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Research Article
In vitro Inhibitory Effects of Glucosinolate from Tumorous Stem Mustard Against H1299.A549 Lung Cancer Cells

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Abstract

Background and Objective: Glucosinolates are converted by the gut microbiota into their bioactive form, which has potent antimicrobial anti-tumour activity. In this study, the inhibitory effects of glucosinolates from tumorous stem mustard against H1299 and A549 lung cancer cell lines were investigated to explore anticancer properties. **Materials and Methods:** Cells were treated with different concentrations of purified glucosinolates for different durations and cell viability was probed by CCK-8. In addition, apoptotic rate, cell cycle and Reactive Oxygen Species (ROS) were investigated using flow cytometry and expression of cytochrome c and Bcl-2 was assessed by western blotting. **Results:** The activity of cells treated with glucosinolates was significantly decreased compared with the control group. Cell proliferation was lowest after treatment with 2 mg mL^{-1} glucosinolates for 48 hrs and the inhibitory effect increased with increasing concentration and duration. After treatment, cells displayed typical apoptotic features, with increased ROS levels. Additionally, glucosinolates blocked the cell cycle in the S phase and G2/M phase and this may be related to the mechanism of inhibitory action. Glucosinolates also inhibited the expression of the antiapoptotic protein Bcl-2, thereby promoting intracellular apoptosis signal transduction and stimulating the release of the apoptosis-inducing factor cytochrome c from mitochondria to the cytoplasm. **Conclusion:** Glucosinolates from tumorous stem mustard showed a strong antitumor effect against lung cancer cells, further studies will be aimed at the possible application as anti-cancer agents for the reduction of lung cancer.

Key words: Tumorous stem mustard, glucosinolates, lung cancer cells, *in vitro* tests, tumorigenesis, *Semen sinapis*, pharmacodynamic, traditional Chinese medicine, myrosinases

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Contemporary society is in a period of rapid economic and technological development and this is associated with complex global medical and health issues, including epidemics that require intervention. As the economy develops, people's environments, lifestyles and dietary structures can change dramatically, which can increase the incidence of malignant diseases such as cancer. The high mortality rate of malignant tumours leads to panic and stress. By 2012, there were approximately 14.1 million people with cancer worldwide, of which 8.2 million have since died and lung, stomach and liver cancers have the highest mortality rate[1]. Faced with this reality, the development of drugs that can inhibit tumorigenesis is important, hence the wealth of research on tumours and antitumour drugs.

Many researchers in the field of anticancer drug research have reported that cruciferous plants can prevent certain cancers[2]. For example, *Semen sinapis* is a cruciferous plant and traditional Chinese medicine[3] that can promote qi, eliminate phlegm, warm lungs, dredge collaterals and relieve pain[4]. *Semen sinapis* contains various components including sinapine, sinalbin and white mustard volatile oil that dominate the pharmacodynamic action[5]. Among these, the glucosinolate sinalbin has been purified in high yield. After treating tumour cells for a given period, indicators of cell morphology and apoptosis were investigated, glucosinolates were found to decrease cell viability of various tumour cells and induce apoptosis[6].

Glucosinolates are mixtures of carbohydrates and glycosides that are found widely in the roots, stems and fruits of cruciferous Brassica plants such as cabbage, broccoli, cauliflower, kale, white radish and carrots[7,8]. According to the literature, 350 genera and 3000 species can synthesis glucosinolates, of which many are cruciferous plants[9]. In many plant cells, glucosinolates and myrosinases are present at the same time but glucosinolates are located in vacuoles, while myrosinases bind to specific proteins, hence when the cell structure is intact, glucosinolates are not degraded by myrosinases. However, when the cell structure is destroyed, glucosinolates are released and degraded by myrosinases to reduce damage[10]. Various physiological and biochemical properties of glucosinolates and their degradation products have been reported, including inhibiting the growth of tumour cells[11,12]. Research on the antitumour effects of glucosinolates indicates that the long-term consumption of cruciferous Brassica vegetables lowers the incidence of lung, stomach, liver and other cancers[13]. For example, an in-depth study on glucosinolates found that hydrolysate isothiocyanate compounds of glucosinolates play an important role in their antitumour effects[13]. Additionally, other natural products present in cruciferous plants, such as folic acid, carotenoids, vitamins and dietary fibre may play a significant role in inhibiting tumours. The antitumour effects of glucosinolates were first reported in the 1970s, showed that the degradation products of glucosinolates can accelerate the metabolism of carcinogens, which leads to a decrease in tumours in mice[14,15].

Tumorous stem mustard is a cruciferous Brassica vegetable that has evolved from wild mustard and it is used widely for mustard production. This plant is rich in glucosinolates, in the seeds and glucosinolates possess anticancer activity[16]. To date, there have been few specific studies on tumorous stem mustard glucosinolates and their effects on various physiological indices associated with tumour cells.

Therefore, in the present study, we extracted glucosinolates from the seeds of tumorous stem mustard and tested their activity against H1299 and A549 lung cancer cell lines[17]. Based on the effects of glucosinolates on cell proliferation tested using CCK-8 assays, changes in various physiological indices of H1299 and A549 cells were assessed to explore the specific mechanism of the antitumour effects. The findings provide support for anti-cancer applications and highlight medicinal value beyond its food function.

MATERIALS AND METHODS

Study area: The study was carried out Cell Laboratory of Chongqing Medical University, China from May, 2019-January, 2020.

