Flowers of *Camellia nitidissima* cause growth inhibition, cell-cycle dysregulation and apoptosis in a human esophageal squamous cell carcinoma cell line

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Abstract. The present study aimed to investigate the chemopreventive effect of *Camellia nitidissima* flowers water extract (CNFE) on the Eca109 human esophageal squamous cell carcinoma (ESCC) cell line. The antiproliferative effect on Eca109 cells was determined using the trypan blue exclusion assay. The effects of CNFE on apoptosis and cell cycle arrest were investigated by flow cytometry. CNFE inhibited cell growth in both a dose- and time-dependent manner in Eca109 cells. CNFE also caused dose- and time-dependent apoptosis of these cells. Treatment of cells with CNFE resulted in dose-dependent G_0/G_1 phase arrest of the cell cycle. The data demonstrated that CNFE serves antiproliferative effects against human ESCC Eca109 cells by inducing apoptosis and interrupting the cell cycle. These results suggested that CNFE has the potential to be a chemoprotective agent for ESCC.

Introduction

Esophageal cancer (EC) ranks as the eighth most common cancer type, with the sixth highest mortality rate worldwide (1). Esophageal squamous cell carcinoma (ESCC) contributed to 80% of all EC. Chemoprevention is the chronic administration of a synthetic, natural or biological agent to reduce the concurrency, or delay the occurrence of malignancy, and is a fast evolving field (2). Great efforts have being made to identify effective chemoprotective agents. However, toxicity is always present alongside efficacy in the common preventive agents, including non-steroidal anti-inflammatory drugs. Therefore, there is a requirement to develop highly efficient chemopreventive agents with fewer side-effects, and natural compounds, including polyphenols and antioxidants, are regarded as important sources for this objective.

Camellia nitidissima Chi (CN), is distributed in a narrow region of Southern China and North Vietnam (3,4). The leaves, flowers and seed oils of CN are used in foodstuffs and Chinese traditional medicines (5). CN has several similar constituents as other *Camellia sinensis* species, including green tea, however, it also has some unique phytochemicals as well (5,6). Ethanol extracts of the seeds of CN exhibit cytotoxicity against human lymphoma cells, and cervical and prostate cancer cells (7). The amount of bioactive components in CN flowers is reported to be higher compared with that in the leaves (6). A previous study reported that flavonoid glycoside extracted from the flowers of CN slowed down the proliferation of human lymphoma U937 cells (8). The toxicity of CN is quite low. A previous study by Peng *et al* recently showed that fresh CN leaf water extracts caused no acute, subacute or genetic hazards in the mouse acute oral toxicity test, 90-day feeding in male Wistar rat, Ames test, mouse teratospermia test and mice sperm abnormality test (9). Another previous study showed that the lethal dose, 50% (LD_{50}) on mice oral toxicity test was up to 106.7 g crude drug/kg (10).

However, the effect of CN flower extracts on the prevention of ESCC remains to be studied. Drinking is the most common way for humans to consume teas. Therefore, water extracts of the CN flower represents the components that would be consumed by humans through daily tea drinking.

In the present study, the CN flower water extract (CNFE) was used to investigate possible chemopreventative effects of CN on an ESCC cell line, Eca109. The cell viability change and apoptosis induced by CNFE were first evaluated, and the effect of CNFE on the cell cycle was further analyzed using flow cytometry.

Materials and methods

Preparation of CNFE. Dry CN flowers were provided by Guangxi Nongyi Organic Agriculture Company (Guangxi, China). A total of 10 g dry CN flowers were steeped

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in 100 ml near boiling double distilled H_2O for 1 h. The infusion was filtered twice through a 0.45 μm polyvinylidene difluoride (PVDF) filter disk (EMD Millipore, Billerica, MA, USA) and vacuum freeze dried at -80˚C to produce a powdered crude extract, which was stored at -20˚C until use. Prior to the experiments, the powdered crude extract was dissolved in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a concentration of 100 mg/ml and filtered twice through a 0.22 μm PVDF filter disk (EMD Millipore).

