

Histological and Molecular Evaluation of the Antiproliferative Activity of *Allium ampeloprasum* Water Extract Against Oral Mucosa Cell Line (Gingival Cancer)

Maryam Hameed Alwan^{1*}, Zainab Hameed², Satria Mandala³

¹Department of Oral Diagnosis, College Of Dentistry, Baghdad University, Iraq

²Department of Pharmacology/ Iraqi Ministry of Health, Baghdad, Iraq

³Human Centric (HUMIC) Engineering & School of Computing, Telkom University, Bandung, Indonesia

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ABSTRACT

Gingival carcinoma is a malignant neoplasm affecting the oral mucosa and is associated with significant morbidity and mortality. *Allium ampeloprasum* var. porrum water extracts have gotten a lot of attention because of their bioactive components, such as polyphenols, flavonoids, and alkaloids, which have a variety of pharmacological activities, including antiproliferative actions. This study aimed to evaluate the histological and molecular effects of *Allium ampeloprasum* (leek) water extract on the proliferation of the murine gingival cancer cell line. Histological evaluation was conducted to examine morphological changes induced by extract treatment. Molecular mechanisms underlying the observed histological changes were investigated using real-time polymerase chain reaction (PCR). Expression levels of key genes associated with cell proliferation and apoptosis were assessed. Histological findings revealed a dose-dependent decrease (100, 50, 25, 12.5, and 6.25 µg/ml) in cell density and altered cell shape in the treated cell line. Also, the percentage of inhibition for the oral mucosa cell line was high, with a significant P of 0.006, in the treated group compared to the control group. Additionally, water extract has an IC₅₀ value of 61 g/ml. The P53 fold increment of gene expression is 0.6, which means the expression level in the experimental condition is 60% higher than the control. This study provides evidence for the potential antiproliferative activity of *Allium ampeloprasum* water extract on the oral mucosa cell line. The observed histological changes, coupled with the modulation of key genes involved in proliferation and apoptosis, suggest that leek water extract may have therapeutic implications in managing oral cancer.

1. Introduction

Cancer is the leading cause of death in the whole world; according to the World Health Organisation (WHO), it was responsible for over 10 million deaths in 2020, or approximately one in every six. Many tumors can be cured if they are diagnosed early and treated properly (Ferlay *et al.* 2020). Various treatment strategies have been employed for cancer, such as chemotherapy, surgery, radiation, and immunotherapy using monoclonal antibodies. However, chemotherapy is the most common choice for cancer treatment. Its drawbacks are the severe

side effects, which reduce the quality of life of cancer patients, and they have been a major issue in cancer treatment (Schirmacher 2019).

Gingival cancer, commonly known as oral cancer, is a malignant tumor of the oral cavity that affects the gums, tongue, and mouth mucosa. It is a global health concern with a high death rate, necessitating the development of novel therapeutic ways to combat it (Andisheh-Tadbir *et al.* 2008; Tuominen and Rautava 2021).

Allium ampeloprasum var. porrum water extracts have gotten a lot of attention because of their bioactive components, such as polyphenols, flavonoids, and alkaloids, which have a variety of pharmacological activities, including antiproliferative actions.

* Corresponding Author

E-mail Address: maryam.h@codental.uobaghdad.edu.iq

Researchers are very interested in these extracts' capacity to inhibit cancer cell growth and division (Zamri and Hamid 2019; Asemami *et al.* 2019)

Medical plants have grown significant sources of active substances that are used in the preparation of drugs; they contain the secondary metabolic products they produce as natural products known for their essential biological and pharmacological activity and as chemical agents (Süntar 2020). Natural products and their derivatives have a wide range of pharmacological effects and are used to treat and prevent various common human diseases. Infectious diseases, cancer, and peptic ulcers are examples, as are their applications as immunomodulators, anticoagulants, antioxidants, therapies for respiratory, digestive, and circulatory system disorders, and antidiabetic medicines (Najmi *et al.* 2022).

