






RESEARCH ARTICLE

Synergistic effect of the combination of *Chrysanthemum cinerariifolium* (Trev.) and doxorubicin in inhibiting PI3K and Cyclin D in oral squamous cell carcinoma: *in vitro* study

[version 1; peer review: awaiting peer review]

Anik Listiyana ^{1,2}, Yuanita Lely Rachmawati³, Hani Susianti⁴,
Nurdiana Nurdiana ⁵, Hidayat Sujuti⁶, Roihatul Mutiah⁷,
Agustina Tri Endharti ^{2,8}

¹Department of Medical Education, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, East Java, 65144, Indonesia

²Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

³Department of Preventive and Public Health Dentistry, Faculty of Dentistry, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

⁴Department of Clinical Pathology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

⁵Department of Pharmacology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

⁶Department of Biochemistry and Biomolecular, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

⁷Department of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, East Java, 65144, Indonesia

⁸Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, 65145, Indonesia

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Abstract

Background: The most common type of lips and oral cavity cancer is oral squamous cell carcinoma (OSCC). Doxorubicin (DX) is commonly used as a chemotherapy drug, but its use is limited due to risk factors and drug resistance. *Chrysanthemum cinerariifolium* (Trev.) (CC) has potential as an anticancer agent. Combining the plant extract and chemotherapy drug might prevent OSCC proliferation by inhibiting PI3K and cyclin D protein. Therefore, the present study aimed to determine the synergistic effect of the combination of *C. cinerariifolium* (Trev.) and doxorubicin in inhibiting PI3K and Cyclin D protein.

Methods: Human oral squamous carcinoma cell lines SCC-9 were used in this study. A cytotoxicity assay was performed to obtain the IC₅₀ value of CC ethanol extract and DX on the SCC-9 cell line. Synergism evaluation of the combination CC and DX was analyzed using CompuSyn software. ELISA and the immunofluorescent assay were performed to determine the level of PI3K and cyclin D in the SCC-9 cell line after being treated with IC₅₀ value of CC, IC₅₀ value of DX and

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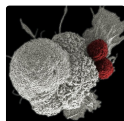
three combinations of CC and DX [7/8 IC₅₀ CC + 1/8 IC₅₀ DX (dose 1), 6/8 IC₅₀ CC + 2/8 IC₅₀ DX (dose 2), and 4/8 IC₅₀ CC + 4/8 IC₅₀ DX (dose 3)].

Results: CC stem ethanol extract and DX inhibited the proliferation of SCC-9 cell lines with the IC₅₀ value of 133.4 µg/mL and 288.3 nM, respectively. The combination of CC and DX at dose 2 (6/8 IC₅₀ CC + 2/8 IC₅₀ DX) exhibited a high decrease in PI3K and cyclin D expression.

Conclusions: The combination of *C. cinerariifolium* and doxorubicin synergistically declined OSCC proliferation by inhibiting PI3K and cyclin D expression.

Keywords

Chrysanthemum cinerariifolium (Trev.), Cyclin D, doxorubicin, IC50, OSCC, PI3K



This article is included in the **Oncology** gateway.

Corresponding author: Agustina Tri Endharti (tinapermana.fk@ub.ac.id)

Author roles: **Listiyana A:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Rachmawati YL:** Formal Analysis, Investigation, Resources, Writing – Review & Editing; **Susianti H:** Investigation, Methodology, Software, Writing – Review & Editing; **Nurdiana N:** Formal Analysis, Investigation, Resources, Writing – Review & Editing; **Sujuti H:** Data Curation, Investigation, Validation, Writing – Review & Editing; **Mutiah R:** Data Curation, Formal Analysis, Methodology, Writing – Review & Editing; **Endharti AT:** Conceptualization, Supervision, Validation, Visualization, Writing – Review & Editing

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Introduction

Cancer is a disease characterized by uncontrolled growth and abnormal cell spread, often resulting in death (American Cancer Society, 2020). According to Global Cancer Statistics, there were approximately 18.1 million new cases of cancer and 9.6 million deaths worldwide from cancer in 2018 (Ferlay *et al.*, 2019). According to the World Cancer Research Fund (2018), lips and oral cavities cancer are the most common type of cancer. More than 90% of cancers in the oral cavity are oral squamous cell carcinoma (OSCC) (World Cancer Research Fund, 2018).

