

Anti-neoplastic effects of *Chrysanthemum indicum* extract on a human hypopharyngeal squamous cell carcinoma cell line

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Abstract. Hypopharyngeal squamous cell carcinoma (HSCC) is an aggressive malignancy associated with poor clinical outcomes and limited therapeutic options. Natural products, such as *Chrysanthemum indicum* (CI) extract, represent potential candidates for anticancer drug development; however, their therapeutic potential against HSCC remains unclear. The present study investigated the antitumor activity of CI crude extract in the human HSCC cell line FaDu and explored the underlying molecular mechanisms. Intracellular oxidative activity following CI treatment was quantified using 2',7'-dichlorodihydrofluorescein diacetate flow cytometry. The expression of proteins associated with cell survival and apoptosis was evaluated using western blotting, while cell viability was assessed using a Cell Counting Kit-8 assay. CI treatment notably reduced FaDu cell viability in a dose- and time-dependent manner. Flow cytometric analysis revealed an acute, concentration-dependent increase in intracellular oxidative activity following CI treatment, which was effectively reversed through pretreatment with the antioxidant N-acetylcysteine. Increased intracellular oxidative activity was accompanied by

reduced expression of the endoplasmic reticulum stress sensor protein kinase R-like endoplasmic reticulum kinase, also known as eukaryotic translation initiation factor 2 α kinase 3 (EIF2AK3), decreased phosphorylated (p)-Akt levels and a reduced p-Akt/total Akt ratio. These changes were accompanied by marked dose-dependent depletion of the anti-apoptotic protein B-cell lymphoma 2. Overall, CI treatment reduced FaDu cell viability and was associated with increased intracellular oxidative activity, reduced EIF2AK3 expression and decreased Akt activation. These findings support the potential antitumor activity of CI in HSCC cells and provide a rationale for further mechanistic and *in vivo* investigation.

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one of the most aggressive malignancies of the upper aerodigestive tract and is frequently characterized by early lymphatic metastasis and a poor prognosis. At the population level, an estimated 86,257 new cases of hypopharyngeal cancer and 40,902 associated deaths occurred worldwide in 2022. A recent systematic review and meta-analysis further reported an annualized recurrence rate of 14.11 per 100 person-years for hypopharyngeal cancer (1,2). The human HSCC cell line FaDu serves as a widely used *in vitro* model due to its invasive phenotype and molecular characteristics relevant to HSCC, including dysregulation of PI3K/AKT signaling, cellular stress-response pathways and apoptosis-related signaling (3-5). Currently, conventional therapeutic modalities, including surgery, radiotherapy and cisplatin-based chemotherapy, often result in suboptimal clinical outcomes, notable treatment-associated toxicities and the development of therapeutic resistance (2,5). Consequently, there is an urgent need to develop novel multitargeted therapeutic strategies with reduced toxicity for the treatment of this aggressive malignancy (3).

In the search for novel anticancer agents, natural products have emerged as potential candidates for drug discovery because of their diverse bioactive constituents and potential to modulate pathways involved in cellular proliferation, survival, oxidative stress and apoptosis (6,7). *Chrysanthemum indicum* (CI) L. (wild chrysanthemum) has

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Abbreviations: CI, *Chrysanthemum indicum*; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; HPV, human papillomavirus; HSCC, hypopharyngeal squamous cell carcinoma; NAC, N-acetylcysteine; PERK/EIF2AK3, protein kinase R-like endoplasmic reticulum kinase/eukaryotic translation initiation factor 2 α kinase 3; ROS, reactive oxygen species; UPR, unfolded protein response

Key words: *Chrysanthemum indicum*, hypopharyngeal squamous cell carcinoma, FaDu cells, reactive oxygen species, protein kinase R-like endoplasmic reticulum kinase, eukaryotic translation initiation factor 2 α kinase 3, Akt signaling

long been used in Traditional East Asian Medicine, primarily for its heat-clearing and detoxifying properties (8). Unlike *Chrysanthemum morifolium* Ramat., which is traditionally used to relieve exterior syndromes and exhibits comparatively weaker detoxifying activity, CI has attracted increasing attention due to its broad pharmacological potential (8-10). Results of previous studies have demonstrated that CI exhibits diverse biological activities, including anti-inflammatory, antioxidant, antimicrobial, immunomodulatory, hepatoprotective and cardioprotective effects (10,11). Notably, its bioactive constituents, such as terpene-rich essential oils, have been reported to disrupt cellular membrane integrity, increase membrane permeability and ultimately impair cellular survival (11-13).

Emerging evidence has suggested that the antitumor effects of numerous natural compounds are closely associated with their ability to disrupt cellular redox homeostasis (14,15). As cancer cells generally exhibit higher basal oxidative stress compared with healthy cells, they may be particularly susceptible to further oxidative damage induced through excessive accumulation of reactive oxygen species (ROS) (14,16). Certain natural products may act as pro-oxidants and induce acute cytotoxic increases in intracellular ROS (15), thereby overwhelming cellular antioxidant capacity, disrupting compensatory survival pathways and promoting programmed cell death (14,16).

