Centipeda minima (Ebuschicao) extract inhibits PI3K-Akt-mTOR signaling in nasopharyngeal carcinoma CNE-1 cells

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Abstract

Background: Centipeda minima (Ebuschicao) has been used for the treatment of various diseases, such as nasal allergies, rhinitis and sinusitis, nasopharyngeal carcinoma, cough, and headache. This study aims to investigate the anticancer activities of Centipeda minima ethanol extracts (CME) against nasopharyngeal carcinoma cell CNE-1 and their underlying mechanism.

Methods: CNE-1 cells were treated with different concentrations (15–50 μg/mL) of CME for different time intervals (24, 48, and 72 h). Cytotoxicity of CME was determined by MTT assay. Cell morphological changes were observed by fluorescence microscopy after HO 33258 staining. Cell cycle status was evaluated by flow cytometry following propidium iodide staining. Apoptosis was detected by flow cytometry following annexin V-FITC/PI staining. The levels of apoptosis-associated and PI3K-Akt-mTOR signaling related proteins were measured by western blotting analysis.

Results: CME (15–50 μg/mL) significantly inhibited the proliferation of CNE-1 in a dose- and time-dependent manner (P = 0.026 for 15 μg/mL, P < 0.001 for 25, 30, 40, and 50 μg/mL, respectively); the IC50 values (μg/mL) were 41.57 ± 0.17, 30.34 ± 0.06 and 24.98 ± 0.08 for 24, 48 and 72 h treatments, respectively. Significant morphological changes of CNE-1 cells displaying apoptosis were observed after CME treatment. CME showed low cytotoxicity toward normal LO2 cells. CNE-1 cells were arrested in the G2/M phase while treated with 15, 25, 40 μg/mL of CME, respectively (P = 0.032, P = 0.0053, P < 0.001). CME (15, 25, 40 μg/mL) down-regulated Bcl-2 expression (P = 0.032, P = 0.0074, P < 0.001), and up-regulated Bax (P = 0.026, P = 0.0056, P < 0.001) with activation of caspase-3, caspase-8, caspase-9, and PARP observed in CNE-1 cells (P = 0.015, P = 0.0067, P < 0.001 for caspase 3; P = 0.210, 0.028, < 0.001 for caspase 8; P = 0.152, 0.082, 0.0080 for caspase 9; P = 0.265, 0.0072, < 0.001 for PARP). CME suppressed the activation of the PI3K-AKT-mTOR pathway (P = 0.03, 0.0007, 0.004, 0.006, 0.022 for p-PI3K, p-Akt-Ser473, p-Akt-Thr308, p-mTOR-Ser2448, p-mTOR-Ser2461, respectively after 40 μg/mL of CME treated for 24 h).

Conclusion: CME inhibited the proliferation of CNE-1 cells and activation of the PI3K-AKT-mTOR signaling pathway.

Background

Nasopharyngeal carcinoma (NPC), a malignant epithelial tumor derived from the nasopharyngeal surface epithelium, has a low incidence in Western countries but a high incidence in Southeast Asia, southern China and the Mediterranean basin [1]. Epstein-Barr virus infection is a critical factor in NPC pathogenesis [2, 3]. Additional etiological factors for NPC include genetic susceptibility, environmental contamination, and dietary habits such as excessive consumption of salt-preserved fish during childhood [1, 4]. Radiotherapy is the standard primary treatment for NPC [5], and a combination of radiotherapy and chemotherapy has been shown to be an effective therapeutic strategy for treating advanced...
NPC [6]. The most prevalent chemotherapeutic drugs used to treat NPC are cisplatin-based drugs, which are highly toxic and prone to drug resistance [7, 8]. Chinese medicine (CM) in combination with conventional cancer therapies has been applied to NPC [9]. Controlled clinic trials demonstrated that CM therapies achieve prolonged survival and improved quality of life in NPC patients [10, 11].