The histological and molecular evaluation of *Allium ampeloprasum* var. porrum water extracts' antiproliferative effect on the gingival cancer cell line (Gingival Ca) has emerged as an important topic of research in oncology.

Moreover, epidemiological studies have found that consuming *Allium* species, such as leeks, lowers the incidence of prostate, colorectal, stomach, and breast cancer. In the bulbs of *Allium ampeloprasum* var. porrum, a new steroidal saponin was found, which displayed hemolytic effects in laboratory testing and served as an immunological adjuvant by boosting the cellular immune response to the ovalbumin antigen. Several additional steroidal saponins and five flavonoids have also been identified with anti-aggregation properties on human platelets (Bastaki *et al.* 2021). The antioxidant content of the green part of the leek is significantly higher than that of the white part. This mismatch can be attributed, at least in part, to the existence of phenolic chemicals, whose synthesis is light-dependent (Bernaert *et al.* 2011).

Through histological and molecular analyses, the present work aims to evaluate the possible antiproliferative activity of *Allium ampeloprasum* var. porrum water extracts on the gingival cancer cell line (Gingival Ca). It is crucial to conduct research that explores inventive methods of treating cancer. Such studies, which take an approach and concentrate on gingival cancer, greatly contribute to the field of cancer research. The findings offer

proof of the effectiveness of natural compounds as a viable treatment option for cancer patients.

2. Materials and Methods

2.1. Plant Extraction Procedure

Allium ampeloprasum var. porrum leaves (around 1.5 kilograms of fresh plant) were collected by hand from the rural area of Baghdad, Iraq. Then, the plant identification was done by a specialist in the National Herbarium of Iraq in Al-Rustumiya/Baghdad, Iraq. Plant leaves were properly washed with tap water to remove dirt and debris before being left to air dry for five days on an open surface and, after complete dryness, processed in a heavy-duty stainless-steel grinder, yielding a fine powder. Water extraction using the maceration technique (digesting procedure) was used to extract the desired components from the fine powder. With 100 g of powder and 400 ml of water, the extraction ratio was 4:1 (weight/volume). The extraction process was done three times. Subsequently, the mixture was then filtered through three layers of sterile gauze to produce a clear extract. The extract was then concentrated under a vacuum using a rotary evaporator until it reached a dry state. The resulting crude extract was carefully stored in a dry, firmly sealed vial (Abubakar and Haque 2020; Hidayat and Wulandari 2021). The crude extracts were stored in a freezer set at -20°C until they were ready for use.

2.2. Preliminary Qualitative Phytochemical Analysis

Chemical tests were carried out using the *Allium ampeloprasum* var. porrum water extract using standard procedures to identify the active constituents (Harborne 1980).

2.2.1. Test for Alkaloids

In a steam bath, plant extracts were mixed with HCL 10/5 ml. Mayer's and Wagner's reagents were used to test for the presence of alkaloids, and white and reddish-brown precipitates were obtained.

2.2.2. Test for Flavonoids

Lead acetate solution was added to plant extracts 0.1/5 ml. The presence of a yellowish-white precipitate was considered a positive test for flavonoids (Mondal and Rahaman 2020).

2.2.3. Tests for Steroids

Liebermann-Burchard test: 3 ml of the extract was treated with chloroform, acetic anhydride, and a few drops of sulphuric acid. The presence of steroids is indicated by the production of dark pink or red color (Namulondo 2023).

2.3. Stock Solution Preparation

The extract is prepared in concentrations of 10 mg/ml in DMSO. The samples were then treated to syringe filtering with a 0.45 µm filter to remove any impurities. (Badgular *et al.* 2017). The cells were exposed to 6.25, 12.5, 25, 50, and 100 µg/ml prepared by diluting the stock solution with the complete growth medium. As a control, DMSO is dissolved in CGM to obtain the final concentration (Papazisis *et al.* 1997).

2.4. Preparation of (DMSO) Solution as a Control

Control was prepared by putting 20 µl DMSO in 980 µl CGM in an Eppendorf tube to make 1 ml.