Despite its broad biological activities, the potential therapeutic activity of CI against HSCC remains poorly understood (12,13). In addition, although disruption of intracellular redox homeostasis has been implicated in the antitumor effects of numerous natural compounds, the molecular mechanisms through which CI may alter redox homeostasis in FaDu cells remain unclear (14). In particular, it remains unknown whether CI-associated changes in intracellular oxidative activity are accompanied by alterations in important cellular survival pathways, such as the protein kinase R-like endoplasmic reticulum kinase/eukaryotic translation initiation factor 2 α kinase 3 (PERK/EIF2AK3) stress-response pathway, which contributes to cellular adaptation to ER stress, and the PI3K/Akt signaling pathway, which promotes tumor cell growth and survival (14).

Therefore, the present study aimed to evaluate the antitumor activity of crude CI extract in FaDu cells and investigate the underlying molecular mechanisms. Specifically, CI-associated changes in intracellular oxidative activity, EIF2AK3/Akt signaling and expression of the anti-apoptotic protein Bcl-2 were evaluated using western blotting. The present study therefore aimed to provide preliminary mechanistic evidence for the antitumor activity of CI in HSCC cells and establishes a basis for further investigation of its therapeutic potential.

Materials and methods

Extraction method. Dried flower heads supplied as CI were purchased from HORN GIN TRADING CO., LTD. Subsequently, 1,004.5 g dried material was pulverized and extracted with 95% (volume/volume) ethanol at room temperature for 24 h. The extraction procedure was repeated three times. The combined filtrates were concentrated under reduced pressure at $\leq 40^{\circ}\text{C}$ using a rotary evaporator (60 rpm) to obtain a dark brown crude extract. After drying, 245.35 g crude extract was obtained, corresponding to an extraction yield of 23.93%

(weight/weight), with a moisture content of 2.63%. The dried extract was stored at -20°C until subsequent biological experiments. Prior to cellular experiments, the dried CI extract was dissolved in absolute ethanol to prepare a 100 mg/ml stock solution and subsequently diluted in culture medium to the indicated final concentrations. These defined extraction parameters were maintained to ensure procedural consistency.

Botanical assessment of the plant material. Plant material supplied as CI was subjected to macroscopic examination, including assessment of its appearance, color and odor. Microscopic analysis of tissue sections was additionally performed using Fast Green staining to visualize tissue morphology and Periodic Acid-Schiff (PAS) staining to detect polysaccharide-containing structures and characterize anatomical features. Plant tissue samples were fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, the samples were washed twice with 1X PBS for 30 min each on ice, dehydrated through a graded ethanol series (10, 30, 50, 70, 85, 95 and 100%), cleared in xylene and infiltrated with molten paraffin at 60°C . The tissues were then embedded in paraffin and sectioned at a thickness of 4-8 μm using a microtome. For Fast Green staining, paraffin-embedded sections were deparaffinized twice in xylene for 5-10 min each and rehydrated through a graded ethanol series (100, 95, 80 and 70%) to distilled water. The sections were stained with 0.05% (w/v) Fast Green FCF (Sigma-Aldrich; Merck KGaA) for 1 min at room temperature. The sections were subsequently rinsed, dehydrated through a graded ethanol series, cleared in xylene and mounted using a resin-based mounting medium. For Periodic acid-Schiff (PAS) staining, deparaffinized and rehydrated sections were stained using a PAS staining kit (Muto Pure Chemicals Co., Ltd.). Briefly, the sections were oxidized with 1% periodic acid for 5 min at room temperature, rinsed thoroughly with distilled water and incubated with Schiff reagent for 10 min at room temperature. The sections were then washed under running tap water for 5 min, counterstained with hematoxylin for 30 sec, dehydrated through a graded ethanol series, cleared twice in xylene and mounted using a resin-based mounting medium. PAS-positive polysaccharide-rich structures were identified by their characteristic magenta staining. Stained sections were examined and photographed using an Eclipse 4,000 light microscope (Nikon Corporation) at x400 magnification. Formal taxonomic identification by a qualified botanist or taxonomist was not performed, and no voucher specimen was deposited in a recognized herbarium or institutional collection.

Cell culture. The human HSCC cell line FaDu was used as the *in vitro* model in the present study. Cells were maintained in EMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO_2 . Upon reaching $\sim 80\%$ confluence, cell monolayers were detached using 0.25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.) and routinely subcultured every 2-3 days to maintain exponential growth.

