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Original Article

Apium graveolens leaf ethanolic extract triggers apoptosis in human tongue cancer cells via caspase-3 and poly(ADP-ribose) polymerase pathways: An in vitro study

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Abstract

Recent advances in cancer treatment have focused on developing alternative therapies with reduced adverse effects. Chemoprevention using natural products derived from plants has gained significant attention. *Apium graveolens* has demonstrated anticancer properties against various cancer cell types, suggesting its potential efficacy against tongue cancer cells. The aim of this study was to evaluate the cytotoxic effects and mechanisms of action of *Apium graveolens* leaf ethanolic extract (AGLEE) on the HSC-3 tongue cancer cell line. The leaves were processed and extracted with 70% ethanol to obtain an ethanolic extract. HSC-3 cells were cultured, subjected to starvation, and pre-treated with or without Z-DEVD-FMK, a caspase-3 inhibitor. Subsequently, the cells were treated with or without doxorubicin or varying concentrations of AGLEE. To assess cell viability and apoptosis, MTT and sub-G1 assays were performed. Additionally, treated HSC-3 cells were collected, lysed, and analyzed for levels of cleaved-caspase-3 and cleaved-poly (ADP-ribose) polymerase (cleaved-PARP) using ELISA. The inhibitory concentration (IC_{50}) value of AGLEE for reducing viable HSC-3 cells was determined to be 48.29 µg/mL. AGLEE significantly decreased HSC-3 cell viability and increased the percentage of apoptotic cells. It exhibited a concentration-dependent reduction in cell viability and an increase in apoptosis. Furthermore, the extract elevated the levels of cleaved-caspase-3 and cleaved-PARP in HSC-3 cells. Pre-treatment with Z-DEVD-FMK reduced the levels of cleaved-caspase-3 and cleaved-PARP induced by AGLEE. Taken together, AGLEE could be proposed as a potential natural therapeutic agent by inducing apoptosis through the caspase-3/PARP pathway in tongue cancer cells.

Keywords: *Apium graveolens*, HSC-3 cells, apoptosis, caspase-3, PARP

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Introduction

Oral squamous cell carcinoma (OSCC) is the most common oral cavity cancer, which primarily affects the tongue, floor of the mouth, and lower lip [1-3]. This malignancy is associated with high recurrence and metastasis rates, leading to poor prognosis [4]. Epidemiological data shows that OSCC accounts for approximately 3% of all cancers worldwide, with over 350,000 new cases and 177,000 deaths annually, highlighting the significant burden of this disease [5]. Standard

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treatment involves surgery, chemotherapy, and radiotherapy [1]. However, these modalities frequently induce nonspecific cell death, leading to significant adverse effects such as nausea, immunosuppression, and damage to healthy surrounding tissues [4].

Recent cancer treatment advancements emphasize alternative therapies with fewer adverse effects [6]. Plant-derived chemoprevention has gained attention [7], with species such as *Brucea javanica* and *Curcuma xanthorrhiza* recognized for their anticancer properties due to bioactive compounds such as flavonoids, polyphenols, and terpenes [8,9]. One of the key mechanisms involves apoptosis induction, activating components such as caspase-3 [7,10]. Inducing apoptosis in OSCC cells can selectively target cancerous cells for programmed cell death without harming surrounding healthy tissues, which is a key advantage over necrosis or other forms of cell death. However, OSCC cells are often resistant to apoptosis, making caspase-3 a significant therapeutic target [11].

Apium graveolens, a species in the Apiaceae family, is widely used in traditional medicine to treat conditions such as hypertension, asthma, diabetes, hepatitis, bronchitis, and visceral spasm [12]. Experimental studies have shown that *A. graveolens* possesses antioxidant, anti-inflammatory [13], antihypercholesterolemic [14], hepatoprotective [15], and antimicrobial properties [10]. Extracts derived from the whole plant or specific parts of *A. graveolens* have demonstrated anticancer activity by inducing apoptosis in various cancer types, including prostate cancer (lymph node carcinoma of the prostate (LNCaP)) cell line [16], Dalton's lymphoma ascites [17], and stomach cancer (BGC-823 cell line) [18]. The antioxidant properties of *A. graveolens* help reduce oxidative stress [19], which is linked to cancer initiation and progression, while its anti-inflammatory effects may inhibit tumor growth [20].

Despite these promising findings, the cytotoxicity and apoptotic effects of *A. graveolens* have not been investigated in human tongue cancer cell lines. Therefore, the aim of this study was to evaluate the cytotoxic effects of *A. graveolens* leaf ethanolic extract (AGLEE) on the human squamous cell carcinoma (HSC)-3 tongue cancer cells. Cell viability and apoptosis were assessed as key parameters for evaluating the anticancer potential of the extract. Cell viability was measured to determine the impact of the extract on the proliferation of cancer cells, while apoptosis assays were conducted to explore the mechanisms of cell death induced by the extract. To further investigate these mechanisms, this study focused on caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage. Caspase-3 is a central effector in the apoptotic pathway, playing a crucial role in the execution phase of apoptosis, while PARP is involved in DNA repair and is cleaved during apoptosis, marking the irreversible transition to cell death [21].

Additionally, varying concentrations of the extract were used to assess the dose-response relationship, with higher concentrations expected to exert stronger effects. A 24-hour treatment period was chosen, as it is a commonly used duration in similar studies to assess cellular responses such as viability and apoptosis, providing a standard context for comparison. It is hypothesized that AGLEE induces apoptosis in HSC-3 cells through activation of the caspase-3 and PARP pathways.

