kit's manual. The extracted DNA (1 µg/mL) was analyzed on gel electrophoresis of 1% gel containing 3 µL/100 mL of SYBR Safe dye (Vivantis, Malaysia). The genomic DNA (1 µg/mL) was mixed with 6X loading dye in the ratio of 5:3 prior to running on agarose gel electrophoresis at 100V for 40 min. DNA maker (100 ng) was used and DNA bands were viewed using gel documentation.[14]

Statistical analysis of data

Data were collected in triplicate and results were reported as means ± standard deviation (SD). Statistical analysis was performed using One-way analysis of variance (ANOVA) and Duncan multiple range's test by the software SPSS (demo version). Statistically significant differences were considered if $p< 0.05$.

RESULTS

Total ITC content in Se-KS

It is found that the total ITC content of Se-KS was 1.02 mmol/100 g DW which was significantly lower than that of 7-day old broccoli microgreens as Cruciferous vegetable reference (Table 1).

Cytotoxicity of Se-KS extract on cancer cells

It was found that Se-KS extract had cytotoxic effects on three types of cancer cells; Caco-2, MCF-7 and HepG2 cells in both time- and dose-dependent manner (Figure 1). It seems Se-KS extract was least cytotoxic towards MCF-7 cells based on lower cytotoxicity percentage at most

extract concentrations and at most of the incubation time intervals (Figure 1) when compare to other cancer cell types. In addition, it had the most cytotoxic capacity towards HepG2 cells (Figure 1).These results were in accordance with the calculated IC50 values of Se-KS extract on cancer cells.

It was shown that the lowest IC50 values at all incubation time intervals were derived from HepG2 cells (Table 2) and the highest IC50 values were from MCF-7 cells. The IC50 values were also dependent upon the incubation times. That means the longer time Se-KS extract was in contact with cancer cells, the more cytotoxic effects were observed (Table 2).

Apoptosis in cancer cells

Apoptosis, is an active, gene regulated form of programmed cell death that is distinct from cell necrosis regarding its morphology, biochemistry, pharmacology and biological significance. Several types of mammalian cells undergo apoptosis during normal development or in response to a plethora of stimuli, including growth factor deprivation, DNA damage, and abnormal expression of oncogene or tumor suppressor genes. [15] Apoptosis is a widely accepted, important mechanism that contributes to cell growth reduction. [16] The present study was undertaken to explore whether Se-KS induces apoptosis in three types of cancer cell lines. Apparently, the Se-KS extract in this work was able to induce apoptosis leading to cancer cell death as detected by characteristics of apoptotic bodies in increased concentrations of Se-KS extract over 24, 24 and 72 h (Figures 2-4).

DNA fragmentation analysis

To elucidate whether the Se-KS extract (250 µg/mL) inhibits cancer cell proliferation through induction of apoptosis; the cell death by DNA fragmentation at 24 h was examined. It was revealed that the Se-KS extract (250 µg/mL) was optimum enough to induce fragmentation in Caco-2, MCF-7 and HepG2 cells as observed by initial signs of DNA ladder patterns (Figure 5) when compared to intact DNA in the untreated cells as negative controls.

DISCUSSION

Cruciferous plants are well-known as rich sources of glucosinolates and thus ITCs. The previous work showed that mature broccoli had the mean total ITC content of 38.6 µmol/100 g fresh weight (FW) across Singapore[17] while mature kale had much lower amount at 18.2 µmol/100 g FW across USA[18] which were lower than the total ITC contents found in our broccoli microgreens and Se-KS suggesting that microgreens are richer in ITCs than mature plants. In addition, it was found that white cabbage sprouts (*Brassica oleracea* var. *capitata)* had the total ITC content of 0.25 ± 0.03 µmol/g DW, Brussel sprouts (*B. oleracea* var. *gemmifera*) had 0.37 ± 0.02 µmol/g DW and radish (*Raphanus sativus* var. *sativus*) had the highest 9.67 ± 0.03 µmol/g DW[19] which were all lower than the total ITC content found in our broccoli microgreens and Se-KS suggesting that total ITC content varied amongst Cruciferous plant microgreens. The total ITC content was a correlation to the total glucosinolates present in these plants and once they were crushed, endogenous plant myrosinase was released to hydrolyze glucosinolates to ITCs.[20] That means higher ITC content may be due to higher glucosinolate content in the plants.