Detection of intracellular oxidative activity using flow cytometry. To quantitatively assess changes in intracellular

oxidative activity, FaDu cells were analyzed using the cell-permeable, ROS-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Cayman Chemical Company) according to established redox biology protocols (14,16). Briefly, FaDu cells were seeded in 6-cm culture dishes and allowed to adhere overnight at 37°C in a humidified atmosphere containing 5% CO₂, reaching ~80% confluence prior to treatment. Cells were subsequently treated with CI extract at concentrations of 1 or 2 mg/ml for 24 h. Vehicle-control cells were treated with an equivalent volume of absolute ethanol. For the ROS-scavenging control, a subset of cells was pre-incubated with 5 mM N-acetylcysteine (NAC; Sigma-Aldrich; Merck KGaA) for 1 h prior to CI treatment. Following the 24-h treatment period, cells were harvested using 0.5% Trypsin-EDTA without phenol red (cat. no. 15400054; Gibco; Thermo Fisher Scientific, Inc.) for 5 min at 37°C and transferred to centrifuge tubes. Cell pellets were washed twice with ice-cold PBS through centrifugation 300 x g for 5 min, resuspended and incubated with 10 µM DCFH-DA for 30 min at 37°C in the dark. Fluorescence signals were subsequently detected using a BD FACSLyric™ flow cytometer (BD Biosciences). Cellular debris was excluded through appropriate gating and a minimum of 10,000 events were acquired per sample. Intracellular oxidative activity was quantified according to the mean fluorescence intensity using FACSuite software (BD Biosciences).

Western blotting. The expression levels of key proteins involved in endoplasmic reticulum (ER) stress and cell survival pathways were evaluated using western blotting. Following 24 h of CI treatment, FaDu cells were harvested, washed with ice-cold PBS and lysed in RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) supplemented with 1% protease and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) on ice for 30 min. Total protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein (30 µg per lane) were separated using 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma; Merck KGaA). Nonspecific binding sites were blocked with 5% BSA in TBS containing 0.1% Tween 20 (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The membranes were subsequently incubated overnight at 4°C with primary antibodies against EIF2AK3 (cat. no. 24390-1-AP; 1:1,000; Proteintech Group, Inc.), Bcl-2 (cat. no. 26593-1-AP; 1:1,000; Proteintech Group, Inc.), phosphorylated (p)-Akt (cat. no. 4060; 1:1,000; Cell Signaling Technology, Inc.), total Akt (cat. no. 4691; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. AM4300; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.), with GAPDH used as the loading control. The membranes were washed three times with TBST for 10 min each and incubated with the appropriate HRP-conjugated secondary antibodies (Goat Anti-Mouse IgG cat. no. 115-035-003; and Goat Anti-Rabbit IgG; cat. no. 111-035-003; 1:5,000; Jackson ImmunoResearch, Inc.) for 1 h at room temperature. Following three additional washes with TBST, immunoreactive protein bands were visualized using an ECL detection kit (Revvity, Inc.) and captured using a ChemiDoc MP digital imaging system (Bio-Rad Laboratories, Inc.). Densitometric analysis was performed using ImageJ (Version 1.53; National Institutes of Health) and the relative

expression levels of target proteins were normalized to the corresponding GAPDH loading controls.

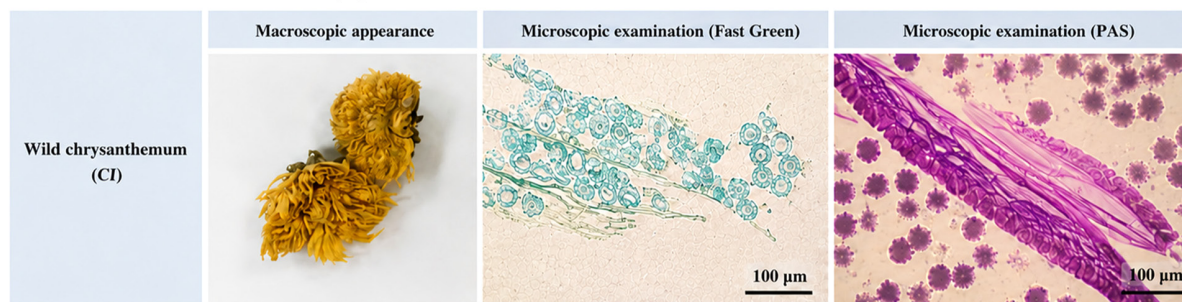
Cell viability assay (CCK-8). The cytotoxic activity of CI extract was evaluated using a CCK-8 assay (Sigma-Aldrich; Merck KGaA). To exclude potential cytotoxic effects of the solvent, cells in the control groups for all *in vitro* assays were treated with an equivalent volume of absolute ethanol, with a maximum final concentration of 2% (v/v), as the vehicle control. Preliminary experiments determined that this ethanol concentration did not induce marked cytotoxicity compared with untreated cells. FaDu cells were seeded into 96-well flat-bottom plates at a density of 6x10³ cells/well and incubated overnight to allow cell attachment. The culture medium was subsequently replaced with fresh medium containing the indicated concentrations of CI extract, and cells were incubated for an additional 48 or 72 h. At the indicated time points, 10 µl CCK-8 reagent was added directly to each well, followed by incubation for 1.5 h. Absorbance at 450 nm was measured using a microplate reader (BioTek; Agilent Technologies, Inc.). Cell viability was calculated relative to the vehicle control group, which was designated as 100%. IC₅₀ values were calculated through nonlinear regression analysis of concentration-response curves using GraphPad Prism (version, 10.0; Dotmatics).