The genetic and epigenetic mutations in oncogenes or tumor suppressor genes lead to cell cycle dysregulation, inhibition of growth suppressor factors, and resistance to apoptosis (Lopez and Lopez, 2020). Mutations in the PI3K/Akt pathway contribute to the development of OSCC (Aali *et al.*, 2020). The PI3K pathway involves cellular cell functions, including growth, angiogenesis and proliferation (Lakshminarayana *et al.*, 2018). Alteration of this pathway leads to cell cycle dysregulation and contributes to the development of OSCC (Li *et al.*, 2018). The activated PI3K pathway promotes cell proliferation and inhibits apoptosis. PI3K regulates the Akt protein, which then phosphorylates p21, causing the complex interaction of cyclins and cyclin-dependent kinases (CDK's) and resulting in cell proliferation (Kidacki *et al.*, 2015).

Cell proliferation also involves genes that play a role in cell cycle control (Saawarn *et al.*, 2012). The cell cycle is a process of regulation of cell proliferation with several stages, including S, G2, M and G1 phases. This process requires cyclin/CDK interactions (Jain, 2019). Cyclin D1 is a protein that is overexpressed to more than 50% in the incidence of cancer (Qie and Diehl, 2016). Overexpression of cyclin D1 causes a shortening of the G1 phase, which results in abnormal cell proliferation and additional genetic lesions (Abid and Merza, 2014). Poor prognosis of OSCC is characterised by the low cell differentiation associated with overexpression of cyclin D (Ramos-García *et al.*, 2019).

The rapid proliferation of OSCC causes most OSCC to be diagnosed at an advanced stage. Various treatments have been used to treat OSCC, but long-term survival is less than 50% (Kumar *et al.*, 2015). Various treatments of OSCC that are frequently used are surgery, radiotherapy or a combination of radiotherapy and surgery, and chemotherapy. One chemotherapy drug commonly used for OSCC is doxorubicin (DX). DX works by inhibiting topoisomerase II, causing the termination of the cell cycle's G2/M phase, which can subsequently induce apoptosis. However, chemotherapy drugs sometimes cause side effects, such as drug resistance (Mansoori *et al.*, 2017). Due to these side effects, the treatment of OSCC requires combination therapy (Dasari and Tchounwou, 2014).

Reducing the dose of DX is required to minimize the side effect of doxorubicin (Fan *et al.*, 2017). Combination chemotherapy can be applied in OSCC treatment to increase the therapeutic effect and reduce the side effects of chemotherapy drugs such as DX. The combined use of chemotherapy drugs and herbal plant compounds such as polyphenols have been shown to have low toxicity, which is particularly advantageous as it can reduce the dose of chemotherapy drugs (Mostafa *et al.*, 2020). *C. cinerariifolium* (Trev.) (CC) extract can inhibit the growth of the T47D breast cancer cell line by inhibiting the cell cycle at G0-G1 and S phases (Mutiah *et al.*, 2020). A previous study by Listiyana *et al.* (2019) revealed that the best cytotoxic activity against T47D cells was observed in CC stems. Therefore, the present study aimed to investigate the effect of the combination of ethanol extract of CC stems and DX in inhibiting PI3K and cyclin D, which are proteins that play a role in increasing cell proliferation in OSCC.

Methods

Plant collection and extraction

CC was obtained from Punten Village, Batu City, East Java and identified at UPT Materia Medica Batu, East Java, Indonesia (no: 074/153/102.20-A/2-22). CC were harvested and the stems cut. The stems were cleaned, dried in the sun, and sorted. The dried stem samples were ground to a powder and then added with 96% ethanol in a ratio of 1:20. The mixture was extracted using UAE (Ultrasonication Assisted Extraction) for 2 min with three replications. Next, the filtrate was evaporated using a rotary evaporator at 50°C to produce a crude extract and concentrated using an oven at 40°C.