2.5. Preparation of Mouth Mucosa Cell Line (Gingival Ca) for Cytotoxicity Assays

The murine cancer cell lines (Kerafast, From the laboratory of Ravindra Uppaluri, USA) were grown in RPMI-1640 media (Capricorn, Germany) containing 10% foetal bovine serum (Capricorn, Germany), 100 units/ml penicillin, and 100 g/ml streptomycin (Gibco, Life Technology, UK). The cell cultures were kept at 37 °C in a humidified atmosphere with 5% CO₂ (Al-Shammari *et al.* 2016).

2.6. MTT Assay

To perform the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Bio-World, USA) test, cells are planted in a multiwell plate 1 × 10⁴ cells/well. They were treated according to the experimental design. Following the treatment period of 2.5 hours at 37°C in 5% CO₂, the cells are incubated with MTT solution to allow the MTT to be taken up by the live cells. Following the incubation period, the formazan crystals were solubilized with detergent, and the absorbance was measured with a spectrophotometer (Genex laboratories, USA) was occupied at 492 nm and the reference at 650 nm by the micro-plate reader; the assay was performed at triplicate (Burton 2005).

2.7. Preparation of the Tissue for Histological Assessment

To examine the histological morphology under an inverted microscope, 200 µl of cell suspensions were seeded at a density of 1 × 10⁴ cells ml⁻¹ into each well of a 96-well microtitration plate. The plate was then incubated for 48 hours at 37°C. The culture medium was carefully removed after incubation, and a 100 µg/ml water extract of *Allium ampeloprasum* var. porrum was applied. After another 24-hour incubation period, the cells were stained by adding 50 µl of crystal violet solution to each well. To allow for staining, the plate was incubated at 37°C for 15 minutes. The stain was then carefully rinsed away with tap water until the dye was gone. The cells were examined with an inverted microscope (Optika, Italy) (Jabir *et al.* 2019).

2.8. Determination IC₅₀

The concentration that inhibits 50% from the cell growth (IC₅₀) was analyzed and calculated for *Allium ampeloprasum* var. porrum water extract by linear regression equation: $y = mx + b$, where y is the percentage of inhibition and is set to be 50%, m is the slope of the standard curve, x is the concentration of the compound tested in µg/ml, and b is the y -intercept of the line of the standard curve.

2.9. Determination of Gene Expression in Mouth Mucosa Cell Line (Gingival Ca)

Once the cells were exposed to the predetermined inhibitory concentration (50%) of the plant extract for the designated incubation period in tissue culture (Nunclon TM, Denmark), the cells, along with the media, were transferred to a 10 ml centrifuge tube and centrifuged at 1,500 rpm for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) (Lot019k8205, Sigma (USA)). After the completion of the centrifugation, the supernatant was discarded, and the cell pellet was resuspended in 200 µl of PBS. The cell suspension was then stored in deep freeze (-85°C) until the day of RNA extraction (Lee *et al.* 2005).

The real-time polymerase chain reaction (PCR) was performed using the AriaMx Real-Time PCR (qPCR) Instrument from Agilent Technologies, United States. The Delta Delta Ct method was utilized to

determine the expression level of the P53 gene and the fold change in mRNA levels, both in cancer cells treated with a plant extract and untreated cancer cells. In this study, the 18rS gene (or muGAPDH) was used as the housekeeping gene (Rao *et al.* 2013).

2.10. Statistical Analysis

A statistical study was performed by using the GraphPad Prism software, version 8.2.1 for Windows 10 and SPSS ver.28. The statistic design for this study was presented as mean \pm SD (standard deviation). Person correlation was utilized to find a correlation between variables, with the P value considered significant at $p < 0.05$.

3. Results

3.1. Phytochemical Analysis

The presence of flavonoids and alkaloids was detected in the aqueous extract of *Allium ampeloprasum* var. porrum leaves Table 1.