Methods

Study design and setting

This experimental laboratory study was conducted at the Prodia Stem Cell Laboratory, Jakarta, Indonesia, between August 2021 and August 2023. The aim of this study was to evaluate the cytotoxic effects of AGLEE on HSC-3 tongue cancer cells. The study began with the preparation of AGLEE and HSC-3 cell culture. The cells were exposed with three doses of AGLEE (1, 10, and 100 µg/mL) or doxorubicin (positive control) for 24 hours after which the cell viability, apoptosis, and specific apoptotic markers were then assessed. The measurement of cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, apoptosis was assessed via sub-G1 assay, and cleaved-caspase-3 and cleaved-PARP levels were quantified using enzyme-linked immunosorbent assay (ELISA).

Sample size, allocation, and randomization

The sample size for this study was determined using the Federer formula for in vitro experiment. This study involved five groups: untreated, positive control (doxorubicin), and three

concentrations of AGLEE (1, 10, and 100 µg/mL). Therefore, a minimum of six replicates per treatment group was required. Accordingly, sextuplicate were used for the MTT and sub-G1 assays, while triplicate was used for the ELISA assays.

Allocation of treatments was conducted under standardized experimental conditions for all groups, including nutrient deprivation and incubation times. Randomization was not applied in this study, as the experimental design involved the use of an in vitro cell line model under controlled laboratory conditions, thereby minimizing environmental variability.

Apium graveolens leaf ethanolic extract (AGLEE) preparation

A. graveolens was sourced from the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Bogor, Indonesia, and authenticated by a specialist botanist at the Research Center for Biology, National Research and Innovation Agency, Jakarta, Indonesia (Specimen identification number: B-478/V/DI.05.07/10/2021). Young leaves were selected as they are known to contain higher concentrations of bioactive compounds compared to mature leaves, which may influence the phytochemical profile and biological activity of the extract, particularly in anti-cancer assays. Leaves of *A. graveolens* were processed at IMACRI using the maceration extraction method, as previously described [22]. Briefly, *A. graveolens* leaves were cut into small pieces, dried, and soaked in 70% ethanol for 24 hours. The extract was then filtered and concentrated using a rotary evaporator, after which the AGLEE was stored at 4°C.

HSC-3 cell culture

HSC-3 cells were cultured using a previously reported method [23]. Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, Missouri, USA), supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin, and 10% fetal bovine serum, was utilized for cell culture. The cells were maintained in an incubator at 37°C with 5% CO_2. Upon reaching 80% confluency, the cells were detached using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, Missouri, USA) and subsequently subcultured.

MTT assay

The MTT assay was performed to quantitatively assess viable cell counts, following previously outlined methods [23]. HSC-3 cells (5×10^3 cells) were transferred into a 96-well plate, subjected to 12 hours of nutrient deprivation to synchronize them in a quiescent state, allowing for a more consistent response to AGLEE and doxorubicin treatment. The cells were then treated with or without 1 µM doxorubicin (Dankos Farma, Jakarta, Indonesia) as a positive control [23] or with various concentrations of AGLEE (1, 10, and 100 µg/mL) for 24 hours. A 24-hour time point was selected based on its common use for evaluating the short-term cytotoxic and apoptotic effects of chemotherapeutic agents on cancer cells [22]. Doxorubicin was chosen as the positive control due to its well-established role as a chemotherapeutic agent in OSCC and other cancer types. It is widely recognized for its efficacy in inducing cancer cell death, particularly through apoptosis, and has been extensively validated [24]. Following treatment, 100 µL of MTT solution (Sigma-Aldrich, St. Louis, Missouri, USA) was added to each well, and the cells were incubated for 4 hours. Subsequently, the culture medium was removed, and 100 µL of dimethyl sulfoxide was introduced to dissolve the formed formazan crystals. The absorbance of the samples was measured at OD_{570} nm using a spectrophotometer (Bio-Rad, Hercules, California, USA).

Sub-G1 assay

Sub-G1 assay was conducted to evaluate the cytotoxic effects of AGLEE on HSC-3 cells by measuring the percentage of apoptotic cells, following previously established methods with slight modifications [25,26]. A total of 5×10^3 HSC-3 cells were seeded in a 12-well plate, subjected to 12 hours of nutrient deprivation, and treated with or without 1 µM doxorubicin or three concentrations of AGLEE (1, 10, and 100 µg/mL) for 24 hours. Following treatment, the cells were harvested and resuspended in 450 µL of a hypotonic fluorochrome solution containing 50 µg/mL propidium iodide (Sigma-Aldrich, St. Louis, Missouri, USA), 0.1% sodium citrate (Wako, Osaka, Japan), and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA). This suspension was incubated for 4 hours at room temperature in the dark. The fluorescence of the HSC-3 cell nuclei

was analyzed using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) at a rate of 400 events per second.