In similar work, mature Chinese kale leaf extract at 50 µg/mL and 500 µg/mL was found to reach cell viability of A549 alveolar basal epithelial cells at 57% and 59% of the untreated control[21] indicating that Chinese kale extracts exhibit moderate cell growth inhibition, *ca.* 60–70% cell survival at 500 µg/mL. Compared to our findings, Se-KS extract at 150 µg/mL was able to reach *ca.* 60% cytotoxicity (40% cell survival) towards Caco-2 cells at 72 h, *ca.* 50% cytotoxicity (50% cell survival) towards MCF-7 cells and *ca.* 80% cytotoxicity (20% cell survival) towards HepG2 cells (Figure 1) suggesting Se-KS extracts were more effective towards cancer cells than mature Chinese kale leaf extract. We hypothesized that Se-KS had greater content of GSLs and thus the corresponding ITCs than mature counterparts and thus

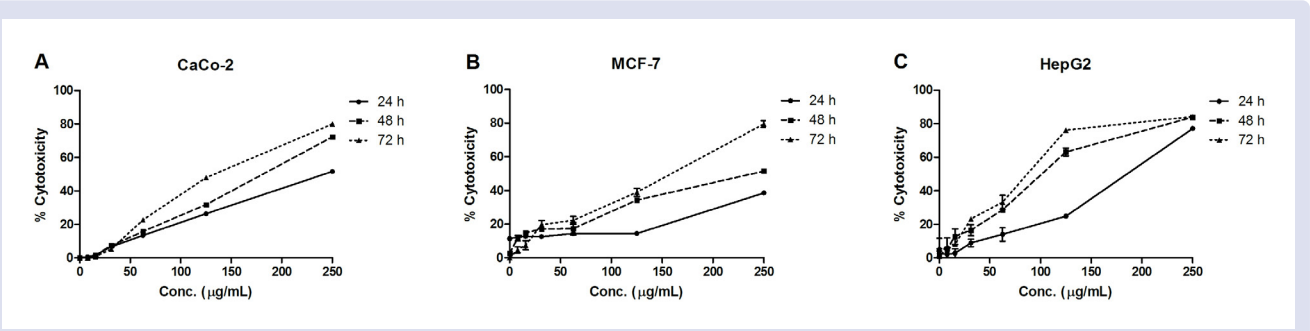

Figure 1: Cytotoxicity of Se-KS extract on cancer cells. (A) Caco-2 cells. (B) MCF-7 cells. (C) HepG2 cells.

Table 1: Total ITC content of selenium-enriched Chinese kale.

Sample	Total ITC content (mmol/100 g DW)
Selenium-enriched Chinese kale	1.02 ± 0.10^{B}
Broccoli microgreen	1.60 ± 0.10^{A}

Capital letters indicate statistical differences in the column ($p < 0.05$).

Table 2: IC50 values of selenium-enriched Chinese kale extract against cancer cells.

		IC_{50} (µg/mL)		
Sample	Time (h)	Caco-2	MCF-7	HepG2
Se-KS	24	345.60 ± 10.71^{Cb}	345.60 ± 10.71^{Cb}	178.27 ± 0.91^{Ca}
	48	234.92 ± 4.09^{Bb}	234.92 ± 4.09^{Bb}	100.56 ± 3.11^{Ba}
	72	151.33 ± 1.76^{Ab}	164.00 ± 7.39^{Ac}	82.83 ± 2.92^{Aa}

Capital letters and small letters indicate statistical differences in the column and rows ($p < 0.05$), respectively.