Reagents: Tumorous stem mustard seeds (Fuzha no. 2) were purchased from Fuling Seed Company (Fuling, Sichuan, China). Glucosinolates were isolated from seeds as described previously (ref). Dimethylsulfoxide (DMSO), glycine and Sodium Dodecyl Sulphate (SDS) were purchased from Jianglai Bio (Shanghai, China). CCK-8 and Phosphate-Buffered Saline (PBS) were from Shenzhen Yuke Scientific Instrument Co. Ltd. (Shenzhen, China). ECL and PVDF were from Millipore (Bedford, MA). Goat anti-rabbit HRP-Ig (H+L) was from Jackson ImmunoResearch Laboratories (West Grove, PA). The protease inhibitor was from Nanjing Di'an Bio (Nanjing, China).

Cell culture: H1299 and A549 human lung carcinoma cell lines were purchased from ATCC (Rockville, Maryland). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)

supplemented with 10% Foetal Bovine Serum (FBS). The medium was changed every 3 days and passaged every 7-10 days. Cells at the logarithmic growth phase were harvested by trypsinization.

Viability assays: To assess the number of live and dead cells after treatment, H1299 or A549 cells were seeded in 96-well plates and cultured for 24 hrs. Subsequently, cells were treated with glucosinolates at different concentrations (0.125, 0.25, 0.5, 1 or 2 mg mL^{-1}) for different time intervals (0, 6, 12, 24 or 48 hrs). At the end of each experiment, cells were incubated with CCK8 reagent at 37°C for 2 hrs and the absorbance at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT).

Apoptosis assay: Apoptosis was assayed by Annexin V and Propidium Iodide (PI) staining and analysed by Fluorescence-Activated Cell Sorting (FACS) using a dedicated instrument (Becton Dickinson, USA). H1299 or A549 cells were treated with glucosinolates at different concentrations for different time intervals as described above. At the end of each experiment, cells were harvested by trypsinization and subjected to FACS analysis.

ROS level determination: To assess intracellular ROS levels, H1299 or A549 cells were cultured for 24 hrs. Subsequently, cells were treated with 100 mg mL^{-1} glucosinolates for different time intervals (0, 6, 12, 24 or 48 hrs). Cells were then washed with serum-free DMEM, stained with the ROS-sensitive probe dihydroethidium (DHE) for 1 hr and fluorescence levels were measured using a flow cytometer and CellQuest software (BD Biosciences). Intracellular ROS levels were calculated from the mean fluorescence intensity.

Cell cycle analysis: H1299 or A549 cells were treated with 100 mg mL^{-1} glucosinolates for different time intervals (0, 6, 12, 24 or 48 hrs) and collected by trypsin treatment. Cells were washed with PBS followed by centrifugation and pellets were resuspended in ice-cold 70% ethanol for 30 min. Cells were collected by centrifugation, washed with PBS and resuspended in 20 µg mL^{-1} Propidium Iodide (PI) in PBS with 2% FBS for 3 min in the dark. The cell cycle was investigated by flow cytometry at an excitation wavelength of 488 nm.

Western blotting assay: Cytochrome C and Bcl-2 levels after glucosinolate treatment were measured by western blotting. H1299 or A549 cells were treated with 100 mg mL^{-1} glucosinolates for different time intervals (0, 6, 12, 24 or 48 hrs). Aliquots of cell lysates containing 50 µg of proteins were separated by 15% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked with TBST buffer (10 mM TRIS-HCl pH 8.0, 0.15 M NaCl, 0.05% Tween-20) containing 5% skimmed milk, incubated with antibodies recognising Cytochrome C (1:1000) or Bcl-2 (1:2000) at 4°C overnight, then treated with horseradish peroxidase-linked anti-mouse IgG and ECL. Expression of glyceraldehyde-3-phosphate dehydrogenase GAPDH was used as an internal control to normalise the expressions of other proteins.

RESULTS

Effects glucosinolates on the viability of H1299 and A549 cells: The effects of tumorous stem mustard glucosinolates on the cell viability of H1299 and A549 cells were investigated by CCK-8 assay and the results are shown in Fig. 1. The results for each time showed that with a gradual increase in the concentration of glucosinolates the absorbance or Optical Density (OD) value tended to decrease, indicating that the viability of H1299 cells gradually weakened and the toxicity of tumorous stem mustard glucosinolates toward H1299 cells gradually increased. At the same concentration, the OD values for 0.125, 0.25 and 0.5 mg mL^{-1} treatment groups increased with increasing treatment duration but OD values for 1 and 2 mg mL^{-1} groups decreased with increasing treatment duration (Fig. 1a). With increasing concentration of glucosinolates and treatment duration, the viability of A549 cells decreased, while the viability of cells in the control group without glucosinolate treatment increased. It can be seen that at 6 hrs, the inhibitory effect was greater than at the other timepoints at a glucosinolate concentration of 2 mg mL^{-1} and inhibition of cell viability was more pronounced with increasing treatment duration (Fig. 1b).

Effects of glucosinolates on apoptosis in H1299 and A549 cells: The effects of glucosinolates on apoptosis in H1299 cells and A549 cells were investigated by flow cytometry. Representative FACS profiles of H1299 cells reveal increasing percentage in apoptotic cells following glucosinolates treatment at 0 hr (Fig. 2a), 6 hrs (Fig. 2b), 12 hrs (Fig. 2c), 24 hrs (Fig. 2d), 48 hrs (Fig. 2e). The results showed that H1299 cells underwent apoptosis under the action of tumorous stem mustard glucosinolates and there was a positive correlation between the treatment duration and the apoptosis rate