Statistical analysis. All *in vitro* experiments were performed using three independent biological replicates (n=3). For the CCK-8 assay, each condition was analyzed in triplicate wells within each independent experiment. Comparisons between two groups were performed using an unpaired Student's t-test, while comparisons among multiple groups were performed using ANOVA followed by Tukey's multiple comparisons test. For CCK-8 experiments involving both concentration and time as independent variables, a two-way ANOVA followed by Tukey's post hoc test was used. Data are presented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

CI exhibits characteristic morphological and anatomical features and yields a crude extract. To characterize the morphological and anatomical features of the raw material, dried flower heads of CI were subjected to macroscopic and microscopic evaluation. Macroscopic examination revealed a capitulum structure, comprising yellow tubular central florets and outer ray florets, together with a distinct aromatic odour, consistent with the morphological features expected for the material supplied as CI (Fig. 1A). Histological cross-sections stained with Fast Green and PAS further demonstrated the anatomical architecture of the floral tissues. PAS staining delineated polysaccharide-rich cell walls and secretory glandular trichomes in magenta, whereas Fast Green provided contrasting visualization of cytoplasmic components in green. Collectively, these findings documented the morphological and anatomical characteristics of the plant material (Fig. 1A). Following morphological and histological assessment, 1,004.5 g pulverized flower heads was extracted with 95% ethanol. Following evaporation of the solvent under reduced

A Characterization of raw material (CI)



B Extraction process of CI



Figure 1. Characterization and crude extract preparation of CI. (A) Macroscopic examination and histological analysis using Fast Green and PAS staining demonstrated the morphological and anatomical features of the plant material. (B) A viscous, dark-brown crude extract was obtained following extraction with 95% ethanol and subsequent solvent removal through rotary evaporation and was used for all subsequent *in vitro* assays. PAS, Periodic Acid-Schiff; CI, *Chrysanthemum indicum*.

pressure, a dark brown, viscous crude extract was obtained (Fig. 1B). The resulting crude extract was aliquoted and stored for subsequent *in vitro* pharmacological and molecular experiments.

CI treatment increases intracellular oxidative activity in FaDu cells. To characterize the early cellular response to CI treatment, changes in intracellular oxidative activity were evaluated using the cell-permeable fluorogenic probe, DCFH-DA. Following cellular uptake, DCFH-DA is deacetylated through intracellular esterases and subsequently oxidized to the fluorescent compound DCF. Flow cytometric analysis revealed a notable concentration-dependent rightward shift in green fluorescence in FaDu cells treated with 1 or 2 mg/ml CI for 24 h (Fig. 2A). Quantitative analysis demonstrated that the relative mean fluorescence intensity was significantly higher in CI-treated cells compared with vehicle-treated controls (Fig. 2B), indicating increased intracellular oxidative activity following CI treatment ($P < 0.01$; Fig. 2).

To determine whether the CI-induced increase in DCF fluorescence was sensitive to antioxidant pretreatment, a rescue experiment was performed using NAC, an antioxidant and ROS scavenger. FaDu cells were pre-incubated with 5 mM NAC for 1 h before CI treatment. NAC pretreatment markedly attenuated the CI-induced increase in DCF fluorescence, reducing the fluorescence signal toward baseline levels (Fig. 2). Collectively, these findings support the involvement of increased intracellular oxidative activity in the cellular response to CI treatment.

CI treatment reduces EIF2AK3 expression and Akt activation. To further characterize the molecular alterations associated

with CI treatment, proteins involved in ER stress adaptation and cell survival were evaluated. FaDu cells were treated with 1 or 2 mg/ml CI for 24 h, followed by western blotting of whole-cell lysates. EIF2AK3, also known as PERK, exhibited a significant concentration-dependent reduction following CI treatment, as supported through densitometric semi-quantification normalized to GAPDH (compared with control; Fig. 3). The activation status of Akt signaling was subsequently evaluated. CI treatment significantly decreased p-Akt levels and the p-Akt/total Akt ratio in FaDu cells (compared with control; Fig. 3). These findings indicated that CI treatment is associated with reduced EIF2AK3 expression and decreased Akt activation in FaDu cells.