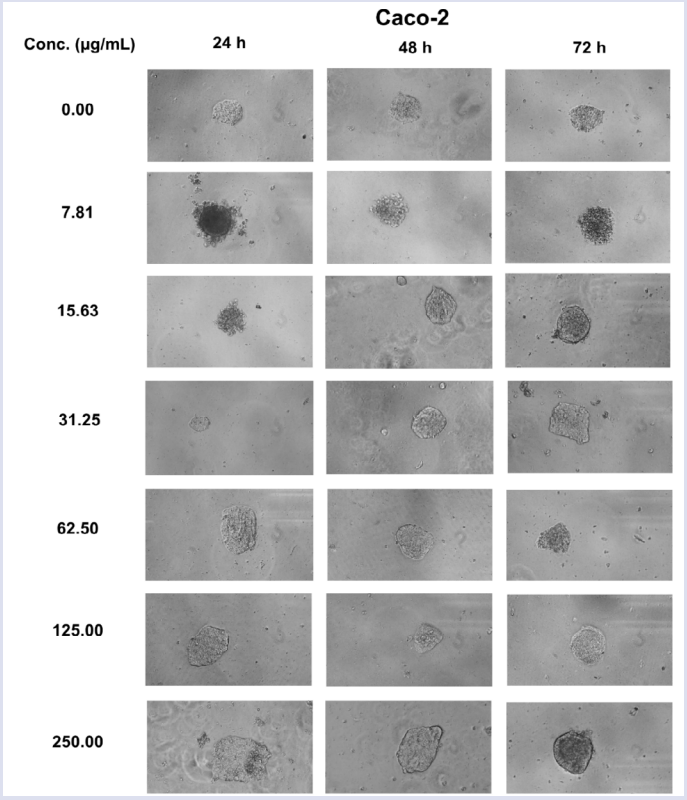

Figure 2: Cell morphology of Caco-2 cells after incubation with various concentrations of Se-KS extracts over 24, 48 and 72 h.

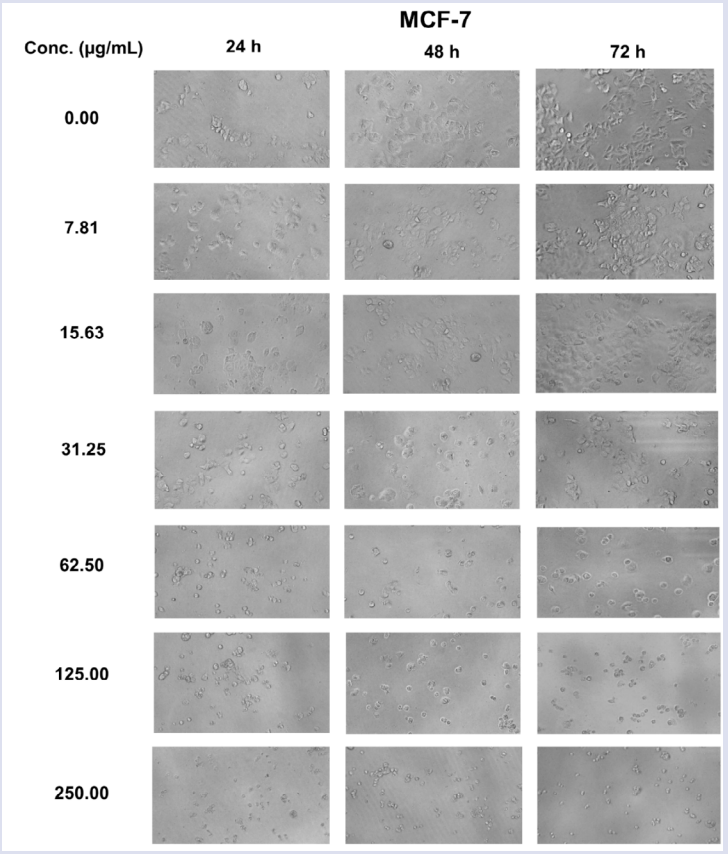

Figure 3: Cell morphology of MCF-7 cells after incubation with various concentrations of Se-KS extracts over 24, 48 and 72 h.