Cell culture and treatment

The *in vitro* study was conducted at the Biomedical Central Laboratory, Universitas Brawijaya. Human oral squamous carcinoma cell lines SCC-9 were purchased from American Type Culture Collection/ATCC, Virginia (catalog number: CRL-1629). Cells were cultured in a complete medium that consisting of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate), supplemented with 90% of 400 ng/mL hydrocortisone and 10% fetal bovine serum.

Cytotoxicity assay

This study used five concentration series of CC stem extract (700, 350, 175, 87.5, 43.5 and 21.875 µg/mL) and DX (800, 400, 200, 100, 50 and 25 nM). SCC-9 cell line was maintained with a complete medium in 96-well plates and then

incubated for 24 h. After 24 h of incubation, the medium was removed and washed using Phosphate Buffered Saline (PBS). Then, each concentration of CC and DX was added into each well with three replications and incubated for 24 h. After 24 h, the medium was removed and washed using PBS, then added with Cell Counting Kit-8 (CCK-8) Reagent (Dojindo Laboratories, Japan). The absorbance of each sample was determined at 450 nm using a microplate reader. The IC₅₀ value of CC and DX was determined using GraphPad Prism 8 (Graphpad Software, La Jolla, Canada, USA).

Synergism evaluation of combination *C. cinerariifolium* (CC) and Doxorubicin (DX)

A combination dose test was carried out based on the IC₅₀ value of CC and DX with seven combinations including 7/8 IC₅₀ CC + 1/8 IC₅₀ DX, 6/8 IC₅₀ CC + 2/8 IC₅₀ DX, 5/8 IC₅₀ CC + 3/8 IC₅₀ DX, 4/8 IC₅₀ CC + 4/8 IC₅₀ DX, 3/8 IC₅₀ CC + 5/8 IC₅₀ DX, 2/8 IC₅₀ CC + 6/8 IC₅₀ DX, 1/8 IC₅₀ CC + 7/8 IC₅₀ DX.

SCC-9 cells were grown in 96-well plates and then incubated for 24 h. After 24 h, the medium was removed and washed using PBS. Each combination of CC and DX was added into each well with three replications and incubated for 24 h. The medium was then removed and washed using PBS. Cell Counting Kit-8 (CCK-8) Reagent (Dojindo Laboratories, Japan) was added to each well. The absorbance of each sample was determined at 450 nm using a microplate reader. CompuSyn software was used to evaluate the synergistic combination of CC and DX. The results from this software were combination index (CI) values. The interpretation of the CI value is <0.1 = Strong synergist, 0.1–0.3 = Powerful synergist, 0.3–0.7 = Synergist, 0.7–0.9 = Light synergist, 0.9–1.1 = Additives, 1.1–1.45 = Light antagonist, 1.45–3.3 = Antagonist, >3.3 = Powerful antagonist.

Measurement of PI3K levels and cyclin D expression

The combination dose for this test was based on the synergism evaluation of the CC and DX combination, which showed synergistic results. This study used six treatment groups, including: control cells without treatment, IC₅₀ value of CC, IC₅₀ value of DX and three combinations of CC and DX [7/8 IC₅₀ CC + 1/8 IC₅₀ DX (dose 1), 6/8 IC₅₀ CC + 2/8 IC₅₀ DX (dose 2), and 4/8 IC₅₀ CC + 4/8 IC₅₀ DX (dose 3)].