3.2. Histological Evaluation of the Anti-Proliferative Activity of *Allium ampeloprasum* var. Porrum Leaves Water Extract

Histological evaluation of the cells under the microscope showed the inhibitory effect of *Allium ampeloprasum* var. porrum leaf extracts on mouth mucosa cell lines after 24 hours. Cells treated with water extract showed signs of shrinkage, irregular cell borders, and changes in nuclear morphology (Figure 1 Photomicrographs of crystal violet-stained oral mucosa cell line (Gingival Ca)).

Table 1. Phytochemical screening of *Allium ampeloprasum* var. porrum water extract

Active constituents	ater extract
Alkaloids	+
Flavonoids	+
Steroids	-

+, - represent the presence and absence of phytoconstituents, respectively

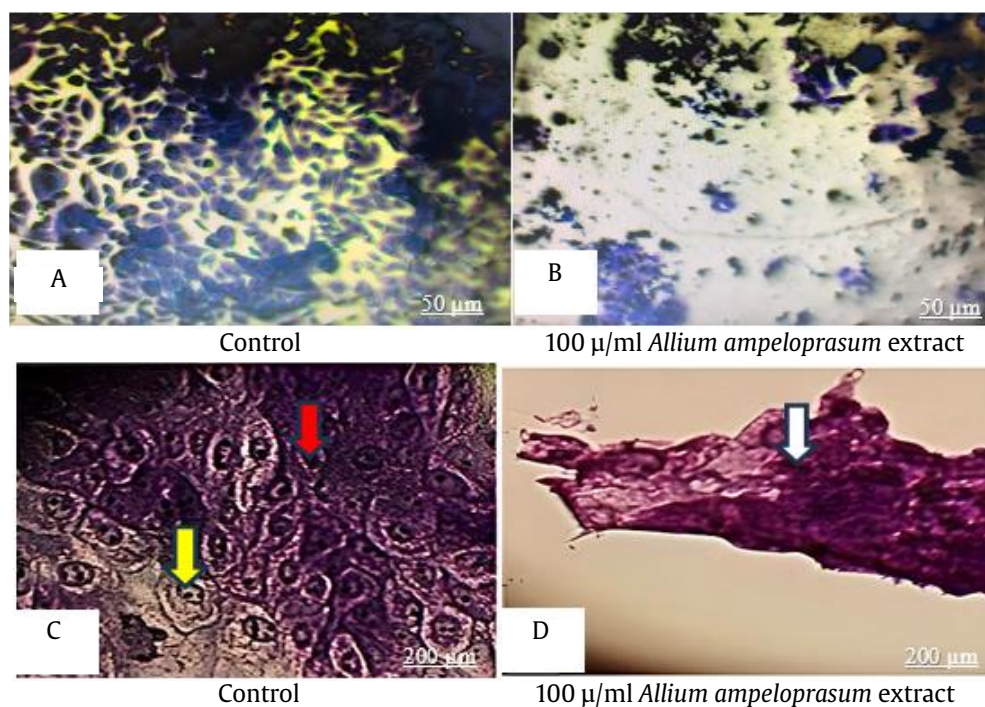


Figure 1. Photomicrographs of a crystal violet-stained mouth mucosa cell line (Gingival Ca). (A) Control untreated cells, high cell density, (B) cell treated with *Allium ampeloprasum*, diminished density, and altered morphology, (C) control, pleomorphic cells with several nucleoli (yellow arrow). Some cells are large with one prominent, dark-stained nucleoli (red arrow), (D) cells treated with water extract show signs of shrinkage, irregular cell borders, and changes in nuclear morphology (white arrow)

3.3. Percentage of Inhibition for the Oral Mucosa Cell Line (Gingival Ca) About Serial Concentrations of Water Extract of *Allium ampeloprasum* var. Porrum

Using the following linear regression equations, the IC_{50} value for water extract of *Allium ampeloprasum* var. porrum leaf was assumed from the graph 1: $[y = 12.068 \ln(x) + 0.3881]$, where Y represents the percentage of inhibition and X represents the concentration. Water extract has an IC_{50} value of 61 $\mu\text{g/ml}$. The IC_{50} values used to categorize the activity of *Allium ampeloprasum* var. porrum aqueous extract were adjusted as follows: $IC_{50} =$ extremely active, $IC_{50} =$ moderately active, $IC_{50} = 201-500 \text{ g/ml} =$ weakly active, and $IC_{50} > 50 \text{ g/ml} =$ inactive (There was significant difference between water extract and control, Figure 2, Table 2).