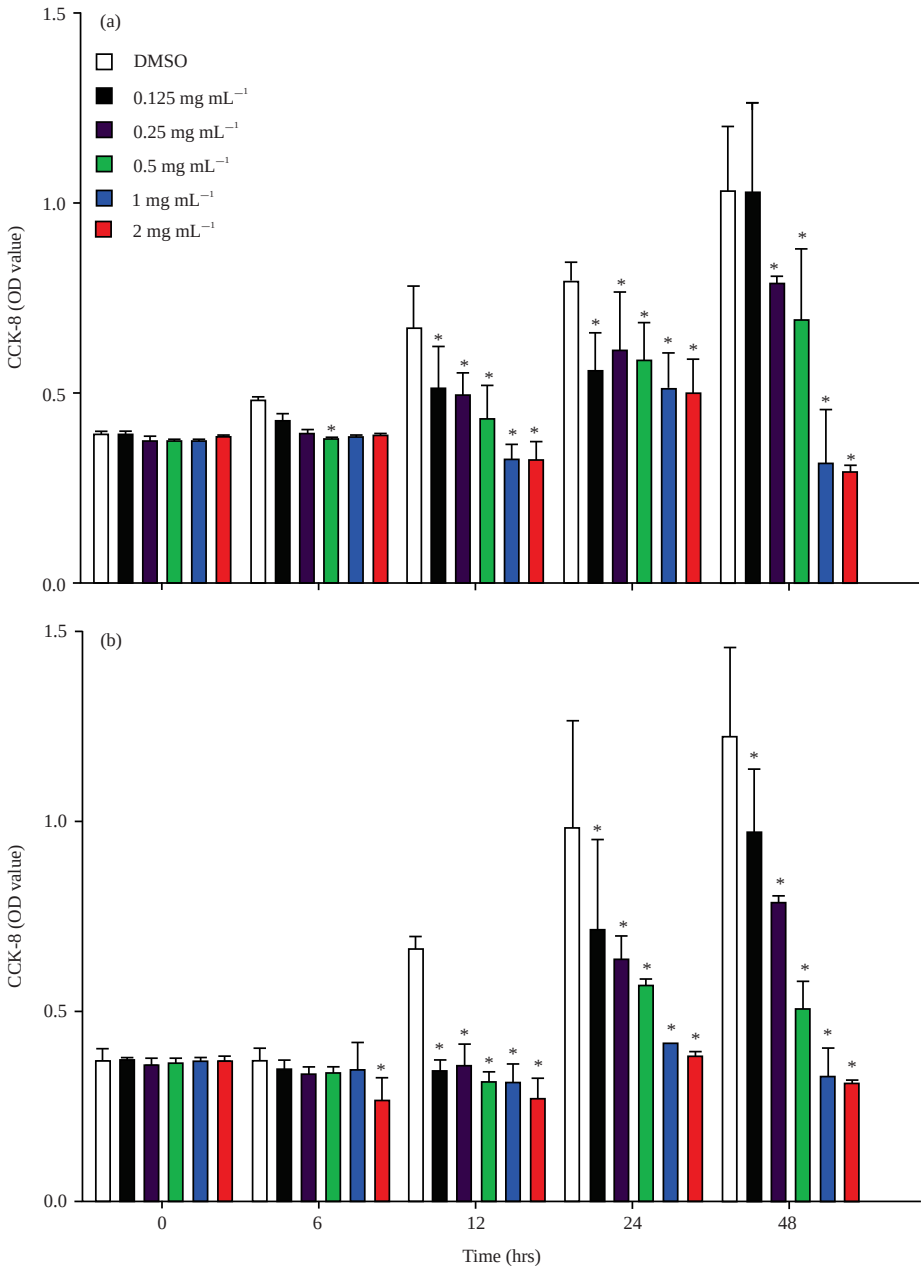

Fig. 1(a-b): Effects of different glucosinolate concentrations and treatment durations on the viability of (a) H1299 and (b) A549 cells

Data are the Mean±SEM for three separate experiments. *p<0.05 compared to the group 0 hr, respectively

(Fig. 2f). Representative FACS profiles of A549 cells reveal increasing percentage in apoptotic cells following glucosinolates treatment at 0 hr (Fig. 3a), 6 hrs (Fig. 3b), 12 hrs (Fig. 3c), 24 hrs (Fig. 3d), 48 hrs (Fig. 3e). The results showed that A549 cells underwent apoptosis under the action of tumorous stem mustard glucosinolates and there was a positive correlation between the treatment duration and the apoptosis rate (Fig. 3f).

Effects of glucosinolates on ROS levels in H1299 and A549 cells: ROS levels in H1299 cells and A549 cells were compared after treatment with glucosinolates for different durations. The peak of the fluorescence curve representing ROS levels shifted to the right with increasing glucosinolate treatment duration. Thus, the ROS content in H1299 cells increased with increasing treatment duration (Fig. 4a-e). Additionally, the relationship between time and