Cell culture. The ESCC cell line, Eca109, was purchased from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The Eca109 cells were cultured in DMEM, supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and incubated at 37˚C in an atmosphere of 5% CO_2.

Trypan blue exclusion assay. Eca109 cells were cultured in 6-well plates ($8x10^5$ cells/well) for 24 h. When the cells reached ~70% confluence, they were treated with CNFE at five different concentrations (100, 200, 300, 400 and 500 μg/ml) for 24, 48 or 72 h. Then the cells were trypsinized and mixed 1:1 with 0.4% trypan blue solution for 2-3 min. The cells were counted on a hemocytometer using an inverted phase contrast microscope. Viable cells exclude trypan blue, while dead cells stain blue due to trypan blue uptake. The ratio of the numbers of dead cells divided by the total number of cells is calculated as the percentage of cell death. At each time point, the effect of CNFE was compared with the non-treated control.

Quantification of apoptosis by flow cytometry. The Eca109 cells were plated at a density of $4x10^5$ cells in 6-well plates and treated with CNFE at 100, 200, 300, 400 and 500 μg/ml. The cells were harvested at the indicated time points, washed twice with cold phosphate-buffered saline, centrifuged for 5 min at 300 x g at room temperature. The cells were subsequently stained with Annexin V and propidium iodide (PI) using the fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection Kit I (BD Bioscience, Franklin Lakes, NJ, USA). Briefly, the cells were incubated at room temperature with 3 μl FITC-Annexin V staining solution for 10 min and 2 μl PI staining solution for 5 min in sequence. A total of 100 μl 1X binding buffer was added at room temperature in the dark and the labeled cells were subsequently analyzed by flow cytometry.

DNA cell cycle analysis. The Eca109 cells (70% confluence) were serum starved for 36 h to synchronize in G_0 phase. They were treated with CNFE at 100, 200, 300, 400 and 50 0 μg/ml in complete culture medium for 24 h. The cells were subsequently trypsinized, washed twice with buffer solution (BD Biosciences) and centrifuged for 5 min at 300 x g at room temperature each time. The pellet was re-suspended in buffer and frozen at -80˚C until analysis. The frozen samples were thawed rapidly in a water-bath at 37˚C. The cells were centrifuged for 5 min at 400 x g at room temperature and stained with the Cycle TEST™ PLUS DNA Reagent kit (BD Biosciences), according to the manufacturer's instructions. The labeled cells were subsequently analyzed by flow cytometry.

Statistical analysis. The probit regression model was applied to estimate the half-maximal inhibitory concentration (IC_{50}) values of CNFE on Eca109 cells. χ^2 was applied to determine the event probability of the cytostatic or cytotoxic effect of CNFE, and to assess the effects of CNFE on the induction of apoptosis and cell-cycle perturbation. All statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

CNFE inhibited the growth of Eca109 cells. Firstly, the present study investigated the effects of CNFE on the viability of Eca109 cells by trypan blue exclusion assay (Fig. 1). As shown in Fig. 1, the percentage of dead cells increased both in a time- and dose-dependent manner. Compared with the non-treated control, the percentage of dead cells when Eca109 cells were treated with 300 μg/ml of CNFE for 24 h was significantly increased (34.43 vs. 6.43%). The cell death of Eca109 cells increased gradually when treated with CNFE at 300, 400 and 500 μg/ml for 24 h ($P<0.001$, compared with the control; Fig. 1). The percentage of dead cells when Eca109 cells were treated with CNFE (300, 400 or 500 μg/ml) for 24 h was 34.42, 37.30 and 39.43%, respectively. Treatment with 200 μg/ml CNFE for 48 or 72 h significantly reduced the cell viability ($P<0.001$, compared with control; Fig. 1). The antiproliferative effect of CNFE was also time-dependent. The IC_{50} of CNFE on Eca109 cells, calculated accordingly at 24, 48 and 72 h, was 513.64, 326.88 and 217.31 μg/ml, respectively (Fig. 1).