Cleaved-caspase-3 and cleaved-PARP ELISA

HSC-3 cells (5×10^5) were plated in a 60 mm dish, subjected to 12 hours of nutrient deprivation, and pre-treated with or without 100 µM Z-DEVD-FMK (R&D Systems, Minneapolis, Minnesota, USA) for 2 hours. Z-DEVD-FMK is a well-established, potent inhibitor of caspase-3, commonly used in apoptosis assays to specifically block caspase-3 activation [27]. This inhibitor was chosen based on previous studies and product datasheet recommendations, which have demonstrated its effectiveness in inhibiting caspase-3 activity and facilitating the evaluation of caspase-3 involvement in apoptotic processes. Given the pivotal role of caspase-3 in apoptosis and the well-documented involvement of PARP in DNA repair and cell death, especially in the downstream of apoptotic signaling pathway, these markers were chosen to investigate the mechanisms of apoptosis induced by the treatments. The initial findings in HSC-3 cells showed evidence of apoptosis, which further supported the selection of these markers for this study [21]. The cells were then treated with or without 1 µM doxorubicin or AGLEE (1, 10, and 100 µg/mL) for 6 or 12 hours. Following treatment, the cells were harvested and washed twice with phosphate-buffered saline. After discarding the supernatant, the cell pellet was collected. The cells were lysed on ice for 30 minutes using Invitrogen Cell Extraction Buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA), supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF), with intermittent vortexing every 10 minutes. The cell extract was then transferred to microcentrifuge tubes and centrifuged for 10 mins at 13,000 rpm at 4°C to obtain the cell lysate. The HSC-3 cell lysate was subsequently used for ELISA to measure cleaved-caspase-3 and cleaved PARP levels, utilizing the Invitrogen Human Caspase-3 (active) ELISA Kit and the Invitrogen Cleaved PARP (214/215) ELISA Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA), in accordance with the manufacturer's instructions. The absorbance of the samples was measured at 450 nm, with sensitivities of the ELISA kits being 0.033 ng/mL for caspase-3 and <0.062 ng/mL for cleaved PARP.

Study variables

The independent variable in this study was the concentration of AGLEE, with three concentrations, 1, 10, and 100 µg/mL. These concentrations were chosen to cover a wide range of potential biological effects. The dependent variables included cell viability, apoptosis, and levels of cleaved caspase-3 and cleaved PARP. Cell viability was defined as the proportion of HSC-3 cells that remained alive after AGLEE treatment. This was measured using the MTT assay, which quantifies mitochondrial activity, with absorbance readings at 570 nm was directly proportional to the number of viable cells. The cutoff value for cell viability was the half-maximal inhibitory concentration (IC_{50}). Apoptosis was defined as the percentage of cells in the sub-G1 phase, indicating DNA fragmentation, and was measured by flow cytometry. The levels of cleaved caspase-3 and cleaved PARP, key markers of apoptosis, were measured using ELISA, with absorbance values at 450 nm indicating the concentration of these proteins in cell lysates.

Statistical analysis

Statistical significance was determined using one-way ANOVA followed by Tukey's HSD test for multiple comparisons. For non-parametric data, the Mann-Whitney test was used. A p-value <0.05 was considered statistically significant. The SPSS Statistic for Macintosh, version 23.0 (IBM, Armonk, USA) was used for all analyses.

Results

Effect of *Apium graveolens* leaf ethanolic extract (AGLEE) on viability of HSC-3 cells

The number of viable HSC-3 cells in the doxorubicin-treated group was significantly lower (p=0.004) than that in the untreated group (**Figure 1A** and **Table 1**). In the AGLEE-treated groups, a clear concentration-dependent reduction (Kruskal-Wallis, p<0.001) in cell viability was observed. The 1 µg/mL AGLEE-treated group showed a non-significant reduction (p=0.109) in

cell viability compared with the untreated group, while the 10 µg/mL AGLEE-treated group exhibited a significant decrease in cell viability compared with both in the 1 µg/mL AGLEE-treated group (p=0.004) and the untreated group (p=0.004). Furthermore, the 100 µg/mL AGLEE-treated group showed a significant reduction in cell viability compared to 1 µg/mL AGLEE-treated group (p=0.004) and the untreated group (p=0.004). The results indicated that AGLEE reduced HSC-3 cell viability in a concentration-dependent manner, with progressively higher concentrations resulting in lower cell viability. The IC_{50} value was 48.29±2.4 µg/mL, as determined from sextuplicate experiments (**Figure 1B**), suggested that AGLEE exhibited moderate inhibitory effects [28].