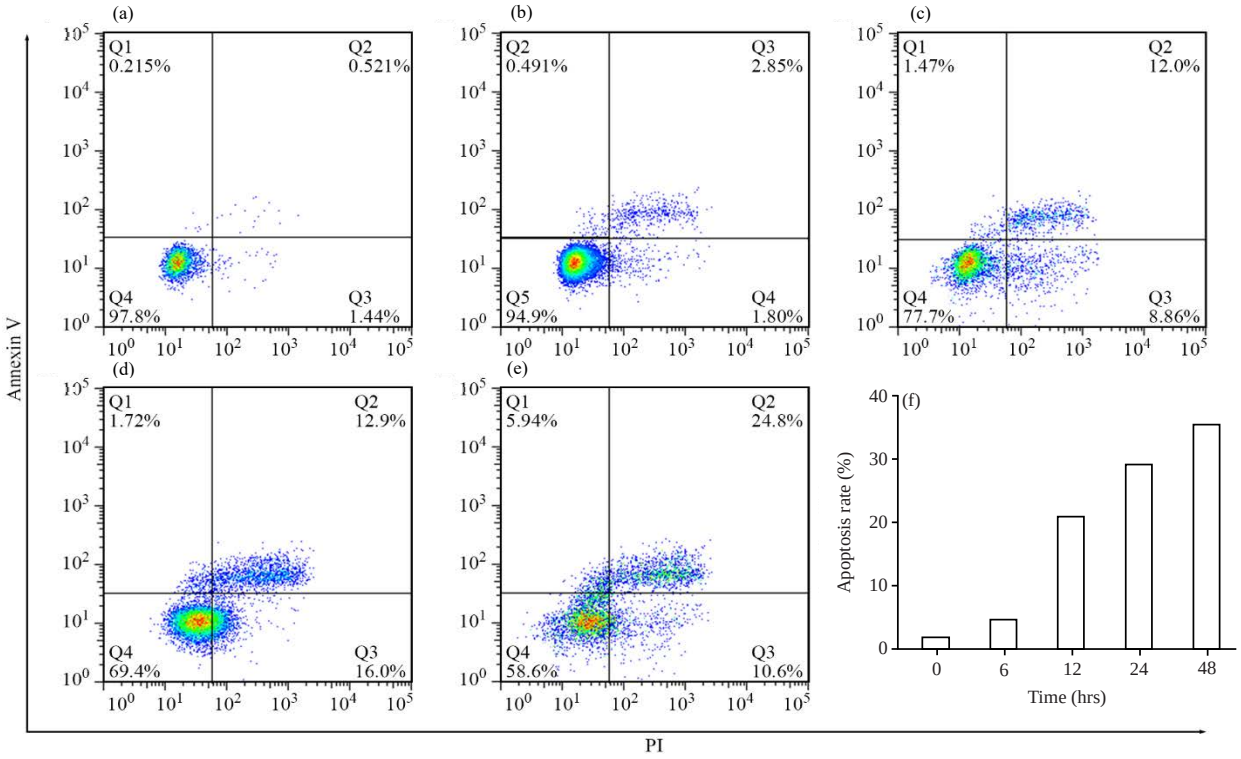

Fig. 2(a-f): Apoptosis of H1299 cells assessed by annexin V-FICT/PI double staining flow cytometry following treatment with different concentrations of glucosinolates, (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Average apoptotic rate of H1299 cells

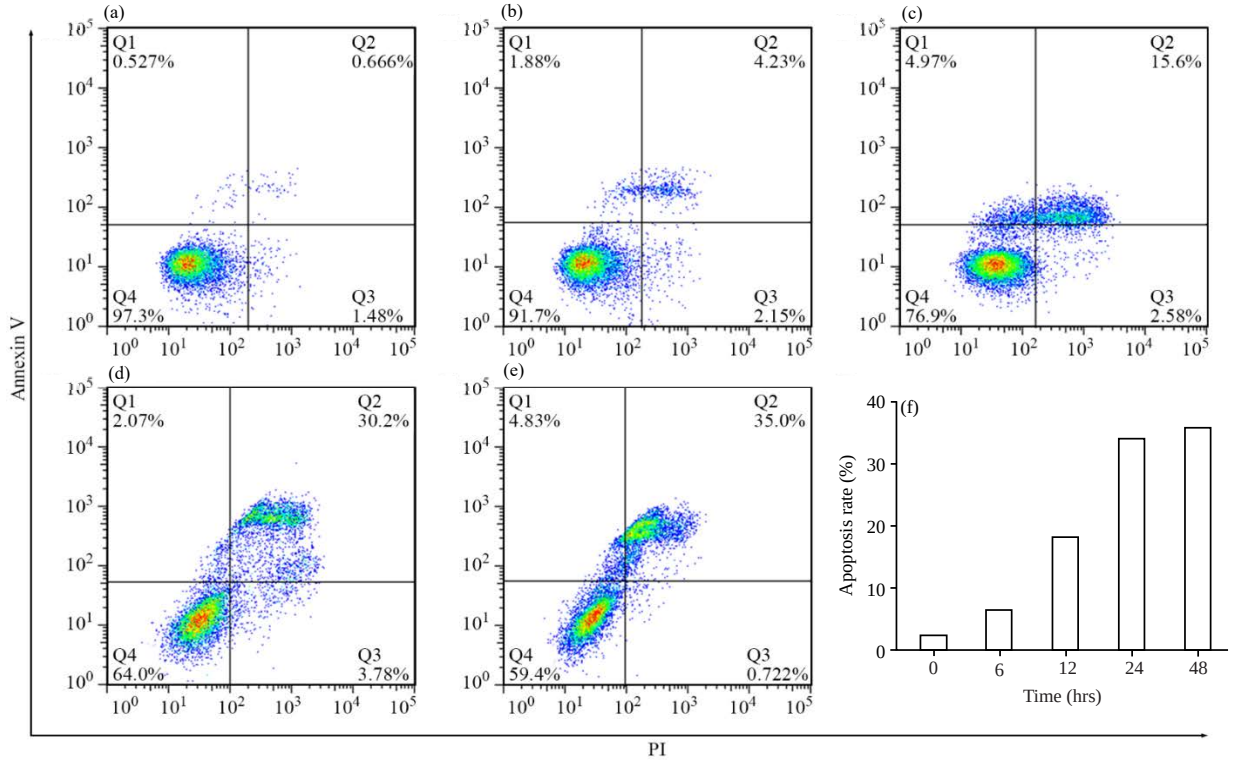

Fig. 3(a-f): Apoptosis of A549 cells assessed by annexin V-FICT/PI double staining flow cytometry following treatment with different concentrations of glucosinolates (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Average apoptotic rate of A549 cells