CI treatment decreases Bcl-2 protein levels. Given the observed alterations in EIF2AK3 expression and Akt activation, the effect of CI treatment on the expression of the anti-apoptotic protein Bcl-2 was subsequently investigated. Western blotting of FaDu cells treated with 1 or 2 mg/ml CI for 24 h revealed a notable concentration-dependent reduction in Bcl-2 protein levels compared with vehicle-treated controls (Fig. 4A). Densitometric analysis further determined the significant progressive reduction in Bcl-2 expression (Fig. 4B), indicating that CI treatment is associated with decreased expression of this anti-apoptotic protein.

CI decreases FaDu cell viability. To determine whether the observed molecular alterations were accompanied by changes in cell viability, FaDu cells were evaluated using a CCK-8 assay. Cells were exposed to increasing concentrations of CI, and cell viability was assessed after 48 and 72 h of treatment. CI treatment notably reduced FaDu cell viability in a

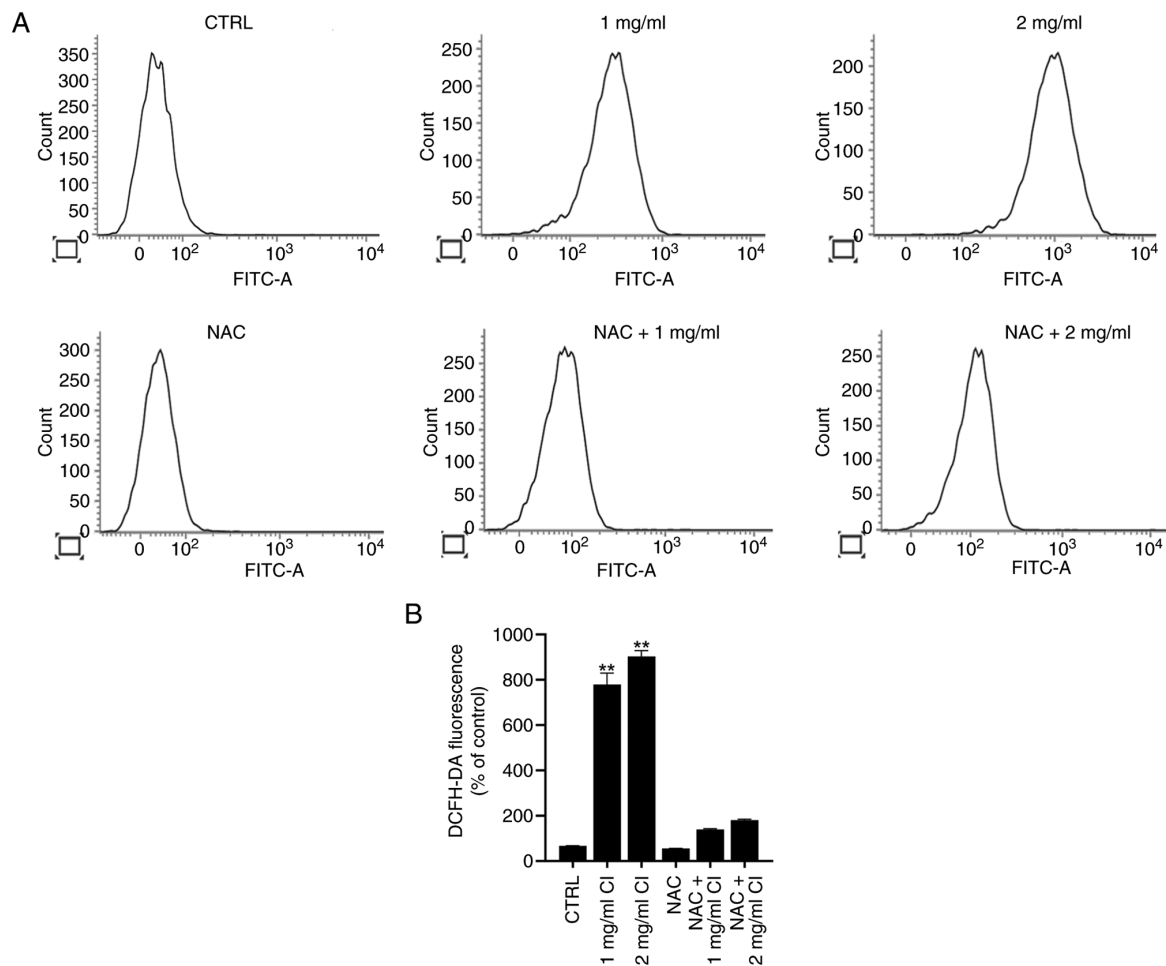


Figure 2. CI treatment increases intracellular oxidative activity in FaDu cells. Intracellular oxidative activity was quantified using the reactive oxygen species-sensitive fluorescent probe DCFH-DA through flow cytometry. (A) FaDu cells were treated with CI at 1 or 2 mg/ml for 24 h, resulting in a concentration-dependent increase in green fluorescence intensity compared with the vehicle control. In the rescue experiment, cells were pretreated with 5 mM NAC for 1 h before CI exposure. (B) Quantification of the relative mean fluorescence intensity. Data are presented as the mean $\pm$ SD of three independent biological replicates. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. ** $P < 0.01$ vs. vehicle control. CI, *Chrysanthemum indicum*; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; NAC, N-acetylcysteine; CTRL, control.