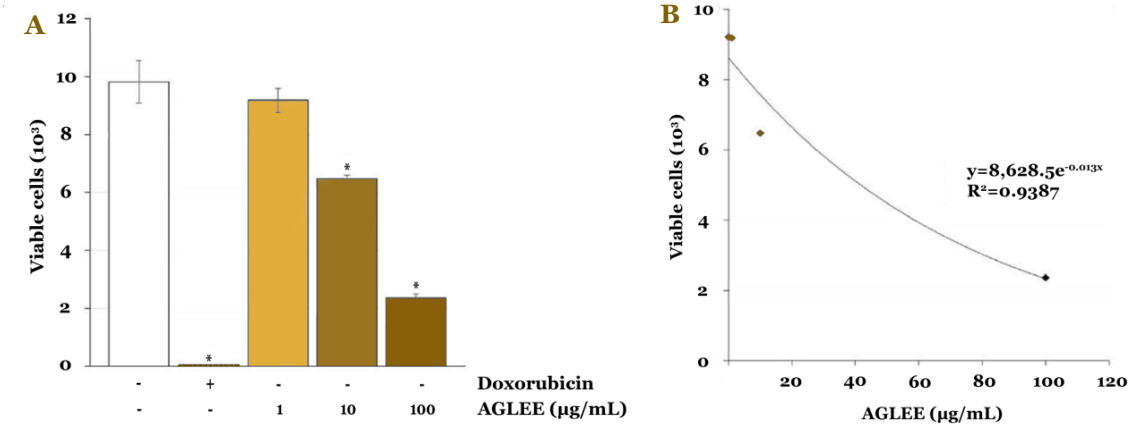

Figure 1. *Apium graveolens* leaf ethanolic extract (AGLEE) reduced the numbers of viable HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, and exposed to either doxorubicin or different concentrations of AGLEE for 24 hours as specified. Subsequently, MTT assay was performed on HSC-3 cells. (A) The number of viable HSC-3 cells. (B) Trendline of IC_{50} value. The results are presented in mean ± standard deviation (n=6). Mann-Whitney test, * statistically significant at p<0.05 compared with the untreated group.

Table 1. Detailed comparisons of *Apium graveolens* leaf ethanolic extract (AGLEE) effects on viability of HSC-3 cells

Group	Viable cells	p-value	
Untreated	9822.02±730.03	vs Doxorubicin	0.004*
		vs 1 µg/mL AGLEE	1.000
		vs 10 µg/mL AGLEE	0.004*
		vs 100 µg/mL AGLEE	0.004*
Doxorubicin	56.42±11.12	vs 1 µg/mL AGLEE	0.004*
		vs 10 µg/mL AGLEE	0.054
		vs 100 µg/mL AGLEE	0.004*
1 µg/mL AGLEE	9185.73±410.51	vs 10 µg/mL AGLEE	0.004*
		vs 100 µg/mL AGLEE	0.004*
10 µg/mL AGLEE	6477.27±112.86	vs 100 µg/mL AGLEE	0.055
100 µg/mL AGLEE	2365.79±132.66		

The results were analyzed using Mann-Whitney test
*Statistically significant at p<0.05

Effect of *Apium graveolens* leaf ethanolic extract (AGLEE) on apoptosis of HSC-3 cells

The percentage of apoptotic HSC-3 cells in the doxorubicin-treated group was significantly higher (p<0.001) than that in the untreated group (**Figure 2** and **Table 2**). The results showed a clear concentration-dependent increase (p<0.001) in apoptosis, with higher concentrations of AGLEE inducing significantly more apoptotic cells. In the 1 µg/mL AGLEE-treated group, the percentage of apoptotic cells was significantly higher (p<0.001) than in the untreated group. The 10 µg/mL AGLEE-treated group induced a significantly higher percentage of apoptotic cells compared with both in the 1 µg/mL AGLEE-treated group (p<0.001) and the untreated group (p<0.001). Additionally, the 100 µg/mL AGLEE-treated group induced significantly more apoptosis compared with the 10 µg/mL AGLEE-treated group (p<0.001), 1 µg/mL AGLEE-treated group (p<0.001) and the untreated group (p<0.001). These findings indicated that AGLEE could induce

apoptosis in HSC-3 cells in a concentration-dependent manner, with significant increases in apoptosis at each higher concentration tested.