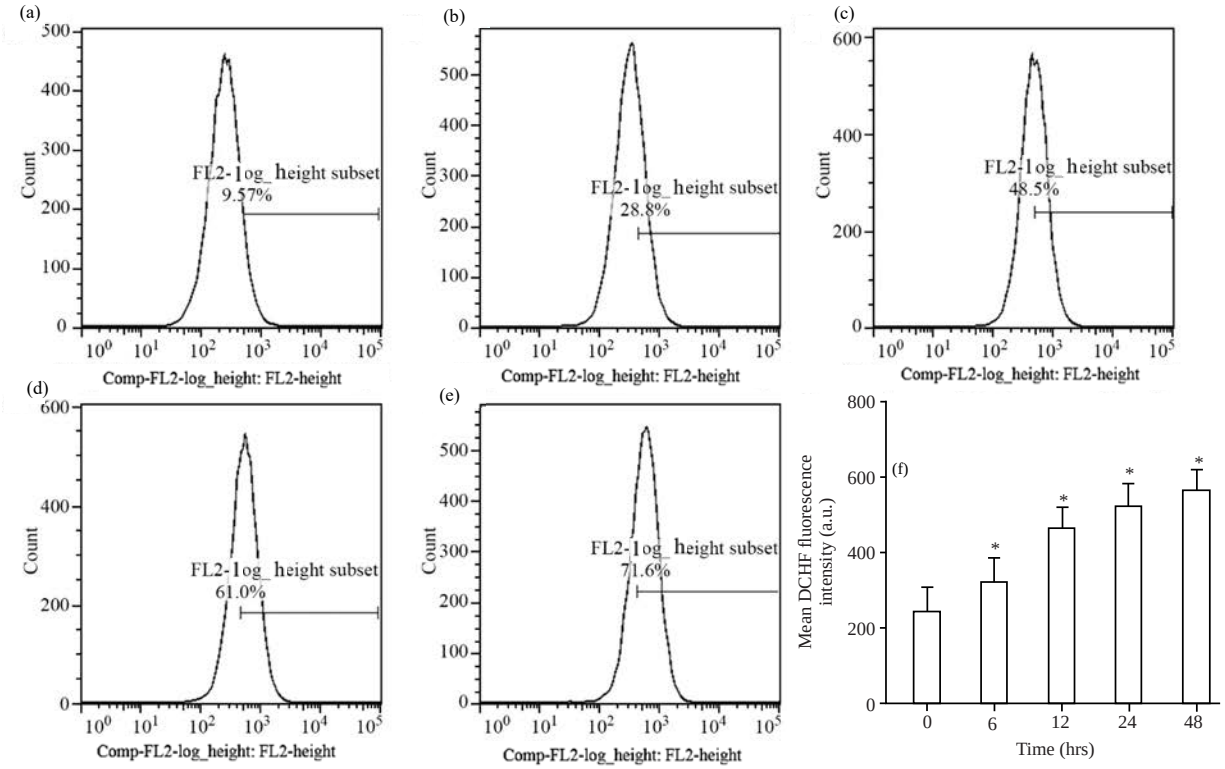

Fig. 4(a-f): Effects of 100 mg mL^{-1} glucosinolates on ROS levels in H1299 cells after different treatment durations, ROS levels at (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Mean DCHF fluorescence intensity

mean fluorescence intensity clearly showed that the average fluorescence intensity increased with increasing treatment duration, indicating that ROS levels in H1299 cells were positively correlated with the action duration of glucosinolates (Fig. 4f, <0.05).

ROS levels were also measured in A549 cells after treatment with mustard glucosinolates at 100 mg mL^{-1} for different durations (Fig. 5a-e). ROS levels were significantly higher in the 6, 12, 24 and 48 hrs treatment groups than in the 0 hr group (Fig. 5f, <0.05).

Effects of glucosinolates on the cell cycle in H1299 and A549 cells: Cell cycle analysis presented additional information on cell behaviour post mustard glucosinolates treatment. H1299 were treated with 100 mg mL^{-1} glucosinolates for different time intervals (Fig. 6a, 0 hr, Fig. 6b, 6 hrs, Fig. 6c, 12 hrs, Fig. 6d, 24 hrs, Fig. 6e, 48 hrs). After treatment of H1299 cells with mustard glucosinolates for 1-6 hrs, the proportion of cells in G0/G1 was increased, while the proportion in the S phase decreased. After 48 hrs of treatment, there were fewer cells in the G0/G1 phase but more in the G2 phase and the S phase (Fig. 6f). A549 cells treated with 100 mg mL^{-1} glucosinolates for different time intervals (Fig. 7a, 0 hr, Fig. 7b, 6 hrs, Fig. 7c, 12 hrs, Fig. 7d, 24 hrs, Fig. 7e, 48 hrs). The number of cells in the G2 phase increased with time and was highest at 48 hrs (Fig. 7f). Therefore, glucosinolates block A549 cells in the G2 phase, which prevents cells from entering mitosis and prolongs the cell cycle to a certain extent but its effect is not obvious.