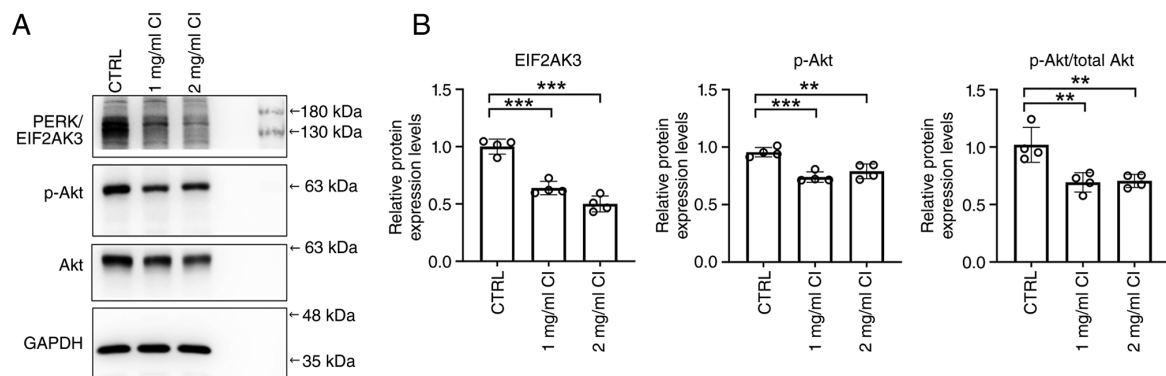


Figure 3. CI-mediated modulation of EIF2AK3 and Akt signaling in FaDu cells. (A) Western blot analysis of EIF2AK3, p-Akt and total Akt protein levels following treatment with CI at 1 or 2 mg/ml for 24 h. GAPDH was used as the loading control. (B) Densitometric semi-quantification of protein levels relative to GAPDH and the p-Akt/total Akt ratio. Data are presented as the mean $\pm$ SD of three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle control. CI, *Chrysanthemum indicum*; PERK, protein kinase R-like endoplasmic reticulum kinase; EIF2AK3, eukaryotic translation initiation factor 2 α kinase 3; p-, phosphorylated; CTRL, control.

concentration-dependent manner. Compared with the vehicle control, CI significantly reduced cell viability at 1 mg/ml ($P < 0.05$) and at 2 and 5 mg/ml ($P < 0.01$) at both 48 and 72 h.

The IC_{50} values of CI extract were 1.83 mg/ml at 48 h and 1.19 mg/ml at 72 h. The reduction in cell viability was also time dependent, with 72 h of treatment producing a greater decrease