PI3K levels were measured using the enzyme-linked immunosorbent assay (ELISA). SCC-9 cells were grown in 24-well plates and then incubated for 24 h. After 24 h, the medium was removed and washed using PBS. Then, each treatment was treated to the cells with three replications and incubated for 24h. The medium was removed and washed using PBS. RIPA Lysis Buffer (RIPA Lysis Buffer-MB-030-0050, Rockland) was added and incubated at 2–8°C for 5 min. Cells were scraped rapidly and transferred to tubes on ice. Cells were centrifuged at 8,000 × g for 10 min at 4°C. The supernatant was then analyzed using the Human Phosphoinositide-3-kinase-interacting Protein 1, PIK3IP1 ELISA Kit (BT Lab, Cat No. E5870Hu, Shanghai Korain Biotech Co., Ltd, China) to measure PI3K levels in ng/mL.

The cyclin D expression was observed by the immunofluorescent assay. The medium was aspirated, incubated with 4% formaldehyde in PBS for 15 min at room temperature, and then rinsed three times with PBS. The first step for immunostaining was blocking the buffer for 60 min. During this step, cyclin D primary antibody (Cat No. bs-0623R, Bioss Antibodies Inc., USA) was prepared by diluting it with antibody dilution buffer, then aspirating the buffer solution. Cyclin D primary antibody was added and incubated for 24 h at 4°C. Then, the samples were rinsed three times with PBS for 5 min. Then, samples were added with fluorochrome-conjugated secondary antibody diluted in antibody dilution buffer and incubated for 1–2 h at room temperature in the dark. Samples were rinsed with PBS and then coated with Prolong Gold Antifade Reagent (#9071) or Prolong Gold Antifade Reagent with DAPI (#8961). Cyclin D expression was visualized using Olympus IX71 Fluorescent Microscope with 40x magnification, then photographed with Olympus Cell Sens software version 3.2. The pixel intensity in the cell nucleus reflecting the expression level was quantified using Image J software (Fiji) and presented as fluorescence (signal) intensity or integrated density (IntDen) value.

Statistical analysis

Data were reported as means ± standard deviation. Statistical significance was analyzed using one-way ANOVA (p < 0.05), then continued with the Post Hoc Tukey test.

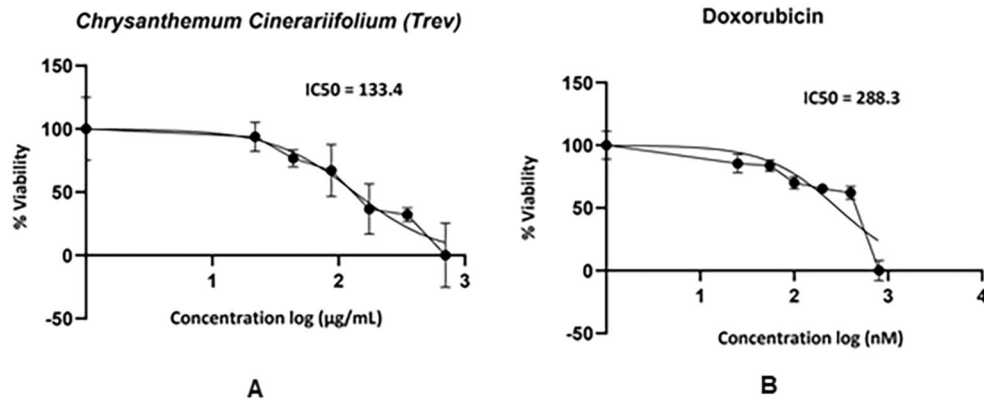
Results

Anticancer activity of CC and DX on SCC-9 cell line

The absorbance value of the SCC-9 cell line after being treated with CC and DX can be seen in [Table 1](#). The higher concentration exhibited a lower absorbance value. It was indicated that the higher concentration of CC and DX decreased the cell viability of SCC-9 cell lines. The results also showed that IC₅₀ values of CC and DX were 133.4 µg/mL and 288.3 nM, respectively ([Figure 1](#)).

Table 1. Absorbance value of SCC-9 cell line after treated with CC and DX.