Expression of cytochrome C and Bcl-2 in H1299 and A549 cells treated with glucosinolates: Western blotting was used to detect the release of Cytochrome C from mitochondria into cytoplasm and expression of the Bcl-2 protein in cells treated with glucosinolates was also investigated to explore the molecular mechanism of apoptosis induced by glucosinolates. The amount of Cytochrome C in H1299 cells (Fig. 8a) and A549 cells (Fig. 8b) was increased significantly with increasing glucosinolate treatment duration. Additionally, the content of the Bcl-2 protein in H1299 cells and A549 cells decreased with increasing intervention time and was

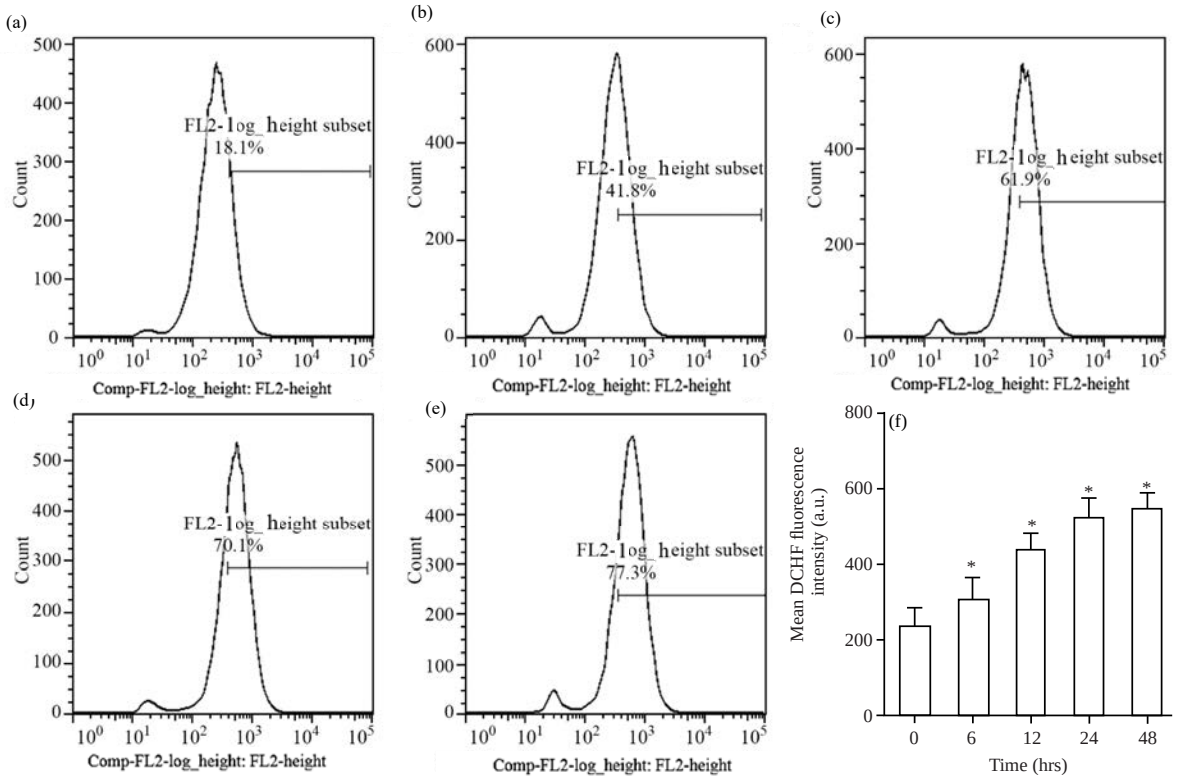

Fig. 5(a-f): Effects of 100 mg mL^{-1} glucosinolates on ROS levels in A549 cells after different treatment durations, ROS levels at (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Mean DCHF fluorescence intensity