CNFE induced apoptosis of Eca109 cells. The apoptosis of Eca109 cells was further analyzed by flow cytometry using FITC-Annexin V and PI (Fig. 2). In control cells, the percentage of early apoptotic cells was 3.01% and the percentage of late apoptotic/dead cells was 5.91% (Fig. 2). Following incubation with 100 μg/ml CNFE for 48 h, the percentages of early apoptotic cells and late apoptotic cells increased to 14.16 and 7.27%, respectively ($P<0.01$; Fig. 2). The apoptosis of Eca109 cells after treatment with 100-500 μg/ml CNFE for 48 h was significantly increased in a dose-dependent manner (Fig. 2). The percentage of apoptotic cells (early and late apoptosis in total) gradually increased as the incubating time was extended. The percentage of apoptotic Eca109 cells following treatment with 300 μg/ml CNFE for 24, 48 and 72 h were 16.55, 25.14 and 30.72% respectively, each significantly increased compared with that of the control cells (9.09, 8.92 and 15.07%, respectively; $P<0.001$; Fig. 3).

CNFE resulted in G_0/G_1 arrest in Eca109 cells. To study whether CNFE can interrupt the cell cycle, flow cytometric analysis with PI staining was used to analyze the distribution of the cell cycle. After treatment with CNFE at 200 μg/ml for 24 h, the number of cells in G_0/G_1 phase was 61.67%, which was significantly more compared with the 51.66% observed in control cells ($P<0.001$; Fig. 4). The S phase cell population and G_2/M phase cell population were significantly reduced to 34.30 and 4.04%, compared with the 36.44 and 11.91%

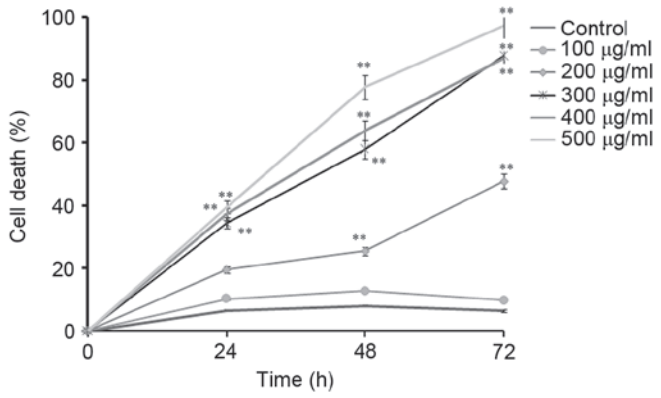

Figure 1. Viability of Eca109 cells following treatment with CNFE. Eca109 cells were exposed to different concentrations of CNFE for up to 72 h. The percentage of dead cells was determined using a trypan blue exclusion assay. Each experiment was performed independently three times. The data are presented as the mean ± standard deviation (**P<0.001, compared with the control at 24, 48 and 72 h. CNFE, *Camellia nitidissima* flowers water extract; h, hours.