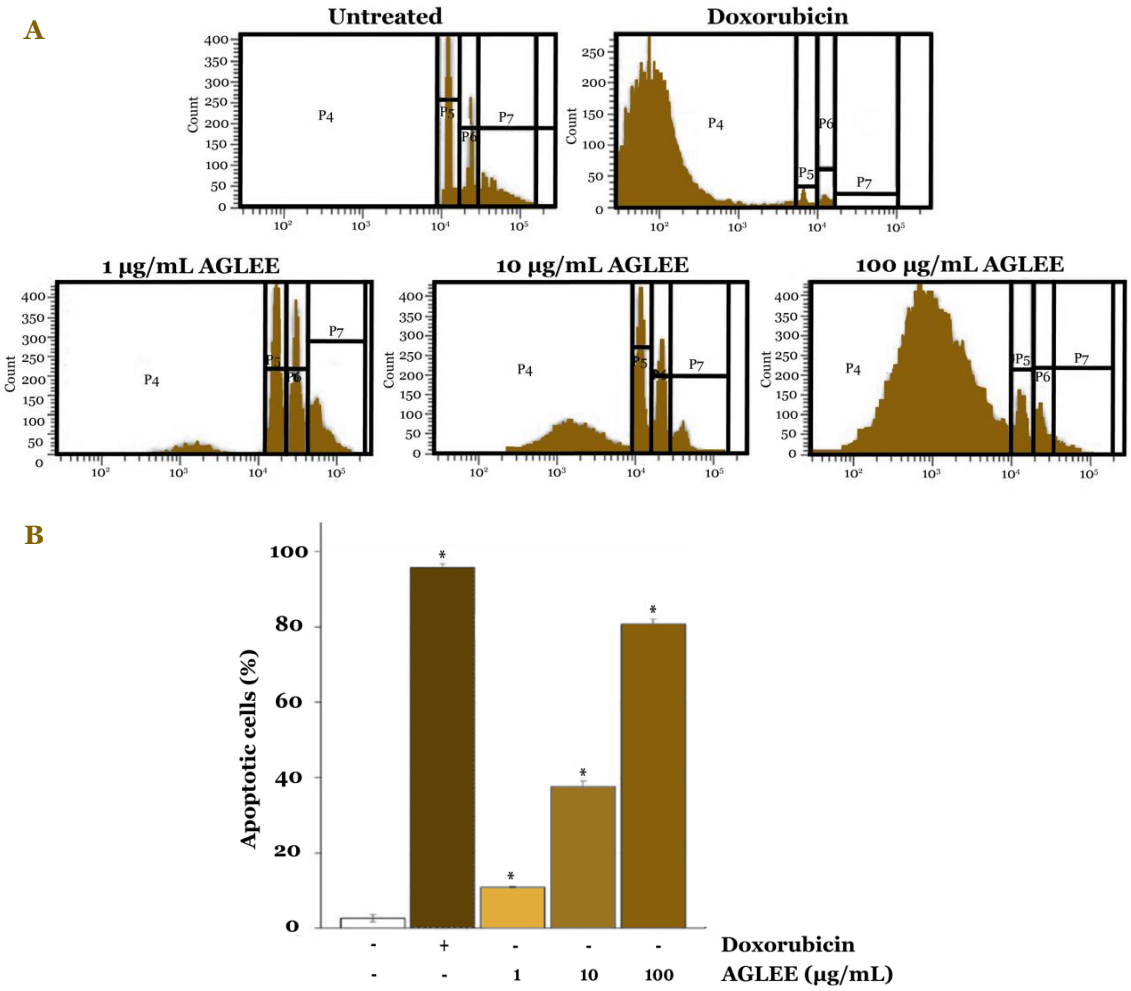

Figure 2. *Apium graveolens* leaf ethanolic extract (AGLEE) increased the percentage of apoptotic HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, and exposed to either doxorubicin or different concentrations of AGLEE for 24 hours. Subsequently, sub-G1 assay was performed on HSC-3 cells. (A) Histogram representing the flow cytometric findings. (B) Mean percentage of apoptotic HSC-3 cells. The results are presented in mean ± standard deviation (n=6). Tukey's HSD, * statistically significant at $p<0.05$ compared with the untreated group.

Table 2. Detailed comparisons of *Apium graveolens* leaf ethanolic extract (AGLEE) effects on apoptosis of HSC-3 cells

Group	Apoptotic cells (%)	*p*-value	
Untreated	2.59±0.26	vs Doxorubicin	<0.001*
		vs 1 µg/mL AGLEE	<0.001*
		vs 10 µg/mL AGLEE	<0.001*
		vs 100 µg/mL AGLEE	<0.001*
Doxorubicin	95.73±0.48	vs 1 µg/mL AGLEE	<0.001*
		vs 10 µg/mL AGLEE	<0.001*
		vs 100 µg/mL AGLEE	<0.001*
1 µg/mL AGLEE	10.82±0.20	vs 10 µg/mL AGLEE	<0.001*
		vs 100 µg/mL AGLEE	<0.001*
10 µg/mL AGLEE	37.62±1.45	vs 100 µg/mL AGLEE	<0.001*
100 µg/mL AGLEE	80.74±1.29		

The results were analyzed using Tukey's HSD test
*Statistically significant at $p<0.05$

Effect of *Apium graveolens* leaf ethanolic extract (AGLEE) on cleaved-caspase-3 level in HSC-3 cells

In the doxorubicin-treated group, a marked increase in the level of cleaved-caspase-3 in HSC-3 cells was observed at both 6 and 12 hours. Treatment with AGLEE also significantly elevated the level of cleaved-caspase-3, beginning at a concentration of 1 µg/mL, with further increases noted at both 6 and 12 hours in cells treated with 10 and 100 µg/mL of AGLEE. The results indicated that AGLEE induced cleaved-caspase-3 in a concentration-dependent manner at both 6 hours (p<0.001) and 12 hours (p=0.015). In each group, similar levels of cleaved-caspase-3 were exhibited at both 6 and 12 hours of treatment with AGLEE, suggesting that the effect of the treatment was not time-dependent (**Figure 3** and **Table 3**).

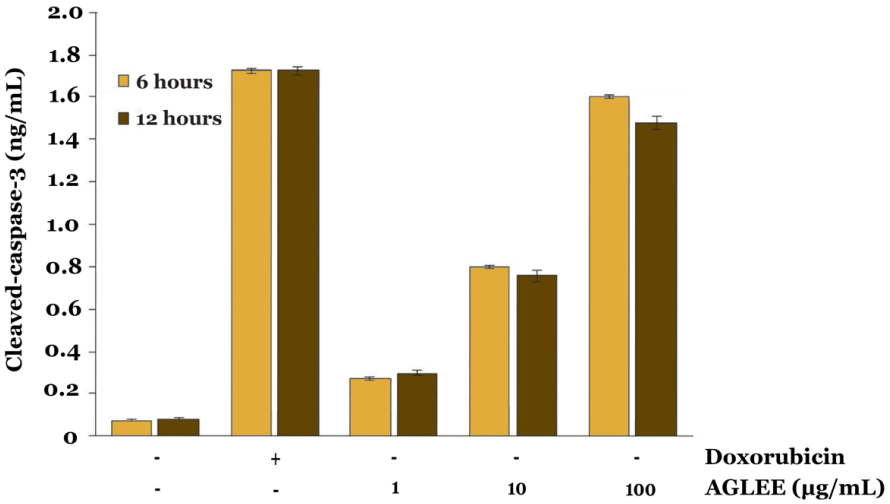

Figure 3. *Apium graveolens* leaf ethanolic extract (AGLEE) increased cleaved-caspase-3 level in HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, and exposed to either doxorubicin or different concentrations of AGLEE for 6 or 12 hours as specified. HSC-3 cells were collected, lysed, and processed to obtain cell lysate then subjected to ELISA. The results are presented in mean ± standard deviation (n=3).