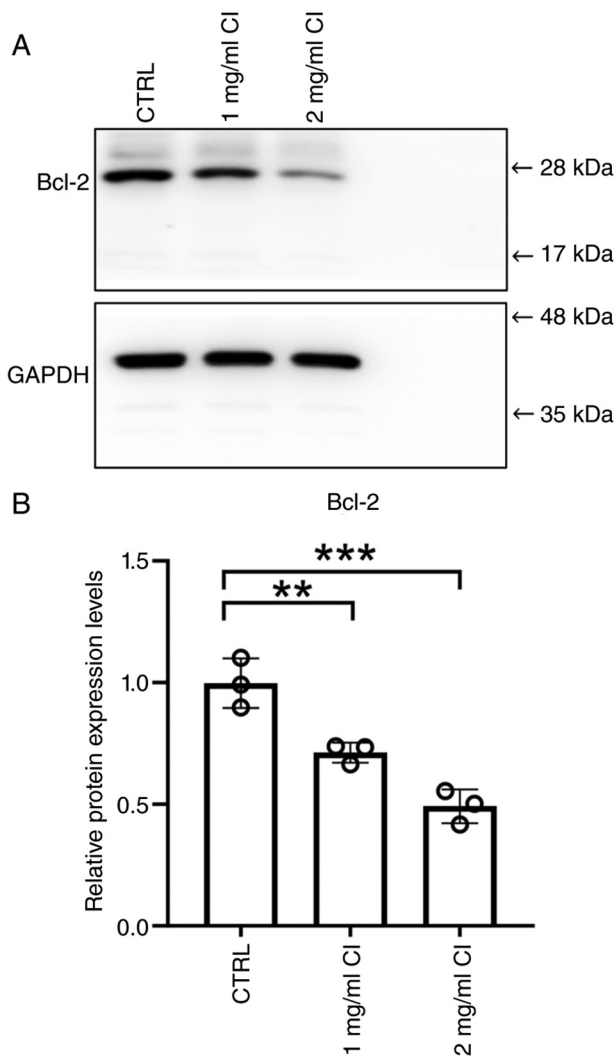


Figure 4. Concentration-dependent effect of CI on the anti-apoptotic protein Bcl-2. (A) Representative western blot analysis of Bcl-2 protein levels in FaDu cells following treatment with CI at 1 or 2 mg/ml for 24 h. (B) Densitometric analysis of Bcl-2 protein levels normalized to GAPDH, demonstrating a significant concentration-dependent reduction following CI treatment. Data are presented as the mean $\pm$ SD of three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle control. CI, *Chrysanthemum indicum*; CTRL, control.

than 48 h of treatment relative to the vehicle control, which was defined as 100% viability (Fig. 5). Collectively, these findings demonstrated that CI decreases FaDu cell viability in a concentration- and time-dependent manner and further support its potential cytotoxic activity against HSCC cells.

Discussion

The present study investigated the antitumor activity and underlying molecular mechanisms of crude CI extract in human HSCC cells. CI treatment was shown to reduce FaDu cell viability in a concentration- and time-dependent manner and was accompanied by increased intracellular oxidative activity. Notably, pretreatment with the antioxidant NAC markedly attenuated the CI-induced increase in DCFH-DA fluorescence, supporting the involvement of oxidative activity in the cellular response to CI treatment.

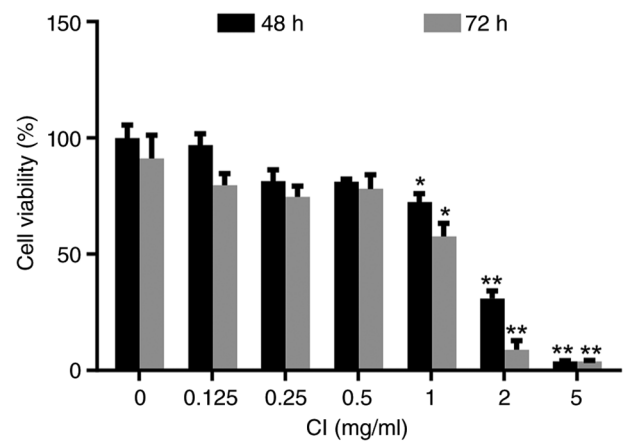


Figure 5. Effect of CI on FaDu cell viability. FaDu cells were treated with vehicle control or CI at 0.125, 0.25, 0.5, 1, 2 or 5 mg/ml for 48 or 72 h, and cell viability was assessed using a Cell Counting Kit-8 assay. Absorbance was measured at 450 nm and cell viability was expressed as a percentage relative to the vehicle control, which was designated as 100%. Data are presented as the mean $\pm$ SD of three independent experiments. Statistical significance was assessed using two-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$ and ** $P < 0.01$ vs. vehicle control at the corresponding time point. CI treatment reduced FaDu cell viability in a concentration- and time-dependent manner. CI, *Chrysanthemum indicum*.

The present study primarily focused on the human HSCC cell line FaDu. Despite evaluation across multiple cell lines perhaps providing a broader assessment of the antitumor activity of CI, FaDu cells were selected as the *in vitro* model because they exhibit characteristics relevant to aggressive HSCC (4,17). FaDu cells display an invasive phenotype and molecular heterogeneity that make them a useful model for investigating the biology of advanced HSCC (2,5,17). Clinically, hypopharyngeal cancer is associated with poor prognosis, in part because of its propensity for early lymphatic spread (2). Thus, the use of FaDu cells provides an appropriate initial model for evaluating the potential antitumor activity of CI. Despite this, validation in additional HSCC cell lines will be necessary to determine the generalizability of the present findings.

Molecular analyses demonstrated that decreased cell viability following CI treatment was accompanied by a significant concentration-dependent reduction in the anti-apoptotic protein Bcl-2 (18,19). Bcl-2 is a key regulator of mitochondrial apoptosis and contributes to the maintenance of mitochondrial outer membrane integrity (18,19). However, conventional assays directly assessing apoptosis, such as Annexin V/PI flow cytometry or terminal deoxynucleotidyl transferase dUTP nick-end labelling, were not performed in the present study. Therefore, although the reduction in Bcl-2 expression together with decreased cell viability is consistent with the potential involvement of apoptotic signaling, the present findings did not establish apoptosis as the mechanism of CI-induced cytotoxicity. Additional studies assessing apoptotic cell populations, caspase activation and other markers of mitochondrial apoptosis are required to demonstrate and quantify this process.