Some CMs exhibit antitumor activities toward NPC cells via growth inhibition, apoptotic induction and cell cycle arrest [12, 13]. *Centipeda minima* (L.) A. et Aschers. (Ebushicao) (Asteraceae) has been used to treat nasal allergies, rhinitis, sinusitis, cough, headache [14, 15] and NPC [16]. Recent researches have focused on its anti-NPC activities, leading to the isolation of a few sesquiterpene lactones against NPC cells [17, 18]. Volatile oils from *C. minima* extracted by steam distillation and supercritical fluid extraction induced CNE cell death via induction of intrinsic apoptosis by regulating the expression of the Bcl-2 family of proteins [16]. Ethyl acetate extracts of *C. minima* exhibited anti-proliferative activity against CNE-2 cells [19]. However, the anticancer activities of *C. minima* ethanol extracts (CME) against the NPC cell line CNE-1, and their underlying mechanism, remain unclear.

This study aims to investigate the anticancer activities of CME against the nasopharyngeal carcinoma cell line CNE-1 and determine their underlying mechanism.

Methods

**Plant materials and sample preparation**

*Centipeda minima* was collected in August 2010 in Xiangfan, Hubei Province, China (latitude, 32°04′ N; longitude, 112°05′ E), and authenticated by Si-bao Chen based on morphological features. A voucher specimen (EBSC-016-09) was deposited at the herbarium of the State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University. The herb was air-dried and ground to coarse powder. The powder (30 g) was macerated with 0.5 L of 95 % ethanol at room temperature for 72 h. The extraction was repeated twice. After extraction, the ethanol extracts were combined, filtered through Whatman filter paper (Whatman, Maidstone, UK), and evaporated to dryness using a rotary evaporator (Precision MLG3, Heidolph, Germany) on a water bath at 40 °C, and further lyophilized to dried powder (3.80 g). CME samples with a yield of 12.65 % were stored in the refrigerator at 4 °C until further use. CME was dissolved in DMSO and filtered to 0.22 μm to obtain the stock concentration of 20 mg/mL. Finally, a serial dilution to concentrations of 15, 25, 30, 40, and 50 μg/mL CME was performed.

**Chemicals**

3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33258 and cisplatin were obtained from Sigma (St. Louis, MO, USA). The annexin V-FITC apoptosis detection kit and cell cycle analysis kit were purchased from Beyotime (Invitrogen, Carlsbad, CA, USA). Precision Plus Protein Standards (Dual Color) and Immun-Star™ WesternC™ Chemiluminescent Kit were purchased from Bio-Rad (Hercules, CA, USA). Antibodies against procaspase 8, cleaved caspase 9, cleaved PARP, PI3K p110α, Akt, p-Akt (Thr308 and Ser473), mTOR and p-mTOR (Ser2448 and Ser2481) were obtained from Cell Signaling Technology (Beverly, MA, USA). All other primary antibodies, as well as anti-rabbit and anti-mouse secondary horseradish peroxidase antibodies, were purchased from Abcam (Cambridge, MA, USA). All other common chemicals were reagent grade.

**Cell culture**

CNE-1 human nasopharyngeal carcinoma cells (depository no. CBP60002) were purchased from the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). LO2 human normal liver cells (depository no. GNHu 6) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10 % fetal bovine serum (Gibco, Life Technologies, Grand Island, NY, USA), 100 IU/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific, Madison, WI, USA) at 37 °C in a humidified incubator with a 5 % CO₂ atmosphere. The medium was renewed three times a week. Cells in logarithmic growth phase were used for all experiments.

**Cell viability assay**

The effects of CME on the viability of CNE-1 and LO2 cells were evaluated by MTT assay [20]. Cells (3 × 10⁴ cells/mL) were seeded into the wells of 96-well culture plates (Thermo Fisher Scientific) in 100 μL of medium per well and then allowed to adhere for 24 h at 37 °C in a 5 % CO₂ atmosphere. After incubation, the cells were treated with the target concentrations of CME (15–50 μg/mL), 0.1 % DMSO as the vehicle control, and cisplatin (4 μg/mL) as a positive control, for 24 h. After incubation for 24, 48, and 72 h at 37 °C in a CO₂ incubator, 10 μL of MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well and incubated for a further 4 h. Then, excess medium was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density in each well was
measured using a microplate spectrophotometer (BMG POLARstar Galaxy, Offenburg, Germany) at 490 nm. Triplicate experiments were performed for treatment with each concentration. The percentage cell viability (%) was calculated by comparison with a sample’s corresponding control. IC\textsubscript{50} values were calculated to evaluate the cytotoxic effects of treatments on CNE-1 cells.