Table 3. Detailed comparisons of *Apium graveolens* leaf ethanolic extract (AGLEE) effects on the cleaved-caspase-3 levels of HSC-3 cells

Duration	Groups	p-value
6 hours	Untreated vs doxorubicin	<0.001#*
	Untreated vs 1 µg/mL AGLEE	<0.001#*
	1 µg/mL AGLEE vs 10 µg/mL AGLEE	<0.001#*
	10 µg/mL AGLEE vs 100 µg/mL AGLEE	<0.001#*
12 hours	Untreated vs doxorubicin	0.046$*
	Untreated vs 1 µg/mL AGLEE	0.046$*
	1 µg/mL AGLEE vs 10 µg/mL AGLEE	<0.001#*
	10 µg/mL AGLEE vs 100 µg/mL AGLEE	<0.001#*

#Analyzed using independent t-test
$Analyzed using Mann-Whitney test
*Statistically significant at p<0.05

Pretreatment with Z-DEVD-FMK significantly inhibited the cleaved-caspase-3 levels induced by AGLEE and doxorubicin at both 6 and 12 hours, as evidenced by lower levels of cleaved-caspase-3 compared to those without Z-DEVD-FMK pretreatment (**Figure 4** and **Table 4**). These findings indicate that Z-DEVD-FMK irreversibly inhibited the AGLEE-induced cleaved-caspase-3 activity.

Effect of *Apium graveolens* leaf ethanolic extract (AGLEE) on cleaved-PARP level in HSC-3 cells

Doxorubicin treatment for 6 hours significantly increased the level of cleaved-PARP in HSC-3 cells. A further increase in cleaved-PARP was observed when the treatment duration was extended to 12 hours (p<0.001). Treatment with AGLEE significantly induced cleaved-PARP

starting at a concentration of 1 µg/mL over a 6-hour period. Notably, HSC-3 cells treated with 100 µg/mL of AGLEE exhibited the highest levels of cleaved-PARP. The induction of cleaved-PARP by AGLEE occurred in a concentration-dependent manner at both 6 hours (p=0.015) and 12 hours (p=0.016). Additionally, the levels of cleaved-PARP at 12 hours were higher than those at 6 hours across all groups, particularly at the 100 µg/mL concentration (p<0.001). These findings indicated that AGLEE induced cleaved-PARP in a time-dependent manner (**Figure 5** and **Table 5**).

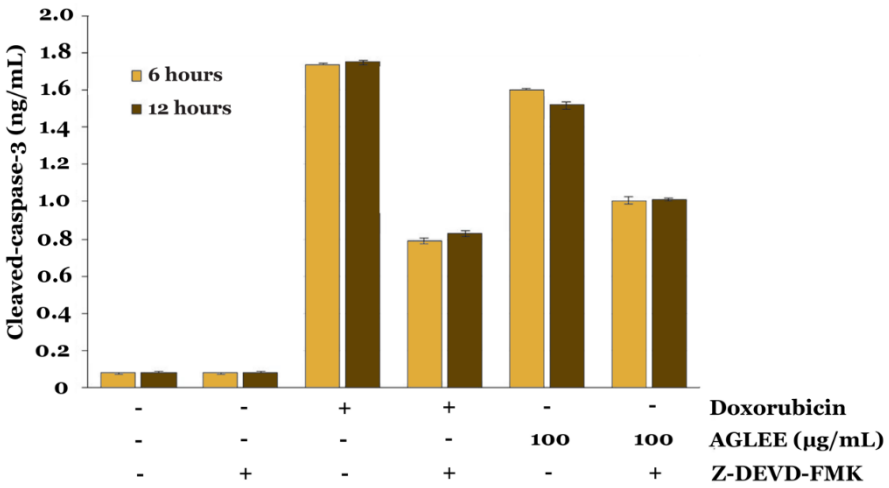

Figure 4. Z-DEVD-FMK decreased *Apium graveolens* leaf ethanolic extract (AGLEE)-induced cleaved-caspase-3 level in HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, pretreated with/without Z-DEVD-FMK for 2 hours, and exposed to either doxorubicin or 100 µg/mL AGLEE for 6 or 12 hours as specified. HSC-3 cells were collected, lysed, and processed to obtain cell lysate then was subjected to ELISA. The results are presented in mean ± standard deviation (n=3).