An additional notable finding was the concentration-dependent reduction in EIF2AK3 expression following CI treatment. EIF2AK3 is an important component of the unfolded protein response (UPR) and contributes to cellular adaptation under

conditions of ER and oxidative stress (20-22). Accordingly, the observed reduction in EIF2AK3 expression may reflect altered cellular stress adaptation following CI treatment. However, changes in total EIF2AK3 expression alone are insufficient to establish either inhibition or collapse of the UPR. Further investigation of EIF2AK3 activation and additional canonical UPR components, including inositol-requiring enzyme 1 α , activating transcription factor 6 and CCAAT/enhancer-binding protein homologous protein, will therefore be necessary to determine how CI affects ER stress signaling.

CI treatment also significantly decreased p-Akt levels and the p-Akt/total Akt ratio, consistent with reduced Akt activation. The PI3K/Akt pathway is frequently dysregulated in squamous cell carcinomas and contributes to tumor cell proliferation and survival (23,24). Despite this, the present data demonstrate an association between CI treatment and reduced Akt activation rather than a direct causal role for Akt signaling in CI-induced cytotoxicity. Functional experiments involving pharmacological or genetic modulation of Akt signaling are therefore required to establish its mechanistic contribution. Collectively, the reductions in EIF2AK3 and Bcl-2 expression and Akt activation, were associated with decreased FaDu cell viability, suggesting that CI treatment affects a number of stress- and survival-associated pathways (24,25).

A number of limitations within the present study should be acknowledged. First, the experiments were conducted exclusively using a single *in vitro* model, the FaDu cell line, limiting the generalizability of the findings to other HSCC models. FaDu represents a human papillomavirus (HPV)-negative hypopharyngeal carcinoma model, and HPV-negative head and neck cancers are generally associated with less favourable clinical outcomes than their HPV-positive counterparts (26). Future studies incorporating additional HPV-negative and HPV-positive models will be necessary to determine whether the effects of CI vary according to HPV status or tumor context. In addition, as a preliminary *in vitro* investigation, the present study lacks *in vivo* efficacy, toxicological and pharmacokinetic data. Therefore, the present findings should be interpreted as preliminary evidence of biological activity in FaDu cells and do not establish tumor selectivity, a therapeutic window, systemic safety or tolerability. Further evaluation using non-malignant cellular models and *in vivo* toxicological and pharmacokinetic studies will be required before translational conclusions can be drawn. In addition, formal taxonomic authentication of the plant material by a qualified botanist or taxonomist was not performed, and no voucher specimen was deposited. Although macroscopic and histological assessments were performed to characterize the plant material, these approaches alone do not provide definitive species-level authentication.

The absence of formal taxonomic verification and a voucher specimen limits retrospective traceability and independent confirmation of the botanical material and should therefore be considered when interpreting and reproducing the present findings. Furthermore, although the extraction procedure was controlled with respect to the amount of starting material, ethanol concentration, extraction duration, number of extraction cycles, evaporation temperature, extraction yield, moisture content and storage conditions, comprehensive chemical standardization of the crude extract was not performed. Specifically, chromatographic fingerprinting and

quantitative analysis of major constituents were not performed for the extract used in the present study. Consequently, chemical batch-to-batch consistency could not be established, which limits reproducibility, comparison with extracts prepared under different conditions and identification of the constituents responsible for the observed biological effects. Accordingly, the present findings should be interpreted as being specific to the crude extract prepared under the conditions described in the present study and should not be generalized to all CI preparations. Future studies should aim to incorporate comprehensive phytochemical profiling and quantitative characterization of major constituents to improve chemical standardization and batch-to-batch reproducibility. Finally, formal physicochemical solubility profiling of the crude CI extract was not performed. Therefore, parameters such as saturation solubility and the physicochemical behavior of the extract in culture medium remain undefined, which may affect the reproducibility and interpretation of the concentration-response findings. Formal solubility characterization should therefore be incorporated into future studies.

In conclusion, the present findings demonstrated that CI treatment increases intracellular oxidative activity and is accompanied by reduced EIF2AK3 and Bcl-2 expression, decreased Akt activation and reduced FaDu cell viability. The attenuation of CI-induced DCFH-DA fluorescence through NAC pretreatment further supports the involvement of oxidative activity in the cellular response to CI. Collectively, these findings may provide preliminary evidence supporting the potential antitumor activity of CI in HSCC cells and warrant further mechanistic investigation using additional cellular models and *in vivo* studies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CWL was responsible for conceptualization, methodology, investigation (plant material preparation and histological assessment), data curation, data analysis and writing the original draft. LCC contributed to conceptualization and methodology. TTL contributed to the investigation (cell culture, CI treatment and cell viability assays). HCL was responsible for conceptualization, methodology, investigation (western blotting, flow cytometric experiments and data acquisition), data curation, data analysis, writing the original draft and supervision. CWL and HCL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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