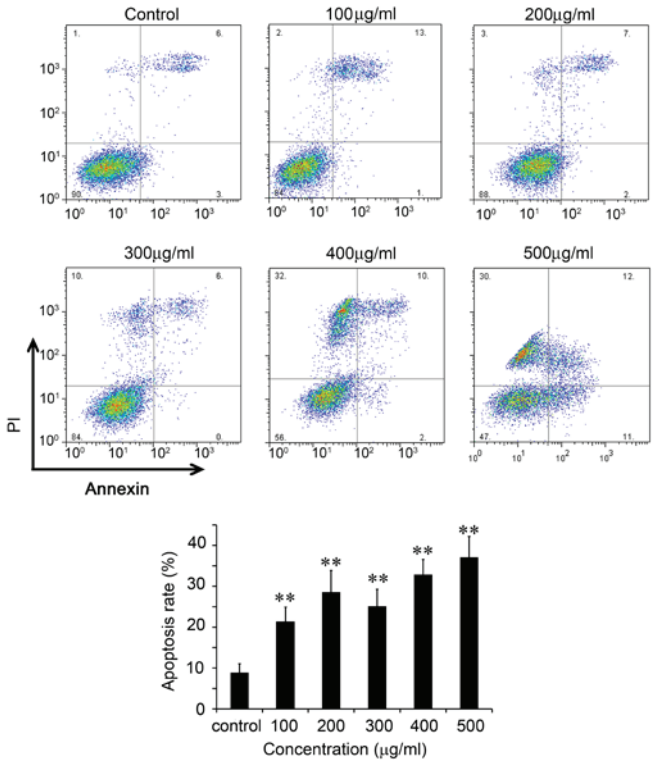

Figure 2. Apoptosis of Eca109 cells induced by CNFE at different concentrations. Eca109 cells were treated with different concentrations of CNFE for 48 h, labeled with fluorescein isothiocyanate-Annexin V and PI, and analyzed by flow cytometry. Data shown are from a representative experiment which was repeated three times. **P<0.01 vs. the control. CNFE, *Camellia nitidissima* flowers water extract; PI, propidium iodide.

observed in control cells (P<0.001; Fig. 4), respectively. The G_0/G_1 arrest effect of CNFE on Eca109 cells in G_0/G_1 phase was dose-dependent (P<0.001; Fig. 4). However, this effect appeared not to be time-dependent. Even when the incubation time was extended to 48 h, the cell cycle distribution revealed no significant change when compared with that at 12 or 36 h (Fig. 5).

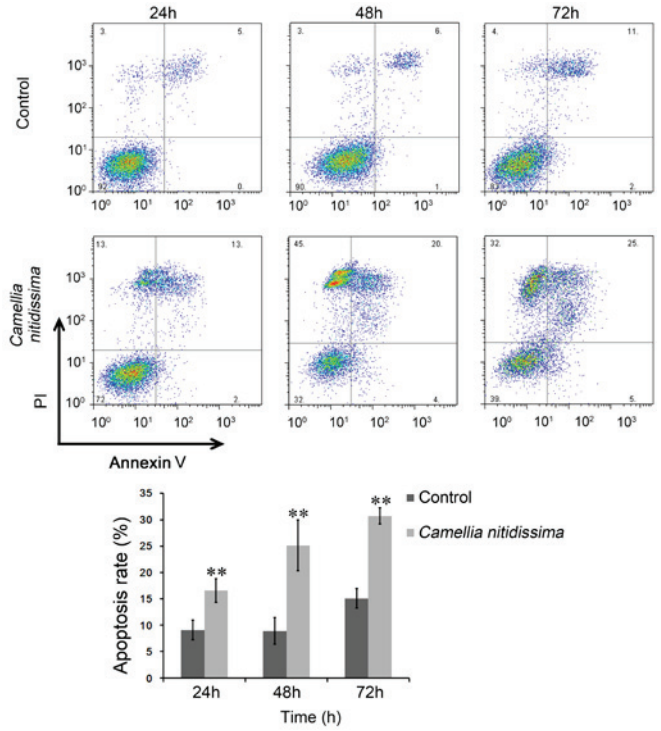

Figure 3. Apoptosis of Eca109 cells induced by CNFE at different time points. Eca109 cells were treated with vehicle or CNFE (300 μg/ml) for 24, 48 and 72 h, labeled with fluorescein isothiocyanate-Annexin V and PI, and analyzed by flow cytometry. Representative data are from three independent experiments. **P<0.01 vs. the control. CNFE, *Camellia nitidissima* flowers water extract; PI, propidium iodide; h, hours.

Discussion

As a part of the ongoing program of investigating the CN flower in relation to cancer chemoprevention, the present study evaluated the cancer preventive effect of CNFE on the human ESCC cell line, Eca109. It was demonstrated that CNFE was able to reduce Eca109 viability both in a time- and dose-dependent manner. CNFE induced the apoptosis of Eca109 cells and caused G_0/G_1 arrest.