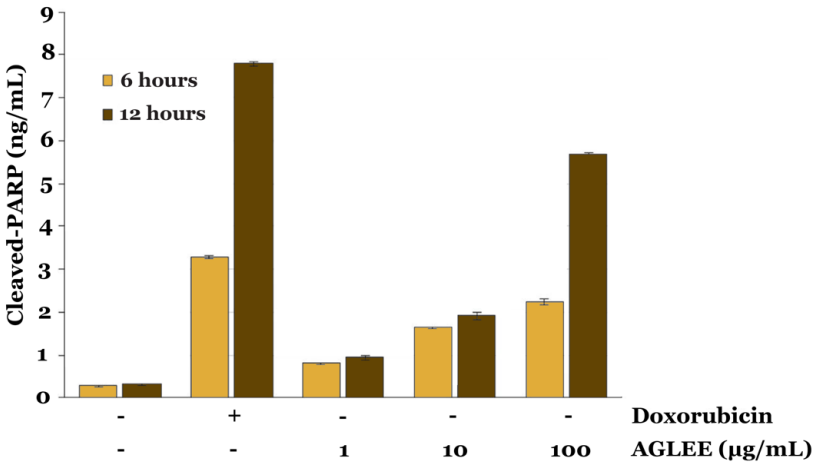

Figure 5. *Apium graveolens* leaf ethanolic extract (AGLEE) increased cleaved-PARP level in HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, and exposed to either doxorubicin or different concentrations of AGLEE for 6 or 12 hours as specified. HSC-3 cells were collected, lysed, and processed to obtain cell lysate then was subjected to ELISA. The results are presented in mean ± standard deviation (n=3).

Table 5. Detailed comparisons of *Apium graveolens* leaf ethanolic extract (AGLEE) effects on cleaved-PARP of HSC-3 cells

Duration	Groups	*p*-value
6 hours	Untreated vs doxorubicin	<0.001#*
	Untreated vs 1 µg/mL AGLEE	0.046$*
	1 µg/mL AGLEE vs 10 µg/mL AGLEE	0.046$*
	10 µg/mL AGLEE vs 100 µg/mL AGLEE	0.004#*
12 hours	Untreated vs doxorubicin	<0.001#*
	Untreated vs 1 µg/mL AGLEE	0.002#*

Duration	Groups	*p*-value
	1 µg/mL AGLEE vs 10 µg/mL AGLEE	<0.001#*
	10 µg/mL AGLEE vs 100 µg/mL AGLEE	<0.001#*

#Analyzed using independent t-test
$Analyzed using Mann-Whitney test
*Statistically significant at $p<0.05$

Pretreatment with Z-DEVD-FMK significantly inhibited the production of cleaved-PARP induced by AGLEE and doxorubicin at both 6 and 12 hours (**Figure 6** and **Table 6**). Given that Z-DEVD-FMK effectively inhibited the AGLEE-induced cleaved-PARP at the 12-hour mark, it can be concluded that Z-DEVD-FMK exhibited irreversible activity against the cleaved-PARP induced by AGLEE.

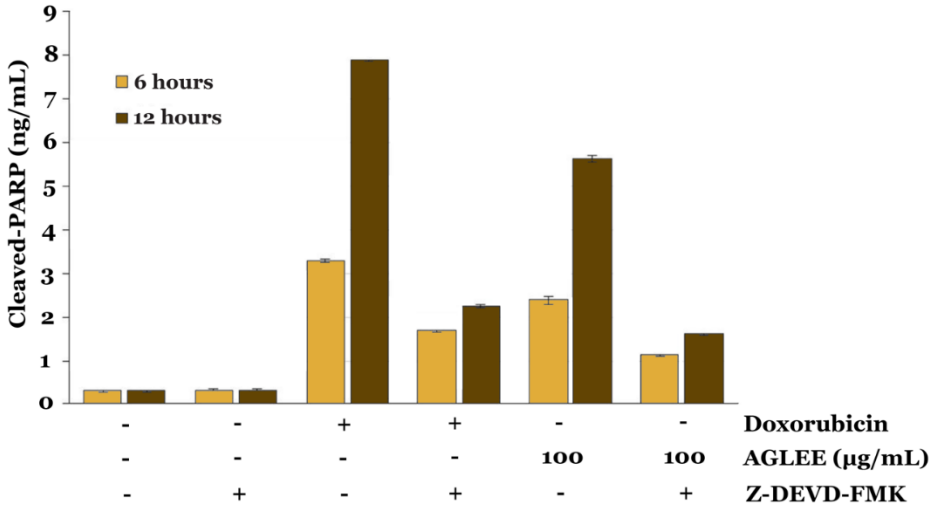

Figure 6. Z-DEVD-FMK decreased *Apium graveolens* leaf ethanolic extract (AGLEE)-induced cleaved-PARP level in HSC-3 cells. HSC-3 cells were cultured, subjected to 12 hours of nutrient deprivation, pretreated with/without Z-DEVD-FMK for 2 hours, and exposed to either doxorubicin or 100 µg/mL AGLEE for 6 or 12 hours as specified. HSC-3 cells were collected, lysed, and processed to obtain cell lysate then was subjected to ELISA. The results are presented in mean ± standard deviation (n=3).

Table 6. Detailed comparisons of *Apium graveolens* leaf ethanolic extract (AGLEE) effects on cleaved-PARP of HSC-3 cells with/without Z-DEVD-FMK

Duration	Groups	*p*-value
6 hours	Untreated vs untreated + Z-DEVD-FMK	0.343#
	Doxorubicin vs doxorubicin + Z-DEVD-FMK	<0.001#*
	100 µg/mL AGLEE vs 100 µg/mL AGLEE + Z-DEVD-FMK	0.002#*
12 hours	Untreated vs untreated + Z-DEVD-FMK	0.338#
	Doxorubicin vs doxorubicin + Z-DEVD-FMK	<0.001#*
	100 µg/mL AGLEE vs 100 µg/mL AGLEE + Z-DEVD-FMK	<0.001#*

#Analyzed using independent t-test
*Statistically significant at $p<0.05$

Discussion

In the present study, AGLEE reduced the viability of HSC-3 cells in a concentration-dependent manner, indicating a cytotoxic effect. The results of the sub-G1 assay further demonstrated that this cytotoxic effect was correlated with the induction of apoptosis. These findings confirmed that AGLEE exhibited a cytotoxic effect on HSC-3 tongue cancer cells, consistent with reports of similar effects in various cancer cell lines, including BGC-823 stomach cancer cells [15], Dalton's lymphoma ascites cells [16], and LNCaP prostate cancer cells [17]. Therefore, the current study contributes to the growing body of evidence supporting the anticancer potential of *A. graveolens*.