**Morphological assessment**
The cells (1 \times 10^6 cells/mL) were seeded into 6-well plates, and then treated with different concentrations (15, 25, 30, 40, or 50 \mu g/mL) of CME for 24 h. Similarly, cells were treated with 25 \mu g/mL CME for 24, 48, and 72 h, respectively. DMSO (0.1 %)-treated cells were employed as controls. Subsequently, the cells were washed twice with cold PBS, fixed with 4 % paraformaldehyde solution for 10 min, stained with Hoechst 33258 (10 \mu g/mL) for 15 min in the dark, and then observed under an inverted fluorescence microscope (Leica DMRB, Weitzl, Germany) at the excitation wavelength of 352 nm and emission wavelength of 461 nm.

**Apoptosis analysis by annexin V-FITC/PI staining**
The numbers of apoptotic cells were assessed using an annexin V-FITC apoptosis detection kit (Invitrogen, USA) according to the manufacturer’s protocol. Cells (1 \times 10^6 cells/mL) were treated with various concentrations of CME for 24, 48, and 72 h. Cells were then harvested, and re-suspended in binding buffer. Subsequently, cells were stained with 5 mL of annexin V-FITC and 5 mL of propidium iodide (PI) for 15 min at room temperature in the dark. The apoptotic cell percentages were then immediately detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

**Cell cycle analysis by PI staining**
Cells (1 \times 10^6 cells/well) were treated with various concentrations of CME (15, 25, and 40 \mu g/mL) for 24, 48, and 72 h. Cells were then collected and washed twice with 1 mL of ice-cold PBS, suspended in 1 mL of ice-cold 70 % ethanol (containing 1 \times 10^5 cells) and kept overnight at 4 °C. The next day, cells were washed with PBS and incubated with 500 \mu L of PI staining solution (containing PI 40 \mu g/mL, RNase A 100 \mu g/mL and PBS) for 30 min in the dark at 37 °C. Then, samples were filtered using 70-\mu m filters into test tubes and detected by flow cytometry (FACSCalibur, Bio-Rad). Analysis of cell cycle status was performed using ModFit LT 3.0™ software (BD Biosciences).

**Western blot analysis**
CNE-1 cells (1 \times 10^6 cells/well) were seeded into 100 mL culture dishes for 24 h and then treated with various concentrations of CME (15, 25, and 40 \mu g/mL). After incubation for 24 h, the cells were harvested and prepared in NP-40 lysis buffer (2 mM Tris–Cl pH 7.5, 150 mM NaCl, 10 % glycerol and 0.2 % NP-40 plus a protease inhibitor cocktail) for 30 min on ice. Cell lysates were ultra-centrifuged (Allegra™ 21R Centrifuge, Beckman Coulter, Krefeld, Germany) at 14,000 \times g for 10 min at 4 °C, and then the supernatant was collected as the total cellular proteins. Protein concentrations were determined using a BCA protein assay kit (Beijing ComWin Biotech Co., Ltd., Beijing, China). Proteins (10 \mu g for β-actin and Bax, 20 \mu g for PARP and cleaved PARP, 30 \mu g for caspase-3, -9, and -8, PI3K p110\alpha, Akt, and p-Akt, 50 \mu g for Bcl-2, mTOR, and p-mTOR) were resolved on 12–15 % SDS-PAGE gels, then transferred to PVDF (polyvinylidene difluoride) membranes (Roche Applied Science, Basel, Switzerland) and blocked with 5 % non-fat dry milk in Tris-buffered saline containing 0.1 % Tween-20 (T-TBS) overnight at 4 °C. After washing with T-TBS, the membranes were incubated with primary antibodies against procaspase-3, -8, and -9; caspase-3, -8, and -9; Bcl-2, Bax, PARP, cleaved PARP; PI3K p110\alpha, Akt, p-Akt (Thr308 and Ser473), mTOR, p-mTOR (Ser2448 and Ser2461), and β-actin overnight at 4 °C. Subsequently, the membranes was incubated with goat anti-rabbit or horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Merck Millipore) at room temperature for 2 h at dilutions of 1:5000 and 1:10,000, respectively. Antibody-bound proteins were detected, normalized to β-actin and quantified in a ChemiDoc™ XRSTM system (Universal Hood II, Bio-Rad) using an Immun-Star™ HRP Chemiluminescence Kit (Bio-Rad).