Similar to other *C. sinensis* species (e.g. green tea), CN is rich in antioxidants, including numerous polyphenols such as gallocatechin, epicatechin, epigallocatechin-3-gallate and gallocatechin gallate (4,6). Only a few previous studies have investigated the possibility of CN extracts in preventing cancer (4,6,7). Water extracts of CN leaf reduce the viability of breast cancer cells (7), and ethanol extracts from the flowers of CN inhibit proliferation and induce apoptosis in human lymphoma cells (8). CN flowers contain more phytochemicals compared with those in the leaves and these include flavonoids, tea polyphenols and saponin (6). Peng *et al* (8) and his colleagues reported that a unique acylated flavonoid glycoside in the ethanol extract of CN flowers induced the apoptosis of human lymphoma cells (8). These bioactive components may serve a role in the anticancer ability of CNFE. Previous studies have confirmed that the water extract of CN leaves and flowers can inhibit the proliferation of hepatoma cells and the diethylnitrosamine-induced precancerous lesions in rat liver (11,12). In the present study, CNFE resulted in loss of Eca109 cell viability in a dose- and time-dependent manner,

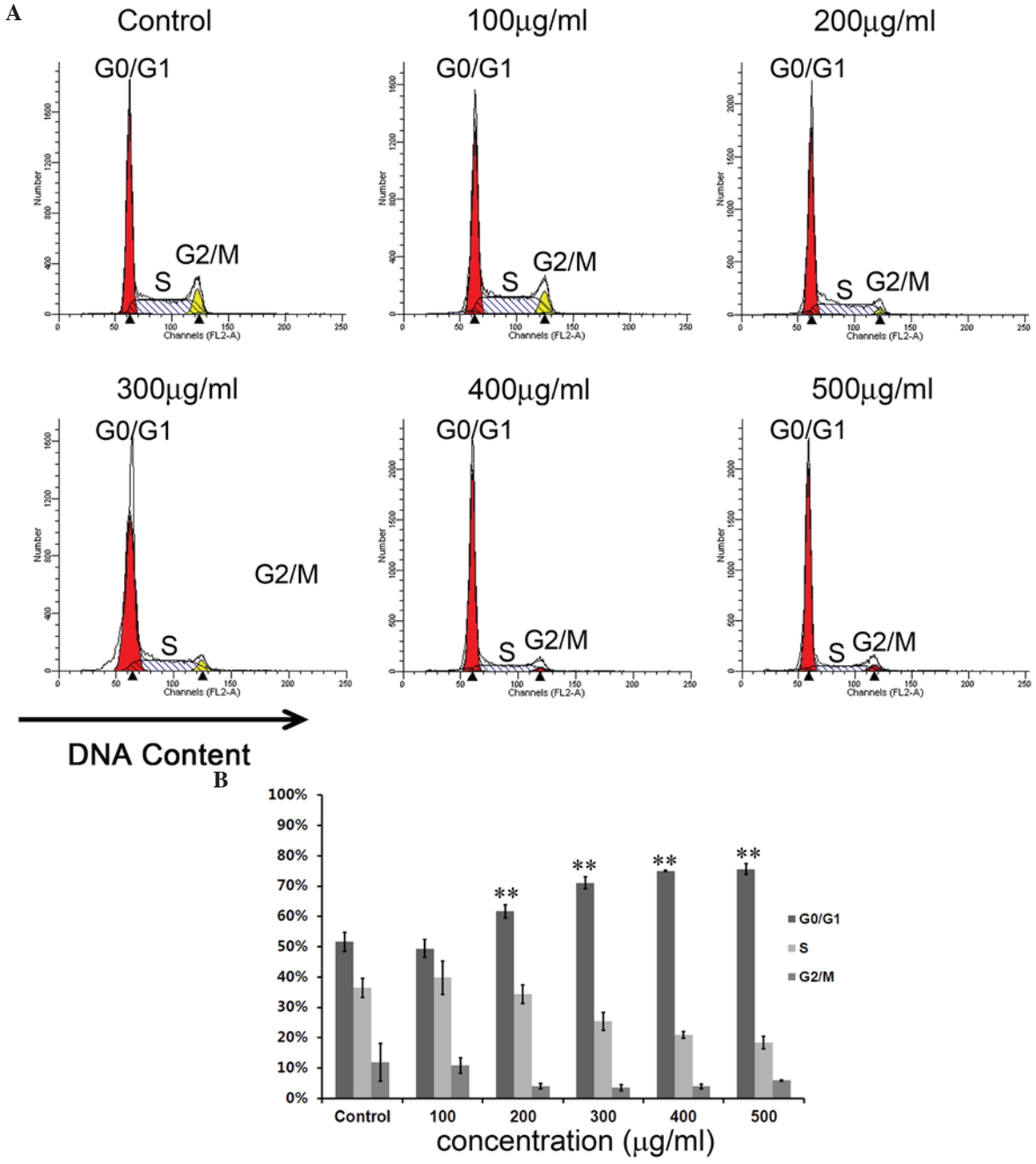

Figure 4. Effect of CNFE on cell-cycle perturbation at different concentrations. (A) Eca109 cells were treated with vehicle or different concentrations of CNFE for 24 h, labeled with propidium iodide and analyzed by flow cytometry. The first peak represents G_0/G_1 phase cell population and the second peak represents G_2/M phase cell population. The data shown are from a representative experiment, which was repeated three times with similar results. (B) The histograms were obtained by PI staining ($^{**}P<0.01$). CNFE, *Camellia nitidissima* flowers water extract.

and also induced apoptosis in a dose- and time-dependent manner.

Cells can be arrested in G1, S and G2/M phases of the cell cycle to prevent replication of damaged DNA or to prevent aberrant mitosis. The present study also confirmed that CNFE was able to arrest Eca109 cells in G_0/G_1 phase, dose-dependently. It was similar to that of green tea and tea polyphenols, which most likely arrest cells in G_0/G_1 phase (13).

However, the cycle arrest and the apoptosis were affected differently in a time-dependent manner. When the Eca109 cells were treated with CNFE at 200 μg/ml, the apoptosis rate increased sharply from 24 to 72 h, however, the percentage of cells arrested in G_0/G_1 phase appeared to remain the same. It is possible that the cell cycle arrest is not the predominant cause of the CNFE-induced the Eca109 cell death. A previous study demonstrated that compounds in CN flower EtOAc-soluble fraction can activate caspase 3 (8). Therefore, CNFE may possibly cause cell death by directly activating the caspase pathway; however, this requires further investigation.

The CNFE exerted its effect by inhibiting cell proliferation and inducing apoptosis; however, its role in chemoprevention on ESCC and its targets were predominantly unknown. Further studies on the underlying mechanisms may assist with highlighting the chemopreventive potential of this rare plant.