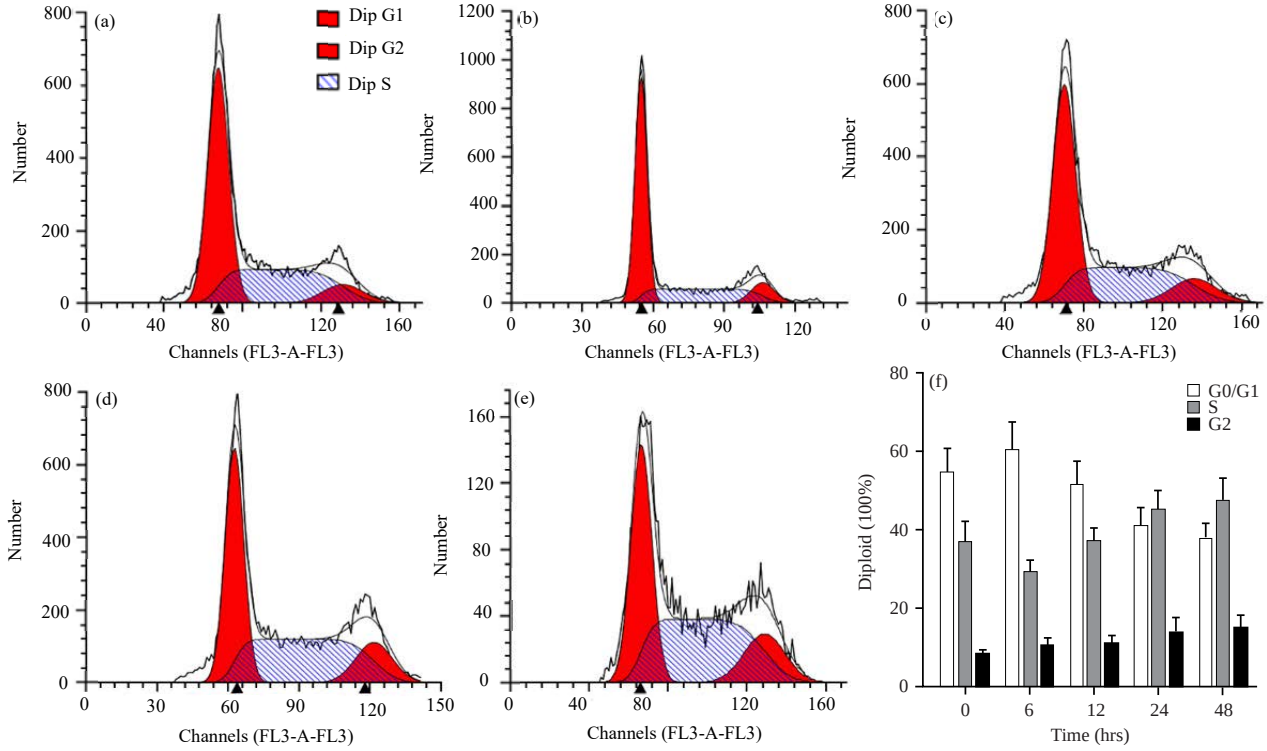

Fig. 6(a-f): Effects of 100 mg mL^{-1} mustard glucosinolates on the cell cycle of H1299 cells after different treatment durations, changes in cell cycle at (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Percentage of diploid cells

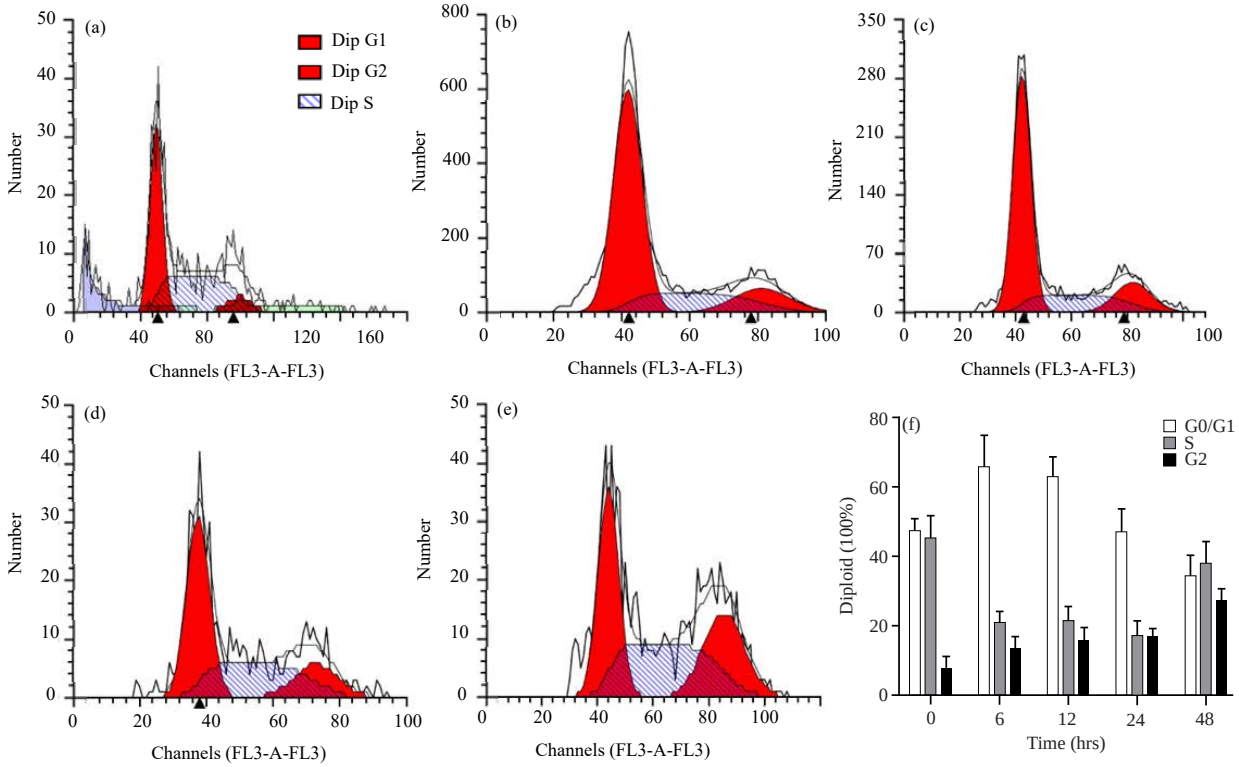

Fig. 7(a-f): Effects of 100 mg mL^{-1} mustard glucosinolates on the cell cycle of A549 cells after different treatment durations, changes in cell cycle at (a) 0, (b) 6, (c) 12, (d) 24, (e) 48 hrs and (f) Percentage of diploid cells

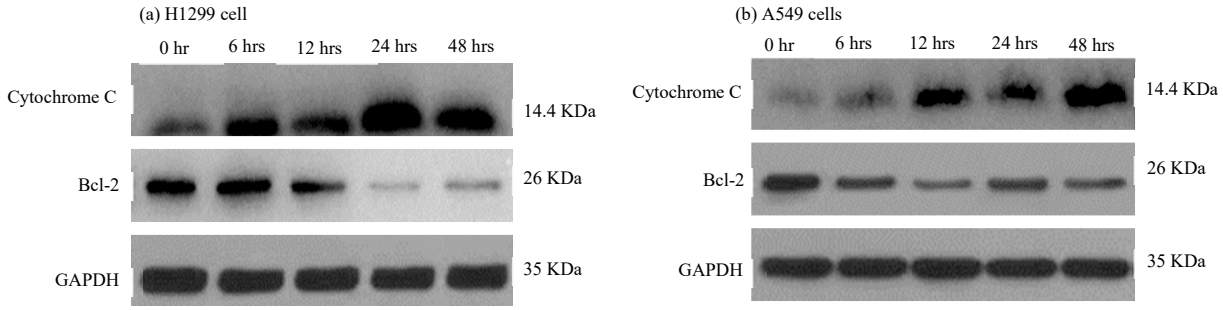

Fig. 8(a-b): Western blotting analysis of the expression of cytochrome C and Bcl-2 in (a) H1299 cells and (b) A549 cells treated with 100 mg mL^{-1} glucosinolates for different durations

negatively correlated with the action duration of glucosinolates. By contrast, GAPDH (control) expression levels were essentially stable.