CC concentration (µg/mL)	Absorbance value (Mean ± SD)	DX concentration (nM)	Absorbance value (Mean ± SD)
700	1.354 ± 0.063	800	1.284 ± 0.044
350	1.433 ± 0.014	400	1.481 ± 0.029
175	1.445 ± 0.049	200	1.492 ± 0.021
87.5	1.520 ± 0.051	100	1.506 ± 0.027
43.75	1.544 ± 0.017	50	1.550 ± 0.025
21.875	1.586 ± 0.029	25	1.555 ± 0.040

**Figure 1.** Log curve graph of CC and DX concentration to cell viability percentage SCC-9 cell line (A. *C. cinerariifolium* (Trev.), B. Doxorubicin).**Table 2.** Synergism evaluation results of CC and DX combination using CompuSyn Software.

Combination dose	CI value	Interpretation
7/8 IC ₅₀ CC + 1/8 IC ₅₀ DX	0.694	Synergist
6/8 IC ₅₀ CC + 2/8 IC ₅₀ DX	0.634	Synergist
5/8 IC ₅₀ CC + 3/8 IC ₅₀ DX	1.004	Nearly additive
4/8 IC ₅₀ CC + 4/8 IC ₅₀ DX	0.698	Synergist
3/8 IC ₅₀ CC + 5/8 IC ₅₀ DX	1.285	Moderate antagonism
2/8 IC ₅₀ CC + 6/8 IC ₅₀ DX	1.948	Antagonist
1/8 IC ₅₀ CC + 7/8 IC ₅₀ DX	2.479	Antagonist

Synergism evaluation of the CC and DX combination, obtained from CompuSyn software, is presented in Table 2. From the seven combinations of doses analyzed, only three combinations showed synergistic effects, including 7/8 IC₅₀ CC + 1/8 IC₅₀ DX, 6/8 IC₅₀ CC + 2/8 IC₅₀ DX, and 4/8 IC₅₀ CC + 4/8 IC₅₀ DX with CI values respectively 0.694, 0.634 and 0.698. CompuSyn analysis also showed that the combination of CC and DX had a dose reduction index (DRI) > 1, indicating a mutual strengthening effect.

PI3K levels in the SCC-9 cell line after being treated with CC and DX combination

The results revealed that the SCC-9 cell line without treatment (control group) had the highest levels of PI3K (4.483 ± 0.59 ng/mL) (Figure 2). PI3K levels decreased significantly ($p < 0.05$) in all treatment groups. Interestingly, dose 2 showed the lowest levels of PI3K compared to all treatment groups, with PI3K levels of 0.715 ± 0.22 ng/mL. PI3K levels in the CC and DX combination group significantly decreased compared to the single CC and DX treatment group. Doses 1, 2 and 3 all were significantly different to the IC₅₀ DX group ($p = 0.001$, 0.000 and 0.002, respectively), while when compared to the IC₅₀ CC group, only doses 1 and 2 were significantly different ($p = 0.001$ and 0.000, respectively). From these results, CC and DX could reduce PI3k levels in single and combined treatments.