Based on IC_{50} value obtained, AGLEE was considered to possess moderate cytotoxic activity [28] against HSC-3 cells, with an IC_{50} of 48.29 µg/mL. This value is notably lower than that of the ethanolic extract from the whole plant of *A. graveolens*, which demonstrated an IC_{50} of 2,840

μg/mL against LNCaP prostate cancer cells [15]. In contrast, the IC_{50} of AGLEE was higher than that of the methanolic extract derived from *A. graveolens* seeds, which showed an IC_{50} of 29.79 μg/mL against Dalton's lymphoma ascites cells [16]. These differences in IC_{50} values can be attributed to several factors, including variations in the sensitivity of the cancer cell lines, the plant parts used (leaves, seeds, or whole plant), the solvents, and the methods employed for extraction [29]. These factors critically influence the bioavailability and composition of the bioactive compounds of *A. graveolens*, such as flavonoids, phenolic acids, and terpenoids [11], which contribute to their cytotoxic potential [29].

Cleavage of caspase-3 is crucial in the execution of apoptosis. In the presence of apoptotic signals, caspase-3 is cleaved either by caspase-8 in the extrinsic apoptotic pathway or by the apoptosome complex in the intrinsic apoptotic pathway [26,27]. In the present study, the concentration-dependent effects of *A. graveolens* resulted in an increase in cleaved-caspase-3 levels. To confirm the induction of caspase-3 cleavage by *A. graveolens*, the caspase-3-specific inhibitor Z-DEVD-FMK was employed. The cleavage of caspase-3 induced by *A. graveolens* was diminished in the presence of Z-DEVD-FMK, thereby affirming the role of caspase-3 in the apoptosis induced by *A. graveolens*. Z-DEVD-FMK is a cell-permeable synthetic peptide that has been extensively utilized to inhibit apoptosis induction in various cancer cell lines in experimental research [30,31]. Z-DEVD-FMK selectively and irreversibly binds to the active site of caspase-3 [33,34]. Consequently, the application of this specific caspase-3 inhibitor underscores the critical role of caspase-3 activation in apoptotic cell death within cancer cells.

Cleaved-caspase-3 catalyzes the cleavage of PARP, which subsequently induces cell death. PARP cleavage is recognized as one of the hallmarks of apoptosis [35]. The data obtained in the present study demonstrated that *A. graveolens* elevated the levels of cleaved-PARP in a concentration- and time-dependent manner. The inhibition of PARP cleavage by Z-DEVD-FMK pretreatment indicated that *A. graveolens* induced apoptosis through a caspase-3-dependent pathway. The caspase-3/PARP signaling pathway has been examined to confirm the occurrence of apoptosis in both physiological and pathophysiological processes. In cancer cells, the activation of this signaling pathway has been associated with apoptotic cell death [10,30]. This pathway can be activated by specific signaling components, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [36]. However, cancer cells may exhibit resistance to these components; thus, natural products may serve as alternatives for inducing apoptosis through the activation of the caspase-3/PARP pathway [10,37,38].

The present study showed the apoptotic effects of AGLEE specifically in tongue cancer cells (HSC-3), positioning it as a promising natural compound with potential therapeutic applications for tongue cancer. This novel finding adds value to the broader field of cancer research, particularly in the exploration of alternative or adjunct therapies for oral cancer. Given that most chemotherapeutic drugs lead to necrotic cell death, which can result in inflammation and damage to surrounding tissues, the ability of AGLEE to induce apoptosis rather than necrosis makes it a particularly attractive candidate for further development. Doxorubicin has been shown to induce apoptosis and controlled cell death [24]. Doxorubicin's ability to intercalate DNA and generate oxidative stress further enhances its apoptotic effects [24]. Given the rising prevalence of tongue cancer and the limitations of current treatments, AGLEE may offer a natural alternative or adjunct to conventional treatments, thus warranting further exploration.

While the results of this study provide important insights into the cytotoxic effects of *A. graveolens* on HSC-3 cells, the findings are limited by several factors, such as the absence of in vivo validation and the inability to identify the specific bioactive compounds responsible for these effects. Additionally, future studies should investigate the caspase-3 and PARP upstream of apoptotic signaling pathway, which would provide a more comprehensive understanding of the molecular mechanisms underlying the anticancer effects of AGLEE and its potential as a therapeutic agent.

Conclusion

A. graveolens was able to reduce viable HSC-3 cells and induce apoptosis in a concentration-dependent manner, while also increasing the levels of cleaved-caspase-3 and cleaved-PARP. This

suggests that *A. graveolens* may act as a potential inducer of the caspase-3/PARP apoptotic signaling pathway in tongue cancer cells.

Ethics approval

Not required.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Data supporting the findings of this study can be obtained from the corresponding author upon request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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