DISCUSSION

The main purpose of this study was to investigate the effects of tumorous stem mustard glucosinolates on growth inhibition and apoptosis in H1299 and A549 lung cancer cells *in vitro*. The occurrence of apoptosis is accompanied by changes in the content of certain substances in cells[18-21]. There were a few cells in the Q_1 region (Annexin V^+/PI^-) indicating early apoptosis and a small number in the Q_2 region (Annexin V^+/PI^+) at the beginning of the experiment. Cells in the Q_3 region (Annexin V^-/PI^+) indicated partial necrosis or mechanical damage. However, there was no significant change in cells in the Q_3 region with prolonged treatment duration but there was a significant increase in the Q_1 and Q_2 regions. It can be seen that a longer treatment duration induced a higher apoptotic rate. Therefore, it can be inferred

that mustard glucosinolates promoted apoptosis of tumour cells and the longer the treatment duration, the more obvious the apoptosis. To explore the mechanism, changes in ROS levels and the cell cycle in H1299 and A549 cells treated with mustard glucosinolates were investigated by flow cytometry. ROS levels in normal cells are relatively stable and the antioxidant enzyme activity in tumour cells is lower than that in normal cells, the efficiency of scavenging ROS is low. Therefore, if the ROS content is the same in tumour cells and normal cells, tumour cells are more sensitive to damage, indicating that ROS can selectively kill tumour cells, while damage to normal cells is relatively low[22]. This indicates that the apoptosis rate increased with increasing treatment duration, which may be due to an increase in ROS content in H1299 and A549 cells caused by glucosinolates. Since CCK-8 assays revealed that mustard glucosinolates inhibited cell proliferation in a time-dependent manner, it can be concluded that H1299 were blocked at the G2/M and S phases of the cell cycle followed prolonged action of mustard glucosinolates resulting in inhibition of growth and cell division[23]. Additionally, western blotting was used to further investigate the effects of glucosinolates on the expression of Cytochrome C and Bcl-2 proteins in H1299 and A549 cells. Cytochrome C is an important respiratory chain protein in mitochondria. When mitochondria sense an apoptotic signal, Cytochrome C is released into the cytoplasm, indicating that the cell is undergoing apoptosis, hence Cytochrome C can be used as an apoptosis-inducing factor to determine whether a cell is in the apoptotic state[24]. As an anti-apoptotic protein, Bcl-2 plays an important role in the development of cancer. Excessive expression of Bcl-2 can disrupt the normal mechanism of apoptosis and create favourable conditions for the survival of tumour cells, resulting in insensitivity to apoptotic signals[25]. Therefore, if the expression of Bcl-2 in cells is increased, cells may become cancerous. The results showed that glucosinolates promoted an increase in ROS levels in H1299 cells, stimulated the release of Cytochrome C from mitochondria, inhibited the expression of the anti-apoptotic protein Bcl-2 and slightly retarded the cell cycle in the S and G2/M phases to slow cell proliferation. However, mustard glucosinolates caused no obvious cell cycle arrest in A549 cells and the inhibition mechanism was not confirmed. During apoptosis, ROS and Ca^{2+} levels in cells rise considerably[26-29]. Herein, H1299 lung cancer cells accumulated high levels of ROS that are known to damage cells. Glucosinolates promoted the release of the apoptosis-inducing factor Cytochrome C and the expression of the anti-apoptotic protein Bcl-2 in both H1299 and A549 cells, which may account for the anti-tumour effects of mustard glucosinolates. Although arrest at S and G2/M phases of the cell cycle was not obvious in H1299 cells, there was a small increase with prolonged treatment duration, indicating that mustard glucosinolates prolonged the cell cycle to reduce cell proliferation. However, other indices related to apoptosis were more obviously affected. In a previous study, increasing the concentration of Fenggang zinc selenium tea enhanced the inhibition of A549 lung cancer cell growth and although the initial inhibition effect was not obvious, inhibition increased with increasing dose and was linearly correlated[30-31]. However, when the concentration was greater than 800 μg mL^{-1}, the inhibition rate increased slowly and decreased compared to a dose of 400-700 μg mL^{-1}. Similarly, in the present study, the inhibitory effect of mustard glucosinolates on cells also increased with increasing doses. However, the highest concentration in this experiment was 100 mg mL^{-1}, which may not have reached the optimal concentration for inhibition of A549 cells. Therefore, further research is needed to determine the optimal concentration of mustard glucosinolates for anti-tumour activity and to explore the mechanisms involved.

CONCLUSION

Anti-tumour drug research uses physiological indicators to investigate inhibitory effects on tumour cells, including detecting effects on cell proliferation, the size of DNA fragments, observing cell morphology by electron microscopy, caspase activity assays and measuring Cytochrome C and Bcl-2 protein expression levels. Herein, physiological indicators showed that tumorous stem mustard glucosinolates induced apoptosis in H1299 and A549 lung cancer cells by arresting and prolonging the cell cycle through a series of mechanisms. This study involved investigated anticancer effects in two cell lines. Further studies are needed on the specific anticancer mechanisms in other tumour cells.

SIGNIFICANCE STATEMENT

This study discovers the possible anticancer effect of tumorous stem mustard glucosinolates. The findings provide support for anti-cancer applications and highlight medicinal value beyond its food function.

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