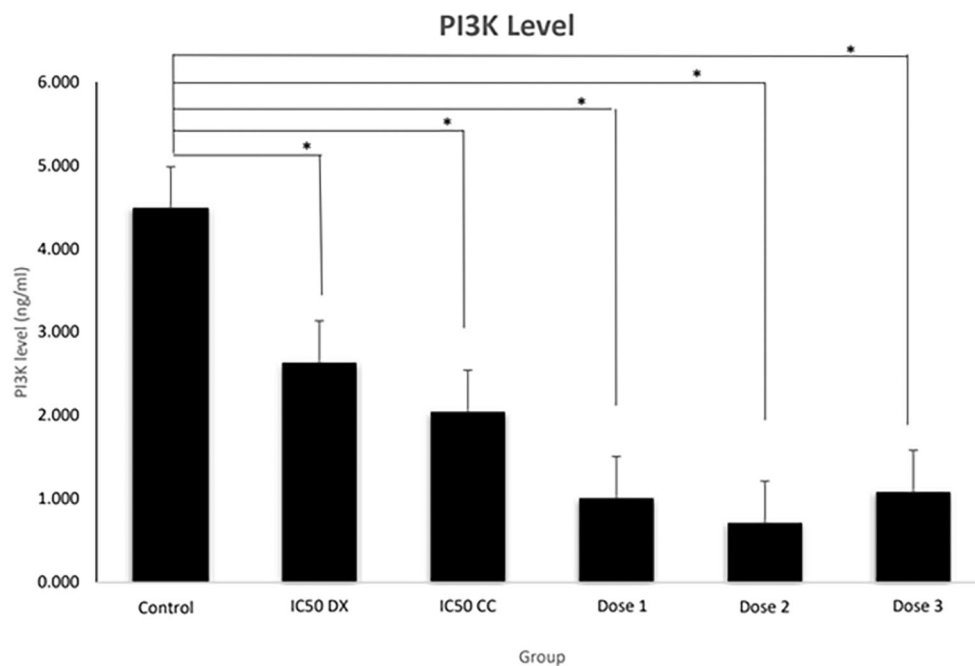


Figure 2. PI3K levels in the SCC-9 cell line after 24 h treatment with combination of CC and DX. PI3K levels (in ng/ml) were measured by ELISA. Control = medium + SCC-9 (without treatment), IC₅₀ DX = medium + SCC-9 + IC₅₀DX. IC₅₀ CC = medium + SCC-9 + IC₅₀ CC, Dose 1 = medium + SCC-9 + (7/8 IC₅₀ CC + 1/8 IC₅₀ DX), Dose 3 = medium + SCC-9 + (6/8 IC₅₀ CC + 2/8 IC₅₀ DX), Dose 3 = medium + SCC-9 + (4/8 IC₅₀ CC + 4/8 IC₅₀ DX). Data are expressed as mean ± SD, *p < 0.05.

Cyclin D expression in SCC-9 cell line after being treated with CC and DX combination

Cyclin D expression was observed from pixel intensity in the cell nuclei of the SCC-9 cells and presented as fluorescence (signal) intensity or integrated density (IntDen) value (Figure 3A). The results showed that CC and DX significantly decreased cyclin D expression in both single and combination treatments. The control group has the highest IntDen value, indicating that the control group expressed the highest Cyclin D. Cyclin D expression significantly declined ($p < 0.05$) in all treatment groups except the IC₅₀ of DX. The lowest cyclin D expression was observed in dose 2 (Figure 3B).

Discussion

DX is a chemotherapeutic agent that is widely used in cancer treatment, but this drug produces toxicity and drug resistance. In this study, DX, in combination with herbal plants, minimized the side effects and increased the therapeutic effect. Based on Table 1, the higher concentration of DX and CC caused a decrease in the cell viability of SCC-9 cells. El-Hamid *et al.* (2019) showed that DX could increase caspase-3 levels, thereby increasing cell apoptosis in the OSCC cell line. Several research studies also proved that CC could be used as an anticancer agent. *C. cinerariifolium* extract contained flavonoids and terpenoids (Mutiah *et al.*, 2020). Flavonoids could suppress the proliferation of OSCC by stopping the cell cycle and inducing apoptosis (Listiyana *et al.*, 2023a). Terpenoid compounds have anticancer activity in breast cancer (Bishayee *et al.*, 2011). Listiyana *et al.* (2019) revealed that CC has a cytotoxic activity on T47D breast cancer cells.

The present study showed that the IC₅₀ value of CC stem extract on the SCC-9 cell line was 133.4 µg/mL. Costa *et al.* (2017) stated that the strong anticancer activity of the extract is indicated by an IC₅₀ value <500 µg/mL and weak anticancer activity is indicated by an IC₅₀ value >500 µg/mL. The stem extract of CC and DX has strong anticancer activity on the SCC-9 cell line due to IC₅₀ value <500 µg/mL. The IC₅₀ value of DX is close to Abdolmohammadi *et al.* (2008) research, which stated that the IC₅₀ value of doxorubicin on T47D cells was 250 nM.