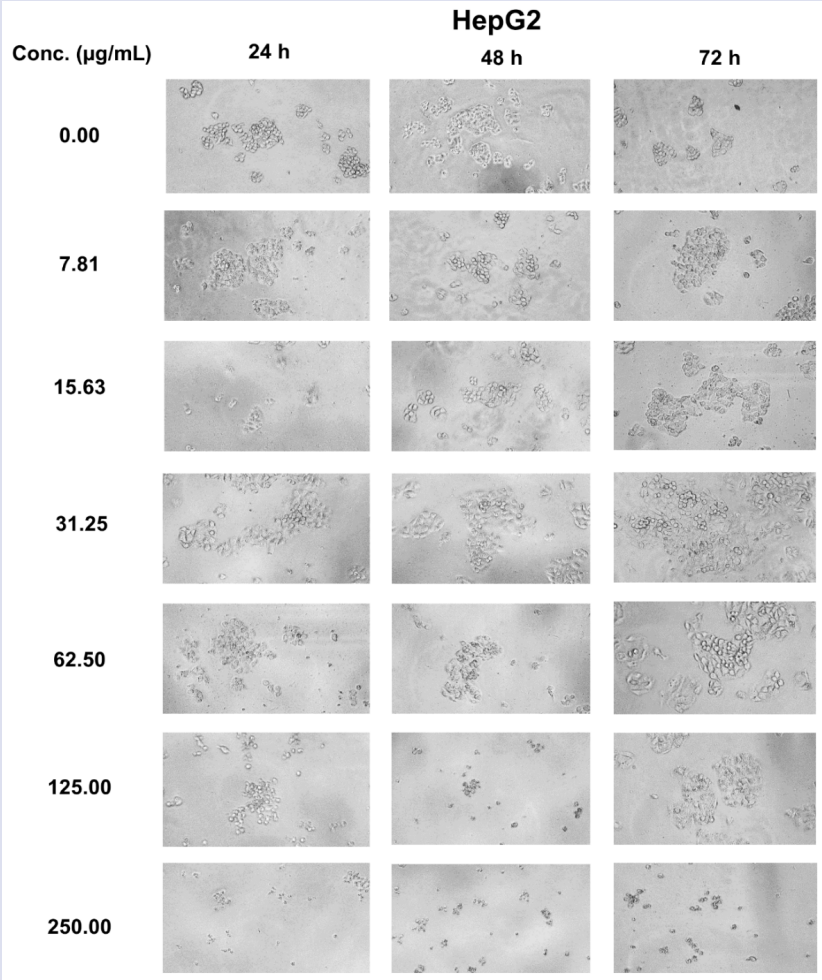

Figure 4: Cell morphology of HepG2 cells after incubation with various concentrations of Se-KS extracts over 24, 48 and 72 h.

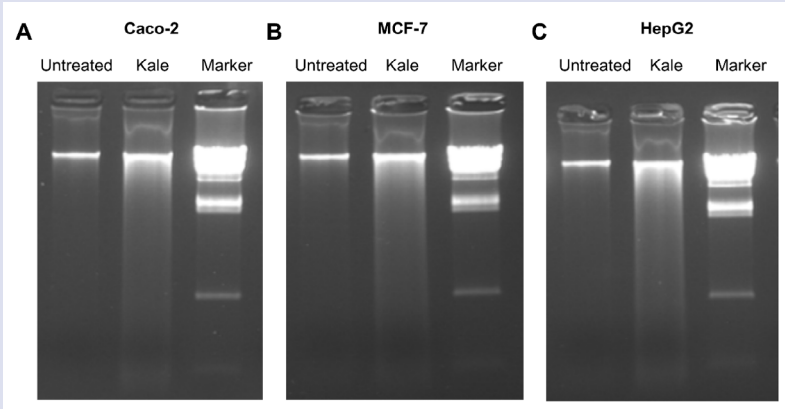

Figure 5: DNA fragmentation of cancer cells by Se-KS extract (250 µg/mL) incubated for 24 h on agarose gel electrophoresis. (A) Caco-2 cells. (B) MCF-7 cells. (C) HepG2 cells.