**Statistical analysis**
All data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Analyses of dose-, concentration- and time-dependent effects were performed by sigmoidal non-linear regression using GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). The percentage cell viability (%) after CME treatment with different concentrations and time intervals was used for the fitting. The two-tailed Student’s t test was used for comparisons of two groups, and multi-group differences were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. P values < 0.05, 0.01, or 0.001 were considered statistically significant.

**Results**

**Cytotoxic effects of CME on CNE-1 cells**
CME-1 cells were treated with various concentrations (15–50 \mu g/mL) of CME for 24, 48, and 72 h, and the cell viability was determined by MTT assay. CME decreased CNE-1 cell viability in a dose- and time-dependent manner (Fig. 1; Table 1). After treatment with CME (15, 25, 30,
and 50 μg/mL) for 24 h, the percentage of live cells were 95.41, 81.27, 60.10, 50.0 and 42.41 %, (P = 0.026 for 15 μg/mL, P < 0.001 for 25, 30, 40, and 50 μg/mL), respectively. Meanwhile, those were 51.14, 34.0, and 25.05 % (P < 0.001 for each test), while CNE-1 cells were treated with CME (40 μg/mL) for 24, 48, and 72 h, respectively. The IC50 values were 41.57 ± 0.17, 30.34 ± 0.06 and 24.98 ± 0.08 μg/mL for 24, 48, and 72 h incubations, respectively. Despite its potency in CNE-1 cells, CME exhibited no significant cytotoxic effect on normal LO2 cells (data not shown due to no significant variation versus vehicle control).

Morphological changes in CNE-1 cells after CME treatment
Morphological changes and cell death in CNE-1 cells were observed under an inverted fluorescence microscope (DFC 420 C, Leica, Germany) after HO 33258 staining. Vehicle-treated (control) cells showed normal cell architecture with clear cytoskeletons (Fig. 2). Typical morphological changes associated with apoptosis, including chromatin condensation, apoptotic body formation and nuclear degradation, were observed in CME-treated cells. These morphological changes were enhanced with increasing concentrations of CME (Fig. 2a), and with increasing treatment duration (Fig. 2b).

Apoptosis analysis by flow cytometry
The timing of induction of apoptosis was determined by flow cytometry following annexin V-FITC/PI double staining. After exposure to CME (15, 25, or 40 μg/mL) for 48 h, the proportion of apoptotic cells dramatically increased in a dose-dependent manner; the percentages of apoptotic cells were 39.53, 56.43, and 76.42 %, respectively (P = 0.0086, P < 0.001, P < 0.001) (Fig. 3a). Similarly,
CME induced CNE-1 cell apoptosis in a time-dependent manner. The percentages of apoptotic cells were 43.30, 76.42, and 91.29 % after treatment with 40 μg/mL CME for 24, 48, and 72 h, respectively (P < 0.001 for each test) (Fig. 3b).

**Flow cytometric analysis of the cell cycle status of CNE-1 cells**

Cell cycle progression was analyzed by flow cytometry to determine whether the cytotoxic effect of CME toward CNE-1 cells was associated with the induction of cell cycle arrest. After treatment with 15, 25, and 40 μg/mL CME for 48 h, the percentages of cells in the G2/M phase were 16.00, 24.71, and 60.85 %, respectively (P = 0.032, P = 0.0052, P < 0.001); while the percentages of those in the G0/G1 phase were 50.74, 47.67, and 21.94 %, respectively (P = 0.102, 0.067, P < 0.001) (Fig. 4a). Meanwhile, after treatment with CME (40 μg/mL) for 24, 48, and 72 h, the percentages of cells in the G2/M phase were 29.38, 35.21, and 41.13 %, respectively (P < 0.001 in each test); while the percentages of those in the G0/G1 phase were 42.69, 30.20, and 25.64 %, respectively (P = 0.0072, P = 0.0047, P < 0.001) (Fig. 4b). No significant variations in the percentages of cells in S phase were observed after CME treatment. These results indicated that the inhibition of CNE-1 cell proliferation induced by CME could be attributed to cell cycle arrest in the G2/M phase.