An increase in PI3K and cyclin D can cause excessive cell proliferation of OSCC. Figure 2 showed that untreated SCC-9 cell lines had the highest PI3K levels. Excessive cell proliferation in OSCC may also be due to gene mutations that encode various components of signalling pathways in proliferation, such as PI3K and cyclin D. In OSCC, mutations and amplification of the PI3K gene occur, especially at advanced stages (Kozaki *et al.*, 2006). Ferreira *et al.* (2017) found that PI3K expression was seen in >90% of OSCC patients, and there was an increase in the gingival tissue, hard palate, and

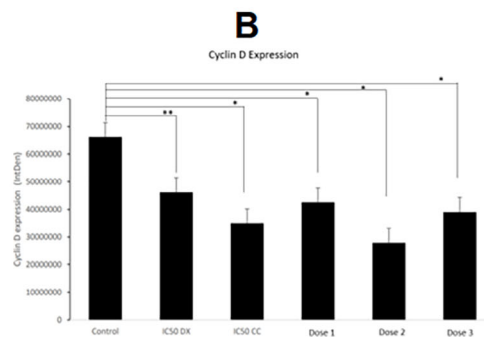
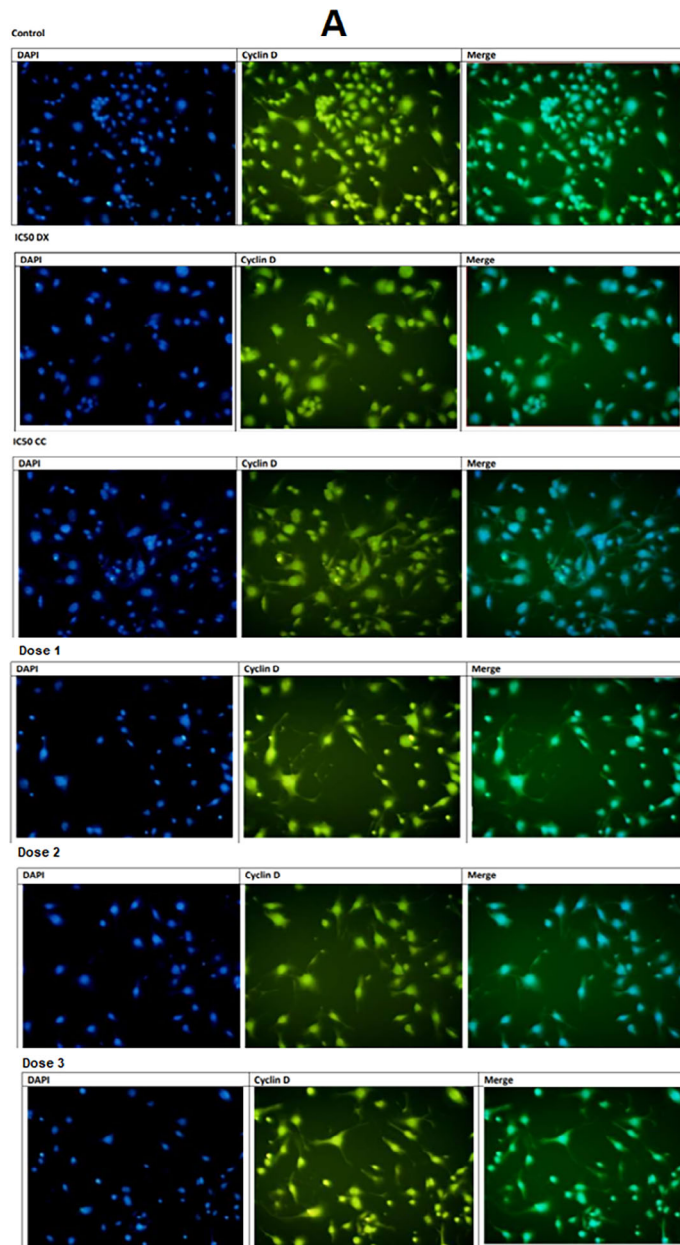