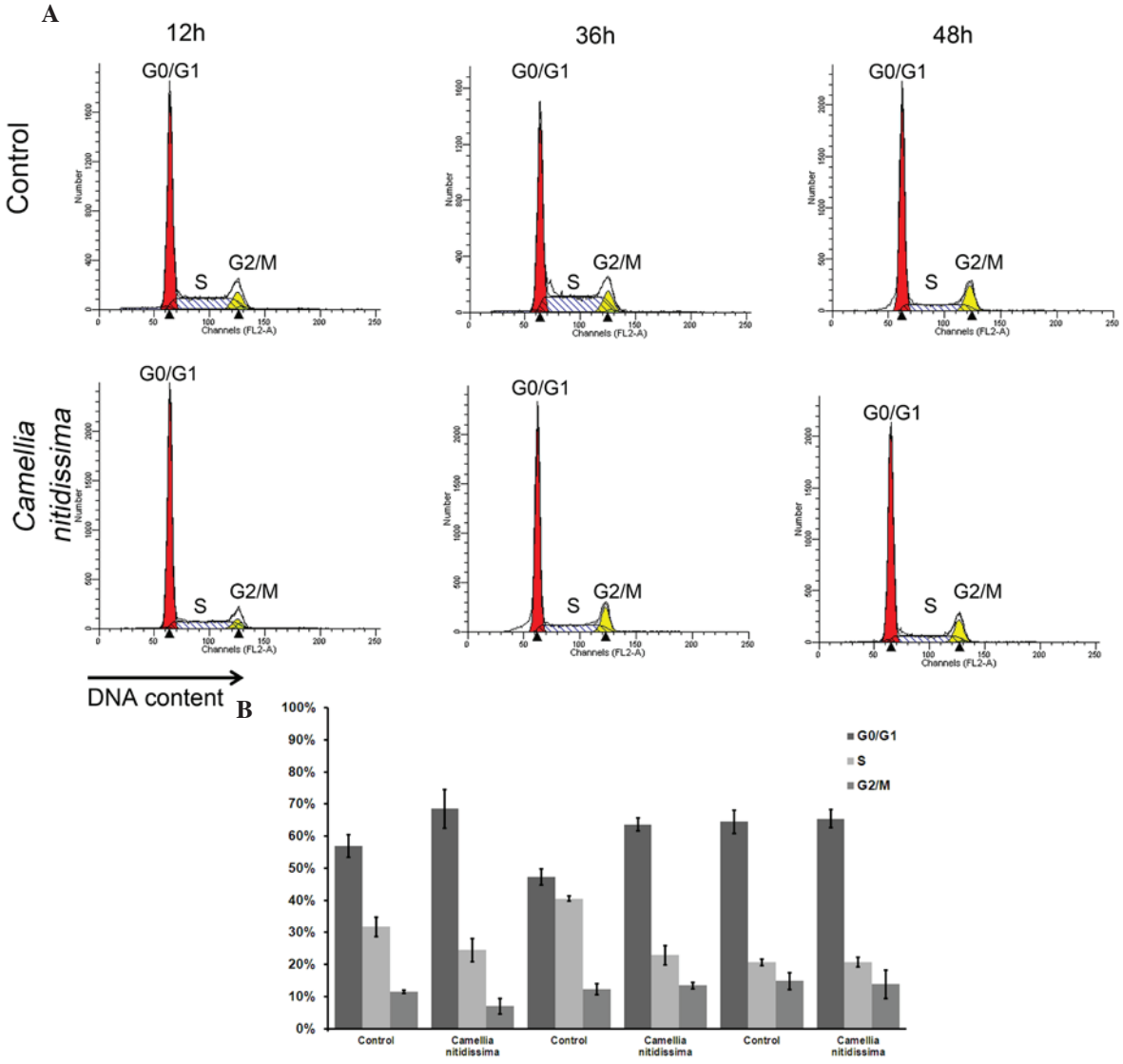

Figure 5. Effect of CNFE on cell-cycle perturbation at different time points. (A) Eca109 cells were treated with vehicle or CNFE (200 μg/ml) for 12, 36 and 48 h, labeled with PI and analyzed by flow cytometry. The data shown are from a representative experiment repeated three times with similar results. (B) The histograms were obtained by PI staining. CNFE, *Camellia nitidissima* flowers water extract; PI, propidium iodide.

Furthermore, as CNFE contains a variety of water-soluble constituents, the individual constituent that is the most important in preventing cancer remains unknown. Future studies must focus on the most effective antioxidant fractions and their functions *in vivo*, and this may assist in developing effective chemoprevention agents from CN extracts.

In conclusion, the present study has demonstrated that CNFE can reduce the viability of Eca109 cells by affecting the cell cycle and by inducing apoptosis *in vitro*. The results of the present study suggested that CNFE or some of its constituents may be potential chemopreventive agents for ESCC in the future.

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