3.4. Determination of P53 Gene Expression

Table 3 represents the Cycles threshold Ct values of both the housekeeping gene HKG and the gene of interest GOI obtained from real-time RT-PCR.

4. Discussion

The global demand for natural products and bioactive derived from plants has undeniably increased, leading pharmaceutical, food, and

cosmetics industries to extensively investigate the realm of medicinal and natural products (Patra *et al.* 2018). To this day, phytochemical studies of different kinds of the *Allium* genus have indicated that 16 species exhibit promising anti-cancer capabilities. This is due to the presence of sulfur and organic chemicals such as S-allyl mercaptocysteine, quercetin, flavonoids, and alkaloids. These chemicals inhibit the cell cycle, affect signaling pathways, induce apoptosis, and have antioxidant activity. As a result, they intervene in numerous stages of cancer cell creation, development, differentiation, and metastasis (Asemani *et al.* 2019).

Moreover, *Allium ampeloprasum* var. porrum has been recognized as a rich source of secondary metabolites, including phenolic acids, flavonoids, and flavonoid polymers (proanthocyanidins or

Table 2. The impact of different doses of *Allium ampeloprasum* var. porrum aqueous extract on the Gingival Ca cell line

Concentration ($\mu\text{g/ml}$)	% of inhibition(m) \pm SD	T-test	P Value
100.00	58.41 \pm 0.004	.972	.006**
50.00	45.39 \pm 0.035		
25.00	37.3 \pm 0.033		
12.50	31.58 \pm 0.029		
6.25	23.49 \pm 0.004		

Correlation is highly significant at the 0.01 level (2-tailed)

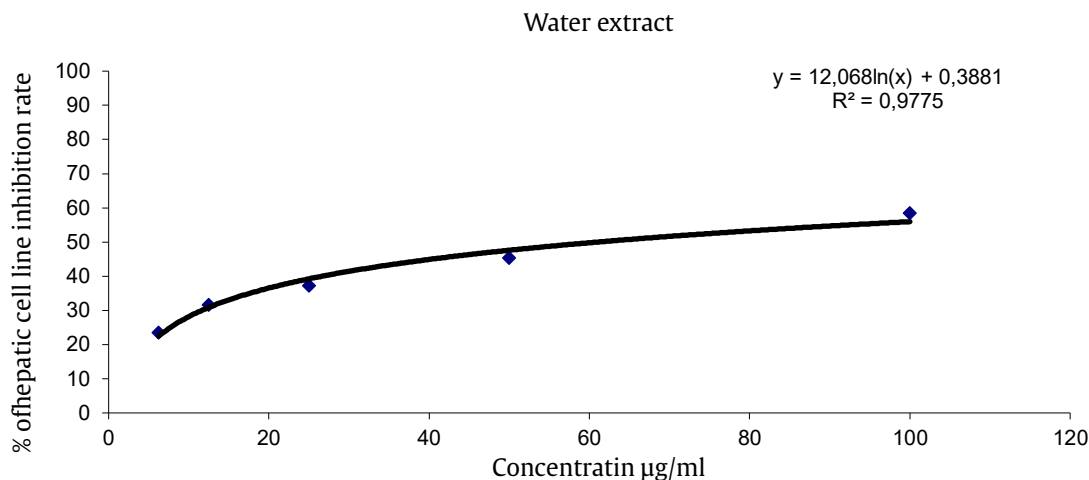


Figure 2. Dose-response curve of water extract of *Allium ampeloprasum* var. porrum for mouth mucosa cell line (Gingival Ca)