Figure 3. The expression of Cyclin D in the SCC-9 cell line after 24 h treatment with the combination of CC and DX. A) The fluorescence image was obtained from the fluorescent microscope. Cyclin D was expressed in the nucleus. Blue indicates cell nuclei with DAPI staining, and yellow indicates Cyclin D expression with FITC. The expression of Cyclin D is indicated by pixel intensity in the cell nucleus and fluorescence (signal) intensity or integrated density (IntDen) value. B) The graph shows the average Cyclin D expression. Control = medium + SCC-9 (without treatment), IC₅₀ DX = medium + SCC-9 + IC₅₀DX. IC₅₀ CC = medium + SCC-9 + IC₅₀ CC, Dose 1 = medium + SCC-9 + (7/8 IC₅₀ CC + 1/8 IC₅₀ DX), Dose 3 = medium + SCC-9 + (6/8 IC₅₀ CC + 2/8 IC₅₀ DX), Dose 3 = medium + SCC-9 + (4/8 IC₅₀ CC + 4/8 IC₅₀ DX). Data are expressed as mean±SD, *p < 0.05.

alveolar ridge by immunohistochemistry methods. The untreated SCC-9 cell line expresses the highest cyclin D. Excessive expression of cyclin D1 causes a shortening of the G1 phase, resulting in abnormal cell proliferation (Saawarn *et al.*, 2012). The poor prognosis in OSSC is markedly associated with low cell differentiation and overexpression of cyclin D (Ramos-García *et al.*, 2019).

PI3K levels decreased significantly in all treatment groups, indicating that the ethanol extract of CC stem and DX could inhibit PI3K expression. PI3K levels at the combined dose significantly decreased compared to the CC and DX single treatment group. CC significantly reduced cyclin D expression in a single treatment and combined with DX. Interestingly, the lowest PI3K and cyclin D expression levels were observed in dose 2. Furthermore, the combination in dose 2 had the best synergistic value (CI=0.634) compared to doses 1 and 3, based on CompuSyn analysis (Table 2). Therefore, it can be concluded that dose 2 is the best combination dose for inhibiting the proliferation of the SCC-9 cells.

CC and DX have the same mechanisms of action in inhibiting cell proliferation, especially in cell cycle inhibition. CC inhibits the G0-G1 and S phases of T47D cells (Mutiah *et al.*, 2020), whereas DX cause G2/M phase termination in T47D cells (Abdolmohammadi *et al.*, 2008). Dose 2 was a combination dose with a lower IC50 percentage of doxorubicin than CC. This study revealed that the combination of CC and DX could inhibit OSCC proliferation through the inhibition of PI3K and cyclin D. The use of the combination of CC and DX which have a synergistic mechanism of action, is expected to reduce the dose DX needed in OSCC therapy.

Conclusions

C stem ethanol extract and DX inhibited SCC-9 cells with IC₅₀ values of 133.4 µg/mL and 288.3 nM, respectively. The combination of CC and DX for dose 2 (6/8 IC₅₀ CC + 2/8 IC₅₀ DX) exhibited a high decrease in PI3K and cyclin D expression in SCC-9 cells. Therefore, the combination of CC and DX synergistically declined OSCC proliferation by inhibiting PI3K and cyclin D expression.

Ethical considerations

This research received ethical approval from the Health Research Ethics Commission (KEPK) of the Faculty of Medicine and Health Sciences, State University of Maulana Malik Ibrahim Islamic Malang, on April 21, 2022, with numbers 091/EC/KEPK-FKIK/2022.

Data availability

Figshare: Synergistic effect of the combination of Chrysanthemum cinerariifolium (Trev.) and doxorubicin in inhibiting PI3K and Cyclin D in oral squamous cell carcinoma in vitro study, <https://doi.org/10.6084/m9.figshare.22584580.v1> (Listiyana *et al.*, 2023b).

This project contains the following underlying data:

- Synergistic effect of the combination of Chrysanthemum cinerariifolium (Trev.) and doxorubicin in inhibiting PI3K and Cyclin D in oral squamous cell carcinoma in vitro study.xlsx

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

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