**Expression of CME-regulated apoptosis-related proteins**

The expression levels of apoptosis-related proteins were analyzed by western blotting to study CME-induced apoptosis of CNE-1 cells in more detail. After treatment with CME (15, 25, 40 μg/mL) for 24 h, the levels of cleaved caspase 3, 8, 9 and cleaved PARP increased in a dose-dependent manner (P = 0.020, P = 0.0060, P < 0.001 for caspase 3; P = 0.210, 0.028, < 0.001 for caspase 8; P = 0.152, 0.082, 0.0082 for caspase 9; P = 0.265, 0.0060, P < 0.001 for PARP), while the levels of pro-caspases 3, 8, 9 and PARP decreased (P = 0.0045, P < 0.001, P < 0.001 for pro-caspase 3; P = 0.147, 0.015, 0.036 for pro-caspase 8; P = 0.250, 0.031, 0.0085 for pro-caspase 9; P = 0.092, < 0.001, 0.0001 for pro-PARP) (Fig. 5a). Meanwhile, the anti-apoptotic protein Bcl-2 was down-regulated (P = 0.035, P = 0.0055, P < 0.001), while the pro-apoptotic protein Bax was up-regulated (P = 0.020, P = 0.0035, P < 0.001) (Fig. 5b), and the Bax/Bcl-2 ratio was significantly increased as the concentrations of CME increasing (P = 0.072, 0.034, 0.0005) (Fig. 5c).

**Inhibition of PI3K-Akt-mTOR signaling in CME-treated CNE-1 cells**

We investigated whether CME treatment could also down-regulate the expression levels of related proteins in the PI3K-Akt-mTOR pathway. After treatment with CME (15, 25, 40 μg/mL) for 24 h, the levels of PI3Kinase P110α, the catalytic subunit of PI3K (P = 0.042, 0.040, 0.030), and the amount of phosphorylation of Akt at

![Flow cytometry analysis of the induction of apoptosis in CNE-1 cells by CME.](image-url)
Fig. 4 Cell cycle distribution of CNE-1 cells in the flow cytometric analysis after CME treatment. 

**a** CNE-1 cells were treated with 0.1 % DMSO (control) and 15, 25, and 40 μg/mL CME for 48 h; 

**b** CNE-1 cells were treated with 40 μg/mL CME for 0 (control), 24, 48, and 72 h.

Fig. 5 Effects of CME on the expression levels of apoptosis-related proteins in CNE-1 cells after 24 h of treatment. 

**a** Expression levels of procaspase-3, -8, and -9; caspase-3, -8, and -9; PARP and cleaved-PARP. 

**b** Expression level of Bcl-2 and Bax. 

**c** Bax/Bcl-2 ratios; each value is based on three independent experiments. *P = 0.034, **P = 0.0005 versus control.
Ser473 ($P = 0.047, 0.0008, 0.0007$) and Thr308 ($P = 0.021, 0.005, 0.004$) were remarkably decreased in a concentration-dependent manner, while the amount of total Akt remained constant following CME treatment (Fig. 6). Consistently, phosphorylation of the downstream effector mTOR at Ser2448 ($P = 0.037, 0.025, 0.006$) and Ser2481 ($P = 0.046, 0.039, 0.022$) also showed a dose-dependent decrease, and the amount of total mTOR was slightly down-regulated upon CME treatment of CNE-1 cells.

**Discussion**

In the present study, the anti-cancer effects of CME toward human nasopharyngeal carcinoma CNE-1 cells in terms of cytotoxicity, apoptosis induction and cell cycle arrest were investigated. CME possessed significant cytotoxic activities against CNE-1 cells, in a time- and dose-dependent manner, but very low cytotoxicity toward normal LO2 cells. These results suggest that CME has potential selective anti-cancer effects on CNE-1 cells.

Apoptotic induction in cancer cells is a crucial therapeutic strategy for cancer treatment. CME induced morphological changes including cell shrinkage, the appearance of apoptotic vacuoles, and chromatin condensation, detected by fluorescence microscopy after CME treatment followed by Ho 35288 staining. This apoptotic induction was confirmed by flow cytometry using an annexin V-FITC/PI double-staining approach.