Table 3. Ct (Cycles threshold) of the housekeeping gene (GAPDH) and the gene of interest (P53)

Gene name	Ct control untreated cells	Ct Cells treated with <i>Allium ampeloprasum</i> var. porrum extract	Δ Ct	$\Delta\Delta$ Ct	Fold increment In P53 gene expression
GAPDH	36.44	38.22	10.11 (control)	1.68	0.6
P53	26.33	29.88	8.43 (treatment)		

condensed tannins), with related health benefits. Organosulfur compounds and flavonols have tumor-inhibiting properties that can block several stages of carcinogenesis. *Allium* species are characterized by their rich content of sulfur compounds that are responsible for the organoleptic parameters and contribute to the antioxidant and antimicrobial activities of these vegetables (Khazaei *et al.* 2017; Strati *et al.* 2018).

The histological finding showed the inhibitory effect of *Allium ampeloprasum* var. porrum leaf extracts on mouth mucosa cell lines (Figure 1). Cells treated with water extract showed signs of shrinkage, irregular cell borders, and changes in nuclear morphology, which suggest that the extract may have caused cellular damage or death. This agrees with the finding of Rose *et al.* (2005). The ability of *Allium* species to impact cell cycle arrest and induce apoptosis has been observed in cancer cell models studied *in vitro* model (Rose *et al.* 2005).

Moreover, the presence of flavonoids, like quercetin, found mostly in *Allium* species, proved to have the ability to increase p53 expression, consequently speeding up tumor cell apoptosis in the experimental group. This is supported by the finding of the He *et al.* study, which concluded that the curcumin treatment improves the general health of patients with colorectal cancer via the mechanism of increased p53 molecule expression in tumor cells and consequently speeds up tumor cell apoptosis (He *et al.* 2011; Chan *et al.* 2013).

Real-time RT-PCR (or kinetic RT-PCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range. Real-time PCR allows precise quantification of specific nucleic acids in a complex mixture even if the starting amount of material is at a very low concentration, and this is accomplished by monitoring the amplification of a target sequence in real-time using fluorescent technology, how quickly the amplified target reaches a threshold detection level correlates with the amount of starting material present (Pfaffl 2004).

The *in vitro* screening of *Allium ampeloprasum* var. porrum water extracts on cancer cell lines showed a dose-dependent inhibition for cancer cell growth after 72 hr (Table 2). The IC₅₀ value was deduced from the graph for water extract of *Allium ampeloprasum* var. porrum leaf. The IC₅₀ value for water extract was 61 µg/ml. The MTT assay revealed that the growth of cancer cells was inhibited in a dose-dependent manner. The criteria used to categorize the activity of *Allium ampeloprasum* var. porrum leaves water extract against cancer cell line based on IC₅₀ values were modified from those of NCI and Geran *et al.* as the IC₅₀ of *Allium ampeloprasum* var. porrum leaves water extract between 21-200 µg/ml, so can be considered as moderately active (Srisawat *et al.* 2013)

In particular, the water extract of the *Allium ampeloprasum* var. porrum was used for the real-time RT-PCR, and the results showed that the fold increment in P53 gene expression was 0.6; here, this could be explained by the molecular mechanism responsible for the antiproliferative activity of *Allium ampeloprasum* var. porrum leaves water extract against mouth mucosa cell line (Gingival Ca) through upregulation of P53. Polyphenols use the p53 signaling pathway to produce anti-cancer activity through apoptosis in a variety of cancers. Polyphenols Isolated from *Allium cepa* Linn (onion, *A. cepa*), a member of the family Liliaceae up-regulating p53 protein (Khan *et al.* 2020).

The statistics reveal that *Allium ampeloprasum* var. porrum has the potential to be a natural treatment agent for gingival cancer. These findings add to the expanding body of knowledge about plant extracts' medicinal capabilities and highlight the need for additional research and clinical trials to investigate their therapeutic uses in cancer treatment fully. Overall, the work represents an important step forward in leveraging the power of natural chemicals to generate innovative treatments for gingival cancer.

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