Se-KS extracts showed higher cytotoxic effect than mature Chinese kale extract. Our hypothesis was supported by the previous reports. The total glucosinolate content was significantly decreased in mature leaves.[22] The concentration of sulforaphane in broccoli sprouts (1,153 mg/100 g DW) is about 10 times greater than mature broccoli (44-171 mg/100 g DW).[23]

It was well-known that abiotic stress, such as supplementation of Se to plants, can lead to increased production of phenolic compounds.[24], but the mechanism is still not fully understood. It is hypothesized that the Se enhances accumulation of glucose which is an important substrate in many metabolic pathways.[25] Likewise, the previous work showed that total phenolic content in Se-KS was significantly higher than that in regular Chinese kale seedlings (R-KS) (30.1 ± 0.1 mg GE/g DW vs.

18.5 ± 0.7 mg GE/g DW).[8] Similarly, broccoli seedlings with Se were shown to have lower GI50 value (concentration that produces 0% cell growth or totally cytostatic effect) at 68.1 µg/mL against MCF-7 cells than that of broccoli seedlings without Se (94.6 µg/mL).[26] However, broccoli seedlings with selenium in the previous work seem to be more cytotoxic towards MCF-7 cells than our Se-KS with IC50 value of 164 µg/mL (Table 2). This was the first report of the effect of Se-KS extract on DNA fragmentation in cancer cells. In literature, it was found that sulforaphane at 5 µM induced partial internucleosomal DNA fragmentation in MCF-7 cells[27] similar to the effect of 250 µg/mL Se-KS extract in this work.

CONCLUSION

It was suggested that the Se-KS extract may be efficacious in treating Caco-2, MCF-7 and HepG2 cancer cells. However, further research will be required to fully delineate the part it plays in cancer and its molecular mechanism, which is not yet clearly understood. Se-KS appears to have potential as a chemopreventive functional food.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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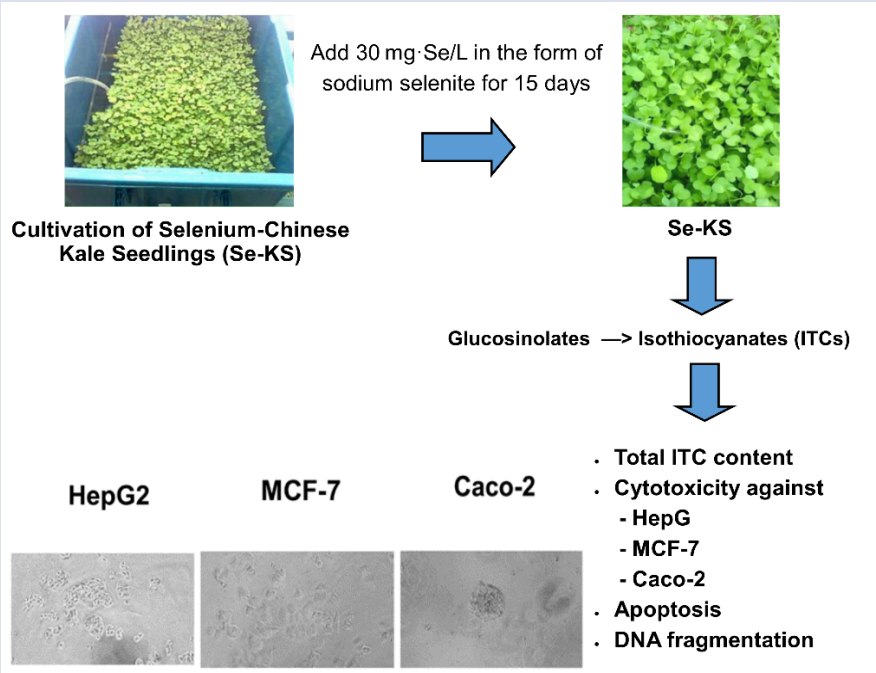
GRAPHICAL ABSTRACT



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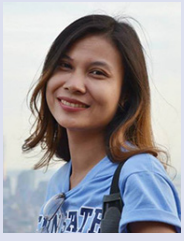

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