In this study, changes in cell cycle progression were analyzed by flow cytometry. CME induced obvious G2/M phase arrest in CNE-1 cells in a dose- and time-dependent manner. G2/M, the most important checkpoint for DNA damage, is critical to cell cycle progression, and strictly regulated by specific genes [21]. If phase arrest cannot be repaired, cells may undergo apoptosis.

Changes in the expression levels of apoptosis-related proteins in CNE-1 cells after CME treatment were evaluated by western blotting to explore the mechanism underlying the induction of apoptosis by CME in CNE-1 cells. The activation of caspase 9 through apoptotic protease activating factor-1 (APAF-1) and cytochrome c release [22] initiates activation of the caspase cascade, cleaves executioner caspase-3, and eventually results in apoptosis [23]. Cleaved caspase 8 activates downstream caspases such as caspase-3, -6, and -7 [24], which cleave specific intracellular substrates, such as polyADP-ribose polymerase (PARP), lamins and inhibitor of caspase activated DNase (ICAD), finally resulting in programmed cell death [25]. This study revealed that CME could significantly induce CNE-1 cell apoptosis via the activation of caspase-8, -9, -3, as well as PARP, through both extrinsic and intrinsic pathways.

The B cell lymphoma 2 (Bcl-2) family proteins, including pro-apoptotic members such as Bax, Bak and anti-apoptotic members such as Bcl-2, Bcl-xL, play important roles in the regulation of mitochondrial-mediated apoptosis [26, 27]. In this study, CME treatments downregulated the expression of Bcl-2, and upregulated the expression of Bax, resulting in CNE-1 cell apoptosis. Furthermore, elevated intracellular ratios of Bax/Bcl-2 were observed during a period of increased apoptotic cell death. These experimental findings suggested that CME induced CNE-1 cell apoptosis via a mitochondrial pathway.

The PI3K-AKT-mTOR pathway is a major signaling pathway in cancer [28]. This pathway is frequently mutated and overactivated in human cancers [29]. The PI3K-AKT-mTOR pathway promotes survival through inhibition of proapoptotic factors and activation of anti-apoptotic factors [30]. Through phosphorylation of pathway components, this pathway inhibits the activity of proapoptotic members while activating anti-apoptotic members [31]. Therefore, blockade of the PI3K-AKT-mTOR pathway has become a cancer therapy strategy. Our present results indicate for the first time that CME downregulates the PI3K-AKT-mTOR-mediated signaling in CNE-1 cells (Fig. 7). Additionally, 14-thienyl methylene matrine (YYJ18), a derivative of matrine, has been reported to induce apoptosis in NPC cells by targeting PI3K-Akt [32], which also provides evidence for the involvement of this pathway in NPC carcinogenesis.
Conclusion
CME inhibited the proliferation of CNE-1 cells and activation of the PI3K-AKT-mTOR signaling pathway.

Abbreviations
CME: Centipeda minima ethanol extracts; CM: Chinese medicine; CNE-1: high differentiated human nasopharyngeal carcinoma cells; CNE-2: low differentiated human nasopharyngeal carcinoma cells; LO2: human hepatocytes; NPC: nasopharyngeal carcinoma; MTT: 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; DMEM: dexamethasone; PBS: phosphate buffered saline; PI: propidium iodide; FITC: fluorescein isothiocyanate; SDS: sodium dodecyl sulphate; PAGE: polyacrylamide gel electrophoresis; TBS: tween-20; PVDF: polyvinylidene difluoride; RNase A: ribonuclease A; ICAD: lamins and Bcl-2: B cell lymphoma/leukemia-2; Bax: Bcl-2 associated X protein; YYJ18: 14-thienyl methylene matrine (YYJ18); Caspase: cysteiny1 aspartate specific proteinase.

Authors’ contributions
SBC and ZLY conceived and designed the study. YQG, HYS, BBL and JHW performed the experiments. YQG, HYS and SWC performed the statistical analysis. YQG and HYS wrote the manuscript. COC, DKWM and AKWT revised the manuscript. All the authors read and approved the final version of the manuscript.

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Compliance with ethical guidelines
Competing interests
The authors declare that they have no conflict of interests.

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